Electrophoretic characterization of protein interactions suggesting limited feasibility of accelerated shelf-life testing of ultra-high temperature milk

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Abstract

Accelerated shelf life testing is applied to a variety of products to estimate keeping quality in a short period of time. The industry has not been successful to apply this approach to UHT milk due to a number of chemical and physical changes of milk proteins that take place during processing and storage. These changes were investigated applying accelerated shelf life principles on UHT milk samples containing 2 different fat levels using native- and SDS-polyacrylamide gel electrophoresis. UHT skim (SM) and whole milk (WM) samples were stored at 20, 30, 40 and 50 °C for 28 days. Irrespective of fat content, UHT treatment had a similar effect on electrophoretic patterns of milk proteins. At the start of testing, proteins were crosslinked mainly through disulphide and non-covalent interactions. However, storage at and above 30 °C enhanced protein crosslinking more via covalent interactions. Extent of aggregation appeared to be influenced by fat content as WM contained a greater amount in comparison to that in SM implying aggregation via melted and/or oxidised fat. Based on reduction in loss in absolute quantity of individual proteins, covalent crosslinking in WM was facilitated manly through deamidated residues and products of lipid oxidation, while Maillard and dehydroalanine products were the main contributors involved in protein changes in SM. Protein crosslinking appears to follow a different pathway at higher temperatures ($\geq$ 40 °C) to that at lower temperatures, which may make it very difficult to extrapolate these changes to predict protein crosslinking at lower temperatures.

Keywords: Storage, aggregation, PAGE, protein crosslinking, UHT, whole milk, skim milk

INTRODUCTION

Production of ultra high temperature (UHT) milk involves heating of milk at a high temperature (usually 130-140 °C) for 3-5 seconds (s) followed by aseptic packaging to produce a 'commercially sterile' product with minimal changes in quality[1]. Thermal treatment enables storage of UHT milk at room temperature up to 9 months, thus, eliminating the requirement for cold chain and refrigeration facilities in the distribution chain. Such a long shelf life at room temperature has made UHT milk an important food product from a nutritional, technological and economical point of view. However, high temperature treatment induces chemical changes such as, protein denaturation, Maillard reaction and mineral imbalances, which also progress during subsequent storage of UHT milk[2, 3]. These chemical changes may lead to crosslinking of proteins resulting in storage instabilities, including sedimentation of proteinaceous material at the bottom of the storage container or gel formation (age-gelation) [4-6]. These storage instabilities limit shelf life and hence, the market potential of UHT milk. Consequently, these changes have been of considerable interest to the dairy industry.

Protein crosslinking can occur primarliy via the formation of disulphide bonds, advanced Maillard products, dehydroalanine and deamidated proteins[1, 7-9]. However, the exact sequence and crosslinking mechanisms that lead to build up of sediment or gel is not known. Exploring the progression of these changes by applying full length shelf life tests could be very time and resource consuming for the industry. Therefore, accelerated shelf life tests could be an alternative for predicting storage changes for products such as UHT milk. In fact, accelerating the rate of deterioration of sensory attributes by exposing the product to elevated storage temperatures, shelf life was predicted in a comparably short time[2]. Besides deterioration in sensory attributes, protein crosslinking is also enhanced at higher storage temperatures[10]. Therefore, using elevated storage temperatures, protein crosslinking reactions

could also be accelerated. These results can then be extrapolated to predict and elucidate aggregation mechanisms at lower storage temperature, thus avoiding long real-time analysis.

Additionally, UHT milk is sold with different fat contents. The fat content of milk influences the shelf life of stored UHT milk[11-13]. Whole milk (WM) appears to undergo lower levels of proteolysis[11, 12] and contain less Maillard reaction products[13] during storage as compared to skim milk (SM). However, the effect of fat content on protein crosslinking during storage at moderate or elevated temperatures has not been described in the literature. Herein, we investigated the chemical changes in milk proteins during storage of UHT milk with different fat content at room, as well as, at elevated temperatures in order to establish feasibility of using an accelerated testing for predicting shelf life of UHT milk.

MATERIALS AND METHODS

Materials. Commercial UHT full cream (whole) and skim milk were kindly provided by a local manufacturer (Murray Goulburn Co-operative Co. Ltd., Victoria, Australia). All milk packs originated from the same batches. The packs were produced on the same day using an established process on an indirect tubular processor (SPX Flow Technology, Mulgrave, Australia) with a 9,000 l/hour capacity at 138 °C for 6 s. The composition of UHT full cream milk as reported by the manufacturer was 33 g/l protein, 34 g/l fat and 53 g/l sugars, 0.55 g/l Na and 1.2 g/l Ca. The composition of UHT skim milk was 34 g/l protein, 1 g/l fat and 53 g/l sugars, 0.55 g/l Na and 1.2 g/l Ca. The electrophoresis chemicals were obtained from BioRad Laboratories, Richmond, CA, USA. The reducing agent 2-mercaptoethanol, bovine milk standards α-lactalbumin (α-la), β-lactoglobulin (β-lg) and bovine serum albumin (BSA) were purchased from SIGMA (Castle Hill, NSW, Australia). Pre-stained SDS-PAGE standard (SeeBlue® Plus2) was procured from Thermo Fisher Scientific (Australia).

Storage of UHT milk. The UHT milk packs were stored at four different temperatures including 20, 30, 40 and 50 °C for 28 days in incubators (Thermoline Scientific Pty Ltd, Wetherill Park, NSW, Australia). The first 3 temperatures represented storage of UHT milk packs under ambient conditions in cold and relatively hot areas. The temperature of 50 °C was chosen to accelerate the development of storage instabilities. Milk packs were analysed on the first day of delivery (day 0), and then at two fortnightly intervals (14 and 28 days) during storage for formation of crosslinked proteins.

Analysis of formation of crosslinked proteins. The crosslinking of milk proteins during storage of UHT milk packs was investigated using gel electrophoresis i.e. native- and SDS-PAGE under reducing as well as non-reducing conditions as previously described[14] with some minor modifications. For native-PAGE, milk from each pack was diluted with the native sample buffer (0.11 M Tris-HCl buffer (pH 6.8), 8.8 % (v/v) glycerol, 2.22 % (v/v) of 0.4 % w/v bromophenol blue solution). A working volume of 10 µl was then loaded on 12.5 % gels and was run for 85 min at 210 V and 70 mA in a BIO-RAD Protean® II xi cell, filled with tank buffer solution (0.025 M Tris, 0.19 M glycine, pH 8.3). Gels were rinsed in Milli Q water for 15 minutes and stained using staining solution (0.15 % Coomasie Brilliant Blue R250 dye, 72 % isopropanol and 3 % acetic acid) by slowly shaking for 1 hour. The stain was completely removed and the gel was destained by shaking slowly in destaining solution (10 % iso-propanol, 10 % acetic acid) overnight on a shaking platform.

