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ARTICLE

3-Deoxygalactosone, a "New" 1,2-Dicarbonyl Compound in Milk Products

Michael Hellwig, Julia Degen, and Thomas Henle*

Institute of Food Chemistry, Technische Universität Dresden, D-01062 Dresden, Germany

1,2-Dicarbonyl compounds are formed in food during Maillard and caramelization reactions. 3-Deoxy-D-*threo*-hexos-2-ulose (3-deoxygalactosone, 3-DGal) and galactosone, two 1,2-dicarbonyl compounds originating from the degradation of galactose, were synthesized and converted to the respective quinoxalines, which were characterized by NMR spectroscopy. Analytical separation of the quinoxalines from the epimeric glucose-derived quinoxalines of 3-deoxyglucosone (3-DG) and glucosone was achieved by RP-HPLC on an RP-phenyl column. This method was used to study the relevance of galactose-derived 1,2-dicarbonyl compounds in a variety of foods. 3-DG and 3-DGal were quantified besides 3-deoxypentosone, methylglyoxal, and glyoxal after derivatization with *o*-phenylenediamine in lactose-hydrolyzed UHT milk, ranging from 2.5 to 18 mg/L and from 2.0 to 11 mg/L, respectively. The concentrations of both compounds tended to be higher in other lactose-hydrolyzed food items as well. During storage of lactose-hydrolyzed milk, the concentrations of the 3-deoxyhexosones first increased, but especially the concentration of 3-DGal tended to decrease on prolonged storage, pointing to lower stability of the compound. 3-DGal was also detected in galactose-free food items such as apple juice and beer. The possible formation of 3-DGal from 3-DG by 3,4-dideoxyglucosone-3-ene as an intermediate is discussed. Compared to the relatively high concentrations of 3-DG and 3-DGal, 3-deoxypentosone, methylglyoxal, and glyoxal were of only minor quantitative importance in all foods studied.

KEYWORDS: 3-Deoxyglucosone; 3-deoxygalactosone; galactosone; methylglyoxal; quinoxaline; phenyl phase; HMF

INTRODUCTION

During heating and/or prolonged storage of foods, 1,2-dicarbonyl compounds are mainly formed from monosaccharides by enolization and dehydration reactions (*1*) and also from disaccharides such as maltose or lactose following specific reaction pathways (*2*−*4*). These carbohydrate degradation products can react readily with the N-termini as well as lysine and arginine side chains of proteins during the Maillard reaction (glycation) to generate "advanced glycation end products" (AGEs) (*5*). Furthermore, 1,2-dicarbonyl compounds are precursors of volatile aroma-active compounds (*6*, *7*) and high molecular weight melanoidins (*8*). Several 1,2-dicarbonyl compounds originating from mono- and disaccharides have been isolated from model mixtures (*3*, *4*, *9*), but only very little information is available about the amount of 1,2-dicarbonyl compounds in complex foods. Small amounts of methylglyoxal (MGO) and glyoxal (GO) ranging between 0.02 and 1.3 mg/kg (mg/L), probably resulting from enzymatic processes, can be found in fermented foods such as yogurt, wine, and beer (*10*, *11*). Surprisingly high amounts of MGO of up to 760 mg/kg were reported for samples of Manuka honey from New Zealand (*12*). Roasted coffee may contain glyoxal and methylglyoxal up to 140 and 210 mg/kg, respectively (*13*). Data about further 1,2-dicarbonyl compounds such as 3-deoxyglucosone (3-DG) in food are scarce. Values ranging from 79 to 1451 mg/kg 3-DG were determined in several commercial honey samples (*12*, *14*, *15*), along with 0.6−75 mg/kg 5-hydroxymethylfurfural (HMF), which is a thermodynamically stable end product of caramelization (*12*, *14*). Recently, the occurrence of GO, MGO, and 3-DG in carbonated soft drinks and high-fructose corn syrup (*16*) has led to questions concerning the impact of ingested 1,2-dicarbonyl compounds on their levels measured in human circulation, as the concentrations of 3-DG or MGO measured in blood, plasma, and urine are higher in diabetic or uremic states than under normoglycemic conditions (*17*−*19*).

As it was shown that GO, MGO, and 3-DG are cytotoxic (*20*), concerns about pathophysiological consequences have led to strategies of minimizing 1,2-dicarbonyl compounds, for example, 3,4-dideoxyglucosone-3-ene (3,4-DGE), in solutions for peritoneal dialysis (*21*, *22*). At low concentrations, however, glyoxal and methylglyoxal were postulated to have beneficial effects in cellular systems (*23*). Methylglyoxal was identified as the main antibacterial principle in Manuka honey (*12*). Due to limited data about their concentrations in most commonly consumed foods, however, it is impossible to assess the physiological role of other dietary 1,2-dicarbonyl compounds.

The purpose of the present work was to increase basic knowledge about chemical reactions occurring during food processing. In particular, our studies aimed to better understand degradation reactions of sugars and the formation of 1,2-dicarbonyl compounds

*Corresponding author (phone +49-351-463-34647; fax +49-351-463-34138; e-mail Thomas.Henle@chemie.tu-dresden.de).

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in complex food matrices. For this, it was necessary to set up a viable chromatographic assay to quantify 1,2-dicarbonyl compounds in milk products. The dicarbonyl compounds were analyzed as the corresponding quinoxalines together with 5-hydroxymethylfurfural (HMF) via RP-HPLC with UV detection on a phenyl-modified column, using the independently synthesized quinoxalines as reference material. Special attention was paid to lactose-hydrolyzed products, containing galactose as an "unusual" monosaccharide besides glucose and lactose. We followed the hypothesis that in galactose-containing milk products the formation of 3-deoxygalactosone must be of particular importance. Formation and degradation mechanisms of 3-deoxyhexosones are presented and discussed.

MATERIALS AND METHODS

Materials. The following chemicals of the highest purity available were purchased from the mentioned suppliers: benzaldehyde, DETAPAC, disodium hydrogen phosphate, phenylhydrazine (Merck, Darmstadt, Germany); *o*-phenylenediamine (OPD), methanol, *p*-toluidine, HMF, quinoxaline, 2-methylquinoxaline (SAF, Steinheim, Germany); D-glucose, D-galactose, benzoylhydrazine (Alfa Aesar, Karlsruhe, Germany); sodium dihydrogen phosphate (Grüssing, Filsum, Germany). Mixed-bed ion exchanger Serdolit MB-2 was from Serva (Heidelberg, Germany). 3-DG and 3-deoxypentosone (3-DPs) were prepared according to methods described in the literature (*24*, *25*). The water used for the preparation of buffers, solutions, and HPLC solvents was obtained using a Purelab plus purification system (USFilter, Ransbach-Baumbach, Germany).

