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α-Dicarbonyl Compounds Formed by Nonenzymatic Browning during the Dry Heating of Caseinate and Lactose

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A method using high-performance liquid chromatography with UV and electrospray ionization mass spectrometry detection was developed for monitoring the α-dicarbonyl compound profiles generated from nonenzymatic browning using *o*-phenylenediamine (OPD) as a trapping agent. The α-dicarbonyl compounds were generated by the "dry" reaction of sodium caseinate and lactose heated at various relative humidities (RHs). The proportions of α-dicarbonyls formed were different for samples heated at low, intermediate, and high RHs. This study shows that relatively large amounts of 3-deoxypentosulose and galactosyl 2-pentosulose are produced under high RHs, while galactosyl hexosulose and 1,4-dideoxyhexosulose are elevated under low RH conditions. Both caramelization and Maillard reaction pathways contributed to the generation of α-dicarbonyls.

KEYWORDS: HPLC-UV-ESI-MS; α-dicarbonyl; nonenzymatic browning; caramelization; Maillard reaction; lactose; caseinate; dry reaction

INTRODUCTION

α-Dicarbonyl compounds are generated when reducing sugars are heated with amine-containing compounds, such as amino acids or proteins (*1*). For a recent review of the Maillard reaction, see ref *2*. The α-dicarbonyls are responsible for the browning and cross-linking of proteins, and their importance, both in food and in the human body, has been recognized (*3*, *4*). Efforts have been made in their structural elucidation and quantification (*5*). α-Dicarbonyls are highly reactive (*6*), and consequently, a trapping reagent, such as *o*-phenylenediamine (OPD), has been used to convert the α-dicarbonyls to their stable quinoxaline derivatives (*4*, *7*, *8*). Gas chromatography−mass spectrometry and high-performance liquid chromatography (HPLC) are two common approaches for the analysis of α-dicarbonyls (*4*, *6*, *7*−*10*). In the present study, we report a HPLC−UV−electrospray ionization mass spectrometry (ESI-MS) method, in which the dicarbonyls were trapped with OPD and the quinoxaline derivatives were monitored by UV and ESI-MS detectors simultaneously.

Water activity (relative humidity, RH) has a major influence on the Maillard reaction and food browning (*11*). In milk powder, browning reactions are maximal at an a_w value of 0.6−0.7 (*11*). Moreover, careful control of the relative humidity is essential when "dry" heating caseinate and reducing sugars to produce novel glycoproteins (*12*, *13*). Because α-dicarbonyls are important browning precursors, we expected that the α-dicarbonyl contents would be affected by RH. However, little research has been done in this area. In this study, the influence of RH on the formation of α-dicarbonyls by nonenzymatic browning was investigated using caseinate and lactose as model reactants in dry-heated mixtures. This is apparently the first time that the α-dicarbonyl profiles generated from nonenzymatic browning have been monitored by HPLC-MS, and this is the first time that the α-dicarbonyl contents at different RHs have been studied.

MATERIALS AND METHODS

Materials. Sodium caseinate (Alanate 180) was manufactured by Fonterra Co-operative Group Ltd. (New Zealand). Lactose monohydrate and OPD were purchased from Sigma (St. Louis, MO). Glyoxal solution (40% aqueous solution) and diphenyl quinoxaline were purchased from Lancaster (Morecambe, United Kingdom).

RHs. Saturated salt solutions of $MgCl_2$, NaBr, $NaNO_3$, NaCl, KCl, and Na_2CO_3 were used to achieve the desired RHs (at 60 °C) of 29, 50, 67, 75, 80, and 90%, respectively. The procedures used were based on previous work (*12*, *13*) on dry heating under controlled RH of caseinate with mono- and oligosaccharides.