The same protocol of native-PAGE was followed for SDS-PAGE with the exception of using a SDS sample buffer (0.0625 M Tris-HCl buffer (pH 6.8), 10 % (v/v) glycerol, 2.5 % (v/v) of 0.4 % w/v bromophenol blue solution, 20% (v/v) of 10 % (w/v) SDS). In reducing SDS-PAGE, just prior to being loaded to gels, 20 µl of 2-mercaptoethanol was added per ml of milk and SDS buffer mix, vortexed and heated in a boiling water bath for five minutes and cooled before running on SDS-PAGE gels in SDS running buffer (0.1 % SDS, 0.025 M Tris

and 0.191 M glycine, pH 8.6). Pre-stained SDS-PAGE standard (3-200 kDa) was used to compare the molecular weights. Milk samples from different packs were analysed in duplicate. Representative gel patterns are shown in the Figure (1-3). Native-PAGE and SDS-PAGE gels were scanned using the ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, Richmond, CA).

Quantitation and kinetics of formation of crosslinked protein. The intensities of the major protein bands in reducing SDS–PAGE were determined using the Image Lab 5.2.1 software associated with the densitometer. The absolute quantity of reduced proteins was calculated from their respective band intensity. As the gels were run in duplicate, the absolute quantity was averaged. The average loss in absolute quantity of reduced milk proteins and increase in amount of aggregates obtained at different storage temperature was plotted versus time.

Statistical analysis. The experiments were arranged in a block, split plot in time design with fat content as the main factor and time as a subplot. The design was replicated on a separate occasion which served as a block. All samples were additionally subsampled at least twice giving at least 4 independent observations. The data was analysed using a GLM procedure of the SAS. The level of significance was pre-set at 0.05.

RESULTS AND DISCUSSION

Protein crosslinking during storage of UHT milk was demonstrated by change in electrophoretic mobility. The type and extent of interactions resulting in protein crosslinking at different storage conditions was explored using native, non-reducing and reducing SDS-PAGE assays. Different bands in patterns were identified by concurrent electrophoresis of standards and by comparing the results with similar reported patterns in the literature[15].

Effect of storage conditions on loss of native proteins. The effect of storage time and temperature on native-PAGE patterns in SM and WM is presented in Figure 1. The patterns show thick bands at the entry of stacking gel corresponding to high molecular mass protein aggregates, which were not able to penetrate the gel. Aggregates probably correspond to denatured whey proteins associated with themselves and caseins via thiol-disulphide exchange[16, 17], non-covalent interactions[18] and/or covalent interactions[19]. Faint bands corresponding to native-like whey proteins namely α-la and β-lg further supported this suggestion. Patterns also exhibited bands corresponding to native like κ-casein (κ-CN), β-casein (β-CN) and α_{s1}-casein (α_{s1}-CN). A diffusely stained region between κ-CN and β-CN bands was observed which has been attributed previously to crosslinked milk proteins[15]. Native-PAGE patterns in both SM and WM at the start of storage i.e. on day 0 appeared to be similar (Figure 1A&D). However, more prominent changes were observed between these samples during storage depending on the storage temperature.

Storage of SM and WM at 20, 30 or 40 °C for 14 days did not alter intensity of bands corresponding to individual native-like caseins (Figure 1B&E). However, after 28 days of storage at same temperatures, intensity of all native protein bands appeared to decrease for both, SM and WM (Figure 1C&F). Notably, loss in 'native-like' band intensities of individual milk proteins appeared to be higher in WM at 40 °C after 28 days than those for SM. This could be due to liquefaction of milk fat around 40 °C[20]. Fat globules are surrounded with caseins and whey proteins on its surface upon homogenisation, thus, when melted, they may act as 'glue' between adjacent proteins and hence increase their chance of interactions. Another explanation could be that at higher storage temperatures lipids undergo oxidation. The intermediates (e.g., free radicals and hydroperoxides) and end products (e.g. malondialdedyde) from lipid peroxidation interact with amino acid residues, such as cysteine, lysine, histidine, valine, methionine and phenylalanine, resulting in protein crosslinks[21, 22].

Although only WM indicated accelerated aggregation of milk proteins at 40 °C, native-PAGE patterns of both SM and WM samples stored at 50 °C were substantially different from those at other temperatures. After 14 days of storage at 50 °C in both SM and WM, bands corresponding to β-lg and α-la completely disappeared with a concomitant decrease in intensity of α_{s1}-CN, κ-CN and β-CN bands (Figure 1B&E). The intensity of diffuse region between κ-CN and β-CN bands also diminished. The complete disappearance of bands corresponding to β-lg and α-la indicated greater reactivity of whey proteins in formation of crosslinks. This was likely caused by denaturation of β-lg and α-la and exposure of lysine residues to a great level in these proteins. These characteristics render them a preferable reactant for lactosylation over caseins[21]. Lactosylated whey proteins may further be converted to advanced Maillard products at higher storage temperatures, which then also crosslink caseins and result in loss of native-like caseins[21]. Further fading out of bands occurred after 28 days of storage indicating even greater extent of crosslinking of proteins and at a far higher rate as compared to other storage temperatures in both SM and WM (Figure 1C&F). Besides increasing concentration of advanced Maillard products, higher storage temperatures also accelerate other protein crosslinks through lipid oxidation intermediates and end products (in case of WM), dehydroalanine, deamidated proteins and hydrophobic or other non-covalent interactions [22, 23]. Dehydroalanine is produced by heat- induced elimination of phosphate from phosphoserine residues in caseins. It subsequently reacts with amino group of lysine residues, imidazole group of histidine or thiol group of cysteine resulting in intra or intermolecular lysinoalanine, histidinoalanine or lanthionine crosslinks, respectively[24]. Deamidation involves conversion of asparagine and glutamine residue to aspartate or glutamate, respectively[1], resulting in degradation of proteins and their aggregation[25]. Additionally, higher storage temperature also induces changes in secondary structure of milk

proteins[25]. This results in exposure of hydrophobic regions, which may crosslink via non-covalent interactions[1, 18, 26, 27].

To obtain information regarding the type of aggregation (covalent and/or non-covalent) and involvement of particular milk proteins, milk samples were further analysed using dissociating agent SDS without reducing agent. SDS dissociates intermolecular and intramolecular non-covalently linked aggregates. Thus, non-reducing SDS-PAGE could reveal involvement of hydrophobic interactions in formation of high molecular mass aggregates observed in native-PAGE patterns.

Effect of storage temperature on loss of SDS monomeric proteins. Figure 2 shows SDS-PAGE non-reducing patterns for both SM and WM. These patterns showed intense bands corresponding to monomeric α_{s1}-CN, κ-CN and β-CN, α_{s2}-CN dimer and BSA. Bands corresponding to SDS monomeric α-la and β-lg were faint but comparatively had a higher intensity than native bands, implying that these proteins were linked via non-covalent interactions in addition to disulphide or other covalent bonds[18, 28]. Reduction in the intensity of high molecular mass aggregates at the top of the stacking gel further supported this suggestion (Figure 1-2). However, noticeable bands corresponding to high molecular mass aggregates and diffuse regions at the top of the stacking gel and resolving gel respectively (Figure 2) indicated that these aggregates were either disulphide linked or covalently bonded. Furthermore, SDS-PAGE patterns of SM and WM at the start of storage were similar (Figure 2), suggesting that effect of UHT treatment was independent of fat content. This contradicts Pellegrino theory that milk fat protects other components of milk from heat-induced changes[29]. However, the effect of fat content appeared more relevant during storage at different temperatures.

