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Maillard Reaction and Protein Cross-Linking in Relation to the Solubility of Milk Powders

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ABSTRACT: Protein changes in relation to solubility, Maillard reaction (MR), and protein cross-linking in whole milk powder (WMP), skim milk powder (SMP), and whey protein concentrate (WPC) stored at different relative humidities (RHs) were investigated by chemical and electrophoretic methods. WMP and SMP reached minimum solubility rapidly, while WPC showed no change in solubility. The loss of solubility corresponded with development of high-molecular-weight protein complexes observed by two-dimensional electrophoresis. The maximal MR rate occurred at 66% RH for WMP and SMP (high lactose/protein ratios) and 84% RH for WPC (low lactose/protein ratios) based on the furosine and hydroxymethylfurfural contents. However, browning was greatest at 84% RH in all powders. The minimum solubility corresponded with the casein and fat contents. The retention of solubility and minimal protein cross-linking of WPC compared to casein-containing powders suggest that the casein content and cross-linking strongly influence the decrease in the solubility of milk powder.

KEYWORDS: Maillard reaction, protein cross-linking, milk powders, solubility, furosine, HMF, 2D electrophoresis

INTRODUCTION

Whole milk powder (WMP) and skim milk powder (SMP) are manufactured by similar processes, in which raw whole or skim milk is sequentially pasteurized, preheated, evaporated, and spray-dried to obtain WMP or SMP. Consequently, WMP consists of protein (25−27%), carbohydrate (37−38%), and fat (25−28%), while SMP contains 35−37% protein, 49−52% carbohydrate, and 0.7−1.3% fat.[1] The high proportion of carbohydrate (predominantly lactose) and protein (and fat in WMP) makes milk powders more sensitive to thermal processing and chemical changes during prolonged storage.[2,3] The Maillard reaction (MR) is a major cause of changes in milk powders during storage. It results in the formation of lactulosyllysine (measured as furosine), hydroxymethylfurfural (HMF), and brown pigments (melanoidins).[4,5] The decrease in the solubility of skim milk powder during storage at high temperature and humidity[6] may be due to changes in the protein structure and/or the MR.[7]

Whey protein concentrate (WPC) is a byproduct of cheese manufacture and is obtained by ultrafiltration and spray drying. It is largely used as a food ingredient because of its functional and nutritional properties. The lactose content in WPC varies from 5 to 51%, resulting in 80−35% protein in WPC80 and WPC35, respectively.[8] WPC is used to fortify cereals, beverages, infant formulas, and sports supplements.[9] It can also improve functional properties, such as emulsifying, foaming, thickening, and water-binding of food products.[10] Similar to other types of milk powder, WPC undergoes physical and chemical changes during processing and storage, e.g., lactose crystallization and MR.[8] Some functional properties (solubility, foaming, and emulsification) in WPC containing 52% protein show little change with time, temperature, and humidity conditions.[11]

Data on milk protein concentrate containing 80% protein (MPC80) obtained in our previous study[12] showed a correlation between the MR and solubility loss. Whether or not this correlation applies to other milk powders was not known. The present study was designed to investigate chemical and physical changes, particularly the MR, protein cross-linking, and solubility, of various commercial milk powders during storage at different humidity conditions. Products representative of the three stages of the MR (furosine, HMF, and browning) as well as cross-linked protein complexes were analyzed and correlated with solubility changes over time. A comparison of these changes was made between milk powders to examine the effect of the composition of the milk powders on the physical and chemical modifications.

MATERIALS AND METHODS

Materials. WMP (24% protein, 35% lactose, and 26% fat), SMP (32% protein, 48.5% lactose, and 0.6% fat), WPC (80% protein, 6% lactose, and 7% fat, hereafter termed WPC80), and MPC80 (81.1% protein, 4.2% lactose, and 1.4% fat) were obtained from Murray Goulburn Co-op (Melbourne, Victoria, Australia). Immobiline DryStrips (pH 4−7, 24 cm) and IPG buffer (pH 4−7) were obtained from GE Healthcare (Sydney, New South Wales, Australia). Acrylamide/*N*,*N*′-methylenediacrylamide solution (29:1) was obtained from Bio-Rad Laboratories (Hercules, CA).

