This is an Accepted Manuscript of an article published by Taylor & Francis in **DRYING TECHNOLOGY** on 22.09.2020, available online: https://www.tandfonline.com/doi/full/10.1080/07373937.2020.1824188

Postprint of: Martysiak-Żurowska D., Rożek P., Puta M., The effect of freeze-drying and storage on lysozyme activity, lactoferrin content, superoxide dismutase activity, total antioxidant capacity and fatty acid profile of freeze-dried human milk, DRYING TECHNOLOGY (2020), pp. 1-11, DOI: 10.1080/07373937.2020.1824188

The effect of freeze-drying and storage on lysozyme activity, lactoferrin content, superoxide dismutase activity, total antioxidant capacity and fatty acid profile of freeze-dried human milk

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ABSTRACT

Pooled human milk samples were freeze-dried and stored for 6 weeks at a temperature of 5 °C and 25 °C. Freeze-drying decreased the water content of milk by 86.5%, and the obtained lyophilizate was readily soluble in water. The freeze-drying process did not affect superoxide dismutase (SOD) activity, fatty acid (FA) profile or lactoferrin (LF) content, but it decreased total antioxidant capacity (TAC) of human milk by 22.1% and induced a minor increase in lysozyme (LZ) activity, by approximately 9.8%. Storage of freeze-dried milk did not show significant influence on TAC, LF, FA and LZ levels, while after six weeks of storage SOD activity decreased by around 27% relative to the level noted immediately after lyophilization. These findings and the remaining state of knowledge imply, that freeze-drying can be a useful method of human milk storage.

KEYWORDS

Human milk; freeze-drying; total antioxidant capacity; enzymes; fatty acids

HIGHLIGHTS

- Lyophilization does not significantly affect the level of SOD and LF of human milk.
- In freeze-dried milk, bioactive substances: TAC level, LF, FA and LZ, remain stable even at room temperature.
- During storage of freeze-dried milk SOD activity decreases significantly.

1. Introduction

Breastfeeding provides newborns with the nutrients and bioactive components essential for growth, development, and immunologic protection. The qualitative composition of milk is similar in every healthy mother, but certain variations are subjected to the mother's diet. The concentrations of almost all milk components change during lactation to meet the child's nutritional needs in various stages of growth. Breast feeding guarantees the optimal development of newborns, which is a particularly important consideration in preterm, low birth weight (LBW, <2500 g) and very low birth weight (VLBW, <1500 g) infants. In preterm, LBW and VLBW children, breast milk, especially colostrum, is not only a food source, but also has therapeutic value.[1] Colostrum, the milk secreted in the first five days after delivery, is characterized by lower fat content than transitional and mature milk as well as a higher content of protein, in particular functional proteins, such as lactoferrin (LF), lysozyme (LZ), cytokines, immunoglobulins, antioxidants and digestive enzymes.[2] Feeding preterm infants with human milk decreases the risk of many diseases, including necrotizing enterocolitis, retinopathy, bronchopulmonary dysplasia and sepsis.[3-7] Human milk also delivers health benefits for adults, including for patients recovering from acute malnutrition, liver transplants [8] and oncological therapy.[9]

When the mother's breast milk is not available, pasteurized donor milk from human milk banks (HMB) is recommended.[10] Breast milk from HMB donors is subjected to low-temperature long-time (LTLT) pasteurization, which is also known as Holder pasteurization. During pasteurization, milk is heated at 62.5°C for 30 minutes, which guarantees its microbiological safety, but also degrades many bioactive components.[11] After pasteurization, human milk is frozen and stored at -20°C, which further worsens its quality. Therefore, long-term storage methods that minimize the loss of valuable components are needed to preserve the nutritional value of pasteurized human milk. Storing freeze-dried milk could be an effective solution.

Freeze-drying (lyophilization) is a process during which freezing is accompanied by dehydration and sublimation of ice crystals to induce minimal changes in the frozen product's components. Lyophilization reduces the activity of water, which prevents the growth of microflora and slows down adverse enzymatic processes. Freeze-drying is a suitable method for preserving all organic substances and foods, including fruit, vegetables, mushrooms, meat, herbs and ready meals, without the use of preservatives. Lyophilization preserves the natural aroma, flavor, color, shape, cellular structure and, most importantly, the nutritional value of food products. Lyophilized foods can be quickly and easily reconstructed (rehydrated).[12,13,14]

The lyophilization process can also be successfully used to dehydrate human milk. The effects of freeze-drying on the concentrations of fat, triglycerides, fatty acids (FAs), proteins, glucose, polyphenols, lipase, B-vitamins, vitamin C, catalase (CAT), lysozyme (LZ), oligosaccharides and immunoglobulins as well as total antioxidant capacity (TAC) and lipid peroxidation in human milk have been evaluated by numerous authors in studies analyzing the consequences of food processing operations.[15-20] The potential use of freeze-dried human milk as a nutritional supplement for infants, in particular preterm neonates, has also been researched.[21,22]