At and below 40 °C, storage for the first 14 days did not change the intensity of protein bands in SM (Figure 2). However, in WM, the intensity of bands corresponding to whey

proteins decreased with a concomitant increase in intensity of aggregate bands at the top of resolving gel. This is in line with similar observations made in native-PAGE patterns of WM after 14 days of storage at 40 °C, implying likely involvement of fat. After 28 days of storage for SM and WM at 20, 30 and 40 °C, a decrease in the intensity of different SDS monomeric bands were observed (Figure 2). Thus, covalent crosslinking of proteins proceeded at a slower rate even at lower storage temperatures[30]. However, in WM, the bands corresponding to whey proteins were diffuse and faint as compared to SM at the same temperature. Decrease in band intensity of whey proteins further exacerbated in WM at 50 °C. After 28 days of storage at this temperature, whey protein bands disappeared and only a diffuse region (smear) with very faint bands corresponding to high molecular mass protein aggregates, α_{s1}-CN and β-CN could be noticed (Figure 2). This diffuse region had been previously attributed to non–disulphide covalent crosslinking of proteins[7]. SM also exhibited an intense aggregate band at top of the resolving gel and faint bands corresponding to β-CN, α_{s1}-CN but also of whey proteins at 50 °C. Faster and complete disappearance of whey proteins in WM at and above 40 °C could be due to enhanced covalent crosslinking due to change in physical state of fat and oxidation of lipids. Additionally, only α_{s1}-CN and β-CN were present in both SM and WM in SDS-PAGE non reduced at 50 °C after 28 days and this could be due to their comparatively higher amounts in milk[31]. Furthermore, due to more hydrophobic nature and lack of cysteine residues, α_{s1}-CN and β-CN may be prone to be involved in non-covalent interactions compared to other caseins.

The nature of the aggregates and smear (disulphide or other type of covalently bonded) produced at higher temperatures in SDS PAGE non-reducing patterns were further explored by using SDS-PAGE under reducing conditions. SDS dissociated hydrophobic interactions and with the addition of β-mercaptoethanol reduced disulphide bonds. Hence, the bands

corresponding to aggregates in SDS-PAGE reducing patterns would be entirely due to non-disulphide covalent interactions.

Effect of storage conditions on loss of reduced proteins. SM and WM had similar patterns on day 0 observed for native-PAGE and SDS-PAGE non-reducing patterns (Figure 1-3). This reconfirmed previous observation that the effect of UHT treatment on electrophoretic mobility of milk proteins was independent of fat content. At the start of storage, high molecular mass aggregates were not present on top of the stacking gel (Figure 3). Only a faint band demonstrative of non-reducible protein aggregates were present at the top of resolving gel as also shown earlier by non-reducing SDS-PAGE. Furthermore, patterns on day 0 had intense bands corresponding to caseins, β-lg and α-la, suggesting thiol-disulphide exchange and hydrophobic interactions as major aggregation pathways at the start of storage[32]. There was also a diffuse region between non-reducible aggregate band and α_{s2}-CN dimer and between BSA and α_{s2}-CN dimer. This region probably also had non-disulphide covalently linked caseins and whey proteins as it was present in native-PAGE as well as in non-reducing SDS-PAGE patterns. Therefore, it appeared that at the start of storage the order of different interactions in both SM and WM was disulphide > non-covalent > covalent (non-disulphide). Besides similar patterns on day 0 in SM and WM, storage conditions affected SM and WM differently in the context of extent of aggregation and consequently loss of different individual milk proteins.

Kinetics of aggregate formation. SM did not exhibit an increase in the amount of non-reducible and high molecular mass aggregates after 28 days of storage at 20 °C (Figure 3-4). However, after 28 days of storage at 30 and 40 °C, an increase in non-reducible aggregates was noted, although not in high molecular mass aggregates. In WM, the trend was slightly different with the non-reducible aggregates starting to build up only after 14 days of storage at 20, 30 or 40 °C. However, like SM there was no apparent increase in the concentration of

high molecular mass aggregates even after 28 days of storage (Figure 3, 6). At 50 °C, the amount of non-reducible and the high molecular mass aggregates increased to a great extent in both SM and WM (Figure 4). The proteins were most likely aggregated by covalent interactions to form high molecular mass aggregates and rate of these interactions was enhanced sharply above 40 °C. Furthermore, the extent of formation of high molecular mass aggregates was greater in WM as compared to SM following 28 days of storage (Figure 4), suggesting enhanced protein aggregation at higher temperatures in the presence of fat. Besides the difference in amounts of aggregates formed, SM and WM also differed in sequence in which individual caseins and whey proteins participated in the formation of covalently bound aggregates.

In SM, no change in the quantity of whey proteins and caseins at 20 °C (Figure 5A), supporting earlier stated observation of no change in reducible and high molecular mass aggregates in skim milk at 20 °C (Figure 3, 4A). At 30 and 40 °C in SM, α_{s2}-CN, β-CN and β-lg decreased during the first 14 days storage followed by α_{s2}-CN and α-la in the next 14 days of storage (Figure 5B-C). However, in WM the reduction in quantity of proteins started at 20 °C (Figure 6A-C). β-Lactoglobulin was one of the first proteins to initiate non-disulphide aggregation in WM at 20, 30 and 40 °C as evident by decrease in its concentration after first 14 days of storage (Figure 6A-C). A reduction in quantity of α_{s1}-CN, α_{s2}-CN and β-lg in next 14 days at 20 and 30 °C was evident in WM (Figure 6 A-B). Nevertheless, at 40 °C, α-la also participated in aggregation through non-disulphide covalent interactions in WM like SM. Loss in quantity of proteins was much higher at 50 °C as compared to 20, 30 and 40 °C in both SM and WM. In addition, κ-CN participated in covalent aggregation only in later half of storage in both WM and SM at 50 °C, overruling proteolysis by bacterial proteinases. It seemed that all the proteins in milk were actively involved in crosslinking at 50 °C. Higher storage temperatures increase intra micellar crosslinking, which might result in increased

electrostatic repulsion inside the micelle resulting in dissociation and sedimentation of cross-linked proteins[33]. However, β-CN and α_{s2}-CN were main players in SM whereas α_{s1}-CN, α_{s2}-CN and α-la were central in protein-protein interactions in WM. The difference in type of caseins involved in WM and SM at different temperatures suggested preference of specific pathways of covalent (non-disulphide) crosslinking.