Reaction of Caseinate and Lactose with OPD as Trapping Agent. Lactose monohydrate (53 mg) and sodium caseinate (1 g) were dissolved in water (100 mL). OPD (17.8 mL; 1 mg/mL) was added to the solution (pH 6.94). The molar ratio of lysine (in caseinate):lactose:OPD was 3.6:1:1. An aliquot (377 μL) of the above solution was added to each 2 mL vial. The aliquots were lyophilized and transferred to desiccators containing various saturated salt solutions. The desiccators were held at room temperature (17 °C) for 24 h to pre-equilibrate the samples and then heated at 60 °C in an oven at 29, 50, 67, 75, 80, and 90% RHs. Vials were removed after 12, 24, 48, and 96 h of heating and stored at −18 °C for HPLC analysis. Control samples containing the same amounts of caseinate, lactose, and OPD were lyophilized and without heating stored at −18 °C for HPLC analysis. All samples were prepared in duplicate.

Water (1 mL) was added to each sample, which was shaken for 30 min and then transferred to a Centricon YM-10 (molecular mass cut

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off, 10 kDa) Centrifugal Filter Device (Millipore, Bedford, MA) and centrifuged at 4000 rpm for 1 h. An aliquot of the filtrate was injected into the HPLC.

Reaction of Lactose and OPD. Lactose monohydrate (500 mg) and OPD (167 mg) were dissolved in 50 mL of water and adjusted to pH 7.0 with dilute acetic acid. The molar ratio of lactose to OPD was 1:1. An aliquot (1 mL) of the solution was transferred to a 10 mL vial, and the samples were treated and analyzed the same way as the mixture of caseinate, lactose, and OPD except that the samples were heated for only 48 h. Before analysis, 8 mL of water was added to each sample. The samples were prepared in duplicate.

Preparation of Caseinate−Lactose Conjugate. Lactose can be conjugated to caseinate via the Maillard reaction, and it is assumed that lactulosyl lysine (as part of caseinate) will be formed as a stable intermediate. To produce the caseinate−lactose conjugate, 64 mg of sodium caseinate and 3.4 mg of lactose monohydrate were dissolved in 10 mL of water and lyophilized. The lyophilized sample was transferred to a desiccator containing saturated $NaNO_3$ solution, which was held at room temperature for 24 h to allow the sample to pre-equilibrate, and then heated for 48 h at 60 °C and 67% RH. The reaction mixture was membrane-filtered with the Centricon YM-10 Centrifugal Filter Device to remove the unreacted lactose and low molecular mass products. The residue was collected and lyophilized; it was regarded as the caseinate−lactose conjugate.

Reaction of Caseinate−Lactose Conjugate and OPD. The caseinate−lactose conjugate was dissolved in 10 mL of water, OPD (1.14 mL, 1 mg/mL) was added, and the pH was adjusted to 7.0 with dilute acetic acid. Water was added to give the final volume of 20 mL, and aliquots of 1 mL were transferred to 2 mL vials and lyophilized. The samples were treated and analyzed the same way as the caseinate, lactose, and OPD except that only one time interval (96 h) was used for this model reaction.

Reaction of Lactose and Caseinate with OPD Added after the Reaction. The reactions of caseinate and lactose or lactose alone were also carried out without the initial addition of OPD. Lactose monohydrate (53 mg) and sodium caseinate (1 g) were dissolved in 100 mL of water, and the pH was adjusted to 7.0 with dilute acetic acid. An aliquot of the solution (320 μL) was transferred to a 2 mL vial and lyophilized. Similarly, lactose solution (1 mL; 10 mg/mL) was transferred to a 10 mL vial and lyophilized, and this was used as the control. The lyophilized samples were equilibrated at room temperature for 24 h and heated at 60 °C and various RHs for 48 h. After the samples were heated, OPD (1 mL; 50 mg/L) was added to each vial containing caseinate and lactose. The samples were reacted at 30 °C for 12 h and then membrane-filtered using the Centricon YM-10 Centrifugal Filter Device. The filtrate was examined by HPLC.

Treatment of Standards. Diphenyl quinoxaline standard (10 mg) was dissolved in methanol (100 mL, 90% v/v). An aliquot (10 mL) was diluted to 50 mL with water and analyzed by HPLC. The peak area of diphenyl quinoxaline was used to quantify other quinoxaline derivatives of α-dicarbonyls in the samples. The standard solution was prepared in triplicate. Glyoxal (8 mL; 40% water solution) standard was mixed with 5 mL of OPD (50 mg/mL) solution and reacted at 30 °C for 12 h and analyzed by HPLC.

