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Article

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Lysine-Derived Protein-bound Heyns Compounds in Bakery Products

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Abstract

Fructose and dicarbonyl compounds resulting from fructose in heated foods have been linked to pathophysiological pathways of several metabolic disorders. Up to now, very little is known about the Maillard reaction of fructose in food. Heyns rearrangement compounds (HRCs), the first stable intermediates of the Maillard reaction between amino components and fructose, have not yet been quantitated as protein-bound products in food. Therefore, the HRCs glucosyllysine and mannosyllysine were synthesized and characterized by NMR. Protein-bound HRCs in cookies containing various sugars and in commercial bakery products were quantitated after enzymatic hydrolysis by RP-HPLC-ESI-MS/MS in the multiple reaction monitoring mode through application of the standard addition method. Protein-bound HRCs were quantitated for the first time in model cookies and in commercial bakery products containing honey, banana and invert sugar syrup. Concentrations of HRCs from 19 to 287 mg/kg were found, which were similar to or exceeded the content of other frequently analyzed Maillard reaction products, such as *N*-ε-carboxymethyllysine (10–76 mg/kg), *N*-ε-carboxyethyllysine (2.5–53 mg/kg) and methylglyoxal-derived hydroimidazolone 1 (10–218 mg/kg) in the analyzed cookies. These results show that substantial amounts of HRCs form during food processing. Analysis of protein-bound HRCs in cookies is therefore useful to evaluate the Maillard reaction of fructose.

Keywords

Maillard reaction; glycation; fructose; Heyns compound; bakery product; HPLC-MS/MS

Introduction

During storage and thermal processing of food, reducing sugars and amino components may react to ultimately form aroma and color compounds due to the Maillard reaction. Aldoses, such as glucose, react with amino groups to Amadori rearrangement compounds (ARCs) as the first stable intermediates. The formation of ARCs is well described, and quantitation of lysine derivatization is widely used to estimate the extent of early stage Maillard reactions in food and in physiological systems.[1] By contrast, the reaction of amino components and ketoses in food has hardly been investigated. Analogous to ARCs, the epimeric Heyns rearrangement compounds (HRCs, figure 1) *N*-ε-glucosyllysine and *N*-ε-mannosyllysine are the first stable intermediates of the Maillard reaction of fructose and lysine.[2] Both HRCs and ARCs degrade to 1,2-dicarbonyl-compounds, which react with amino components under formation of advanced glycation endproducts (AGEs). *N*-ε-carboxymethyllysine (CML) is a major AGE on lysine side chains.[3] It is formed via oxidative degradation of ARCs and proposedly of HRCs[4] and during the reaction of lysine and glyoxal.[5] The reaction of lysine and methylglyoxal leads to the formation of *N*-ε-carboxyethyllysine (CEL). A typical AGE on arginine side chains is methylglyoxal-derived hydroimidazolone 1 (MG-H1) which originates from the reaction of arginine and methylglyoxal.[6]

Heyns was the first to describe the synthesis of the products named after him.[2] Free HRCs were found in stored lyophilized apricots,[7] tobacco,[8] raw licorice,[9] tomato powder[10] and garlic[11] via paper chromatography,[7,8] thin layer chromatography,[9] cation exchange chromatography with refractive index detection[10] and HPLC-MS/MS.[11] Reduced protein-bound HRCs were found in human ocular lens protein after acid reduction and hydrolysis using RP-HPLC.[12] By adding *N*-α-hippuryllysine to a cookie dough, it was shown that peptide-bound HRCs form under baking conditions.[13] However, the direct analysis of protein-bound HRCs in food has yet to be described. Since the use of high fructose corn syrup

(HFCS) has replaced sucrose in the United States by almost 50% in the last 40 years, free fructose has become far more available in foods.[14] Thus, the potential of fructose-induced Maillard reaction in food has increased. To evaluate the extent of the early stage of the Maillard reaction in foods containing honey, HFCS and fruits, analysis of protein-bound HRCs is necessary.

The essential amino acid lysine is not available for nutrition, when bound in ARCs and AGEs, however, the human colonic microbiota is able to degrade fructosyllysine, CML and pyrraline.[15] Dietary AGEs are discussed to have adverse metabolic effects through their accumulation in human tissue and the postulated interaction with AGE-receptors, which leads to inflammation.[16] Thus, dietary HRCs and other fructose derived MRP could have pathophysiological effects due to the loss of lysine and because of the inflammatory effects of dietary AGEs.

It was therefore the purpose of this study to obtain information about the role of fructose in glycation, more precisely to quantitate protein-bound HRCs in food.

Materials and methods

Chemicals. Ethanol and HPLC gradient grade acetonitrile were purchased from VWR Prolabo (Leuven, Belgium). Nonafluoropentanoic acid (NFPA), *N*,*N*-dimethylformamide (DMF, <0.01% water), deuterium oxide, hydrochloric acid, ammonia solution (25%), prolidase (138 U/mg protein), pepsin (3555 U/mg protein), and leucine aminopeptidase (18 U/mg protein) from Sigma-Aldrich were employed (Steinheim, Germany) and acetic acid, fructose, glucose, and sucrose from Roth (Karlsruhe, Germany). *N*-*α*-*tert*-butyloxycarbonyllysine (Boc-lysine), petroleum ether (boiling point range: 40–60 °C), and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Fluka (St Gallen, Switzerland) and triphenyl tetrazolium chloride from Kallies (Sebnitz, Germany). Pronase E (4000 PU/mg

protein) from Merck (Darmstadt, Germany) was used and pyridine from Acros (Geel, Belgium). Formic acid and sodium hydroxide were purchased from Grüssing (Filsum, Germany) and ninhydrin from Serva (Heidelberg, Germany). The water used for the preparation of solutions and buffers was prepared by a Purelab Plus purification system (USF Elga, Ransbach-Baumbach, Germany).Water for HPLC-MS/MS was obtained by a Bi 18 E double distillation system (QCS, Maintal, Germany). Fructosyllysine,[17] CML[18], CEL[18] and MG-H1[18] (Figure 1) were synthesized and characterized in our laboratory according to the literature stated. The substances met the spectroscopic and chromatographic characteristics published in the respective protocols.