Methods. Samples of commercial WMP, SMP, and WPC80 powders were stored in desiccators containing saturated salt solutions (K_2CO_3, $NaNO_2$, and KCl) to achieve relative humidities (RHs) of 44, 66, and 84%, respectively. These desiccators were placed in incubators at 30 °C for up to 12 weeks. Milk powder samples were analyzed for solubility, furosine, HMF, and brown color after 1, 2, 4, 6, 8, 10, and 12 weeks of storage, as described previously.[12] The term "solubility" refers to the percentage of milk powder that dissolves under the conditions of the test, i.e., 30 °C for 30 min using a 5% solution;[14] the

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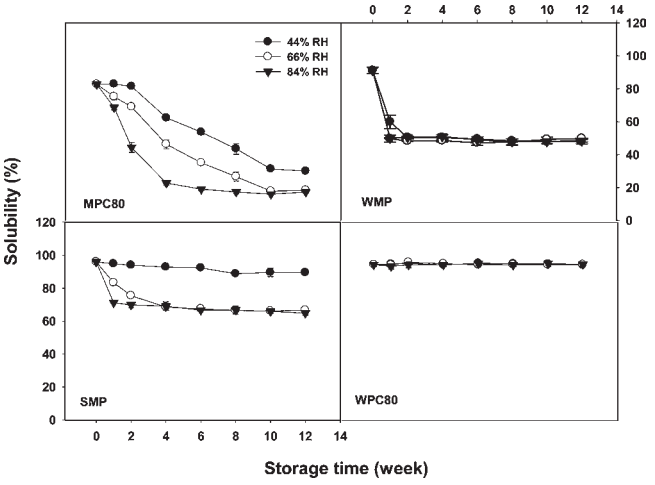

Figure 1. Solubility (%) of MPC80, WMP, SMP, and WPC80 during storage at 30 °C and different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

insoluble portion in this test may become soluble under more severe solubilization conditions. Control samples were fresh milk powders stored at −20 °C. The whole trial was performed in duplicate using two different batches of WMP, SMP, and WPC80.

Changes in the proteins of these milk powders during storage were analyzed by two-dimensional gel electrophoresis (2DE). Isoelectric focusing on immobilized pH gradient strips (pH 4−7) was used in the first dimension, and 25 × 20 cm, 14% polyacrylamide gels were in the second dimension, as described previously.[13] Reducing 2DE was used to eliminate disulfide cross-linking of proteins in milk powder.

Statistical Analysis. Statistical analysis was performed with the general linear model procedure in Minitab Release15 (Minitab, Inc., Chicago, IL) for a completely randomized design. The p values were obtained by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Solubility. The solubilities of WMP, SMP, and WPC80 during storage for 12 weeks are shown in Figure 1. For comparison purposes, the solubility of MPC80 during storage under the same conditions, which we have previously reported,[12] are also shown in Figure 1. The solubilities of control WMP, SMP, WPC80, and MPC80 samples were 91, 96, 95, and 82%, respectively. WMP rapidly decreased in solubility and reached a minimum (approximately 48−52%) after 2 weeks at 44% RH or 1 week at 66 or 84% RH. The percentage of insoluble material (around 48−52%) could be accounted for by the combination of fat (26%) and protein (24%). Therefore, the decrease in solubility of WMP could be correlated with the protein and fat fractions. It has been suggested that the loss in solubility of high-protein milk powders is due to the formation of a hydrophobic cross-linked protein network on the surface of powder particles, which prevents their dispersion in water.[14,15] The protein cross-linking results in stored milk powders obtained by 2DE are shown in the next section. MR and lipid oxidation are two of the main causes of protein cross-linking. Advanced Maillard products, such as glyoxal and methylglyoxal, can react with lysyl, arginyl, and tryptophanyl residues of the proteins to form cross-links. The intermediates (e.g., free radicals and hydroperoxides) and end products (e.g., malondialdedyde) from lipid peroxidation can also interact with amino acid residues, such as cysteine, lysine,

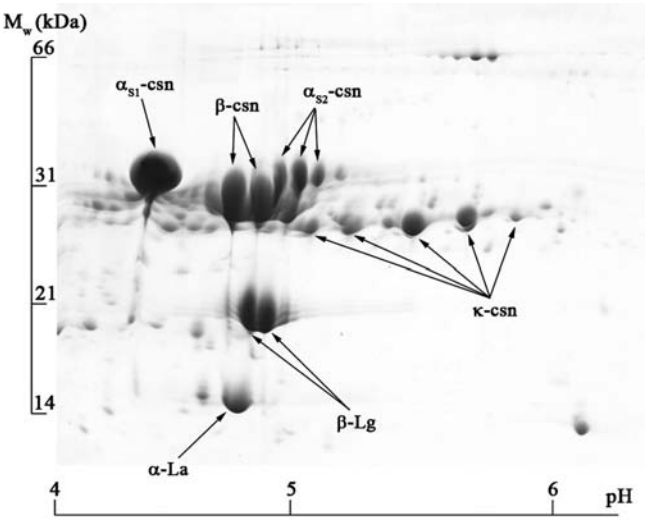

Figure 2. Reducing 2DE of control SMP stored at −20 °C. The sample was separated on IPG strips (pH 4−7) in the first dimension and on 25 × 20 cm, 14% polyacrylamide gels in the second dimension. The vertical scale shows apparent molecular mass in kDa, and the horizontal scale shows pH.

