Chapter 43

The liophilization process in raw human milk and after pasteurization for glutamine analysis

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ABSTRACT

Objective: to evaluate the concentration of the amino acid glutamine in breast milk, in the mature lactation stage and after its pasteurization and lyophilization process. Methods: Twenty-six postpartum women were initially recruited, of which 18 were eligible, 8 were excluded, and 10 postpartum women participated. Each sample of 100ml of milk in the mature stage (15th day of puerperium) was collected individually at each address. The samples were divided into two fractions of 50 ml each, one fraction intended for measurement of glutamine in the stage of lyophilized mature breast milk (LHML) and the other fraction for measurement in lyophilized and pasteurized mature breast milk (LHMPL). Results: the average age of the mothers was 22 to 34 years old, the BMI of 26.79 Kg/m2, the gestational age of 38.95 weeks, normal delivery occurred in 60% of the patients the profile of the newborns 70% female and 30% male with an average weight of 3.29 kg and height of 51.85 cm. The concentration of glutamine in the LHML group was 1.80 µMOL + 0.33 and in the LHMPL group it was 1.93 µMOL + 0.44 ($p > 0.05$). Conclusion: pasteurization and lyophilization processes will not change the concentration of glutamine in breast milk.

1 INTRODUCTION

Breast milk (LM) is a complex biological fluid sufficient to supply all the nutritional needs of the newborn (NB) during the first six months of life. Although maternal feeding varies enormously, THE, surprisingly, presents a similar composition for all women in the world who breastfeed, being the exception only for mothers with severe malnutrition. The balanced nutritional composition consists of proteins, lipids, carbohydrates, vitamins and minerals [1] .

From birth to discontinue breastfeeding, the mother produces the necessary milk and physiologically adapted for each stage of development, being divided into three main phases: colostrum, transition milk and finally a production with volume in average of 700 to 900 ml/day from the 15th day of mature milk [2].

According to the authors Valentine CJ et al [4], the current recommendations emphasize that the preterm newborn should receive breast milk or breast milk given to the Human Milk Bank, in addition to commercial fortifying to improve milk composition, since human milk is the only nutritional source of lactating.

For authors Snoj Tratnik J et al [5], breast milk provides ideal nutrients and protective factors that improve the immune and gastrointestinal systems, as well as support the long-term neurodevelopment of premature babies.

For a response to the infectious process, a modulation of the immune response is fundamental, an action that involves numerous organs and components, especially immunonutrients and glutamine (GLN) [7].

GLN represents the main amino acid transferred to the fetus by transplacental means between mother, placenta and fetus, and constitute one of the most abundant amino acids in breast milk. However, babies born prematurely suffer an abrupt interruption of the placental supply of glutamine, which results in exclusive dependence on nutritional therapy, and the concentration of free glutamine is naturally found in the composition in breast milk [8].

The pasteurization process is capable of inactivating up to 100% of pathogenic microorganisms, 99.9% of the saprophyte microbiota of breast milk are totally destroyed by heat, which is why pasteurized breast milk is safe for introduction to newborns in the ICU [9].

According to Savino F et al [10], products that undergo a freeze-drying process are characterized by low weight, due to water withdrawal, are preserved even at room temperature and when reconstituted recover their original properties.

A recent study conducted by Néia V. et al [11], from the State University of Maringá (EMU), analyzed samples of raw human milk from donors from the Human Milk Bank of the University Hospital of Maringá. The aim of this study was to obtain human milk powder by different technologies, evaluate nutritional and immunological components after processing and evaluate the viability of the use of human milk powder in the Network of Human Milk Banks. The study was carried out with samples of raw human milk (colostrum, transition and mature) from donors from the Human Milk Bank of the University Hospital of Maringá. The samples were subdivided into twelve groups being classified after processing as: raw colostrum, pasteurized colostrum, lyophilized colostrum, *spray-drying colostrum, raw transition*, pasteurized transition, lyophilized transition, *spray drying transition*, *raw mature*, pasteurized mature, lyophilized mature and *mature spray-drying* . The samples were submitted to fatty acid composition analysis by Gas Chromatography with Flame Ionization Detector (CG-DIC), triacylglycerol profile by Mass Spectrometry with Direct Infusion Electrospray Ionization (ESI-MS) and serum concentrations of cytokines by Flow Cytometry.