Food Samples. Food samples were purchased from different supermarkets. Raw cow's milk was obtained from a local farm. A storage experiment was performed with one conventional UHT-treated milk and two lactose-hydrolyzed UHT-treated milks from different suppliers. For this, 1 L packages of milk were stored either at 4 °C in a refrigeration room, at room temperature, and at 37 °C in an incubator. Storage was started at 3 months before the expiry date. Samples were withdrawn at 0.5, 1, 2, 3, and 4 months, respectively, and analyzed for their contents in 1,2-dicarbonyl compounds, furosine, and HMF. In a preliminary experiment, 10 mL of conventional and lactose-hydrolyzed milk, respectively, was heated at 80 °C in a water bath. Samples taken during 5 h of heating were subjected to 1,2-dicarbonyl analysis.

Preparation of Food Samples for Analysis. Liquid samples were analyzed without further preparation, except for carbonated soft drinks and beer, which were decarbonated by use of an ultrasonic bath. Five hundred microliters of the solutions was mixed with 1 mL of methanol and allowed to precipitate for 1 h at −20 °C. Semisolid samples (e.g., yogurt) were directly mixed with methanol to a concentration of 1 g/3 mL. The mixtures were centrifuged at 10000*g* for 15 min, and the supernatant was directly used for HMF analysis after additional membrane filtration (0.45 μm, hydrophilic polypropylene). For the analysis of 1,2-dicarbonyl compounds, 500 μL of the supernatant was mixed with 150 μL of 0.5 M sodium phosphate buffer, pH 7.0, and 150 μL of a 0.2% (w/v) OPD solution containing 18.5 mM DETAPAC. The mixture was kept in the dark overnight and membrane filtered (0.45 μm) before chromatographic analysis. To evaluate interferences from the matrix, each precipitated sample was also measured without derivatization. For the analysis of furosine, 4.55 mL of 12 N hydrochloric acid was added to 3.00 mL of milk samples. The mixtures were hydrolyzed at 110 °C for 23 h and then filtered. The filtrates were subjected to solid phase extraction as described in the literature (*26*).

Analytical High-Pressure Liquid Chromatography (HPLC). All analytical HPLC analyses were performed using a high -pressure gradient system from Amersham Pharmacia Biotech (Uppsala, Sweden), consisting of a pump P-900 with an online degasser (Knauer, Berlin, Germany) and a UV detector UV-900. Peaks were evaluated using the software UNICORN V 4.00.

HPLC Analysis of Quinoxalines Derived from 1,2-Dicarbonyl Compounds. Quinoxalines were separated on a stainless steel column filled with Prontosil 60 Phenyl material (250 mm × 4.6 mm, 5 μm, Knauer) with an integrated guard column (5 mm × 4 mm) filled with the same material and an online filter (3 μm) between the sample loop and column. Gradient optimization in view of high resolution of the quinoxalines of 3-DG and 3-DGal and the simultaneous analysis of other quinoxalines yielded the following conditions: The mobile phases were 0.075% acetic acid (solvent A) and a mixture of 80% methanol and 20% solvent A (solvent B). The flow rate was 0.7 mL/min, and the separation was performed at room temperature. Fifty microliters of the derivatized solutions was injected. The gradient started with 10% solvent B and was changed linearly to 50% B over a period of 25 min. After 5 min at 50% B, the proportion of solvent B was elevated to 70% within 4 min, held there for 10 min, and then changed to 10% in 4 min. The column was equilibrated with 10% solvent B for 12 min at a flow rate of 0.5 mL/min. UV detection was performed at 280 and 312 nm, simultaneously. For external calibration, a stock solution of quinoxalines in water was prepared (c = 0.87−1.22 mmol/L), which was diluted to the appropriate concentrations.

The limits of detection (LOD) and quantification (LOQ) were calculated as the concentrations of the analyte necessary to show a peak at a signal-to-noise ratio of 3 and 10, respectively. For the determination of the interday repeatability, the samples of lactose-hydrolyzed milk were derivatized five times on different days. The recoveries of 3-DG, 3-DGal, 3-DPs, GO, and MGO were calculated from the slope of the recovery function after spiking raw cow's milk with ascending concentrations (6−40 μM) of dicarbonyl compounds and derivatization with OPD.

Analysis of HMF and Furosine. HMF and furosine were analyzed by RP-HPLC-UV according to the methods of refs *14* and *26*, respectively.

Semipreparative HPLC. This was performed with a low-pressure gradient pump system consisting of a Smartline pump 1000 with a 50 mL pump head, a Smartline manager with online degasser, and a UV detector 2500 (all from Knauer). All separations were performed at room temperature using an RP-18 column (Eurospher 100, 100 mm × 16 mm, 10 μm, Knauer) with a guard column (30 mm × 16 mm) filled with the same material. The flow rate was 8 mL/min, and UV detection was at 312 nm. The raw synthesis solutions were membrane filtered (0.45 mm), and samples of 1 mL were injected manually. The quinoxalines were isolated using the solvents mentioned above for the analytical HPLC with a linear gradient from 40 to 100% B in 18 min. The quinoxaline of 3-DGal was additionally purified with a linear gradient from 25 to 100% B in 23 min.

Mass Spectrometry. For mass spectrometry analyses, a PerSeptive Biosystems Mariner time-of-flight mass spectrometry instrument equipped with an electrospray ionization source (ESI-TOF-MS, Applied Biosystems, Stafford, TX) working in the positive mode was used. Calibration of the mass scale was established using a mixture of bradykinin, angiotensin I, and neurotensin. After appropriate dilution of the samples with a mixture of methanol, ethylacetate, and acetic acid (50:50:1, v/v), the sample was injected at a flow rate of 5 μL/min into the ESI source by a syringe pump. Spray tip potential, nozzle potential, quadrupole RF voltage, and detector voltage were adjusted to 4812.3, 80, 1000, and 2400 V, respectively.

HPLC-DAD-ESI-MS Analysis of Quinoxalines Derived from 1,2-Dicarbonyl Compounds. This was performed on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) consisting of a high-pressure gradient pump system, an autosampler, and a diode array detector, which was coupled to the above-mentioned ESI-MS instrument working in the positive mode. The same column, gradient, and solvents as described for the HPLC analysis of quinoxalines in food samples were used. MS conditions were as follows: quadrupole RF voltage, 999.76 V; nozzle temperature, 140.01 °C; reflector potential, 1549.99 V; detector voltage, 1750.06 V; first mass, 100; last mass, 1200.

Nuclear Magnetic Resonance (NMR) Spectrometry. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 500 instrument (Rheinstetten, Germany) at 500 and 125 MHz, respectively. Deuterium oxide was used as the solvent. All chemical shifts are given in parts per million (ppm), those of protons relative to the internal HOD signal (4.70 ppm) and those of carbon atoms relative to external standard tetramethylsilane. Assignments of ^{1}H and ^{13}C signals are based on ^{1}H−^{1}H COSY (correlation spectroscopy), heteronuclear single quantum coherence (HSQC), and distortionless enhancement by polarization transfer (DEPT) experiments.

