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ORIGINAL ARTICLE

Enolization and racemization reactions of glucose and fructose on heating with amino-acid enantiomers and the formation of melanoidins as a result of the Maillard reaction

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Abstract This study investigated the enolization and racemization reactions of glucose and fructose on heating with amino acid enantiomers and the formation of melanoidins as a result of the Maillard reaction. The study measured reducing sugars and L- and D- amino acids using HPLC as an index for the amount of enolization of the sugars and isomerization of the amino acids. Additionally, the absorption of melanoidins was measured at different wavelengths (420, 450, 470, 490 nm); the UV–Vis spectra and the extinction coefficient were determined for the formation of melanoidins. Melanoidins were, rather arbitrarily, defined by a high-molecular-weight (HMW) if it was above a lower limit of 12.4 kDa, which was the nominal cut-off value in the dialysis system used. A remarkable enolization reaction of the sugars was observed in the course of the Maillard reaction. Especially, in the Fru/D-Asn model system, the degree of sugar enolization was more than in the other model systems. All of the FDAA (1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide) amino acids were separated by TLC. The racemization of the amino acids was higher in the fructose-amino acids systems. Isomer formation was the highest in the Fru/D-Asn system. The L- and D- isomers showed different absorptions in the UV–Vis spectra, although these had similar shapes. The absorption of the melanoidins formed from glucose was higher than that formed from fructose. In particular, the sugar–asparagine system showed different characteristics according to the L- and D-isomers. The differences in the extinction coefficients of the melanoidins was significant ($P < 0.05$), except for the sugar–lysine system.

Keywords Enolization · Extinction coefficient · Maillard reaction · Melanoidins · Racemization

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Introduction

The reaction between reducing sugars and amino acids is known as the Maillard reaction or non-enzymic browning reaction (Maillard 1913). The Maillard reaction is a complicated reaction that produces a large number of the so-called Maillard reaction products (MRPs) such as aroma compounds, ultra-violet absorbing intermediates, and dark-brown polymeric compounds named melanoidins (Wijewickreme et al. 1997). Melanoidins are widely distributed in foods (Delgado-Andrade and Morales 2005) and have different functional properties such as antioxidant, antimicrobial and metal-binding activities (Morales and Jiménez-Pérez 2004; Rufián-Henares and Morales 2005; Morales et al. 2005).

Enolization reaction known as the "Lobry de Bruyn–Alberda van Ekenstein transformation" produces enediol anion species. Through the reactions of sugar transformation due to the Lobry de Bruyn–Alberda van Ekenstein transformation, aldoses are converted into ketoses and vice versa. Similarly, an aminoketose is converted into aminoaldose in the presence of the free amino acids. In other words, glucose and fructose can isomerise from one into the other through the Lobry de Bruyn–Alberda van Ekenstein transformation (Speck 1958). Sugar isomerization 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). Because these sugar reactions occur simultaneously with the Maillard reaction and the sugar reaction products subsequently take part in the Maillard reaction, the Maillard reaction becomes even more intricate. Therefore, to be able to control the Maillard reaction, it is necessary to study the reactions of interest quantitatively.

The conversion of free or protein- or peptide-bound physiological L-amino acids into their mirror images (enantiomers) named D-amino acids is of great interest from the nutritional and physiological point of view (Friedman 1999). This process of the change of chirality ("handedness") of amino acids is commonly referred to as racemization or epimerisation if several chiral centers are involved, although, in the strict sense, racemic amino acids contain equal amounts of D- and L-amino acids (Brückner et al. 2001). The Maillard reaction can also explain the formation of D-amino acids in food. Brückner et al. (2001) have recently pointed out that D-amino acids are formed on heating aqueous solutions of L-amino acids (2.5 mM) together with an excess (278 mM) of saccharides (glucose, fructose, and saccharose) at 100°C for 24–96 h in aqueous solutions of pH 2.5 (AcOH) or pH 7.0 (NaOAc). Thus, the formation of D-amino acids in many foods of plant and animal origin are the results of nonenzymic browning since the presence of amino acids together with saccharides is common. As for the racemization mechanism, it is postulated that the reaction of amino acids with glucose or fructose starts with the reversible formation of Schiff bases. The degree of racemization depends in particular on steric and electronic properties of the amino acid side chains. It should be noted that the early stages of the Maillard reaction proceeds already under mild conditions (Brückner et al. 2001; Ledl and Schleicher 1990) and do not require alkaline or acidic condition. This new racemization mechanism based on the relatively stable Amadori compounds has been used to explain the generation of free D-amino acid in foods such as dried fruits, concentrated plant juices and fortified wines (Pätzold et al. 2003). It might also explain the occurrence of D-amino acids in biological systems which is not depending on microorganisms or racemazes (Brückner and Schieber 2000; Erbe and Brückner 2000a, b). Recently, heating experiments of synthetic Amadori compounds proved that they are sources of amino acid-enantiomers (Pätzold and Brückner 2005, 2006a, b). Amino acid racemization, however, is very much dependent on temperature, pH, and presence of catalysts (Bada, 1972). The situation in foodstuffs, however, is very complex, since it had been realized in recent years that microbial fermented foods are rich on D-amino acids owing to the presence of bacteria and their racemases (Friedman 1999). Furthermore, convincing evidence has been recently established that D-amino acids are formed in the course of the Maillard reaction, i.e. reaction of reducing sugars and amino compounds such as amino acids (Brückner et al. 2001; Pätzold and Brückner 2005, 2006a).