histidine, valine, methionine, and phenylalanine, to form protein cross-links.[16] Lipid oxidation occurs at very low water activity (a_w) because of the independence of large hydrophobic molecules (fat) on water mobility.[17] This could explain why the loss in solubility of WMP during storage was not affected by RH conditions.

The trend of solubility change in SMP during storage was similar to that of WMP at 66 and 84% RH. However, it was very different at 44% RH, where the solubility decreased slowly to 89% over the 12 weeks of storage (Figure 1). In contrast, the solubility decreased rapidly at 84% RH and more gradually at 66% RH. Both reached a plateau of solubility of approximately 68%, which is equal to the total solids content of SMP minus the protein content. Thus, the significant decrease ($p < 0.001$) in solubility of SMP at high RH can be accounted for by the protein (chiefly casein micelles) alone. A similar correlation between the loss of solubility and the protein content was observed in our previous study on MPC80, in which nearly 80% of the powder was insoluble when the plateau was reached after storage for 4 weeks at 30 °C and 84% RH.[12] Anema et al. indicated that casein micelles could be responsible for the insoluble material formed in MPC85 during storage based on a progressive loss of casein from the soluble component.[15] Mimouni et al., using electron microscopy, showed an increase in micelle−micelle interactions in stored MPC85 that could hinder redispersion and, therefore, play a direct role in the decrease in solubility.[18]

In contrast to WMP, SMP, and MPC80,[12] the solubility of WPC was stable over the 12 weeks of storage at the three relative humidities (Figure 1). This result is similar to that reported by Hsu and Fennema.[11] They concluded that the functional properties (foaming and solubility) of WPC containing 52% protein were not significantly affected by storage conditions (moderate temperature, humidity, and prolonged time). The stability of WPC80 during storage provides support for the assumption that the casein fraction accounts for the insoluble material of aged milk powder containing casein micelles. Thus, the solubility of the milk powders, except WPC80, decreased substantially during

