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Formation of α-Dicarbonyl Compounds in Beer during Storage of Pilsner

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With the aim of determining the formation of α-dicarbonyl intermediates during beer aging on the shelf, α-dicarbonyls were identified and quantified after derivatization with 1,2-diaminobenze to generate quinoxalines. The sensory effects of α-dicarbonyls were evaluated by the quantification of key Strecker aldehydes and by GC-olfactometry (GCO)analysis of beer headspace using solid phase microextraction. Four α-dicarbonyls, reported here for the first time, were detected in fresh and aged beers, three were derived from the 2,3-enolization pathway of mono- and disaccharides, and the fourth was derived from the epimerization of 3-deoxy-2-hexosulose. Ten α-dicarbonyls were quantified during beer processing and during different periods of beer aging at 28 °C. The aging periods were from 15 to 105 days. During beer aging, 1-deoxydiuloses were produced and degraded, while 1,4-dideoxydiuloses were produced at the highest rates. The GCO analysis indicated that forced beer aging increased the amounts of furaneol, *trans*-2-nonenal, and phenylacetaldehyde. The blockage of α-dicarbonyls inhibited the accumulation of sensory-active aldehydes in the beer headspace.

KEYWORDS: α-Dicarbonyl quantification; beer aging; 1,4-dideoxydiuloses; 1-deoxydiuloses; GC-olfactometry

INTRODUCTION

The most appreciated characteristic of beer by consumers is its fresh flavor. This appealing characteristic is progressively lost on the shelf due to flavor changes. These flavor changes deteriorate the beer flavor and are referred to as aging. A technological solution to this important practical problem, however, is not in sight because of the incomplete knowledge concerning the mechanisms involved in beer aging and the flavor-active compounds responsible for flavor deterioration. Until now, most of the approaches considered in the brewing industry have been oriented toward the elimination of oxygen uptake throughout the brewing process to eliminate *trans*-2-nonenal or its precursors from malt and wort (*1*). Nevertheless, despite all efforts concerning this subject, only a relatively small improvement in beer flavor stability has been attained (*2*). Reducing the oxygen content in packaged beer only gives a product of limited flavor stability (*3*). Therefore, other chemical mechanisms and/or pathways different from the oxidative pathway that also contribute to flavor formation should be considered, among them the Maillard reaction, which may have an important role in flavor stability.

In our previous work, the formation of a group of α-dicarbonyl intermediates, arising most likely from carbohydrate degradation, were determined in beer for the first time (*4*). In that study, aging took place at 28 °C, which can be considered a mild to low temperature for most model Maillard reaction studies. It was observed that the formation of 5-hydroxymethylfurfural (5-HMF) from the dehydration of 3-deoxy-2-hexosulose (3-DH) correlated well with beer aging. However, it was not clear as to how α-dicarbonyl intermediates originating from 2,3-enolization, 1-deoxydiuloses, were involved as precursors of aroma-active compounds such as Strecker aldehydes and cyclic enolones. Our hypothesis was that these α-dicarbonyls may play an important role in beer flavor deterioration even at this mild condition since a significant increase in the concentration of 1,4-DDP and 1,4-DDH was observed in beer stored at 28 °C, 1,4-DDP and 1,4-DDH being derived from the Strecker degradation of 1-DP and 1-DH in the presence of amino acids (*5*). An alternative mechanism for 1,4-DDH formation, the so-called "peeling off" mechanism, was proposed for the quasi-water-free thermolysis of maltose (*6*). In our previous work, the formation of 1-DH also was detected in beers, (*4*) but its concentration could not be estimated and neither could the

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presence of 1-DP be determined. This would have allowed us to identify 1-deoxydiulose degradation pathways in beer.

Most of the studies in the literature indicating the formation of α-dicarbonyls in model systems were performed using mixtures of carbohydrates and amino acids at high concentrations (0.5−1 M) and high reaction temperatures (>90 °C) (*7, 8*). Yet, these conditions are far from those found in beer during aging. Beer, as a Maillard system, consists mainly of a mixture of hexose-derived Amadori compounds (*9*), 3-deoxyosones (*4*), and some monosaccharides and amino acids, all of them at micromolar concentrations. Furthermore, the relatively low storage temperatures (20−30 °C) relevant for beer aging on the shelf decrease the reaction rates of α-dicarbonyl formation as compared to typical Maillard model systems. This situation makes it necessary to expand the observation time from a few hours in a Maillard system to weeks in the case of beer aging.

In our previous study, it was observed that the addition of aminoguanidine to beer decreased flavor deterioration, specifically the development of bread and caramel flavor notes (*4*). However, it could not be ascertained as to whether this effect was due to the inactivation of α-dicarbonyls (*4*). In the current work, the ability of aminoguanidine to block the degradation of α-dicarbonyls in beer during storage was studied and compared to that of 1,2-DAB. The aim of this study was to select an effective reagent to block α-dicarbonyl degradation during beer aging and to carry out sensorial studies by GCO.

Therefore, in this work, the isolation and identification of four α-dicarbonyls that had not been detected in beer before are described. Second, a thorough quantitative follow-up of eight α-dicarbonyls during beer aging is reported. Finally, a study of the relevance of α-dicarbonyls as flavor precursors of beer aging was performed by employing the blockage strategy with 1,2-DAB. The effects on the flavor were measured by both quantifying key Strecker aldehydes and by GCO analysis of the beer headspace.

The low reactant concentration in fresh beer makes it necessary to use analytical methods that include efficient concentration and isolation steps. The diversity of known α-dicarbonyls also makes necessary the employment of high-resolution chromatographic techniques such as capillary GC that allow sensitive determinations. A HRGC-MS method also was developed for the quantification of α-dicarbonyls as quinoxalines. The MS detection was necessary to ensure adequate selectivity.

EXPERIMENTAL PROCEDURES

Beer. The samples for the present study were obtained from commercial Venezuelan Pilsner beer. Beer samples were taken after bottling and pasteurization. The dissolved oxygen content was <0.20 mg/L and had a pH of 4.3. Storage of samples for the aging process was carried out either in a cool room at 5 °C or in a temperature controlled room at 28 °C.

Materials. ACS grade chemicals were obtained from Aldrich (St. Louis, MO), Sigma (St. Louis, MO), Sylon BFT Supelco (Bellefonte, PA), and Merck (Darmstadt, Germany). The reference compounds of the odorants were obtained commercially: ethyl butyrate, ethyl hexanoate, ethyl-3-methylbutanonate, ethyl octanoate, furaneol, 3-methylbutanoic acid, 2-methylpropanol, 3-(methylthio)-propionaldehyde, and (*E*)-2-nonenal (Aldrich, St Louis, MO); 3-methylbutanol, ethyl-3-methylbutanoate, 3-methylbutanoic acid, and linalool (Sigma, St. Louis, MO); and γ-nonanoic lactone, 2-phenylethyl acetate, and DMS (Chem Service, West Chester, PA).

Syntheses of Reference Material. *3-DH.* The preparation was performed without modifications following the method reported by Madson and Feather (*10*).

2-(2′(S),3′(R),4′-Trihydroxybutyl) Quinoxaline (3-DH Quinoxaline). 3-DH quinoxaline was prepared following the method reported by Hofmann et al. (*11*) and was purified by RP-HPLC. 3-DH (6.2 mmol) and 1,2-DAB (6 mmol) were stirred in water (20 mL) for 1 h at 60 °C. The reaction mixture was extracted with methylene chloride (5 × 100 mL), and then the combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated in vacuo. The crude products were purified by column chromatography (3.0 cm × 20.0 cm) over silica gel (100 g, TLC 60 GF_{254}, 15 μm, Merck) by elution with acetonitrile/water (9:1, v/v). The fraction containing 3-DH quinoxaline was dried in vacuo. The solids were resuspended in a mixture of acetonitrile and water (20:80, v/v) and separated by semipreparative isocratic RP-HPLC (acetonitrile/water, 4:96, v/v). The target quinoxaline eluted at t_R = 14−16 min. Acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 0.34 mmol): ^{1}H NMR (400 MHz, in DMSO-d_6, COSY, HSQC) δ 3.00 [dd, 1H, ^2J = 13.6 Hz, ^3J = 9.7, -CHaHb-CH(OH)-], 3.32 [dd, 1H, ^2J = 13.6 Hz, ^3J = 2.9, -CHaHb-CH(OH)-], 3.45 [m, 2H, -CH(OH)-CHaHb(OH)], 3.64 [dd, 1H, ^2J = 13.4, ^3J = 6.8, -CH(OH)-CHaHb(OH)], 3.91 [m, 1H, -CH(OH)-CH(OH)-], 4.56 [m, 1H, -CH_2(OH)], 4.81 [m, 2H, -CH(OH)-CH(OH)-], 7.80 [m, 2H], 8.04 [m, 2H], 8.86 [s, 1H, 2 × *N*=CH−]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 450 (2, $[M]^{+\cdot}$), 435 (8), 360 (26), 347 (23), 245 (100), 205 (16), 147 (30), 73 (72).

