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THE EFFECT OF LYOPHILIZATION ON SELECTED BIOLOGICALLY ACTIVE COMPONENTS (VITAMIN C, CATALASE, LYSOZYME), TOTAL ANTIOXIDANT CAPACITY AND LIPID OXIDATION IN HUMAN MILK

S u m m a r y

Human milk is rich in polyunsaturated fatty acids, as well as lysozyme, vitamin C and other bioactive compounds. The effect of lyophilization on the content of antioxidants (vitamin C and catalase CAT), bactericidal compounds (lysozyme), total antioxidant capacity (TAC) and lipid peroxidation in human milk was investigated in this study. Samples of mature human milk were collected from five healthy women who gave birth on the scheduled date and without complications. Freeze drying resulted in the removal of 88.2 % of the initial water content from milk. The human milk lyophilizate was readily soluble in water. Lyophilization had no effect on the content of primary (lipid peroxides LP) and secondary (thiobarbituric acid reactive substances TBARS) products of lipid oxidation. Freeze-drying led to a significant decrease in the vitamin C content and TAC values of milk (by 31 % and 16.5 %, respectively). Catalase and lysozyme were resistant to freeze-drying. Lyophilization induced a decrease in lysozyme content (9 %) and catalase activity (11 %) but these changes were not statistically significant. Low-temperature dehydration and rehydration of human milk lyophilizates promote satisfactory retention of biologically active ingredients and prevent the oxidation of human milk lipids. The results of this study indicate that lyophilization can be considered as an effective method for prolonging the shelf life of human milk.

Słowa kluczowe: human milk, lyophilization, vitamin C, catalase, lysozyme, lipid oxidation

Introduction

Human milk contains all the nutrients that are required for the healthy growth and development of infants and small children. It is also an abundant source of biologically

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active ingredients, including antimicrobial and antioxidant compounds [13]. Human milk banks (HMB) offer a solution to infants who cannot be fed mother's own milk. Infants that were born prematurely or with health conditions are the main beneficiaries of HMB. Human milk is characterized by unique composition and highly available nutrients, therefore, it can also be used to treat various diseases in adults. Human milk has been found to deliver positive effects in patients recovering from acute malnutrition, liver transplants [9] and oncological therapy [12].

Human milk can be effectively stored by freezing (-20 ºC). The concentrations and activity of bioactive milk compounds decrease during freeze storage subject to the applied temperature [4]. For this reason, scientists are searching for new methods that would effectively prolong the shelf life of human milk and minimize the degradation of its biologically active components.

Lyophilization is an alternative method for preserving human milk. Water is removed from milk at low temperatures to protect thermally sensitive components against degradation. Lower water activity in milk prevents the growth of microflora and slows down adverse enzymatic processes [6]. Recent research focuses on the effects of lyophilization on the concentrations of lipids, proteins, lactose [11], fatty acids [3] and immunoglobulins [2] in human milk.

Materials and methods

Materials

Mature milk was collected from five healthy mothers who gave birth on the scheduled date and without complications at the Department of Obstetrics of the Clinical Hospital in Gdańsk. All newborns were in good health (Apgar score of 9 - 10) with normal birth weight (3100 ÷ 3800 g). The mothers expressed breast milk at home under hygienic conditions. Milk samples were pooled, divided into 50 ml samples and frozen at -80 ºC.

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

Lyophilization

Frozen milk samples were lyophilized in the Alpha 2-4 LD Plus freeze drier (Martin Christ, Germany) under the following process parameters: pressure of 0.94 mBar, lyophilization temperature of -20 ºC and condenser temperature of -80 ºC. The lyophilization process was continued until the achievement of constant weight, but not longer than 48 h. The freeze drying process was carried out in triplicate.

Determination of the moisture content of lyophilized milk

The moisture content of lyophilized milk was determined with the Radwag Max 50 laboratory scale (Poland). The product was dried at a temperature of 102 ºC until the achievement of constant weight.

Dilution of human milk lyophilizates

Milk lyophilizates were diluted in ultra-pure distilled water with a temperature of 37 ± 1 ºC. Lyophilized samples were diluted by adding sufficient amounts of water to achieve the initial volume before freeze-drying.

Determination of total antioxidant capacity

The total antioxidant capacity (TAC) of milk was determined in the ABTS assay [8]. TAC values were expressed as Trolox equivalent antioxidant capacity (TEAC) from a calibration curve of Trolox concentrations in standard solutions vs. their absorbance.

Determination of vitamin C content

The total vitamin C content of lyophilized milk was determined by reversed-phase high-performance liquid chromatography with UV detection (RP-HPLC/UV) according to the method proposed by Romeu-Nadal et al. [10]. Dehydroascorbic acid (DHsA) was converted to ascorbic acid (AsA) in the presence of DL-Dithiothreitol (DTT) as the reducing agent. The AsA was stabilized with 0.56 % meta-phosphoric acid solution.

Determination of catalase activity

Catalase (CAT) activity was determined with the use of the commercial spectrophotometric Catalase Assay Kit (Cayman Chemicals, Ellsworth Rd, USA).