Non-disulphide covalent crosslinking in UHT milk during storage occurs via advanced Maillard products (AMP), dehydroalanine, deamidated residues and lipid oxidation products (in WM)[7, 21]. Crosslinking via AMP involves lactosylation of lysine residues as the first step[34], implying that proteins with a higher number of lysine residues would be preferentially involved in this reaction. Lysine content of different milk proteins vary following the order α_{s2}-CN> β-lg>α_{s1}-CN>α-la>β-CN>κ-CN[31]. α_{s2}-CN being one of the major player in covalent aggregation in SM suggested higher levels of AMP mediated crosslinking. Additionally, disappearance of α-la in SM even at 30 °C also suggested a higher rate of Maillard reaction in the former. α-la is actively involved in lactose synthesis[35] thus its preference for lactose was demonstrated by lactosylation of almost all its lysine residues during storage of UHT milk[1]. Moreover, α-la may also preferentially engage in this reaction[21] likely due to conformational changes and denaturation that take place during UHT treatment [36]. Greater extent of Maillard reaction in SM was also supported by comparatively higher increase in 'b' value of colour in SM than WM at temperatures above 30 °C (data not shown). Valero, Villamiel, Miralles, Sanz and Martínez-Castro [13] have also reported higher levels of Maillard products in SM than WM during storage. Greater AMP mediated crosslinking in SM also explained the reduction in quantity of β-CN in SM. Lactosylation results in increased hydrophillicity of the casein micelle interior, which might have weakened hydrophobic associations[37] and hence released β-CN to the solution (Figure 7A). Pellegrino, et al. [38] observed an increase in covalent aggregation of β-CN in a model system containing glucose with the extent of the advanced

Maillard reaction, which further supports our suggestion. Besides lactosylation, α_{s2}-CN also appears to be most reactive candidate for dehydroalanine linked proteins due to a high number of phosphoserine residues and also the presence of two cysteine residues[39]. This indicates that SM with greater involvement of α_{s2}-CN and β-CN implied higher rate of AMP and dehydroalanine mediated crosslinking (Figure 7A).

In WM, α_{s1}-CN along with α_{s2}-CN and α-la were the main proteins involved in non-disulphide covalent crosslinking. α_{s1}-CN has been reported to be the most abundant protein in aggregates and had been attributed mainly to deamidation of its asparagine residues besides AMP and dehydroalanine mediated protein crosslinking [1]. Furthermore, higher amount of aggregates in WM than SM can be attributed to crosslinking via lipid oxidation products and melted fat globules at and above 40 °C in WM. The modified fat globule membrane also consists of whey proteins and caseins besides its native components. Therefore, the difference in involvement of different proteins in SM and WM might be due to differences in their access and availability. Thus, covalent crosslinking in UHT whole milk facilitates majorly through deamidated residues and lipid oxidation products, while Maillard and dehydroalanine products are the main contributors in UHT skim milk (Figure 7B).

In conclusion, protein crosslinking was accelerated at higher storage temperatures. However, at temperatures above 30 °C in WM and above 40 °C in SM the rate and type of these reactions were very different from those at 20 °C. Especially in case of WM, due to change in state and/or lipid oxidation, protein crosslinking appeared to follow a different pathway that may not occur when stored at lower temperatures. This may make a task to extrapolate these reactions to predict protein crosslinking at a lower temperature very difficult. These results suggest that testing at higher temperatures (40 and 50 °C) thus seem unsuitable for accelerating shelf life of UHT milk in terms of protein aggregation. In addition, in context of sensory attributes, greater levels of browning at 40 or 50 °C could not possibly

predict the time to achieve the same extent of browning at 20 and 30 °C. Thus accelerating deterioration of sensory attributes by using accelerated storage temperature may not be applicable to predict quality changes at lower storage temperatures.

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Notes

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ABBREVIATIONS USED

αs_1-CN, αs_1-casein; β-CN, β- casein; κ-CN, κ-casein; α-la, α- lactalbumin ; β-lg, β-lactoglobulin; PAGE, Polyacrylamide Gel Electrophoresis; SDS, Sodium Dodecyl Sulphate. SM, skim milk; UHT, ultrahigh temperature; WM, whole milk; BSA, bovine serum albumin; AMP, advanced Maillard products.

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Figure Captions

Figure 1. Native-PAGE patterns of UHT skim milk (SM) and whole milk (WM) on day 0, 14 and 28. In the patterns, L1, L2, L3, L4, L5 and L6 represents protein standards of β-lactoglobulin (β-lg), α-lactalbumin (α-la), calcium caseinate, bovine serum albumin (BSA), SM and WM on day 0, respectively. The numbers 20, 30, 40 and 50 stand for samples stored at 20, 30, 40 and 50 °C at a given storage time of SM (A, B and C) and WM (D, E and F). The identities of different bands are marked on patterns.

Figure 2. Non-reducing SDS-PAGE patterns of UHT skim milk (SM) and whole milk (WM) on day 0 (Control), 14th and 28th day of storage. In the patterns, L1, L2, L3 L4 and L5 represent molecular weight marker and protein standards of α-lactalbumin (α-la), β-lactoglobulin (β-lg), calcium caseinate and bovine serum albumin (BSA) respectively. SM and WM are samples stored on 0 day and 20, 30, 40 and 50 denote samples stored at 20, 30, 40 and 50 °C at a given storage time. The identities of different samples are marked on patterns.

Figure 3. Reducing SDS-PAGE patterns of UHT skim milk (SM) and whole milk (WM) on day 0, 14 and 28 of storage. Storage samples were reduced with β-mercaptoethanol and electrophoresed on SDS gels (4-12.5%). In the patterns, L1, L2, L3, L4, L5 and C represent molecular weight marker, protein standards of α-lactalbumin (α-la), β-lactoglobulin (β-lg), calcium caseinate and bovine serum albumin (BSA), and SM and WM samples stored on 0 day respectively. The numbers 20, 30, 40 and 50 denote samples stored at 20, 30, 40 and 50 °C at a given storage time. The identities of different samples are marked on patterns.

Figure 4. Change in absolute quantity of non-reducible (A&B) and high molecular mass aggregates (C&D) during storage of skim UHT milk (SM) and whole milk (WM) at storage

temperature of 20 °C ( ), 30 °C ( ), 40 °C ( ) and 50 °C ( ) as obtained from image analysis of reducing SDS-PAGE.

Figure 5. Change in absolute quantity of caseins (α_{s1}-CN; α_{s2}-CN; β-CN; κ-CN), whey proteins (β-lg and α-la) during storage of UHT skim milk (SM) at storage temperature of 20 °C (A), 30 °C (B), 40 °C (C) and 50 °C (D) as obtained from image analysis of reducing SDS-PAGE.

Figure 6. Change in absolute quantity of caseins (α_{s1}-CN; α_{s2}-CN; β-CN; κ-CN), whey proteins (β-lactoglobulin (β-lg) and alpha-lactalbumin (α-la) during storage of UHT whole milk (WM) at storage temperature of 20 °C (A), 30 °C (B), 40 °C (C) and 50 °C (D) as obtained from image analysis of reducing SDS-PAGE.

Figure 7 . Advanced maillard product (AMP), dehydroalanine (DHA), deamidated residues and lipid oxidation products mediated protein crosslinking in (A) skim milk (SM) at and above 30 °C and (B) whole milk (WM) at and above 40 °C (α_{s1}-CN; α_{s2}-CN; β-CN; κ-CN; α-la; β-lg; colloidal calcium phosphate (CCP); Lactose; L lysine amino acid residues; AMP; DHA; aggregated whey proteins; deamidated residues; fat globules; oxidised fat globules; melted fat globules; crosslinked aggregates; crosslinked aggregates along with melted and oxidised fat.

