Effects of Carbonylation on the Freshness of Milk and Carbonylation Prevention Measures

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Research Article

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Abstract

Objective: To study the effects of carbonylation on freshness during milk storage and develop methods for keeping milk fresh.

Methods: The effects of storage temperature, time, vacuum and repeated freeze-thaw on milk carbonylation were determined. Levels of carbonylation were measured.

Results: The carbonylation content increased with storage time at room temperature. Vacuum protection and antibacterial protection decreased the protein carbonylation content. The carbonylation content increased with the number of freeze-thaw cycles under low-temperature storage conditions.

Conclusion: Vacuum freezing delays oxidative damage to milk protein.

Introduction

Milk is a very important food in people's daily lives and is rich in protein, fat, sugar, vitamins and minerals. Maintaining milk freshness is important for general preservation [1] and more so for preventing non-enzymatic and irreversible carbonyl modification of proteins in milk. The freshness of milk directly affects the quality of milk, and the quality of milk is controlled by detecting the freshness of milk. At present, the main detection methods of milk freshness are milk acidity, relative density, viscosity, electronic tongue technology, electronic nose technology.

However, due to the masking effect of milk proteins, the results obtained in the international standard acidity test are often larger than the actual acidity, and the evaluation error of milk quality is large, because milk acidizing is due to the degradation and acidizing of lactose to form lactic acid due to bacterial action, and the structure and function of protein are changed by acidified milk environment. The strategy used in this study reveals an intuitive and effective measure of the denaturation degree of protein carbonylation, and the changes of milk protein under different freeze treatment conditions efficiently and accurately in the dairy industry.

Milk protein carbonylation alters the appearance, physical properties and chemical properties of milk, and eventually result in a loss of edibility[2,3].

Protein carbonylation in milk is an irreversible and non-enzymatic modification of proteins and is the most common indicator of protein oxidation. [4].

The pathway of carbonyl protein generation is divided into two categories depending on the presence or absence of active oxygen radicals; reactive oxygen radicals are highly reactive oxygen radicals capable of oxidizing proteins and nucleic acids[5].Side chains of amino acids generate carbonyl proteins, which are prone to free radical oxidation[6]; proteins undergo lipid oxidation and non-enzymatic glycosylation, thus, forming carbonyl proteins (i.e., through glycosylation)[7,8].

Therefore, indicators to evaluate the freshness of milk have important practical importance in research on milk quality, including the development of freshness detection methods, comprehensive understanding of the deterioration process of milk, and analysis of changes in various indicators and the problems existing in various applications[9].Preventing increased protein carbonylation prolongs milk freshness[10,11].

Various ingredients in milk have different levels of tolerance to repeated freeze-thaw cycles. Related data on this issue are limited, and published papers often do not have high statistical capacity[12,13]. The effects of storage temperatures, repeated freeze-thaw cycles, or delays in fresh milk under different storage temperatures and protection methods[14,15]. We measured the effects of various protection methods on the detected carbonylation of protein components of cow milk under different storage temperatures.

Materials And Methods

Sample source

All selected dairy products were made from Dalian Xinle Dairy Company (Dalian, Liaoning Province, food production license number: SC10521021302324) and after collection were kept in plastic bags; information about date of production and shelf life were available.

Table 1
Nutrient composition table

Item	Per 100 ml	Nutrient reference value (%)
Energy	253 KJ	3%
Protein	3.0 g	5%
Adipose	3.3 g	6%
Carbohydrate	4.7 g	2%
Sodium	60 mg	3%
Calcium	100 mg	13%

Dispensing of milk

Fresh milk samples that were non-clotted, odorless (not bitter, sour and so forth), transparent liquids, and were not in sacks, were collected in groups.The samples were grouped and numbered, and the total protein TP concentration in fresh milk, biochemical indicators and the protein carbonylation content were immediately detected and considered as the original levels. Each sample was divided into 5 mL after measurement, placed in autoclaved plastic EP test tubes and sealed with sealing film to prevent evaporation.

