Effect of sodium azide addition and aging storage on casein micelle size

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Abstract. Casein micelles affected most of milk properties, therefore the use sodium azide as milk preservation is not expected to alter milk properties during storage, including the casein micelle size. The aim of this study was to analyse casein micelle size after the addition of sodium azide during storage. The experiment was performed as a complete block randomised design with three replications. The addition of 0.02-0.10% Na-azide do not lead to any noticeable differences in average casein size at the same day and show similar trend after 14 day-storage. At concentration of 0.02% sodium azide (Na-azide), the size of pasteurised milk did not change up to 12 days, while the size of raw skim milk slightly increased by ageing time at day 5. The treated concentration did not affect the size distribution, except for milk with 0.02% Na -azide which had narrower distribution compared to other treated and control milk. The finding from this study suggests that the role of Na -azide in this experiments during storage at 4°C is only for preventing the microbial growth.

1. Introduction
Milk contains high amount of proteins, lipids and minerals, along with carbohydrates and vitamins. However, it is believed that the change in protein state is a basis for creating new dairy ingredients or products [1], because milk proteins mainly affect the properties of many dairy products [2]. Consequently, the properties of casein, comprises nearly 80% of the milk protein, have great impact on the functional behaviour of dairy products.

Based on the milk composition, it can be assumed that the main structural components of milk are fat globules and casein micelles (CMs). The white colour of skim milk is caused by the colloidal casein micelles scattering significant amount of light. Casein micelles exist in milk as aggregates of the casein proteins and a proportion of mineral calcium phosphate [3]. The casein micelles have an extremely variability of size and are sterically stabilised by a hairy layer of κ-casein molecules and perhaps β-casein [4, 5].

There are some components that contribute to the stability of casein micelles, including the integration of κ-casein, colloidal calcium phosphate and hydrophobic interactions [6] (Roach and Harte 2008). The collapse of surface protein κ-casein will affect the micellar solubility and generate aggregation [7]. On the other hand, the removal of calcium phosphate induces the dissociation of the micelles [8]. Hydrophobic interaction is important in the link between sub-micelles, so the distraction of the interactions might cause the micellar dissociation or aggregation [7]. Therefore, modification or
disruption of the components might initiate either micelle aggregation or dissociation into submicelles and lead to the change in casein micelle size.

Various study reported that sodium azide (Na-azide), in various concentrations, was added to prevent microbial growth at experiments on casein micelle size [9,10,11]. However, it was not reported whether the addition would have an impact on the parameter measured. Sodium azide, also known as sodium trinitride, is the inorganic compound with the formula NaN₃. Hydrazoic acid or azomide is a colourless liquid, which boils and solidifies at 37°C and -80°C, respectively. The azomide salt of sodium is stable and dissociate in solution [12]. In laboratories, Na-azide is useful for a bacteriostatic and also as a preservative. It is also a metabolic inhibitor, which inhibits oxidative phosphorylation. The role of sodium azide has been studied many years ago, not only in milk but also other products. Other research found that sodium azide can inhibit yeast and mold activities, caused by genus Candida in tomato juice. However, the effect significantly decreased, if the tomato juice is refrigerated for more than 5 days [13].

Limited research has been performed which relates to casein micelle size on milk during experiment. Therefore, the research findings are expected to provide more information on casein micelle size after the addition of sodium azide and how long the preservative can prolong shelf life of the milk.

2. **Materials and methods**

2.1. **Materials**

Fresh pasteurised skim milk was purchased from local supermarket. For comparison, raw skim milk from local farm was used.

2.2. **Methods**

In order to understand effects that might relate to the size measurements, we carried out the experiments on the addition of Na-azide (antimicrobial agent) with various concentrations and also ageing of the skim milk. Then, the samples were stored cold at 4°C until further treatments. The initial pH was around 6.6 and the average size and size distribution of the casein micelles were determined. All experiments were repeated three times on different batches of milk samples.

2.3. **Sodium azide**

Pasteurised milk was divided into 4 parts of 100 ml sample. Na-azide was added to the each sample at the concentrations of 0, 0.02, 0.05 and 0.1%. The sub-samples were stored in refrigerator at 4°C then subjected to analysis for casein micelle size at 0, 3, 5, 7, 10, 12 and 14 days of storage. Check batches were milk sub-sample without preservative and were analysed only until 7 days.

2.4. **Effect of storage**

Pasteurised and raw milk were stored in refrigerator at 4°C for 14 days. Sodium azide (w/v) was added at day 0. The casein micelle size was measured every 2 or 3 days for 14 days of storage.

2.5. **Experimental Design and Statistics**

All experiments were performed as a complete block randomised design with two or three replications. Statistical analyses to determine any treatment effects were done using Minitab 16.0. The corresponding data were determined by analysis of variance (ANOVA) using the General Linear Model. Tukey simultaneous test at the level of P = 0.05 was carried out to assess whether different treatments resulted in statistically significant differences.