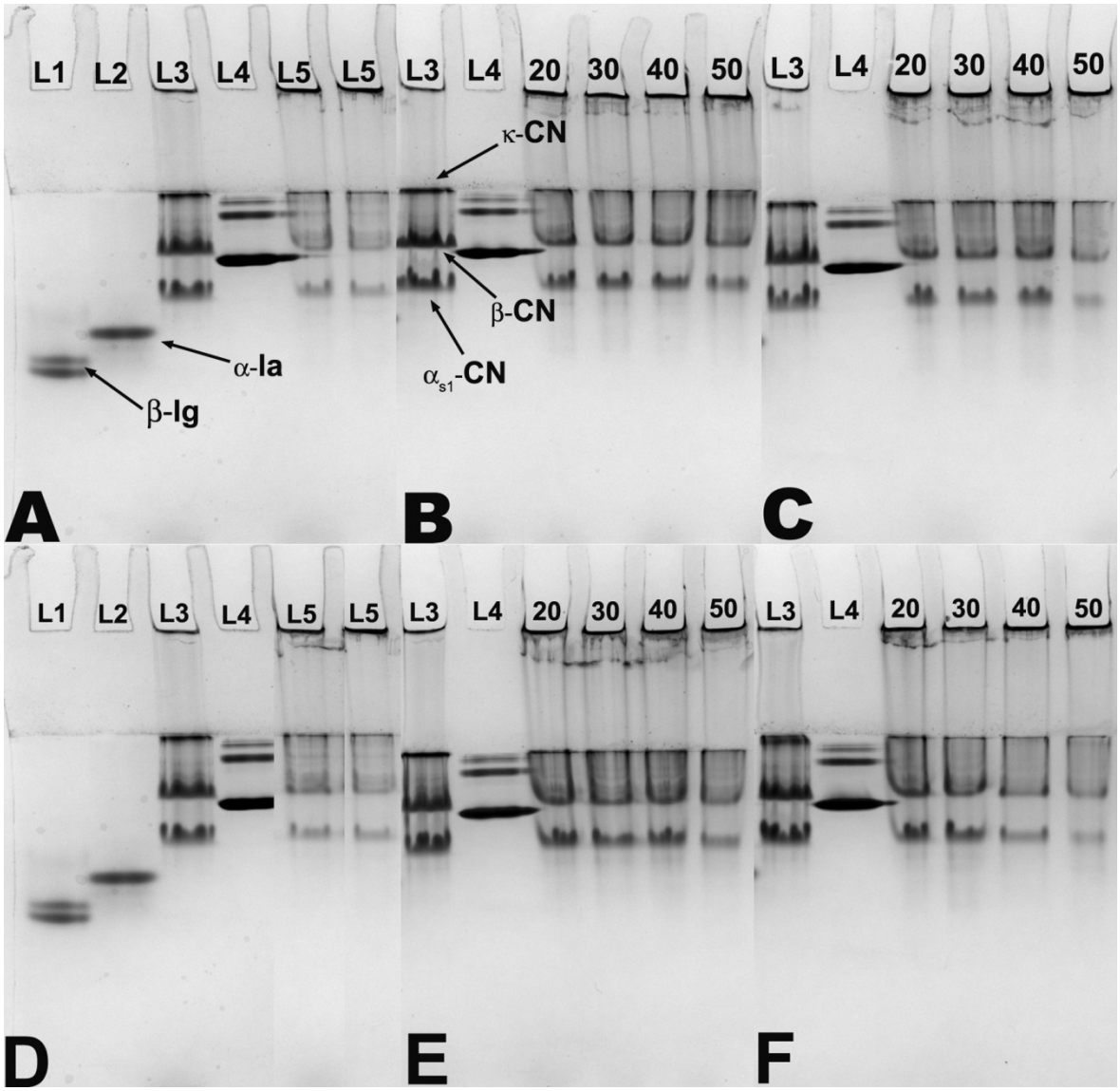

L1
L2
L3
L4
L5
L5
L3
L4
20
30
40
50
L3
L4
20
30
40
50
κ-CN
β-CN
α-la
β-lg
α_{s1}-CN
A
B
C
L1
L2
L3
L4
L5
L5
L3
L4
20
30
40
50
L3
L4
20
30
40
50
D
E
F

Figure 1.

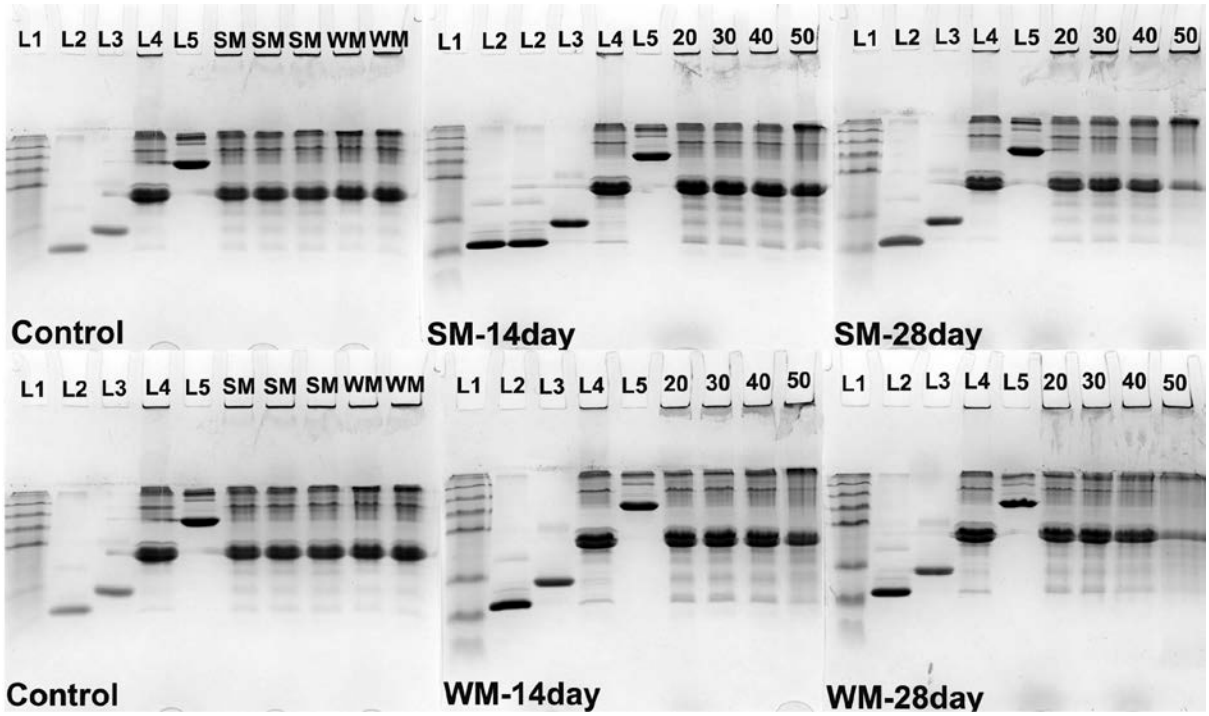

L1 L2 L3 L4 L5 SM SM SM WM WM
Control
L1 L2 L2 L3 L4 L5 20 30 40 50
SM-14day
L1 L2 L3 L4 L5 20 30 40 50
SM-28day
L1 L2 L3 L4 L5 SM SM SM WM WM
Control
L1 L2 L3 L4 L5 20 30 40 50
WM-14day
L1 L2 L3 L4 L5 20 30 40 50
WM-28day

Figure 2.