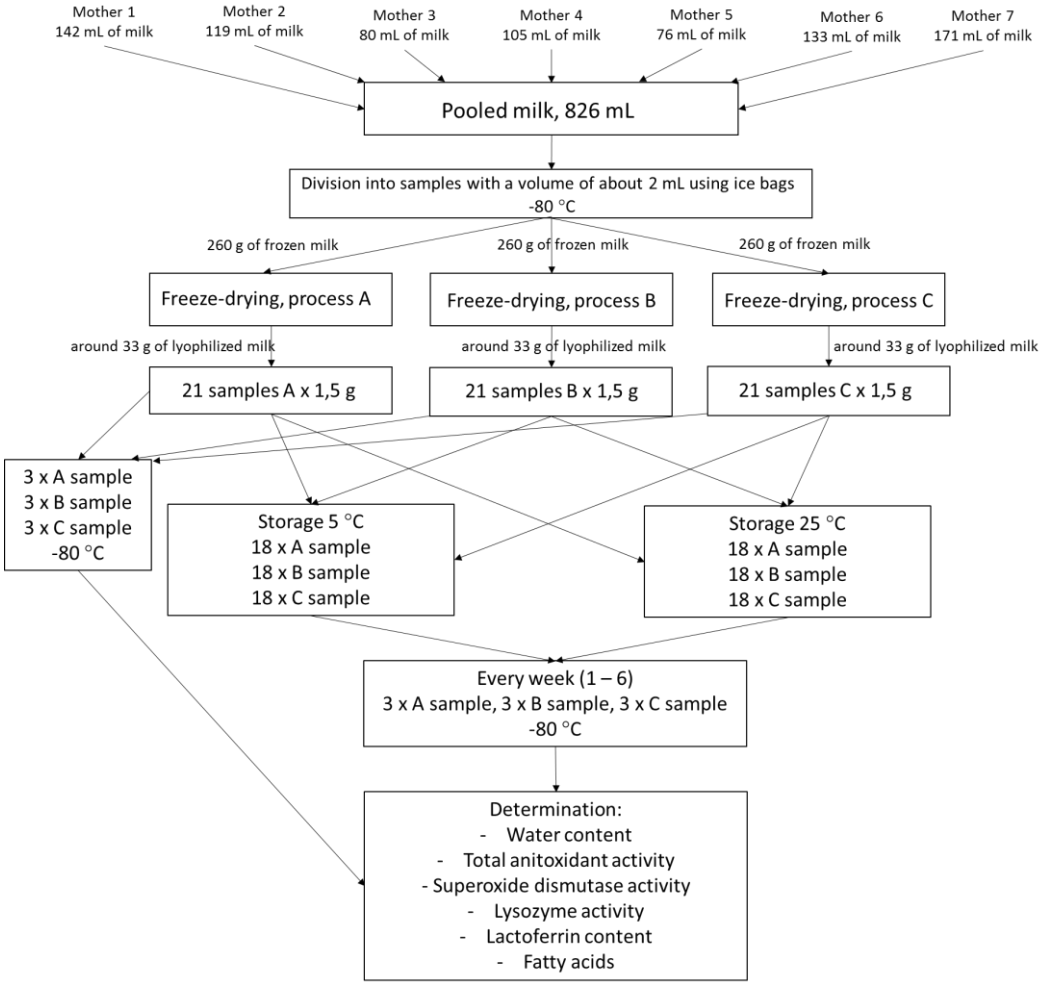

Figure 1. Experimental design of the study

The impact of freeze-drying on the composition of human milk has been relatively well documented, whereas changes in lyophilizate composition during storage remain insufficiently investigated. Considering the applicability of freeze-drying for the long-term conservation of human milk and the use of freeze-dried human milk as multi-nutrients fortifier for infants it is

extremely important to determine how storage of lyophilizates effects on nutrients and bioactive compounds of milk.

Therefore, the aim of this study was to determine the effect of freeze-drying and storage of freeze-dried milk on the activity and content of selected bioactive components in human milk. This is the first study to investigate the effect of freeze-drying on LF content and superoxide dismutase (SOD) activity in human milk, and the influence of six-week storage at refrigeration (~5°C) and room (~25°C) temperatures on the activity of SOD and LZ, the content of LF and FAs, and TAC in freeze-dried human milk.

2. Materials and methods

2.1. Sample collection

Samples of mature human milk were collected from seven healthy, non-smoking women residing in Gdańsk (Poland) and the surrounding areas, between days 21 and 30 after birth. All pregnancies were full-term and occurred without complications. All newborns were in good health (Apgar score ≥ 9 in the first minute of life), and their body weights were within the norm (3100 - 3800 g). Breast milk was sampled by the mothers at home with an electric breast pump (Symphony, Medela) into sterile containers designed for food contact, according to standard hygiene requirements. The samples were collected by the mothers within 24 h and stored in a refrigerator at around 5°C. Milk samples from different mothers were delivered to the laboratory at the same time; they were immediately pooled and divided into smaller samples of approximately 2 mL with the use of ice bags. Milk was stored at -80 °C until lyophilization, but not longer than one week (Figure 1).

All of the experimental procedures were approved by the Local Ethics Committee of the Medical University of Gdansk. The patients gave their written consent to participate in the study.

2.2. Lyophilization

Breast milk samples were frozen at -80°C and lyophilized in the Alpha 2-4 LD Plus freeze drier (Martin Christ, Germany). The freeze-drying process was continued until the achievement of constant weight, but not longer than 72 hours. Milk samples were freeze-dried in portions of approximately 260 g in triplicate, labeled as freeze-drying A, B and C (Figure 1).

2.3. Storage of human milk lyophilizates

The lyophilizates from each repetition of the process were divided into samples of 1.5 g each. Eighteen samples from each freeze-drying process were stored in sealed containers at

room (25°C) and refrigeration temperature (5°C). The samples were stored at the above temperatures for up to six weeks. Every week, nine samples were deep-frozen at -80°C to preserve their composition, properties and structure until analysis.