Determination of lysozyme concentration

Lysozyme concentration was determined in the ELISA assay with the use of the Lysozyme ELISA Kit (Immundiagnostik AG, Bensheim, Germany).

Lipid peroxidation assay

Lipid peroxidation in milk was determined by the method described by Turoli et al. [14]. The content of lipid peroxides (LP) [μM] was calculated based on the value of ε for J^{3-} which is set at 2.19×10^4 /M/cm at 353 nm [1].

Determination of the content of thiobarbituric acid reactive substances

The content of thiobarbituric acid reactive substances (TBARS), the indicators of polyunsaturated fatty acid (PUFA) oxidation, was determined by the method proposed

by Turoli et al. [14]. TBARS were expressed as malondialdehyde (MDA) based on the calibration curve of MDA concentrations in standard solutions vs. their absorbance.

Statistical analysis

The results were processed statistically in the Statistica 12.0 program. The significance of differences between analyte concentrations in untreated human milk and in diluted lyophilizate was evaluated by one-way ANOVA and Tukey's post-hoc test at a significance level of $p \leq 0.05$.

Results and discussion

Lyophilization led to effective dehydration of human milk, and 88.2 % of initial water content was sublimated. The moisture content of freeze-dried milk was determined at 2.3 ± 0.19 %. The initial content of solids in the analyzed milk was estimated at 9.5 ± 0.26 %. The obtained lyophilizate was easily rehydrated.

Table 1. Concentrations of selected compounds in raw (control sample) and lyophilized (rehydrated) human milk

Tabela 1. Stężenie wybranych składników w surowym (próba kontrolna) oraz liofilizowanym (roztworzonym) mleku ludzkim

Compound Składnik	Human milk / Mleko ludzkie	
	Raw Surowe ($\bar{x}$ ± SD)	Lyophilized Liofilizowane ($\bar{x}$ ± SD)
TAC [mg TE/100 ml]	$31.4^{a} \pm 2.70$	$26.2^{b} \pm 2.19$
Vitamin C [mg AsA/L]	$39.4^{a} \pm 1.83$	$27.0^{b} \pm 2.27$
CAT [nmol/min/ml]	$46.0^{a} \pm 5.76$	$40.8^{a} \pm 6.31$
Lysozyme [µg/ml]	$87.7^{a} \pm 11.83$	$79.7^{a} \pm 16.68$
LP [µM/L]	$7.1^{a} \pm 0.88$	$6.9^{a} \pm 0.96$
TBARS [µg MDA/100 ml]	$58.0^{a} \pm 5.76$	$57.9^{a} \pm 5.81$

$\bar{x}$ ± SD – mean value ± standard deviation / wartość średnia ± odchylenie standardowe;
a, b – mean values followed by different letters within rows are significantly different ($p \leq 0.05$) / średnie wartości oznaczone w tym samym wierszu różnymi literami różnią się statystycznie istotnie ($p \leq 0.05$)

The results of analyses were used to determine the effect of lyophilization on the concentrations and activity of bioactive milk components (tab. 1). Lysozyme was most resistant to lyophilization, and its content in freeze-dried milk was reduced by 9.1 ± 1.37 %. Vitamin C was most sensitive and lyophilization decreased the vitamin C content of milk by 31.5 ± 7.85 %. The TAC of diluted milk lyophilizate was lowered by 16.6 ± 2.05 %. Catalase is relatively resistant to freeze-drying, and its activity in diluted lyophilizate decreased by 11.3 ± 5.22 % relative to the initial value. Lyophilization

led to a minor (non-significant) decrease (2.82 %) in the content of primary lipid peroxidation (LP) products. Freeze-drying did not affect the content of secondary lipid oxidation products (TBARS) in the analyzed milk.

Dehydration prolongs the shelf life of food products by significantly inhibiting microbial growth and slowing down enzymatic processes. In this study, freeze-drying effectively removed water from human milk and produced lyophilizates with 2.3 % moisture content. The initial content of solids in the analyzed milk was estimated at 9.5 %. The obtained lyophilizate was easily rehydrated.

Lyophilization is not a method of pasteurization, but it decreases the counts of vegetative bacterial cells. Salcedo et al. [11] demonstrated that lyophilization significantly reduced the counts of *mesophilic aerobes*, *Staphylococcus aureus* and *Enterococci spp.* bacteria in human milk. Their study also revealed that freeze-drying does not affect the bactericidal properties of human milk, which indicates that lyophilized milk is characterized by a high content of bactericidal compounds. Similar observations were made in our study which demonstrated that lyophilization did not exert a significant influence on the lysozyme content of human milk. Vincenzetti et al. [15] evaluated the influence of low-temperature dehydration on the content and activity of lysozyme in donkey's milk. In the cited study, lysozyme content decreased by 15.5 %, but its antimicrobial properties were not affected. However, it should be noted that lysozyme content and activity in donkey's milk are significantly lower than in human milk.