Elemental Analysis. Elemental analysis data were obtained on a Euro EA 3000 elemental analyzer (Eurovector, Milano, Italy). We used elemental analysis to calculate the true product content of the quinoxaline preparations. The percentage of nitrogen in the preparation was divided by the theoretical percentage of nitrogen of the target substance and the content expressed in percent by weight.

Synthesis of 3-Deoxy-D-*threo*-hexos-2-ulose (3-Deoxygalactosone, 3-DGal). Synthesis methods described in the literature (*25*, *27*) were applied with minor modifications. Twenty grams of D-galactose and 11 g of *p*-toluidine were added to 450 mL of ethanol containing 22 mL of acetic acid. After 30 min of refluxing, 33 g of benzoylhydrazine was added and the mixture was refluxed for a further 7 h. The mixture was cooled slowly and left standing overnight at room temperature. Precipitation of crude 3-DGal bis(benzoylhydrazone) was completed by further cooling the mixture to −20 °C overnight. The product was collected by suction and first washed three times with 100 mL of ice-cold ethanol and then three times with 100 mL of diethyl ether. The white crystals were air-dried in a hood (yield = 21.3 g).

Ten grams of 3-DGal bis(benzoylhydrazone) was suspended in 800 mL of 37.5% aqueous ethanol. After the addition of 22 mL of acetic acid and 16 mL of benzaldehyde, the mixture was stirred under reflux for 2 h. The precipitate was filtered off after cooling to room temperature and the ethanol evaporated in vacuo. The mixture was left to stand at 4 °C overnight and filtered. The filtrate was first stirred with 60 g of Serdolit MB-2 for 15 min and then concentrated to 100 mL using a rotary evaporator at a bath temperature of 35 °C. The solution was extracted six times each with 50 mL of diethyl ether, evaporated to dryness, and dissolved in a mixture of 5 mL of water and 50 mL of 96% ethanol. The mixture was stirred with 10 g of Serdolit MB-2 for 10 min and filtered through filter paper. The filtrate was evaporated to a light yellow oily residue, taken up in water, and lyophilized to yield 2.05 g of 3-DGal. The product was characterized by the above-mentioned HPLC method after derivatization with OPD and directly utilized for quinoxaline synthesis.

Synthesis of D-*lyxo*-Hexos-2-ulose (Galactosone). D-Galactose (4.04 g) was added to a solution of 10 mL of phenylhydrazine in 70 mL of 2.5 N aqueous acetic acid. The mixture was heated under reflux in an oil bath until after 30 min the bis(phenylhydrazone) of D-galactosone began to crystallize. After cooling, the mixture was filtered, and the product was crystallized once from 150 mL of boiling 96% ethanol, filtered again, and finally dried in vacuo to yield 1.83 g of yellow D-galactosone bis(phenylhydrazone). According to the method of Mayer (*28*), 1.5 g of the bis(phenylhydrazone) was first heated to 100 °C with 12.8 mL of benzaldehyde under intense stirring in a round-bottom flask. The hot suspension was transferred into 150 mL of boiling water and the round-bottom flask rinsed with a total of 50 mL of ethanol. The mixture was intensely stirred under reflux for 2 h and then cooled to room temperature. The reddish-brown oil, which separated, was filtered off from a turbid yellow solution. The oil was subjected to the cooking and filtration steps two more times. The remaining red oil was discarded, and the pooled filtrates were filtered after standing overnight at room temperature. The filtrate was evaporated to dryness and taken up in 80 mL of water. The yellow solution was extracted three times, each with 80 mL of diethyl ether and 80 mL of ethyl acetate. Ten milliliters of ethanol and 2.0 g of activated charcoal were added to the aqueous phase. After 10 min of stirring, the mixture was filtered; the clear filtrate was evaporated to dryness, taken up in water, and lyophilized to yield 102.6 mg of D-galactosone as an off-white powder.

Synthesis of 2-(2′(*S*),3′(*R*),4′-Trihydroxybutyl)quinoxaline (3-DG-quinoxaline). This was carried out according to the method given in ref *29*. Three hundred and fifty milligrams of 3-DG and 270 mg of OPD were dissolved in 35 mL of methanol and stirred overnight in the dark. The solution was filtered and the filtrate evaporated to dryness in vacuo. The residue was taken up in 4−5 mL of HPLC eluent A, membrane filtered (0.45 μm), and subjected to semipreparative HPLC. The eluate of the prominent peak was collected, evaporated to dryness in vacuo, and then lyophilized to yield the quinoxaline as a yellow fluffy powder.

ESI-MS, positive mode, $[M + H]^+$ *m*/*z* 235.1; ^{1}H NMR (500 MHz, D_2O), δ 2.93 (dd, 1H, *J* = 14.2 Hz, *J* = 9.9 Hz, H1A′), 3.13 (dd, 1H, *J* = 14.2 Hz, *J* = 3.3 Hz, H1B′), 3.55 (dd, 1H, *J* = 11.5 Hz, *J* = 7.0 Hz, H4A′), 3.61 (m, 1H, H3′), 3.72 (dd, 1H, *J* = 11.6 Hz, *J* = 3.2 Hz, H4B′), 3.96 (m, 1H, H2′), 7.60 (m, 2H, H7, H8), 7.70 (m, 2H, H6, H9), 8.52 (s, 1H, H3). Elemental analysis: $C_{12}H_{14}N_2O_3$ (MW = 234.26) requires C, 61.53%; H, 6.02%; N, 11.96%. Found: C, 59.86%; H, 6.65%; N, 11.74%. Content = 98.2%, based on nitrogen. Yield = 120 mg.

Synthesis of 2-(2′(*R*),3′(*R*),4′-Trihydroxybutyl)quinoxaline (3-DGal-quinoxaline). This was carried out accordingly, but using 350 mg of the 3-DGal preparation instead of 3-DG. Due to a coeluting impurity, the isolate of the first semipreparative run was additionally purified by a gradient with a weaker solvent strength.