2.4. Determination of water content

The water content of freeze-dried samples was determined by using a moisture analyzer (MAX 50/1 Radwag, Poland) according to ISO 5537:2004.[23] The measurements were conducted at 102°C until the weight of the sample did not change in the course of three consecutive minutes. The measurements were performed in duplicate, separately for freeze-drying and storage.

2.5. Reconstitution of liquid milk

Milk samples were reconstituted by adding sterile redistilled water to freeze-dried milk in an amount corresponding to the amount of water removed during freeze-drying.

2.6. Determination of total antioxidant capacity

The TAC of milk was determined in the oxygen radical absorbance capacity assay with fluorescein (ORAC-FL) based on the procedure described by Saenz, Elisia, Innis, Friel & Kitts.[24] In the ORAC method, peroxyl radicals are generated during the thermal decomposition of 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) dissolved in a phosphate buffer (pH= 7.4). The free radical damage to fluorescein (FL) leads to a decrease in fluorescent intensity. The presence of antioxidants inhibits free radical damage to the fluorescent compound. Reduction the degradation rate of fluorescein over time is proportional to the antioxidant content of the solution. Milk samples were diluted 1:199 with a phosphate buffer. All measurements were performed with a microplate reader (Synergy HT, BIOKOM). The value of TAC was expressed in μM of Trolox (TE) per mL of milk.

2.7. Determination of superoxide dismutase activity

The activity of SOD in milk samples was determined with the Cayman kit (Superoxide Dismutase Assay Kit, Item No. 706002). Milk samples were centrifuged at 10 000 rpm (1 118 x g) at 4°C for 10 min. The resulting supernatant was collected, and the samples were diluted 1:4 with a sample buffer (50 mM Tris-HCl in HPLC-grade water, pH 8.0). Superoxide radicals are generated during the reaction of xanthine with xanthine oxidase, and the produced radicals react with tetrazolium salt to produce formazan dye, a colored compound with maximum absorbance at 450 nm. The SOD activity of the samples was calculated using a calibration curve

according to the Cayman procedure. The activity of SOD was expressed in unit (U) per mL, where one activity U denotes the amount of enzyme that reduces the formation of superoxide radicals by 50%.

2.8. Determination of lysozyme activity

Enzyme antimicrobial activity was determined spectrophotometrically by measuring the absorbance of the *Micrococcus lysodeikticus* cell suspension in 0.075 mM potassium phosphate buffer at pH 6.3 according to the method described by Viazis, Farkas & Allen.[25] Milk samples were diluted 1:19 with potassium phosphate buffer. All assays were performed using the JENWAY 3600 spectrophotometer. Lysozyme activity was expressed in activity units (U) per mL of milk.

2.9. Determination of lactoferrin content

The LF content of human milk was determined according to the method of Dračkova et al.[26] Milk samples were centrifuged (4°C, 12 000 rpm (1 610 x g), 15 min). The clear supernatant was collected into Eppendorf tube and diluted 1:5 (v:v) with ultrapure water. The samples were analyzed by High-Performance Liquid Chromatography in the Perkin Elmer series 200 chromatograph with a UV-VIS detector. Separation was performed at room temperature on the Kinetex C18 chromatographic column (4.0x150 mm, 5 µm, 100A, Phenomenex) with a guard column SB-C18 (4.6 x 12 mm) and 10 µL injection volumes. The analysis was carried out in linear gradient elution mode from 65% A : 35% B (0 min) to 50% A : 50% B (20 min), where eluent A was 0.1% trifluoroacetic acid in distilled water, and eluent B was 0.1% solution of trifluoroacetic acid in acetonitrile. Volume flow was 1 mL/min, and the chromatograms were monitored at 210 nm. Lactoferrin recovery was determined at 98.18% by the internal standard method, using of three independent solution of lactoferrin in five replicate and the same sample of human milk as a matrix.[27] The LF content of milk was calculated with a calibration curve in the concentration range of 0.02–0.85 mg /mL where peak area was plotted against LF concentration in standard solutions.

2.10. Determination of fatty acid composition

Lipids were extracted from human milk samples by the Roese-Gottlieb method.[28] Fatty acid methyl ester (FAME) profiles were determined according to standard EN:ISO 5509:2000.[29] FAMEs were separated by high-performance gas chromatography (HP-GC) based on the length of the hydrocarbon chain and the degree of FAME unsaturation. Analyses were carried out using a Hewlett-Packard GC system (Hewlett-Packard, Palo Alto, CA, USA)

with a split/splitless injector, Rtx 2330 column (100 m × 0.25 mm, Restek, Bellefonte, PA, USA) and a flame ionization detector (FID). FID and injector temperature were maintained at 250°C. The initial column oven temperature was 155 °C for 30 min, after which it was gradually increased to 210°C at the rate of 1.5 °C/ min and held at this temperature until the end of the analysis. Qualitative and quantitative analyses of FAs in the evaluated samples were performed with FAME standards (Sigma-Aldrich). The results were expressed as the percentage of the individual FAs in total FAs.

2.11. Statistical analysis

The statistical analysis was conducted in GraphPad Prism 11.0 (La Jolla, CA, USA). The results were presented as mean values and standard deviations (mean ± SD). The normality of all variables was evaluated with the Shapiro-Wilk test. The significance of differences in the content or activity of the compounds measured in raw and lyophilized human milk was determined by one-way analysis of variance (ANOVA). The analysis of variance was performed with the use of Tukey's multiple comparison test. Differences between means were considered statistically significant at $p < 0.05$.