3. **Results and discussion**

3.1. **Effect of sodium azide on casein micelle size**

Casein micelles size at different concentration of Na-azide are presented in Figure 1. Although the CM size ranged between 251-258 nm, analysis result of the size among all samples showed no statistical
difference until day 7 of storage. Moreover, the addition of 0.02 - 0.1% Na-azide do not lead to any noticeable differences in average casein size at the same day. It was clear that the milk without Na-azide addition (0% Na-azide) became more concentrated, so that CM measurements after day 7 was terminated for this samples. Milk with various Na-azide concentration showed similar trend after 14-day of storage.

![Figure 1](image)

**Figure 1.** The particle size of skim milk with Na-azide addition after 14-day storage at 4°C

Figure 2 reported the size distribution of casein micelles at day 0, 7 and 14 days. At day 0, all untreated and treated milk has similar size distribution. In agreement with the identical trend amongst the Na-azide addition, only little differences were observed between micelle size distributions in the treated samples, especially after 14 day of storage. However, at day 7, although the addition of 0.02, 0.05, 0.1% Na-azide did not affect the average size of casein (255, 251, 254 nm, respectively), the 0.02% Na-azide had narrower distribution compared to other treated and control milk.

![Figure 2](image)
Figure 2. The size distribution of skim milk with Na-azide addition at day 0, 7 and 14 stored at 4°C

Na-azide is an ionic solid, soluble in water with the formula NaN₃. This colourless azide has been used as a common preservative agent in stock solution in laboratory and also applied in milk samples [14, 15, 16, 17, 18, 19]. Although some papers did not mention the type and the concentration of preservative being employed in the milk samples, the amount of Na-azide being used is noted in the range of 0.01% [20, 21], 0.02% [22], 0.04%, 0.05% [23] and up to 0.1%.

Result of this study indicated that the preservation concentrations mentioned can, therefore, be classified to be equally good, as they did not influence the parameter measured. Therefore, throughout the experiments, 0.02% Na-azide will be used to prevent the microbial growth.

3.2. Effect of ageing of skim milk

The average casein size of skim milk (SM) and raw skim milk (RSM) during 14-day storage are presented in Table 1. Storage time had significant effect on the parameter measured, either for SM and RSM. For both milk samples, the size tended to increase during the storage, rise up to 20 nm in SM and 30 nm in RSM.

For skim milk, the size after 2-day storage was similar with the control milk. However, there was a noticeable increase in the size at prolong storage up to 14 days, with the maximum size at day 5, increase from 243 nm to 266 nm. Although a milder, linear-like decrease occurred after 7 days, the size increased again at day 14.

Similarly, the size of raw skim milk increased over the storage time, with the maximum size was after day 5. The Na-azide addition has no significant effect on CM size until 14-day of storage. This study indicated that the trend for CM size of SM (skim milk) and RSM (raw skim milk) was not affected by the application of pasteurisation for skim milk.

Table 1. Casein micelle size of skim milk and raw skim milk during ageing storage for 14 days

| Day | 0  | 2  | 5  | 7  | 9  | 12 | 14  |
|-----|----|----|----|----|----|----|-----|
| SM  | 243±2.0d | 245±2.0d | 266±2.4a | 257±3.3abc | 250±1.7bcd | 249±1.9bcd | 260±2.0ab |
| RSM | 242±2.0b | 245±2.0ab | 262±2.0ab | 255±2.0ab | 258±2.0ab | 261±2.0ab | 270±2.0a |

SM: skim milk RSM: raw skim milk
Figure 3. The particle size of skim milk (SM) and raw skim milk (RSM) after 14-day of storage at 4°C

The size distribution of skim milk and raw skim milk are presented in Figure 4 and 5, respectively. Raw skim milk was used without any pasteurisation treatment after milk separation from cream. However, only little differences were observed between size distributions in skim milk and raw skim milk under the same day of storage. Both of milk types had similar size and size distribution.

Figure 4. The size distribution of skim milk after 9-day (A) and 14-day (B) storage at 4°C
Figure 5. The size distribution of raw skim milk after 9-day (A) and 14-day (B) storage at 4°C

The size of casein micelles in fresh milk is well established, however, it seemed that the casein behaviour during storage of milk cannot be neglected. This result is in agreement with result from previous study, which noted the slight increase in particle size after 8-day storage [24]. He found that the aging process leads to an increase in particle size by 20 nm at day 7 and 30 nm at day 8. Other research reported that on routine determination of urea in milk, the addition of Na-azide at ten times higher level than recommended did not affect the added urea content [25].