2-(1′,2′,3′-Trihydroxypropyl)-3-methyl Quinoxaline (1-DH Quinoxaline). 1-DH quinoxaline was prepared following the method reported by Hofmann et al. (*11*) and purified by RP-HPLC. A mixture of glucose (98 mmol), alanine (91 mmol), and 1,2-DAB (74 mmol) in phosphate buffer (400 mL, 0.5 mol/L, pH 6.8) was refluxed for 12 h. After cooling at room temperature, the reaction mixture was extracted with methylene chloride (5 × 100 mL), and the combined organic extracts were dried over anhydrous $MgSO_4$ and after concentration separated by column chromatography (3.0 cm × 30.0 cm) over silica gel (100 g, TLC 60 GF_{254}, Merck), conditioned with ethyl acetate. Elution was performed with ethyl acetate (200 mL), followed by ethyl acetate/methanol (50:50, v:v, 300 mL), affording the target compound as a crude product. Further fractionation by semipreparative RP-HPLC allowed the isolation of the target compound as well as 2-(2′,3′-dihydroxypropyl)-3-methyl quinoxaline. The separation was performed isocratically (acetonitrile/water, 4:96, v/v). The target quinoxaline eluted at t_R = 12−14 min. The acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 0.27 mmol): ^{1}H NMR (400 MHz, in DMSO-d_6, COSY, HSQC) δ 2.80 (s, 3H, -CH_3), 3.63 [m, 1H, -CH(OH)-CHaHb(OH)], 3.77 [m, 1H, -CH(OH)-*C*HaHb(OH)], 3.96 [m, 1H, -CH(OH)-CH_2(OH)], 4.89 [dd, 1H, ^3J = 8.24, $J_{H,OH}$ = 6.1, -CH(OH)-*C*H(OH)-], 4.56 [m, 1H, -CH_2(OH)], 4.66 [d, 1H, $J_{H,OH}$ = 5.6, -CH(OH)-CH(OH)-], 5.52 [d, 1H, $J_{H,OH}$ = 6.1, -CH(OH)-CH(OH)-], 7.77 [m, 2H], 8.01 [m, 2H]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 450 (1, $[M]^{+\cdot}$), 435 (6˙), 347 (3), 246 (100), 205 (36), 147 (42), 73 (91).

2-(2′,3′-Dihydroxypropyl)-3-methyl Quinoxaline (1,4-DDH Quinoxaline). 1,4-DDH quinoxaline was isolated from the same crude fraction that contained 1-DH quinoxaline by semipreparative RP-HPLC (as explained previously) and eluted at t_R = 22−24 min. The acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 0.26 mmol): ^{1}H NMR (300 MHz, in chloroform-d_3) δ 2.72 (s, 3H, −C=C−CH_3), 3.14 [m, 2H, −C=C−CH_2−CH(OH)−], 3.72 [dd, 1H, ^2J = 11.24, ^3J = 5.69, -CH(OH)-CHaHb(OH)], 3.84 [dd, 1H, ^2J = 11.24, ^3J = 3.68, -CH(OH)-CHaHb(OH)], 4.48 [m, 1H, -CH(OH)-CH_2(OH)], 7.68 [m, 2H], 7.97 [m, 2H]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 362 (<1, $[M]^{+\cdot}$), 347 (24˙), 272 (100), 259 (87), 158 (60˙), 205 (7), 147 (22), 73 (64).

2-(1′,2′-Dihydroxyethyl)-3-methyl Quinoxaline (1-DP Quinoxaline). 1-DP quinoxaline was prepared following the method reported by Hofmann (*12*) and purified by RP-HPLC. A mixture of xylose (19.6 mmol), alanine (18.3 mmol), and 1,2-DAB (14.8 mmol) in phosphate buffer (80 mL, 0.5 mol/L, pH 6.8) was refluxed for 10 h. After cooling to room temperature, the reaction mixture was extracted with CH_2Cl_2 (5 × 20 mL), and the combined organic extracts were dried over anhydrous $MgSO_4$ and after concentration were separated by column chromatography (3.0 cm × 35 mm) over silica gel (120 g, TLC 60 GF_{254}, Merck) conditioned with ethyl acetate. Elution was performed

with ethyl acetate (300 mL), followed by ethyl acetate/methanol (1:1, 500 mL) affording the target compound as a crude material. Further fractionation by semipreparative RP-HPLC allowed the isolation and purification of the target compound as well as 2-(2′-hydroxyethyl)-3-methyl quinoxaline (1,4-DDP quinoxaline) and 2-(2′,3′-dihydroxypropyl) quinoxaline (3-DP quinoxaline). Separation was performed isocratically using acetonitrile/water (4:96, v/v). The target quinoxaline eluted at $t_R = 20-22$ min. Acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 3.28 mmol): ^{1}H NMR (400 MHz, in D_2O) δ 2.60 (s, 3H, -C$\underline{H}_3$), 3.71 [dd, 1H, $^2J = 11.96$, $^3J = 7.08$, -CHaHb(OH)], 3.80 [dd, 1H, $^2J = 11.96$, $^3J = 4.12$, -CHa$\underline{H}$b(OH)], 5.05 [dd, 1H, $^3J = 7.08$, $^3J = 4.12$, -C$\underline{H}$(OH)-], 7.42 [m, 2H], 7.64 [m, 2H]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 348 (25, $[M]^+$), 333 (17), 258 (26), 245 (100), 169 (7), 147 (18), 73 (55).

2-(2′-Hydroxyethyl)-3-methyl Quinoxaline (1,4-DDP Quinoxaline). 1,4-DDP quinoxaline was isolated from the same fraction containing 1-DP quinoxaline by semipreparative RP-HPLC. Separation was performed isocratically using a mixture of acetonitrile and water (4:96, v/v). The target quinoxaline eluted at $t_R = 65-67$ min. The acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 0.23 mmol): ^{1}H NMR (300 MHz, in DMSO-d_6) δ 2.72 (s, 3H, —C═C—C$\underline{H}_3$), 3.14 [t, 2H, $^3J = 6.90$, —C═C—C$\underline{H}_2$—CH$_2$(OH)], 3.90 [dt, 2H, $^3J = 6.90$, $^3J_{H\text{-}OH} = 5.49$, -CH$_2$-C$\underline{H}_2$(OH)], 4.73 [t, 1H, $^3J_{H\text{-}OH} = 5.49$, -CH$_2$-CH$_2$(O$\underline{H}$)], 7.72 [m, 2H], 7.96 [m, 2H]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 260 (34, M^+), 245 (60), 170 (100), 143 (19), 73(63).

2-(2′,3′-Dihydroxypropyl) Quinoxaline (3-DP Quinoxaline). 3-DP quinoxaline was isolated from the same fraction containing 1-DP quinoxaline by semipreparative RP-HPLC. The separation was performed isocratically using a mixture of acetonitrile and water (4:96, v/v). The target quinoxaline eluted at $t_R = 17-19$ min. The acetonitrile was eliminated by evaporation in vacuo and then freeze-dried (white powder, 0.07 mmol): (400 MHz, in D_2O) δ 3.05 [dd, 1H, $^2J = 14.1$ Hz, $^3J = 8.8$, -CHaHb-CH(OH)-], 3.16 [dd, 1H, $^2J = 14.1$ Hz, $^3J = 4.4$, -CHa$\underline{H}$b-CH(OH)-], δ 3.58 [dd, 1H, $^2J = 11.7$ Hz, $^3J = 4.0$, -CHaHb(O$\underline{H}$)], 3.69 [dd, 1H, $^2J = 11.7$ Hz, $^3J = 6.2$, -CHa$\underline{H}$b(OH)], 4.16 [m, 1H, -C$\underline{H}$(OH)], 3.64 [dd, 1H, $^2J = 13.4$, $^3J = 6.8$, -C$\underline{H}$(OH)-CHa$\underline{H}$b(OH)], 7.76 [m, 2H], 7.90 [m, 2H], 8.70 [s, 1H, 2 × *N*═C$\underline{H}$—]; HRGC-MS (MS/EI, trimethylsilylated quinoxaline) *m/z* 348 (8, $[M]^{+\cdot}$), 333 (29$^{\cdot}$), 258 (100), 245 (2), 169 (48), 147 (29), 144 (35), 73 (67).