Milk freezing protection status

The procedures and steps of cryopreservation are also important factors affecting the cryopreservation of samples. According to the method of cryopreservation, ice crystals are formed inside and outside the cells during the process of cryopreservation, which causes physical damage to the milk when the temperature drops. Some studies have shown that ice crystallization can only be formed by slowly cooling under the temperature range of -60 and 0℃. The slower the cooling, the larger the ice crystals. The ice crystals formed at about -25℃ are the most and have the largest impact on the liquid. Therefore, in the process of cryopreservation, try to avoid the harmful temperature zone or accelerate the speed of cryopreservation to reduce the impact of physical injury on the experimental results, in order to better preserve the liquid.The samples including the following groups were randomly selected and numbered: -20℃ ordinary frozen storage group, -20℃ vacuum protected frozen storage group, slow frozen instant protection group, quick frozen slow soluble protection group, quick frozen instant protection group, and freeze-thaw protection group.

For the -20℃ ordinary frozen storage group, fresh milk was divided into aliquots in high-pressure sterile EP tubes, marked, placed in a -20℃ freezer, and repeatedly frozen and thawed every 30 minutes. The freezing and thawing times were divided into groups in which the samples were repeatedly mixed, the sealing film was removed, and the samples were centrifuged at 2000 r/min for 5 minutes and equilibrated to room temperature. Each freeze and thaw time was performed in three groups of parallel samples for group identification.In the process of gradually cooling, the cooling process avoids the thermal shock caused by rapid freezing and melting, which causes physical damage to the components in the liquid. It is better to use 1℃ per minute. During the experiment, it was found that the milk could be cooled to -20℃ in a -20℃low-temperature refrigerator at 30 minutes, and this time was used to carry out the freezing cycle for subsequent test grouping.

For the -20℃ frozen storage group under vacuum protection, the test milk was placed in an EP tube after autoclaving, labeled, and subjected to food-grade vacuum standards used for vacuum storage and frozen storage. The compressor was evacuated for storage. Samples were placed in a freezer at -20℃, and freeze-thaw cycles were repeated every 30 minutes. The samples were grouped according to the number of freeze-thaw cycles. The samples were repeatedly mixed each time, the sealing film was removed, and samples were centrifuged at 2000 r/min for 5 minutes and equilibrated to room temperature. Each number of fusions was performed in three sets of parallel samples, which were identified by grouping.

For the slow-freezing and fast-dissolving protection group, fresh milk was placed in an EP tube after autoclaving, then incubated at 4℃, -20℃ and -80℃ in order, for 0.5 h at each temperature. The samples were then warmed in a 37℃ water bath immediately after freezing. Repeated freeze-thaw was performed according to this method, and the samples were repeatedly mixed each time. The sealing film was removed, and samples were centrifuged at 2000 r/min for 5 minutes and equilibrated to room temperature. Each freeze-thaw cycle was performed in three groups that included parallel samples for group identification.

For the quick-freezing and slow-dissolving protection group,

Four different conditions of freeze-thaw protection were investigated: quick-freezing, quick-freezing, slow-freezing, slow-freezing, and slow-freezing. Under different frozen storage conditions, ordinary cryopreservation used as a control group to compare with other groups in protein content changes, the protein carbonylation content index under various frozen storage conditions increased with the number of freeze-thaw cycles, and the protein carbonylation content also increased.milk was divided into aliquots in high-pressure sterile EP tubes, labeled, and immediately placed in liquid nitrogen and then stored in a -80℃ freezer. This was followed by storage at -20℃, 4℃ and 37℃ for 0.5 h at each temperature. The samples were repeatedly mixed each time, the sealing film was removed, and the samples were centrifuged at 2000r/min for 5min and equilibrated to room temperature. Three sets of parallel samples were examined.

For the quick-frozen and instant-protected group, fresh milk was divided into aliquots in plastic EP tubes after autoclaving, randomly marked, immediately frozen in liquid nitrogen and transferred to a -80℃ freezer. After freezing for 0.5 h, samples were transferred to a 37℃ water bath. The samples were repeatedly mixed each time, the sealing film was removed, and samples were centrifuged at 2000 r/min for 5 min and equilibrated to room temperature. Three sets of parallel samples were examined and identified in groups.