Table 1. ^{13}C and ^{1}H NMR Data of 2-(1′(*R*),2′(*R*),3′(*R*),4′-Tetrahydroxybutyl)quinoxaline (Quinoxaline of Galactosone)

C atom (**Figure 1**)	δ_C[a]	δ_H[b]	COSY coupling
2	156.51 (Ci)		
3	144.75 (CH)	8.83 (s, 1H)	
5/8	128.19 (CH)	7.89 (m, 2H)	6/7
	128.29 (CH)		
6/7	131.12 (CH)	7.71 (m, 2H)	5/8
	131.45 (CH)		
9/10	141.08 (Ci)		
	141.13 (Ci)		
1′	72.65 (CH)	4.94 (d, 1H; 8.4)	2′
2′	73.22 (CH)	3.92 (dd, 1H; 2.0; 8.4)	1′; 3′
3′	70.50 (CH)	3.98 (ddd, 1H; 1.9; 5.6; 7.0)	2′; 4′
4′	63.24 (CH_2)	Ha 3.61 (d, 1H; 5.6)	3′ 3′
		Hb 3.61 (d, 1H; 7.1)	

[a] Type of signal deduced from ^{13}C-DEPT measurement is given in parentheses. [b] Atom assignment is based on HSQC experiments; ^3J coupling constants are expressed in hertz.

ESI-MS, positive mode, $[M + H]^+$ *m*/*z* 235.1; ^{1}H NMR (500 MHz, D_2O), δ 3.06 (d, 2H, *J* = 6.7 Hz, H1′), 3.62 (m, 3H, H2′, H3′, H4A′), 4.04 (dt, 1H, *J* = 3.1 Hz, *J* = 6.9 Hz, H4B′), 7.66 (m, 2H, H7, H8), 7.79 (m, 2H, H6, H9), 8.59 (s, 1H, H3). Elemental analysis: $C_{12}H_{14}N_2O_3$ (MW = 234.26) requires C, 61.53%; H, 6.02%; N, 11.96%. Found: C, 59.13%; H, 5.90%; N, 11.53%. Content = 96.4%, based on nitrogen. Yield = 161 mg.

Synthesis of 2-(2′(*R*),3′-Dihydroxypropyl)quinoxaline (3-DPs-quinoxaline). This was carried out as described for 3-DG-quinoxaline, starting with 350 mg of 3-DPs instead of 3-DG.

ESI-MS, positive mode, $[M + H]^+$ *m*/*z* 205.1; ^{1}H NMR (500 MHz, D_2O), δ 2.93 (dd, 1H, *J* = 14.1 Hz, *J* = 9.1 Hz, H1A′), 3.02 (dd, 1 H, *J* = 14.1 Hz, *J* = 4.4 Hz, H1B′), 3.50 (dd, 1H, *J* = 11.8 Hz, *J* = 6.5 Hz, H4A′), 3.59 (dd, 1H, *J* = 11.8 Hz, *J* = 4.0 Hz, H4B′), 4.06 Hz (m, 1H, H2′), 7.63 (m, 2H, H7, H8), 7.74 (m, 2H, H6, H9), 8.55 (s, 1H, H3). Elemental analysis: $C_{11}H_{12}N_2O_2$ (MW = 204.23) requires C, 64.69%; H, 5.92%; N, 13.72%. Found: C, 62.58%; H, 5.46%; N, 13.47%. Content = 98.2%, based on nitrogen. Yield = 182 mg.

Synthesis of 2-(1′(*R*),2′(*R*),3′(*R*),4′-Tetrahydroxybutyl)quinoxaline (Galactosone-quinoxaline). This was carried out as described for the 3-DG-quinoxaline starting from 51.2 mg of galactosone and 36.4 mg of OPD in 4.7 mL of methanol.

ESI-MS, positive mode, $[M + H]^+$ *m*/*z* 251.1; NMR data, see **Table 1**. Elemental analysis: $C_{12}H_{14}N_2O_4$ (MW = 250.25) requires C, 57.59%; H, 5.64%; N, 11.19%. Found: C, 56.81%; H, 5.84%; N, 10.76%. Content = 96.2%, based on nitrogen. Yield = 36.2 mg (molar yield = 48.0%).

RESULTS AND DISCUSSION

Synthesis and RP-HPLC Analysis of Galactose-Derived Quinoxalines. Structures of the quinoxalines synthesized in this study are shown in **Figure 1**. This study aimed at analyzing 1,2-dicarbonyl compounds in "lactose-free" milk products dedicated to lactose-intolerant consumers. In such products, lactose is enzymatically hydrolyzed to glucose and galactose, which may induce the formation of 3-deoxygalactosone (3-DGal) and the oxidation product galactosone together with the corresponding derivatives resulting from glucose, namely 3-deoxyglucosone (3-DG) and glucosone. For the synthesis of 3-DGal, a protocol published by El Khadem et al. (*27*) was followed with minor modifications (*25*). During synthesis, the solutions of 3-DGal tended to discolor more strongly than those of 3-DG, and in the first attempts, byproduct appeared. Any heat impact during and following transhydrazonation had to be controlled carefully to minimize byproduct formation. The resulting product was directly used for the synthesis of the quinoxaline of 3-DGal, which met the spectroscopic properties published by Bravo et al. (*7*). For the synthesis of galactosone, we adapted a synthesis protocol for glucosone (*28*), which is based on the transhydrazonation of the

osone bis(phenylhydrazone) with a 30-fold molar excess of benzaldehyde. The synthesis afforded galactosone as an off-white powder in 13.7% molar yield from the bis(phenylhydrazone), being less than the yield published for glucosone (*28*), which could be due to poor solubility of galactosone bis(phenylhydrazone) in benzaldehyde and water. HPLC analysis after derivatization with *o*-phenylenediamine (OPD) revealed that the preparation was chromatographically pure with only a small amount (5.1%) of 3-DGal as a byproduct. For further structural elucidation, the respective quinoxaline (**Figure 1**) was synthesized and subjected to one- and two-dimensional NMR experiments, the results of which are presented in **Table 1**.

3-DG and 3-DGal are epimeric sugars forming epimeric quinoxalines on derivatization with OPD and, thus, interact very similarly with stationary phases during chromatographic separations.

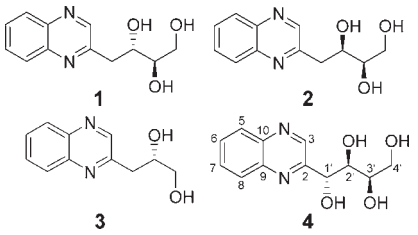

Figure 1. Chemical structures of the quinoxalines synthesized in this study. The quinoxalines were prepared from **1** 3-deoxyglucosone (3-DG), **2** 3-deoxygalactosone (3-DGal), **3** 3-deoxypentosone (3-DPs), and **4** galactosone.