There have been two basic approaches for the chromatographic resolution of enantiomers of amino acids: a direct and an indirect method (Bhushan and Brückner 2004). The direct approach requires no chemical derivatization prior to separation process. Resolution is possible through reversible diastereomeric association between the chromatographic chiral environment and the solute enantiomers. The enantiomers may interact during the course of chromatographic process with a chiral stationary phase (CSP) or a chiral selector added to the mobile phase (CMPA) or a chiral selector mixed with/immobilized (especially in TLC) on the stationary phase (Bhushan and Martens 1997, 2003). Direct methods have certain critical disadvantages. Protein stationary phases are not durable over time and pH, and also have low sample capacity. Besides, the correct elution order is difficult to be predicted because of the complexity of interactions with the protein (Pirkle and Pochapsky 1989). On the other hand, the separation of diastereomeric pair via the indirect method is sometimes simpler to perform and often has better resolution than with a direct method because chromatographic conditions are much easily optimized. There is little doubt that of the various methods available for the indirect HPLC resolution of enantiomers of amino acids use of Marfey's reagent has been most successful. Marfey's reagent provides a very simple and effective analytical method for the chromatographic resolution of enantiomers of amino acids. It has been widely used for structural characterization of peptides, confirmation of racemization in peptide synthesis, and detection of small quantities of D-amino acids. Marfey's method uses amino acid derivatization with 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDAA) to form diastereomers of amino acids. The separation of diastereomers of the resulting FDAA amino acids is performed by HPLC using a reversed-phase C18 column (Marfey 1984). Szókán et al. (1988) separated FDAA D- and L-amino acids by HPLC using a reversed phase column. In addition, one of the most sensitive fluorogenic reagents used for amino acid and amine determination is *o*-phthaldialdehyde (OPA) (Morales et al. 1996; Vigo et al. 1992). For fluorometry, OPA is more sensitive and easier to use than fluorescamine and ninhydrin (Roth 1971). OPA is, 1 order of magnitude, more sensitive than fluorescamine and 10 times more sensitive than ninhydrin (Joseph and Marsden 1986).

In the Maillard reaction, melanoidins are known as the main end product of the reaction. These brown polymers have significant effect on the quality of food, since color is an important food attribute and a key factor in consumer acceptance. Browning is usually measured spectrophotometrically and expressed in absorbance units, which gives

qualitative information in terms of color formation but cannot be related in quantitative terms to molecular concentration. Studies on color formation have been summarized in different review articles (Feather 1985; Friedman 1996). Hashiba (1982) concluded that browning was directly proportional to the reducing power of the sugar and to the amounts of glycine consumed, by comparing different sugars with one single amino acid. On the other hand, Rizzi (1997) stated that many colored products appear to be (retro) aldolization/dehydration products of sugars which may or may not be attached to proteins or other sources of amino nitrogen. The mechanism of the formation of brown color is not fully understood and the structure of melanoidins is largely unknown, which makes it difficult to quantify these compounds. However, this quantification is necessary when trying to predict or optimize browning in processed foods from a known molecular composition.

According to the Lambert–Beer equation ($A = \varepsilon \times c \times l$), there is a direct linear relation between absorbance (A) and concentration (c), through the extinction coefficient (ε), if the factor l, the length of the cuvette, is constant. Previous studies, not only in a sugar/amino acid system (Leong 1999; Wedzicha and Kaputo 1992) but also in a sugar/protein system (Brands et al. 2002), have shown that it is possible to relate absorbance caused by nondialysable melanoidins to the number of sugar molecules incorporated in those melanoidins, by heating radioactive glucose with an amino acid and/or protein. Experiments in a glucose/glycine system at 55 and 90°C, pH 5.5 (Wedzicha and Kaputo 1992) suggested that the chromophores in melanoidins with Mr > 12,000 Da formed in the early stages of heating are similar to those at later stages. However, under these conditions the amount of material with Mr > 12,000 Da is believed to be very small (Hofmann 1998). In a similar study (55°C, pH 5.5) but with the melanoidins cut off at 3,500 Da, it was also reported that the extinction coefficient remained constant throughout the heating time in glucose/amino acid systems (Leong 1999). Values of ε at 470 nm were estimated to range from 0.34 l $mmol^{-1}$ cm^{-1} for alanine to 0.94 l $mmol^{-1}$ cm^{-1} for glycine. On the other hand, in sugar/casein systems (120°C, pH 6.8), where ε also remained constant throughout the heating period, independently of the sugar, glucose or fructose, a constant value of 0.3 l $mmol^{-1}$ cm^{-1} was obtained (recalculated to ε at 470 nm) (Brands et al. 2002).

It may be speculated that melanoidins are formed as a result of random polymerization of carbohydrate degradation products or adducts of those with amino compounds. The reaction conditions can determine the type of products that are formed during the Maillard reaction and as a consequence the followed pathways in the melanoidins formation (Tressl et al. 1995).

