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Article

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Formation of Reactive Intermediates, Color, and Antioxidant Activity in the Maillard Reaction of Maltose in Comparison to D-Glucose

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ABSTRACT

In this study the Maillard reaction of maltose and D-glucose in presence of L-alanine was investigated in aqueous solution at 130 °C and pH 5. The reactivity of both carbohydrates was compared in regards of their degradation, browning and antioxidant activity. In order to identify relevant differences in the reaction pathways, the concentrations of selected intermediates such as 1,2-dicarbonyl compounds, furans, furanones, and pyranones were determined. It was found, that the degradation of maltose pre-dominantly yields 1,2-dicarbonyls that still carry a glucosyl moiety and thus subsequent reactions to HMF, furfural, and 2-acetylfuran are favored due to the elimination of D-glucose, which is an excellent leaving group in aqueous solution. Consequently, higher amounts of these heterocycles are formed from maltose. The only relevant C_6-1,2-dicarbonyls 3-deoxyglucosone and 3-deoxygalactosone in maltose incubations are produced in nearly equimolar amounts during the first 60 min of heating as by-products of the HMF formation.

KEYWORDS

Maillard reaction; maltose; antioxidant activity; reductones; color formation.

INTRODUCTION

The majority of food items is processed today, and thus heat-treated for preservation reasons and/or to develop certain organoleptic characteristics that meet the expectations of the consumer.[1] A main reason for the changes in qualities such as taste, flavor, texture, and – most obviously – color are non-enzymatic browning reactions, caused by the thermal degradation of reducing carbohydrates. In presence of amino components the reactions taking place are summarized under the term of Maillard reaction.[2–4] Besides colorants and aroma-active compounds reducing substances are formed in the course of the Maillard reaction, that are able to influence the oxidative stability of foods.[5] In our previous publications, we could identify 1,2-dicarbonyls,[6; 7] such as 1-deoxyglucosone and glucosone, as well as heterocyclic intermediates,[8] such as Furaneol, 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one (DHHM), maltol, and isomaltol as reductones. The heterocyclic reductone ethers show considerably higher antioxidant capacities than the 1,2-dicarbonyls and in analogy to ascorbic acid these compounds are prooxidants in presence of redox-active metal ions.

But before the impact of these intermediates on complex matrices, such as food or living cells, can be discussed, the general reaction pathways and possible key compounds have to be identified. Although the Maillard reaction of mono- and disaccharides might be well described in principle, most studies are based on analysis of either 1,2-dicarbonyls or their subsequent degradation products in form of furans, furanones, and pyranones. In our approach, we analyzed both groups of early intermediates starting from maltose (Mal) in presence of L-alanine (Ala) in a closed, aqueous system at 130 °C and a pH value of 5 in direct comparison to D-glucose (Glc). The focus on Mal was chosen, because of the disaccharide's high relevance in food systems. Browning, antioxidant activity, degradation of the respective carbohydrate component, formation of 1,2-dicarbonyl compounds, and heterocycles were investigated in systems of both carbohydrates. Additionally, incubations of

Mal in combination with L-proline (Pro) and L-lysine (Lys) as well as without addition of an amino component were analyzed. As in previous investigations,[6; 7] Ala was chosen as reference amino acid because of its simple, chemically inert side-chain. Lys with its two amino functions (in α- and ε-position) and the secondary amine Pro were used to investigate the influence of amines with higher or lower nucleophilicity than Ala. Because of its additional amino function in the side chain, Lys is able to form cross-links between different intermediates and reacts even if it is bound in peptides or proteins, for which reason Lys has a special role in the Maillard reaction.

To gain further knowledge about their stability and their contribution to color and antioxidant properties the heterocyclic intermediates HMF, maltol, isomaltol, DHHM, and Furaneol were incubated with Ala under identical conditions as carbohydrate and amino acid mixtures and analyzed in regards of reactant degradation, browning, and antioxidant activity.

MATERIALS AND METHODS

Chemicals. 2-acetylfuran, 5-hydroxymethylfurfural, ethylmaltol, Furaneol, piperidine, L-alanine, L-lysine, and L-proline were purchased from Acros organics (Geel, Belgium); aqueous hydrogen chloride solution (1 M) was purchased from Bernd-Kraft-GmbH (Duisburg, Germany); *ortho*-phenylenediamine was purchased from Fluka (Steinheim, Germany); aqueous sodium hydroxid solution (1 M), iron(III) nitrate nonahydrate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sodium chloride, and *para*-toluidine were purchased from Merck (Darmstadt, Germany); acetic acid, and D-glucose were purchased from Roth (Karlsruhe, Germany); 1,10-phenanthroline, 2-acetylpyrrole, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), disodium edetate dehydrate, Furaneol, furfural, maltol, potassium persulfate, sodium bathocuproinsulfonate, Trolox, and maltose monohydrate were purchased from Sigma-Aldrich (Steinheim, Germany); methanol was

purchased from VWR chemicals (Darmstadt, Germany); tetrabutylammonium hydrogensulfate was purchased from TCI (Eschborn, Germany).

Synthesis. The syntheses of DHHM and isomaltol were carried out as described in our previous publication.[8] Maltose phenylosazone was synthesized according to Oikawa et al.,[9] and used for the synthesis of the maltosone quinoxaline derivative (maltosone-Q) as described by Smuda et al.[10] 3-Deoxymaltose bis(benzoylhydrazone) was synthesized as described below and used for the synthesis of 3-deoxymaltosone-Q according to Smuda et al.[10] The isolation of 1-deoxymaltosone-Q from a reaction mixture of Mal, Lys, and *ortho*-phenylenediamine (OPD) was performed as described by Smuda et al.[10] Spectroscopic data of all obtained quinoxaline derivatives with maltose backbone are in line with ref. [10]. 3-Deoxygalactose bis(benzoylhydrazone) and 3-deoxygalactosone-Q were synthesized according to Hellwig et al.[11] The spectroscopic data of 3-deoxygalactosone-Q are in line with ref. 10.