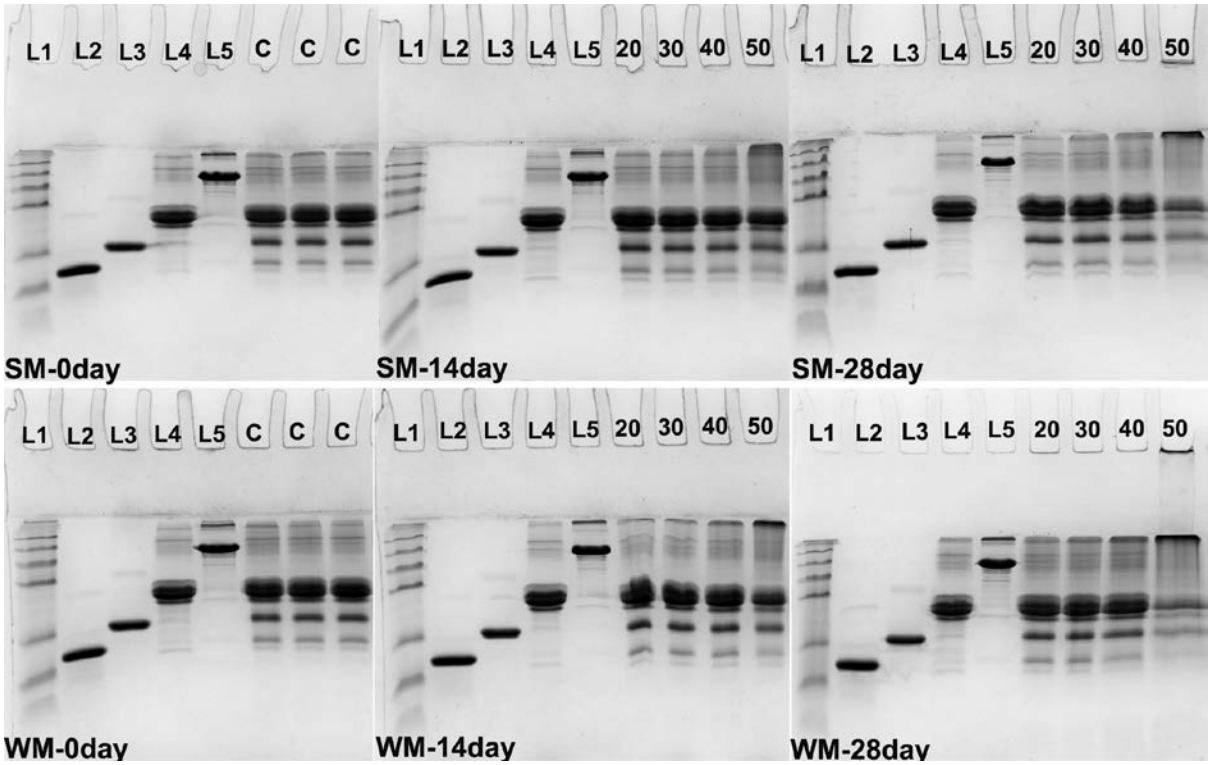

L1 L2 L3 L4 L5 C C C
L1 L2 L3 L4 L5 20 30 40 50
L1 L2 L3 L4 L5 20 30 40 50
SM-0day
SM-14day
SM-28day
L1 L2 L3 L4 L5 C C C
L1 L2 L3 L4 L5 20 30 40 50
L1 L2 L3 L4 L5 20 30 40 50
WM-0day
WM-14day
WM-28day

Figure 3.

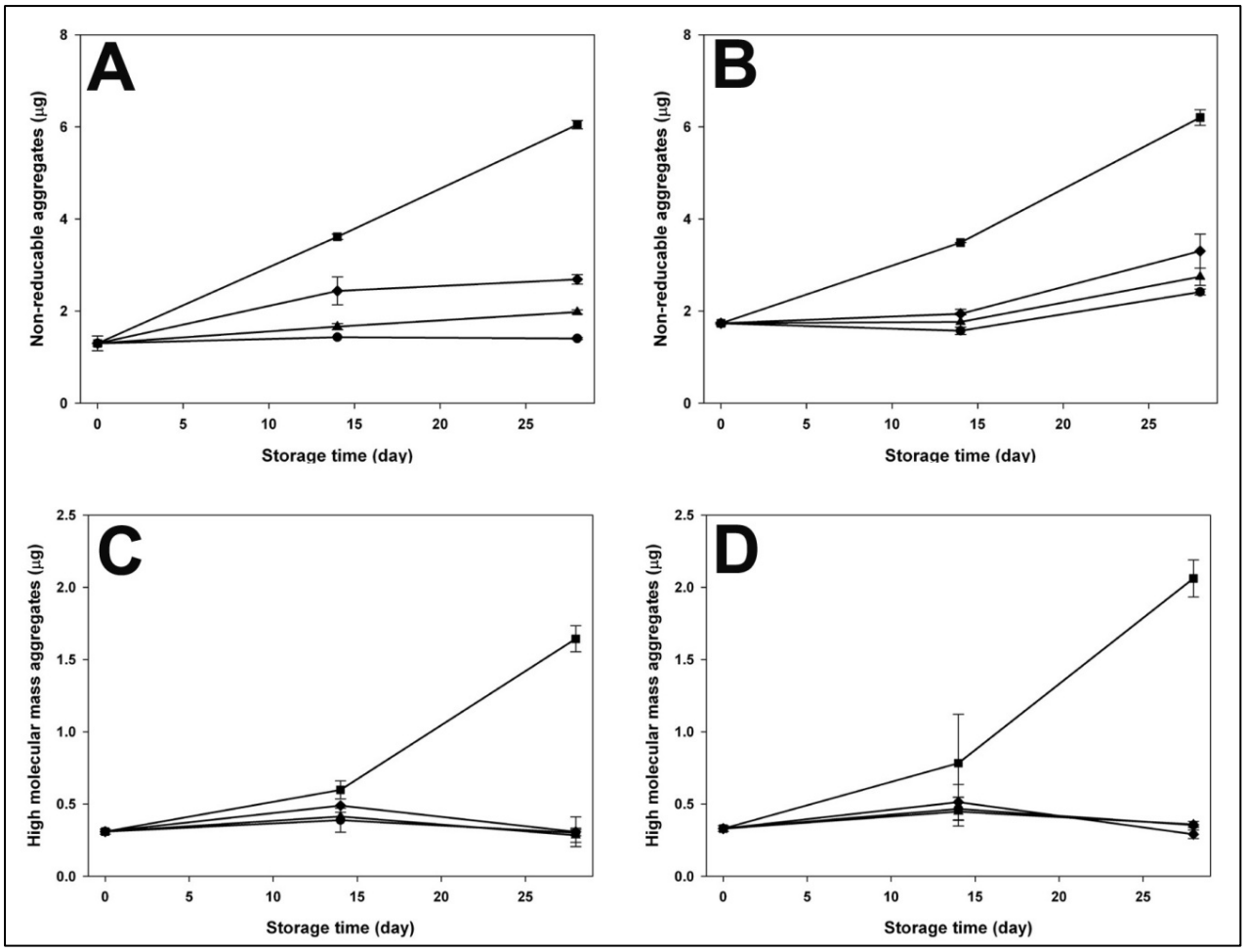

A
B
C
D
Non-reducable aggregates (μg)
High molecular mass aggregates (μg)
Storage time (day)
0
5
10
15
20
25
0
2
4
6
8
0.0
0.5
1.0
1.5
2.0
2.5

Figure 4.

