BREASTFEEDING MEDICINE
Volume 7, Number 4, 2012

DOI: 10.1089/bfm.2011.0079

Effect of Freezing Time on Macronutrients and Energy Content of Breastmilk

Nadia Raquel García-Lara,[1] Diana Escuder-Vieco,[1,2] Oscar García-Algar,[2,3] Javier De la Cruz,[4] David Lora,[4] and Carmen Pallás-Alonso[1,2]

Abstract

Background: In neonatal units and human milk banks freezing breastmilk at less than –20°C is the choice for preserving it. Scientific evidence in relation to the loss of nutritional quality during freezing is rare. Our main aim in this study is to determine the effect of freezing time up to 3 months on the content of fat, total nitrogen, lactose, and energy. Our secondary aim is to assess whether ultrasonic homogenization of samples enables a more suitable reading of breastmilk macronutrients with a human milk analyzer (HMA) (MIRIS®, Uppsala, Sweden).
Methods: Refrigerated breastmilk samples were collected. Each sample was divided into six pairs of aliquots. One pair was analyzed on day 0, and the remaining pairs were frozen and analyzed, one each at 7, 15, 30, 60, and 90 days later. For each pair, one aliquot was homogenized by stirring, and the other by applying ultrasound. Samples were analyzed with the HMA.
Results: By 3 months from freezing with the two homogenization methods, we observed a relevant and significant decline in the concentration of fat and energy content. The modification of total nitrogen and lactose was not constant and of lower magnitude. The absolute concentration of all macronutrients and calories was greater with ultrasonic homogenization.
Conclusions: After 3 months from freezing at –20°C, an important decrease in fat and caloric content is observed. Correct homogenization is fundamental for correct nutritional analysis.

Background

In neonatal units and human milk banks freezing breastmilk at less than –20°C guarantees its microbiological safety and hinders the growth of microorganisms. However, the enzyme activity inherent to breastmilk may remain at this temperature. Scientific evidence in relation to the loss of nutritional quality during this procedure is rare.

Some studies[1,2] consider that freezing at less than –80°C is the "gold standard" of long-term freezing of breastmilk as this minimizes the loss of its properties. However, this procedure is too expensive, and most neonatal units and human milk banks freeze exclusively at less than –20°C. In international guidelines the maximum period for freezing time at less than –20°C recommended for both breastmilk and donor milk (both pre- and post-pasteurization) is highly variable, ranging between 1 and 12 months.[3–9]

The effect of freezing time at less than –20°C on macronutrients (fat, proteins, and lactose) and energy content has rarely been studied.[2,10–18]

Our main aim in the present study is to determine the effect of freezing time up to 3 months on the content of fat, total nitrogen, lactose, and energy of a sample of raw human milk using a human milk analyzer (HMA) (MIRIS®, Uppsala, Sweden).

Freezing breastmilk gives rise to a series of physical changes in its principal components such as rupture of the fat globule membranes and alteration of casein micelles. As mentioned in other studies, the importance of achieving representative samples of unfrozen human breastmilk is very important for its correct analysis. In previous studies, ultrasonic homogenization has been used to optimize homogenization and avoid loss of fat in infusion systems.[19–21] Our secondary aim is to assess whether ultrasonic homogenization of samples enables a more suitable reading of breastmilk macronutrients with the HMA.

Materials and Methods

Two investigators participated in the collection, preparation, and analysis of the samples. There was no blinding in any of the study phases.

Departments of [1]Neonatology and [4]Epidemiology and Research, Hospital 12 de Octubre, Madrid, Spain.
[2]SAMID Spanish Collaborative Research Network, Spain.
[3]Department of Pediatrics, Hospital del Mar, Barcelona, Spain.

Samples

Breastmilk samples were collected with a volume of ≥ 35 mL extracted manually or with a pump. Immediately following extraction, the samples were stored in a refrigerator at < 5°C for a maximum period of 24 hours prior to their analysis.