In this other study Oliveira MM et al [12], analyzed from the direct lyophilization of breast milk and concentrated breast milk (LHM) demonstrating microbiological safety, acceptable osmolarity and adequate nutritional composition of the main macro and micronutrients, in addition to the significant increase only in the essential elements Manganese and Selenium. The content of essential micronutrients in the LHM

concentrate was similar or higher than that of preterm breast milk, suggesting that it is feasible for nutritional support of premature babies.

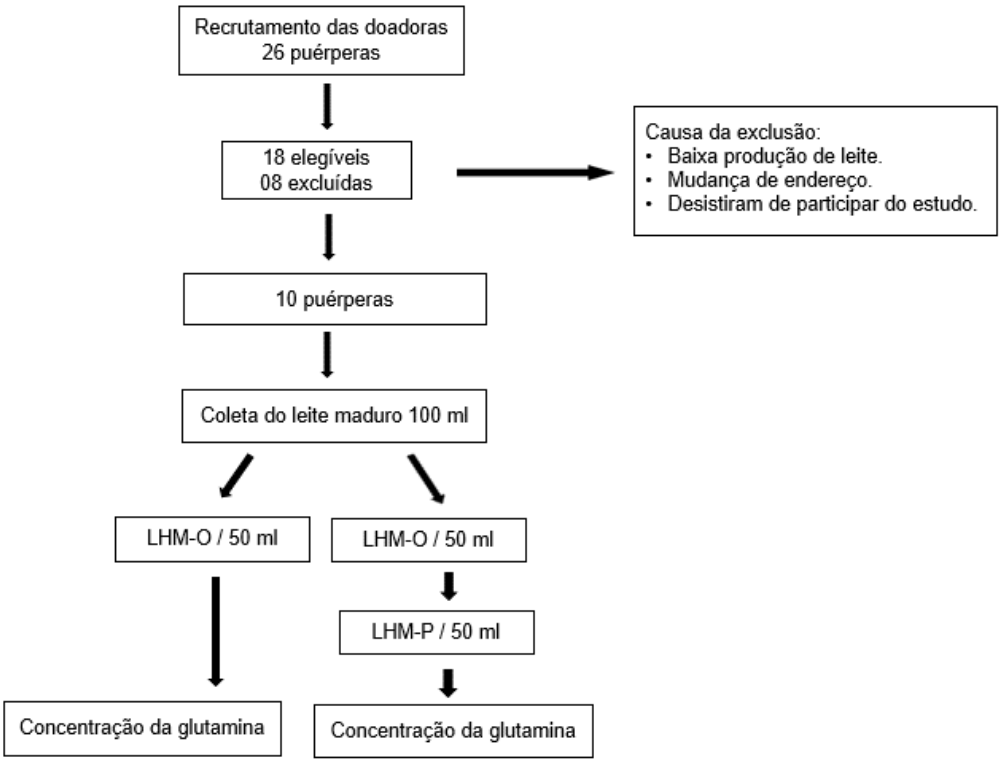
Although there are studies on the amount of glutamine in the milk of mothers of full-term newborns, no studies evaluating the concentrations of this amino acid in pasteurized and freeze-dried human milk have been found in the literature.

In this context, our study should evaluate the concentration of glutamine in lyophilized mature human milk and raw mature human milk and after the pasteurization and freeze-dried process.