HPLC-UV-ESI-MS. The analyses were performed using a HP1100 HPLC (Agilent Technologies, Wilmington, DE) with a diode array detector (DAD) and a Luna C18 column (250 mm × 4.60 mm, 5 μm; Phenomenex, Torrance, CA). Water and methanol were used as eluents with an elution program that has been reported in a previous study (*7*). The detection wavelength was 317 nm; the injection volume was 50 μL. For the MS analysis, the outlet from the DAD detector was connected to an on-line splitter, attached to a Mariner mass spectrometer (PerSeptive Biosystems, Framingham, MA), with an electrospray source in positive ion mode and time-of-flight mass analyzer. The splitting ratio was 20:1. The operative parameters were set as follows: spray tip potential, 3804 V; spray chamber temperature, 0 °C; acceleration potential, 4000 V; scan range, *m*/*z* 99−1200; 1.00 s per spectrum; and nebulizer gas pressure, 42 psi.

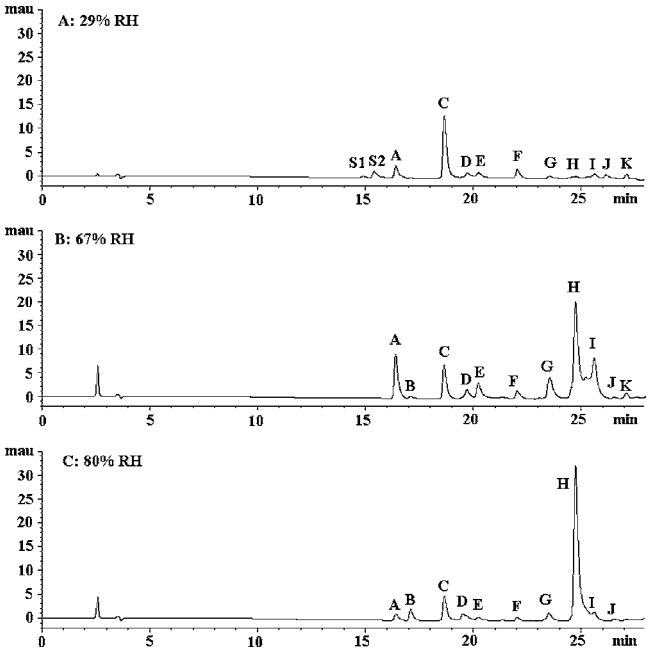

Figure 1. HPLC chromatograms of sugars (in the form of imines with OPD) and α-dicarbonyls (quinoxaline derivatives) from the caseinate, lactose, and OPD mixture at 60 °C at various RHs; reaction time, 24 h; detection wavelength, 317 nm; Mau, milliabsorbance unit. Key: S1, an isomer of lactose; S2, lactose; A, galactosyl 1-deoxyhexosulose; B, galactosyl 2-pentosulose; C, galactosyl hexosulose (galactosyl 2-hexosulose or galactosyl 2,3-hexodiulose, which could not be differentiated); D, hexosulose isomer 1 (2-hexosulose or 2,3-hexodiulose); E, hexosulose isomer 2 (2-hexosulose or 2,3-hexodiulose); F, galactosyl 3-deoxyhexosulose; G, deoxyhexosulose isomer; H, 3-deoxypentosulose; I, deoxyhexosulose isomer; J, 1,4-dideoxyhexosulose; and K, methylglyoxal.

RESULTS AND DISCUSSION

Peak Identification. Samples were detected with DAD and MS detectors simultaneously. Typical UV chromatograms representing samples at low, intermediate, and high RHs are shown in **Figure 1**.

The peaks in the UV chromatograms were identified by their pseudomolecular peaks ($[M + H]^+$, $[M + Na]^+$, or $[M + NH_4]^+$), as detected by MS. The mass spectra of these peaks correspond to a wide range of α-dicarbonyl compounds as their quinoxaline derivatives. Among them, several pairs of peaks have the same *m*/*z* values, such as peaks A and F, D and E, as well as G and I (**Figure 1**). Putatively, these pairs are isomers.