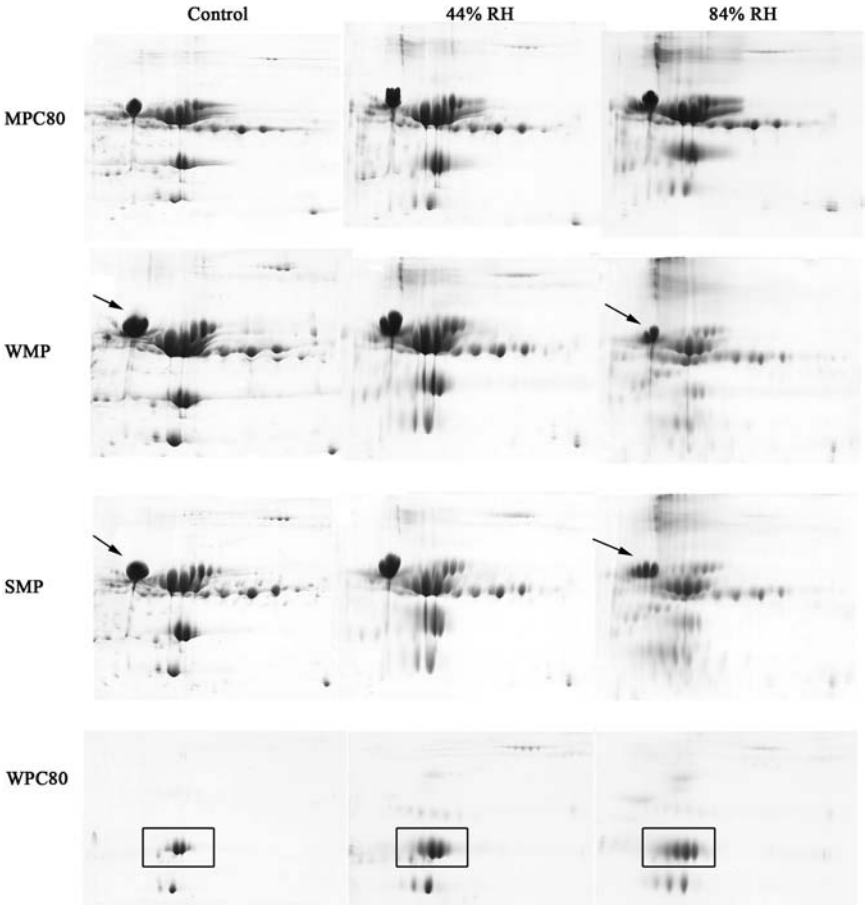

Figure 3. Reducing 2DE of control MPC80, WMP, SMP, and WPC80 and samples of these powders stored for 12 weeks at 30 °C and 44 and 84% RH. The samples were separated on IPG strips (pH 4−7) in the first dimension and on 25 × 20 cm, 14% polyacrylamide gels in the second dimension.

storage. MPC80 showed a rapid loss within 4 weeks at high RH,[12] while WMP and SMP reached a solubility plateau under the same conditions. From these data, it appears that insolubility of MPC80, WMP, and SMP mainly occurs in the casein micelles (and fat in WMP), whose structure is chemically or physically modified under these storage conditions.

2DE Analysis. 2DE was used to investigate chemical changes of proteins in milk powder during storage, particularly MR and protein cross-linking. Milk proteins were separated by their isoelectric point and molecular weight. Figure 2 shows the separation of the major proteins, including α_{S1}-casein (α_{S1}-csn), α_{S2}-casein (α_{S2}-csn), β-casein (β-csn), κ-casein (κ-csn), β-lactoglobulin (β-Lg) A and B variants, and α-lactalbumin (α-La), in a control sample of SMP.

2DE gels of control and MPC80, WMP, SMP, and WPC80 samples stored at 30 °C (44 and 84% RH) for 12 weeks are shown in Figure 3. Several major changes were observed. The appearance of additional acidic spots of α_{S1}-csn in 2DE gels of WMP and SMP stored at 30 °C and 84% RH (indicated by arrows) compared to those in control samples could be due to deamidation.[13] The conversion of an asparagine residue to an aspartate (or glutamine to glutamate) causes an increased negative charge on the protein, thus reducing its isoelectric point (pI). This chemical modification was also observed by the increase in the number of spots to the left of the original β-Lg spots, particularly in WPC (see boxes in Figure 3). Lactosylation of whey proteins was also observed on 2DE in all types of powder and is characterized by vertical spot stacking, as described previously for stored ultra-high-temperature (UHT) milk samples.[13] This is more apparent in Figure 4, which shows enlargements of the region around α-La in the gels of WMP, SMP, and WPC80 from Figure 3. The vertical spot stacking is clearest for the samples stored at 44% RH for 12 weeks. The samples stored at 84% RH for the same time show such extensive changes that the patterns became ill-defined (analysis of the lactosylation pattern for MPC80 will be the subject of a separate paper). The third major change was the formation of high-molecular-weight protein complexes, visible as diffuse staining above the casein monomers in Figure 3. They were observed in MPC80, WMP, and SMP but none or very little in WPC80. α_{S1}-csn has been confirmed as the most abundant protein in the spots of the cross-linked protein.[13] Of the four types of milk powder, WMP, SMP, WPC80, and MPC80, the proteins in WMP and SMP showed the most degradation under high-humidity storage conditions. The decrease in the size of the spots of proteins on 2DE gels from WMP and SMP stored at 84% RH could also indicate the formation of very high-molecular-weight complexes that could not enter the gel. This could be due to the high fat and lactose contents of WMP and SMP, respectively, that may accelerate the formation of protein cross-linking via fat oxidation or advanced MR products.[16]

The formation of high-molecular-weight protein complexes was correlated with the solubility loss of milk powder. For example, the solubility of SMP was relatively stable for 12 weeks