All the donors had their child admitted to the Department of Neonatology of the 12 de Octubre Hospital (Madrid, Spain). All the women signed informed consent.

The samples came from just one or various extractions over 24 hours and were stored in a glass flask with a sterile plastic cap provided. No extraction instructions different from those provided in the unit were given.

Data were also collected on the variables maternal age, gestational age of the infant, stage of lactation (time after infant birth when the sample was collected), number of children, and occurrence of prior breastfeeding.

Preparation of the samples and homogenization

For correct homogenization, each sample was initially heated at 40°C in a thermostatic bath. The temperature was verified by means of a data logger thermometer with certified calibration. Subsequently, the sample was stirred by means of rocking the sample in an arc-like fashion 10 times; 12 aliquots of 2.5 mL were prepared and placed in polypropylene test tubes. The 12 aliquots were in turn distributed into six pairs. Each tube was sealed and identified. Aliquots were identified based on days frozen—day 0 (or "raw milk"), 7, 15, 30, 60, and 90—and the kind of homogenization they were going to receive ("manual" or "ultrasonic"). Aliquots other than day 0 (raw) were frozen immediately at less than –20°C.

Each aliquot from the pair was homogenized differently. If it was identified as manual, it was heated at 40°C in a thermostatic bath and subsequently stirred by rocking in an arc-like fashion twice. For the "ultrasound" aliquots an ultrasound processor was applied (model VCX 130PB, Sonics and Materials, Newtown, CT). The instrument has two different settings: PROC1 for manually homogenized samples and PROC2 for ultrasonically homogenized ones.

The parameters used with the ultrasound processor were 75% amplitude and 1.5 seconds/mL. These parameters revealed suitable homogenization of the breastmilk samples (preliminary unpublished data). A Coulter counter (particle size analyzer by laser diffraction technology) (Beckman Coulter, Brea, CA) was used to measure the size of breastmilk fat globules. This was performed in "manually" homogenized samples and ultrasonically homogenized ones using different parameters from the ultrasound processor. We verified that by applying the aforementioned parameters the size of the globules was standardized; > 99% of these were < 1 μm in size.

Analysis of the samples

HMA was used to analyze the samples. The equipment requires 2 mL of breastmilk and provides the reading of fat, total nitrogen, lactose, dry material, and energy contents. We used the setting PROC1 for aliquots identified as "manual" (both raw and thawed ones) and PROC2 for "ultrasonic" ones (both raw and thawed ones).

For the technical data, the instrument provided repeatability values of < 0.05%. In our laboratory we performed a repeatability study in which we obtained similar results ($n = 10$ samples of raw breastmilk and $n = 10$ samples of unfrozen breastmilk; intraclass correlation coefficient for fat and kcal = 0.99).

The HMA uses technology based on the transmission of mid-infrared spectroscopy, designed specifically for the determination of macronutrients in the breastmilk. A very thin layer of breastmilk from the sample (< 100 μm) is exposed to infrared radiation. For the calculation of each macronutrient the instrument uses the amount of radiation absorbed by the different functional groups at specific wavelengths, and it performs an estimate referring to the amount of infrared light absorbed by the distilled water at the same wavelength.

The "fat" value provided by the instrument corresponds to the total lipid-soluble fraction of the sample including human milk triglycerides, diglycerides, free fatty acids, phospholipids, and cholesterol. The "total nitrogen" content includes protein and non-protein nitrogen. This value is multiplied by the conversion factor 6.25. The "lactose" value corresponds to the total constituted by its content and the oligosaccharides. To calculate total energy content we used the formula total energy (in Kcal/dL) = 9.25 × "fat" + 4.40 × "total nitrogen" + 3.95 × "lactose."

The instrument was calibrated by the manufacturer to optimally measure breastmilk with its normal biological variation. The manufacturer used a set of reference breastmilk samples with different fat, protein, and lactose contents that were analyzed with benchmark biochemical methods: the Roese–Gottlieb method (fat), the Kjeldahl method (total nitrogen), and high-performance liquid chromatography (lactose). This was performed separately for human milk that was manually homogenized and human milk that was ultrasonically homogenized. The habitual use of MIRIS CHECK solution provided by the manufacturer for normal use of the instrument avoids the need for recalibration.