For the slow-freezing and slow-dissolving protection group, fresh milk was divided into aliquots in high-pressure sterile EP tubes, labeled and incubated at 37℃, 4℃, -20℃ and -80℃ sequentially for 0.5 h each. The samples were then incubated at -20℃, 4℃ and 37℃ for 0.5 h each. Freeze-thaw cycles were repeated five times according to this method. Three sets of parallel samples were examined for each number of freeze-thaw cycles for group identification.

Differences in protection status of milk at room temperature

Samples were placed in a constant temperature water bath equilibrated to 25℃ for room temperature time measurements. Among them, nine samples were randomly selected as the vacuum protection group, and nine randomly selected samples had 0.1% per kilogram concentration of streptomycin and penicillin added. The groups included fresh milk with antibiotic at room temperature and ordinary milk left at room temperature.

In the vacuum protection group, nine randomly selected samples were vacuum protected for vacuum preservation. Starting from 0 hours, the protein content and protein carbonylation content of the milk were continuously monitored every 4 hours for 24 hours, three times per hour.

Sets of parallel samples

Antibiotics were added in the milk group at room temperature. Nine randomly selected samples of fresh milk were mixed with 1:1 streptomycin and penicillin at a concentration of 1:1 per kilogram. Starting from

0 hours, the milk protein content and protein carbonylation were continuously monitored every 4 hours for 24 hours, and three sets of parallel samples were collected every hour.

In the ordinary room temperature milk group, starting from 0 hours, the milk protein content and protein carbonylation content were continuously monitored every 4 hours for 24 hours, and three groups of parallel samples were collected every hour.

Determination of protein carbonylation

The total protein TP detection kit was provided by Mindray. The testing was performed with a Mindray BS-800 automatic biochemical analyzer. The protein carbonyl content was determined according to the method of Levine (1994)[16] with a protein carbonylation content determination kit provided by Beijing Solibao Technology Co., Ltd. in a 1.5 mL centrifuge tube, to which 0.1 mL of protein solution and 0.5 mL of 0.02 mol/L 2,4-dinitrophenylhydrazine in 2 mol/L HCl solution were added and reacted at 25℃ for 40 min. For blank samples, 0.5 mL of a 2 mol/L HCl solution without 2,4-dinitrophenylhydrazine was added. Then 0.5 mL of 20% trichloroacetic acid was added to the above reaction solution. Samples were centrifuged (11000 × g, 5 min, 4℃), the supernatant was discarded, and 1 mL of ethanol-acetic acid was used for protein precipitation. The ethyl acetate solution (volume ratio 1:1) was washed three times. After the solvent was evaporated, the protein was suspended in 1 mL of a 6 mol/L guanidine hydrochloride solution and incubated in a water bath at 37℃ for 30 minutes. The blank sample was used as a control, and the absorbance at 370 nm was measured. The content of the protein carbonyl derivative (nmol/mg protein) was calculated by using the molar absorption coefficient of 22000 M-1 cm-1. Three replicates of each sample were measured, and the median value was taken.

The formula for calculating the carbonyl content was:

carbonyl content (nmol/g myofin) = $(A \times n)/(\varepsilon \times \rho) \times 10^9$

In the formula, A represents the absorbance at the wavelength of 370 nm; n represents the dilution factor; ε represents the molar absorption coefficient of 22000/(L/(mol · cm)); and ρ represents the protein mass concentration (mg/mL).

Results

Results of carbonylation of proteins with or without antibiotics at room temperature

The effect of streptomycin on the carbonyl content of milk is shown in Figure 1. With increasing room temperature standing time, the carbonyl content of milk protein increased. Starting from the 8th hour in milk left at room temperature (see Figure 1), we compared the protein carbonylation index content in milk with added antibiotics in the bacteriostatic condition versus ordinary pasteurized milk without antibiotics. The protein carbonylation content increased. The degree of milk protein carbonylation with and without

streptomycin differed. Although the protein carbonylation content increased, the protein carbonylation content of fresh pasteurized milk with antibiotics was always higher than that at room temperature. Less protein carbonylation was observed in fresh pasteurized milk. After 12 hours, the increase and the trends in the carbonylation content of protein in milk without antibiotics at room temperature, compared with milk with antibiotics at room temperature, were clearest, reaching 127.28 μmol/g by 24 hours. The detected carbonylation values in pasteurized fresh milk with antibiotics at the 20th hour were lower and different from that of milk placed at room temperature. During the entire measurement process, antioxidant measurements of the protective effects of antibiotics were collected. Bacteria promoted the carbonylation of proteins. Under room temperature storage, streptomycin had a protective effect on the antioxidant status of milk.