The aim of the present study was to investigate the enolization and racemization reactions of glucose and fructose on heating with amino acid enantiomers, can influence the formation of melanoidins as a result of the Maillard reaction. Melanoidins were, rather arbitrarily, defined by a high-molecular-weight (HMW) if it was above a lower limit of 12.4 kDa, which was the nominal cut-off value in the dialysis system used.

Materials and methods

Materials

Glycine, L-asparagine, D-asparagine, L-lysine, D-lysine, D-glucose, D-fructose, 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey's reagent) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydrogen carbonate ($NaHCO_3$) was purchased from Shimakyu Chemical Co. (Osaka, Japan). HPLC-grade water was purchased from J. T. Baker (Phillipsburg, USA). A reagents were of highest reagent grade and used without further purification.

Preparation of MRPs

An aqueous solution of 2 mol l^{-1} of sugar, 2 mol l^{-1} of amino acid and 0.2 mol l^{-1} of $NaHCO_3$ were dissolved in 100 ml of doubly distilled water. Ten model systems were prepared: glucose–glycine (Glc/Gly), glucose–L-asparagine (Glc/L-Asn), glucose–D-asparagine (Glc/D-Asn), glucose–L-lysine (Glc/L-Lys), glucose–D-lysine (Glc/D-Lys), fructose–glycine (Fru/Gly), fructose–L-asparagine (Fru/L-Asn), fructose–D-asparagine (Fru/D-Asn), fructose–L-lysine (Fru/L-Lys) and fructose–D-lysine (Fru/D-Lys). Model solutions were refluxed without pH control in an oil bath at 145°C for 2 h.

Dialysis

A portion of the mixture of MRPs (10 ml) was added to a wet cellulose dialysis tube (33 mm of flat width, 21 mm of diameter, 12.4 kDa of molecular weight cutoff (MWCO), Cat. No. D9652, Sigma). Batch dialysis was performed against 1,500 ml of doubly distilled water for 168 h at 4°C. Water was changed every 3 h for further of 12 h and then every 10–12 h for the rest of the dialysis time. After dialysis, samples were freeze-dried and stored in desiccators over silica gel (Fujimaki et al. 1979; Lee et al. 1987). Dialysis was only used for formation of melanoidins.

Determination of sugars in MRPs

The reducing sugars in MRPs before dialysis were determined using an HP 1100 liquid chromatograph (Hewlett Packard, Wilmington, USA). An agilent quaternary pump connected to a refractive index detector (Hewlett Packard, Model: G1362A) was used with a Zorbax carbohydrate column (4.6 ± 250 mm, 5 μm particle size, Agilent Technologies, Wilmington, USA). The mobile phase consisting of acetonitrile:water (75:25, v/v) was delivered at a flow rate of 2.0 ml min^{-1}. The column temperature was 30°C and 1 μl portions were injected into the HPLC system. The data analysis was performed using Chemstation software (Hewlett Packard).

Derivatization of amino acids with FDAA in MRPs

Amino acids were derivatized with FDAA reagent according to Marfey's methods (Marfey 1984). A measure 10 μl of MRPs before dialysis in 20 μl of H_2O and 8 μl of 1 mol l^{-1} $NaHCO_3$ was mixed with 400 μg of FDAA in 40 μl acetone and incubated at 40°C for 1 h with occasional shaking. The reaction was terminated by adding 4 μl of 2 mol l^{-1} HCl. Acetone, water and HCl were removed by evaporation under reduced pressure in a centrifugal evaporator. After evaporation, 20 μl of methanol was added to dissolve the resultant FDAA amino acid. FDAA amino-acid solution (2 μl) thus prepared (0.5%, w/v) was spotted on a reversed phase pre-coated TLC plate (RP-18, $F_{254}S$, 5 cm × 10, from Merck, Darmstadt, Germany), and developed with acetonitrile/triethylamine-phosphate buffer (50 mM, pH 5.5) at 25/75 (v/v) in a pre-equilibrated glass chamber at 25°C. The FDAA amino acid spots were yellow and visible. When the ascending solvent front neared the top margin, the plate was removed from the chamber and dried with a hair-drier. The TLC was completed in 20 min at 25°C. A trial for quantitative analysis was made by varying the amount of DL-amino acid derivatives to the plate. The yellow spots were scraped off the plate after the chromatography, and extracted with methanol/water (1/1, v/v). Since FDAA is sensitive to light, the FDAA amino acids were not exposed to the light during all procedures.

