Temperature Effect on Lactose Crystallization, Maillard Reactions, and Lipid Oxidation in Whole Milk Powder

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Whole milk powder with an initial water content of 4.4% (w/w) and a water activity of 0.23 stored in hermetically sealed vials for up to 147 days below (37 and 45 °C) and above (55 °C) the glass transition temperature (T_g determined to have the value 48 °C) showed a strong temperature dependence for quality deterioration corresponding to energies of activation close to 200 kJ/mol for most deteriorative processes. The glass transition was found not to cause any deviation from Arrhenius temperature dependence. Lactose crystallization, which occurred as a gradual process as monitored by isothermal calorimetry, is concluded to liberate bound water (a_w increase to 0.46) with a modest time delay (approximately 2 days at 55 °C) and with concomitant surface browning as evidenced by an increasing Hunter *b*-value. Browning and formation of bound hydroxymethyl-furfural determined by HPLC seem to be coupled, while formation of another Maillard reaction product, furosine, occurred gradually and was initiated prior to crystallization. Initiation of lipid oxidation, as detected by lipid-derived radicals (high *g*-value ESR spectra), and progression of lipid oxidation, as detected by headspace GC, seem not to be affected by lactose crystallization and browning, and no indication of browning products acting as antioxidants could be determined.

KEYWORDS: Whole milk powder; lactose crystallization; lipid oxidation; Maillard reactions; nonenzymatic browning; ESR spectroscopy; furosine; HMF; radicals

INTRODUCTION

Whole milk powder consists mainly of lactose (approximately 38%), whey proteins (approximately 4%), caseins (approximately 20%), and milk fat (approximately 26%). Milk powder particles consist of a continuous mass of amorphous lactose and other low molar mass components in which fat globules and proteins are embedded (*1*). Physical processes involving mainly lactose and milk fat together with chemical reactions including these and other components are limiting for the shelf life of whole milk powder and other dry products based on milk powder such as infant formula and instant powders for cocoa, coffee, and chocolate-flavored beverages. Aging of milk powder affects the flavor of the reconstituted milk and the nutritive value, mainly due to loss of lysine, as well as the physical and functional properties important for the use of milk powder as a food ingredient (*2*).

Three types of reactions are deteriorative and determine the shelf life of milk powder in practice: lactose crystallization, lipid oxidation, and Maillard reactions (nonenzymatic browning). Residual activity of enzymes such as β-galactosidase may also play a role for quality deterioration especially in low-heat powders (*3*). As whole milk powder usually is dried to a water content low enough to ensure that the amorphous matrix is in the glassy state, the materials can be regarded as being inert

toward crystallization of lactose throughout even very long storage periods. On the other hand, moisture or elevated storage temperatures can dramatically increase the rate of lactose crystallization. Lactose crystallization will increase water activity due to decreased binding of water resulting in an acceleration of nonenzymatic browning reactions, lipid oxidation, and other deteriorative chemical reactions (*4*). Lactose crystallization will, however, also decrease lactose available for browning reactions, but since crystallization has been found to occur gradually (*5*), an increased rate of browning is expected at least at some stage of crystallization. Lipid oxidation involves oxidation of unsaturated fatty acids in phospholipids and triglycerides, leading to the formation of volatile secondary oxidation products. These oxidation products may bind to proteins but are generally considered responsible for the cardboard-like off-flavor in oxidized whole milk powder, although also decarboxylated β-keto acids may contribute (*6*). The high concentration of lactose and lysine-rich proteins in milk makes milk, and especially milk products with intermediate water activity, sensitive to thermally induced nonenzymatic browning (*7*). In milk products such Maillard reactions are induced by heating during processing and long time storage at moderate to high temperature (*8*). The browning reactions thus have been found to cause the main changes in milk powders during storage (*9*).

Temperature is important for both lactose crystallization and for Maillard reactions leading to browning. These two types of temperature-sensitive reactions may further interact with lipid

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oxidation, since increasing water activity from lactose crystallization or the initial steps in the Maillard reactions, where reaction conditions liberate water, will accelerate lipid oxidation due to higher molecular mobility, while certain Maillard reaction products are known as antioxidants. This complex interaction, which involves elements of both negative and positive feedback, needs detailed studies in order to optimize production and storage conditions.

The present study was accordingly designed to follow the progression of three types of processes mainly responsible for quality deterioration of milk powder under storage at mildly accelerated temperature conditions. A variety of analytical methods were used for the same samples in order to follow the different changes simultaneously and to provide rate data for a mechanistic discussion of possible relationships between lactose crystallization, lipid oxidation, and Maillard reactions. Three different storage temperatures were chosen in order to estimate activation energies for key processes and to investigate the temperature dependence of the chemical and physical deterioration in a temperature range around the glass transition temperature, where the most dramatic effects are expected.

MATERIALS AND METHODS

Whole Milk Powder. Spray-dried, high-heat whole milk powder, Milex, was obtained from Arla Foods Ingredients (Arinco, Videbæk, Denmark). The whole milk powder contained 33−42% lactose, 26−29% milk protein, and 26% milk fat according to product specifications and was produced as part of a full-scale standard production. The powder used for this experiment was produced approximately 5 months before the start of the experiment and was stored at 5 °C in nitrogen atmosphere packed tins until use.

Glass Transition Temperature. The glass transition temperature, T_g, was determined in the fresh powder prior to storage using differential scanning calorimetry (DSC). T_g was determined using a DSC 820 Mettler Toledo (Schwerzenbach, Switzerland), which is based on the heat flux principle and cooled with liquid nitrogen. Calibration of heat flow and temperature was performed with indium as the standard (mp = 156.6 °C, Mettler Toledo calibration kit, ME 119442). The linearity of the calibration was verified with zinc (mp = 419.5 °C, Mettler Toledo calibration kit, ME 119442), decane (T_m = −29.66 °C), and cyclohexane (T_m = 6.47 °C). Approximately 10 mg of whole milk powder was hermetically sealed into a 40 μL aluminum DSC crucible. An empty sealed crucible was used as the blank. The sample was scanned at a rate of 5 °C/min from −20 to 110 °C. T_g was determined as the onset temperature of the endothermic baseline shift (*10*).