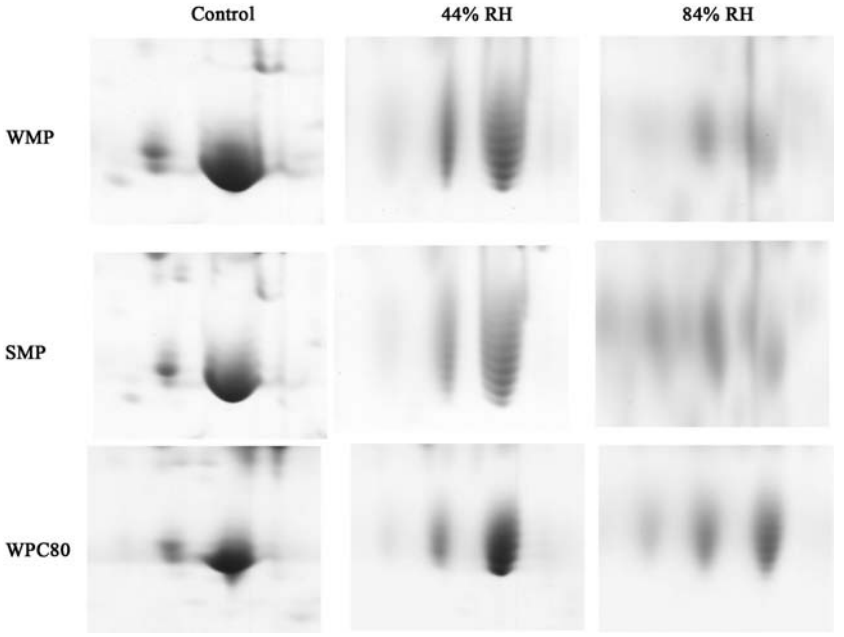

Figure 4. Lactosylation of α-La. Enlargement of the 2DE gels of WMP, SMP, and WPC80 from Figure 3, highlighting the region corresponding to α-La and showing the changes in the spot pattern arising from lactosylation.

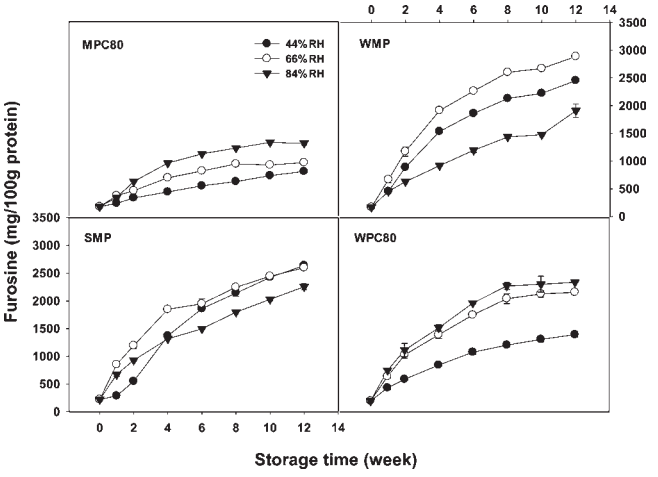

Figure 5. Furosine content of MPC80, WMP, SMP and WPC80 during storage at 30 °C and different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

in samples stored at 44% RH, while those stored at the 84% RH showed a substantial loss (Figure 1). This correlates with low and high amounts of protein cross-linking, respectively (Figure 3). WPC showed no loss of solubility during storage, corresponding to the formation of no or very little protein complexes of molecular mass around 50 kDa (Figure 3). Therefore, it could be concluded that the formation of high-molecular-weight protein complexes is closely associated with the loss in solubility of milk powder. Dehydroalanine, which can be formed by the loss of phosphate from phosphoserine via a β-elimination reaction, could be responsible for protein cross-linking. This intermediate can react with other amino acid residues (e.g., lysine and histidine) to form cross-links, such as lysinoalanine or histidinoalanine. In addition, advanced MR products (e.g., dicarbonyl compounds) have the ability to cross-link proteins. These two mechanisms could account for the formation of the protein cross-linking in milk powder.

Analysis of MR Products. *Furosine.* Furosine is a good indicator of the first stage of the MR, which is considered to be a temperature-, humidity-, pH-, and time-dependent reaction. Figure 5 shows the furosine levels of WMP, SMP, and WPC80 as a function of the storage time. Again, the corresponding data previously published for MPC80[12] are included in this figure for comparison purposes. There was an increase in the furosine content of stored WMP samples. The amount of furosine in WMP directly after manufacture ranged from 159 to 163 mg/100 g of protein, which is slightly higher than data reported by Thomsen et al.[4] of approximately 127 mg/100 g of protein (0.5 mmol/100 g of protein). This could be due to compositional or manufacturing differences. Furosine in WMP increased to almost 3000 mg/100 g of protein after 12 weeks of storage at 66% RH; this is about twice the amount reached in MPC80 under the same conditions.[12] This could be explained by the higher lactose/protein ratio in WMP.