2-Propyl-3-methyl Quinoxaline (IS). 2,3-Hexanedione (44 mmol) and 1,2-DAB (46 mmol) were stirred in methanol (20 mL) for 1 h at 60 °C. The reaction mixture was extracted with chloroform (3 × 10 mL), the combined organic layers were washed with 0.1 M HCl (3 × 10 mL) and dried over anhydrous $MgSO_4$. The chloroform was evaporated until dryness in vacuo. Quinoxaline was obtained as a reddish brown liquid: ^{1}H NMR (400 MHz, chloroform-d_3) δ 1.06 ppm (t, 3H, $^2J = 7.3$), 1.85 (tc, 2H, $^2J = 7.3$ and 6.5), 2.75 (s, 3H), 2.96 (t, 2H, $^2J = 6.5$), 7.65 (m, 2H), 7.98 (m, 2H).

Quantification of α-Dicarbonyls by HRGC-MS. A 2.2 mL aliquot of 460 mM 1,2-DAB (Sigma, St. Louis, MO) in methanol was added to 222 mL of beer kept in a bottle and under a CO_2 stream at ambient temperature. The bottle was immediately recapped with crown caps using a bench capper. The beer pH increased to 4.5 after 1,2-DAB addition. No further pH adjustment was carried out. Two derivatization procedures were carried out, the reaction time being the difference between them. In the derivatization procedure 1 (DE1), the reaction time with 1,2-DAB varied with the aging period. In this case, bottles were stored at 28 °C up to 3 months. In the derivatization procedure 2 (DE2) 1,2-DAB was allowed to react in beer at 28 °C for 24 h. After the storage period, 15 μL of 0.1006 M 2-propyl-3-methyl quinoxaline (synthetic material, see Syntheses of Reference Material) in methanol was added to a 25 mL portion of each beer sample as an IS, and the beer was extracted with chloroform (3 × 10 mL) to obtain the chloroform and aqueous soluble quinoxalines.

Quantification of Chloroform Soluble Quinoxalines. The combined chloroform extracts were dried over anhydrous $MgSO_4$ and evaporated in vacuo until dry. The solid obtained was resuspended in BSTFA and TMCS, 99:1 (Supelco, Bellefonte, PA) (400 μL) and heated (1 h at 60 °C), and then 1 μL of the solution was injected into the GC instrument. The α-dicarbonyls methylglyoxal, 2,3-butanedione, 1,4-DDP, 1-DP, and 1,4-DDH were detected as their corresponding quinoxaline derivatives at the t_R given in parentheses: 2-methylquinoxaline (Aldrich) (10.2 min), 2,3-dimethylquinoxaline (Aldrich) (12.3 min), 1,4-DDP quinoxaline (synthetic material) (19.7 min), 1-DP quinoxaline (synthetic material) (21.4 min), and 1,4-DDH quinoxaline (synthetic material) (23.9 min). Calibration curves were prepared by treating standard solutions containing known amounts of each pure, synthetic reference compound as described previously for beer samples. The peak areas were determined at the target ions *m/z* 144, 158, 170, 245, and 272, respectively, for the mentioned quinoxalines and *m/z* 158 for the IS quinoxaline.

Quantification of Water Soluble Quinoxalines. After extraction with chloroform, 10 mL of the remaining aqueous phase was spiked with the IS (0.1006 M, 60 μL) and passed through a preconditioned LC-18 solid phase extraction cartridge (6 mL, Supelclean, LC18, Supelco). The cartridge was washed with distilled water (10 mL), and quinoxalines were eluted with methanol (HPLC grade, 4 mL) and dried under a stream of nitrogen. The solid was resuspended in BSTFA and TMCS, 99:1 (Supelco) (500 μL) and acetonitrile (500 μL) and heated (1 h, 60 °C), and then 1 μL of the solution was injected into the GC-MS. The α-dicarbonyls 3-DP, 1-DH, and 3-DH were detected as their corresponding quinoxaline derivatives at the t_R given in parentheses: 3-DP quinoxaline (synthetic material) (23.3 min), 1-DH quinoxaline (synthetic material) (24.7 min), and 3-DH quinoxaline (synthetic material) (26.8 min). The peak areas were determined at the target ions *m/z* 245, 246, and 245, respectively, for the mentioned quinoxalines and *m/z* 158 for the IS. To verify the performance of the developed HRGC-MS method for α-dicarbonyls, parameters such as limit of detection (LOD), linearity, and recovery were evaluated. LOD limits were calculated following the IUPAC approach (*13*), which consists of analyzing blank beer samples ($n = 10$), calculating the standard deviation, and expressing the results as the blank average value plus 2.26 (the *t* value for 95% probability and 9 degrees of freedom) times the standard deviation. The IS was added to each blank sample to correct for the injection and extraction errors. The recoveries of α-dicarbonyls from beer were assessed by spiking the corresponding pure quinoxaline (25, 60, 9, 15, 120, 9.8, 20, and 25 μM for 1,4-DDP, 1,4-DDH, 1-DP, 3-DP, 3-DH, 1-DH, diacetyl, and methylglyoxal, respectively). Three replicates were prepared for the recovery study.

Quantification of Strecker Aldehydes by SPME-HRGC-MS. The aldehydes 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal (Aldrich) were quantified by the method developed by Vesely et al. (*14*) with slight modifications. Briefly, a 65 μm PDMS/DVB SPME fiber (Supelco) was exposed to a PFBHA solution (6 g/L) for 5 min at 40 °C. The loaded fiber was then exposed (30 min at 50 °C) to the headspace of 20 mL beer samples placed in 40 mL vials containing the IS (3-methyl-2-butenal, 200 μL, 2 mg/L). Derivatized aldehydes were desorbed from the fiber in the HRGC-MS injection port.

Semipreparative RP-HPLC. A Waters HPLC System LC Module I Plus was used, consisting of a 600 pump, Wisp 717 autosampler, UV—vis 486 detector operating at 320 nm, and an automatic fraction collector from Waters. Separations were performed on a stainless steel column (6 μm, 60 Å, 19 mm × 300 mm i.d., C18 Prep Nova Pak HR, Waters Corp.) at a flow rate of 10 mL/min. Chromatographic data were acquired using Millennium 2010 Chromatography Manager (Waters Corp, v. 2.15).

HRGC-MS. HRGC-MS was performed using a Hewlett-Packard HP6890 GC (Agilent Technologies, Palo Alto, CA) instrument by using a capillary HP-5MS column (30 m × 0.25 mm i.d., 0.25 μm, Agilent Technologies) and a split/splitless inlet at 220 °C. The quinoxaline samples were applied in the split mode (5:1), and the oven temperature was 70 °C. The temperature of the oven was raised at 6 °C/min to 260 °C, then raised at 25 °C/min to 280 °C and held for 2 min. For Strecker aldehyde analysis, the PDMS/DVB fiber was desorbed in the injection port by being heated in the pulsed splitless mode for 1 min at 250 °C (9.9 psi for 0.5 min). The oven temperature program was as follows: 70 °C (1 min), then 6 °C/min to 200 °C, and 25 °C/min to 265 °C (5 min). For both methods, MS analysis was performed with a Hewlett-Packard 5973A mass selective detector in the electron impact mode (70 eV), and the mass range was between *m/z* 15 and 550.

Table 1. NMR data for **1** (Dimethylsulfoxide-d_6)

position	δ_C (ppm)	DEPT	δ_H[a,b] (ppm)	COSY	HMBC
2	149.90	C			H2′
3	154.69	C			H20
5	128.62	CH	8.05 (m)		
6	131.04	CH	7.85 (m)		H8
7	129.91	CH	7.85 (m)		H5
8	129.43	CH	8.05 (m)		
9	141.48	C			H8, H7
10	139.95	C			H6, H5
1′	151.79	C			H13a, H13b H12, H14
2′	110.51	CH	5.74 (t; 7.30)	H13a, H13b	
3′	57.43	CH_2	Ha, 3.75 (dd; 7.70; 12.00)	H12, H13b	
			Hb, 3.80 (dd; 7.00; 12.00)	H12, H3′a	
1″	98.93	CH	5.30 (d; 3.64)	H15	H12
2″	72.12	CH	3.32 (m)	H14	
3″	74.42	CH	3.479 (m)	H17	H1″
4″	70.22	CH	3.19 (t; 9.1)	H16, H18	
5″	73.47	CH	3.41 (m)	H17	H14
6″	61.13	CH_2	Ha, 3.55 (dd; 4.8; 10.10)	H17, H19b	
			Hb, 3.60 (d; 10.27)	H19a	
1‴	23.09	CH_3	2.77 (s)		

[a] Assignments based on HMQC. [b] Coupling constants are given in hertz.