Determination of amino acids in MRPs

The amino acids in ten MRPs before dialysis were analyzed using HP 1100 liquid chromatograph (Hewlett Packard) with a variable wavelength detector VWD HP 1100 operating at 338 nm (Excitation = 340 nm). Separation was carried out with a Zorbax Eclipse AAA Rapid Resolution column (150×4.6 mm I.D., 5 μm particle size, Agilent Technologies, USA). A linear gradient profile of mobile phase, comprising 40 mM Na_2HPO_4, pH 7.8 (solvent A) and ACN:MeOH:water 45:45:10 (v/v) (solvent B), 0% B (0–1.9 min), 0–57% (1.9–18.1 min), 57–100% (18.1–18.8 min), 100% (18.8–22.3 min), 100–0% (22.3–23.2 min) and 0% (23.2–26 min) was applied at a flow rate of 2.0 ml min^{-1}. The column was equilibrated for 5 min under initial conditions prior to injection of the next samples. The column temperature was 40°C. In order to determine amino acids from MRPs, precolumn derivatization with *o*-phthalaldehyde (OPA) was used and 0.5 μl portions were injected into the HPLC system. The data analysis was performed using Chemstation software (Hewlett Packard). Relative quantities of amino acid enantiomers were calculated from peak areas of derivatives : %L = 100l/(D + L), %D = 100D/(D + L), where %L and %D represents relative amounts of L and D-amino acids with regard to the sum of (D + L) amino acids and D and L represent the peak areas of the respective enantiomer determined by HPLC.

Wavelength spectra of melanoidins

Absorption spectra of melanoidins were recorded by a UV–Vis spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan) with the wavelength ranging from 200 to 700 nm. Samples were dissolved in deionized water at a concentration of 0.1 mg ml^{-1}.

Spectrophotometric analysis

Browning was measured by a spectrophotometer (Shimadzu UV 160A, Shimadzu Co.) with the absorbance at 420, 450, 470 and 490 nm. Optical pathlength of the cuvette was 1 cm (Sara and Van Boekel 2003). The melanoidins extinction coefficient is calculated according to the Lambert–Beer equation ($A = \varepsilon \times c \times l$), there is a direct linear relation between absorbance (A) and concentration (c), through the extinction coefficient (ε), if the factor l, the length of the cuvette, is constant. The chosen wavelengths result from the fact that in literature reports concerning brown color, usually one of these four wavelengths are used, since there is no maximum in the visible spectrum (400–500 nm approximately) for brown color (Sara 1997).

Statistical analysis

Each experiment was carried out twice, and Duncan's multiple range tests in the SPSS statistical package (SPSS 12.0 for windows, SPSS Inc, Chicago, IL) was used to detect significant differences ($P < 0.05$).

Results

The loss and enolization of sugar in MRPs

After heating for 2 h, the concentration of the reactants was decreased. Moreover, the loss of glucose was more than the loss of fructose, regardless of the amino acid enantiomers (Fig. 1.). In both the glucose and fructose-amino acid systems, the sugars were almost completely destroyed in the MRPs resulting from L- and D-lysine. However, the MRPs from the Fru/Gly, Fru/L-Asn, and Fru/D-Asn model systems, respectively remained at 2.18, 2.20, and 2.00% of the original sugar. Through the sugar transformation reactions due to the Lobry de Bruyn–Alberda van Ekenstein transformation, glucose was converted into fructose and vice versa. In the present study, the degree of sugar enolization was higher in fructose-amino systems than in glucose-amino acid systems. However, no sucrose was detected. In the glucose-amino acid systems, the degree of sugar enolization was D-Asn > L-Asn > Gly > L-Lys > D-Lys. In the fructose-amino acid system, the degree of sugar enolization was D-Asn > L-Asn > Gly > D-Lys > L-Lys. Especially, in the Fru/D-Asn model system, the degree of sugar enolization was greater than in the other model systems.

The racemization of amino acids in MRPs

Figure 2 shows chromatograms for the FDAA amino acids. The spots of L-L-diastereosisomers move faster than those of the corresponding D-enantiomers, i.e. the FDAA L-D-diastereosisomers have greater affinity for the C18 silica gel than the corresponding FDAA L-L-diastereosisomers. A better separation was obtained by repeating the chromatography, i.e. by putting the developed and dried plate back into the glass chamber for further development. The enantiomers were well-separated after the third development. All of the diastereosisomers of the FDAA amino acids were separated except for a chiral glycine. The quantities of the L- and D- amino acids determined by HPLC in the MRPs, and the relative quantities of their isomers, are shown in Table 1 and Fig. 3. D-amino acid

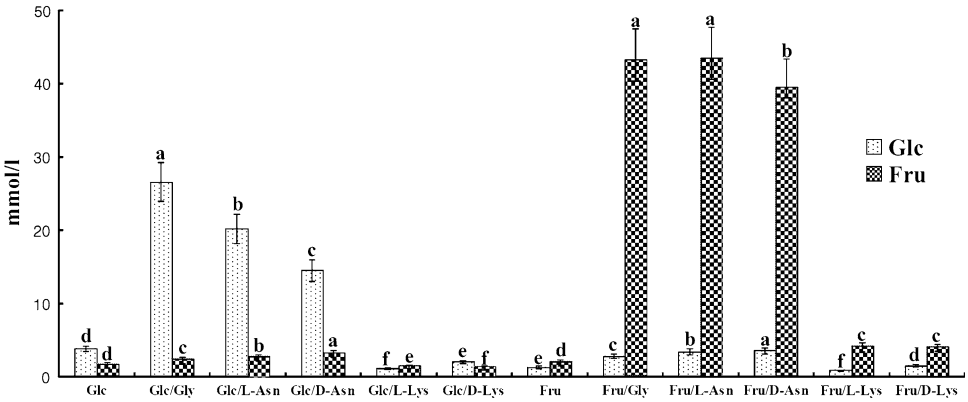

Fig. 1 Quantities of sugars by enolization reaction in Maillard reaction heated at 145°C and pH 6.8. The error bars represent the standard deviation for each observation