3-Deoxymaltose bis(benzoylhydrazone). The synthesis was carried out as described by El Khadem et al.[12] and Madsen et al.[13] with some modifications. 50.0 g of maltose monohydrate (139 mmol), 13.75 g of *para*-toluidine (128 mmol), and 25.0 g of sodium chloride were suspended in 475 mL of water and 25 mL of acetic acid. The mixture was stirred for 12 h at 60 °C under argon atmosphere. Subsequently, 13.75 g of benzhydrazide (303 mmol) were added, the reaction mixture was stirred for additional 48 h at 60 °C under argon atmosphere, cooled down, and stored at –21 °C overnight. The residue was filtered off and washed with ice-cold water.

HPLC-DAD Analysis of 1,2-Dicarboyl Compounds. The following system and settings were used: pump, Shimadzu LC20AD; auto sampler, Shimadzu SIL-10AF; column oven, Shimadzu CTO-20A; detector, Shimadzu SPD-M20A; communication module, Shimadzu CBM-20A; software, Shimadzu LCsolution v1.22 SP1; column, Machery-Nagel EC250/4.6 Nucleodur C18 100-5; injection volume, 40 µL; flow, 0.5 mL/min; column temperature, 35 °C; eluent A, water; eluent B, methanol; gradient: 0 min, 17.5 % B; 15 min, 17.5 % B;

35 min, 28 % B; 55 min, 49 % B; 60 min, 95 % B; 65 min, 95 % B; 70 min, 17.5 % B; detector range, 190–600 nm; quantitation wavelength, 318 nm. The 1,2-dicarbonyl compounds in the samples were trapped with OPD and analyzed as quinoxaline derivatives. For derivatization 400 µL freshly prepared sample were incubated with 400 µL OPD solution (50 mM in methanol/water, 1:1, v/v) for 24 h at room temperature. The samples were stored at –20 °C prior to analysis.

Glucosone-Q (t_R = 28.3 min), 1-deoxyglucosone-Q (t_R = 36.0 min), 3-deoxyglucosone-Q (t_R = 40.6 min), 3-deoxypentosone-Q (t_R = 46.0 min), and 1.4-dideoxyglucosone (t_R = 53.8) are part of the mix standard used in our working group.[14; 6; 7] Maltosone-Q (t_R = 26.2 min) and 3-deoxymaltosone-Q (t_R = 34.7 min) were synthesized as standards for quantitation. 1-Deoxymaltosone-Q (t_R = 25.4 min) and 3-deoxygalactosone-Q (t_R = 41.2 min) were synthesized as authentic references, but quantified as maltosone-Q and 3-deoxyglucoson-Q, respectively.

HPLC-DAD Analysis of Heterocyclic Compounds. The following system and settings were used: pump, Shimadzu LC9A; auto sampler, Shimadzu SCL-6B; column oven, Shimadzu CTO-6B; detector, Shimadzu SPD-M10A; communication module, Shimadzu CBM-10A; software, Shimadzu Class-LC10 v1.64A; column, Machery-Nagel EC250/4.6 Nucleosil C18 120-5; injection volume, 10 µL; flow, 0.5 mL/min; column temperature, 40 °C; eluent A, phosphate buffer (5 mM, pH 6.0) with tetrabutylammonium hydrogensulfate (2.5 mM) and disodium edetate (1 mM); eluent B, methanol; gradient: 0 min, 5 % B; 5 min, 5 % B; 15 min, 20 % B; 20 min, 20 % B; 25 min, 95 % B; 35 min, 95 % B; 40 min, 5 % B; detector range, 190–500 nm; quantitation wavelength, 285 nm. The samples were diluted and filtered (syringe filter, nylon, 0.45 µm) prior to analysis.

The formation of HMF (t_R = 21.0 min) and furfural (t_R = 24.2 min) in carbohydrate incubations and the degradation of maltol (t_R = 26.3 min), Furaneol (t_R = 26.3 min), DHHM

(t_R = 16.2 min), HMF, and isomaltol (t_R = 30.0 min) in incubations of heterocyclic intermediates was analyzed by HPLC-DAD.

HPLC-PAD Analysis of Carbohydrates. The system and method described in a publication of Wegener et al.[15] were used.

GC-MS Analysis of Heterocyclic Compounds. The system and settings described in our previous publication[8] were used. 800 µL sample were mixed with 100 µL internal standard (aqueous solution of ethylmaltol with a concentration of 10 mM) and extracted three times with 800 µL ethyl acetate. The solvent was reduced to around 200 µL under nitrogen stream and the samples were subjected to GC-MS analysis.

The determination of DHHM (t_R = 24.3 min), maltol (t_R = 23.4 min), isomaltol (t_R = 18.5 min), and 2-acetylfuran (t_R = 16.1 min) in carbohydrate incubations was performed with GC-MS.

UV/Vis Measurements. For the browning measurements, a Bio-Tek Instruments UVIKON XL photospectrometer was used. The absorbance at 420 nm was measured in a quartz cuvette against water. The samples were diluted with water when necessary (absorbance > 1.4) and turbid samples were syringe filtered (nylon, 0.2 µm) before analysis.

Microplate Assays. For the TEAC and phenanthroline assay, a Tecan Infinite M200 microplate reader was used. Micro plates with 96 wells were purchased from TPP (Trasadingen, Switzerland).

TEAC Assay. The aqueous 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical cation solution was prepared by mixing 25 mL of ABTS solution (10 mM) and 25 mL of potassium persulfate solution (3.5 mM). Before use, the mixture was incubated overnight at room temperature in the dark and diluted 12:100 in PBS buffer (5 mM phosphate, pH 7.2–7.4) to prepare the radical working solution. The calibration was performed with seven trolox standards (0.010−0.100 mM; diluted in PBS buffer). 100 µL of sample were filled in each well and the initial absorbance was measured at 734 nm. After addition of

144 100 µL radical working solution, the samples were incubated for 120 min and the final
145 absorbance was measured at 734 nm.