Sample Preparation. Experiments were performed under mildly accelerated conditions at three different temperatures: 37, 45, and 55 °C. The whole milk powder was repacked into 20 mL clear glass headspace vials (Mikrolab, Aarhus, Denmark) with approximately 10 g of milk powder in each. The vials were sealed with aluminum press caps and incubated for 17 days at 55 °C and for 150 days at 45 and 37 °C. The powder stored at 37 °C remained white throughout the duration of the experiment, whereas the powder stored at the higher temperatures became brown toward the end of the storage periods.

Color. The surface color was measured according to the Hunter *L*, *a*, and *b* tristimulus color system as described in refs 11 and 12 using a Byk-Gardner color guide 45/0 (Byk-Gardner CB-6692, Geretsried, Germany). The color changes were expressed by the Hunter *b*-value, which measures yellowness (+) or blueness (−), where an increase in the *b*-value reflects a change in color toward yellow and brown (*13*). The results are presented as the mean of three measurements.

Furosine. A Waters HPLC (Alliance 2695, Waters, Milford, MA) with an Alltech furosine-dedicated column (Alltech, Capelle aan den Ijssel, Netherlands) and UV detector operating at 280 nm (996 PDA, Waters, Milford, MA) was used for the analysis of furosine (*N*-furoylmethyl-L-lysine) content in whole milk powder by the IDF standard method (IDF 193|ISO 18329: 2004). A sample of 6.5 g of milk powder was mixed with 50 mL of water, and 2 mL of this mixture

was hydrolyzed in 8 mL of 10.6 N HCl at 110 °C for 23 h. The hydrolyzed mixture was purified through a C18 cartridge (solid-phase extraction, J. T. Baker, Philipsburg, NJ) using 3 N HCl. For the HPLC analysis two solvents were used: (A) 0.4% acetic acid in water (v/v) and (B) 0.3 potassium chloride in solvent A (w/v). A standard solution of furosine (1 nM/mL in 3 N HCl) was analyzed in parallel with the samples to determine the concentration of furosine in the samples. The content of furosine was expressed as mg of furosine per 100 g of powder in order to compare with the HMF results and is presented as the mean of two measurements.

5-Hydroxymethyl-2-furfuraldehyde (HMF). The amount of bound 5-hydroxymethyl-2-furfuraldehyde (B-HMF) was determined by use of an RP-HPLC method described by Morales et al. (*7*). To measure the amount of B-HMF, lactose was separated from the reconstituted milk sample (3.25 g of powder per 25 mL of water) by loading the sample on an NAP-10 (Sephadex G-25) disposable column (1.3 × 2.6 cm^2) as described by Morales et al. (*7*). Then, 1 mL of the recovered eluate was digested with 0.5 mL of 0.3 N oxalic acid for 1 h at 100 °C. After rapid cooling on ice the mixture was deproteinized with 0.5 mL of trichloroacetic acid (TCA) solution (40%, w/v) and centrifuged at 10 000 rpm for 12 min. After filtration through a 0.45 μm filter, the sample was ready for HPLC analysis. Forty microliters of the sample was injected into a Waters Alliance 2695 HPLC (Waters, Milford, MA) with a Spherisorb column (5 μ, 4.6 mm × 250 mm) (ODS2, Waters, Milford, MA) and UV detector operating at 280 nm (996 PDA, Waters, Milford, MA). The amount of HMF was expressed as μmol per 100 g of powder and is presented as the mean of two measurements.

Heat of Crystallization, ΔH. The heat associated with crystallization of lactose in whole milk powder was measured with isothermal differential scanning calorimetry (Micro-DSC III, Setaram, Caluire, France) according to Knudsen et al. (*14*) with minor modifications. Calibration of heat flow and temperature was performed with naphthalene (mp = 80.3 °C). An amount of 100.0 mg of whole milk powder was placed in the sample vessel and scanned at 1 °C/min from 10 to 74 °C, after which the heat production of the sample was measured in the isothermal mode at 74 °C for 12 h, which was sufficient for crystallization to be complete for all samples. The evolved heat, ΔH (J/g), obtained by integration was taken as a measure of the fraction of lactose not crystallized and used to follow the progression of crystallization. Due to the long time requirement for each ΔH measurement, only one run was performed for every sample.

Radical Concentration. The radical concentration was measured by electron spin resonance (ESR) spectroscopy according to Thomsen et al. (*12*). A JES FR30 free radical monitor (JEOL, Tokyo, Japan) was used with the following parameters: sweep time 2 min, sweep width 5 mT, microwave power 4 mW, modulation width 0.1 mT, and time constant 0.3 s. A built-in Mn^{2+} marker was used as the instrument control and to relate to the sample signal from day-to-day measurement. The results were expressed as the area under the powder signal relative to the area under the signal from the standard and divided by the density of the powder in the tube determined by weighing a fixed volume of the actual sample. The relative radical concentration was determined as a mean of two measurements. *g*-Values to characterize the radicals were determined relative to the *g*-value of the right Mn^{2+} marker signal (g = 1.981) and the distance (ΔH_{Mn} = 8.69 mT) between the two Mn^{2+} marker signals.