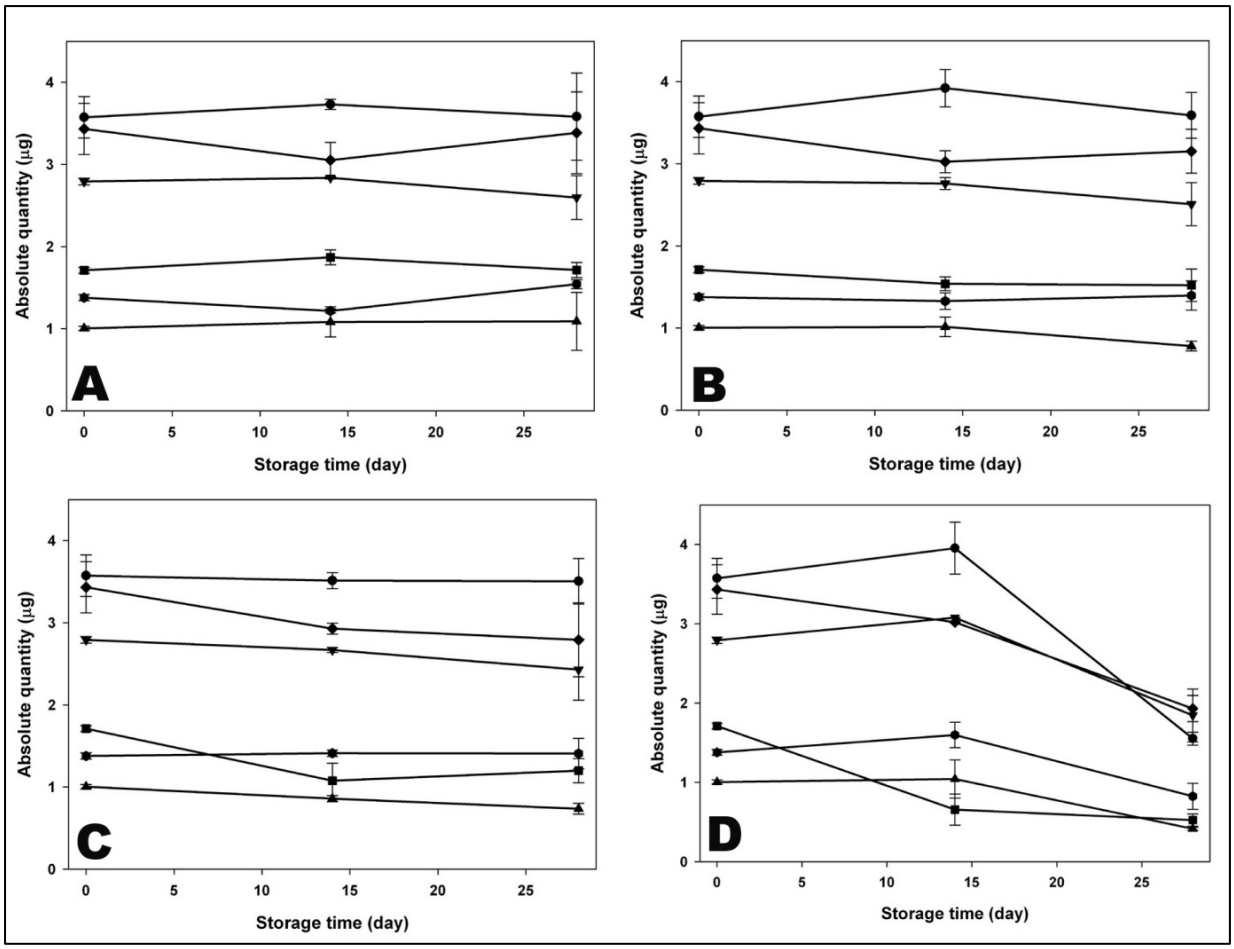

A
B
C
D
Absolute quantity (μg)
Storage time (day)
0
1
2
3
4
0
5
10
15
20
25

Figure 5.