3. Results and discussion

Freeze-dried human milk was a powdery, homogeneous substance that was relatively susceptible to caking and readily rehydrated, but did not show hygroscopicity. The storage temperatures of both groups of samples were monitored every day for six weeks using digital thermometers for laboratory use (LB-710DT, Lab-El, Poland). The average storage temperatures were 25.24 ±0.75°C and 4.91±0.51°C. Milk was stored at room temperature to verify whether freeze-dried human milk should be stored only at refrigeration temperature, which generates additional cost.

Apart from the relative humidity, storage temperature is the main parameter that significantly impacts the quality of powdered milk. According to the literature,[30] storage of model infant milk formula (IMF) powders, mimicking human milk, at ambient temperature (25°C), and low relative humidity levels (11% and 44%) was found to avoid lactose crystallization and caking. The storage of these powders at higher temperature and higher relative humidity caused increased lactose crystallinity and increased surface fat content what induced caking.

3.1. The effect of freeze-drying and storage on water content

The amount of water that can be evaporated during lyophilization depends on the product's properties and drying conditions which should be selected appropriately for the tested product. In the present research, high freeze-drying efficiency was maintained, and water loss was determined based on the difference in milk weight before and after freeze-drying. On average, freeze-drying reduced the water content of milk by 86.51 ± 0.49% compared to fresh milk. The moisture content of the analyzed samples was measured immediately after freeze-drying and weekly during storage. The average moisture content of powdered human milk was determined at 3.92 ± 0.53% directly after lyophilization, whereas in a study by Castro-Albarran et al.[16], the final moisture content of freeze-dried milk reached 2.48 ± 0.61%.

Table 1. The content of water in human milk and moisture of freeze-dried mature breast milk after lyophilization and during storage.

Sample	Moisture (%)	
Raw milk (water content)	90.43 ± 0.913[a]	
Freeze-dried milk	3.92 ±0.53[b]	
Storage time (weeks)	Storage temperature	
	5 °C	*25 °C*
1	3.91 ±0.31[b]	3.93 ±0.52[b]
2	3.83 ±0.32[b]	3.91 ±0.31[b]
3	3.86 ±0.10[b]	4.09 ±0.22[b]
4	3.95 ±0.35[b]	4.03 ±0.08[b]
5	3.82 ±0.05[b]	4.06 ±0.25[b]
6	3.84 ±0.13[b]	4.01 ±0.21[b]

[a, b] – Mean values in columns with different superscript differ significantly at the level of $p \leq 0.05$

Only minor changes in the water content of lyophilizates were observed at both storage temperatures. The moisture content of powdered human milk decreased by approximately 2% during six weeks of storage at about 5°C (i.e., by 80 mg per 100 g of powdered milk) and increased by approximately 2.3% during storage at about 25°C (by 90 mg of water /100g of the sample) (Table 1). The changes in moisture content were not statistically significant. However, they indicated the potential need for better protection of lyophilized milk samples during storage, for example, using vacuum sealing. The absence of an increase in the moisture content of powdered milk is very important. Masumi et al. [31] showed that uncontrollable moisture uptake by IMF powders triggers many undesirable effects including lowering of the glass transition temperature, crystallization of lactose, formation of free fat, undesirable modification of surface composition and morphology, and reduced solubility.

3.2. The effect of freeze-drying and storage on TAC

The ORAC-FL assay is a highly sensitive method for measuring the total antioxidant capacity of biological samples, based on the hydrogen atom transfer (HAT) mechanism.[24] In the tested samples of raw human milk, TAC was determined at 9.32 μmol TE/ mL in the ORAC-FL assay (Table 2). Lyophilization directly influenced the antioxidant capacity of human milk. The TAC of freeze-dried human milk was approximately at 22.04 ±4.67% lower than that of raw milk. Similar results determining the decrease in antioxidant capacity of human milk as a result of freeze-drying at the level of 17% were noted in a study where TAC was determined using the ABTS method. [20]

Storage of freeze-dried human milk at refrigeration temperature (approx. 5 °C) does not affect the milk TAC. We have indicated that after 6 weeks of storage, the value of TAC was identical to that determined immediately after freeze-drying. In a study by Lozano, Castellote, Montes & Lopez-Sabater[32], the antioxidant capacity remained practically constant in freeze-dried human milk stored at 4°C for 90 days.

The antioxidant capacity of freeze-dried milk changed slightly during storage at room temperature. After six weeks of storage at 25°C, the TAC of freeze-dried milk was further reduced by 6.9% compared to the values measured immediately after lyophilization. The most significant changes in the TAC value occurred between the second and third weeks of storage. Higher storage temperature promotes the acceleration of chemical reactions, including oxidation and antioxidation reactions, which consecutively leads to a decrease in the content of antioxidants in milk, e.g. after 5 days of storage at 40°C Lozano et al.[32] showed a 24% decrease in TAC, and after 30 days - nearly a 42% decrease.