Several dicarbonyls generated from the Maillard reaction, such as 1-deoxyhexosulose, 3-deoxyhexosulose, 1,4-dideoxyhexosulose, and 2-pentosulose, have been reported (1, 4, 6, 8, 9, and 14−16). However, no other dicarbonyls corresponding to the above *m*/*z* values have been reported. Therefore, peaks G and H in **Figure 1** are likely to be quinoxaline derivatives of 1-deoxyhexosulose and 3-deoxyhexosulose (which could not be distinguished without reference standards), while peaks I and J are tentatively identified as quinoxaline derivatives of 3-pentosulose and 1,4-dideoxyhexosulose, respectively.

From the *m*/*z* values, two peaks corresponding to galactosyl deoxyhexosuloses were detected. In a previous HPLC study (*9*) on the Amadori products of maltose, glucosyl 1-deoxyhexosulose and glucosyl 3-deoxyhexosulose were reported. Both maltose and lactose are α-(1→4) linked, so we tentatively identified peaks A and F as the quinoxaline derivatives of galactosyl 1-deoxyhexosulose and galactosyl 3-deoxyhexosu-

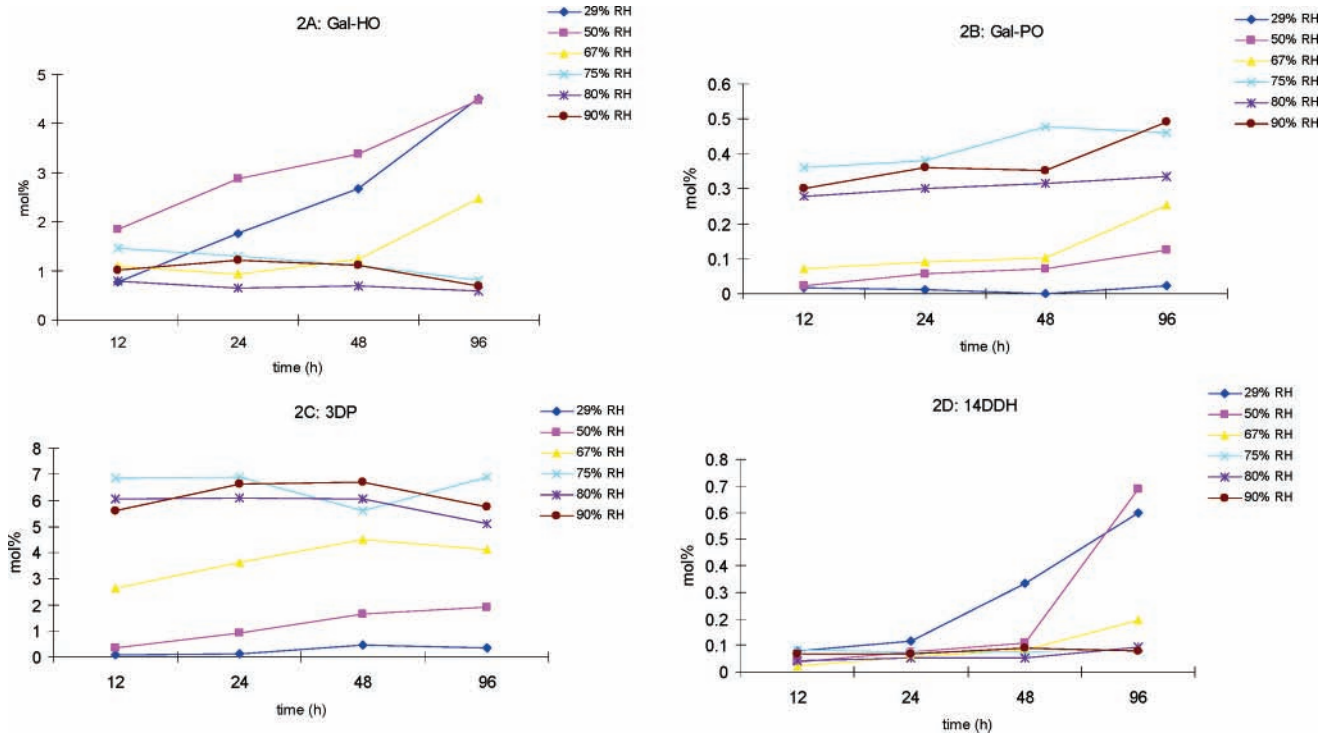

Figure 2. Selected α-dicarbonyls formed from the mixture of caseinate, lactose, and OPD and incubated at 60 °C, various RHs, and time intervals (12, 24, 48, and 96 h), detected as quinoxalines (mol %; α-dicarbonyl/lactose), and expressed as diphenyl quinoxaline equivalent. (**A**) Gal-HO, galactosyl hexosulose (galactosyl 2-hexosulose or galactosyl 2,3-hexodiulose, which could not be differentiated); (**B**) Gal-PO, galactosyl 2-pentosulose; (**C**) 3DP, 3-deoxypentulose; and (**D**) 14DDH, 1,4-dideoxyhexosulose.

lose, respectively. Again, reference standards are needed to confirm the identification and to distinguish them.

Two peaks (D and E) were found corresponding to the pseudomolecular ($[M + H]^+$) masses of the quinoxaline derivative of hexosulose. A possible explanation is that they are hexosulose isomers, i.e., one of them is 2-hexosulose, and the other is 2,3-hexodiulose, which are interconverted through enolization. In addition, the generation of other isomers such as 3,4-hexodiulose could not be excluded due to the interchangeability of carbonyl positions in glycosuloses.

Peaks B and C were identified as quinoxaline derivatives of galactosyl 2-pentosulose and galactosyl hexosulose, respectively, based on their pseudomolecular mass peaks. Peak C could be galactosyl 2-hexosulose or galactosyl 2,3-hexodiulose, which could not be differentiated in this study.