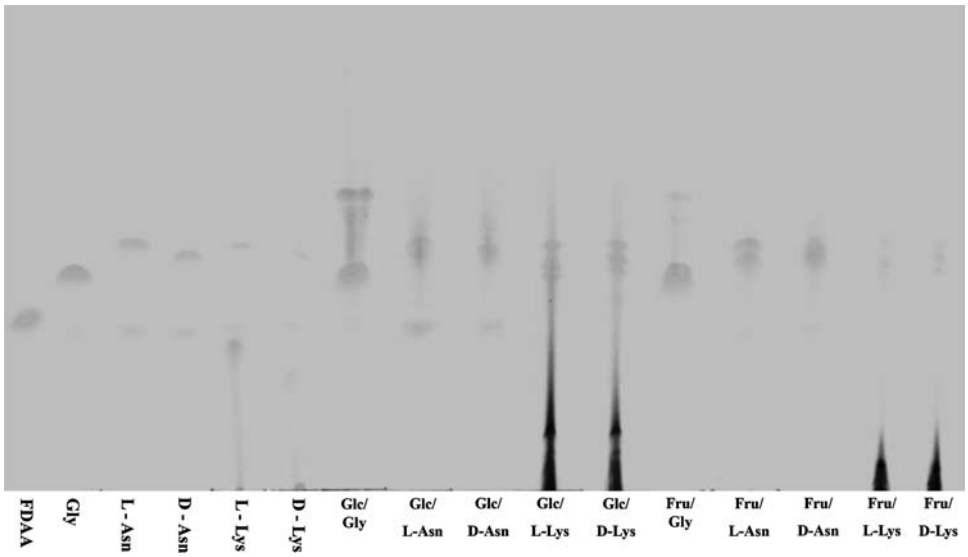

Fig. 2 TLC separation of the FDAA amino acids in the model systems

Table 1 Quantities of L- and D-amino acids (mmol/l) and relative amounts of isomers (%L and %D) resulting from the Maillard reaction (at 145°C and pH 6.8)

	Glc/Gly	Glc/L-Asn	Glc/D-Asn	Glc/L-Lys	Glc/D-Lys	Fru/Gly	Fru/L-Asn	Fru/D-Asn	Fru/L-Lys	Fru/D-Lys
L-AAs	1.19[a] ± 0.03	0.77 ± 0.02b	0.08 ± 0.01c	0.94 ± 0.02a	0.09 ± 0.01c	3.75 ± 0.03	2.61 ± 0.03a	0.29 ± 0.02c	0.61 ± 0.01b	0.08 ± 0.01d
D-AAs		0.13 ± 0.02d	0.80 ± 0.06b	0.27 ± 0.01c	1.05 ± 0.04a		0.09 ± 0.01d	0.59 ± 0.01b	0.23 ± 0.02c	0.77 ± 0.02a
%L	–	–	9.26 ± 0.37	–	7.96 ± 0.72	–	–	32.26 ± 2.20	–	9.49 ± 0.90
%D		14.73 ± 1.19	–	22.34 ± 0.34	–		3.08 ± 0.44	–	26.95 ± 1.28	–

Values are means of three replicates ± standard deviation. Means in a column followed by different letters are significantly different at the $P < 0.05$ level

[a] Remaining achiral glycine

was detected in L-amino acid systems. Similarly, L-amino acid could also be observed in D-amino acid systems. For example, 0.27 mmol l^{-1} of isomer were detected in the Glc/L-Lys system. And 0.29 mmol l^{-1} of isomer were detected in the Fru/D-Asn system. Formation of the isomer was the highest in the Fru/D-Asn system. The relative quantities of the isomers formed decreased Fru/D-Asn > Fru/L-Lys > Glc/L-Lys > Glc/L-Asn > Fru/D-Lys > Glc/D-Asn > Glc/D-Lys > Fru/L-Asn. Notably, high relative amounts of opposite enantiomers, approaching 26.95% D-Lys in the Fru/L-Lys system and 32.26% L-Asn in the Fru/D-Asn system, could be detected in fructose-amino acid systems. Therefore, the racemization of amino acids was higher in the fructose-amino acid systems. It is assumed that D-amino acids were generated from the fructose-amino acids (Amadori rearrangement products), which were formed in the course of the Maillard reaction.

Formation of melanoidins

Figure 4 shows the UV–VIS spectra of the melanoidins. The L- and D- isomers show different absorptions in the UV–VIS spectra, although these have similar shapes. Every peak had a maximum absorbance that appeared in the range between 260 and 320 nm, which is characteristic of melanoidins. However, the absorption intensities for the two isomers were different: low absorption intensities were formed for the L-isomer, whereas higher absorption intensities were formed for the D-isomer (see Fig. 4b). The absorbance of melanoidins with glucose was higher than that of fructose (see Fig. 4a, b). Glucose and fructose, however, showed little difference, when the UV spectra of L- and D-Lys were compared (see Fig. 4c).