2 METHODS

The study is in accordance with the ethical principles approved by the research ethics committee of UFMT, registered under protocol number 23108.049789/14-9 and submitted to the Research Ethics Committee Plataforma Brasil, opinion no. 1,083,955. Data collection was performed, a sample of the material under study in the Gynecology and Obstetrics services and in the Human Milk Bank of the Júlio Muller University Hospital, evaluating the breast milk of mothers exclusively breastfeeding to newborns. As shown in Figure 1, 26 puerpered women were recruited, 18 of whom were elected. However, several factors have occurred, dropouts or exclusions from research such as: change of address, unknown telephone number and low milk production. Thus, 10 puerperable women remained in the study; for glutamine analysis, the volume of 50 ml of each sample in each stage was standardized.

Figure 1 presents the first flow of the study for the recruitment of puerperum women, breast milk collection and sample preparation.



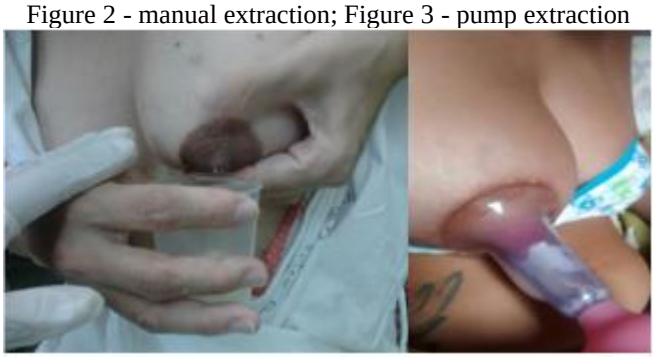
The study was conducted from an instrument, used to recruit donors by a structured questionnaire and adapted from a guideline for the application of the research [13] **(Appendix 1).** Subsequently, the medical records and the pregnant woman's card were analyzed to evaluate the tests performed during prenatal care. Anthropometric data were evaluated such as: pre-gestational weight, gestational weight gain and height; eating habits of food frequency through recall during pregnancy. After hospital discharge, we continued to accompany them in the outpatient clinic and/or residence to milk breast milk from the 15th day of the postpartum period.

The study was conducted in three stages:

1) collection of raw human milk; 2) preparation of samples: a) pasteurization and b) lyophilization; 3) glutamine analysis.

Respecting a standardized [technique 9], a second sample with 50 ml per donor was collected by manual and pump extraction, a first sample with 50 ml each per donor, and from the 15th day of the puerperium a second sample with 50 ml per donor for the pasteurization process was performed according to the standardization protocol and recommended by the Brazilian HBL Network. (available at: http://www.redeblh.fiocruz.br).

Figure 2 - manual extraction; Figure 3 - pump extraction



a) Pasteurization process

In accordance with the standardized techniques, two samples were collected with 100ml of the total of each donor, the first on the fifteenth day and the second from the 15th day. The samples were transported in a thermal box with the temperature in constant measurement with digital thermometer, maintaining the temperature between +2ºC and +8ºC. From each collection per donor a sample with 50 ml was pasteurized (LHP) according to the protocol standardized by the Milk Bank and recommended by the Brazilian HBL Network (available at: http://www.redeblh.fiocruz.br).

a.1) Freeze-dried process

The lyophilization process to which the samples were submitted, an LHC sample and another LHP sample, occurred in a continuous process lasting 72 hours, using the equipment "LS 3000 lyophilizer" from

the manufacturer Terroni Ltda, according to the photo, in Figure 2, of the equipment used. The result was the solid sample with reduced granulometry.

Photo 4: Process of freeze-dried raw mature human milk

b) Glutamine Analysis

b.1) Preparation of samples

As a first step, each of the lyophilized samples (LHC and LHP) were weighed, from each one 2g were taken and **Figure 5** was identified; for the removal of all milk fat, the washing process with petroleum ether **Figure 6** and filtering on filter paper **Figure 7 was applied** , for higher quality of washing, the samples were submitted to two washes, the first in 10ml of petroleum ether and the second in 5ml; after the filtration process, the samples were rested for 30 minutes for complete solvent volatization, after the samples were stored in tubes (eppendorf's**) Figure 8**. The entire process of handling the samples was carried out in an exhaust chapel, also using sterile equipment, following the biosafety standards provided for the procedure.