Vacuum protection affects protein carbonylation at room temperature

The vacuum protection method had a protective effect on milk stored at room temperature under the influence of the single factor of storage time. See Figure 2 for details. After standing at room temperature for 12 hours, milk was placed at room temperature under vacuum protection. Compared with that in stored milk, the carbonylation content of room temperature milk protein increased significantly and reached 537.273 μmol/g by 24 hours. Compared with the carbonylation of milk at room temperature, that of pasteurized fresh milk under vacuum protection at the 20th hour was markedly different. During the entire measurement process, anti-oxidation measurements of the protective effect of vacuum protection were collected. Vacuum promoted the carbonylation of proteins. Under the room temperature storage condition, the vacuum protection method had a protective effect on the antioxidant effects in milk and prolonged the freshness.

Determination of carbonylation of proteins in normal freeze-thaw and vacuum conditions

When the temperature was kept at -20° C (Figure 3),repeated freeze-thaw affected the stability of biochemical indicators. That is, used fresh milk at room temperature as the initial protein carbonylation value for comparisonthe single effect of repeated freeze-thaw increased the protein carbonylation content. From the second repeated freeze-thaw cycle, the repeated freeze-thawed and vacuum-protected milk showed increasing protein carbonylation content as the number of freeze-thaw cycles increased. We examined the protein carbonylation content index under the vacuum protection condition, compared with normal frozen storage. In vacuum-protected fresh pasteurized milk, compared with fresh milk, the protein carbonylation content also increased with prolonged storage time, but the protein carbonylation content remained less than that in ordinary frozen fresh pasteurized milk. Therefore, the stability of PC is affected by the interaction of repeated freezing and thawing, and the fluctuation of the indicator is due to the single factor of freezing time. In addition, as the number of vacuum freeze-thaw cycles increases, the protein carbonylation content of vacuum freeze-thawed milk is significantly lower than that of regular fresh freeze-thawed fresh milk. The vacuum condition had a protective effect on milk freshness.

Protection of four frozen conditions: quick-freezing, quick-freezing, slow-freezing, slow-freezing, and slow-freezing

Four different conditions of freeze-thaw protection were investigated: quick-freezing, quick-freezing, slow-freezing, slow-freezing, and slow-freezing. Under different frozen storage conditions,ordinary cryopreservation used as a control group to compare with other groups in protein content changes,the protein carbonylation content index under various frozen storage conditions increased with the number of freeze-thaw cycles, and the protein carbonylation content also increased. The degree of carbonylation and oxidation of milk proteins under various frozen storage conditions increased with the number of freeze-thaw cycles. Although the protein carbonylation content significantly increased, we compared the protein carbonylation content of fresh pasteurized milk in the slow-freezing and slow-thawing and repeatedly freeze-thawing groups with that of the other three freeze-thaw conditions (see Figure 4 for details). The milk protein carbonylation content under different protection methods and repeated freezing and thawing is shown in Figure 4, along with the repeated freeze-thaw control group and the number of repeated freeze-thaw cycles. As the number of freeze-thaw cycles increased, the milk protein carbonyl content significantly increased. Freezing and thawing accelerated protein denaturation, the degree of protein oxidation and the degree of protein carbonylation. Therefore, the slow-freezing and slow-freezing conditions are damaging to milk protein.

Discussion

This study analyzed the effects of different storage temperatures, storage times and freeze-thaw cycles on the total protein and protein carbonylation content of milk biochemical indicators, and explored the stability of frozen milk biochemical indicators during production and transportation.