Synthesis and Isolation of *N*-ε-(2-Deoxy-D-glucosyl)-L-lysine (Glucosyllysine) and *N*-ε-(2-Deoxy-D-mannosyl)-L-lysine (Mannosyllysine). The compounds were synthesized[2,13] and isolated[17,19] according to literature methods with minor modifications. Boc-lysine (0.62 g, 2.5 mmol) and anhydrous fructose (4.5 g, 14 mmol) were refluxed in 37 mL of dry dimethylformamide for one hour. After evaporation of the solvent, the residue was dissolved in 100 mL of water. Black precipitate was removed by filtration. The pH value was adjusted to 2.0 with 6 M hydrochloric acid. For removal of the Boc protecting group and uncharged by-products, the solution was applied to a 200x25 mm glass column filled with 80 mL of cation exchange resin Lewatit S 100 (20–50 mesh) in the H^+ form, previously equilibrated with 250 mL of 6 M hydrochloric acid and 250 mL of water. The sugar was eluted with 250 mL of water and after one night of incubation, material bound to the exchanger was eluted with 250 mL of 2 M ammonia. The eluate was evaporated to dryness and dissolved in 20 mL of 0.1 M pyridinium formate buffer, pH 3.0. The pH value was adjusted to 3.0 with formic acid. The solution was applied to a 750x15 mm semipreparative ion-exchange column filled with DOWEX 50 W-X8 (100-200 mesh) in the pyridinium form, previously

equilibrated with 360 mL of 6 M hydrochloric acid, 360 mL of water, 360 mL of 2 M aqueous pyridine, 360 mL of water and 360 mL of 0.1 M pyridinium formate buffer (pH 3.00). Separation was achieved with elution by gravity of 360 mL of 0.25 M pyridinium acetate buffer (pH 4.05) and 360 mL of 0.3 M pyridinium acetate buffer (pH 4.05) at a flow rate of 0.4 mL/min. A fraction collector (RediFrac, Pharmacia Biotech, Sweden) was used and fractions of 10 mL were collected. Of each fraction, 1 μL was spotted on two TLC plates. Spraying with 1% triphenyl tetrazolium chloride in 1 M sodium hydroxide and 0.1% ninhydrin in ethanol, respectively, indicated that the HRCs eluted with 70–220 mL of the last buffer. An aliquot of those fractions (5 μL) was diluted in 495 μL of the loading buffer and subjected to amino acid analysis. Fractions eluting with 70–190 mL of 0.3 M pyridinium acetate buffer (pH 4.05) contained glucosyllysine, and mannosyllysine eluted with 130–220 mL of this buffer. The fractions that contained only one HRC were pooled, dried in vacuo, repeatedly dissolved in water and evaporated, lyophilized and characterized.

N-ε-(2-deoxy-D-glucosyl)-L-lysine (glucosyllysine): HPLC-MS/MS: see Figure 2 A; elemental analysis: $C_{12}H_{24}N_2O_7$ (MW = 308.32), calculated: C 46.75%, H 7.85%, N 9.09%; found: C 39.59%, H 6.84%, N 7.75%; content = 76.3%, based on nitrogen and chromatographic purity from LC-MS/MS (ratio of glucosyllysine to mannosyllysine and fructosyllysine). Yield = 281 mg (molar yield = 27.8%). NMR data: see Table 1 and supporting information Figure S1–S5.

N-ε-(2-deoxy-D-mannosyl)-L-lysine (mannosyllysine): HPLC-MS/MS: see Figure 2 B; elemental analysis: $C_{12}H_{24}N_2O_7$ (MW = 308.32), calculated: C 46.75%, H 7.85%, N 9.09%; found: C 41.19%, H 7.44%, N 6.91%; content = 64.5%, based on nitrogen and chromatographic purity from LC-MS/MS (ratio of mannosyllysine to glucosyllysine and fructosyllysine). Yield = 23 mg (molar yield = 2.1%). NMR data: see Table 2 and supporting information Figure S6–S10.

Characterization of Glucosyllysine and Mannosyllysine. ^{1}H and ^{13}C NMR spectra were recorded on an Avance III HDX 500 MHz Ascend instrument from Bruker (Rheinstetten, Germany) at 500.13 MHz and 125.75 MHz, respectively. Glucosyllysine (7.1 mg) and mannosyllysine (5.4 mg) were each dissolved in 770 μL of deuterium oxide and stored at room temperature overnight to allow mutarotation. All chemical shifts are given in parts per million (ppm), those of protons relative to the internal HOD signal (4.70 ppm), those of carbon atoms relative to external standard tetramethylsilane. Assignments of ^{1}H and ^{13}C signals are based on ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC); ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC) and ^{13}C distortionless enhancement by polarization transfer (DEPT) experiments. Elemental analysis was performed on a vario MICRO cube CHNS elemental analyzer (Elementar, Hanau, Germany).

Bakery Products. All bakery products, wheat flour, margarine, honey, bananas and baking powder were purchased in local retail stores. Cookie doughs were prepared from 10.00 g of wheat flour, 5.90 g of margarine, 0.30 g of baking powder, 7.5 mL of water and 5.00 g of either fructose, glucose, or sucrose. For a honey cookie dough, 6.60 g of honey and 5.5 mL of water and for a banana cookie dough, 10.00 g of banana and 3.20 g of sucrose were added to wheat flour, margarine and baking powder. Cookies were formed manually to a thickness of about 5 mm and a diameter of about 5 cm. The cookies were baked in a laboratory drying oven for 10 min at 175 °C. After cooling, the cookies were homogenized by mortar and pestle. A sample of 160 mg of a baked or purchased cookie was weighed into a 2 mL plastic tube and defatted using 1 mL of petroleum ether as described previously.[13] The sample was mixed vigorously by vortex, centrifuged (10,600 x *g*, 10 min) and the petroleum ether was discarded. After repeating this procedure, 500 μL of water was added to the precipitate. The suspension was mixed by vortex and dialyzed against water at 4 °C for 2 days (cellulose