The browning, which was defined as the absorption measured at 470 nm, was determined for each studied system after dialysis (see Fig. 5). The browning increased linearly as the concentration increased for all model systems. The Fru/Gly system showed the highest degree of browning among all the model systems (see Fig. 5a). For the sugar–asparagine systems, the fructose–asparagine system showed a lower degree of browning than the glucose–asparagine system (see Fig. 5b). On the other hand, for the sugar–lysine systems, the glucose–lysine system showed a lower degree of browning than the fructose–lysine system (see Fig. 5c). Especially, glucose-amino acid systems showed different characteristics according to the L- and D-isomers. Table 2 shows the values of ε for different wavelengths. In the present study, in the Glc/Gly system the average molar extinction coefficient was 0.72 l $mmol^{-1}$ cm^{-1} at 420 nm. In the Glc/L-Asn system ε was 0.54 l $mmol^{-1}$ cm^{-1}. In the Glc/D-Asn system ε was 0.57 l $mmol^{-1}$ cm^{-1}. In the Glc/L-Lys system ε was 0.57 l $mmol^{-1}$ cm^{-1}. In the Glc/D-Lys system ε was

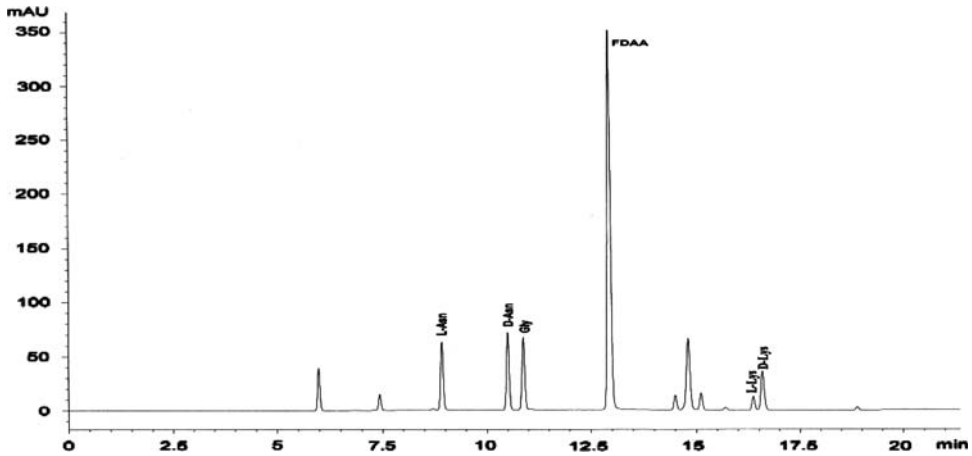

Fig. 3 HPLC chromatograms of derivatived amino acid enantiomers results from the Maillard reaction at 145°C and pH 6.8