Volatile Oxidation Products. Volatile oxidation products, pentanal and 2-heptanone, in the headspace of whole milk powder were determined by headspace GC according to the method described by Shahidi and Pegg (*15*) with minor modifications. A 2.0 g sample of whole milk powder was transferred to a 20 mL headspace vial along with 50 μL of solution of the internal standard, 5-methyl-2-hexanone in 1,2-propanediol (1600 ppm). To keep the milk powder dry the standard solution and powder were kept separate with the standard solution in a 200 μL insertion tube on top of the powder within the headspace vial. The vials were closed with aluminum press caps. The samples were preheated in an HP 7694 headspace sampler (Hewlett-Packard, Palo Alto, CA) for 45 min at 90 °C before the vapor phase was transferred to a 3 cm^3 loop (321-056 HSP) under the following conditions: carrier gas, helium; vial pressure, 0.90 bar; tr line, 110 °C; loop temp, 100 °C; vial eq time, 45 min; GC cycle time, 25 min;

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inject time, 0.40 min; loop eq time, 0.02 min; loop fill time, 0.04 min, and pressurize time, 0.13 min. The chromatographic separation was performed by an HP 6890 headspace GC (Hewlett-Packard, Palo Alto, CA). From the loop, the vapor phase was injected (injection temperature, 200 °C; injection time, 0.4 min) onto a high-polarity HP19095X-123 HP wax bonded polyethylene glycol column (30.0 m × 530 μm × 1 μm) (Hewlett-Packard, Palo Alto, CA). Helium was used as the carrier gas (0.66 bar) with a splitting of 7:1. The oven temperature was 50 °C for 5 min, followed by 115 °C for 1 min (after heating at 10 °C/min), and finally 200 °C for 1 min (after heating at 30 °C/min). The flame ionization detector temperature was 250 °C. Concentrations of volatile oxidation products (ppm) were determined by comparing peak areas of each product to that of the internal standard.

Water Content. The water content of the whole milk powder was determined on all samples at each temperature by incubating samples of 0.500 g of whole milk powder in 5.0 mL of water-free methanol (max 0.005% H_2O) for 24 h and subsequently determining the water content of 1.00 mL of the powder−methanol slurry by Karl Fischer titration (Mettler Toledo DL 18, Switzerland). The water content was determined as the mean of three measurements.

Water Activity. The water activity of the milk powder was measured at room temperature using an Aqua Lab CX-2 (Aqua Lab, Pullman WA, U.S.A.). The water activity was determined as the mean of two measurements.

RESULTS

Physical Changes. To characterize the specific whole milk powder under investigation and in order to select the storage temperatures, the glass transition temperature was determined, using conventional temperature-scanning calorimetry, to have the value of 48 °C. On the basis of this value for T_g, the three temperatures 37, 45, and 55 °C were chosen as storage temperatures in order to include the effect of glass transition on the physical and chemical processes occurring in the actual product.

Lactose in whole milk powder has previously been shown to crystallize more gradually instead of through an "all or none" process as observed for amorphous lactose and infant formula (*14*). To monitor the progress of the crystallization in whole milk powder during storage at mildly accelerated conditions, a semiquantitative calorimetric method was developed. In this calorimetric method the temperature of the sample was scanned at 1 °C/min from 10 to 74 °C, after which the heat production of the sample was followed in isothermal mode at 74 °C for 12 h. At this high temperature the amorphous state of lactose is kinetically highly unstable, and crystallization will be completed within a relatively short time scale. The heat released during the isothermal crystallization can be considered to be proportional to the amount of lactose undergoing crystallization.

Figure 1 shows three isothermal time traces of heat flow for three samples of whole milk powder measured after storage for 0, 71, and 126 days in closed vials at 45 °C. At day 0, and after 71 days of storage at 45 °C, evolution of heat was observed immediately after reaching 74 °C in the calorimeter, as seen by the steep rise of the heat flow curves. Crystallization was complete after 2 h of isothermal measurement for both samples, after which the curves leveled off for both samples. On the contrary, for the sample stored for 126 days only a gradual leveling off of the heat flux curve was observed corresponding to the transition from scanning to isothermal mode indicating that crystallization was already completed during storage of the sample.

By subtraction of the appropriate baselines and integration of the curves, the heat of crystallization, ΔH (J/g), was calculated. This quantity is a direct measurement of the processes taking place during the calorimetric measurements,
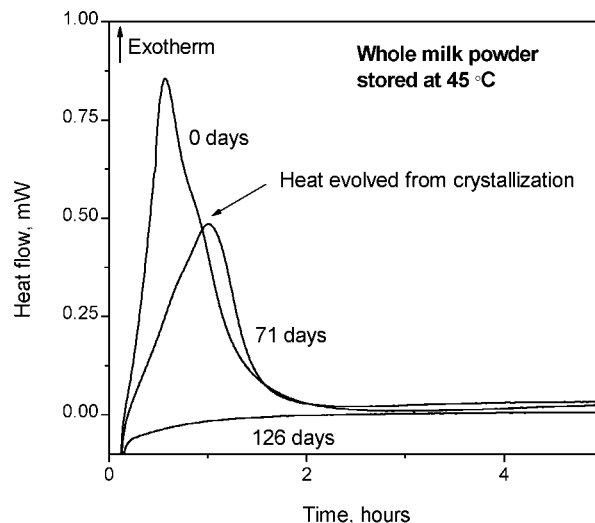

Figure 1. Isothermal time traces at 74 °C of heat flow for samples of whole milk powder stored in closed vials at 45 °C for 0, 71, and 126 days prior to calorimetric measurement.

which is easily converted to quantification of the degree of crystallization in the milk powder before calorimetric measurements, i.e., at the specific point during the time course of storage. For the samples under consideration in **Figure 1**, ΔH was found to be −16.2, −10.9, and 0 J/g, respectively, for the same powder stored for 0, 71, and 126 days. Accordingly, most lactose crystallized in the fresh samples during the isothermal calorimetric measurements, less lactose crystallized after 71 days of storage, and no lactose crystallized after 126 days. Notably, the decreasing heat evolution indicated by these numbers for ΔH reflects that the amount of amorphous lactose becomes smaller with storage time, when milk powder is exposed to a temperature of 45 °C. Thus, lactose crystallizes gradually during the 126 days of storage, and the progression of the crystallization process can be monitored by isothermal calorimetric measurements.