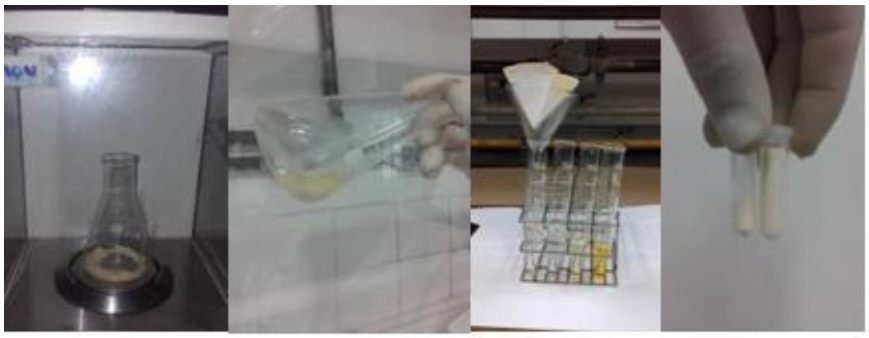


Figure 5 **Figure 6** **Figure 7** **Figure 8**

b.2) Glutamine Analysis

For amino acid analysis (AA) the sigma-aldrich manufacturer's kit was used to determine glutamine/glutamate, it was maintained at storage temperature 2-8°c, according to the manufacturer's guidance. Each of the samples was reconstituted in **water Figure 8** and divided into three equal parts (wells) into two reactions A and B, **Figure 9** according to the protocol. The measurement occurred through l-glutamine spectrophotometry and/or L-glutamate by enzymatic determination of L-glutamine and l-glutamate dehydrogenation with nad conversion to NADH **Figure 10.** Absorption was read at 340 nm after 40 minutes and/or until absorbance remains constant. Subtracting bottom absorbance from this reading to liquid absorbance. Glutamine Standard Curve: L-glutamine patterns using mmoles vs. absorbance.

Figure 8; Figure 9

Figure 10

3 FINDINGS

Table 1 : Demographic data and type of pair and gestational age of postpartum women.

PUÉRPERA	AGE (years)	WEIGHT (Kg)	HEIGHT (m)	IG (weeks)	BMI (kg/m^2)	T P
P1	22	75	1,62	41,0	28,57	N
P2	20	57	1,57	39,0	23,12	N
P3	21	59	1,59	40,0	23,33	N
P4	22	72	1,63	37,0	27,09	N
P5	34	68	1,56	39,0	27,94	C
P6	24	67	1,57	39,5	27,18	N
P7	26	60	1,53	38,0	25,63	C

P8	18	64	1,66	37,0	23,22	C
P9	33	74	1,62	40,0	28,19	C
P10	34	69	1,71	39,0	23,59	N
Average EPM	25,40 + 1,93	66,50 + 2,01	1,60 + 0,01	38,95+ 0,41	25,79 + 0,71	N=60% C= 40%

GA: Gestational age; BMI: Body mass index; TP: Type of delivery; C: Cesarean section; N= Normal; Kg: Kg; m: meters; EPM: Standard error of the mean.

The mean age was 22 to 34 years, BMI 26.79 kg/^{m2}, gestational age was 39.95 weeks, and compared to type of normal delivery and cesarean section, data were 60% and 40% respectively **(table 1).**

Table 2: Profile of newborns in the study.

VARIABLE	AVERAGE + EPM
Weight (Kg)	3,29 + 0,08
Height (cm)	51,85 + 1,09

Kg: Kg; cm: centimeter; m: meter; EPM: Standard error of the mean.