We found that the quinoxaline derivative of glyoxal was eluted at 28 min, but no corresponding mass peak was detected in any of the samples tested. The absence of glyoxal is consistent with the experimental conditions, in which the molar ratio of sugar to lysine (in casein) was chosen to be much less than 1.0, in order to initiate the reaction while minimizing subsequent nonenzymatic browning reactions.

Both peaks S1 and S2 in **Figure 1** correspond to *m*/*z* of 433 as detected by MS; thus, it is concluded that S1 and S2 are the imines formed between the carbonyl group of lactose (or its isomer) and one of the amine groups of OPD. The peak area of S2 is considerably larger than that of S1, so it is likely that S2 corresponds to the imine formed between lactose and OPD (this was confirmed by the MS result for the control sample). Similarly, S1 corresponds to the imine formed between an isomer of lactose and OPD. Such lactose isomers could be generated during the heating of lactose at 60 °C. The reaction of lactose and OPD could not be used for quantitation purposes because the reactive efficiency between OPD and lactose is much lower as compared to that between OPD and α-dicarbonyls. The imine derivative formed between one carbonyl group of galactosyl hexosulose and one amine group of OPD was also detected as its pseudomolecular peak (*m*/*z* 431). It was eluted immediately prior to peak C (galactosyl hexosulose quinoxaline) in **Figure 1**. Although it was not detected in the UV chromatogram, it produced a disproportionately large peak in the mass chromatogram (result not shown). The implication is that only a negligible amount of this compound was formed, as compared with the major product formed during the α-dicarbonyl and OPD derivatization, the quinoxaline derivative of galactosyl hexosulose. However, the alkalinity of the remaining amine in the OPD is stronger than that of the imine groups formed after the derivatization and hence is easier to protonate in the positive ionization mode of ESI; thus, a much stronger signal in the MS was observed.

Figure 2A−D shows that at low RHs (29 and 50%), the contents of α-dicarbonyls increased with time. However, at high RHs, such as 75, 80, and 90%, there is a general trend that the α-dicarbonyl contents decreased between 48 and 96 h. After 48 h at high RHs, the color of the dry mixtures was amber and their solubilities had decreased. It is inferred that at high RH conditions, the quinoxaline derivatives of α-dicarbonyls at 96 h were not fully extracted into solution, probably due to cross-linking with the caseinate.