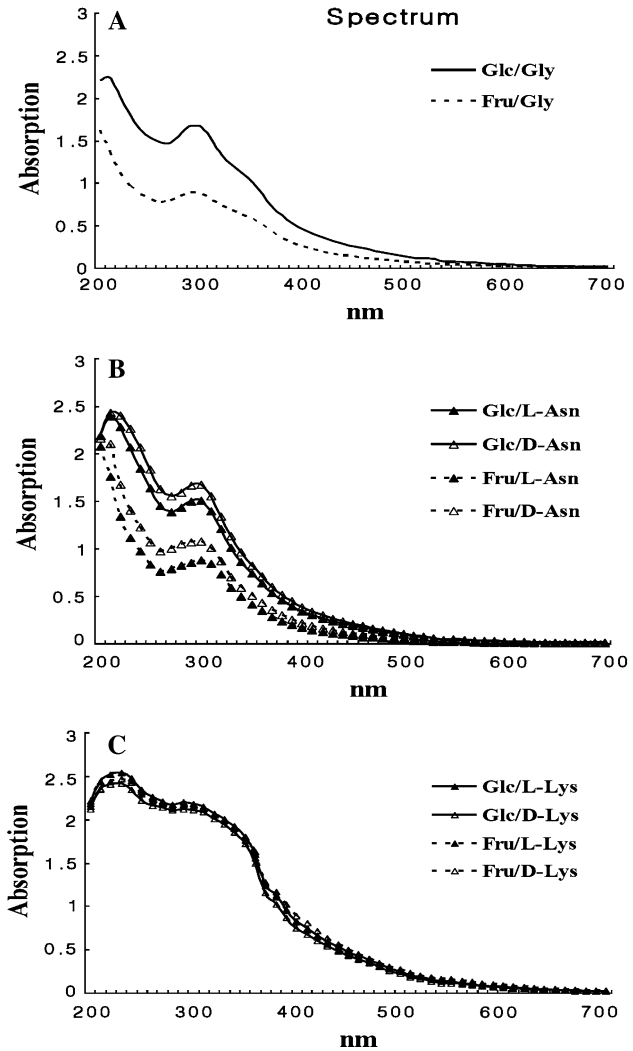

Fig. 4 The UV–VIS spectra of the melanoidins

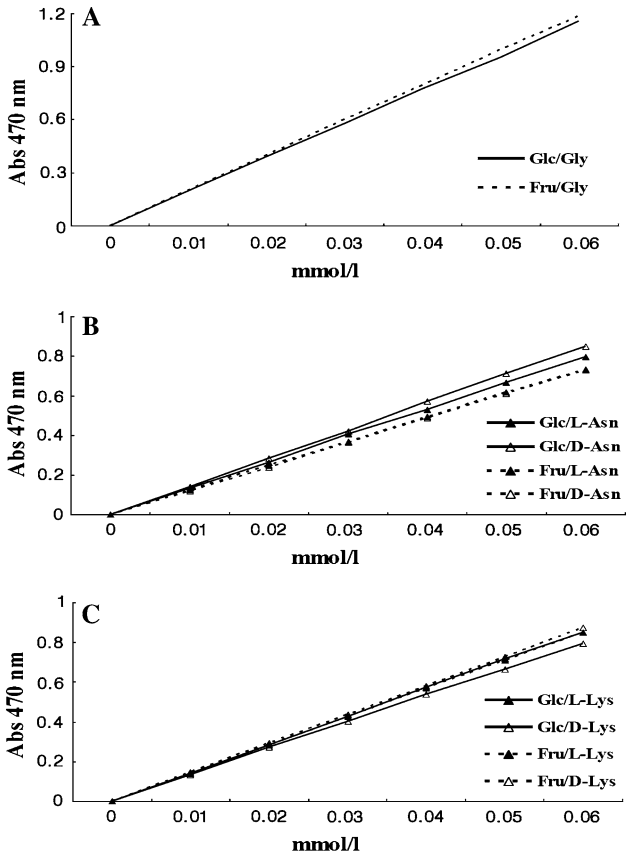

Fig. 5 Browning [measured as absorbance (Abs) at 470 nm] as function of the melanoidin concentration (measured as incorporated sugar)

0.54 l mmol^{-1} cm^{-1}. On the other hand, in the Fru/Gly system ε was 0.76 l mmol^{-1} cm^{-1}. In the Fru/L-Asn system ε was 0.51 l mmol^{-1} cm^{-1}. In the Fru/D-Asn system ε was 0.50 l mmol^{-1} cm^{-1}. In the Fru/L-Lys system ε was 0.58 l mmol^{-1} cm^{-1}. And in the Fru/D-Lys system ε was 0.60 l mmol^{-1} cm^{-1}. The differences in the extinction coefficients of the melanoidins formed in the ten model systems were significant, at the 5% level, except for the sugar–lysine systems. This indicates that in each of the systems different reaction intermediates might be formed,

Table 2 Extinction coefficient (ε) of the melanoidins measured for different wavelengths under different reaction conditions

Wavelength (nm)				
	420	450	470	490
Glc/Gly	0.72 ± 0.05	0.57 ± 0.04	0.48 ± 0.03	0.40 ± 0.03
Glc/L-Asn	0.54 ± 0.04	0.40 ± 0.02	0.33 ± 0.03	0.27 ± 0.01
Glc/D-Asn	0.57 ± 0.06	0.43 ± 0.05	0.35 ± 0.03	0.29 ± 0.02
Glc/L-Lys	0.57 ± 0.05	0.43 ± 0.03	0.36 ± 0.02	0.29 ± 0.02
Glc/D-Lys	0.54 ± 0.04	0.40 ± 0.04	0.33 ± 0.03	0.27 ± 0.02
Fru/Gly	0.76 ± 0.05	0.59 ± 0.04	0.50 ± 0.03	0.41 ± 0.03
Fru/L-Asn	0.51 ± 0.04	0.38 ± 0.02	0.31 ± 0.03	0.25 ± 0.01
Fru/D-Asn	0.50 ± 0.06	0.38 ± 0.05	0.30 ± 0.03	0.24 ± 0.02
Fru/L-Lys	0.58 ± 0.05	0.43 ± 0.03	0.35 ± 0.02	0.29 ± 0.02
Fru/D-Lys	0.60 ± 0.04	0.44 ± 0.04	0.36 ± 0.03	0.30 ± 0.02

Values are means of three replicates ± standard deviation

giving rise to melanoidins with different average extinction coefficients.

Discussion

Enolization of sugars

The enolization reaction known as the 'Lobry de Bruyn–Alberda van Ekenstein transformation' produces enediol anion species. Glucose and fructose can isomerise into one another this transformation (Speck 1958). In the present study, in both the glucose and fructose-amino acid systems, isomerisation products were formed in considerable amounts (Fig. 1). According to the Lobry de Bruyn–Alberda van Eckenstein rearrangement all sugars, glucose and fructose are in equilibrium with the same intermediate, the 1, 2-endiol. However, fructose is also in equilibrium with the 2, 3-endiol. The formation of the 1, 2-endiol from the respective enaminol is not so likely to happen. However, by the release of the amino acid, the enaminol can form its 2, 3-endiol, which through enolization can lead to the formation of sugars, in particular fructose (Anet 1964). This indicates that fructose can be formed from DFG (*N*-(1-deoxy-D-fructos-1-yl)-glycine) by its 2, 3-enolization step, whereas mannose and glucose can only be formed via the 1, 2-enaminol, through the Schiff base in the Maillard reaction. Moreover, it could also be argued that the sugars might arise by aldol-type condensations between smaller sugar fragments generated from the decomposition of DFG (Martins and Van Boekel 2003). Thus, sugar isomerisation can also be formed in the Maillard reaction by L- and D-amino acids.