Breast milk contains many antioxidants, that inhibit lipid and protein oxidation, which causes a decrease in antioxidant capacity of milk. TAC is a measure of the activity and content of non-enzymatic antioxidants in the tested samples. Tijerina-Sáenz, Innis & Kitts [42] showed a clear relationship between the alpha-tocopherol content in human milk and the TAC level of human milk. Most importantly, removing water as ice during freeze-drying does not generate the markers of undesirable biochemical processes, such as nitrites, superoxide anions, hydroperoxides, lipoperoxides and γ-glutamyl transpeptidase.[17] This means that a significant decrease in the TAC level during lyophilization was due to the protective activity of antioxidants rather than the direct degradation of antioxidant.

3.3. The effect of freeze-drying and storage on SOD activity

The SOD activity in raw human milk samples collected from different women can vary significantly depending on the stage of lactation and individual characteristics of mothers.[34] The enzyme activity in the pooled laboratory samples (raw milk, control) was determined at 1.01 ± 0.60 U/mL.

The present study is the first study to describe the effect of lyophilization and storage on SOD activity in human milk. SOD activity was not influenced by freeze-drying, but it decreased during the storage of freeze-dried human milk. After six weeks of storage at 5°C and 25°C, SOD activity decreased by approximately 26% and 31%, respectively, compared to the values measured immediately after lyophilization (Table 2). The greatest and a statistically significant decrease in SOD activity was observed during the first week of storage. The evaluated parameter remained fairly stable during the remaining weeks of storage, and a minor and temporary increase was noted after 3 weeks of storage. Concerning other enzymes of human milk, Friend et al. [18] pointed out that freeze-drying significantly decreased lactoperoxidase activity (by approx. 77%) and completely degraded it after storing the freeze-dried milk for 1 month, even at 4°C. But, neither the freeze-drying process nor the storage of freeze-dried milk for 6 months at 25°C had a significant effect on the activity of lipase and protease.

Table 2. Effect of freeze drying and storage freeze-dried milk on antioxidative properties (Total Antioxidant Capacity TAC) and enzymes (superoxide dismutase SOD, lactoferrin LF and lysozyme LZ) of mature breast milk (means ±SD).

Sample	TAC (µmol TE/ mL)		SOD (U/ mL)		LF (g /L)		LZ (U /mL)	
Raw milk	9.33 ± 0.58^{a}		1.01 ± 0.60^{a}		2.29 ± 0.11^{a}		18267 ± 355^{a}	
Freeze-dried milk	7.27 ± 0.64^{b}		1.05 ± 0.08^{a}		2.34 ± 0.10^{a}		20054 ± 546^{b}	
Storage time	Storage temperature							
(weeks)	5 °C	25 °C	5 °C	25 °C	5 °C	25 °C	5 °C	25 °C
1	7.76 ±0.63^{b}	7.55 ±0.39^{b}	0.86 ±0.16^{b}	0.88 ±0.10^{b}	2.44 ±0.08^{a}	2.39 ±0.09^{a}	19527 ±380^{b}	20289 ±440^{b}
2	7.79 ±0.56^{b}	7.82 ±0.31^{b}	0.81± 0.12^{b}	0.84 ±0.11^{b}	2.42 ±0.09^{a}	2.31 ±0.08^{a}	19170 ±320^{a}	18741 ±421^{a}
3	7.52 ±0.61^{b}	7.31 ±0.35^{b}	0.83 ±0.28^{b}	0.92 ±0.33^{b}	2.23 ±0.02^{a}	2.33 ±0.08^{a}	19782 ±415^{a}	19544 ±504^{a}
4	7.73 ±0.51^{b}	7.22 ±0.43^{c}	0.79 ±0.20^{b}	0.81 ±0.05^{b}	2.27 ±0.09^{a}	2.23 ±0.05^{a}	19004 ±575^{a}	19779 ±404^{a}
5	7.21 ±0.61^{b}	7.27 ±0.30^{b}	0.75 ±0.20^{b}	0.79 ±0.09^{b}	2.32 ±0.05^{a}	2.30 ±0.07^{a}	19546 ±513^{a}	19187 ±363^{a}
6	7.27 ±0.28^{b}	6.85 ±0.13^{c}	0.79 ±0.14^{b}	0.74 ±0.07^{b}	2.34 ±0.06^{a}	2.37 ±0.09^{a}	19630 ±605^{a}	19213 ±548^{a}

$^{a, b}$ – Mean values in columns with different superscript differ significantly at a given storage temperature ($p \leq 0.05$).

The influence of physical processes and storage on SOD activity in human milk has been insufficiently investigated in the literature. Marinović et al.[35] reported that Holder

pasteurization decreased SOD activity by approximately 67%, but according to other authors, convective heating at a temperature of 62.5°C for 30 min did not induce significant changes in SOD activity.[36] On the other hand, human milk samples exposed to moderate pressure and sub-zero temperature (193 MPa, -20ºC), the significant increase in SOD activity was observed, which is probably due to the release of SOD from human milk cells under pressurization.[36]

3.4. The effect of freeze-drying and storage on LF concentration

The LF content of mature human milk ranges from approximately 2.2 g /L (29-56 days) to 3.3 g /L (57 - 84 days).[37] In this study, the LF content of raw human milk was determined at 2.29 ±0.11 g /L, which was within the reference range. The LF content of freeze-dried milk increased by 2.3% compared to raw milk, but the difference was not statistically significant. LF concentration in freeze-dried human milk did not change during the 6 weeks of storage, regardless of the storage temperature.