146 **Phenanthroline Assay.** The phenanthroline assay was carried out as described in our
147 previous publication.[8]

148 **Incubation of Carbohydrates and Heterocyclic Intermediates.** An overview of the
149 prepared reaction mixtures is given in table 1. Prior to incubation the pH value of the mixtures
150 was adjusted to (5.0 ± 0.1) with aqueous solutions of hydrochloric acid or sodium hydroxide.
151 The pH adjusted mixtures were sealed in ampules (each 2.5 mL) and heated at (130 ± 1) °C in
152 a heating block for 0, 30, 60, 120, 180, and 300 min. Every sample was prepared in triplicate
153 (all results are given as means ± standard deviation).

154

155 ## RESULTS & DISCUSSION

156 **Degradation, Formation and Transformation of Carbohydrates.** The samples of the
157 carbohydrate and amino acid mixtures obtained after 300 min of heating were analyzed with
158 HPLC-PAD for degradation of the used carbohydrate component as indication of the
159 reactivity of the respective system, for the hydrolysis of maltose, and for transformation of
160 Glc to D-fructose (Fru) (table 2). Without amino component, 6 % of the initial amount of Mal
161 degraded, wherein around 4 % was cleaved to Glc. The conversion of Mal was accelerated
162 under amino catalysis leading to a degradation of 10 % (Pro), 26 % (Ala), and 32 % (Lys),
163 respectively. In all systems considerable amounts of Glc (between 3 to 9 % of the initial Mal
164 concentration) could be found, originating from hydrolysis of Mal or Mal specific Maillard
165 reaction intermediates. Of course, these concentrations do not represent the accumulated
166 amount of Glc formed in these systems, because Glc undergoes Maillard reaction as well.
167 Under identical conditions Glc is even more reactive than Mal, showing a slightly higher
168 conversion of 29 % in combination with Ala.

In samples obtained from Glc/Ala, around 5 % of the initial Glc amount is converted to Fru via isomerization.[16] Only trace amounts of Fru can be found in Mal systems, because of the much lower concentrations of Glc present in these samples.

Color Formation and Antioxidant Activity of Carbohydrate Incubations. Maillard reaction mixtures are typically associated with the formation of color, mostly measured as absorbance at 420 nm, and an increasing antioxidant activity, determined by different photometric assays, depending on the chosen reaction conditions.[5] In general, both properties tend to increase over the course of the heating time, resulting in a direct, linear correlation. Such a trend was observed with two different antioxidant assays for all samples prepared for this study. The color formation and the antioxidant activities did correspond to the degradation of the used carbohydrate component in each system. For example, Mal/Lys showed the strongest degradation of Mal out of all Mal systems and the highest browning/antioxidant activity, followed by Mal/Ala, Mal/Pro, and Mal. The same trends in color for the used combinations of carbohydrates and amino components could be observed under different conditions by Kwak et al.[17]

The results of the correlation between antioxidant activity (measured with the TEAC assay and expressed as mmol trolox equivalents (TE) per L) and color are shown in Figure 1. The same plot for the phenanthroline assay is to be found in the supporting information in Fig. S-1 (all values for color and the antioxidant activity are given in Table S-1).

The benefit of using both methods is, that it is possible to distinguish between the total antioxidant activity of the sample, based on the radical scavenging ability determined with the TEAC assay, and the metal reducing properties, measured with the phenantroline assay. Whereas the first method detects all reductones formed in the Maillard reaction, the latter excludes reductones with complexing abilities, such as maltol, isomaltol, or various pyridin-4-ones.[8] Both properties correlate linear with the browning of the samples, but the metal reducing substances in the samples caused antioxidant activities that are around 75 % lower

than the total reducing abilities. This suggests that complexing substances in Maillard reaction mixtures have a huge impact on the overall antioxidant abilities of the corresponding samples. Considering, only metal reducing reductones initiate redox cycling and consequently cause radical generation, these results indicate that antioxidants formed during the Maillard reaction are mostly beneficial to the oxidative stability of foods and only a fraction bears the risk of prooxidative effects. In addition, the amount of radical generation caused by metal reducing reductones is most likely compensated by the antioxidant activity of the sample anyways.

Formation of 1,2-Dicarbonyl Compounds in Carbohydrate Incubations. The general reaction pathways concerning the formation of 1,2-dicarbonyl compounds from Mal were thoroughly investigated by Smuda et al.[10] These experiments were carried out in phosphate buffer (pH 7.4) at moderate temperatures (50 °C) and with long incubation times (up to 7 days) with Lys. The focus of the present work was to compare the reactivity of Mal and Glc in presence of Ala in an unbuffered aqueous model system (initial pH value of 5.0) at elevated temperatures (130 °C) and short reaction times (up to 300 min) to investigate the reaction pathways without influence of a complex matrix and to induce strong color formation. Without buffer the pH value dropped depending on the reactants to pH 4.7 ± 0.0 (Glc/Ala), 4.4 ± 0.0 (Mal/Ala), 3.9 ± 0.1 (Mal), 4.5 ± 0.1 (Mal/Pro), and 3.7 ± 0.0 (Mal/Lys) after 300 min. There were significant differences in the formed amount of reaction intermediates to expect in comparison to the results of Smuda et al.,[10] because pH value,[18] temperature,[19] and phosphate[20; 21] are known to drastically affect the Maillard reaction.