Figures 2A and **3A** show the time development in ΔH for samples of whole milk powder stored at the three temperatures, 37 °C (**Figure 2A**), 45 °C (**Figure 2A**), and 55 °C (**Figure 3A**). For the powder stored at 55 °C (**Figure 3A**) crystallization (and other possible exothermic reactions) of the powder was completed after 13 days of storage, as concluded from the fact that no heat was released in the calorimetric assay after this storage period. In comparison, the powder stored at the lower temperature, 45 °C (**Figure 2A**), reached the same point after 126 days of storage. For samples stored at both 55 and 45 °C the development of ΔH was similar except for the time development in the two storage experiments. The lactose crystallized gradually during storage, as was concluded from the gradual increase seen for both curves. For the powder stored at 37 °C, crystallization was not observed to any significant degree within the time scale of 146 days (**Figure 2A**).

The development in the water content was monitored using Karl Fischer titration. The water content of samples stored at any of the three temperatures decreased slightly from about 4.4 to about 3.5% (w/w). This small and hardly significant decrease in water content may indicate that the vials used for storage were not perfectly closed systems at the slightly elevated temperatures, and differences in water activity may have caused some interchange of water with the surroundings.

The water activity, a_w, measured for the milk powder during storage clearly depended on crystallization of lactose (*12*, *16*). The storage experiments were performed in closed vials at
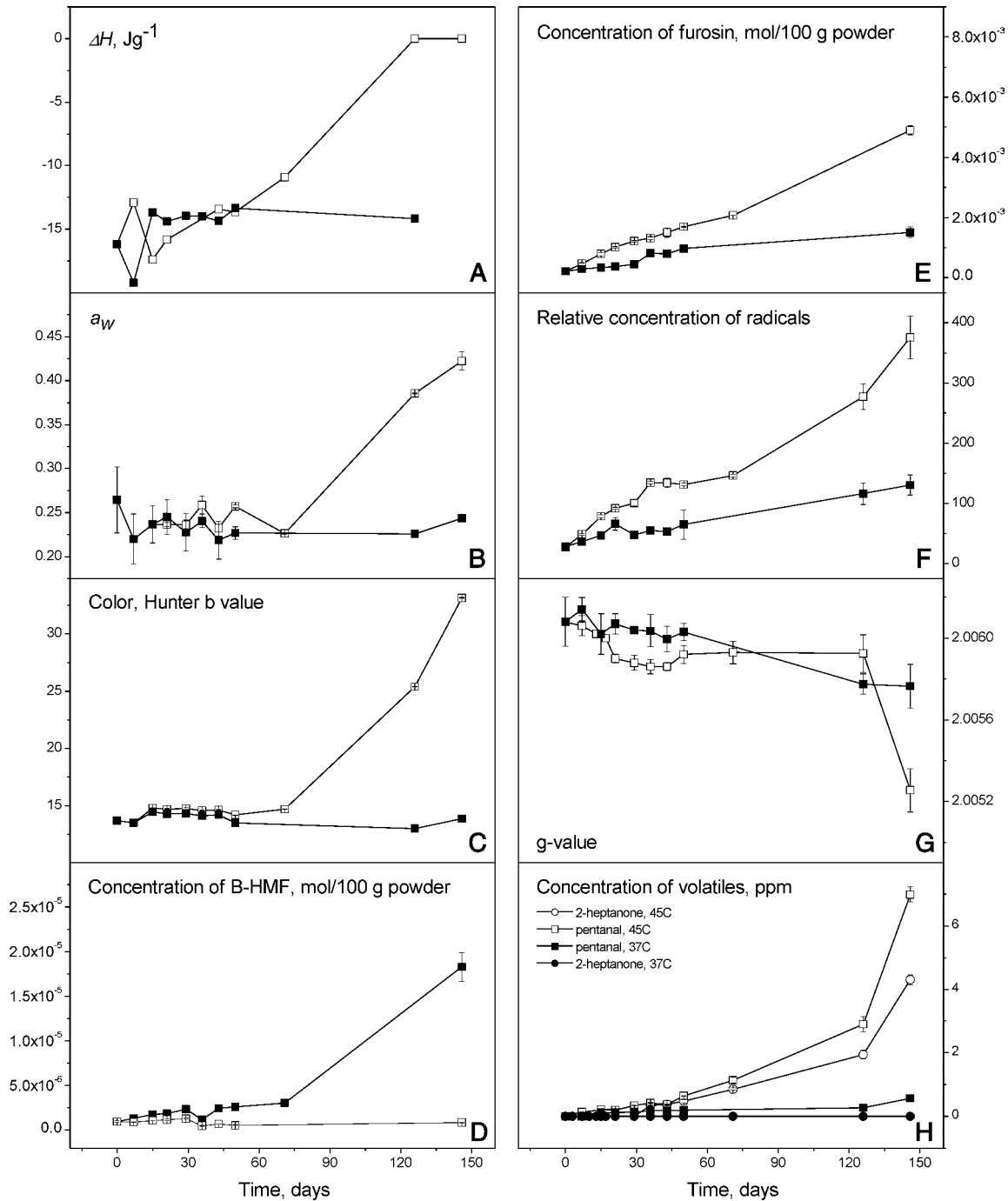
Figure 2. Physical and chemical changes in whole milk powder stored in closed vials at 37 °C (■,●) or at 45 °C (□,○). (**A**) Heat of crystallization at 74 °C determined by isothermal calorimetry (**Figure 1**). (**B**) Water activity, a_w, determined at room temperature. (**C**) Surface browning measured as the Hunter *b* tristimulus parameter. (**D**) Bound hydroxymethyl-furfural (B-HMF) as determined by HPLC. (**E**) Furosine as determined by HPLC following hydrolysis. (**F**) Relative radical concentration as determined by ESR spectroscopy (signal intensity). (**G**) *g*-Value of the radical detected by ESR spectroscopy. (**H**) 2-Heptanone (circles) and pentanal (squares) as detected by headspace GC.