4. Conclusions
It is concluded that Na-azide did not change the CM size during ageing storage. The addition of Na-azide up to 1% was only used as a preservative for preventing the microbial growth. It is also suggested that preserved milk samples should be kept in the refrigerator and treated or analysed within less 5 days, to minimise the effect of storage time.

References
[1] Onwulata C, Phillips J, et al. 2010 Texturized dairy proteins." Journal of Food Science 75(2): E100.
[2] Phadungath C 2005 "Casein Micelle Structure: A concise Review." Songklanakarin J. Sci. Technol. 27(1): 201-212.
[3] Walstra P 1999 "Casein sub-micelles: do they exist?" International Dairy Journal 9(3-6): 189-192.
[4] Slattery CW 1976 Review: Casein micelle structure - Examination of models." Journal of Dairy Science 59(9): 1547-1556.
[5] Muller-Buschbaum P, Gebhardt R, et al. 2007 Effect of calcium concentration on the structure of casein micelles in thin films." Biophysical Journal 93(3): 960-968.
[6] Roach A. and Harte F 2008 Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization." Innovative Food Science & Emerging Technologies 9(1): 1-8.
[7] de Kruif CG 1999 Casein micelle interactions," International Dairy Journal 9(3-6): 183-188.
[8] Panouillé M, Nicolai T and Durand D 2004, ‘Heat induced aggregation and gelation of casein submicelles’, International Dairy Journal, vol. 14, no. 4, pp. 297-303.
[9] Sinaga H, Bansal N and Bhandari, B 2016. Partial renneting of pasteurized bovine milk: Casein micelle size, heat and storage stability. *Food Research International*. Vol 84 pp 52-60
[10] Sinaga H, Bansal N and Bhandari, B. 2017. Gelation properties of partially renneted milk. *International Journal of Food Properties*, 20 (8): 1700-14
[11] Sinaga H, Bansal N and Bhandari B 2016 Effects of milk pH alteration on casein micelle size and gelation properties of milk. International Journal of Food Properties. DOI 10.1080/10942912.2016.1152480

[12] Slawson MS, Snyder ML 1952 Mechanism of action sodium azide on the genus Candida. Journal of Dental Research vol 31 (1): 47

[13] Graham JDP 1949 Actions of sodium azide Brit. J. Pharmacol. 4: 1

[14] Hamann J, Gyodi P, et al. 1991. "On the Influence of Varying Preserving Agents and Storage Times on Somatic-Cell Counting in Milk Using the Fossomatic-360 and Fossomatic-180." Kieler Milchwirtschaftliche Forschungsberichte 43(4): 273-295.

[15] Hanus O, Gencurova V, et al. 1992 Tests of Milkofix, a New Preservative Substance for Milk Samples Used for the Purposes of an Infrared-Analysis of Basic Milk-Composition .2. Checks of Preservative Effects in Relation to the Infrared-Analysis." Veterinarni Medicina 37(1): 33-43.

[16] Chalermsan N, Vijchullata P, et al. 2003 Influence of preservatives on raw milk components and somatic cell counts analysis. Proceedings of 41st Kasetsart University Annual Conference, 3-7 February, 2003. Subject: Animals and Veterinary Medicine. Kasetsart University Annual Conference: 127-135.

[17] Gallaher JJ, Hollender R, et al. 2005 Effect of composition and antioxidants on the oxidative stability of fluid milk supplemented with an algae oil emulsion International Dairy Journal 15(4): 333-341.

[18] Haddadi K, Moussaoui F, et al. (2005). "E.-coli proteolytic activity in milk and casein breakdown." Reproduction Nutrition Development 45(4): 485-496.

[19] Besse NG, Cauquil A, et al. (2008). Comparative Study of Different Milk Samples Preservation Procedures for Bacteriologic Examination Food Analytical Methods 1(1): 36-42.

[20] Madadlou A, Mousavi ME, et al. 2009 Alkaline pH does not disrupt re-assembled casein micelles Food Chemistry 116(4): 929-932.

[21] Knudsen JC and Skibsted LH 2010 High pressure effects on the structure of casein micelles in milk as studied by cryo-transmission electron microscopy." Food Chemistry 119(1): 202-208.

[22] Sandra S and Dalgleish DG 2007 The effect of ultra high-pressure homogenization (UHPH) on rennet coagulation properties of unheated and heated fresh skimmed milk." International Dairy Journal 17(9): 1043-1052.

[23] Huppertz, T., P. F. Fox, et al. (2004a). "Properties of casein micelles in high pressure-treated bovine milk." Food Chemistry 87(1): 103-110.

[24] Lieske B 1998 Effects of ageing of raw milk on some structural properties of native casein micelles." Milchwissenschaft-Milk Science International 53(10): 562-565.

[25] Luzzana M and Giardino R 1999 Urea determination in milk by differential pH technique. Lait vol 79 (2): 261-67.

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