α-Dicarbonyls produced via nonenzymatic browning have previously been studied, and a selected number of α-dicarbonyls were monitored (*4*, *7*−*10*). In the present study, we attempted to monitor all of the dicarbonyls generated using HPLC-MS. Considering the complexity of dicarbonyls in the caramelization or Maillard reaction, the HPLC-UV-ESI-MS method has been shown to be a very useful tool. A limitation of the method is that the identity of α-dicarbonyl isomers cannot be confirmed.

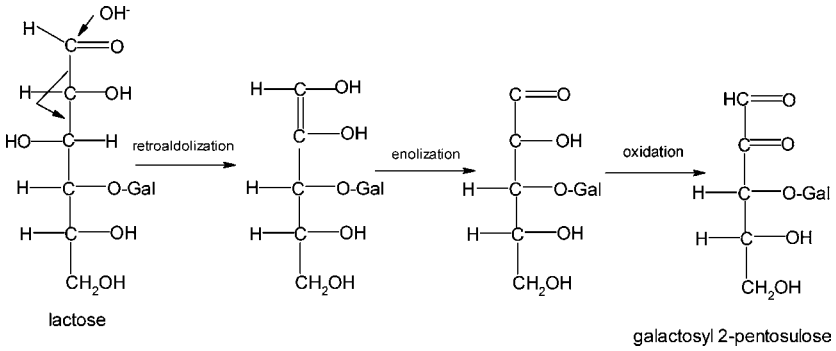

Figure 3. Schematic description of the formation of galactosyl 2-pentosulose.

Further work to confirm the identification of the dicarbonyls using authentic standards or HPLC-MS-MS is advisable.

α-Dicarbonyl Levels at Various RHs. These gave distinctly different patterns at low (29 and 50%), intermediate (67%), and high (75, 80, and 90%) RH conditions (see **Figures 1** and **2**). A large galactosyl hexosulose peak (C) was detected in samples heated at low RH (**Figure 1A)**. In contrast, a large 3-deoxypentosulose peak (H) existed in samples heated at high RH and the galactosyl 2-pentosulose (B) amount was also relatively increased at high RHs as compared to low RHs. (**Figure 1C)**. The amounts of galactosyl hexosulose (peak C), galactosyl 2-pentosulose (peak B), and 3-deoxypentosulose (peak H) in samples heated at 67% RH were intermediate (**Figure 1B)**. The above trends were consistent for samples measured at each time interval (**Figure 2**).

3-Deoxypentosulose has been reported to be the predominant α-dicarbonyl produced from the heating of maltose, glycine, and OPD solution (Maillard reaction conditions) and maltose and OPD solution (caramelization conditions) (*8*). 3-Deoxypentosulose was not generated when the glucose, glycine, and OPD solution was heated (*8*); thus, it was formed through a pathway specific for the heat-induced degradation of oligo- and polysaccharides with α-(1→4) linkages (*8*). In an earlier study (*7*) on the heating of maltose, glycine, and OPD in a "dry reaction" model, no 3-deoxypentosulose was detected and 1,4-dideoxyhexosulose was the predominant α-dicarbonyl. Hollnagel and Kroh (*8*) suggested that 3-deoxypentosulose was formed via retroaldolization cleavage between C1 and C2 of the Amadori product. However, 3-deoxypentulose was formed during caramelization of maltose, presumably also by retroaldolization cleavage (*8*). Moreover, they (*8*) considered that the essential role of hydroxyl ion or carboxylate ion in retroaldolization explained why this reaction only predominates in aqueous systems. Therefore, it was expected that the 3-deoxypentosulose contents would be higher and predominant at high RH as compared to low RH conditions. In the present study, a dideoxyhexosulose (peak J in **Figure 1**) was also detected and we assume that it is 1,4-dideoxyhexosulose, which has been reported in a number of studies (*7*, *8*, *14*). It has been suggested (*5*) that 1,4-dideoxyhexosulose can be formed in the presence of aliphatic amines via 1-amino-1,4-dideoxyhexosulose. Using glycine, maltose, and OPD as reactants, the formation of 1,4-dideoxyhexosulose is not favored in aqueous solution (*7*, *8*). From **Figure 2D**, it can be seen that generally speaking, 1,4-dideoxyhexosulose contents are higher at low rather than high RH, which also agrees with previous studies (*7*, *8*).