149 tubings, MWCO, 14 kDa, Sigma-Aldrich). The water was changed twice per day. The
150 retentates were lyophilized.
151
152 **Enzymatic Hydrolysis.** Samples containing 2–3 mg of protein (8–40 mg of lyophilized
153 cookie, depending on protein content) were used according to the literature.[20] Hydrochloric
154 acid (1.05 mL, 0.02 M) containing 356 U of pepsin was added. After 24 h incubation in a
155 drying chamber at 37 °C, 300 μL of 2 M TRIS buffer, pH 8.2 containing 400 PU of pronase E
156 was added. Following 24 h of further incubation, 22 μL of 2 M TRIS buffer containing 0.3 U
157 of leucine-aminopeptidase and 2 U of prolidase was added. After 48 h, the samples were
158 frozen. Centrifugation (10,600 x *g*, 10 min) and membrane filtration (0.45 μm) of thawed
159 samples were performed prior to HPLC analysis. An aliquot of the hydrolysate (150 μL) was
160 suspended in 450 μL of the loading buffer for amino acid analysis (0.12 N lithium citrate, pH
161 2.20). After membrane filtration (0.45 μm), samples were subjected to amino acid analysis.
162
163 **Amino Acid Analysis.** Amino acids separation was performed on a PEEK column filled with
164 the cation exchange resin LCA K07/Li (150 mm × 4.6 mm, 7 μm) using the amino acid
165 analyzer S 433 (Sykam, Fürstenfeldbruck, Germany) with a gradient program utilized
166 previously.[21] Loading and running buffers for this lithium system were purchased from
167 Sykam. After post-column derivatization with ninhydrin, the absorbance of the effluent was
168 recorded with a two-channel photometer simultaneously working at 440 nm and 570 nm,
169 respectively. A commercial amino acid mixture was obtained (Sigma-Aldrich, Steinheim,
170 Germany) and used for external calibration.
171
172 **High-Pressure Liquid Chromatography (HPLC) with Tandem Mass Spectrometric**
173 **Detection.** Protein-bound MRPs were analyzed as described before[15,21,22] on a high pressure
174 gradient system 1200 Series (Agilent Technologies, Böblingen, Germany), consisting of a

175 binary pump, an online degasser, an autosampler, a column oven, and a diode array detector.
176 Chromatographic separation was performed on a Zorbax SB C-18 column (50 mm x 2.1 mm,
177 3.5 μm, Agilent), with 10 mM NFPA in double-distilled water (solvent A) and 10 mM NFPA
178 in acetonitrile (solvent B) as the mobile phases. The injection volume was 2 μL. Analysis was
179 conducted at 35 °C using a flow rate of 0.25 mL/min. A gradient of 5% B (0 min) to 32% B
180 (15 min), then to 85% B (16–19 min), and finally to 5% B (20 min) was used. An
181 equilibration time of 8 min before the next run was implemented. The HPLC was coupled to
182 the mass spectrometer 6410 Triple Quad (Agilent) working in the positive mode with a source
183 temperature of 350 °C and a capillary voltage of 4000 V. Nitrogen was used as the nebulizing
184 gas (nitrogen generator 5183-2003, Agilent) with a flow rate of 11 L/min and a pressure of
185 35 psi. The multiple-reaction monitoring mode (MRM) was used for mass spectrometric
186 detection with the conditions listed in table 3. These conditions were optimized by scan and
187 product ion scan measurements of the respective standards (Figure 2). Data acquisition and
188 evaluation was conducted with the software Mass Hunter B.02.00 (Agilent).
189 The MRPs were quantitated by the standard addition method with three runs. For each run,
190 100 μL of enzymatic hydrolysate was mixed either with 20 μL of water, or with 10 μL of
191 water and 10 μL of a standard solution, or with 20 μL of standard solution. The standard
192 solution contained *N*-ε-glucosyllysine (19.0 μg/mL), *N*-ε-fructosyllysine (70.1 μg/mL), CML
193 (5.0 μg/mL), CEL (5.0 μg/mL), and MG-H1 (15.0 μg/mL) dissolved in water. Limits of
194 detection (LOD) and quantitation (LOQ) for the compounds are shown in table 4. For a
195 matrix calibration, the standard solution was added to an enzymatic hydrolysate of water.
196 Concentration ranges of the calibration are shown in table 4.
197
198 **Statistical Treatment**. All samples were analyzed at least in duplicate. Baking experiments
199 were done one time. Limits of detection (LOD) and quantitation (LOQ) were calculated as the
200 concentrations of MRPs necessary to show peaks at signal-to-noise ratios of 3 and 10,

respectively. Coefficients of determination were calculated for standard addition regression lines. The intercept accuracy was calculated as the percentage difference between the area of the sample peak without addition of standards and the intercept of the regression line. The linearity of the matrix calibration was determined with the Mandel's fitting test for $\alpha = 0.05$.

Results and discussion

Synthesis of Heyns Rearrangement Compounds (HRCs). The aim of this study was to quantitate protein-bound HRCs in bakery products containing fructose in order to evaluate the early stage of the Maillard reaction with fructose. Therefore, the epimeric HRCs glucosyllysine and mannosyllysine (Figure 1) needed to be synthesized and characterized as reference material for chromatographic analysis. Protocols for synthesis of HRCs were adapted[2,13] with some modifications, whereas isolation and separation were performed as described for fructosyllysine[17] and formyline.[19] The peaks of glucosyllysine and mannosyllysine overlapped during semi-preparative ion-exchange chromatography as determined by amino acid analysis. Only fractions containing one product were pooled and processed further. One- and two-dimensional NMR spectroscopy (Tables 1 and 2, supporting information Figures S1–S10) confirmed the structural identity of the compounds. Residual acetic acid from the chromatography eluent was identified as an impurity. Chemical shifts of glucosyllysine were in accordance with those published in the literature.[13,23,24] In pyranose sugars, coupling constants $J_{1,2}$ of two vicinal axial protons are higher than 7 Hz, while the coupling constants of vicinal protons of which at least one is in equatorial position are smaller than 5 Hz.[25] Mannosyllysine had similar chemical shifts and coupling constants as glucosyllysine. However, the pyranosyl form of glucosyllysine has an axial proton at C_2 and therefore the coupling constant of the protons at C_2 and C_3 is 10.5 Hz and that of the protons at C_2 and C_1 of the β-pyranosyl moiety is 8.5 Hz. In mannosyllysine those coupling constants