Lozano et al. [7] noted stable levels of vitamin E, α-, γ- and δ-tocopherol and TAC (determined with ferrous iron Fe^{2+}) in human milk that was lyophilized and stored at 4 ºC for 90 days. Freeze-drying significantly reduced the concentrations of vitamin C (by around 11 %) and ascorbic acid (by around 15 %) in milk. However, in the cited study, the results reported after lyophilization were not compared with the initial values in raw milk. The influence of lyophilization on the vitamin C content of human milk has not been analyzed to date, but the above parameter has been studied in donkey's milk. Donkey's milk and human milk are characterized by similar levels of vitamin C (around 57 mg/l), and the vitamin C content of donkey's milk was reduced by around 10.5 % after freeze-drying [15]. In our study, lyophilization was responsible for a 30 % drop in vitamin C concentration.

Catalase is also an important milk component with antioxidant properties. In our study, this enzyme was relatively resistant to freeze-drying, and its initial activity was preserved in 89 % in lyophilized milk. Vitamin C and antioxidant enzymes, including catalase, are only two of the numerous antioxidant compounds present in human milk. Their overall concentrations and antioxidant activity are described by TAC values. In the present study, the TAC of lyophilized milk, determined in the ABTS assay, was equivalent to nearly 84 % of its initial value.

In a study by Cortez and Soria [5], lyophilization did not influence protein, glucose, triglyceride or polyphenol concentrations in human milk. Lyophilization does not affect the fatty acid composition of human milk [3], but it significantly decreases the diameter of milk fat globules. Human milk fat consists of globules (which form emulsion droplets) with hydrophobic triacylglycerols and cholesterol esters in the interior and an amphipathic exterior layer composed of phospholipids, proteins, cholesterol and enzymes. Human milk fat globules have a diameter of approximately 1 μm, but globules with a diameter of 4 μm are also encountered. The diameter of milk fat globules can be reduced to increase their specific surface area and susceptibility to oxidation. Lyophilization takes place at sub-zero temperatures with no air access, which stabilizes nutrients and bioactive compounds in dehydrated products. Freeze-drying does not increase the concentrations of markers of undesirable biochemical processes, such as nitrites, superoxide anions, hydrogen peroxides, lipid peroxides and γ-glutamyl transpeptidase [5]. In our study, lyophilization did not contribute to the oxidation of human milk lipids. An increase in the content of primary (LP) and secondary (TBARS) products of lipid oxidation was not observed in freeze-dried milk.

Conclusions

Low-temperature dehydration and rehydration of lyophilizates promote satisfactory retention of bioactive milk components, and do not lead to the oxidation of human milk lipids. According to other studies, the stability of milk lyophilizates, including human milk lyophilizates, is preserved during storage at a temperature of 4 ºC. Lyophilization also reduces the weight and volume of milk samples, which significantly facilitates their transport and storage. The results of this study indicate that freeze-drying may be considered as an effective alternative method for prolonging the shelf life of human milk.

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WPŁYW LIOFILIZACJI MLEKA LUDZKIEGO NA WYBRANE BIOLOGICZNIE AKTYWNE SKŁADNIKI (WITAMINA C, KATALAZA, LIZOZYM), OGÓLNĄ ZDOLNOŚĆ PRZECIWUTLENIAJĄCĄ ORAZ UTLENIANIE LIPIDÓW

S t r e s z c z e n i e

Mleko ludzkie zawiera znaczące ilości wielonienasyconych kwasów tłuszczowych, lizozymu, witaminy C oraz innych bioaktywnych składników. W niniejszej pracy zbadano wpływ procesu liofilizacji na zawartość przeciwutleniaczy (witamina C i katalaza CAT), związków bakteriobójczych (lizozym), całkowitą zdolności przeciwutleniającą (TAC) oraz na utlenianie lipidów mleka ludzkiego. Próbki do badań stanowiło dojrzałe mleko ludzkie uzyskane od pięciu zdrowych kobiet, które urodziły o czasie i bez komplikacji. Suszenia sublimacyjne spowodowało usuniecie z mleka 88,2 % wody. Uzyskany liofilizat mleka ludzkiego łatwo rozpuszczał się wodzie. Liofilizacja nie miała wpływu na zawartość pierwszorzędowych (nadtlenki lipidów LP) i drugorzędowych (substancje reagujące w kwasem tiobarbiturowym) produktów utleniania lipidów. Liofilizacja powodowała natomiast znaczący spadek zawartości witaminy C oraz i TAC mleka (odpowiednio o 31 % i 16,5 %). Katalaza i lizozym okazały się enzymami odpornymi na suszenie sublimacyjne. Liofilizacja wywołała obniżenie zawartości lizozymu (o ok. 9 %) i aktywności katalazy (o ok. 11 %), zmiany te nie były jednak statystycznie istotne. Odwodnienie prowadzone w niskich temperaturach oraz rehydratacja liofilizatów mleka ludzkiego pozwala na zadowalającą retencję biologicznie aktywnych składników i zapobiegają utlenianiu lipidów mleka ludzkiego. Wyniki uzyskane

w niniejszej pracy wskazują, że liofilizacja może być brana pod uwagę jako metoda wydłużająca okres przydatności do spożycia mleka kobiecego.

Key words: mleko ludzkie, liofilizacja, witamina C, katalaza, lizozym, utlenianie lipidów