GCO. GCO was performed using a Hewlett-Packard HP6890 GC (Agilent Technologies) instrument, with a capillary HP-5MS column (12 m × 0.32 mm i.d., 0.25 μm, Agilent Technologies), a CIS 4 PTV inlet, and an odor detection port, both from Gerstel (Mülheim, Germany). The DVB/car/PDMS fiber previously exposed to the sample headspace was desorbed in the injection port by being heated in the pulsed splitless mode for 1 min at 250 °C (9.9 psi for 0.5 min). The oven temperature program was as follows: 35 °C (5 min), 35−180 °C (4.5 °C/min), 180−250 °C (35 °C/min), and 250 °C (5 min). Panelists activated an electronic device during the elution of an aroma peak. The device gives rise to an analogical signal. This signal is linearly dependent on time while the panelist is pressing the button and decays exponentially after it is released. The analogical signal was registered directly into the Chemstation software (Agilent Technologies) through a controller board installed in the GC instrument. Panelists also described the odor of the eluting peaks. Two trained panelists were used. Samples were analyzed in quadruplicate by each panelist. Alkane linear retention indices (RI) of the compounds were calculated according to a reported procedure (*15*). Identification of volatiles was performed based on the comparison of RI, EI mass spectra, and odor quality perceived at the sniffing port with reference substances.

HRMS/EI. High-resolution mass spectra were obtained in a Jeol JMS-AX50WA double sector. Samples were introduced through a direct inlet, and they were analyzed in the electron impact mode (70 eV).

HRMS with Liquid Secondary Ionization (HRMS/LSIM). HRMS was run on a VG ZAB-2SE (VG, Manchester, U.K.) instrument with a unit for a liquid secondary ionization matrix.

NMR. NMR experiments ^{1}H, ^{13}C, DEPT, COSY, HMQC, and HMBC were performed on a Jeol NMR spectrometer (Jeol, Peabody, MA) operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using magnetic susceptibility plugs of zirconia for 5 mm tubes (Doty Scientific, Columbia, SC) or on a Bruker NMR spectrometer, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C (Bruker, Rheinstetten, Germany).

RESULTS AND DISCUSSION

New α-Dicarbonyls in Beer. In a previous study, it was found that α-dicarbonyls accumulated in beer during storage. A hypothesis was proposed that α-dicarbonyls were derived from carbohydrate degradations that occur in beer during aging (*4*). Three groups of α-dicarbonyls were detected: (i) α-dicarbonyls with the sugar carbon chains intact, such as 3-DH, 3-DP, and 1-DH; (ii) α-dicarbonyls derived from the Strecker degradation of 1-deoxydiuloses, such as 1,4-DDP and 1,4-DDH, and (iii) fragmentation products such as methylglyoxal and diacetyl.

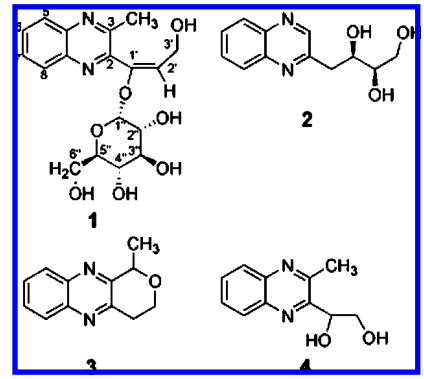

Figure 1. Chemical structures of quinoxalines isolated from beer (**1**−**4**).

Other unknown quinoxaline fractions also were found. In the present work, the identification of four new quinoxalines, three derived from α-dicarbonyls produced from the 2,3-enolization pathway and one diastereoisomer of 3-DH, is reported.

The molecular formula of compound **1**, $C_{18}H_{21}N_2O_7$, was determined by FAB ($[M + K]^+$ at *m/z* 417.2) as well as signal integration of its ^{1}H NMR spectrum and the number of resonance signals of its ^{13}C NMR spectrum (**Table 1**). Signal assignment by homo- and heteronuclear 2-D experiments led to the unequivocal identification **1** as 2-[1′-O-(α-D-glucopyranosyl)-3′-hydroxy-propenyl)-3-methyl quinoxaline (**Figure 1**), the derivative of the α-dicarbonyl 1,5-dideoxy-4-glucopyranosyl-2,3-hexodiulose-4-ene (1,5-DDM). This compound was previously detected as the *β*-pyranone form in heated milk (*16*) and as the main degradation product of the maltose Amadori compound (*17*). This article is the first report on the detection of 1,5-DDM in beer. 1,5-DDM is relevant to this beverage since maltose may represent up to 70% of the reducing sugars.

Compound **2** showed a very similar ^{13}C NMR spectrum to that of the quinoxaline of 3-DH-*erythro* (*4*). However, in **2**, the protons at C1′ were observed as a doublet (3.12 ppm, $^3J = 6.6$ Hz), and the proton at C2′ was detected as a double triplet (4.03, $^3J = 2.3$, $^3J_{H\text{-}OH} = 6.6$) (**Table 2**), while in the 3-DH-*erythro* quinoxaline (see 3-DH Quinoxaline in Experimental Procedures), the diasterotopic protons at C1′ were observed as double−doublets (3.00 ppm, dd, 1H, $^2J = 13.6$ Hz, $^3J = 9.7$,

Table 2. NMR Data for **2** (Dimethylsulfoxide-d_6)

position	δ_C (ppm)	DEPT	δ_H[a,b] (ppm)	COSY
2	157.3	C		
3	147.7	CH	8,85 (s)	
5	129.1	CH	8.04 (m)	
6	130.5	CH	7.80 (m)	
7	129.6	CH	7.80 (m)	
8	129.3	CH	8.04 (m)	
9	142.1	C		
10	141.1	C		
1′	40.2	CH_2	3.12 (d; 6,6)	H2′
2′	71.4	CH	4.03 (dt; 6,6; 2,3)	H1′, H3′, OH-2
3′	74.4	CH	3.42 (m)	H2′, H4′(b), OH-3
4′	63.0	CH_2	Ha 3.43 (m)	H3′, H4′(b)OH-4
			Hb 3.52 (dd, 12.9; 8.5)	H3′, H4′(a)OH-4
OH-2′			4.57	H2′
OH-3′			4.66[c]	H3′
OH-4′			4.47[c]	H4′(a), H4′(b)

[a] Assignments based on HSQC. [b] Coupling constants are given in hertz. [c] Broad band.

Table 3. ^{1}H NMR Data for **3** (300 MHz, Chloroform-d_3)

position	δ_H[a] (ppm)
1	4.99 (c, 6.49)
3	Ha 3.43 (ddd, 17.02; 10.86; 6.16)
	Hb 3.13 (ddd, 17.02; 3.23; 3.22)
4	Ha 4.38 (ddd, 11.74; 6.16; 3.23)
	Hb 4.03 (ddd, 11.74; 10.86; 3.22)
6	7.99 (m)
7	7.69 (m)
8	7.69 (m)
9	7.99 (m)
1′	1.73 (d, 6.49)

[a] Coupling constants are given in hertz.

and 3.32 ppm, dd, 1H, $^2J = 13.6$ Hz, $^3J = 2.9$), and the proton at C2′ appeared as a broad band (3.91 ppm). These spectroscopic data suggest that **2** should correspond to 2-[2′(*R*),3′(*R*),4′-trihydroxybutyl] quinoxaline, the derivative of 3-deoxy-D-*threo*-2-hexosulose. This article is the first report on the detection of 3-DH-*threo* in a food system. The α-dicarbonyl 3-DH-*threo* was originally identified by Anet (*18*), in a synthetic model system of tagatose or galactose and glycine. The detection of 3-DH-*threo* in beer suggests that epimerization of 3-DH-*erythro* could take place during beverage storage or during wort preparation through a possible mechanism that involves the hydration of 3,4-dideoxy-hexosulose-3-ene (3,4-DDH), an intermediate structure in the degradation of 3-DH to generate 5-HMF (*19, 20*). This finding indicates that the dehydration of 3-DH, traditionally considered to be an irreversible step, is a reversible reaction at least in beer. The epimerization of 3-DH-*erythro* is evidence of the formation of 3,4-DDH in beer and is consistent with our previous report concerning the detection of an intermediate compound between 3-DH and 5-HMF that theoretically would correspond to 3,4-DDH (*4*).

Compound **3** revealed a molecular ion at *m*/*z* 200.0389 (HRMS/EI), indicating a molecular formula of $C_{12}H_{12}N_2O$. Signals assignment of the ^{1}H NMR data (**Table 3**) suggested that **3** was 1-methyl-3,4-dihydro-1*H*-pyran[3,4-*b*] quinoxaline (**Figure 1**), the quinoxaline of 2,3-dihydro-5-hydroxy-6-methyl-4*H*-pyran-4-one (2,3-dihydromaltol), a flavor-active compound that was previously detected in caramalt and caramalt beers (*21*) and in aged wines (*22*). 2,3-Dihydromaltol is a product of 1-DH degradation via the intermediate 5-hydroxydihydromaltol (*23*).