Data analysis

Descriptive statistics are presented for the four outcome variables (fat, total nitrogen, lactose, and energy content) and for both homogenization techniques (manual and ultrasound). After normality of distributions was checked, mean and 95% confidence intervals of the mean are presented at each assessment time (raw breastmilk and at 7, 15, 30, 60, and 90 days). Statistical significance of paired comparisons is computed with paired t test.

Mean difference at each assessment time with raw breastmilk (confidence interval) adjusted for homogenization technique and stage of lactation was estimated with linear mixed regression models. Mean difference by day was estimated for the different study periods (1–7, 8–15, 15–30, 30–60, and 60–90 days). The data analysis for this article was generated using SAS software (SAS Institute Inc., Cary, NC).

Results

Description of the sample

Sixty-one breastmilk samples were collected donated by 59 mothers. Mean age of the mothers was 32.55 years (SD 5.27). Sixty-five percent of the mothers were from Spain, 18% were from Central and South America, 10% were from other European countries other from Spain, and 7% were from Africa. Fifty-eight percent of the mothers did not have a previous

child. Mean gestational age of their children was 32.66 weeks (SD 5.82). Forty-six percent ($n = 28$) of the mothers gave birth before week 32 of gestation, 21% ($n = 13$) between week 32 and 36, and 33% ($n = 19$) at week 37 or later.

Regarding the stage of lactation (expressed as days), the percentile distribution shows a minimum value of 5 days and a maximum value of 389 days. The 25th, 50th, and 75th percentile values were 13, 18, and 41 days respectively.

Macronutrient and energy contents considering time of freezing and type of homogenization (Table 1)

Fat. In manually homogenized samples, we observed a significant reduction in concentration relative to raw breastmilk at 7, 15, 30, 60, and 90 days from freezing.

In ultrasonically homogenized samples, we observed a significant reduction in concentration relative to raw breastmilk at 7, 15, 30, 60, and 90 days from freezing.

Total nitrogen. In manually homogenized samples, we only observed a significant reduction relative to the concentration in raw milk at 15, 30, and 90 days from freezing.

In ultrasonically homogenized samples, we did not observe a significant reduction in any time interval relative to the concentration in raw breastmilk.

Lactose. In manually homogenized samples, we only noted a significant reduction relative to the concentration in raw breastmilk at 90 days from freezing.

In ultrasonically homogenized samples, we only observed a significant reduction relative to the concentration of raw breastmilk at 90 days from freezing.

Energy content. In manually homogenized samples, we observed a significant reduction in concentration relative to raw breastmilk at 15, 30, 60, and 90 days from freezing.

In ultrasonically homogenized samples, we observed a significant reduction in concentration relative to raw milk at 7, 15, 30, 60, and 90 days from freezing.

Macronutrient and caloric contents adjusted for freezing time, type of homogenization, and stage of lactation (Table 2)

We considered as the reference group fresh milk samples manually homogenized and with stage of lactation longer than 15 days.

Regarding freezing time, fat content decreases with time. Considering the mean difference by day, the maximum decrease was observed in the period 0–7 days (–0.027 g/dL/day). In following periods mean decrease by day was reduced: For 7–15 and 15–30 days the values were –0.016 and –0.011 g/dL/day, respectively, and for the periods 30–60 and 60–90 days the values were –0.007 and –0.006 g/dL/day, respectively.

For total nitrogen and lactose, small, changeable, and in most cases not significant differences were observed considering both absolute values and also mean difference by day.

Caloric content, as well as the fat content, decreased with freezing time. Considering the mean difference by day, the biggest decrease was observed in the period 0–7 days (–0.22 kcal/dL/day), and afterward the decrease was constant but lower with time: For 7–15 and 15–30 days the values were –0.06 and –0.07 kcal/dL/day, respectively, and for the periods 30–60 and 60–90 days the values were –0.03 and –0.07 kcal/dL/day, respectively.