On the basis of the above discussion, the increase in galactosyl 2-pentosulose as a function of RH, as found from the present study, can be readily explained. Retroaldolization appears to be the sole pathway responsible for the formation of galactosyl pentosulose (**Figure 3**), as in the case of 3-deoxypentosulose. The retroaldolization requires the participation of water, so it will happen to a greater extent at high RH.

Galactosyl 2-pentosulose has not been reported in previous studies on Maillard reaction and sugar caramelization. The quinoxaline derivative of this compound was not detected in the reaction of caseinate−lactose conjugate and OPD. Therefore, it is likely to originate from the caramelization of lactose.

Figure 2A shows that the galactosyl hexosulose content is higher at low RHs (29 and 50%) than at 67% RH or above, and the difference became more pronounced as the heating time increased. The rate of food browning is maximal at intermediate water activity (*11*, *17*). van Boekel (*18*) reported that with decreasing a a_w value, reactants became more concentrated and this results in an increase in rate, but at certain a_w values, the system became so concentrated that diffusion becomes increasingly difficult and the browning rate decreases. There are two competitive decomposition pathways in sugar caramelization: generation of deoxyhexosuloses by enolization and β-elimination, which occurs in aqueous conditions, and the generation of hexosuloses, which requires an oxidative step (*19*−*22*). One study (*23*) proposed that molecular oxygen from air was involved in the initial stage of the Maillard reaction. Oxidation involving molecular oxygen can occur between gas−solid or gas−liquid interfaces and is faster at low RH because of the concentration effect. Moreover, it was observed in our work that the apparent volume of samples incubated at high RH was markedly reduced as compared to that of low RH samples, which was due to the collapse of the sample when more moisture was absorbed. So, samples at high RH have less surface area exposed to oxygen and less oxidation would take place; thus, the galactosyl hexosulose contents are low.

Galactosyl hexosulose was also detected in the mixture of caseinate−lactose conjugate and OPD after 96 h of heating, as shown in **Table 1**. Lactose can be converted to galactosyl hexosulose by a single oxidation step as discussed above. Unlike lactose, a putative Amadori product in the caseinate−lactose conjugate, in addition to oxidation, has to undergo other steps such as enolization and deamination to produce hexosulose (*21*). Moreover, water is required for the final deamination. Therefore, oxidation might not be the rate-limiting step. At 96 h, less galactosyl hexosulose was produced in the caseinate, lactose, and OPD mixture with increasing RH (**Figure 2A**) as compared to caseinate−lactose conjugate and OPD with increasing RH (**Table 1**).

Routes for α-Dicarbonyl Generation. The α-dicarbonyl compounds were produced by two possible pathways: caramelization of lactose or decomposition of the caseinate−lactose conjugate. The caseinate−lactose conjugate with OPD and lactose with OPD were heated under various RHs for 96 or 48 h, respectively, and the quinoxaline derivatives of α-dicarbonyls were analyzed by HPLC-UV-ESI-MS. α-Dicarbonyl compounds

Table 1. Selected α-Dicarbonyls Formed from the Reaction of the Caseinate–Lactose Conjugate with OPD, Incubated at 60 °C and Various RHs for 96 h, Detected as Quinoxalines (mol %; α-Dicarbonyl/Lactose of Caseinate–Lactose Conjugate), and Expressed as Diphenyl Quinoxaline Equivalent[a]

	content of α-dicarbonyls[b] (mol %; α-dicarbonyl/ lactose)		
RH (%)	Gal-HO	3DP	14DDH
29	0.18	0.06	0.02
50	0.25	0.00	0.02
67	0.26	0.61	0.06
75	0.26	0.57	0.06
80	0.26	0.83	0.05
90	0.20	0.53	0.04

[a] Key: Gal-HO, galactosyl hexosulose (galactosyl 2-hexosulose or galactosyl 2,3-hexodiulose, which could not be differentiated); 3DP, 3-deoxypentosulose; and 14DDH: 1,4-dideoxyhexosulose. [b] To calculate the α-dicarbonyl contents, it was assumed that all of the lactose molecules were conjugated to caseinate.