Compound **4** corresponds to 2-(1′,2′-dihydroxyethyl)-3-methyl quinoxaline by comparison of both its GC retention time (sylilated sample) and its EI mass spectrum with those obtained from the pure synthesized compound, 1-DP quinoxaline. This is also the first observation of such a type of α-dicarbonyl detected in beer. The corresponding quinoxaline was previously reported in model reaction systems of xylose with α-amino acids (*5*). The detection of 1-DP is consistent with our previous report on the accumulation in aged beers of its Strecker degradation product 1,4-DDP.

Formation of α-Dicarbonyls in Beer. In a recent paper, it was reported that the α-dicarbonyls methylglyoxal, diacetyl, 1,4-DDP, and 1,4-DDH accumulated in beer from 2- to 9-fold during storage at 28 °C in the presence of 1,2-DAB (*4*). The quantification was performed by HPLC, which allowed good resolution for the group of hydrophobic quinoxalines but rendered limited separation for those quinoxalines that remained in beer after chloroform extraction. Since GC allowed a much better resolution, and MS detection provides selectivity, a new procedure was developed for the quantification of α-dicarbonyls using GC-MS. α-Dicarbonyls were derivatized to quinoxalines. Since several quinoxalines had polyol groups, a silylation step with BSTFA was included to improve quinoxalines' volatility. The mass spectra of the quinoxalines were obtained, and the main ions were chosen for the quantification (**Figures 2** and **3**). Under the chromatographic conditions described, calibration models (IS mode) were constructed by relating peak area ratios versus concentration (**Table 4**). To verify the performance of the developed method for α-dicarbonyls, parameters such linearity, LOD, and recovery were evaluated. Good linearity was achieved in the range between 1 and 250 μM for methylglyoxal and diacetyl, between 0.5 and 50 μM for 1-DP, 3-DP, 1-DH, 1,4-DDP, and 1,4-DDH, and between 10 and 500 μM for 3-DH (coefficient of determination between 99.74 and 99.96%). The values of LOD were in the range between 0.01 and 0.1 μM. To evaluate the accuracy, recovery experiments were carried out, following the method described in the Experimental Procedures. Good recovery efficiencies were achieved within the range between 96 and 105%.

The concentrations of α-dicarbonyls in beer were determined by two derivatization procedures. In the first one, α-dicarbonyls were quantified by allowing 1,2-DAB to react in beer for prolonged periods of time at 28 °C (derivatization procedure DE1) (*5, 6, 12, 24*). This procedure has a disadvantage in that it did not permit us to obtain information about those α-dicarbonyls that degrade during beer aging. Therefore, in the second procedure, α-dicarbonyls were quantified by allowing 1,2-DAB to react either in beer or in wort for 24 h at 28 °C (derivatization procedure DE2).

Table 5 presents the concentration of the primary and secondary α-dicarbonyls determined by DE2 in fresh and aged beer, and for comparison, in unboiled and boiled wort. Primary α-dicarbonyls are defined as those directly derived from the degradation of Amadori compounds such as 3-DP, 1-DP, 3-DH, and 1-DH, while secondary α-dicarbonyls are those produced by the degradation of primary α-dicarbonyls including methylglyoxal, diacetyl, 1,4-DDP, and 1,4-DDH.

Primary α-dicarbonyls were generated during the heating steps of the brewing process and remained in the fresh and aged beer (**Table 5**), suggesting that they are relatively inert during the fermentation step. 3-DH was predominant, reflecting the higher concentration of its precursors (glucose) in wort. 3-Deoxyosones (3-DH and 3-DP) were present in larger amounts than 1-deoxydiuloses (1-DH and 1-DP). These findings are in line with what has been observed in heated glucose−glycine model systems (*25*). Noteworthy was the detection of 1-deoxy-

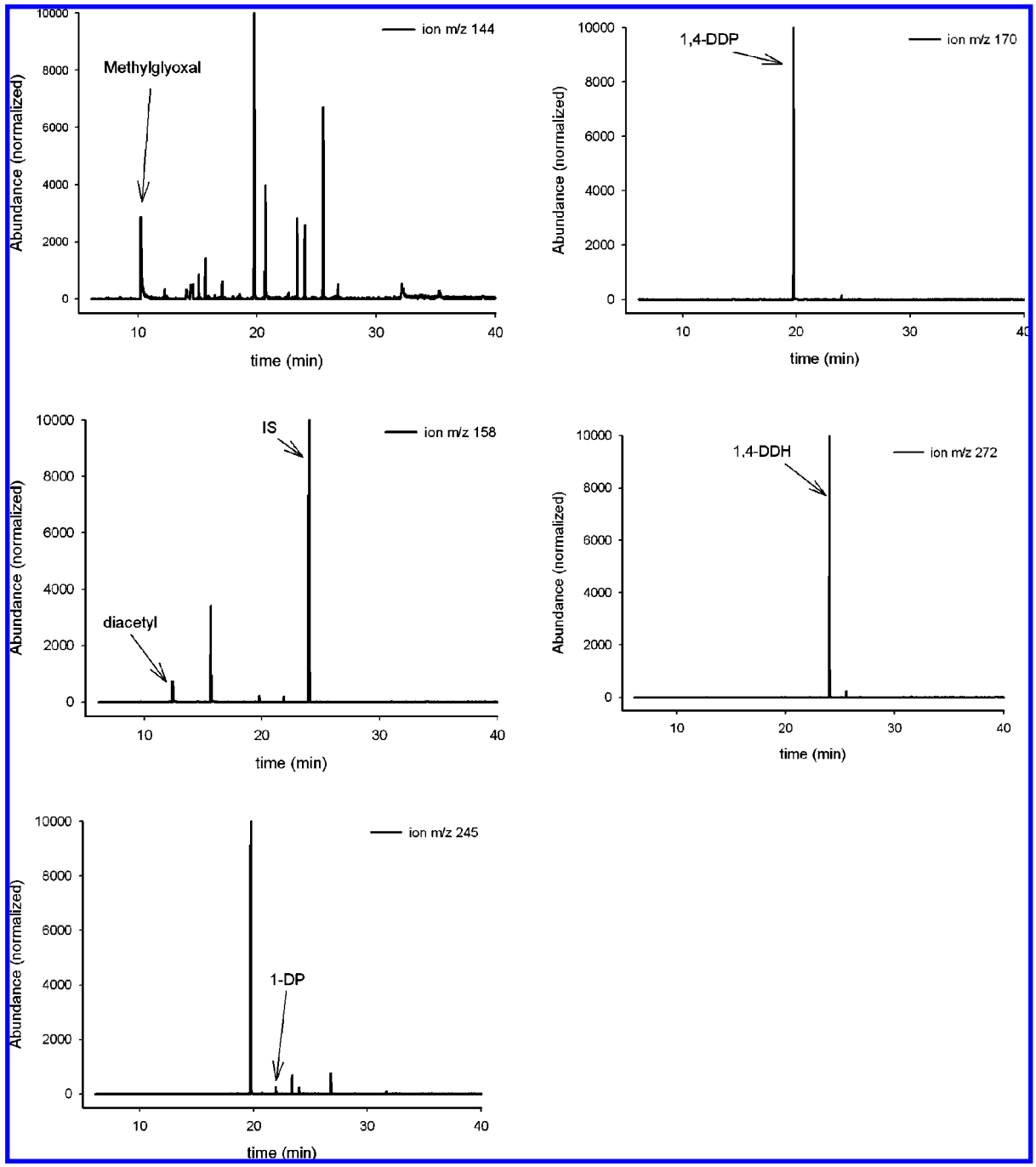

Figure 2. Ion selective chromatograms for chloroform soluble silylated quinoxalines.

diuloses in fresh and aged beer, considering the very short half-life reported for 1-DH of about 0.5 h (*26*).

The concentrations of primary α-dicarbonyls decreased during beer storage at 28 °C (**Table 5**), which suggests both a net decrease in the α-dicarbonyl formation rate and a significant reactivity of α-dicarbonyls in beer. The degradation rate order of primary α-dicarbonyls was 3-DH > 1-DH > 3-DDP > 1-DP. 3-DH degraded at an apparent medium rate of 3.6 μM/week, which seems to correlate well with the medium formation rate of 5-HMF (a byproduct of 3-DH degradation) in beer at the same temperature (2.2 μM/week) (*27*).

Figure 4 shows the concentration profile of the primary α-dicarbonyls during storage at 28 °C. The concentration of 1-DP and 3-DP increased at an average rate of 0.1 and 0.7 μM/week, respectively, which suggests that their corresponding Amadori compounds were rather unstable during beer storage. On the other hand, the concentration of 1-DH did not change significantly. This failure of 1-DH to accumulate during aging may suggest that its degradation reactions were probably faster than its reaction with 1,2-DAB since with the DE2 method a net loss of 1-DH was observed during storage. Conversely, 3-DH showed a significant decrease during this period. This was unexpected based on the high stability of 3-DH quinoxaline observed in aqueous solutions (*25*).