were 4.6 and 1.7 Hz, indicating a proton in equatorial position at C_2. Based on this findings and by comparison to similar structures,[26] mannosyllysine and glucosyllysine were identified. Glucosyllysine was found in α- and β-pyranose isomers (ca. 75% and 25%). In addition to α- and β-pyranose isomers (ca. 46% and 25%), mannosyllysine also formed considerable amounts of α- and β-furanose isomers (ca. 18% and 9%). The H-1 ^{1}H NMR signal of the α-furanose form of mannosyllysine overlapped with the H-1 signal of emerging glucosyllysine which may have been formed during the period of time between dissolution of the compound and the NMR measurement.[27] Therefore, the percentage of α-furanose might be overestimated.

The reaction mixture before ion exchange chromatography contained the HRCs glucosyllysine and mannosyllysine, but also the ARC fructosyllysine. After isolation, 76% of glucosyllysine, 3% of mannosyllysine and 6% of fructosyllysine were present in the glucosyllysine isolate as deduced from amino acid analysis, HPLC-MS/MS and elemental analysis. The mannosyllysine isolate contained 65% of mannosyllysine, 6% of glucosyllysine and 6% of fructosyllysine. Though concentrations of mannosyllysine were higher than those of glucosyllysine in the synthesis mixture after heating (molar yield glucosyllysine: ca. 30%, mannosyllysine: ca. 50%), concentrations of mannosyllysine in the eluted fractions and the yield of isolated mannosyllysine were more than 10 times smaller than those of glucosyllysine (molar yield glucosyllysine: 27.8%, mannosyllysine: 2.1%). Sugars with lower shares of the pyranose forms, such as mannosyllysine, show higher concentrations of the open-chain form and are therefore more reactive via the Maillard reaction and the Lobry de Bruyn-Alberda van Ekenstein transformation.[28] Mannosyllysine, therefore, is less stable than glucosyllysine during the isolation process. Yields and purity of both substances were high enough for chromatographic analysis.

Determination of HRCs in Bakery Products. A method to quantitate the HRCs glucosyllysine and mannosyllysine, as well as the ARC fructosyllysine and the AGEs CML, CEL, and MG-H1, in bakery products was established using enzymatic hydrolysis and LC-MS/MS. Enzymatic hydrolysis has already been used for the quantitation of different MRPs such as fructosyllysine, pyrraline and CML.[21,29] The rate of release after enzymatic digestion was at least 80% of the rate after acid hydrolysis for aliphatic amino acids in roasted peanuts and dried pasta.[29,30] In eye lens protein, the rate of release after enzymatic digestion compared to acid hydrolysis was 52–91% for CML.[31] The quantitative data presented in this study might therefore be slightly underestimated. LC-MS/MS analysis using the standard addition method of enzymatically hydrolyzed standard solutions of glucosyllysine and mannosyllysine showed that mannosyllysine is prone to interconversion during enzymatic hydrolysis. Of mannosyllysine, only 29 ± 8% (SD, n=3) could be recovered unchanged. The larger part of 63 ± 7% had been transformed to glucosyllysine and 9 ± 1% were missing. On the other hand glucosyllysine is stable with a recovery of 88 ± 3%. The missing 12 % of glucosyllysine had been transformed to mannosyllysine. Traces of fructosyllysine were detected, when the glucosyllysine standard was subjected to enzymatic hydrolysis, but CML was not detectable in enzymatic hydrolysates of both HRCs. Thus, the HRCs isomerize during enzymatic hydrolysis, with glucosyllysine as the favored product. As explained above, mannosyllysine is more prone to Lobry de Bruyn-Alberda van Ekenstein transformation, leading to the formation of the more stable glucosyllysine. In the present study, mannosyllysine was only found in traces in bakery products, either due to isomerization to glucosyllysine or due to its absence in the products. Nonetheless, as the quantification of glucosyllysine probably encloses mannosyllysine present in the food product, the results will be given as the sum of both compounds and designated glucosyl/mannosyllysine (LysGlc/Man) this study. Because only trace amounts of mannosyllysine were found in bakery products the 9% loss of mannosyllysine during enzymatic hydrolysis can also be assumed to be negligible.

LysGlc/Man analysis was performed via an HPLC-MS/MS method used previously for ARCs and AGEs.[15,21,22] Product ion spectra of cookie samples were compared to those of standard solutions (Figure 2 A, D). Transitions that were both selective and abundant were chosen for quantitation (Table 3). With this HPLC-MS/MS method, simultaneous quantitation of the two lysine HRCs and the ARC fructosyllysine was enabled (Figure 2 E, F). Fructosyllysine and glucosyllysine also had minor peaks at the transitions of the other compound at their peak maxima, but were separated via their retention time (Figure 2 A, C). This led to ca. 15 μg falsely detected glucosyllysine per mg fructosyllysine and ca. 70 μg falsely detected fructosyllysine per mg glucosyllysine. Consequently, for the ratios glucosyllysine to fructosyllysine found in this study, up to 12% of the quantitated amounts might be overestimated. In addition to HRCs and fructosyllysine, the AGEs CML and CEL as major lysine derivatives and MG-H1 as a major arginine derivative[6] were quantitated. The standard addition method was used because stable isotope labeled standards were not available for HRCs. Linearity for the concentration ranges of the standard additions was verified with matrix calibrations of the analytes (concentrations see Table 4). Coefficients of determination of the standard addition regression lines were $r^2 > 0.99$ for 96% of the samples and $r^2 > 0.98$ for all samples. The intercept accuracy was between 85 and 115% for 96% of all samples and between 75 and 125% for all samples. Such intercept accuracy may be caused by overlapping peaks of the fructosyllysine and glucosyllysine transitions. LODs and LOQs were sufficiently low to enable quantitation of all analytes in most samples (Table 4).