The samples homogenized with the ultrasound processor revealed higher values for fat, total nitrogen, lactose, and energy contents than those homogenized "manually." The biggest differences are observed for fat and caloric concentration.

Considering the stage of lactation, samples of more than 15 days had a significant higher fat content and significantly lower total nitrogen and lactose values.

In summary, freezing time, homogenization method, and stage of lactation independently modified fat content. For caloric content, freezing time and homogenization method independently modified it.

Discussion

Considering freezing time interval, our analysis revealed that 90 days after freezing at less than –20°C, the fat and energy concentrations underwent a clinically relevant decrease in magnitude. In the bivariate analysis, the differences were statistically significant for all time periods, and we observed a cumulative maximum decrease at 90 days. In the multivariable analysis, a cumulative decrease was also observed with freezing time.

On the other hand, we observed the maximum absolute relative decrease at the 0–7-day period for both fat and caloric contents.

Conversely, total nitrogen and lactose contents revealed a variable, low-magnitude, and not constant reduction with freezing time that consequently was clinically not relevant.

Given the major weight of the fat in the overall energy content of the breastmilk and the very inferior reduction in content of total nitrogen and lactose in our results, we believe that the decline in energy content is mainly determined by that of fat.

This is the first study designed to assess the effect of freezing at –20°C over a period of 90 days on the concentration of macronutrients and energy. There have been very few works published in this regard. Tacken et al.[10] studied 30 samples frozen at –18°C for 28 days and did not find any significant differences in the concentration of fat using a colorimetric method. Friend et al.[15] studied in 20 samples (10 frozen slowly and 10 quickly) with the Roese–Gottlieb method the concentration of fat after 1 week, 1 month, and 3 months from freezing at –20°C; this study found a decrease, although this was not statistically significant. Sipralsert et al.[11] studied with the creamatocrit technique 12 samples stored at –20°C for 28 days and did not observe any statistically significant changes in the fat content; no energy content data were published. The studies mentioned have fewer samples and lesser freezing times, and only the concentration of total triglycerides from the sample was studied.

We used HMA for the analysis. We ruled out laboratory methods ("gold standard" ones) because they are costly and time consuming. The instrument uses the mid-infrared spectrometry technology that has been broadly used before in the dairy industry. Two works recently published compared the results obtained by HMA with those obtained by benchmark laboratory methods for fat, proteins, lactose, and caloric

Table 1. Comparison of Fat, Total Nitrogen, Lactose, and Caloric Concentrations in Human Milk at Different Freezing Time Periods Up to 3 Months and Considering Two Types of Homogenization