When the results obtained by both derivatization procedures for the cases of 1-DP and 3-DP are combined, it seems that they were both continuously generated (as shown by the DE1 procedure) and degraded (as shown by the DE2 procedure) during beer aging. These competing reactions have been observed in model reaction systems at 37 °C (*25*). The results

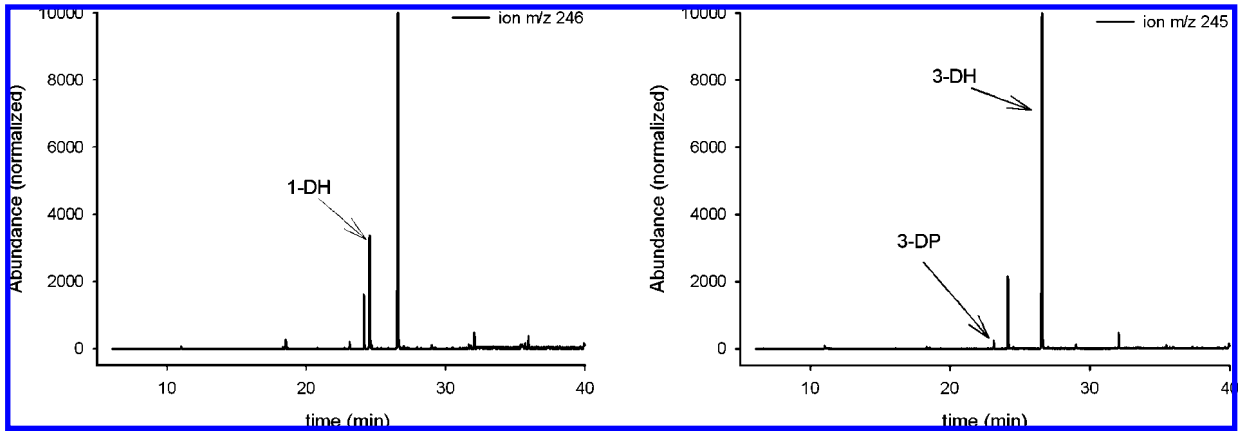

Figure 3. Ion selective chromatograms for water soluble silylated quinoxalines.

Table 4. Figures of Merit for Method of α-Dicarbonyl Quantification[a]

α-dicarbonyl	R^2 (%)	slope ± SE	intercept ± SE	linear range (μM)	LOD (μM)	recovery
methylglyoxal	99.96	0.0050 ± 0.0003	−(0.02 ± 0.01)[b]	1–250	0.02	99 ± 1
diacetyl	99.93	0.0062 ± 0.0002	(0.01 ± 0.01)[b]	1–250	0.03	101 ± 2
1-DP	99.93	0.00248 ± 0.00004	−(0.002 ± 0.001)[b]	0.5–50	0.01	104 ± 3
3-DP	99.91	0.00262 ± 0.00004	−(0.003 ± 0.001)[b]	0.5–50	0.02	96 ± 3
1-DH	99.87	0.0037 ± 0.0001	−(0.03 ± 0.02)[b]	0.5–50	0.02	103 ± 3
3-DH	99.77	0.0037 ± 0.0001	−(0.03 ± 0.02)[b]	10–500	0.06	101 ± 2
1,4-DDP	99.74	0.0140 ± 0.0004	(0.02 ± 0.01)[b]	0.5–50	0.1	101 ± 1
1,4-DDH	99.93	0.0109 ± 0.0002	−(0.002 ± 0.004)[b]	0.5–50	0.01	105 ± 3

[a] 2-Propyl-3-methyl quinoxaline was added as IS. IS linear range was between 1 and 250 μM. [b] Value is not statistically different to zero ($p < 0.05$).

Table 5. Concentrations of Primary and Secondary α-Dicarbonyls Determined in Wort and in Beer Following DE2

	concentration (μM)			
α-dicarbonyl	wort after maceration (2 h at 60 °C)	wort after boiling (2 h at 95 °C)	fresh beer	aged beer (105 days at 28 °C)
3-DP	4.9 ± 0.2	6.9 ± 0.3	2.8 ± 0.4	1.65 ± 0.02
3-DH	80 ± 3	180 ± 4	156 ± 3	104.8 ± 0.8
1-DP	1.5 ± 0.2	2.7 ± 0.3	1.4 ± 0.1	0.63 ± 0.02
1-DH	7.5 ± 0.3	13.9 ± 0.8	12.4 ± 0.4	2.96 ± 0.01
methylglyoxal	6.3 ± 0.7	8.0 ± 0.7	5.0 ± 0.1	*bql*[a]
diacetyl	2.9 ± 0.1	2.6 ± 0.2	4.3 ± 0.1	*bql*
1,4-DDP	*bql*	*bql*	*bql*	*bql*
1,4-DDH	6.3 ± 0.1	8.0 ± 0.1	5.9 ± 0.8	3.0 ± 0.2

[a] *bql*: Below quantification limit.

clearly show the need for using both derivatization procedures to understand the behavior of primary α-dicarbonyls during beer aging.

Since the secondary α-dicarbonyls could hardly be detected at the end of the storage period by the DE2 procedure (**Table 5**), the analysis of their behavior during aging was performed by the DE1 procedure (**Figure 5**). Secondary α-dicarbonyls showed a significant net increase during aging. 1,4-DDH had the highest average rate (6.6 μM/week), followed by 1,4-DDP (3.0 μM/week), diacetyl (1.5 and μM/week), and methylglyoxal (0.8 μM/week).

The difficulty in trapping secondary α-dicarbonyls using the DE2 procedure, as compared to their clear tendency to accumulate when 1,2-DAB is present during aging, is evidence of their high reactivity. Yet, when comparing the degradation rate of 1-deoxydiuloses and the formation rate of 1,4-dideoxydiuloses, the question arises as to if 1,4-dideoxydiulose accumulation can be totally explained by the degradation of 1-deoxydiuloses. The 1,4-DDH accumulation rate (**Figure 5**) in relation to the 1-DH degradation rate (**Table 5**) was 10:1, while in the case of 1,4-DDP, the ratio was even higher, 60:1. Similar behavior was observed in a glucose/glycine/1,2-DAB model system at low water activity, in which 1,4-DDH accumulated at a higher rate than its precursor 1-DH (*6*). This observation and the failure of 1-deoxydiuloses to accumulate in the presence of 1,2-DAB (**Figure 4**) suggest either that their degradation reactions are faster than their reaction with 1,2-DAB through (possibly cyclic) forms not accessible to the trapping reagent (*25*) or that there are other routes for 1,4-DDH formation in beer not involving 1-DH as the precursor.

A second group of secondary α-dicarbonyls that are generated at lower formation rates can be distinguished (i.e., diacetyl and methylglyoxal). Diacetyl was accumulated above its flavor threshold in beer (*28*). It is speculated that both diacetyl and methylglyoxal may be generated through the retroaldolic cleavage of 1-deoxydiuloses.

Sensory Effect of α-Dicarbonyls during Beer Aging. In a previous study, aminoguanidine was added as a trapping reagent of α-dicarbonyls during beer aging, and the effect on flavor deterioration was determined (*4*). It was observed that the addition of aminoguanidine did in fact slow the development of bread-like, caramel, and burnt flavor notes. However, it could not be ascertained that this effect was only due to the inactivation of α-dicarbonyls because aminoguanidine also can react with aldehydes, and the extent of α-dicarbonyl blockage was unknown.

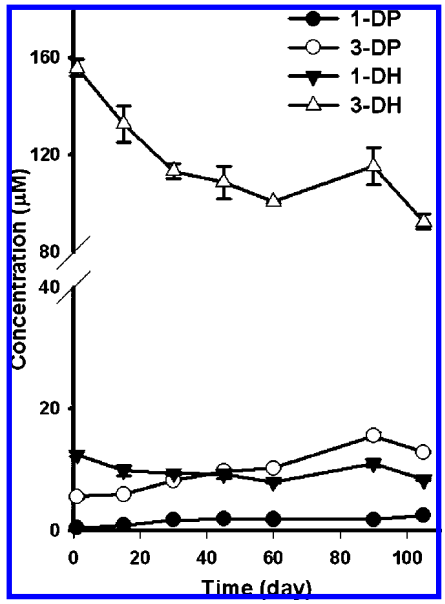

Figure 4. Concentrations of primary α-dicarbonyls determined by DE1 derivatization procedure in beer during storage at 28 °C. Each point represents an average of two determinations.

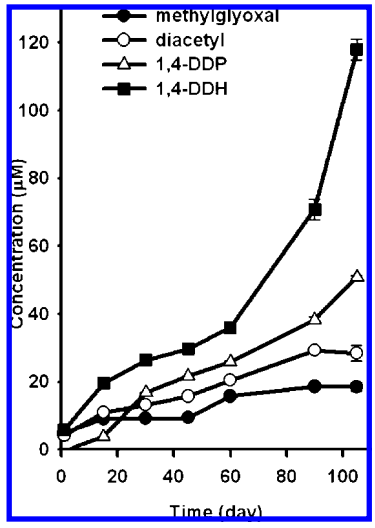

Figure 5. Concentrations of secondary α-dicarbonyls determined by derivatization DE1 procedure in beer during storage at 28 °C. Each point represents an average of two determinations.