The concentrations of the main intermediates obtained from Mal and Glc after 300 min heating time are summarized in Table 3. As already described by Smuda et al.[10] degradation of Mal under Maillard conditions predominantly yields 1,2-dicarbonyl compounds with an intact backbone of Mal, such as 3-deoxymaltosone (3-DM), 1-deoxymaltosone (1-DM), and maltosone. 3-deoxyglucosone (3-DG), 3-deoxygalactosone (3-DGal), and 3-deoxypentosone (3-DP) were the only 1,2-dicarbonyls with C_6- or C_5-body that could be found to a relevant

extend. In addition, small amounts of glucosone and 1,4-dideoxyglucosone were detected in individual samples. Whereas Smuda et al.[10] could only find trace amounts of 3-DM and identified 1-DM as the quantitative most important 1,2-dicarbonyl compound in Mal/Lys incubations under mild conditions, at 130 °C both compounds were equally relevant in systems of Mal containing Ala, Pro, or Lys. The differences in the formation of 1-DM and 3-DM are most likely attributed to the pH dependent degradation of their precursor in form of the Amadori rearrangement product (ARP).[22; 23] Under acidic conditions the ARP is mainly degraded to 3-deoxyosones via 1,2-enolization. On the other hand, the 2,3-enolization and the formation of 1-deoxyosones is favored at pH values higher than 7. In addition, phosphate is known to abstract protons of ARPs[24] and could possibly mediate the 2,3-enolization.

In analogy to the color formation Mal systems with Lys showed the highest 1-DM concentration (272 μM) followed by incubations with Ala (162 μM), Pro (99 μM), and without amino acid addition (2 μM). However, the 3-DM concentrations did not correspond to the browning at 420 nm. The reaction mixture with Lys had the highest concentration of 3-DM (459 μM), but the caramelization model produced higher amounts of 3-DM (310 μM) than the Maillard systems with Ala (139 μM) and Pro (59 μM). Maltosone could only be quantified in the Mal incubations without amino acid (30 μM) after 300 min, but was found in Mal/Ala and Mal/Pro samples between 30 and 180 min and in Lys samples between 30 and 60 min (data not shown).

When the formation of the respective osones, 1-deoxyosones, and 3-deoxyosones in the Mal/Ala system is compared to Glc/Ala, it is evident that Glc produces higher amounts of 3-deoxyosones absolutely and in relation to the osones and 1-deoxyosones. Furthermore, 3-DP was only found in trace amounts in the Glc/Ala incubations, but belonged to the main intermediates in Mal/Ala samples. The importance of 3-DP in the Maillard reaction of Mal was already recognized by Hollnagel et al.[25] and later by Smuda et al.[10] The authors explain the preferred formation from Mal in comparison to Glc with two different mechanisms, but

starting from the corresponding ARP both pathways involve an oxidation step, hydrolytic 1,3-dicarbonyl cleavage, and elimination of the Glc moiety (supporting information Fig. S-2). But the investigations of Smuda et al.[10] strongly suggest the formation via maltosone. However, in both cases the elimination of Glc is the driving force of the reaction, because of its good leaving group ability in aqueous solution. This explains the higher amounts of 3-DP formed in Mal/Ala samples in comparison to Glc/Ala. The substantial differences in the 3-DP concentrations obtained from incubation of Mal with different amino compounds are likely caused by the reactivity of the 3-DP degradation product furfural and are discussed later on.

In contrast to Hollnagel et al.[26] 1,4-dideoxyglucosone (1,4-DDG) could not be identified as dominating 1,2-dicarbonyl compound in the Maillard reaction of maltose in our study. But these investigations were carried out in an open, dry system with L-glycine and most importantly in presence of OPD, because the authors chose to use a pre-derivatization method for the quantification of 1,2-dicarbonyls. As amino compound OPD takes influence on the Maillard reaction and the trapping of the 1,2-dicarbonyls withdrawals them from the reaction equilibrium. Therefore, the results are hard to compare to the present study. But there are indications, that the formation of 1,4-DDG might be favored from Mal over Glc that will be discussed in the section regarding the formation of *O*-heterocycles.

Formation of HMF from 3-Deoxyosones in Carbohydrate Incubations. Generally, the 5-(hydroxymethyl)-2-furaldehyde (HMF) concentration was 2 to 10-fold higher than the summarized concentrations of its precursors. HMF is formed from 3-DM, 3-DG, and 3-DGal after acetalyzation and aromatization through elimination.[27; 28] The crucial intermediate is the unsaturated 3,4-dideoxyglucoson-3-ene (3,4-DGE), which exists as *E* and *Z* isomer, but only the *Z* form will yield HMF.[29] Starting from 3-DM instead of 3-DG or 3-DGal, Glc is eliminated instead of water to form 3,4-DGE (Figure 2). Due to the fact, that Glc is a much better leaving group in an aqueous system, higher amounts of HMF could be found in the incubations of Mal/Ala in comparison to Glc/Ala, even though the concentrations of the

respective precursors in form of the different 3-deoxyosones are higher in the Glc system (Table 3). The favored formation of HMF from oligosaccharides in consequence of the better leaving group ability of the respective carbohydrate moiety was described earlier by Kroh,[30] but under different conditions (caramelization in a dry system).

As side-reaction 3,4-DGE is hydrolyzed producing the C_6-3-deoxyosones 3-DG and 3-DGal.[29; 31] Both compounds might undergo cleavage reactions or form HMF after dehydration. Mal/Ala systems should theoretically yield nearly equal amounts of both C_6-bodies in consequence of the degradation of 3-DM, whereas Glc/Ala should produce an excess of 3-DG and significant smaller amounts of 3-DGal as the only by-product. The latter is clearly shown by the data presented in Table 3, but equal concentrations of 3-DG and 3-DGal were only to be found at the beginning of the reaction (30–60 min) in Mal/Ala incubations (Fig. 3). With increasing heating time the relative quantity of 3-DG rose, indicating that the degradation of Glc is getting more important due to the elimination of the monosaccharide from 3-DM in course of the formation of 3,4-DGE. On the other hand, starting from Glc (Glc/Ala incubation) always an excess of 3-DG was produced.

Investigations in dry systems did show, that Mal/Ala only forms 3-DG.[32] The reason for this observation is the lack of water in these systems and following the reaction scheme in Fig. 2 liberated Glc is the only source of 3-DG and there is no alternative way to form 3-DGal.