Content, type of homogenization	*Raw milk*	*Versus raw milk at freezing time of*				
		7 days	*15 days*	*30 days*	*60 days*	*90 days*
Fat (g/dL)						
Manual	4.88 (4.52, 5.23)	4.69 (4.34, 5.03)	4.54 (4.21, 4.88)	4.54 (4.19, 4.90)	4.37 (4.01, 4.72)	4.19 (3.84, 4.53)
P	—	$p = 0.048$	$p = 0.001$	$p = 0.001$	$p < 0.001$	$p < 0.001$
Ultrasonic	5.23 (4.82, 5.64)	5.04 (4.66, 5.43)	5.08 (4.69, 5.47)	4.96 (4.57, 5.34)	4.87 (4.49, 5.24)	4.76 (4.39, 5.14)
P	—	$p = 0.002$	$p = 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Total nitrogen (g/dL)						
Manual	1.35 (1.28, 1.42)	1.36 (1.29, 1.43)	1.31 (1.24, 1.39)	1.31 (1.23, 1.39)	1.33 (1.26, 1.40)	1.30 (1.23, 1.38)
P	—	$p = 0.438$	$p = 0.012$	$p = 0.009$	$p = 0.132$	$p = 0.006$
Ultrasonic	1.42 (1.34, 1.50)	1.44 (1.36, 1.52)	1.42 (1.35, 1.50)	1.40 (1.33, 1.48)	1.41 (1.34, 1.48)	1.42 (1.35, 1.49)
P	—	$p = 0.180$	$p = 0.885$	$p = 0.333$	$p = 0.404$	$p = 0.892$
Lactose (g/dL)						
Manual	5.94 (5.85, 6.03)	5.99 (5.91, 6.07)	6.03 (5.95, 6.10)	5.96 (5.88, 6.04)	5.98 (5.89, 6.06)	5.86 (5.76, 5.95)
P	—	$p = 0.043$	$p = 0.001$	$p = 0.291$	$p = 0.359$	$p = 0.016$
Ultrasonic	6.09 (6.01, 6.17)	6.10 (6.03, 6.18)	6.11 (6.04, 6.18)	6.07 (5.99, 6.14)	6.06 (5.99, 6.13)	5.98 (5.90, 6.06)
P	—	$p = 0.487$	$p = 0.281$	$p = 0.175$	$p = 0.180$	$p < 0.001$
Caloric (kcal/dL)						
Manual	75.33 (71.96, 78.70)	73.87 (70.60, 77.13)	72.54 (69.37, 75.71)	71.92 (68.56, 75.27)	70.45 (67.08, 73.82)	67.95 (64.56, 71.34)
P	—	$p = 0.101$	$p = 0.002$	$p < 0.001$	$p < 0.001$	$p < 0.001$
Ultrasonic	78.69 (75.07, 82.33)	77.13 (73.72, 80.55)	77.46 (73.99, 80.93)	76.03 (72.52, 79.55)	75.23 (71.84, 78.62)	73.92 (70.55,77.29)
P	—	$p = 0.003$	$p = 0.005$	$p < 0.001$	$p < 0.001$	$p < 0.001$

All values are expressed as mean (confidence interval of the mean) ($n = 61$ samples).
Student's paired t tests are used to compare each freezing time group samples with raw milk group samples.

TABLE 2. MACRONUTRIENT AND CALORIC CONTENTS OF BREASTMILK ADJUSTED FOR FREEZING TIME, TYPE OF HOMOGENIZATION, AND STAGE OF LACTATION

		Difference vs. raw milk for freezing time of					*Difference for ultrasonic vs. manual homogenization*	*Stage of lactation <15 days vs. >15 days*
Content	*Reference group*[a]	*7 days*	*15 days*	*30 days*	*60 days*	*90 days*		
Fat (g/dL)	5.13 (4.68, 5.59)	–0.19 (–0.28, –0.09)	–0.24 (–0.34, –0.15)	–0.31 (–0.41, –0.22)	–0.42 (–0.51, –0.32)	–0.58 (–0.67, –0.48)	0.45 (0.39, 0.50)	–0.72 (–1.40, –0.05)
P	—	$p = 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.0348$
Total nitrogen (g/dL)	1.22 (1.14, 1.30)	0.01 (–0.01, 0.04)	–0.02 (–0.04, 0.01)	–0.03 (–0.05, –0.01)	–0.02 (–0.04, 0.01)	–0.02 (–0.05, 0.01)	0.09 (0.08, 0.1)	0.28 (0.16, 0.40)
P	—	$p = 0.2035$	$p = 0.1202$	$p = 0.0099$	$p = 0.1158$	$p = 0.0391$	$p < 0.001$	$p < 0.001$
Lactose (g/dL)	5.89 (5.8, 5.98)	0.03 (–0.001, 0.07)	0.05 (0.02, 0,09)	0.01 (–0.03, 0.04)	–0.004 (–0.04, 0.03)	–0.09 (–0.12, –0.06)	0.11 (0.09, 0.13)	0.15 (0.01, 0.29)
P	—	$p = 0.0677$	$p = 0.0017$	$p = 0.9355$	$p = 0.8616$	$p < 0.0001$	$p < 0.0001$	$p = 0.0299$
Caloric (kcal/dL)	76.91 (72.66, 81.15)	–1.51 (–2.39, –0.63)	–2.01 (–2.89, –1.12)	–3.12 (–4.01, –2.23)	–3.99 (–4.89, –3.11)	–6.04 (–6.93, –5.15)	4.36 (3.84, 4.88)	–4.87 (–11.18, 1.42)
P	—	$p = 0.0009$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p = 0.1290$