Table 6. Concentrations of Residual α-Dicarbonyls after Beer Aging for 1 Week at 28 °C in the Presence of Aminoguanidine

	concentration[a] (μM)		
α-dicarbonyl	no addition	2 mM AG	10 mM AG
pyruvic acid	400 ± 51[a]	195 ± 33[b]	77 ± 10[c]
glyoxal	3.5 ± 0.7	3.5 ± 0.7	3.0 ± 0.7
methylglyoxal	5.0 ± 0.6	4.5 ± 0.6	3.5 ± 0.6
diacetyl	7.6 ± 0.4[a]	7.0 ± 0.5[a]	3.2 ± 0.5[b]
3-DH	239 ± 5[a]	202 ± 5[b]	100 ± 5[c]
1,4-DDP	5.4 ± 0.4	7.7 ± 0.5	7.0 ± 0.5
1,4-DDH	8.7 ± 0.4[a]	7.6 ± 0.7[a]	2.3 ± 0.4[b]

[a] Values marked with different letters showed a significant difference with $p < 0.05$.

To evaluate the extent of the aminoguanidine reaction with α-dicarbonyls, this trapping reagent was added to beer at two levels (2 and 10 mM), the beer was allowed to age for a week at 28 °C, and the residual amount of α-dicarbonyls was determined after DE2 derivatization procedure and quantification following a previously published HPLC method (*4*). The results are shown in **Table 6**. It can be seen that even using 10 mM aminoguanidine, 1,2-DAB was able to react with all α-dicarbonyls. Only pyruvic acid and 1,4-DDH had a relatively high affinity for aminoguanidine. These results showed that 1,2-DAB is about 4-fold more reactive with α-dicarbonyls than aminoguanidine. This is in agreement with previous results, indicating that the rate of reaction of aminoguanidine in comparison with 1,2-DAB is by far too slow to prevent degradation of the native α-dicarbonyls (*25*). The previous considerations led to the selection of 1,2-DAB as an effective trapping reagent to evaluate the role of α-dicarbonyls on aroma formation during beer storage.

With the aim to assess the extent of 1,2-DAB reaction with aldehydes, 1,4-DAB was selected as a control reagent. The latter reagent cannot cyclize to form stable compounds by reacting with α-dicarbonyls, but it could react with monocarbonyls in a similar way as 1,2-DAB would. The effect of adding 1,2-DAB and 1,4-DAB by the quantification of 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal in the headspace of fresh and aged beers was measured. **Figure 6** shows that heating beer at

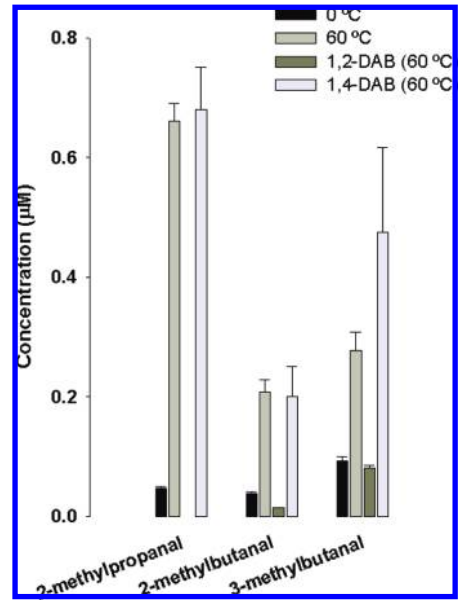

Figure 6. Formation of Strecker aldehydes 2-methylpropanal, 2-methylbutanal, and 3-methylbutanal in beer stored for 3 days with 1,2-DAB or 1,4-DAB.

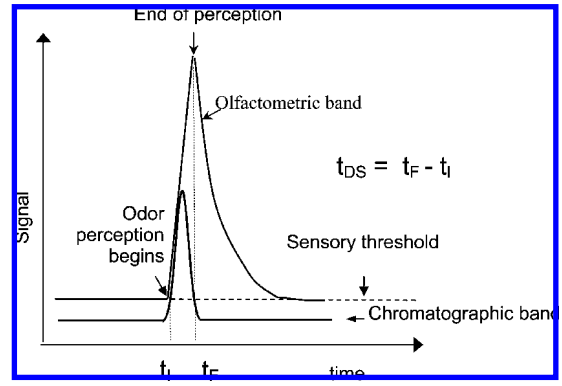

Figure 7. Time dependent profiles of olfactometric and chromatographic signals of a given odorant. The duration of stimulus t_{DS} was determined by subtracting t_I, the time at which the stimulus is first detected from t_F, the time at which the panelist no longer perceives the odor.

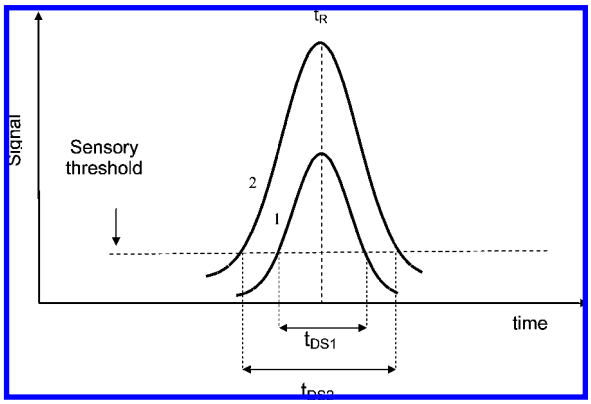

Figure 8. Relationship between t_{DS} and amount of odorant. The graph shows time dependent profiles of two chromatographic signals corresponding to different concentrations of the same odorant. The odorant in signal 1 is at a lower amount than in signal 2. Accordingly, t_{DS1} is lower than t_{DS2}.

60 °C for 3 days increased significantly the formation of all aldehydes. However, in the presence of 1,2-DAB, the aldehyde formation was completely inhibited; actually, the aldehyde concentrations were lower than in fresh beer. Conversely, the addition of 1,4-DAB did not affect the formation of 2-methylpropanal and 2-methylbutanal and increased the concentration of 3-methylbutanal. It is important to mention that the addition of 1,4-DAB to beer accelerated browning and the development of sweet and caramel notes in comparison to the control beer and the beer with 1,2-DAB. This result suggests both that 1,2-DAB may be used as trapping reagent to evaluate the role of α-dicarbonyls in beer, without the complication of possible side reactions with sensory-active monocarbonyls, and that beer α-dicarbonyls may be involved in the generation of Strecker aldehydes.

The impossibility of ingesting the beers spiked with 1,2-DAB to perform sensory analysis was overcome by the use of GCO analysis of the beer headspace using SPME. For registering GCO data, panelists kept an electric switch pressed during the time they perceived an aroma at the sniffing port. The generated analog signal (olfactometric signal) was stored in a computer via chromatographic software. For each odorant, both the time at the beginning of the stimulus (t_I) and the time at the end of the stimulus (t_F) were obtained, as indicated in **Figure 7**. The duration of a stimulus (t_{DS}) was calculated by subtracting t_I from t_F. The olfactometric elution time (t_E) was estimated as follows: $t_E = t_I + 1/2t_{WS}$. Differences in t_{DS} can be related to differences in the amount of chromatographed odorants since the width of the band may be proportional to the amount of an odorant (**Figure 8**). Thus, the contribution of each odorant to the aroma deterioration could be estimated by determining the physical variable t_{DS}. This method is independent of the ability of the panelist to perceive odor intensity changes.

Twenty-seven odor-active zones were detected, and 17 of them were identified with a single chemical compound by comparison with pure standards and by mass spectrometry (**Table 7**). All of them, except dimethyl sulfide, were previously observed in beer by employing GCO (*29*).