The effect of lyophilization on the LF content of human milk has not been widely documented in the literature. There is evidence to indicate that freeze-storage of raw human milk leads to LF degradation. Goldsmith, Dickson, Barnhart, Toledo & Eiten-Miller[38] reported an estimated 11% decrease in the LF content of raw human milk after four weeks of storage at -20°C. Furthermore, the effect of pasteurization used in HMB on the level of LF was determined. Chang et al.[39] stated that the mean concentration of LF in pasteurized milk was 66% lower than that in fresh milk. Freezing caused a decrease in the LF level by about 11.5%.

3.5. The effect of freeze-drying and storage on LZ activity

Lysozyme is an active enzyme whose concentration is approximately 1000 times higher in human milk than in bovine milk. In the present study, LZ activity was determined at 18 267 ±355 U /mL in the pooled laboratory sample. Sousa, Delgadillo & Saraiva[40] estimated LZ activity in the human colostrum at 18 000 ±570 U /mL. Our findings showed that LZ was resistant to lyophilization, and its activity was even slightly higher in freeze-dried milk than in raw milk (Table 2). Our previous study demonstrated that the LZ content of breast milk decreased by approximately 9% after freeze-drying.[20] Interestingly, Vincenzetti et al.[41] observed a similar phenomenon in freeze-dried donkey's milk, where LZ activity increased by approx. 11%, while its content decreased by approx. 15%. Some enzymes retain or even increase their activity against microbicidal after partial denaturation. Düring et al.[42] showed that heat-denatured LZ significantly reduces the enzymatic activity but maintains its antimicrobial functions.

Table 3. Content of selected fatty acids (% of total FA) in fat extracted from raw, freeze-dried and stored freeze-dried human milk.

Fatty acid	Raw milk	Freeze-dried milk	*Storage time (weeks)*											
			1	2	3	4	5	6	1	2	3	4	5	6
			Storage temperature 5 °C						*Storage temperature 25 °C*					
SFA	45.43±1.75	45.80±1.70	45.12±1.80	45.03±1.55	44.47±1.69	44.75±1.79	44.31±1.53	44.23±1.66	45.21±1.69	43.92±1.72	44.21±1.77	43.51±1.50	44.03±1.42	44.18±1.63
including:														
C12:0	4.68±0.22	4.87±0.23	4.64±0.19	4.93±0.19	4.74±0.22	5.09±0.31	4.57±0.21	4.78±0.25	4.66±0.19	4.74±0.26	4.64±0.22	4.86±0.25	4.33±0.23	4.56±0.22
C14:0	6.73±0.28	6.65±0.22	6.42±0.29	6.68±0.25	6.69±0.26	6.38±0.24	6.71±0.24	6.38±0.28	6.79±0.26	5.32±0.25	5.76±0.22	5.82±0.26	6.26±0.27	6.21±0.29
C16:0	23.94±0.32	24.03±1.03	24.68±0.95	24.07±1.10	23.83±1.02	23.34±1.06	23.54±0.93	23.73±0.89	24.11±1.03	24.40±1.05	24.39±1.01	23.72±0.96	24.18±0.98	24.31±1.03
C18:0	7.71±0.33	8.04±0.35	7.17±0.32	6.94±0.31	6.78±0.32	7.26±0.35	7.26±0.35	7.11±0.36	7.31±0.34	7.32±0.35	7.14±0.33	6.91±0.29	6.98±0.32	6.93±0.36
MUFA	40.62±1.26	39.53±1.23	40.72±1.36	40.32±1.15	39.73±1.22	40.96±1.32	40.25±1.21	40.11±1.19	40.16±1.34	40.12±1.41	41.20±1.29	41.57±1.31	41.10±1.32	41.40±1.24
including:														
C16:1 (n-7)	2.35±0.09	2.57±0.08	2.46±0.09	2.59±0.09	2.47±0.08	2.89±0.08	2.24±0.08	2.39±0.07	2.54±0.10	2.23±0.09	2.32±0.12	2.37±0.09	2.34±0.10	2.36±0.09
C18:1 (n-9)	33.11±1.12	32.85±1.22	33.18±1.15	32.12±1.16	31.92±1.20	33.06±1.11	32.68±1.12	32.58±1.11	32.35±1.14	33.25±1.20	33.57±1.18	33.74±1.46	33.33±1.16	33.83±1.13
Σ MUFA trans	1.22±0.05	1.18±0.04	1.13±0.05	1.12±0.05	1.14±0.06	1.25±0.05	1.20±0.04	1.15±0.05	1.11±0.05	1.22±0.04	1.16±0.05	1.11±0.06	1.27±0.06	1.10±0.05
PUFA	13.31±0.53	13.28±0.56	13.26±0.62	13.04±0.66	13.87±0.65	13.86±0.59	13.46±0.42	13.57±0.62	13.40±0.60	13.24±0.58	13.98±0.59	13.70±0.47	13.34±0.52	13.58±0.41
including:														
PUFA (n-6)	11.12±0.46	11.10±0.45	10.80±0.44	10.72±0.38	11.44±0.35	11.36±0.35	11.14±0.37	11.12±0.41	10.91±0.40	10.92±0.23	11.23±0.33	11.06±0.31	10.78±0.42	11.20±0.43
PUFA (n-3)	1.38±0.07	1.40±0.06	1.42±0.06	1.43±0.07	1.37±0.08	1.53±0.08	1.33±0.07	1.36±0.06	1.36±0.07	1.44±0.08	1.47±0.08	1.42±0.07	1.41±0.06	1.43±0.07
C18:2 (n-6)	9.78±0.33	9.75±36	9.35±0.31	9.31±0.29	9.89±0.39	9.89±0.25	9.64±0.35	9.68±0.32	9.24±0.30	9.46±0.29	9.55±0.36	9.39±0.35	9.21±0.30	9.76±0.28
C18:3 (n-3)	1.01±0.04	0.98±0.04	1.00±0.03	1.02±0.04	0.95±0.03	1.07±0.03	0.94±0.04	0.92±0.05	0.99±0.03	1.02±0.05	1.01±0.04	0.99±0.03	0.92±0.03	0.98±0.03
C20:4 (n-6)	0.62±0.02	0.63±0.02	0.59±0.03	0.58±0.01	0.55±0.01	0.56±0.02	0.53±0.02	0.57±0.02	0.58±0.03	0.58±0.03	0.69±0.03	0.61±0.02	0.58±0.03	0.52±0.02
C20:5 (n-3)	0.19±0.01	0.16±0.01	0.15±.02	0.17±0.02	0.16±0.02	0.15±0.02	0.17±0.02	0.16±0.02	0.18±0.03	0.22±0.02	0.21±0.02	0.14±0.01	0.24±0.02	0.22±0.02
C22:6 (n-3)	0.41±0.02	0.36±0.02	0.58±0.02	0.42±0.02	0.58±0.02	0.55±0.02	0.51±0.03	0.58±0.01	0.67±0.02	0.48±0.02	0.73±0.02	0.62±0.03	0.55±0.02	0.55±0.01
Σ CLA*	0.32±0.01	0.36±0.02	0.35±0.02	0.37±0.02	0.36±0.03	0.34±0.03	0.39±0.01	0.42±0.02	0.37±0.02	0.31±0.02	0.47±0.03	0.47±0.02	0.51±0.02	0.32±0.02