Data are mean (95% confidence interval of the mean) ($n = 61$ samples).
Statistical analysis for p values was by regression mixed-model analysis.
[a]Values of the reference group are estimated for samples of raw milk, manually homogenized and with stage of lactation >15 days.

contents.[22,23] Both concluded that it is a practical instrument for daily measurement within the clinical scope of breastmilk macronutrients.

Two hypotheses could justify the reduction in fat content with freezing in accordance with storage time at less than –20°C. First, some studies mentioned above pointed out that at the temperature of –20°C lipase activity is maintained and that therefore there is active lipolysis, which breaks down the triglycerides, reducing their content and increasing the content of diglycerides, monoglycerides, and free fatty acids.[16] However, if we consider that the two wavelength bands used by the instrument for lipids measure both triglycerides and diglycerides and free fatty acids, this should not mean significant differences in the quantification of fat.

Conversely, the reduction in antioxidant activity of breastmilk with its storage, both refrigerated at less than –5°C and frozen at less than –20°C, has been demonstrated.[2,10,17,18,24–26] Consequently, peroxidation of both free fatty acids and those bound to glycerol (tri- and diglycerides) occurred, leading to a physicochemical modification in these. The fatty acids with their structure modified would not be quantified by HMA. We would need to confirm our hypothesis with studies designed to quantify the peroxidation of fatty acids from breastmilk with different freezing periods at less than –20°C and the correlation of this with its reading in the HMA.

In this context, the significant reduction in fat and caloric content observed with freezing time could be due to unread lipid content by the instrument.

The biggest fat and caloric content decrease was observed at 7 days. This could be related to the freezing and thawing effect. In recently published research, a significant decrease in fat content after thawing both slowly and quickly was observed.[27] Regarding the freezing and thawing effect on human milk properties, an increase in lipolysis has been observed, but the influence on the antioxidant properties has not been studied.[12,14]

One limitation of our study is that our samples had a variable refrigeration time (from 0 to 24 hours). Considering the antioxidant loss properties of human milk during refrigeration,[17,18,24,25] this could be a potential confounder if our previous hypothesis as explained below was correct.

Freezing means a series of physicochemical changes in the breastmilk components. A rupture occurs in the fat globule membrane followed by coalescence (formation of "cream"). For the proteins, phenomena also occur that give rise to precipitates (casein micelles are destabilized, and quaternary structure of whey proteins are altered). To "rehomogenize" the breastmilk, the need both to heat refrigerated breastmilk and milk stored at –20°C to 38–40°C and subsequently to stir with gentle inversion to avoid part of the fat remaining adherent to the surfaces has been reported.[28,29] However, this procedure does not break down protein aggregates and does not completely prevent the formation of cream. Ultrasound samples revealed higher values of macronutrients than those manually homogenized. This could be justified by a more optimal homogenization. Correct preparation of the breastmilk samples is very important to minimize errors in measuring their components.

Finally, in the multivariable model, we observed that samples obtained at more than 15 days of lactation had higher fat content and lower total nitrogen and lactose ones. For

caloric content, a decrease is observed but was nonsignificant. These findings are in agreement with previous studies.[30,31] We did not observe a lower decrease with freezing time in fat content in samples containing colostrum (stage of lactation less than 15 days). Refrigeration and prompt freezing could diminish the antioxidant capacity of colostrum. There are no published studies that investigate the change of antioxidant capacity of colostrum and mature milk separately with refrigeration and freezing time.