The Maillard Reaction of Fructose in Cookies Baked with Fructose, Glucose, Honey and Banana. In order to simulate the formation of ARC and HRC under baking conditions, model cookies containing either fructose or glucose were baked. In both cookies, protein-bound LysGlc/Man and fructosyllysine were quantitated (Table 5). Of total lysine, 6.8% were modified to fructosyllysine in glucose-containing cookies, while only 1.6% of total lysine

were modified to LysGlc/Man in fructose-containing cookies. In cookies baked in the presence of hippuryllysine, 33% of hippuryllysine were derivatized to hippuryl-glucosyllysine in a fructose-containing cookie and 63% of hippuryllysine were derivatized to hippuryl-fructosyllysine in a cookie containing glucose.[13] While the ratio between ARCs and HRCs is similar, the yields are 5 times higher in the hippuryllysine cookies. When hippuryllysine is used as a model amine, all lysine amino groups can be glycated, whereas in protein, some lysine side chains are not or less available for derivatization with fructose, causing lower yields of HRCs.[4] In raw cookie dough and in sucrose-containing cookies, fructosyllysine was found in traces and LysGlc/Man was not detectable or found only in traces. Thus, protein-bound HRC form under baking conditions in cookies from fructose in significant concentrations.

Fructosyllysine was also found in fructose-containing cookies and LysGlc/Man in cookies containing glucose with yields of 0.8% and 0.2%, respectively. The formation of ARCs in the presence of fructose and of HRCs in the presence of glucose was described before and was explained via the addition of a second amino moiety to the product followed by elimination of the first.[27,32] These subsequent Amadori and Heyns rearrangement reactions were also observed, when ARCs and HRCs were incubated with additional amino compounds.[27] On the other hand, the isomerization of glucose to fructose and vice versa through the Lobry de Bruyn-Alberda van Ekenstein transformation could lead to this observation (Figure 3). This transformation is described to take place not only in basic solutions, but also in neutral aqueous glucose solutions[33,34] and cookies.[35] Fructose concentrations in heated glucose solutions rose up to 38% of the initial glucose concentrations.[36] This means that HRCs also form from glucose and that they play a minor role in the Maillard reaction with glucose as well. Additionally, the extent of Maillard reaction with fructose can be overestimated through this pathway.

328 To obtain information about the formation of HRCs from fructose in the presence of glucose
329 and to simulate realistic baking conditions, additional cookies containing honey and banana
330 were baked under the same conditions and the same water content as the cookies with
331 fructose and glucose. While in honey cookies the sugar content of the fructose and glucose
332 cookie was completely replaced by honey saccharides, in banana cookies only 36% of the
333 sugar was replaced by banana saccharides, since otherwise the dough would have been too
334 fluid. The remaining sugar was replaced by sucrose. In honey and banana, fructose and
335 glucose occur nearly in the ratio 1:1. Differences between reactivity of glucose and fructose
336 were also found when the molar conversion of the parent saccharides was compared: Per mol
337 of added fructose, the fructose cookie contained 0.21 mmol LysGlc/Man, while the honey
338 cookie had 0.15 mmol and the banana cookie had 0.36 mmol LysGlc/Man. In comparison to
339 that, the glucose cookie contained 0.83 mmol fructosyllysine per mol glucose, the honey
340 cookie 1.36 mmol and the banana cookie 1.42 mmol fructosyllysine per mol glucose.
341 Apparently, fructose has a lower potential to form early stage MRPs. Additionally, the HRC
342 formation from fructose occurs in systems containing both fructose and glucose like in
343 cookies with honey or banana and is similar to the HRC formation in systems without
344 glucose.
345 The AGEs CML, CEL and MG-H1 as important lysine and arginine AGEs were analyzed in
346 all cookies to study differences in the advanced Maillard reaction with fructose and glucose.
347 The CML contents were similar in the cookies containing fructose, glucose, honey and
348 banana (Table 5). This indicates that glucose and fructose do not differ significantly in the
349 formation of CML under baking conditions. An explanation might be that fructosyllysine and
350 glucosyllysine similarly degrade to CML.[4] By contrast, the highest MG-H1 and CEL
351 concentrations were found in the cookies with the highest fructose content. In aqueous sugar-
352 lysine model systems at above 100 °C, the formation of methylglyoxal from fructose was up
353 to three times higher than the formation from glucose.[37,38] Higher concentrations of

354 methylglyoxal explain the increased levels of the methylglyoxal-arginine adduct MG-H1 and
355 the methylglyoxal-lysine adduct CEL. Preliminary experiments have shown that the
356 percentage of arginine modified to MG-H1 and lysine modified to CEL correlates with the
357 concentrations of fructose or HRCs (data not shown). We are currently working on a further
358 exploration of the formation of MG-H1 and CEL from fructose which could be an indicator
359 for the "fructose pathway" of the Maillard reaction. The total loss of detectable lysine was ca.
360 15% in the glucose-containing cookie and ca. 60% in the fructose-containing cookie. While
361 the formation of fructosyllysine, LysGlc/Man, CML, and CEL in the glucose cookie accounts
362 for ca. 48% of the total lysine loss, the formation of the same products accounts for not more
363 than 4% of the total lysine loss in the fructose cookie. Therefore other derivatization or
364 fragmentation pathways have to be of major importance in the Maillard reaction with
365 fructose. Additionally, the nutritional loss of the essential amino acid lysine is much higher in
366 the fructose cookies than in the glucose cookies.
367
368 **The Maillard Reaction of Fructose in Commercial Bakery Products.** To evaluate HRC
369 formation and the degree of fructose-induced Maillard reaction in commonly consumed food,
370 commercial bakery products containing fructose were analyzed. The different cookies, cakes
371 and gingerbreads contained honey, invert sugar syrup and fruits as sweeteners. Protein-bound
372 HRCs could be quantitated in all fructose containing bakery products in the range of 19 to
373 287 mg/kg. As expected, the lowest concentration of HRCs was found in the banana bakery
374 products which had the lowest amount of fructose (Table 5). The highest HRC level was
375 found in a cookie containing invert sugar syrup and honey. The amounts measured lead to a
376 consumption of 0.6 to 9 mg LysGlc/Man per 30 mg serving of the bakery product. This equals
377 16–90% of the ARC consumption with those bakery products.
378 Fructosyllysine, CML, CEL, and MG-H1 were also analyzed in the commercial bakery
379 products. Contents of fructosyllysine (1500–6400 mg/kg protein), CML, CEL and MG-H1