In relation to newborns, 70% were female and 30% male, with an average weight of 3.29kg and height of 51.85 centimeters **(table 2).**

The glutamine concentration in the LHMCL and LHMPL groups was similar, as represented in the data **in (Table 3).**

Groups	Average + EPM
Group - LHMCL	1.80 ± 0.33
Group - LHMPL	1.93 ± 0.44

Student t-test for independent samples with significance level of α = 5%. Equal letters in columns mean no statistical difference (p = 0.825).
LHMCL: freeze-dried raw mature human milk; LHMPL: pasteurized and freeze-dried mature human milk; EPM: Standard error of the mean. **(table 3).**

4 DISCUSSION

The present study was carried out with data from **the type of delivery, gestational age of the postpartum women and profile of the newborns,** we analyzed the concentration of the amino acid in human milk from the lyophilized obtained from raw mature human milk and after pasteurization, presenting data on the concentration of the amino acid glutamine present in the groups cruphilized human milk and pasteurized and lyophilized human milk.

In a neonatal ICU environment, where newborns with some clinical complications lacked nutritional therapy for the essential components naturally present in breast milk that strengthen the immune system [4].

One possibility of lyophilized human milk after the pasteurization process would be to prolong the shelf life of human milk, since human milk has a shelf life of up to six months when frozen, from the pasteurization process following the Human Milk Bank Network (RBLH) [protocol 9].

In the study Néia V. et al [11] researched human milk powder obtained by different technologies (lyophilization and spry drying), demonstrated that fatty acids, triacylglycerols and cytokines were conserved after processing and present viability of use in RBLH, besides the possibility of extending the shelf life of human milk with the use of this process.

According to Pereira [14], the criticalpatient needs specific nutrients, the glutamine present in human milk, due to the numerous actions he/she performs, is considered an immunopharmaceutical nutrient and is now classified as an essential amino acid in this clinical condition. Therefore, its replacement must be guaranteed, and in this way it is essential to concentrate in pasteurized milk for the evolution of the newborn. In Brazil, the process of pasteurization of breast milk is a safe alternative for the conservation of human milk.

The supply of pasteurized human milk or the mother itself is indispensable for these premature infants due to its benefits due to the fragility of this population. For Nyqvist KH[15] early use of human milk has important positive effects for the development of premature infants and contributes to the reduction of hospitalization time.

In 2000 Agostoni C et al [8], they conducted a study analyzing breast milk in several phases, except in pasteurized breast milk. We evaluated 16 puerperal women with full-term newborns on exclusive breastfeeding, and analysis of amino acids in the three phases of breast milk: colostrum, mature milk with thirty days and mature milk at three months. Amino acids were determined using high-efficiency liquid chromatography (HPLC). Concluding that there was a significant increase in glutamine concentration, according to the maturation of breast milk than the AA content of human milk, showed that glutamine and glutamic acid increased in the first 4 to 6 weeks of life, and glutamine increased approximately 2.5 and 20 times, respectively, with lactation progression representing more than 50% of total AA at 3 months.

Neu J et al [16] found that glutamine has an immunomodulatory effect that protects babies from infections.

For Anni Larnkjær et al [17], glutamine is rich in breast milk, but varies considerably among mothers. According to Moura EC and Carvalho RN [18], the chemical variation of human milk changes over time, in order to adapt to the physiological characteristics and nutritional needs of the baby.

Martins EC et al [19], reported that the method developed to freeze human milk aims to preserve its characteristics, even at room temperature, being stored adequately so as not to undergo changes in volume, texture, color, flavor, aroma, vitamin content, mineral salts, proteins, lipid content main caloric-energy source, among other parameters, allowing shelf life to be longer than 1 year and, resist intact for many years. Like Nascimento [20], it demonstrated the efficacy of the freeze-dried process in the conservation of nutritional aspects of human milk, colostrum and pasteurized human milk samples.

In this recent study, the authors Bomfim VS et al [21] analyzed the total lipid content and lipid profile of Human Milk at Baseline (HMB) and Concentrates with Human Milk + lyophilized (with milk freeze in

the immediate period (HMCI), at 3 months (HMC3m) and at 6 months (HMC6m) of storage). Concluding that lyophilized human milk fortified as a human milk concentrate brings potential benefits to the newborn, mainly by conserving the essential nutrients present only in breast milk.