Formation of *O*-Heterocycles in Carbohydrate Incubations. In contrast to HMF, the heterocyclic intermediates furfural, 2-acetylfuran, isomaltol, DHHM, and maltol were formed in concentrations comparable to the 1,2-dicarbonyl compounds. The favored formation of 3-DP from Mal entails higher concentrations of the subsequently formed furfural and in consequence the system Mal/Ala contained the 3-fold amount of furfural (143 μM) than Glc/Ala (43 μM). The lower concentrations of furfural in incubations with Pro (27 μM) and

298 Lys (90 μM) might be attributed to the formation of unique reaction products of the respective
299 amino acids with furfural as reported by Hofmann[33] or Murata et al.[34]

300 DHHM – the main degradation product of 1-deoxyglucosone (1-DG)[35; 36] – was the
301 quantitative most important heterocycle past HMF. Because 1-DG is mainly produced from
302 Glc degradation, the highest DHHM concentration can be found in the Glc/Ala system
303 (487 μM). In Mal incubations without amino acid addition neither 1-DG nor DHHM could be
304 detected. Even though, in Mal/Pro and Mal/Ala the 1,2-dicarbonyl precursor is not to be
305 found, DHHM concentrations of 113 μM and 138 μM, respectively, indicate the intermediate
306 occurrence of 1-DG in these systems. The highest DHHM content of all Mal incubations was
307 detected in combination with Lys (237 μM) and at the same time Mal/Lys was the only Mal
308 system in which 1-DG could be quantified (34 μM).

309 As described in literature[35] and as observed in our experiments, maltol is exclusively
310 formed in incubations containing Mal. Although, the concentration of the respective precursor
311 1-DM increased in the order Mal (2 μM), Mal/Pro (99 μM), Mal/Ala (162 μM), and Mal/Lys
312 (272 μM), there were lower concentrations of the pyran-4-one found in Mal/Lys (112 μM)
313 than in Mal/Ala (171 μM). This suggests that in combination with Lys different degradation
314 pathways of 1-DM are important, for instance the formation of maltosine.[37]

315 Kim et al.[38] postulated the formation of 2-acetylfuran starting from DHHM with 1-DG as
316 intermediate stage. The mechanism can be transferred to Mal or Glc reaction mixtures starting
317 directly from the respective 1-deoxyosone (for 1-DM see Fig. 4). The first steps, including the
318 reduction of 1-DM and the elimination of Glc, resulting in 1,4-DDG are equal to the "peeling
319 off" mechanism described by Hollnagel et al.[26] and Pfeifer et al.[14] But the favored formation
320 of 1,4-DDG from Mal in comparison to Glc, as reported by the named authors, was not
321 observed, as discussed earlier. In fact, its concentration was similar in the incubations of both
322 carbohydrates. However, the amount of the subsequently formed 2-acetylfuran differed
323 strongly and incubations with Mal showed substantially higher amounts of the furan

compound, indicating that the "peeling off" mechanism occurs indeed. The formation of 2-acetylfuran from DHHM, as described by Kim et al.,[38] seems not to be of importance in carbohydrate reaction mixtures. Although the Glc/Ala system contained the highest amount of the pyran-4-one, it did not produce any 2-acetylfuran. On the contrary, the Mal systems formed 2-acetylfuran, despite the fact that they showed lower DHHM concentrations in comparison to Glc/Ala.

Degradation, Color Formation, and Antioxidant Activity in Incubations of *O*-Heterocycles. Since Furaneol,[39] DHHM,[38] and isomaltol[40] are known as highly reactive intermediates, their final concentrations in Maillard reaction mixtures of Mal are of limited value for the estimation of their contribution to relevant properties of the respective systems. To investigate the stability and their influence on color and antioxidant activity, selected heterocycles were incubated with Ala at 130 °C. In consideration of our previous investigations,[8] isomaltol, Furaneol, DHHM, and maltol were chosen to represent different classes of reductone ethers and in addition, HMF was used, because of the high quantities found in Mal reaction mixtures. The starting concentration of the *O*-heterocycles was chosen a decimal power lower (20 mM instead of 200 mM) to reflect the real concentrations and on account of their limited solubility in water in comparison to the carbohydrates.

The most stable *O*-heterocycles under these conditions were the aromatic compounds maltol and HMF, which concentrations were not measurably reduced. Furaneol, DHHM, and isomaltol on the other hand showed a strong degradation. The Furaneol content was reduced by around 78 % after 300 min, the DHHM content by 98 %, and isomaltol was not detected in the respective samples (supporting information Fig. S-3). But in contrast to carbohydrate incubations, there is no general connection between the degradation of the respective reactants and color formation. For instance, both DHHM and isomaltol degraded quickly, but DHHM samples did not produce colorants, whereas isomaltol samples were strongly colored (supporting information Fig. S-4). The browning of isomaltol is most likely caused by

condensation of isomaltol and one of its main degradation products – a C_4-furanone – to a red colored aromatic compound as described in literature.[40] Even though, the concentration of HMF stayed nearly constant in course of the heating time, incubations of HMF/Ala yielded yellow solutions and showed the second strongest browning of all investigated *O*-heterocycles. This might be attributed to the formation of small amounts of high colored polymers through vinylogous aldol addition.[41] Furaneol and DHHM are known to form mostly colorless degradation products, as result of oxidation, reduction, or cleavage reactions. Consequently, both compounds did not show a measurable browning after incubation with Ala.

The reaction mixtures resulting from *O*-heterocycles and Ala were analyzed in regards of their antioxidant activities with means of the TEAC and phenanthroline assay. In contrast to carbohydrate systems, which form various reductones in course of their degradation and consequently exhibited increasing antioxidant activities with increasing heating time and color formation, the samples derived from *O*-heterocycles showed a more complex picture (Fig. 5). Because of their reducing abilities maltol, Furaneol, DHHM and isomaltol showed antioxidant activities even in samples that are not thermally treated (0 min). The presence of Ala and the adjusted pH value did influence the antioxidant properties of the reductones considerably. Whereas isomaltol showed lower antioxidant capacities as the other reductones when tested isolated,[8] the antioxidant activity of the isomaltol/Ala system was much higher than the activities of maltol, Furaneol, and DHHM in combination with Ala.