Our study increases knowledge on the impact of freezing at –20°C, the form of long-term storage used in virtually all neonatology units and human milk banks, on the nutritional quality of breastmilk. Two important aspects should be to take into account: On the one hand, the importance of freezing and thawing deterioration, and on the other hand, the cumulative deterioration as the storage time increases. The latter aspect has been reported with other beneficial properties of breastmilk such as antioxidant capacity or bactericidal activity.[1,17,32]

It is urgent to establish recommendations for periods of storage at –20°C based on the scientific evidence that reasonably ensures the quality of breastmilk even at the cost of increasing losses because of expiration date. To our knowledge, there are no studies about the effect of ingestion of lipid peroxides in the newborn, but it is supposed that even an exogenous source of reactive oxygen species may be dangerous, especially in critical immature premature babies with reduced antioxidant defense capacity.[33]

These results would not be valid for pasteurized human milk as Holder pasteurization leads to a series of alterations in the activities of enzymes present in the breastmilk.

Acknowledgments

The generosity of the human milk donors made this research possible. We also thank L. Fernández (Nutrition, Bromatology, and Food Technology, Universidad Complutense, Madrid, Spain) and SAMID Spanish Collaborative Research Network. This study was funded by Spanish Health Research Funding (grant FIS 09/00040).

Disclosure Statement

No competing financial interests exist.

References

1. Ogundele MO: Techniques for the storage of human breast milk: Implications for anti-microbial functions and safety of stored milk. *Eur J Pediatr* 2000;159:793–797.
2. Silvestre D, Miranda M, Muriach M, et al. Frozen breast milk at –20 degrees C and –80 degrees C: A longitudinal study of glutathione peroxidase activity and malondialdehyde concentration. *J Hum Lact* 2010;26:35–41.
3. *NICE Clinical Guideline 93. Donor Breast Milk Banks: The Operation of Donor Milk Banks*. National Institute for Health and Clinical Excellence, London, 2010.
4. Hartmann BT, Pang WW, Keil AD, et al. Best practice guidelines for the operation of a donor human milk bank in an Australian NICU. *Early Hum Dev* 2007;83:667–673.
5. *Guidelines for the Establishment and Operation of a Donor Human Milk Bank*. Human Milk Banking Association of North America, Fort Worth, TX, 2009.
6. Academy of Breastfeeding Medicine Protocol Committee. ABM clinical protocol 8: Human milk storage information for home use for full-term infants (original protocol March 2004; revision 1 March 2010). *Breastfeed Med* 2010;5:127–30.
7. Omarsdottir S, Casper C, Akerman A, et al. Breastmilk handling routines for preterm infants in Sweden: A national cross-sectional study. *Breastfeed Med* 2008;3:165–170.
8. Gutiérrez D, de Almeida JA. Human milk banks in Brazil. *J Hum Lact* 1998;14:333–335.
9. Arslanoglu S, Bertino E, Tonetto P, et al. Guidelines for the establishment and operation of a donor human milk bank. *J Matern Fetal Neonatal Med* 2010;23(Suppl 2):1–20.
10. Tacken KJ, Vogelsang A, van Lingen RA, et al. Loss of triglycerides and carotenoids in human milk after processing. *Arch Dis Child Fetal Neonatal Ed* 2009;94:F447–F450.
11. Silprasert A, Dejsarai W, Keawvichit R, et al. Effect of storage on the creamatocrit and total energy content of human milk. *Hum Nutr Clin Nutr* 1987;41:31–36.
12. Berkow SE, Freed LM, Hamosh M, et al. Lipases and lipids in human milk: Effect of freeze-thawing and storage. *Pediatr Res* 1984;18:1257–1262.
13. Lawrence RA. Storage of human milk and the influence of procedures on immunological components of human milk. *Acta Paediatr Suppl* 1999;88(430):14–18.
14. Wardell JM, Hill CM, D'Souza SW. Effect of pasteurization and of freezing and thawing human milk on its triglyceride content. *Acta Paediatr Scand* 1981;70:467–471.
15. Friend BA, Shahani KM, Long CA, et al. The effect of processing and storage on key enzymes, B vitamins, and lipids of mature human milk. I. Evaluation of fresh samples and effects of freezing and frozen storage. *Pediatr Res* 1983;17:61–64.
16. Bitman J, Wood DL, Mehta NR, et al. Lipolysis of triglycerides of human milk during storage at low temperatures: a note of caution. *J Pediatr Gastroenterol Nutr* 1983;2:521–524.
17. Miranda M, Muriach M, Almansa I, et al. Oxidative status of human milk and its variations during cold storage. *Biofactors* 2004;20:129–137.
18. Buss IH, McGill F, Darlow BA, et al. Vitamin C is reduced in human milk after storage. *Acta Paediatr* 2001;90:813–815.
19. Rayol MR, Martinez FE, Jorge SM, et al. Feeding premature infants banked human milk homogenized by ultrasonic treatment. *J Pediatr* 1993;123:985–988.
20. Martinez FE, Desai ID, Davidson AG, et al. Ultrasonic homogenization of expressed human milk to prevent fat loss during tube feeding. *J Pediatr Gastroenterol Nutr* 1987;6:593–597.
21. Martinez FE, Davidson AG, Anderson JD, et al. Effects of ultrasonic homogenization of human milk on lipolysis, IgA, IgG, lactoferrin and bacterial content. *Nutr Res* 1992;12:561–568.
22. Menjo A, Mizuno K, Murase M, et al. Bedside analysis of human milk for adjustable nutrition strategy. *Acta Paediatr* 2009;98:380–384.
23. Casadio YS, Williams TM, Lai CT, et al. Evaluation of a mid-infrared analyzer for the determination of the macronutrient composition of human milk. *J Hum Lact* 2010;26:376–383.
24. Bank MR, Kirksey A, West K, et al. Effect of storage time and temperature on folacin and vitamin C levels in term and preterm human milk. *Am J Clin Nutr* 1985;41:235–242.
25. Hanna N, Ahmed K, Anwar M, et al. Effect of storage on breast milk antioxidant activity. *Arch Dis Child Fetal Neonatal Ed* 2004;89:F518–F520.
26. Van Zoeren-Grobben D, Moison RM, Ester WM, et al. Lipid peroxidation in human milk and infant formula: Effect of