Means from triplicate experiments ±SD, * CLA c9,11t and CLA t10,c12

Storage did not exert a significant influence on LZ activity. The studied parameter was nearly identical after freeze-drying and after 6 weeks of storage at 5°C and 25°C. Similar observations were made by Friend, Shahani & Long[18] who found no changes in the LZ content of freeze-dried human milk stored for 6 months at 4°C and 25°C.

3.6. The effect of freeze-drying and storage on FA concentrations

More than 60 different FA have been found in human milk fat. Table 3 presents the content of individual FA groups and the levels of selected FA, including long chain polyunsaturated fatty acids (LCPUFAs) important for the development of a child, in the fat of raw milk, subjected to freeze-drying, and in storage freeze-dried milk. There was no significant effect of lyophilization on the FA profiles of human milk fat.

Stability of FAs in food for infants is very important, in particular concerning the metabolites of both FA families, including arachidonic acid (AA) C20:4 (n-6), eicosapentaenoic acid (EPA) C20:5 (n-3) and docosahexaenoic acid (DHA) C22:6 (n-3). These LCPUFAs are necessary for the development of the central nervous system and the retina in infants. In newborns, the conversion rate of linoleic acid (LA) C18:2 (n-6) and linolenic acid C18:3 (n-3) is not sufficiently high to produce the LCPUFA amounts required for their healthy development.

LCPUFA have many double bonds, which makes them particularly sensitive to oxidation during processing and storage. Despite this theoretical risk, no difference was noted in the FA profiles of raw, freeze-dried and stored powdered human milk (Table 3).
Friend, Shahani & Long[18] and Cavazos-Garduño et al.[15] also reported that lyophilization exerted no significant effect on the FA profile of human milk, but it significantly decreased the diameter of milk fat globules.[15] A reduction in the diameter of globules increases their specific surface area, which in turn can increase their susceptibility to oxidation. However, the content of primary (lipid peroxide, LP) and secondary (thiobarbituric acid reactive substances, TBARS) lipid oxidation products did not increase in freeze-dried milk.[20] Lozano, Castellote, Montes & Lopez-Sabater[32] demonstrated that the most important PUFA remained stable during the storage of freeze-dried human milk 4°C for 90 days and at 40°C for 60 days. In our study, we also did not find a negative effect of the storage of human milk lyophilizates on the content of FA present in milk fat.

3.7. Characteristics and potential uses of freeze-dried human milk

Freeze-drying occurs at sub-zero temperatures with no air access, which stabilizes the nutrients and bioactive compounds in dehydrated products.[43] In a study by Friend, Shahani &

Long[18], freeze-drying had no effect on the content of lipase, protease, biotin, niacin, pantothenic acid, fat or free FA in human milk. Cortez & Soria[17] demonstrated that also proteins, triglycerides and polyphenols of human milk were not affected by the freeze-drying process, as well as human milk oligosaccharides.[19] In the present study, freeze-drying did not exert a significant influence on LZ activity, SOD activity or LF content of human milk. Furthermore, a minor increase in LZ activity was even observed immediately after lyophilization.

Also, studies show that compared to freezing (used for preserving human milk), lyophilization of human milk is reported to better preserve nutrients and several immune components while preventing oxidative deterioration.[17,32]

However, not all substances present in human milk are resistant to lyophilization. Freeze-drying significantly decreased lactoperoxidase activity by approx.77% and catalase by approx. 11%, as well as the content of vitamin C (by approx. 32%), glucose and immunoglobulins (from 8 to 23%).[16,18,20] In the present study, freeze-drying also induced a 22.1% decrease in TAC which is a measure of the activity and content of non-enzymatic antioxidants in the tested samples.