In another Tavares [22] study, we analyzed 30 preterm newborns, with gestational age less than 34 weeks, body mass at birth equal to or less than 1500 grams, in order to determine the amino acids in a modified diet in human milk in three groups (G): G1= human milk from the pasteurized bank with 5% of the FM85 additive; G2= human milk from pasteurized bank with evaporated human milk additive and G3= human milk from pasteurized milk bank with lyophilized human milk additive. By dosing the blood glutamine of the newborns in the different groups, it demonstrated that it increases according to the group (G): (G1<G2<G3), concluding that there is preservation of glutamine concentration after the specific evaporation and lyophilization processes.

In this current study Jarzynka S et al [23] evaluated the efficacy and safety of the new combination of methods selected for the preservation of human donor milk compared to heat treatment, this combination of the two techniques, high pressure processing and lyophilization, presented the best potential for preserving the nutritional value of human milk and was evaluated for microbiological safety.

Based on these reports, our study chose to freeze the collected human milk, and the freeze-dried process allows the maintenance of nutrients in the samples for a long period of time. Ddemonstrated that the concentration of glutamine is maintained even after pasteurization and freeze-dried processes, strengthening the importance of using breast milk for critical and premature newborns in intensive care units ICU. However, we understand that the mastery of the freeze-dried process allows an authentic, faithful conservation of human milk and will allow the dosage of several important nutrients of its composition for replacement in a critical clinical situation for newborns.

The lyophilization process, whether of mature human milk or concentrated human milk, as demonstrated in the experiment, does not interfere with the nutrients present, allowing an authentic and faithful conservation of the LH, allowing it to be reconstituted and served to newborns or even added to the mother's milk complementing the necessary amount when the mother is not able to offer the necessary quantities.

However, more studies are needed to better define the composition of pasteurized and freeze-dried human milk, with regard to nutritional trace elements, such as glutamine, which are fundamental when administering nutritional therapy to critical newborns in ICU, with the possibility of offering pasteurized and lyophilized human milk evaluating the immunomodulatory response.

The limitations of literature presenting methodologies for analysis of human milk, especially in the analysis of glutamine after pasteurization and lyophilization, was one of the complicating factors for our study, another situation is related to the difficulty of obtaining breast milk donors, under the conditions and standardizations that the study requires. After these observations, the results of the analysis provided

reliable information on the finding of glutamine concentration in raw and pasteurized human milk after lyophilization. As an additional result, freeze-dried which is a safe, simple and low-cost process can be used to enable the storage of human milk in milk banks, extending the milk shelf life.

Based on our data, we can conclude that the concentration of glutamine in mature milk did not change after pasteurization and lyophilization processes, keeping the amino acid in a proposed study.

Annex 1 - Questionnaire

Name:_____Leito_____D.N____/_____

Address:_____

City:_____Estado:_____Telefone:_____

Age:_____ Under 15 years ( ) Over 35 years ( )

Literate: Yes ( ) No ( )

Schooling: None ( ) Fundamental ( ) Middle ( ) Upper ( )

Marital Status: Married ( ) Stable Union ( ) single ( ) other ( )

Personal history: cardiopathic ( ) diabetes hypertension ( ) urinary infections ( ) others ( )

Current pregnancy: previous weight: _____ current weight: _____ height: _____

Started prenatal care in: 1st quarter ( ) 2nd quarter ( ) 3rd quarter ( )

Obstetric data:

Gestation:_____

Type of delivery: normal () cesarean section () forceps ()

Gestational age: _____ female ( ) male ( )

Date of birth: ____/_____

Clinical examinations: complete ( ) incomplete ( ) none ( )

Immunizations: complete scheme ( ) incomplete ( ) none ( )

Diet during pregnancy: normal ( ) altered ( )

Supplementation with ferrous sulfate (SF) and folic acid (PA)

SF () yes () no AF () yes () no

Date of collection of breast milk:

First____/_____/_____

Second_____/_____/_____

Date:_____/_____/_____

Signature:_____

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