As a matter of fact, we failed to separate these quinoxalines on a C18 modified silica column even after thorough modification of previously published methods (*12*, *14*). We then transferred the method to a phenyl-modified column providing better selectivity for aromatic compounds such as quinoxalines. With the final optimized gradient, the phenyl column allowed baseline separation (resolution up to 1.4) for the epimeric quinoxalines at room temperature and at a flow rate of 0.7 mL/min. Higher temperature and/or flow rate lowered the resolution. The quinoxalines of glucosone and galactosone were baseline separated as well (resolution = 2.5) (**Figure 2A**). The total analysis time of 60 min might be considered disadvantageous with respect to sample throughput, but the striking advantages of our new method are (i) the separation of two isobaric critical pairs, which is also essential when MS detection is applied, and (ii) a peak shape improvement especially for peaks eluting with higher retention times: The tailing factor of the peaks of the quinoxalines derived from glyoxal and methylglyoxal was between 1.5 and 1.6 on the phenyl column even after 3 months of intense usage, whereas it exceeded 2 on a new RP-18 column. This makes the method better suited for the analysis of a broader spectrum of matrix-burdened samples, because the problem of coeluting UV-active matrix constituents can more easily be solved when peaks remain narrow even after long gradient times.

Derivatization and Quantification of 1,2-Dicarbonyl Compounds. The derivatization of 1,2-dicarbonyl compounds using OPD is usually carried out in aqueous buffered solution after the removal of proteins in the presence of different auxiliary agents, for example, antioxidants or metal chelators. In this study, we

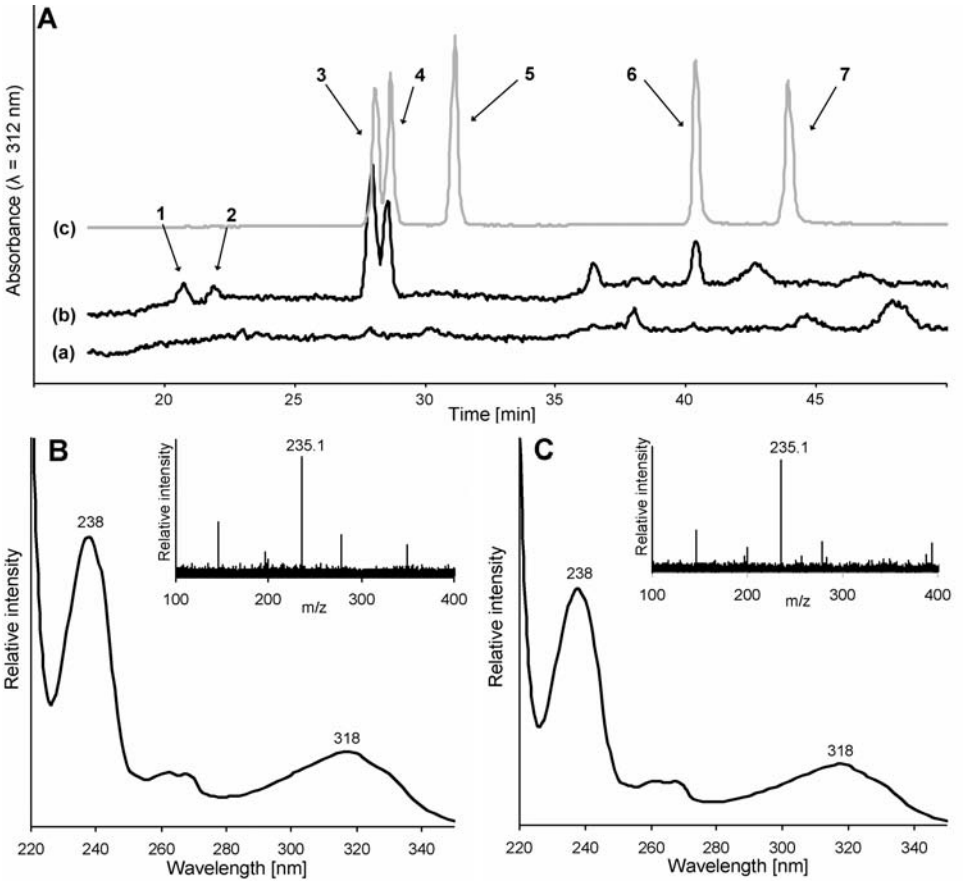

Figure 2. (**A**) Chromatograms of (a) conventional semiskimmed UHT milk, (b) lactose-hydrolyzed semiskimmed UHT milk, and (c) a standard quinoxaline mixture acquired by Phenyl-RP-HPLC with UV (312 nm) and ESI-MS detection. Arrows indicate the peaks of the quinoxalines of (1) galactosone, (2) glucosone, (3) 3-deoxyglucosone, (4) 3-deoxygalactosone, (5) 3-deoxypentosone, (6) glyoxal, and (7) methylglyoxal. (**B**) UV and MS spectra (inset) of the peak eluting at 28.1 min (quinoxaline of 3-DG). (**C**) UV and MS spectra (inset) of the peak eluting at 28.7 min (quinoxaline of 3-DGal).

used diethylenetriaminepentaacetic acid (DETAPAC) to suppress transition metal-catalyzed oxidations. For protein precipitation, methanol was chosen instead of trichloroacetic acid (TCA), because TCA led to a minimal generation of MGO from glucose when OPD derivatization was carried out in a solution of glucose at the concentration found in lactose-free milk (2.3 g/L) (data not shown). Methanolic precipitation using the optimized solvent/sample ratios as given under Materials and Methods proved to yield very reproducible results without any negative effects on the applicability and stability of the phenyl column. However, when a solution of glucose of 2.3 g/L was subjected to derivatization after methanolic precipitation, the formation of the glucosone quinoxaline at a level of 0.1 μmol/mmol of glucose was noted. The formation of glucosone artifacts during derivatization has already been addressed in the literature (*29*). We conclude that glucosone and galactosone cannot be quantified validly by applying the derivatization conditions used in this study. Concentrations of glucosone and galactosone are therefore not reported.

We then determined the method performance for the 1,2-dicarbonyl compounds 3-DG, 3-DGal, 3-DPs, glyoxal, and methylglyoxal. Quantification was performed at λ = 312 nm as a suitable wavelength for a broader spectrum of quinoxalines. The UV maxima of the quinoxalines can vary slightly depending on ring substitution (*15*, *22*); however, using 312 nm instead of the individual UV maxima had no significant influence on peak areas and sensitivity. External calibration was performed with the corresponding quinoxalines. Because the derivatization reaction leads to the complete conversion of the 1,2-dicarbonyl compounds to stable quinoxalines (*13*, *29*), this procedure is often followed during the analysis of 1,2-dicarbonyl compounds (*3*, *7*, *13*, *16*). All calibration curves showed linearity within the calibration ranges between 2 and 300 μM (**Table 2**). LODs for quinoxalines ranged between 0.29 and 0.41 μM, corresponding to 1,2-dicarbonyl concentrations between 0.02 and 0.05 mg/L. LOQs for quinoxalines ranged between 0.60 and 1.08 μM (**Table 2**), equivalent to 1,2-dicarbonyl concentrations between 0.04 and 0.12 mg/L. At the LOQ, peaks could be integrated with a relative standard deviation of < 5%. LOQs were slightly higher when the calibration was performed in raw cow's milk as a matrix. The relative interday repeatability was assessed for the main analytes of this study by repetitive measurements of the same milk sample and was at 5.0% for 3-DG and at 4.8% for 3-DGal, respectively, whereas the intraday repeatability was < 3%. Raw cow's milk was used to assess the recovery of 1,2-dicarbonyl compounds during sample workup under inclusion of all steps of analysis. Even when 1,2-dicarbonyl compounds were added to milk and the spiked samples were allowed to stand for 1 h before methanolic precipitation, the average recovery of the analytes was between 99.9 and 102.7% (**Table 2**), with individual values ranging between 96.2 and 105.3%. We conclude that 1,2-dicarbonyl compounds, even if reversibly bound to proteins, can be fully recovered after methanolic precipitation.