storage, tube feeding and exposure to phototherapy. *Acta Paediatr* 1993;82:645–649.
27. Vieira AA, Soares FV, Pimenta HP, et al. Analysis of the influence of pasteurization, freezing/thawing, and offer processes on human milk's macronutrient concentrations. *Early Hum Dev* 2011;87:577–580.
28. Jensen RG, Clark RM. Methods of lipid analysis. *J Pediatr Gastroenterol Nutr* 1984;3:296–299.
29. Jensen RG, ed. *Handbook of Milk Composition*. Academic Press, San Diego, CA, 1995.
30. Mitoulas LR, Kent JC, Cox DB, et al. Variation in fat, lactose and protein in human milk over 24h and throughout the first year of lactation. *Br J Nutr* 2002;88:29–37.
31. Saarela T, Kokkonen J, Koivisto M. Macronutrient and energy contents of human milk fractions during the first six months of lactation. *Acta Paediatr* 2005;94:1176–1181.
32. Hernandez J, Lemons P, Lemons J, et al. Effect of storage processes on the bacterial growth-inhibiting activity of human breast milk. *Pediatrics* 1979;63:597–601.
33. Turoli D, Testolin G, Zanini R, et al. Determination of oxidative status in breast and formula milk. *Acta Paediatr* 2004; 93:1569–1574.

Address correspondence to:
Nadia Raquel García-Lara, M.D.
Servicio de Neonatología
Hospital 12 de Octubre
Edificio Materno-Infantil
Avenida de Córdoba sin Número
28041 Madrid, Spain

E-mail: nadiaraquelg@yahoo.es