Racemization of amino acids

A procedure is described for separating D- and L-amino acids using reversed-phase TLC. Amino acids were derivatized with 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide (FDAA) and spotted on a reversed phase precoated TLC plate (Marfey 1984; Nagata et al. 2001). Each FDAA amino acid could be separated from the others by two-dimensional TLC and then extracted from the plate with solvent and subsequently analyzed (Nagata et al. 1992). In the present study, the D(L)-amino acid was detected in the sugars-L(D)-amino acid systems. The formation of an isomer in the ten model systems was the highest in the Fru/D-Asn system (Table 1). Brückner et al. (2001) showed that the heating of L-amino acids together with reducing sugars leads to the formation of large amounts of D-amino acids. This reaction has been known as the Maillard reaction (Ledl and Schleicher 1990) or non-enzymic browning reaction (Friedman 1996). Pätzold and Brückner (2004) postulated that D-amino acids are generated from relatively stable intermediates of this reaction, called the Amadori and Heyns compounds. The release of amino acids from Amadori compounds is reversible until the amino acids are finally irreversibly transferred at the advanced stages of the Maillard reaction into heterocyclic or polymeric compounds. Consequently, the generation of D-amino acid from Amadori compounds (or Heyns compounds resulting from fructose and amino acids) is postulated to be a major route for their formation in the Maillard reaction. A tentative mechanism via the formation of a carbanion in the Amadori compound has been presented (Pätzold and Brückner 2004). This general route for the generation of D-amino acids has been extended to other foods rich in reducing sugars and amino acids. The Maillard reaction also explains the occurrence of D-amino acids in roasted coffee and cacao (Casal et al. 2005; Kutz et al. 2004).

Formation of melanoidins

In the present study, every peak has a maximum absorbance that appeared in the range between 260 and 320 nm, which is characteristic of melanoidins. This trend was also described by other researcher in relation to melanoidins-type colorants (Guimaraes et al. 1996; Rafik et al. 1997). The final stage of the browning reaction was monitored by the increase in absorbance at 470 nm (Ajandouz et al. 2001). The browning increased linearly as the concentration increased for all model systems. The Fru/Gly system showed the highest degree of browning among all the model systems. In Fig. 5, the results show that the extinction coefficient of the dialysable melanoidins remained constant, in the ten systems studied. The extinction

coefficient was calculated by taking the average of the repetitions carried out under the same conditions. In Table 2 the values of ε are presented for different wavelengths. Leong (1999) reported that at 470 nm the extinction coefficient for glucose/glycine melanoidins was considerably higher, namely 0.94 l $mmol^{-1}$ cm^{-1} and that it varied according to the type of amino acid. Using the kinetic modeling approach in the glucose/glycine system at pH 5.5 and 55°C, Leong and Wedzicha (2000) estimated ε to be 1.0 l $mmol^{-1}$ cm^{-1} from absorbance measurements without radiolabelling. On the other hand, Wedzicha and Kaputo (1992), by applying the same radiolabelling technique, showed that ε was not affected by the composition molar ratios of glucose and glycine, or by the heating temperature. Values of 0.41 l $mmol^{-1}$ cm^{-1}at 90°C and 0.37 l $mmol^{-1}$ cm^{-1} at 55°C were obtained, at 450 nm. Also, in a recent study Brands et al. (2002) reported that the melanoidins extinction coefficient remained constant in a sugar/casein system, during the observation period (90 min at 120°C, pH 6.8) independently of the sugar. They found values of 0.48 and 0.53 l $mmol^{-1}$ cm^{-1} for the glucose/casein and fructose/casein systems, respectively, at 420 nm.

Conclusion

This study was to investigate how the enolization and racemization reactions of glucose and fructose, on heating with amino acid enantiomers, can influence the formation of melanoidins as a result of the Maillard reaction. The results of this study prove that D(L) -amino acids are formed on heating aqueous solutions of L(D)-amino acids and saccharides. The melanoidins that are formed by D-amino acids are similar to those formed by L-amino acids. The presence of amino acids together with saccharides is common. Thus, the study also provides a feasible explanation for the generation of free D-amino acids in foods or biological systems that is not dependent on microorganisms or racemases (Brückner and Schieber 2000; Erbe and Brückner 2000a, b). Furthermore, it is important to evaluate the color development and melanoidins extinction coefficient in the course of the Maillard reaction. The color of the melanoidins formed in the model systems might be related to the enolization of sugars and racemization of amino acids. Therefore, it could be more useful to the understanding of the Maillard reaction and might be extended to the reaction of amino acids with reactive carbonyl compounds in general.

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