conditions which resulted in a slightly decreasing water content; thus, the observed increase in a_w could not indicate chemical reactions occurring which developed water but rather a change in the moisture sorption isotherm of the powder, which can be assigned to crystallization of lactose (*12, 16*). In **Figures 2B** and **3B**, the development of a_w is shown for storage at different temperatures. In accordance with the results of ΔH, no change in a_w was observed for the powder stored at 37 °C, whereas a clear similarity is seen in the curves for the values of ΔH and a_w during storage of powder at both 45 and 55 °C. For powder stored at 45 °C, a lag period of 71 days was observed during

which both ΔH and a_w were almost constant (**Figure 2B**), after which a gradual rise was seen until a_w was almost doubled concomitant with an increase in ΔH. With regards to the ΔH, the data are somewhat noisy during the lag time, before onset of severe crystallization. However, after 71 days of storage the curve corresponding to 45 °C increases as compared to that of 37 °C, indicating that at this stage crystallization initiates at 45 °C.

For the powder stored at 55 °C, the lag period was reduced to about 7 days followed by a steep increase in a_w from day 7 to 13, after which the values of a_w approached a constant value
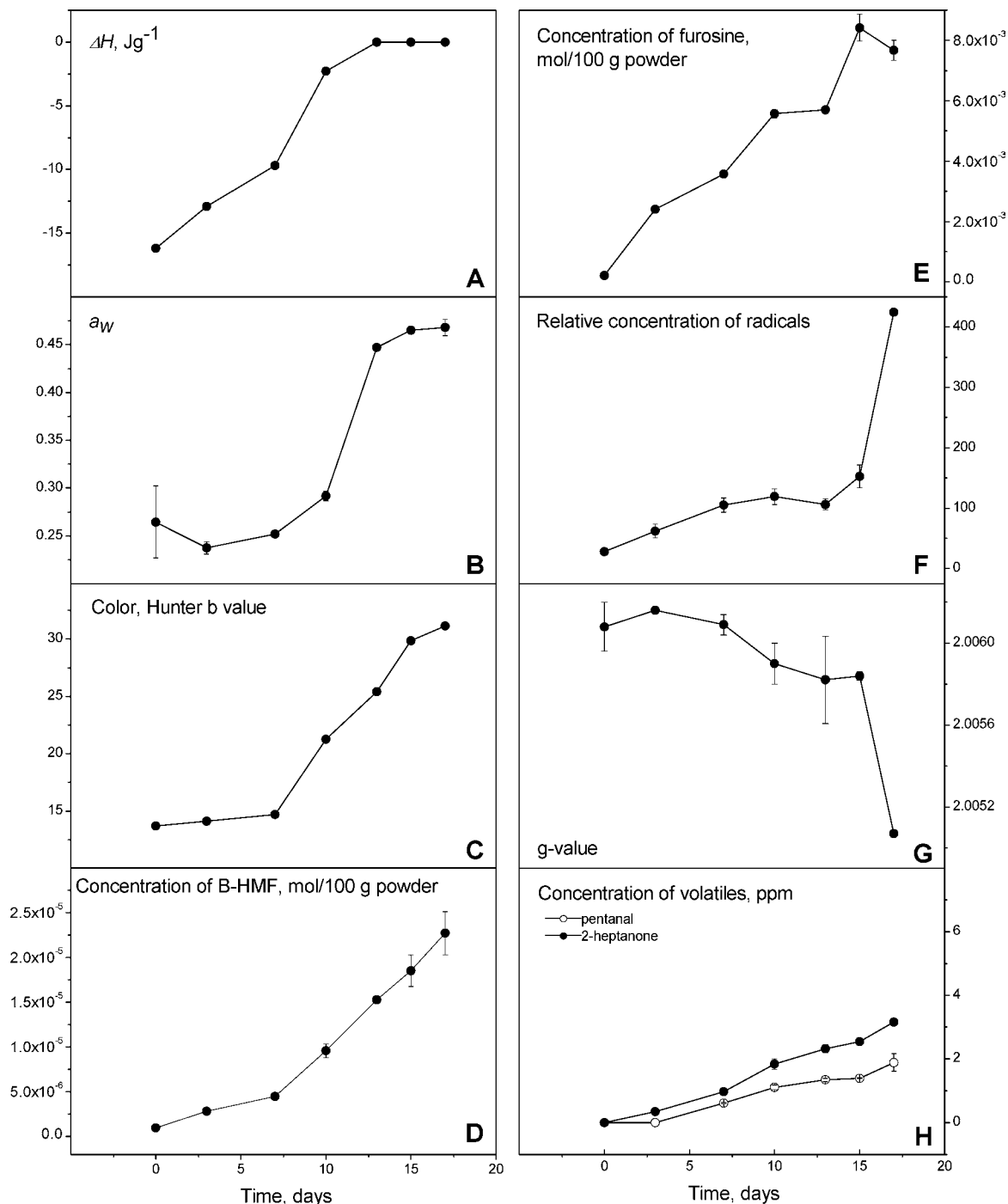
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Figure 3. Physical and chemical changes in whole milk powder stored in closed vials at 55 °C. (**A**) Heat of crystallization at 74 °C determined by isothermal calorimetry (**Figure 1**). (**B**) Water activity, a_w, determined at room temperature. (**C**) Surface browning measured as the Hunter *b* tristimulus parameter. (**D**) Bound hydroxymethyl-furfural (B-HMF) as determined by HPLC. (**E**) Furosine as determined by HPLC following hydrolysis. (**F**) Relative radical concentration as determined by ESR spectroscopy (signal intensity). (**G**) *g*-Value of the radical detected by ESR spectroscopy. (**H**) 2-Heptanone (○) and pentanal (●) as detected by headspace GC.

of 0.46 for days 15 and 17 during which period ΔH indicated that crystallization was completed (**Figure 2B**). For the powder stored at 45 °C, a constant value for a_w was not observed as the storage period after complete crystallization probably was too short to observe this effect, as adjustment of a_w seems to be leaping behind crystallization.