Analysis of changes in the composition of human milk stored in the form of freeze-dried powder also gave promising results. In the conducted study LF, TAC, LZ and FAs remained stable during 6 weeks of storage of freeze-dried milk at both refrigeration and room temperature. The only exception was SOD whose activity was significantly reduced in the freeze-dried samples, mainly during the first week of storage. Additionally, other bioactive components in freeze-dried human milk are also stable during storage. According to Friend, Shahani & Long[18], lipase and protease retained their activity for six months at 4ºC and 25 ºC, and the content of lysozyme and B vitamins did not change. Other studies stated that storage of freeze-dried human milk at 4ºC also did not affect the content of tocopherols and PUFAs, but induced a minor decrease (by approx. 11%) in vitamin C levels after 90 days of storage.[32] Importantly, the bioactive components of human milk were more effectively preserved in freeze-dried samples stored, even at room temperature, than in frozen samples of raw milk. Xavier, Rai & Hagde[44] observed a 25% decrease in the TAC of raw milk stored for one week at -8°C, whereas Hanna et al. [45] reported an 11% decrease in the TAC of raw milk stored for one week at 4°C and a 19% decrease in raw milk stored for one week at -20°C. Freeze storage of raw human milk at -20 °C for 3 months did not influence LZ activity[46,47], but it decreased its LF content by approximately 30% after 4 weeks of storage at -20°C.[38] In raw human milk stored for 7 days at -20°C, SOD activity decreased dramatically by approximately 62%.[35]

In summary, the present research indicates that freeze-dried human milk remains bioactive and retains most of its health benefits during storage, even at room temperature, unlike liquid human milk which properties deteriorate considerably during refrigeration and freeze storage. Freeze-dried human milk can be seen as a milk concentrate, rich in nutrients and bioactive components.

The survival rate and the quality of life of preterm newborns, especially those of low and very low birth weight, have improved significantly due to the recent technical and scientific advancements in neonatal care. The most serious problem in feeding premature infants is the immaturity of the gastrointestinal tract structures, which makes them unable to absorb the right amount of nutrients. Even though the composition of premature mothers' milk is different from that of women who gave birth on time, there are usually deficiencies in protein, calories and micronutrients. The latest recommendations for adequate nutritional care include increasing protein supply, improving quality parenteral lipid formulations, and providing mineral supplementation, in addition to the feeding of human milk.[48]

To prevent the subsequent effects of nutritional deficiencies and ensure the proper growth of this group of newborns, breast milk is usually strengthened by using commercially available fortifier based on cow's milk or based on plant ingredients (lipids, protein hydrolysates). However, the freeze-drying process of human milk can be a more effective alternative for preparing human milk fortifiers.[21] Many preterm, LBW and VLBW infants require fortified milk with protein, calories and micronutrients. We believe that fortification with freeze-dried human milk is a much better solution than spray-dried fortifiers prepared with the use of bovine milk or plant ingredients. The use of human milk, rather than the milk of an unrelated species, for fortification is undoubtedly the best solution.[21,22] It guarantees the child's optimal growth and development, and it could decrease medical treatment costs in the future. Additionally, recent studies show that the production of a concentrate with freeze-dried human milk brings great benefits to the newborns by offering high-quality nutrients and preserving the nutrients present only in breast milk.[21] However, further clinical trials are needed to evaluate the efficacy of fortifiers from human milk for LBW and VLBW infants; the safety and tolerability of such formulations should be assessed.

The lyophilization process is not considered a pasteurization method. Therefore, freeze-drying cannot be regarded as an alternative to Holder pasteurization for the preservation of human milk in milk banks. However, intensive dehydration decreases the rate of enzymatic transformation and slows down microbial growth in stored freeze-dried products. Human milk should be used to fed the children of other women only if it meets stringent microbiological

requirements. The above also applies to human milk fortifiers. Freeze-dried human milk as an alternative or fortifier should only be used if it meets stringent microbiological requirements.

To this day, freeze drying is an expensive process, especially when conducted on a small scale, as in freeze-dried human milk. But should be noted that the welfare and healthy development of newborns, in particular preterm infants and low-birth-weight infants, should be a priority regardless of cost.

4. Conclusions

The results of this study indicate that freeze-drying does not induce significant negative changes in the bioactive quality of human milk, and that the composition of freeze-dried human milk remains stable during storage, even at room temperature.

Freeze-drying of human milk has no effect on SOD activity or LF levels in human milk. More importantly, it has been found that the storage of freeze-dried human milk, even at room temperature, does not cause significant changes in LZ activity, TAC or FA and LF content. Additionally, the decrease in SOD activity noted in freeze-dried milk stored at 25°C is significantly lower than the decrease in SOD activity during freezer storage of liquid human milk, as reported in the literature.

Taking into account the results presented here and the remaining state of knowledge, freeze-drying seems to be a promising method of human milk storage, which may improve the processes of HMB. Moreover, freeze-dried human milk shows significant potential as a multi-nutrient fortifier in the nutrition of preterm neonates.

Acknowledgements

The authors would like to thank the mothers for their time and effort in providing milk samples.

Declarations of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding details

The project was funded by a grant from the National Science Centre, allocated pursuant to decision No. DEC-2013/09 / B / NZ9 / 01779.

Ethical statements

The authors declare that they do not have any conflict of interest. Written informed consent was obtained from all study participants. All experimental procedures have been approved by the

Local Ethics Committee of the Medical University of Gdansk. The subjects gave their informed consent before the start of any procedure.

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