Our study of the effects of the single factor of storage time on the carbonylation content of protein indicated that when the storage temperature was maintained at 25℃, extending the storage time affected the stability of the protein. The content of oxidative carbonylation of proteins increased with time, as did the content of protein carbonylation (that is, the degree of protein oxidation). Simultaneously, the addition of antibiotics inhibited the degree of oxidative carbonylation of proteins by inhibiting the growth of bacteria. When the storage temperature was maintained at 25℃ prolonged storage time affected the stability of the protein, and oxidative carbonylation of proteins occurred over time. The content of chemicals increased, but to a lesser extent than in ordinary room temperature milk. Therefore, bacteria promote the oxidative carbonylation of proteins.

When the storage temperature was maintained at 25℃, vacuum lock protection was used to study the protein carbonylation and vacuum protection method. Over time, the content of oxidative carbonylation of the protein increased, and the protein carbonylation increased over time. Although the degree of protein oxidation increased, it did so to a lesser extent than the ordinary oxidative carbonylation content of milk stored at room temperature. Therefore, milk freshness is prolonged by the vacuum lock protection method, thus decreasing the effect of time on the carbonylation content of protein.

When the storage temperature was fixed at the ordinary freezing temperature, in the frozen condition, the fluctuation of the oxidative carbonylation index of the protein was affected by the single factor of freezing time. As the number of freeze-thaw cycles increased, the protein carbonylation content increased. Therefore, when the storage temperature was fixed at the ordinary freezing temperature and the frozen condition was fixed, ordinary frozen storage under vacuum protection, compared with the ordinary freezing method, resulted in a relatively small increase in protein carbonylation content as the number of freeze-thaw cycles increased.

In the determination of protein carbonylation content, the stability of the oxidative carbonylation content of proteins is affected by the interaction between the freezing temperature and the storage method. The fluctuation of the index is due to the single factor of storage time or to the use of various freezing methods. The storage methods, with increasing freezing and thawing times, can promote an increase in protein carbonylation content, and there was no essential difference among the methods. The slowest increase in the protection status under slow freezing and slow dissolving was detrimental to the storage of milk.

In summary, the total protein TP and protein oxidative carbonylation PC indicators are unstable under various storage conditions. Factors that lead to increased detection results include storage temperature, repeated freeze-thaw cycles, freezing methods, antibiotics and vacuum protection status. The effects of vacuum condition, storage temperature, freezing and thawing times and freezing method also were found to interact. In summary, the conventional milk index values in biological sample banks are unstable. The factors causing the detected content to fluctuate are storage temperature, number of repeated freezing and thawing times, freezing time, and interactions among storage temperature, the number of freeze-thaw cycles and the freezing time effect. Therefore, milk samples should not be stored frozen in biological sample banks. Fresh samples should be selected for the detection of the indicators TP and PC; otherwise, milk freshness and the possibility of protein oxidation will be decreased. Our results provide a theoretical basis for the rapid determination of the freshness of frozen milk through protein carbonylation determination technology.

Declarations

Conflict of interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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Figures