Maillard Reactions. The progress of the Maillard reactions was monitored by measuring the early stage reaction products, furosine (ϵ-*N*-furoylmethyl-L-lysine) and HMF (5-hydroxy-methyl-2-furfuraldehyde), as well as color according to the Hunter Lab system. Since furosine and HMF are not contributing

to any brown color, as they only absorb in the UV spectral region, the combination of measurement of furosine and HMF with color measurement provides information of the progression of different stages of the Maillard reactions, with furosine and HMF as precursors of the brown pigment.

Furosine and HMF are both early markers of the Maillard reactions, since they are derived from the Amadori product, ϵ-*N*-deoxylactolusyl-L-lysine, resulting from the condensation reaction between lactose and lysine, by different pathways. Acid hydrolysis of ϵ-*N*-deoxylactolusyl-L-lysine during the analytical procedure converts it to the more stable furosine, which can be

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directly determined by HPLC. Thus, determination of furosine can be regarded as a convenient indirect quantification of the Amadori product. The process conditions used for the milk before drying may have some influence on the formation of furosine (*17*); however, the drying conditions are critical for the levels of furosine in milk powder, and a significant increase is seen when liquid, pasteurized milk is compared with milk powder. The storage conditions used for milk powder have also been found to affect the level of furosine, and furosine has been concluded to be a relevant indicator of Maillard reactions occurring in milk powder during storage.

HMF (5-hydroxymethyl-2-furfuraldehyde) is formed upon heat treatment of milk by Maillard reactions under acidic conditions via 1,2-enolization of the Amadori product and by isomerization and degradation of sugars also in the absence of amines. The so-called "bound HMF" (B-HMF), which is determined by decomposition of the Amadori compound of a sugar-free (lactose-free) fraction of milk (*7*), provides a good indirect measure of the extent of Maillard reaction in many milk products. As lactose is not present in the crucial digestion step during the analysis, HMF formed by non-Maillard-related sugar degradation is not contributing to the quantification used in the present study, and the results only reflect the amount of Amadori compound formed during storage before separation of sugars.

Figures 2E and **3E** show the development in furosine concentration in the whole milk powder during storage at 55, 45, and 37 °C. It is seen that the concentration of furosine increases gradually, almost linearly, throughout the storage period (corresponding to zero-order kinetics). At the lowest storage temperature, 37 °C, where enthalpy of crystallization and water activity remained constant, indicating that lactose is not crystallizing, significant amounts of furosine were still detected in the samples (about 1.75 mmol/100 g of powder after 128 days of storage). The development of furosine is highly dependent on temperature, as indicated by the observation that after only 18 days of storage at 55 °C approximately 6 mmol of furosine/100 g of powder was formed.

A similar pattern in the development was found for the other Maillard reaction indicator, as the concentration of B-HMF increased gradually from the first day of storage (**Figures 2D** and **3D**). The concentration of B-HMF is somewhat smaller as compared to that of furosine throughout the storage period, and after 18 days of storage at 55 °C the concentration B-HMF was approximately 22.5 μmol/100 g of powder. As both compounds are formed from the same Amadori product during the analytical procedures, the differences in concentration are intriguing. However, for any of the two analytical techniques the degree of conversion has not been documented and cannot be considered to be quantitative. The B-HMF procedure indicates a separation process during which B-HMF from high molecular weight protein-based Amadori products are concentrated, in contrast to the procedure for furosine, which accordingly also will include Amadori products of free lysine or peptides. When the amounts of furosine and B-HMF quantified at the lowest storage temperature are compared, it is further noticed that following the complete storage period of 146 days, a significant formation of furosine was observed in contrast to a small decrease in the B-HMF content relative to the fresh product.

Previous investigations of whole milk powder stored at 60 °C have shown that the increases in a_w and the change in color due to formation of late-stage Maillard products were strongly coupled (*12*). In **Figures 2C** and **3C** the development of color in the powder during storage is shown. For samples stored at 37 °C no change in *b*-value was observed (**Figure 2C**), and the

powder remained white, which was confirmed visually. For the samples stored at higher temperatures a lag phase of 7 days for samples stored at 55 °C (**Figure 3C**) and of 71 days for samples stored at 45 °C (**Figure 2C**) was observed prior to a gradual increase in Hunter *b*-value. For samples stored at any of the two temperatures, the Hunter *b*-value was doubled within the time of storage. The browning seems to increase not reaching any limiting Hunter *b*-value for any of the samples, as was also expected, since the color parameter quantifies formation of late Maillard reaction products (melanoidins), and there is no reason to expect that the amount of reactants would limit the amount of formed end products. When the development in water activity (**Figures 2B** and **3B**) is compared with the browning as measured as the Hunter *b*-value, it is confirmed that the browning occurs simultaneously with the increase in a_w, also at lower temperatures.

Formation of Volatiles Related to Lipid Oxidation and Detection of Radicals. Lipid oxidation was followed by measurements of radicals, as well as detection of two volatile products, pentanal and 2-heptanone. Both products are related to off-flavor of milk products and result directly from oxidation of unsaturated fatty acids naturally present in the milk fat (pentanal) (*18*) and from decarboxylation of β-keto acids (2-heptanone) (*19*). These products represent the two volatiles present in highest concentration in this study. Pentanal is formed by β-scission of position 13 lipid peroxides, which is considered to be a less important pathway as compared to the α-scission pathway forming hexanal. Surprisingly, hexanal was not detected in this study for unknown reasons.