Table 2. Performance Parameters for the Optimized RP-HPLC Method

			LOD		LOQ		
	R^2	linear range[a] (μmol/L)	μmol/L	mg/L[b]	μmol/L	mg/L[b]	recovery[c] (%)
3-DG	0.9987	3−300	0.33	0.05	0.79	0.12	100.0 ± 2.6
3-DGal	0.9987	3−300	0.29	0.05	0.60	0.10	100.0 ± 2.1
3-DPs	0.9987	2−200	0.33	0.04	0.69	0.09	100.0 ± 1.5
GO	0.9978	3−350	0.35	0.02	0.76	0.04	99.9 ± 4.5
MGO	0.9977	3−340	0.41	0.03	1.08	0.08	102.7 ± 5.4

[a] Calibration experiments were performed using quinoxalines as the standards (n = 3-5). [b] Values are expressed for the 1,2-dicarbonyl compounds. [c] Recovery was determined by addition of various concentrations (8−40 μM, n = 3) of 1,2-dicarbonyl compounds to raw cow's milk under inclusion of all steps of analysis. Values were calculated from the slope of the recovery function and are given in percent ± SE.

In summary, a viable method for the analysis of 1,2-dicarbonyl compounds has been set up and was shown to be of appropriate performance. All samples were additionally measured without OPD derivatization to recognize possible coeluting substances from the matrix. Samples of lactose-hydrolyzed milk were also analyzed via HPLC-DAD-ESI-MS for the purpose of peak assignment.

Analysis of Milk and Milk Products. Various samples of conventional semiskimmed UHT milk and milk products were then analyzed for their contents of 1,2-dicarbonyl compounds. 3-DG could be quantified only in one sample at a concentration of 1.4 mg/L (**Table 3**). In most of the other conventional milk products, 1,2-dicarbonyl compounds were hardly detectable with the exception of evaporated milk, in which the contents, however, did not exceed 2 mg/L for all compounds tested.

Table 3. Amounts of 1,2-Dicarbonyl Compounds in Milk Samples[a]

food product	3-DG	3-DGal	3-DPs	GO	MGO	HMF	n
lactose-hydrolyzed milk products							
semiskimmed UHT milk, 1.5% fat	2.5−18	2.0−11	nd	nd−3.2	nd	nd	6
yogurt, 3.5% fat	tr	0.7	nd	nd	0.7	nd	1
fruit yogurt, strawberry	11	tr	nd	tr	2.3	0.8	1
curd cheese, 0.2% fat	tr	tr	nd	nd	nd	nd	1
cream, 30% fat	4.6	5.3	nd	1.0	nd	nd	1
coffee cream, 10% fat	16	19	nd	nd	nd	nd	1
conventional milk products							
semiskimmed UHT milk, 1.5% fat	nd−1.4	nd−tr	nd	nd	nd−tr	nd	3
yogurt, 3.5% fat	nd	nd	tr	tr	tr	nd	1
fruit yogurt, strawberry	3.8	tr	nd	nd	tr	0.8	1
curd cheese, 0.2% fat	nd	nd	nd	nd	nd	nd	1
cream, 30% fat	tr	nd	nd	nd	nd	nd	1
coffee cream, 10% fat	tr	nd	tr	tr	nd	nd	1
evaporated milk	2.0−2.2	0.9−1.0	1.2−1.5	0.6−0.8	nd	nd−0.4	2
ricotta cheese	nd	nd	nd	nd	nd	nd	1
kefir	nd	nd	nd	nd	tr	nd	1
probiotic drink	8.6	nd	nd	1.4	1.6	nd	1
infant milk with added micronutrients	17	nd	nd	nd	0.6	nd	1
whey drink	13	nd	nd	nd	1.1	2.3	1

[a] Data are given as ranges in mg/L and mg/kg, respectively. 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3-DPs, 3-deoxypentosone; GO, glyoxal; MGO, methylglyoxal; HMF, 5-hydroxymethylfurfural; *n*, number of samples; nd, not detected; tr, trace amounts, between LOD and LOQ.

In line with our hypothesis, both 3-DG and 3-DGal were detected in all semiskimmed lactose-hydrolyzed UHT milks. Chromatograms of milk samples are shown in **Figure 2A**. The identity of the two peaks was verified by comparison with the independently synthesized standard quinoxalines and by HPLC-DAD-ESI-MS. Both peaks showed the same characteristic quinoxaline UV spectrum and a prominent peak at m/z 235.1 in the mass spectrum, which could be ascribed to the molecular ion $[M + H]^+$ of the quinoxalines of 3-DG and 3-DGal (**Figure 2B**,**C**). 3-DG and 3-DGal were quantified in semiskimmed lactose-hydrolyzed UHT milks with values ranging between 2.5 and 18 mg/L and between 2.0 and 11 mg/L, respectively (**Table 3**). Interestingly, milk from different batches of the same producer differed more strongly in its 3-DGal than in its 3-DG content. Contents of 3-DG were always about 70−80% higher than those of 3-DGal.

This is the first report on quantitative data for 3-DGal in food after its detection in beer (*7*). Moreover, glyoxal was determined in four of the five lactose-hydrolyzed milk samples. The relatively wide concentration ranges (coefficient of variation was 43.4% for 3-DG and 41.6% for 3-DGal) indicate that differences in the production process of lactose-hydrolyzed milk obviously affect not only heating markers such as furosine, lactulose, or fructose (*30*) but also the 1,2-dicarbonyl contents, although to a lesser extent. The 1,2-dicarbonyl contents of other lactose-hydrolyzed milk products also tended to be higher when compared to their conventional equivalents (**Table 3**). The 3-DG contents of complex milk-based foods with added fruit preparations, micronutrients (e.g., iron), or juices (whey drink) were higher than those of the pure milk products, revealing the possibility of carry-over from the ingredients. 3-DPs, a specific degradation product of lactose (*31*), was detected only in evaporated milk. The concentrations of GO and MGO in fermented products such as yogurt are in agreement with literature values (*10*). In summary, 3-DG and 3-DGal are the most important 1,2-dicarbonyl compounds in all of the milk products tested. The content of both compounds in UHT milk increases, when lactose is hydrolyzed.