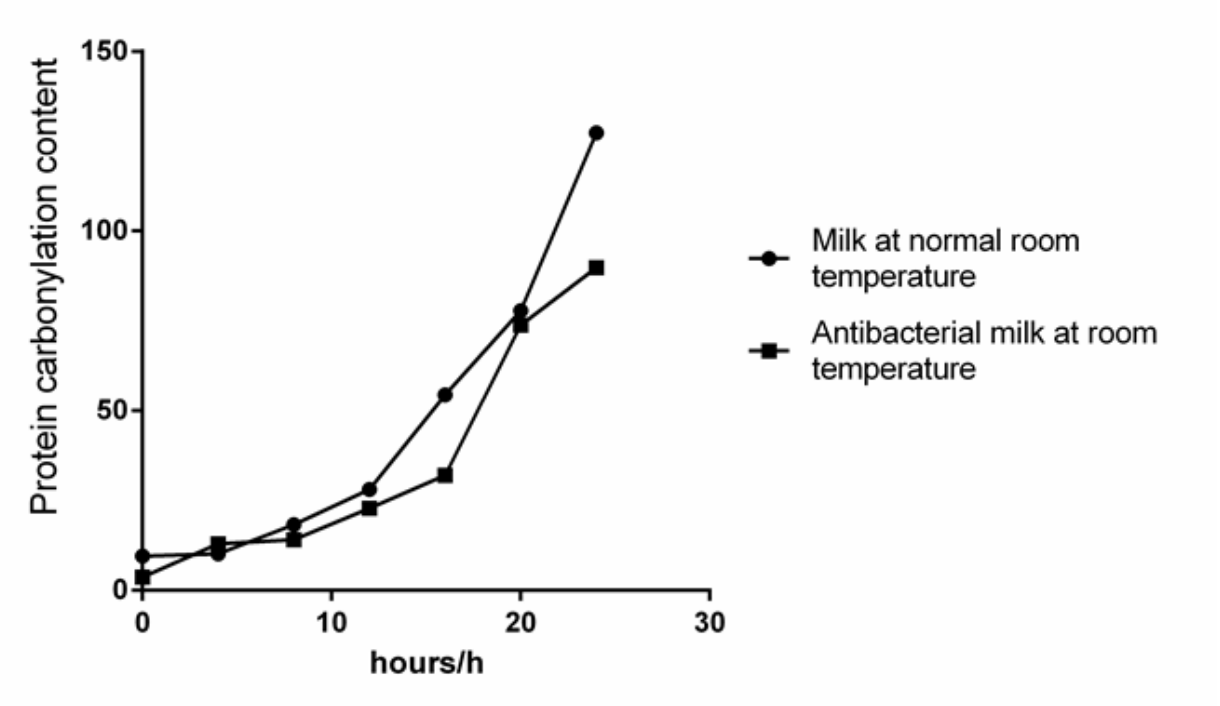

Figure 1

Trends in the presence or absence of antibiotics over time at room temperature

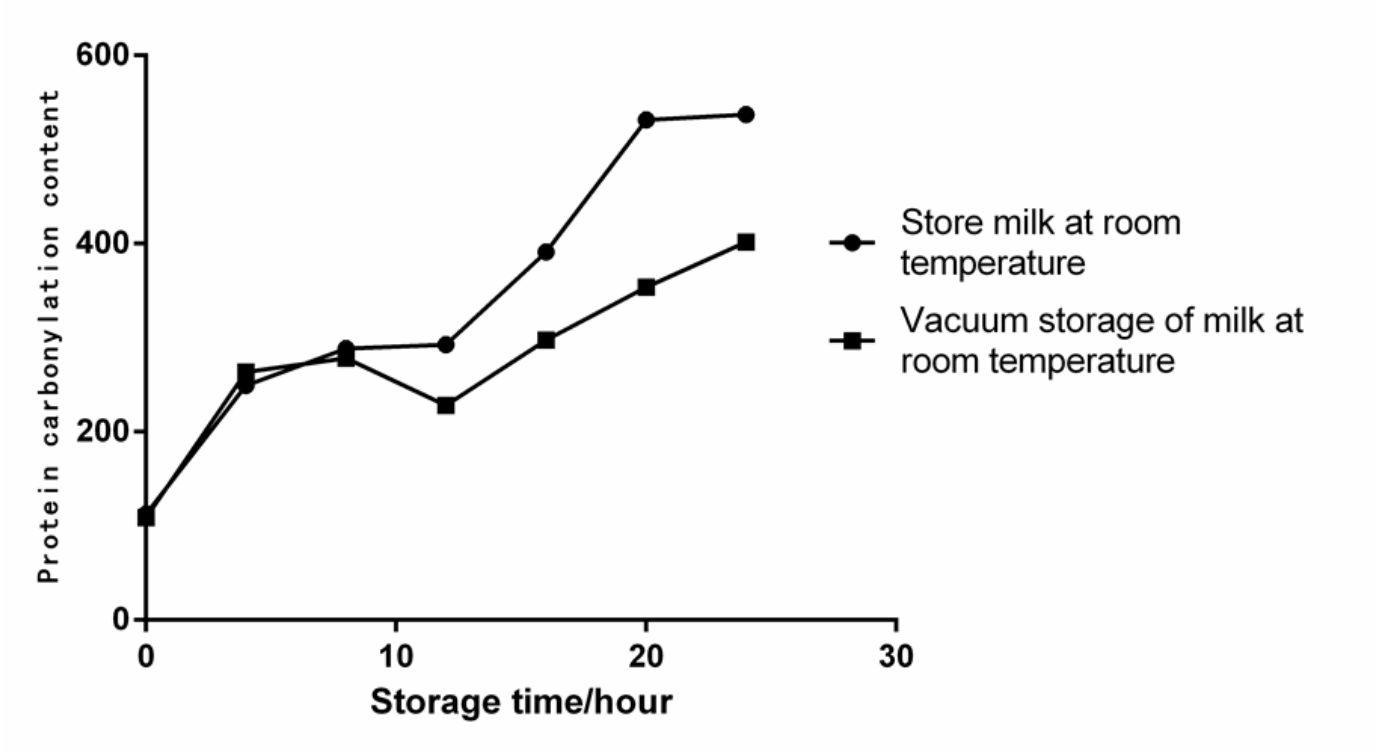