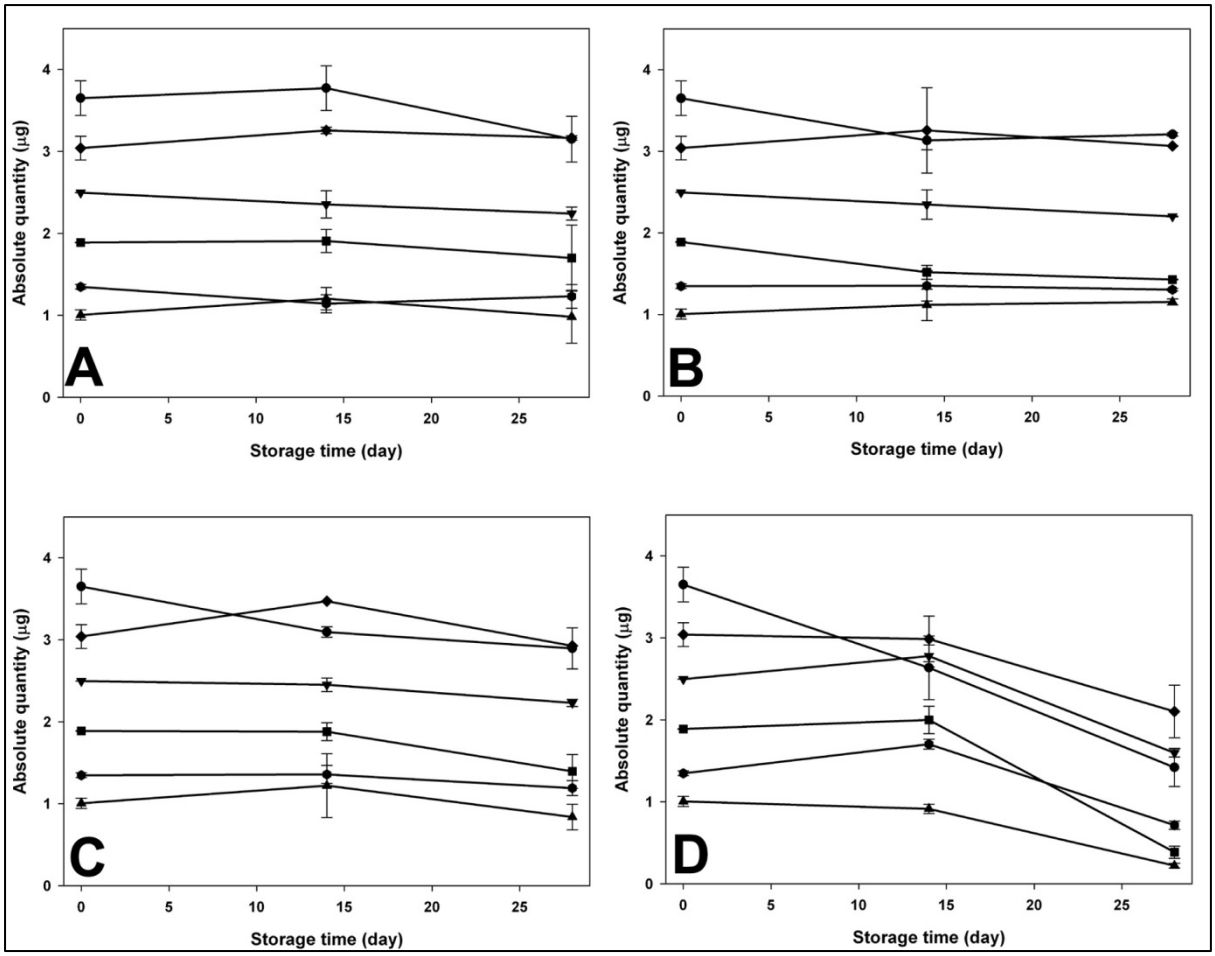
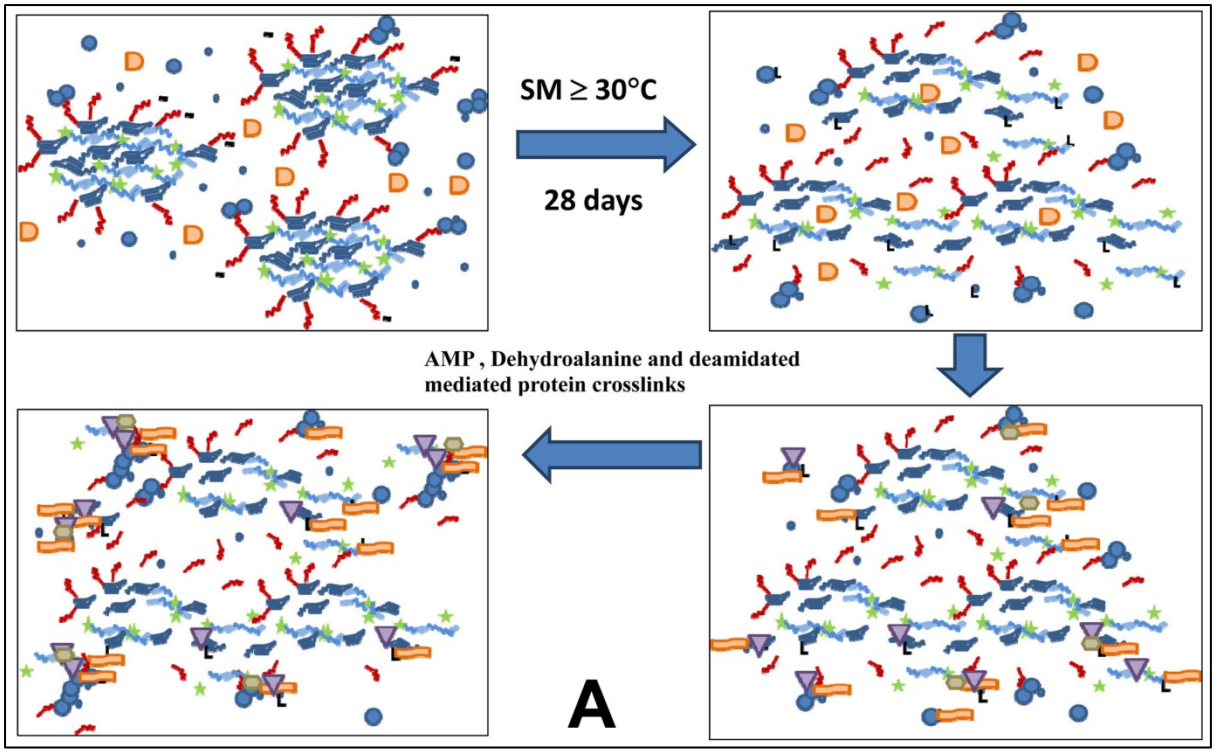

A
B
C
D
Absolute quantity (μg)
Storage time (day)
0
1
2
3
4
0
5
10
15
20
25

Figure 6.


SM ≥ 30°C
28 days
AMP , Dehydroalanine and deamidated mediated protein crosslinks
A

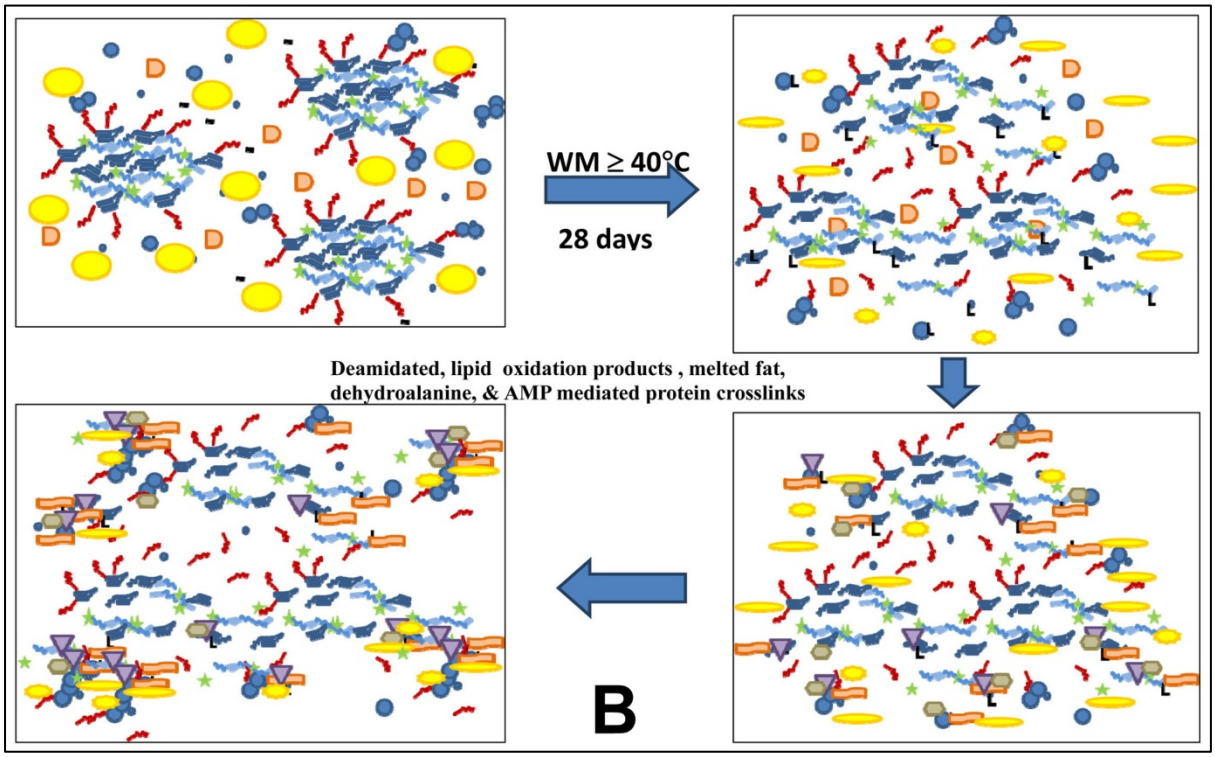

WM ≥ 40°C
28 days
Deamidated, lipid oxidation products , melted fat, dehydroalanine, & AMP mediated protein crosslinks
B

Figure 7.

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