Maltol/Ala incubations did not show any changes in their antioxidant activity in the course of 300 min heating time confirming the thermal stability of maltol. The decreasing antioxidant activities of Furaneol, DHHM, and isomaltol incubations indicate that the reductone function is destroyed in most degradation pathways of reductone ethers. But the antioxidant activities measured after 300 min do not correspond to the remaining concentrations of the used reductones. This is most obvious for isomaltol which degraded completely in course of the

heating, but still exhibited around 36 % of the initial antioxidant activity. Therefore, certain degradation products seem to maintain the reducing properties of isomaltol.

HMF is the only compound that showed a behavior similar to the carbohydrates. Lacking a reductone structure the untreated HMF/Ala samples did not show an antioxidant activity, but with increasing heating time the activity of the samples rose. However, these antioxidant activities are considerably lower than the activities of the samples containing reductone ethers (small diagram in Fig. 5).

In general, the phenanthroline assay showed the same results, except that incubations of metal chelating reductones, such as maltol and isomaltol, did not exhibit measurable antioxidant activities, due to the mechanism of this method, which is based on the reduction of metal ions (supporting information Fig. S-5).

Differences between the Maillard Reaction of Mal and Glc. Looking at the intermediates analyzed in this investigation, the product range of the Ala catalyzed degradation of Mal and Glc bears many similarities. Both systems produced predominantly 1,2-dicarbonyl compounds with an intact carbohydrate backbone. The only relevant C_6-1,2-dicarbonyls in Mal/Ala incubations were 3-DG and 3-DGal, which are most probably by-products of the HMF formation and are not primarily formed from Glc after hydrolysis of Mal. The relevance of Glc degradation in Mal systems increased during the time of incubation, because several reaction pathways liberate Glc, for instance the formation of HMF, furfural, or 2-acetylfuran, and because hydrolysis of Mal gains in importance. The Maillard reactions of both carbohydrates differ mainly in regard of relative and absolute quantities of the various intermediates. The summarized concentration of all analyzed 1,2-dicarbonyl compounds were slightly higher starting from Glc/Ala. 3-DG was found in a 6-fold higher concentration than 1-DG after 300 min (Table 3) in Glc/Ala, whereas in Mal/Ala the ratio of 3-DM to 1-DM was around 0.86:1. The ratios of 3-DG/3-DGal and the respective dependencies were discussed in detail in the section about HMF formation.

The only 1,2-dicarbonyl compounds that could be found in higher concentrations in Mal incubations than in Glc incubations besides the Mal specific 1,2-dicarbonyls was 3-DP. The driving force of this reaction is the elimination of Glc in case of the formation from the disaccharide.[42] Starting from the monosaccharide water has to be eliminated, which is the less favorable leaving group in an aqueous setup. The higher concentration of 3-DP was directly translated into a higher concentration of the subsequently formed furfural.

Besides furfural also HMF and 2-acetylfuran were formed in higher quantities from Mal, as consequence of the elimination of a glucosyl moiety from the respective intermediates. An exception was the degradation of 1-deoxyosones to DHHM or maltol. Maltol was exclusively formed in Mal systems, whereas DHHM was found in both systems, but in significantly higher amounts in Glc incubations. However, even the summarized concentration of both pyranones in Mal/Ala was lower than the DHHM concentration in Glc/Ala. Considering that only low concentrations of 1-DG were found in comparison to subsequently formed DHHM, the reaction seems to be fast. Maltol on the other hand was detected in almost identical concentrations as its precursor 1-DM, indicating a rather slow conversion. In addition, the formation of 2-acetylfuran is a competitive pathway to the maltol formation in the degradation of 1-DM, but seems to be irrelevant starting from 1-DG.

But, even though the concentrations of most *O*-heterocycles, especially of HMF as typical indicator substance for heat treatment, were considerably higher in Mal incubations than in Glc incubations, Glc showed a faster degradation and a stronger color formation under identical conditions. These contradictory observations might be explained by results obtained from the incubations of *O*-heterocycles with Ala. Of course, the degradation of the respective reactants and the color yields in these experiments have to be interpreted carefully, because the behavior of these compounds could drastically change in complex Maillard reaction mixtures. Nevertheless, these results indicate that furans and completely conjugated pyranons preferably formed by Mal, namely HMF, furfural, and maltol, are rather stable compounds,

whereas Glc predominantly produced the highly reactive DHHM. The stability of HMF can also be seen in the carbohydrate incubations, because it does accumulate in amounts roughly a decimal power higher than all other intermediates. Despite its low thermal stability in presence of Ala, DHHM is detected in relatively high concentrations in carbohydrate incubations, indicating that it has to be formed in even higher intermediate concentrations and might have a crucial role in the reaction cascade. And although, the trends in color formation of the incubations of DHHM/Ala and HMF/Ala suggest the contrary, it is likely that DHHM and/or its degradation products contribute to browning reactions. But this hypothesis has to be verified in further investigation through the utilization of more complex model systems.

The absence of other highly reactive heterocycles in the carbohydrate systems, such as furanone derivatives or isomaltol, might indicate that these compounds react too fast under the used conditions to be tracked as intermediates. This could be investigated in following studies by the analysis of their characteristic degradation products. Hence, in order to fully understand the role of heterocyclic intermediates in the Maillard reaction on browning additional work is needed. Especially the isolation and structure elucidation of the formed colorants by means of MS and NMR is of great interest.