Figure 2

Vacuum protection status and protein carbonylation content at room temperature

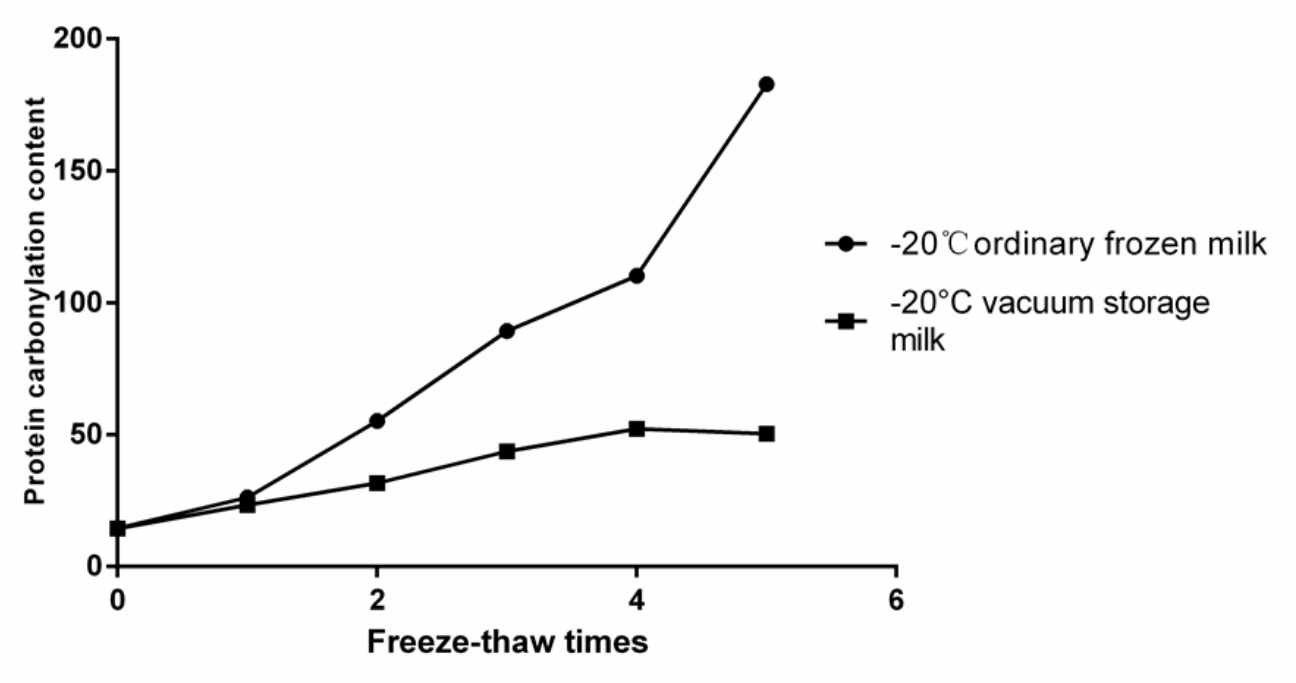

Figure 3

Milk protein carbonylation under repeated freeze-thaw cycles