With the exception of evaporated milk, HMF is only of minor importance in milk products. Contents of HMF measured in fruit yogurts and the whey drink can also be explained by carry-over from fruit preparations, which can contain considerable amounts of HMF (*32*). The peaks of the quinoxalines of glucosone and galactosone are also visible in the chromatograms in **Figure 2A** with peak areas ranging up to 8−13% and 20−31%, respectively, of the peak areas of the corresponding 3-deoxyhexosones. These two quinoxalines, however, were not detectable in all lactose-hydrolyzed milk samples, so that they cannot solely be regarded as derivatization artifacts as stated above. It can tentatively be stated that glucosone and galactosone occur in some lactose-hydrolyzed milks and that their presence must be dependent on the manufacturing process, even though they cannot accurately be quantified. Especially, data on the occurrence of galactosone in foods could be of physiological relevance, because it was found to be an inhibitor of glucuronidation in isolated rat hepatocytes (*33*).

Influence of Storage on 1,2-Dicarbonyl Compounds in Milk. A storage experiment was performed to evaluate if and how the contents of 1,2-dicarbonyl compounds change during storage. UHT-treated milk samples, one conventional from one producer and two lactose-hydrolyzed from two different producers (milks A and B), were stored for up to 4 months at three different temperatures and analyzed for 1,2-dicarbonyl compounds, HMF, and furosine. As lactose-hydrolyzed milk products can contain two main Amadori products, namely, fructoselysine and tagatoselysine, it was necessary to perform the acid hydrolysis in the presence of 7.3 N hydrochloric acid. Only at this acid

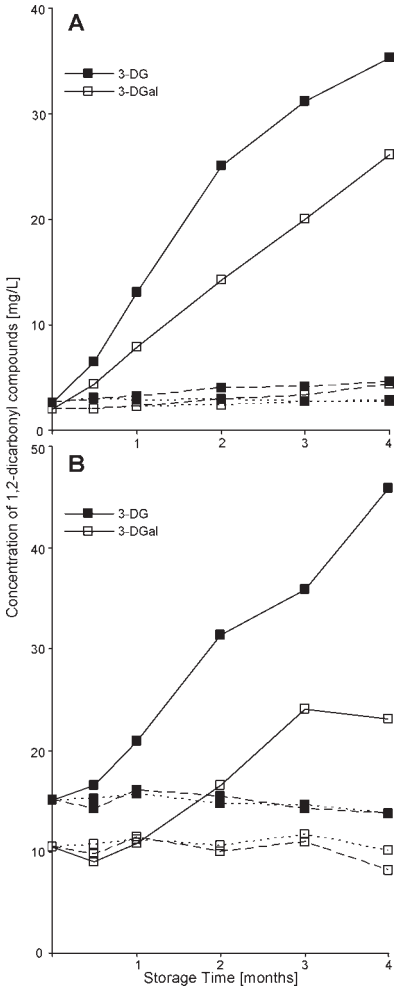

Figure 3. Development of the concentrations of 3-deoxyglucosone (3-DG) and 3-deoxygalactosone (3-DGal) during storage at 4 °C (- - -), 20 °C (— — —), and 37 °C (—) of lactose-hydrolyzed milk from two different producers (**A** and **B**). Data are means ± SD (n = 2−3).

concentration are both products converted to furosine to the same extent (*34*), and the furosine content is proportional to the total Amadori product content.

In conventional UHT milk, the concentrations of all analytes were low. The furosine content of 90.4 mg/100 g of protein measured for the UHT-treated conventional milk was in agreement with published data (*30*). There was a slight increase by 25% in the furosine content after 4 months at room temperature, which was also measured by Messia et al. (*30*), and a 5-fold increase after 4 months at 37 °C (data not shown). This, however, was not paralleled by an increase in the contents of the two 3-deoxyhexosones. The concentration of 3-DPs was below the LOQ at the beginning of the experiment and rose to not more than 0.09 mg/L after 4 months of storage only at 37 °C. In the lactose-hydrolyzed milks, the initial furosine values were higher (167.7 and 310.0 mg/100 g of protein for milks A and B, respectively). Moreover, the increase of furosine during storage was more pronounced (+95% for milk A and +59% for milk B after 4 months at room temperature), as already stated by Messia et al. (*30*), and it correlated with an increase in both the 3-DG and 3-DGal contents. The concentration of Amadori products, however, was still 10−30 times higher than that of the 3-deoxyhexosones. A closer look at the development of the concentrations of the 3-deoxyhexosones during storage (**Figure 3**) reveals that they increase at all temperatures throughout the experiment in milk A, in which the

concentrations were low at the beginning. In milk B, however, which was relatively high in 3-deoxyhexosones at the beginning, the concentrations tended to decrease, especially at lower storage temperatures.

These varying results indicate that 3-DG and in particular 3-DGal are kinetically, but not thermodynamically, stable. Formation and degradation kinetics seem to be differently promoted by different temperatures depending on the concentration. As already noted during synthesis, 3-DGal is less stable than 3-DG, because it is degraded more quickly than 3-DG (**Figure 3**). The same effect was observed when lactose-hydrolyzed milk was heated at 80 °C for up to 5 h. Here, the concentration of 3-DG increased by 160% during the experiment, whereas that of 3-DGal had increased by only 70% after 4 h and then declined (data not shown).

HMF as a possible degradation product of 3-DG and 3-DGal and a widely used marker of heat treatment was found in only one of the two lactose-hydrolyzed milks throughout the storage experiment, namely, in milk A, which showed the lower concentrations of 3-deoxyhexosones and furosine. 3-DG and 3-DGal might serve as more appropriate markers for monitoring heat processing and storage because they appear earlier in the reaction cascade, which finally leads to HMF. For practical purposes, it will be necessary to thoroughly investigate how the formation and degradation of 3-DG and 3-DGal depend on different processing conditions, for example, temperature, time of heating, and pH milieu.