The antioxidant properties of the carbohydrate incubations cannot be explained by the concentrations of the analyzed reductones, indicating that several of the subsequently formed intermediates and end products also contribute to reducing and complexing abilities of the respective samples. However, our data suggest a connection between the formation of colored and antioxidant substances. A possible explanation might be condensation reactions that integrate intact reductones in the skeleton of melanoidin polymers, for instance the aldol condensation of furanones and furan-2-aldehyd derivatives.[43] Furthermore, the incubations of HMF/Ala did show an increase of antioxidant properties indicating that the polymerization of non-reducing Maillard intermediates might lead to the formation of reducing functional groups.

ABBREVATIONS USED

1,4-DDG, 1,4-dideoxyglucosone; 1-DG, 1-deoxyglucosone; 1-DM, 1-deoxymaltosone; 3,4-DGE, 3,4-dideoxyglucoson-3-ene; 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3-DM, 3-deoxymaltosone; 3-DP, 3-deoxypentosone; Abs[420], absorbance at 420 nm; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); Ala, L-alanine; ARP, Amadori rearrangement product; DAD, diode array detector; DHHM, 3,5-dihydroxy-6-methyl-2,3-dihydro-4*H*-pyran-4-one; F, dilution factor; Fru, D-fructose; GC, gas chromatography; Glc, D-glucose; Lys, L-lysine; Mal, maltose; MS, mass spectrometry; nd, not detected; NMR, nuclear magnetic resonance (spectroscopy); Pro, L-proline; OPD, *ortho*-phenylendiamin; PAD, pulsed amperometric detector; PBS, phosphate-buffered saline; Q, quinoxaline; TE, trolox equivalent(s); TEAC, trolox equivalent antioxidant capacity; t_R, retention time.

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577

TABLES

Table 1: Overview of the prepared incubations of carbohydrates and heterocyclic intermediates.

carbohydrate/ heterocycle	concentration (mM)	amino acid	concentration (mM)
Glc	200	Ala	200
Mal	200	–	200
Mal	200	Ala	200
Mal	200	Pro	200
Mal	200	Lys	200
HMF	20	Ala	20
maltol	20	Ala	20
Furaneol	20	Ala	20
DHHM	20	Ala	20
isomaltol	20	Ala	20

Table 2: Carbohydrate concentrations in reaction mixtures of Glc/Ala, Mal, Mal/Pro, Mal/Ala, and Mal/Lys after 300 min of heating at 130 °C and pH 5.

	concentration (mM)				
	Glc/Ala	**Mal**	**Mal/Pro**	**Mal/Ala**	**Mal/Lys**
Mal	nd	188 ± 19	179 ± 12	148 ± 2	135 ± 2
Glc	141 ± 2	16 ± 1	10 ± 1	18 ± 0	35 ± 1
Fru	9 ± 1	nd	nd	1 ± 0	nd

Table 3: Concentrations of 1,2-dicarbonyl compounds and heterocyclic intermediates formed from the degradation of Glc/Ala, Mal, Mal/Pro, Mal/Ala, and Mal/Lys after 300 min of heating at 130 °C and pH 5.

	concentration (μM)				
	Glc/Ala	**Mal**	**Mal/Pro**	**Mal/Ala**	**Mal/Lys**
glucosone	23 ± 3	nd	nd	4 ± 0	nd
1-deoxyglucosone	93 ± 8	nd	nd	nd	34 ± 3
3-deoxyglucosone	548 ± 48	79 ± 9	23 ± 2	137 ± 12	172 ± 3
3-deoxygalactosone	76 ± 9	48 ± 7	12 ± 1	71 ± 3	55 ± 6
3-deoxypentosone	4 ± 6	3 ± 1	35 ± 4	92 ± 3	21 ± 1
1,4-dideoxyglucosone	14 ± 2	nd	nd	16 ± 0	13 ± 5
maltosone	nd	30 ± 9	nd	nd	nd
1-deoxymaltosone	nd	2 ± 2	99 ± 11	162 ± 3	272 ± 16
3-deoxymaltosone	nd	310 ± 35	59 ± 8	139 ± 5	459 ± 13
HMF	1210 ± 113	1694 ± 137	487 ± 57	2064 ± 91	6518 ± 107
furfural	45 ± 13	43 ± 9	27 ± 2	143 ± 10	90 ± 9
DHHM	487 ± 13	nd	113 ± 1	138 ± 3	237 ± 10
maltol	nd	nd	87 ± 7	171 ± 2	112 ± 6
isomaltol	nd	nd	nd	nd	34 ± 5
2-acetylfuran	nd	nd	43 ± 3	54 ± 2	85 ± 2

FIGURE CAPTIONS

Figure 1: Correlation between antioxidant activity (TEAC) and color in all carbohydrate incubations (n = 30, m = 3).

Figure 2: Formation of HMF from 3-DM with 3,4-DGE as relevant intermediate and the C_6-3-deoxyosones 3-DG and 3-DGal as by-products.

Figure 3: Ratio of 3-DG to 3-DGal in incubation of Glc/Ala and Mal/Ala in the course of 300 min heating time.

Figure 4: Formation of 2-acetylfuran from 1-DM through reduction, β-elimination of Glc, cyclization, and elimination of two moles of water adopted from Kim et al.[38] and Hollnagel et al.[26] R' represents a glucosyl moiety.

Figure 5: Changes in the antioxidant activity of maltol/Ala, Furaneol/Ala, DHHM/Ala, HMF/Ala, and isomaltol/Ala at pH 5 and 130 °C measured with the TEAC assay.