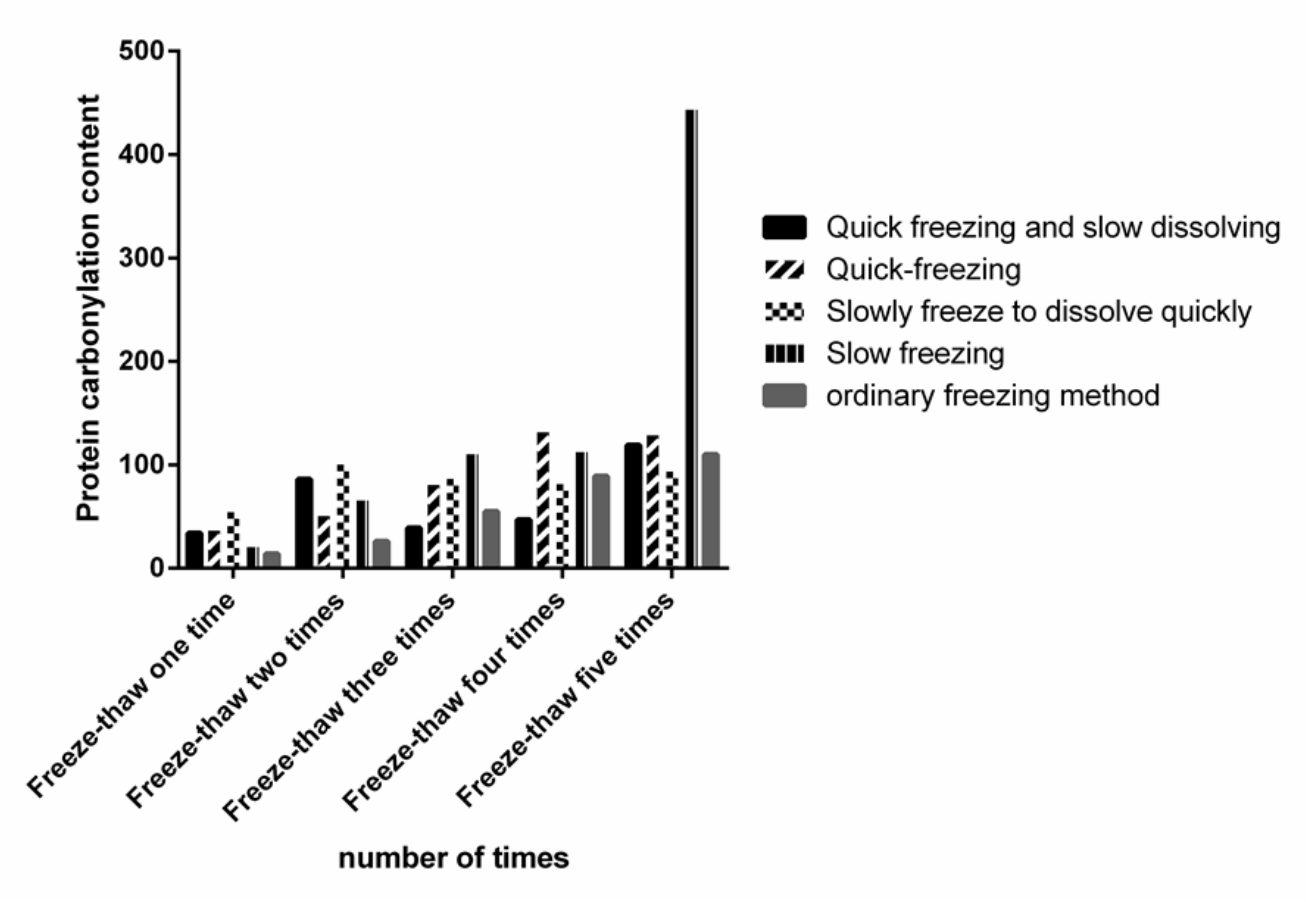

Figure 4

Trends in protein carbonylation content in four protected conditions with different freeze-thaw conditions