The maximal rate of the MR, as given by the furosine data, in WMP occurred at 66% RH (a_w = 0.66). This is comparable to other studies on the effect of a_w on the MR rate.[19,20] However, the physical and chemical nature of the food system can change the effect of a_w.[21,22] For example, in MPC80, the maximal rate of MR was at 84% RH.[12] This is attributable to the high protein content in MPC80. Lactose crystallization in milk powder is delayed in the presence of high-molecular-weight polymers, such as protein, resulting in increased glass transition temperatures (T_g) of the system.[23] A high moisture content or a_w (e.g., 84% RH) lowers the T_g of milk powder;[8] therefore, at a storage temperature of 30 °C and RH of 84%, the reactants could attain a rubbery state and become more flexible and diluted than at lower RHs.

A similar trend in the furosine content was achieved in SMP as for WMP. Furosine increased from 210 to 230 mg/100 g to a maximum of 2400−2600 mg/100 g of protein after 12 weeks of

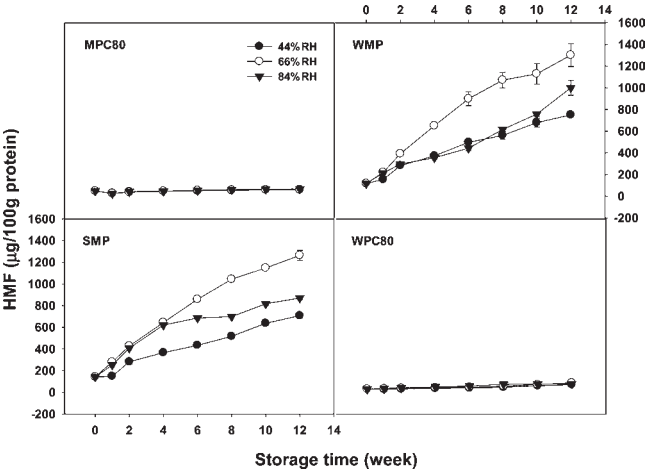

Figure 6. HMF content of MPC80, WMP, SMP, and WPC80 during storage at 30 °C and at different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

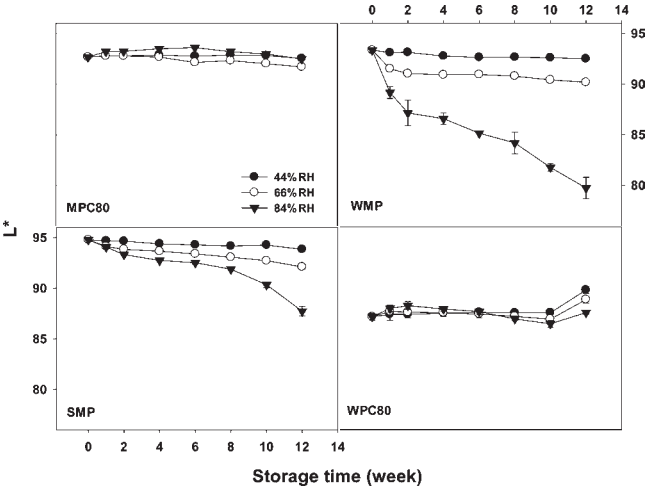

Figure 7. L^* value of MPC80, SMP, WMP, and WPC80 during storage at 30 °C and different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

storage (Figure 5). The maximal rate of furosine formation in SMP was also at 66% RH. The furosine level of SMP samples stored at 44% RH was initially lower but reached the same level as that of the samples stored at 66% RH after 6 weeks. This could be explained by the dilution effect on the MR at higher RH. Malec et al.[22] reported the loss of lysine in SMP over a wide range of a_w (0.32−0.98) during storage. They indicated that the maximal loss was at $a_w = 0.56$ and the rate of loss started to decrease at $a_w = 0.70$. Therefore, water activity in the range of 0.5−0.7 is considered to be optimal for the MR in SMP; this is consistent with the finding in this study.