The aroma of fresh beer and beer stored at 60 °C for 3 days in the presence and absence of 1,2-DAB was studied. The experiment was repeated 3 times with samples taken from independent batches, and each panelist analyzed twice each sample. The data were submitted to analysis of variance to estimate the effects of forced aging and 1,2-DAB addition. It can be observed in **Table 7** that forced aging affected the t_{DS} values of three odorants: furaneol, *trans*-2-nonenal, and phenylacetaldehyde. These compounds had t_{DS} values significantly higher after 3 days at 60 °C than in fresh beer ($p < 0.05$). Thus, several chemical degradation pathways may be associated to beer flavor deterioration during storage. Furaneol is generated

Table 7. Effect of DAB on Amount of Odor Compounds Present in Aged Beer As Determined by GCO

				t_{DS}[a] (s)		
ID[b]	compound	odor	Kovats RI	fresh beer	aged beer[c]	aged beer[c] with DAB
	unknown	sulfurous, fetid	629	1.5 ± 0.1	1.5 ± 0.4	1.5 ± 0.3
I	dimethyl sulfide	sweet corn-like	655	1.3 ± 0.3	1.5 ± 0.4	1.4 ± 0.6
I	3-methyl-butanol	rancid	759	1.7 ± 0.3	1.6 ± 0.3	1.7 ± 0.4
	unknown	alcoholic, greenish	787	1.2 ± 0.4	1.3 ± 0.2	1.3 ± 0.2
I	ethyl butanoate	herbaceous, fruity	820	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.2
	unknown	alcoholic, solvent-like	844	n.d.	1.2 ± 0.2	1.2 ± 0.1
I	ethyl 3-methylbutanoate	fruity	865	1.5 ± 0.3	1.4 ± 0.3	1.4 ± 0.2
I	3-methylbutanoic acid	cheesy, boiled milk-like	872	1.7 ± 0.4	1.8 ± 0.5	2.0 ± 0.4
I	3-methylbutyl-acetate	fruity, banana-like	881	2.0 ± 0.4	2.3 ± 0.7	2.2 ± 0.6
	unknown	sweet, aloe-like	926	n.d.	1.4 ± 0.3[c]	1.5 ± 0.2[c]
I	3-methylthio-propanal	cooked potato-like	930	1.7 ± 0.4	2.2 ± 0.7	2.0 ± 0.6
	unknown	fruity, sweet	988	1.7 ± 0.3	1.6 ± 0.4	1.6 ± 0.0.4
	unknown	old bread, plastic-like	1003	0.9 ± 0.2	1.3 ± 0.2	1.4 ± 0.4
I	ethyl hexanoate	fruity, coconut oil-like	1021	2.0 ± 0.5	2.0 ± 0.3	2.0 ± 0.4
I	phenylacetaldehyde	floral	1058	0.2 ± 0.4[c]	1.8 ± 0.4[b]	0.4 ± 0.6[a]
I	furaneol	caramel-like	1088	0.5 ± 0.6[c]	1.8 ± 0.4[b]	1.8 ± 0.9[b]
I	linalool	citrusy, lemony	1117	1.4 ± 0.3	1.6 ± 0.3	1.6 ± 0.3
I	2-phenyl-ethanol	floral, rose	1128	1.7 ± 0.4	2.2 ± 0.6	2.0 ± 0.7
	unknown	aldehydic, green	1169	1.2 ± 0.1	1.5 ± 0.2	1.3 ± 0.2
I	*trans*-2-nonenal	plastic-like, nonenal-like	1179	n.d.	2.1 ± 0.5[c]	1.2 ± 0.1[b]
I	ethyl octanoate	sweet, plastic-like	1213	1.9 ± 0.4	1.7 ± 0.3	1.9 ± 0.5
I	2-phenylethyl acetate	floral	1266	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.2
I	octanoic acid	smoky	1290	1.4 ± 0.4	1.7 ± 0.3	1.5 ± 0.2
	unknown	tortilla-like, cracker-like	1300	1.2 ± 0.3	1.4 ± 0.3	1.2 ± 0.2
I	γ-nonalactone	peachy, apple-like	1361	1.5 ± 0.1	1.7 ± 0.4	1.6 ± 0.3
T	β-damascenone	peachy, cooked apple-like	1387	1.9 ± 0.4	1.9 ± 0.6	1.9 ± 0.5

[a] Av duration of stimulus (t_{DS}) determined by employing two panelists, $n = 24$. Intervals followed by different letters were different at $p \leq 0.5$. [b] I: Compound was identified by comparing it with the reference substance on the basis of the following criteria: retention index (RI), mass spectra obtained by MS(EI), and odor quality perceived at the sniffing port. T: Compound was tentatively identified by comparing its mass spectrum with a reference library. [c] Beer was aged at 60 °C for 3 days.

by the enolization and dehydration of 6-deoxyhexoses such as rhamnose (*30*); however, it has been demonstrated that yeast metabolites such as fructose-1−6-diphosphate may be an important source of furaneol in food systems (*31*). *trans*-2-Nonenal is associated with a cardboard off-flavor; it is formed from the oxidative degradation of lipids, and it is believed to be liberated from protein adducts during beer storage (*32*). The increase of phenylacetaldehyde upon forced aging supports the importance of Strecker degradation of phenylalanine and its role on beer flavor deterioration. These observations are in agreement with recent results showing that phenylacetaldehyde and methional play a key role in the aroma deterioration of beer (*33, 34*).

When beer was force-aged in the presence of 1,2-DAB, there was only a significant decrease ($p < 0.05$) in the t_{DS} values of both phenylacetaldehyde and *trans*-2-nonenal as compared to t_{DS} values in the absence of 1,2-DAB, as shown in **Table 7**. In the case of *trans*-2-nonenal, the effect of 1,2-DAB on its t_{DS} value is probably due to an increase of free HSO_3^-. α-Dicarbonyls can form relatively stable adducts with HSO_3^- (*35*). After the addition of 1,2-DAB, the fraction of α-dicarbonyls engaged in hydroxysulfonate adducts may be converted to quinoxalines and HSO_3^-. Free HSO_3^- may react with *trans*-2-nonenal to generate hydroxysulfonate, which is the mechanism that has been suggested for its protecting effect on the generation of the cardboard flavor (*36*).

The decrease in the t_{DS} value of phenylacetaldehyde in the presence of 1,2-DAB supports strongly the role of α-dicarbonyls in the generation of this aldehyde. As previously shown (**Table 6**), 1,2-DAB is an efficient trapping reagent for α-dicarbonyls. The addition of 1,2-DAB to beer before forced aging significantly lowered the α-dicarbonyl concentration, and therefore, the rate of amino acid degradation should have been lowered.

This is the first attempt to quantify the amount of α-dicarbonyl intermediates derived from carbohydrate degradation in a food system at its natural aging conditions, with the aim of determining the relevant chemical pathways that may be involved in the formation of aroma compounds that deteriorate beer fresh flavor. The combined use of two derivatization procedures allowed us to demonstrate that primary α-dicarbonyls were both continuously generated and degraded during beer aging at 28 °C. Notable was the detection of 1-DH in fresh and aged beer, considering the very short half-life reported for this α-dicarbonyl. Secondary α-dicarbonyls (1,4-dideoxydiuloses, methylglyoxal, and diacetyl) were detected only after prolonged reaction with 1,2-DAB and showed a significant increase during aging, particularly 1,4-DDH (6.6 μM/week) and 1,4-DDP (3.0 μM/week). However, our results showed that the formation of 1,4-dideoxydiuloses cannot be totally explained by the degradation of their putative precursors 1-deoxydiuloses, suggesting that other unknown chemical pathways should be considered for their formation in beer. The addition of 1,2-DAB to beer before forced aging at 60 °C for 3 days inhibited the formation of phenylacetaldehyde and *trans*-2-nonenal as shown by the decrease in their t_{DS} values, in the GCO analysis, when compared to those values obtained in the absence of 1,2-DAB. Two different mechanisms may account for these effects. The inhibition of phenylacetaldehyde formation may be the result of partial blockage of the α-dicarbonyl reaction with amino acids to generate Strecker aldehydes. Concomitantly, the addition of 1,2-DAB increases the concentration of free HSO_3^-, and more *trans*-2-nonenal may be trapped as hydroxysulfonate.

ABBREVIATIONS USED

1-DH, 1-deoxy-2,3-hexodiulose; 1-DP, 1-deoxy-2,3-pentodiulose; 1,2-DAB, 1,2-diaminobenzene; 1,4-DAB, 1,4-diaminobenzene; 1,4-DDH, 1,4-dideoxy-2,3-hexodiulose; 1,4-DDP, 1,4-dideoxy-2,3-pentodiulose; 1,5-DDM, 1,5-dideoxy-4-glucopyranosyl-2,3-hexodiulose-4-ene; 2,3-dihydromaltol, 2,3-dihydro-5-hydroxy-6-methyl-4*H*-pyran-4-one; 3-DH, 3-deoxy-2-hexosulose; 3,4-DDH, 3,4-dideoxy-hexosulose-3-ene; 5-HMF, 5-hydroxymethylfurfural; BSTFA, *N*,*O*-bistrimethylsilyltrifluoroacetamide; *bql*, below quantification limit; GCO, gas chromatography-olfactometry; PDMS/DVB, poly(dimethylsiloxane)/divinylbenzene; SPME, solid phase microextraction; t_I, time at beginning of odor stimulus; t_F, time at end of odor stimulus; t_E, olfactometric elution time; t_{DS}, duration of stimulus; TMCS, trimethylchlorosilane; IS, internal standard.

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