FIGURES

Figure 1

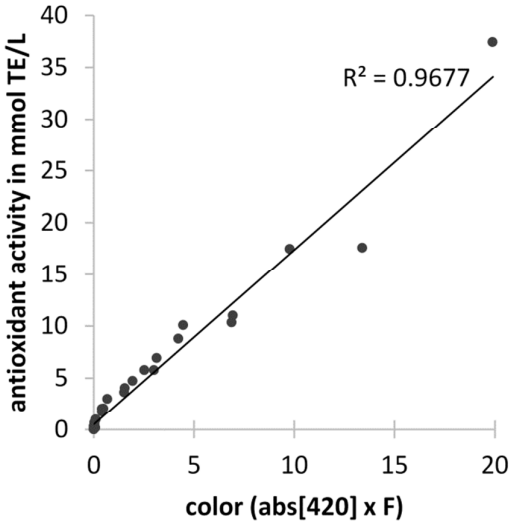


Figure 2

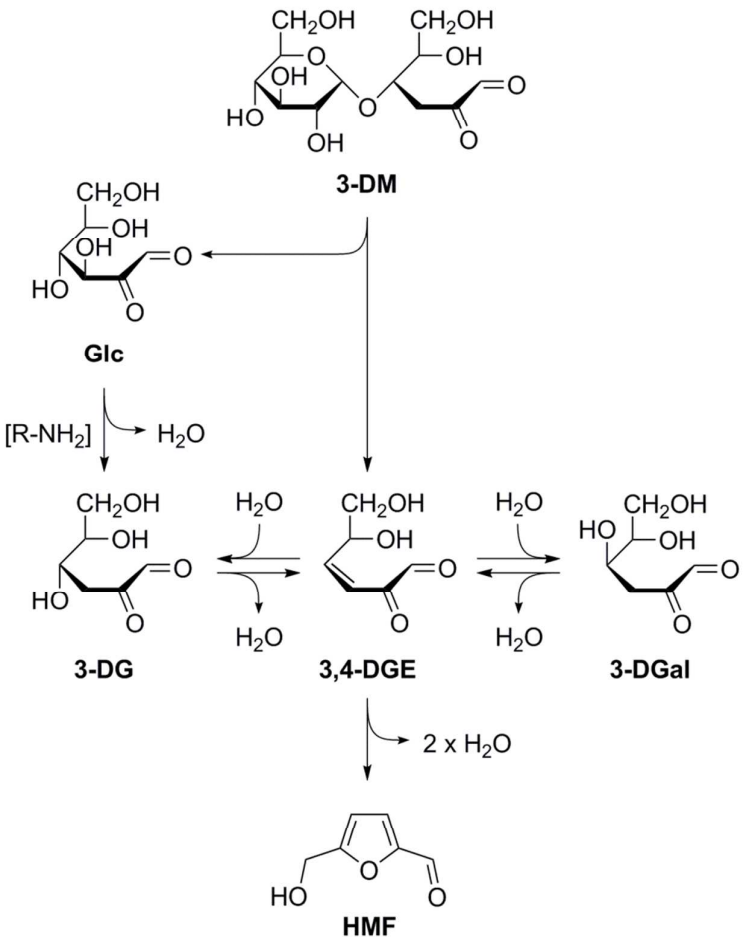


Figure 3

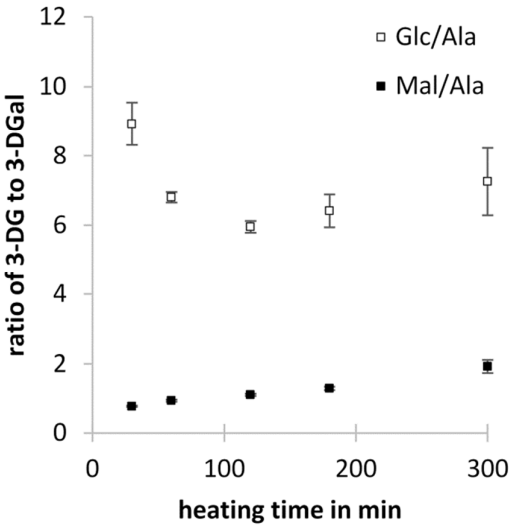

12
10
8
6
4
2
0
0
100
200
300
ratio of 3-DG to 3-DGal
heating time in min
Glc/Ala
Mal/Ala

Figure 4

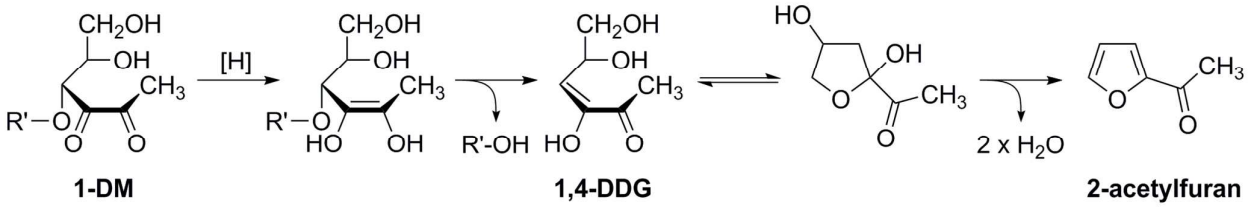
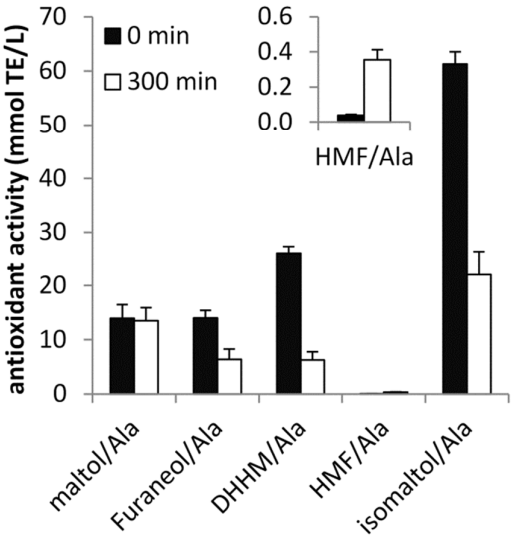


Figure 5


antioxidant activity (mmol TE/L)
70
60
50
40
30
20
10
0
0 min
300 min
0.6
0.4
0.2
0.0
HMF/Ala
maltol/Ala
Furaneol/Ala
DHHM/Ala
HMF/Ala
isomaltol/Ala

GRAPHIC FOR TABLE OF CONTENTS