WPC80 was more similar to MPC80 than other powders in the production of furosine (Figure 5). The furosine content of WPC80 was 176−209 mg/100 g of protein at week 0 and increased to 2300 mg/100 g of protein after 12 weeks of storage at 30 °C and 84% RH. These levels were much higher than those recorded for MPC80.[12] The difference could be that the whey proteins, such as β-lactoglobulin (the main component in WPC80), are more reactive with lactose than casein (the main component in MPC80); therefore, WPC80 would be more vulnerable to the MR than MPC80 at the same protein concentration. Because the maximal rate of MR occurring in various milk powders was obtained at different RH values (e.g., 84% RH for MPC80 and WPC80 and 66% RH for WMP and SMP), the maximal value of furosine was used to compare between milk powder samples. The amounts of furosine in MPC80 and WPC80 were lower than in WMP and SMP. For example, the furosine contents of MPC80 and WPC80 stored at 30 °C and 84% RH for 12 weeks were 1300 and 2300 mg/100 g of protein, while the furosine contents of WMP and SMP stored at 30 °C and 66% RH for 12 weeks were 2880 and 2590 mg/100 g of protein. That suggests that the MR rate is accelerated in WMP and SMP by the high lactose/protein ratio. This was also observed in the study by Morgan et al.[8] on WPC80 with different lactose concentrations.

In summary, there was an overall significant interaction between furosine production and humidity in milk powders upon storage. From the data, it appears that the range of a_w for the maximal rate of MR is not always from 0.5 to 0.7 because it depends upon the protein/lactose ratio in milk powder, which determines their T_g values. The more high-molecular-weight polymers present, the higher the T_g that can be achieved. As long as the reactants are in the right state, which is sufficiently mobile, the MR can attain its maximal rate. The maximal MR rate was attained in MPC80 and WPC80 at 84% RH, whereas in WMP and SMP (up to 6 weeks), it was attained at 66% RH.

HMF. Free HMF is considered as a second-stage MR product because of its formation via decomposition of lactulosyllysine. Although free HMF is present in milk and milk-based products at very low concentrations, it is still a reliable indicator of heat and/or storage damage in milk systems.

Data on the free HMF content of WMP, SMP, WPC80, and MPC80[12] are reported in Figure 6. The free HMF content in WMP and SMP dramatically increased, while its level in MPC80 and WPC80 did not show much change over 12 weeks of storage. Because of the higher lactose concentrations in WMP and SMP compared to MPC80 and WPC80, more MR occurred, leading to larger increases in free HMF content. The levels of free HMF in WMP and SMP were similar. This suggests that milk fat had little or no effect on the MR in milk powders. Nevertheless, Morales and Perez[24] concluded that milk fat does affect free HMF formation in milk during heat treatment. This was explained by the protection of other components by milk fat against heat-induced changes, as reported by Pellegrino.[25] In our study, free HMF was formed during storage at relatively low temperatures, and hence, Pellegrino's theory may not apply.

Free HMF ranged from 110−150 to 1220−1370 μg/100 g of protein for both WMP and SMP stored at 30 °C and 44 and 66% RH (Figure 6). The content of free HMF in UHT whole milk and semi-skim milk was reported to range from 220 to 2000 μg/100 g of protein,[26] which is similar to the levels in milk powders in this study. During milk powder processing, milk experiences heat treatment, especially in preheating and spray drying. Thus, the MR is initiated during processing and continues during storage. Lactulosyllysine, the first Maillard product and source of furosine, continues to form during storage but, at the same time, is degraded through the MR to HMF and other products. The pattern of free HMF formation was similar to that of furosine, in that 66% RH was a maximal condition for Maillard product formation. However, free HMF formation showed no significant change in WPC80, which is similar to that observed for MPC80.

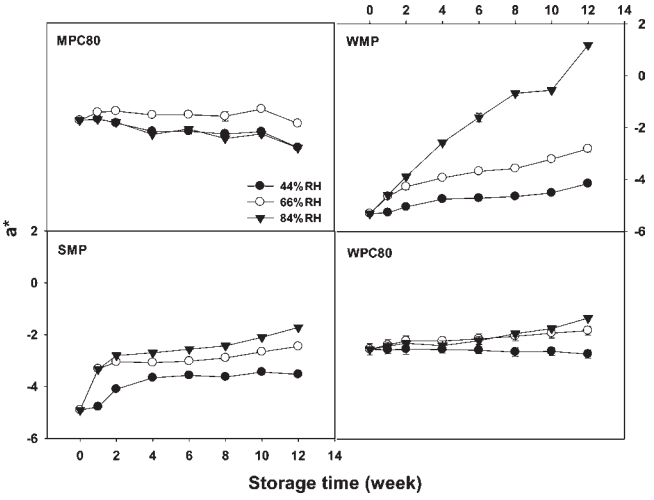

Figure 8. a^* value of MPC80, SMP, WMP, and WPC80 during storage at 30 °C and different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