In **Figures 2H** and **3H** the results from the GC headspace measurements of pentanal and 2-heptanone are presented. For milk powder stored at any of the three temperatures, pentanal was formed prior to 2-heptanone. For the powder stored at 37 °C, no 2-heptanone could be detected during the storage period, and only a small increase in pentanal was observed. At 55 °C a gradual increase in concentration was seen for both volatiles during 17 days of storage, although for 2-heptanone a lag phase of 3 days is noted.

The content of radicals detectable in milk powder has been associated with early stages of lipid oxidation (*11*, *12*). Moreover, it has been shown that at least two types of radicals exist in milk powder differing in *g*-values and in spectral appearance during storage. The *g*-value is a spectral characteristic of radicals, and formation of the radicals with the low *g*-value is closely related to lactose crystallization and browning (*12*). In **Figure 2**, parts **F** and **G**, and **Figure 3**, parts **F** and **G**, the development in radical concentration during storage and changes in the *g*-values of the radicals detected in the powder stored at the three different temperatures are shown. Like the formation of furosine and secondary oxidation products, a gradual increase in concentration of radicals was observed for milk powder stored at each of the three temperatures. For the powder stored at 55 °C, a steep rise in the intensity of the ESR signal was observed from day 15 to 17, where the powder had become brown, and the formation of the low *g*-value radicals, the so-called "brown" radicals (*12*), was progressing strongly. A simultaneous decrease in the *g*-value is noted (**Figure 3G**), as a gradual and slow decrease in the *g*-value was observed from day 0 to 15 (from 2.0061 to 2.0058), followed by a steep fall from day 15 to 17 (from 2.0058 to 2.0051). In contrast, the increase in the total radical concentration was found to occur more gradually during the whole storage period for the samples stored at 45 °C. However, a slightly steeper increase was seen for the ESR signal intensity from day 71 to 146 compared to
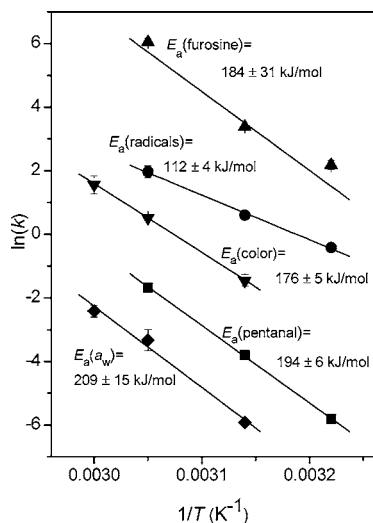
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Figure 4. Arrhenius-type plot based on relative rate of formation of high *g*-value radicals ("white" radicals) related to lipid oxidation (●) corresponding to zero-order kinetics, rate of formation of furosine (▲) corresponding to zero-order kinetics, rate of formation of pentanal (■) corresponding to zero-order kinetics, surface browning (▼) corresponding to linear increase in Hunter *b*-value following an initial lag phase, and rate of linear increase in water activity (◆) following initial lag phase. For each plot the corresponding calculated activation energy is shown.

that from day 0 to 71, although a somewhat similar relation between radical concentration and *g*-value was observed. Thus from day 0 to 126, the *g*-value was almost constant around 2.0059−2.0060, after which a steep decrease to 2.0053 was observed between day 126 and 146. At 37 °C, the *g*-value decreased slowly from 2.0061 (day 0) to 2.0058 (day 146) (**Figure 2G**), which was accompanied by a gradual, but slow, increase in the concentration of radicals (**Figure 2F**). For this "low" temperature, the *g*-value does not reach the low values as observed for the two higher temperatures. Likewise, the concentration of radicals, though increasing steadily, does not reach the high levels seen at the higher temperatures. As lactose crystallization, water activity increase, and increase of Hunter *b*-value were also absent at 37 °C, the results confirm the finding of Thomsen et al. (*12*) that these events are strongly coupled.

The deteriorative processes all showed a strong temperature dependence which was analyzed within the framework of the Arrhenius equation. For the processes which showed a gradual change, rather than an "all-or-none" behavior, apparent energies of activation were estimated from Arrhenius plots as shown in **Figure 4**.

DISCUSSION

Storage of the whole milk powder above the glass transition temperature (T_g = 48 °C) at 55 °C or closer to T_g at 45 °C resulted in significant quality changes as may be seen from **Figures 2** and **3**. Some of the parameters measured in order to follow the product quality showed a clear lag phase prior to abrupt changes taking place. These quantities include the product water activity, Hunter *b*-value, B-HMF, concentration of radicals, and the effective *g*-value of the ESR spectra. The time resolution used for measurements at 45 and 55 °C was different due to the different storage time needed to follow the changes. In some cases this limits the conclusion regarding the onset of some of the individual processes at 45 °C. However, the results found for 45 °C seems in general to support the observations obtained for 55 °C. At 55 °C water activity, Hunter *b*-value,

and B-HMF each showed a lag phase of approximately 6 days, after which time period the various processes showed a sudden acceleration. The development of radicals and the changes in effective *g*-value were leaping somewhat behind, as the abrupt changes for these quantities were observed after 13 to 15 days of storage. The interpretation of the time development of the enthalpy changes related to lactose crystallization, ΔH, is limited by the fact that no information about the possible error associated with the method is available, as only single determinations were possible for this analysis. The numerical value of ΔH seems to decrease quite smoothly without inflection points or discontinuities. However, it cannot be ruled out that the process accelerates slightly after 6 days corresponding to the lag phase for water activity, Hunter *b*-value, and B-HMF. In contrast to these quality parameters, the formation of furosine and the volatile oxidation products, pentanal and 2-heptanone, developed almost linearly with time without any lag phase. Thus, the formation of these compounds seems to be progressing independently of the dramatic event taking place in the powder after about 6 days at 55 °C or 80 days at 45 °C. The quality parameters can accordingly be divided into two groups—one group where changes are gradual and which starts to change immediately and another group where a lag phase precedes a sudden acceleration.