were in similar ranges as stated in the literature for bakery products (fructosyllysine in bakery products: 400–13000 mg/kg protein, calculated from furosine;[17,39] CML in biscuits: 10–26 mg/kg; CEL in biscuits: 4.9–34; MG-H1 in biscuits: 40–369 mg/kg).[40] Contents of HRC exceeded the contents of CML and CEL in all and those of MG-H1 in most analyzed cookies. Therefore, substances of the early stage of Maillard reaction are of major importance in cookies, and ARCs and HRCs are useful indicators for Maillard reaction with glucose and fructose, respectively. To evaluate the relevance of the Maillard reaction with fructose in other fructose containing foods and in pathophysiological pathways, further analysis of HRCs is important. Furthermore, indicators to differentiate the advanced stage of the Maillard reaction with fructose and glucose need to be found.

Fructose, either of dietary origin or formed in the polyol pathway, is discussed as a causative factor for diabetic complications, nonalcoholic fatty liver disease and the metabolic syndrome.[41,42] Because the formation of HRCs under physiological conditions could be observed,[4,12] the Maillard reaction with fructose and fructose metabolites is discussed to play a role in the pathophysiological pathways of the diseases mentioned above.[41,43] The established protocol for analysis of HRCs adapted for pathophysiological material could bring insight into the role of the fructose pathway of the Maillard reaction in those diseases.

With the established method and the synthesized HRCs, protein bound HRCs could be quantitated. Analysis of protein-bound HRCs was used to evaluate the early stage of the Maillard reaction with fructose in bakery products. We could show that HRCs form under baking conditions not only in cookies baked with fructose but also in cookies with glucose or both sugars and in commercial bakery products containing honey, banana and invert sugar syrup. Protein-bound HRCs were quantitated in similar or higher levels (19–287 mg/kg) than the AGEs CML (10–76 mg/kg), CEL (2.5–53 mg/kg) and MG-H1 (10–218 mg/kg). Analysis

405 of protein-bound HRCs is therefore suitable to evaluate the Maillard reaction of fructose in
406 cookies.
407

408 Supporting information

409 1D/2D NMR spectra of glucosyllysine and mannosyllysine. Figure S1: ^{1}H NMR spectrum,
410 Figure S2: ^{13}C NMR spectrum, Figure S3: COSY correlations, Figure S4: HSQC correlations,
411 Figure S5: HMBC correlations of glucosyllysine. Figure S6: ^{1}H NMR spectrum, Figure S7:
412 ^{13}C NMR spectrum, Figure S8: COSY correlations, Figure S9: HSQC correlations, Figure
413 S10: HMBC correlations of mannosyllysine. This material is available free of charge via the
414 Internet at http://pubs.acs.org.
415

416 Abbreviations used

417 AGE, advanced glycation end product; ARC, Amadori rearrangement compound; CEL, *N*-ε-
418 carboxyethyllysine; CML, *N*-ε-carboxymethyllysine; DMF, *N*,*N*-dimethylformamide; HFCS,
419 high fructose corn syrup; HPLC, high pressure liquid chromatography; HRC, Heyns
420 rearrangement compound; LOD, limit of detection; LOQ, limit of quantitation; LysGlc/Man,
421 glucosyl/mannosyllysine; MG-H1, methylglyoxal-derived hydroimidazolone, MRM, multiple
422 reaction monitoring; MRP, Maillard reaction product; MS, mass spectrometry; NFPA,
423 nonafluoropentanoic acid; RP, reversed-phase; TRIS, tris(hydroxymethyl)aminomethane
424

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Notes

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Figure captions

Figure 1. Structures of the Maillard reaction products analyzed in this study.

Figure 2. Product ion spectra of glucosyllysine (A), mannosyllysine (B), and fructosyllysine (C) obtained during HPLC-MS/MS analysis of standard solutions, and of glucosyllysine in a hydrolyzed cookie sample (D). Operating conditions were those for the transition *m/z* 309 → 273 (Table 3) For (D) 5 µL of NFPA were added to 100 µL of the sample for better separation of glucosyllysine and fructosyllysine. Precursor (italic numbers), quantifier (bold numbers) and qualifier ions are denoted. HPLC-MS/MS chromatograms of a mixture of glucosyllysine and fructosyllysine (1) and a hydrolyzed cookie sample (2) with the transitions *m/z* 309 → 210 (E, glucosyllysine) and *m/z* 309 → 273 (F, fructosyllysine).

Figure 3. Reactions of glucose and fructose with amino components: **1** Lobry de Bruyn-Alberda van Ekenstein transformation, **2** formation of Schiff base and Amadori rearrangement compound, **3** formation of Schiff base and Heyns rearrangement compound, **4** formation of Schiff base and subsequent Heyns and Amadori rearrangement compounds

Table 1. ^{13}C and ^{1}H NMR Spectroscopic Data of N^{ε}-(2-deoxy-D-glucosyl)-L-lysine

C Atom (figure 1)	δ_C [ppm]	δ_H [ppm]	J(x,y) [Hz]	^{1}H-^{1}H COSY coupling	^{1}H-^{13}C HMBC coupling
glucose moiety					
1	88.00[a]/93.26 [b] (CH)	5.46[a]/4.87[b] d	3.6 [a]/8.5[b] (1,2)	2[a]	3; 5
2	59.97[a]/2.48[b] (CH)	3.16[a]/2.75[b] dd	3.6[a]/8.5[b] (1,2); 10,6 (2,3)	1; 3[a]	1; 4; 12
3	69.99 (CH)	3.83 dd	10.6 (2,3); 9.1 (3,4)	2; 4	2; 5
4	69.68 (CH)	3.39 dd	9.1 (3,4); 8.6 (4,5)	3	2; 3; 5; 6
5	71.26 (CH)	3.80 m	-	6	3; 4; 6
6	60.28 (CH_2)	3.70 d	5.4 (5,6)	5	5
lysyl moiety					
7	174.55 (Ci)	-	-	-	-
8	54.46 (CH)	3.66 t	6.2	9	7; 9; 10
9	29.84 (CH_2)	1.82 m	-	8; 10	7; 8; 10; 11
10	21.57 (CH_2)	1.40 m	-	9; 11	8; 9; 11; 12
11	25.33 (CH_2)	1.68 m	-	10; 12	9; 10; 12
12	45.35 (CH_2)	3.09 t	7.5	11	2; 10; 11