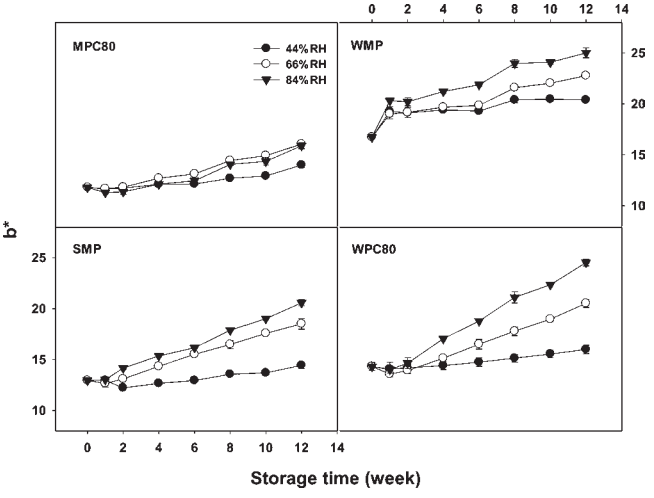

Figure 9. b^* value of MPC80, SMP, WMP, and WPC80 during storage at 30 °C and different RHs: ●, 44%; ○, 66%; and ▼, 84%, as a function of time. Error bars indicate standard deviations.

It could be due to the low MR rate in these high-protein powders with low lactose/protein ratios.

Color Changes in Milk Powder. The formation of melanoidin, which causes brown coloration in milk products via processing and/or storage is categorized as the final stage of MR. Because the structure of melanoidin is not fully characterized, the browning measurement is one of the common ways to investigate progression of the reaction. Figures 7, 8, and 9 show the change in lightness (L^*), the change in color from greenness to redness (a^*), and the change in color from yellowness to blueness (b^*) of WMP, SMP, and WPC80 during storage, respectively; previously published data[12] for MPC80 are also included for comparative purposes. As seen from Figure 7, L^* decreased rapidly in WMP and gradually in SMP at 84% RH, whereas L^* of those samples was quite stable when the powders were stored at low humidity (44 and 66% RH). The decrease in the L^* value represented a decrease in lightness and an increase in brown coloration in these milk powders. The a^* increase (Figure 8) and b^* increase (Figure 9) also indicated brown pigment formation in WMP and SMP during storage. The absolute color values cannot be compared between these different powders. For example, WMP is normally more yellow than SMP because of the β-carotene in the fat component. MPC80 and WPC80 showed no significant changes in L^* and a^* during storage, but b^* in all types of milk powders showed a gradual increase upon storage. Overall, milk color depends upon a variety of factors: feeding regime of the cow, fat and carotene contents, and processing and storage conditions.[27]

Non-enzymatic browning (because of the MR) is considered to be a humidity-dependent reaction. In our study, the maximal rate for the MR was shown to be at 66% RH in WMP and SMP based on furosine and HMF measurements. However, b^* increased with rising RH, leading to 84% RH being a maximal condition for brown pigment formation and color changes. The MR is a complex series of reactions, in which many factors can affect its rate. Therefore, a full explanation of the results obtained in this trial requires more mechanistic studies to be conducted on the effects of environmental factors on the rate of the MR in different types of milk powders.

In conclusion, different milk powders showed different trends in solubility, protein cross-linking, and formation of MR products during storage. MPC80, WMP, and SMP lost solubility, while WPC80 remained stable under high storage humidity conditions for 12 weeks. Casein (and fat in WMP) could account for the insolubility of the powders. Although the increase in MR indicators (furosine, HMF, and b^* value) was associated with the decrease in solubility of MPC80, WMP, and SMP during storage, this did not apply to WPC80. A considerable amount of high-molecular-weight protein complexes was observed on 2DE gels of MPC80, WMP, and SMP but not of WPC80. This showed a strong correlation with and may be largely responsible for solubility loss in MPC80, WMP, and SMP. Cross-linking between individual casein molecules in micelles is unlikely to have much impact on solubility, but cross-links between molecules in different micelles would make it harder for the micelles to disperse, thereby reducing solubility. The precise mechanism of cross-linking remains to be determined. If it is via dicarbonyl compounds produced as advanced MR products, this may explain the correlation between MR and cross-linking. It is also not yet clear whether the covalently cross-linked caseins seen at the molecular level are involved in the micelle−micelle contacts seen at the microscopic level.[18] Further research is required on the relationship between the MR and protein cross-linking to provide a full understanding of the chemical and physical modifications in milk powder during storage.

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