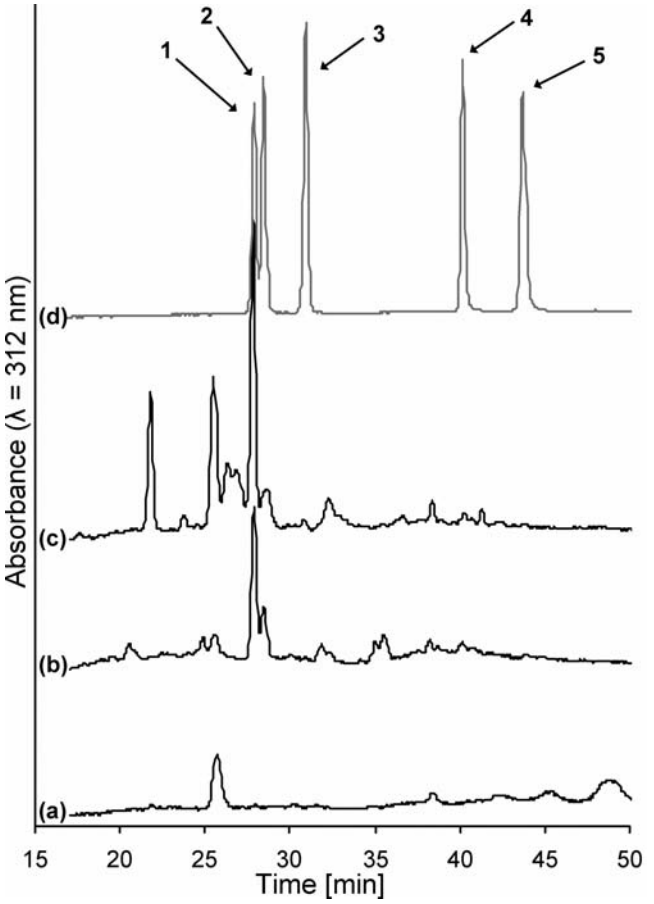

Figure 4. Chromatograms of equally prepared samples of (a) cola drink, (b) Pilsner beer, and (c) apple juice, and (d) standard chinoxaline mixture acquired by Phenyl-RP-HPLC with UV detection. Arrows indicate the peaks of the quinoxalines of (1) 3-deoxyglucosone, (2) 3-deoxygalactosone, (3) 3-deoxypentosone, (4) glyoxal, and (5) methylglyoxal.

Analysis of Other Liquid Food Samples. Encouraged by these findings, we sought to expand the study to other liquid samples such as soft drinks, juice, and beer, which could easily be introduced into the analysis protocol. Chromatograms of three samples are shown in **Figure 4**. Soft drinks such as cola sweetened with sucrose displayed contents of 3-DG of only up to 0.7 mg/L, which is far lower than the values published by Lo et al. (*16*), who found 10−34 mg/L 3-DG in a variety of carbonated beverages. On the other hand, 3-DG was present in four apple juices (11−49 mg/L) and five dark beers (27−41 mg/L). As already discussed above for milk, this can be explained by the highly stable full acetal sucrose in soft drinks compared with less stable monosaccharides in fruit juices and lactose-hydrolyzed milk. The most striking finding, however, was the occurrence of 3-DGal in these samples. 3-DGal was found in three of four apple juices in concentrations up to 3.3 mg/L and in all dark beer samples with concentrations ranging between 11 and 15 mg/L. The small amounts of 3-DGal in fruit juices might originate from traces of galactose, but the galactose content was reported not to exceed 400 mg/kg in most fruits, whereas glucose contents are >100-fold higher (*35*). Moreover, the data concerning lactose-hydrolyzed milk products show that when starting from equimolar mixtures of glucose and galactose, slightly more 3-DG than 3-DGal is formed, resulting in disproportionately high 3-DGal contents in fruit juices and beer. A plausible explanation for these findings is the interconversion of 3-DG and 3-DGal via 3,4-dideoxyglucosone-3-ene (3,4-DGE) as stated by Bravo et al. (*7*). Therefore, two pathways of 3-DGal formation have to be taken into account, one directly from galactose as in lactose-hydrolyzed milk products and the other by epimerization from 3-DG (**Figure 5**). We strongly support this hypothesis, because 3,4-DGE will only react to HMF when it is present in the *Z*-form. The *Z*-form, however, is sterically not

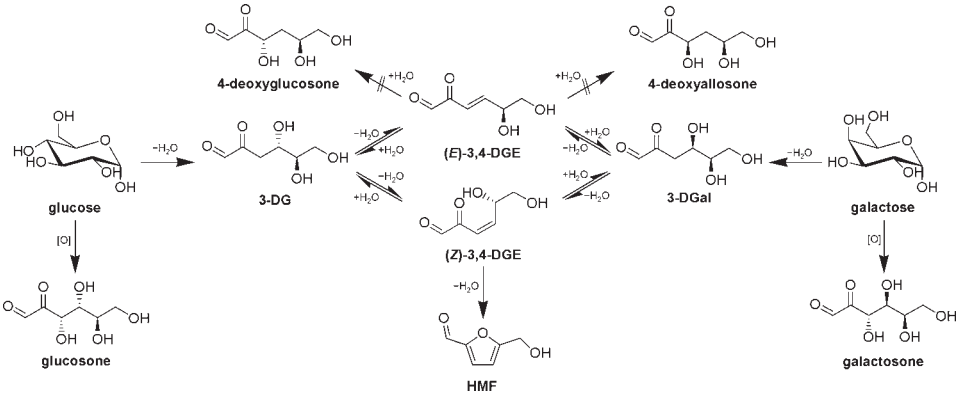

Figure 5. Proposed pathways of generation of 3-deoxygalactosone (3-DGal) and galactosone including the interconversion between 3-DG and 3-DGal during caramelization. 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3,4-DGE, 3,4-dideoxyglucosone-3-ene; HMF, 5-hydroxymethylfurfural.

favored, and therefore, (*E*)-3,4-DGE might accumulate to some extent, thereby also permitting its analysis (*15*, *21*, *22*). Rehydration of the vinylogous system should be favored at the C4 and lead to both 3-DG and 3-DGal, and not at the C3, so that 4-deoxy products (**Figure 5**) will most probably not be formed. We suppose that 3-DG and 3-DGal differ in their tendency to form either (*E*)- or (*Z*)-3,4-DGE and are currently working on the further elucidation of the respective degradation pathways.

In conclusion, RP-HPLC using a phenyl phase represents a promising tool for the analysis of 1,2-dicarbonyl compounds in foods. The application of this method has shed first light on the extent of sugar degradation in lactose-hydrolyzed milk products and other foodstuffs, showing that the occurrence of 1,2-dicarbonyl compounds, among which 3-DG (and 3-DGal) predominate, depends primarily on the stability of the food sugar constituents. With this in mind, high concentrations of 1,2-dicarbonyl compounds, especially 3-DG and 3-DGal, can be expected in sugar-rich food such as jams, sweets, and canned fruits. A thorough survey of 1,2-dicarbonyl compounds in commonly consumed foods will be the subject of our further research.

ABBREVIATIONS USED

3,4-DGE, 3,4-dideoxyglucoson-3-ene; 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3-DPs, 3-deoxypentosone; DETAPAC, diethylenetriaminepentaacetic acid; GO, glyoxal; HMF, 5-hydroxymethylfurfural; HPLC, high-pressure liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MGO, methylglyoxal; NMR, nuclear magnetic resonance; OPD, *o*-phenylenediamine; RP, reversed phase; TCA, trichloroacetic acid; UHT, ultrahigh temperature.

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