Data of [a] α-pyranosyl-conformation (75%) and [b] β-pyranosyl-conformation (25%) of glucosyllysine; percentage of conformation calculated from the peak-area of the proton at C_1

Table 2. ^{13}C and ^{1}H NMR Spectroscopic Data of N^{ε}-(2-deoxy-D-mannosyl)-L-lysine

C Atom (figure 1)	δ_C [ppm]	δ_H [ppm]	J(x,y) [Hz]	^{1}H-^{1}H COSY coupling	^{1}H-^{13}C HMBC coupling
mannose moiety					
1	89.58[a]/91.95[b]/ 98.75[c]/ 93.88[d]/ (CH)	5.36[a]/5.12[b]/5.47[c]/5.43[d] d	1.7[a]/1.7[b]/5.3[c]/5.5[d] (1,2)	2[a]	3[a]; 5[a]
2[a]	61.09(CH)	3.30 m		1; 3	3
3[a]	67.38 (CH)	4.06 dd	4.6 (2,3); 9.5 (3,4)	2; 4	4
4[a]	66.41 (CH)	3.53 dd	9.5 (3,4); 9.5 (4,5)	3; 5	3; 5; 6
5[a]	71.90 (CH)	3.80 m	-	4; 6	4
6[a]	60.27 (CH_2)	3.76 d	3.9 (5,6)	5	4; 5
lysyl moiety					
7	174.62 (Ci)	-	-	-	-
8	54.53 (CH)	3.68 t	6.2	9	7; 9; 10
9	29.94 (CH_2)	1.82 m	-	8; 10	7; 8; 10; 11
10	21.69 (CH_2)	1.40 m	-	9; 11	8; 9; 11; 12
11	26.37 (CH_2)	1.68 m	-	10; 12	9: 10: 12
12	47.21 (CH_2)	2.95 t	7.7	11	10: 11

Data of [a] α-pyranosyl-conformation (46%), [b] β-pyranosyl-conformation (25%), [c] α-furanosyl-conformation (18%) and [d] β-furanosyl-conformation (9%) of mannosyllysine; percentage of conformation calculated from the peak-area of the proton at C_1

Table 3. Transitions Recorded during MRM Measurement of MRPs in bakery products.

Time frame	Compound	Precursor ion [m/z]	Product ion [m/z]	Fragmentor voltage [V]	Collision energy [eV]	Dwell time [ms]	Q/q[a]
4–8.3 min	CML	205	84	100	20	70	Q
		205	130	100	10	70	q
	CEL	219	84	100	20	70	Q
		219	130	100	10	70	q
	Glucosyllysine	309	180	100	20	100	q
		309	210	100	10	100	Q
	Fructosyllysine	309	110	100	20	100	q
		309	273	100	10	100	Q
8.5–19.5 min	MG-H1	229	114	90	20	200	Q
		229	166	90	20	200	q

[a] Q, transition used for quantitation; q, transition used for the confirmation of the presence of the analyte.

Table 4. Performance Parameters of the HPLC-MS/MS Method used for the Analysis of MRPs in bakery products.

MRP	LOD[a]	LOQ	c_V	calibration range
	[mg/kg cookie]	[mg/kg cookie]	[%]	[mg/L]
Fructosyllysine	2.1	5.2	0.4–20.1	0.08–38.9
Glucosyllysine[b]	1.3	3.7	2.0–20.4	0.02–10.4
CML	0.6	1.1	0.5–19.7	0.005–1.4
CEL	0.3	0.7	0.8–19.9	0.005–2.7
MG-H1	1.0	2.6	1.2–20.3	0.02–8.2

[a] Limits of detection (LOD) and limits of quantitation (LOQ) are given in mg/kg cookie for protein-bound MRPs based on the average protein content of all analyzed cookies. Coefficients of variation (c_V) were determined by triplicate analysis of different cookies.

Table 5. Concentrations of protein-bound MRPs in cookie dough, baked cookies and purchased baked products in mg/kg cookie

	sweetener	Fructosyllysine	LysGlc/Man	CML	CEL	MG-H1
dough		tr	nd	nd–tr	nd	nd–tr
baked cookies	sucrose[a]	tr	tr	1.71 ± 0.34	nd	tr
	glucose	459 ± 37	11.28 ± 0.61	7.9 ± 1.5	1.541 ± 0.013	8.8 ± 1.7
	fructose	57.7 ± 1.1	118 ± 24	6.22 ± 0.71	4.76 ± 0.87	137.5 ± 7.3
	honey	312 ± 63	40.4 ± 1.3	6.940 ± 0.057	2.97 ± 0.15	27.7 ± 2.5
	banana	63.65 ± 0.29	15.7 ± 1.7	7.67 ± 0.18	2.45 ± 0.12	14.7 ± 3.0
purchased bakery products[b]	honey (7)[c]	108–469	19–208	13–76	2.5–32	10–93
	banana (1)	61.3 ± 4.4	24.9 ± 1.5	10.5 ± 1.5	23.5 ± 4.2	60.7 ± 3.4
	invert sugar syrup (4)[d]	116–717	82–287	32–42	12–53	45–218

[a] data are mean ± SD ($n \geq 2$) for one kind of product, data are given as ranges for groups of different bakery products, the number of different bakery products is stated in brackets. [c] 4 products also contained glucose syrup [d] 2 products also contained small amounts of honey; nd, not detected (below LOD); tr, traces (between LOD and LOQ)