On the basis of such considerations, it is of interest to identify the main "dramatic event" that takes place after about 6 days of storage at 55 °C and which influenced the various processes to a different extent. We suggest that crystallization of lactose is the main cause of the dramatic changes after 6 days of storage at 55 °C. In this perspective, the differences observed in the pattern of the development of ΔH and a_w with regard to the abruptness of the process need to be examined. The observed differences between the apparent gradual development of ΔH and the delayed and more abrupt change in a_w may reflect a nonlinear relation between the increase of water activity and the degree of crystallization. During crystallization of lactose into an anhydrous form of lactose (β-lactose), the released water is sorbed and concentrated into the remaining amorphous phase. The amount left of this phase as well as the sorption characteristics (isotherm) of this remaining phase and the amount of released water will thus control the increase of water activity. Moisture sorption isotherms are generally nonlinear, and together with the decreasing amount of amorphous material, these effects could very well favor the observation of a nonlinear abrupt increase of water activity. However, more experiments, and theoretical consideration, are required in order to clarify these questions.

The increase in water activity initiated by lactose crystallization clearly induces the formation of late-stage Maillard reaction products after 6 days of storage at 55 °C and 80 days at 45 °C. As observed before at slightly higher temperatures (*12*), the onset of formation of high concentrations of low *g*-value radicals lags behind the onset of color formation. The effect is further increased at the lower temperature of 55 °C used in the present study.

It has been reported in the literature that the rate of lipid oxidation in whole milk powder depends on the water activity (*11*). However, the subsequent increase in water activity induced by crystallization of lactose in the present experiments apparently did not affect the progression of lipid oxidation, as no abrupt changes in the formation of volatiles were observed. This is probably a consequence of the fact that the lipid phase is partly separated from the surrounding amorphous matrix, and thus the event of lactose crystallization does not have a direct

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impact on the chemical reactions in the lipid phase. Radical-generating processes may not be hindered in the amorphous phase and hence initiate lipid oxidation in the lipid phase. Such processes might, however, be influenced by lactose crystallization and changes in water activity and could be responsible for the water activity dependence reported in the literature from some older studies. However, the increase in water activity observed in the present study takes place too late in the process in order to affect the formation of secondary oxidation products.

Late-stage Maillard reaction products have been found to possess antioxidative effects in milk powder (*6*). The results presented in the present paper do not suggest such mechanisms to be of major importance, as the observed rate of formation of secondary oxidation products increased rather than decreased at the stages where browning products were formed. As for the effect of water activity, the possible antioxidative Maillard products are formed in the amorphous matrix, and the effect in the lipid phase is probably too small to change the progress of lipid oxidation at this late stage.

The processes under consideration show strong temperature dependence, as reflected by the very different time scale seen in **Figures 2** and **3**. Roughly the same change of the measured quantities is observed over 17 days at 55 °C and over 150 days at 45 °C. When the temperature is lowered 8 °C to 37 °C, virtually no changes take place within a time scale of 150 days. The only exceptions from this observation are the fact that considerable amounts of furosine and radicals are formed at the lowest temperature. To quantify this temperature dependence further, the energy of activation was derived. Briefly, the development of pentanal, furosine, and radicals could be well described by zeroth-order kinetics, and thus the reaction rate was determined by linear regression. For the changes related to browning and increase of water activity, it was necessary to include data from a previous study, where these changes were studied at 60 °C (*12*) as virtually no changes were observed at 37 °C. For these processes the zero-order rate following the induction period could be determined and used for determination of the activation energies, cf. **Figure 4**.

In **Figure 4** the energy of activation, E_a, for development of high *g*-value radicals formed during the early storage period and related to lipid oxidation are collected together with E_a for formation of furosine and pentanal, which all occurred without a lag phase, and the E_a for color and water activity again determined for the zero-order kinetics observed after the lag phase.

All of the processes, except radical formation, show activation energies somewhere in a narrow interval between 180 and 210 kJ/mol. It has been suggested that the dramatic viscosity changes in a temperature interval in the neighborhood of the glass transition temperature can cause a very strong temperature dependence of chemical reactions. The effective energy of activation due to such effects of glass transitions is expected to be in the range of 200−400 kJ/mol (*20*). In this context it is worth noticing that for the two highest temperatures the main component of the amorphous phase is crystallizing, and thus the properties "host" phase for many of the chemical processes changes dramatically in parallel with the ongoing chemical reactions. The remaining amorphous phase is being concentrated. In case of water this is observed as an increase of the water activity. This behavior is thus incomparable with most other systems where the solvent is behaving more conventionally. It is generally accepted that crystallization kinetics are strongly influenced by molecular mobility and mechanical properties of the material (*20*). Consequently, the change of mechanical

properties of the amorphous matrix could be a likely explanation for the observation of the high activation energies through a direct action on each of the chemical reactions or through an indirect action via up-concentration or water activity effect.

Glass transition temperatures have also been speculated to be an absolute threshold for stability of amorphous foods. For the whole milk powder under consideration, the glass transition temperature was measured to be 48 °C. Thus, the observation of formation of the Maillard product furosine and the formation of radicals and even secondary lipid oxidation products such as 2-heptanone and pentanal prior to crystallization is not in agreement with this more conservative interpretation of the glass transition theory. In this context it is also worth noticing that the glass transition was not introducing any deviation from Arrhenius temperature dependence. Accordingly, we conclude that although the values of energy activation are high for the process studied in milk powder in a temperature interval around the glass transition temperature, we have found no indication of T_g as a critical temperature, below which storage can be prolonged "indefinitely".

In summary, crystallization of lactose has a dramatic impact on many chemical changes in whole milk powder stored at mildly elevated temperatures. Lactose crystallization induces an autocatalytic increase in water activity and triggers the development of the late-stage Maillard reaction products, melanoidins, and the low *g*-value radicals. Development of furosine, lipid-dependent radicals, and secondary oxidation products was not found to be influenced by the water activity increase following lactose crystallization.

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