Table 2. Selected α-Dicarbonyls from the Reaction of Lactose with OPD Incubated at Various RHs for 48 h, Detected as Quinoxalines (mol %; α-Dicarbonyl/Lactose), and Expressed as Diphenyl Quinoxaline Equivalent[a]

	content of α-dicarbonyls (mol %; α-dicarbonyl/lactose)			
RH (%)	Gal-HO	Gal-PO	3DP	14DDH
29	0.142	0.002	0.012	0.254
50	0.090	0.000	0.012	0.238
67	0.093	0.001	0.016	0.262
75	0.062	0.018	0.021	0.090
80	0.061	0.012	0.019	0.002
90	0.074	0.011	0.025	0.011

[a] Key: Gal-HO, galactosyl hexosulose (galactosyl 2-hexosulose or galactosyl 2,3-hexodiulose, which could not be differentiated); Gal-PO, galactosyl 2-pentosulose; 3DP, 3-deoxypentosulose; and 14DDH, 1,4-dideoxyhexosulose.

were similar to those in the mixture of caseinate, lactose, and OPD. The α-dicarbonyl compounds arising from the reactions of the caseinate−lactose conjugate with OPD and lactose with OPD are listed in **Tables 1** and **2**, respectively. Aside from the fact that the 3-deoxypentosulose content is low at low RH conditions (29 and 50%), the results from **Table 1** show that the α-dicarbonyl profiles of caseinate−lactose conjugate with OPD do not resemble those found in the mixture of caseinate, lactose, and OPD. For the lactose-modified casein with OPD, the α-dicarbonyl profiles at various RHs are similar, suggesting that the dicarbonyl contents are not markedly affected by RH. In addition, no galactosyl 2-pentosulose was detected from caseinate−lactose conjugate and OPD. On the other hand, the α-dicarbonyl profile of lactose with OPD has some similarities to that of the mixture of caseinate, lactose, and OPD. At low RH, their galactosyl 2-pentosulose and 3-deoxypentosulose contents were low, whereas galactosyl hexosulose and 1,4-dideoxyhexosulose contents were high and vice versa at high RH. However, the trend was not as pronounced as in the mixture of caseinate, lactose, and OPD. The α-dicarbonyl levels in the mixture of lactose and OPD were considerably lower as compared with that in the mixture of caseinate, lactose, and OPD or the caseinate−lactose conjugate with OPD. In summary, it is likely that both caramelization and Maillard reaction pathways contributed to the α-dicarbonyls in the mixture of caseinate, lactose, and OPD, although to what extent each pathway contributed cannot be determined at present.

It should be noted that in the mixture of caseinate, lactose, and OPD, the decomposition of lactose could be expected to be enhanced by the catalytic effect of amine groups in OPD. Similarly, for the mixture of lactose and OPD, the presence of OPD can also alter the decomposition pathway. Addition of the trapping reagent subsequent to the heat-induced reaction is an alternative. However, α-dicarbonyls are highly reactive compounds and significant amounts of them may be rapidly degraded under the incubation conditions. The pros and cons of the timing of OPD addition have been discussed previously (*4*, *6*−*8*, *10*). Hofmann (*6*) reported that the addition of OPD in advance leads to accumulation of the derivatized target compounds and consequently offers no insight into the actual amounts of these reaction intermediates produced at certain stages of the reaction. In contrast, Hollnagel and Kroh (*8*) suggested that all of the α-dicarbonyls could be analyzed in their accumulated concentration if OPD is added prior to the reaction. In the present study, a mixture of caseinate and lactose was incubated without OPD for 48 h at 60 °C and various RHs. The trapping reagent, OPD, was added after the incubation and before the membrane filtration. The results (not shown) indicated that the amount of dicarbonyl quinoxalines was approximately five times lower than that of the samples incubated in the presence of OPD. The implication is that most of the α-dicarbonyls that were generated had participated in subsequent reactions; hence, their levels would be grossly underestimated if OPD was added after the incubation. Considering that the aim of the present study is to understand the α-dicarbonyl profiles at the early stage of the nonenzymatic browning, we believe that the current approach is appropriate.

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