Figures

Fig. 1 (one-column figure)

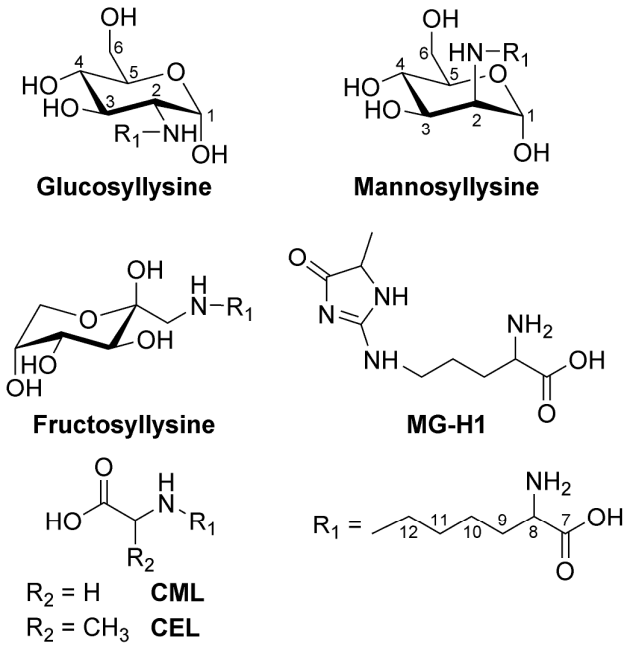


Fig. 2 (two column figure)

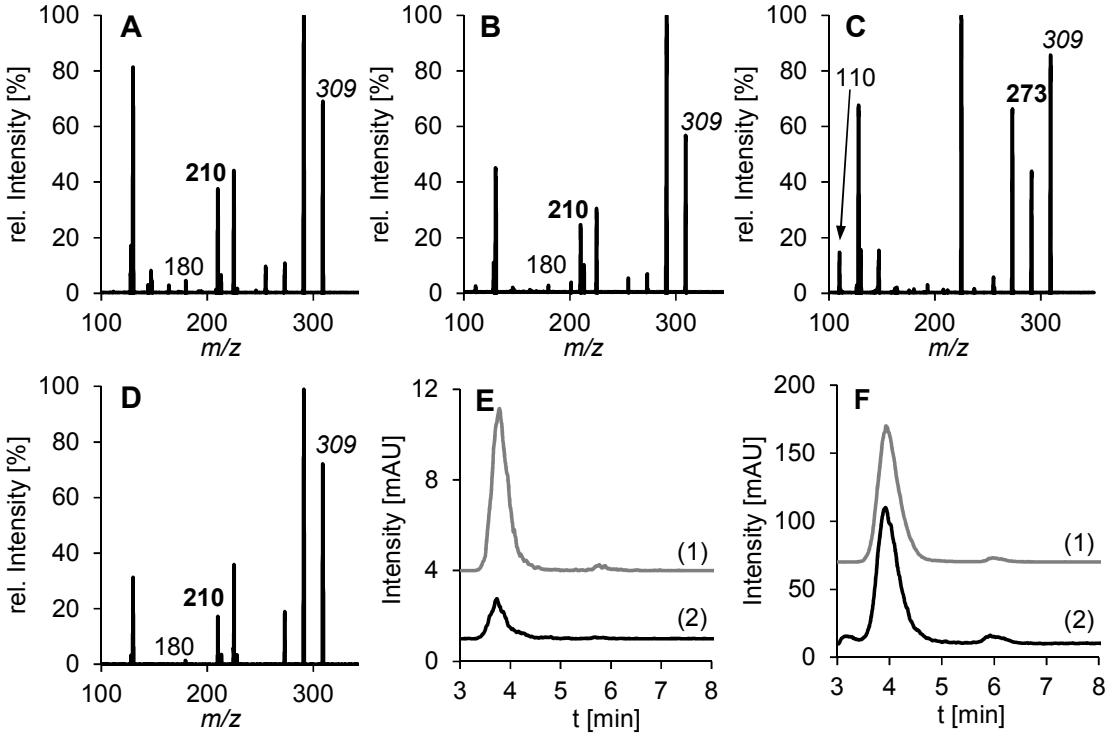
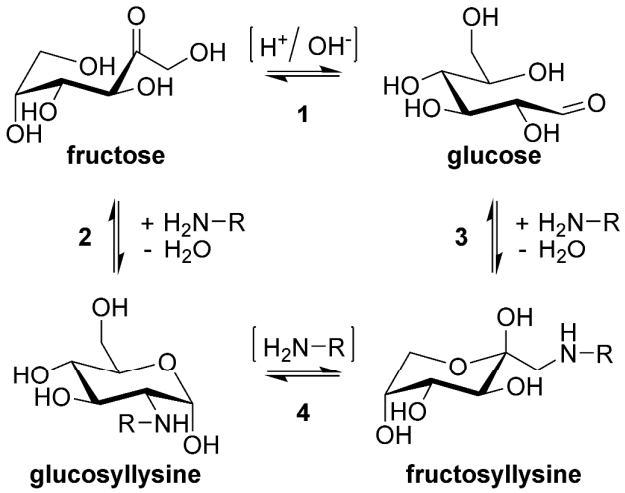
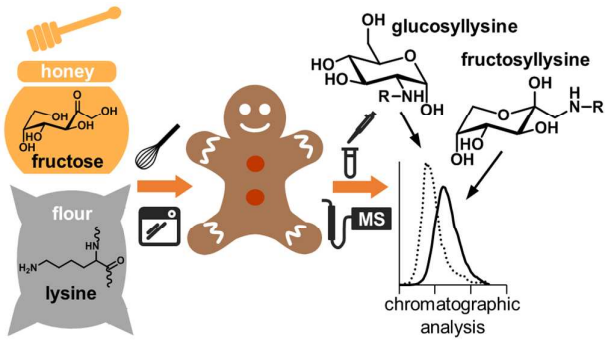


Fig 3. (one column figure)



TOC graphic


honey
fructose
flour
lysine
glucosyllysine
fructosyllysine
MS
chromatographic
analysis