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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

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Summary

A study was initiated to evaluate the effects of several methods of processing and storage on key enzymes, B vitamins, and lipid components of mature human milk. In order to establish standard values for the nutrient components with which to compare processed samples, a total of 30 individual raw samples of mature human milk were analyzed. There was considerable sample to sample variation as indicated by the large range of values for each component. Freezing and frozen storage had little effect on the enzymes of pooled samples of milk. Lactoperoxidase activity decreased from 36 in raw pooled samples to 17 in pooled samples slow frozen and stored for 3 months at −25°C ($P < 0.05$). Similarly, quick freezing and storage for 3 months significantly decreased the lactoperoxidase activity of pooled samples from 93 to 14 ($P < 0.05$). Quick freezing and frozen storage tended to increase lipase activity although the changes were not significant. Freezing and frozen storage did not significantly affect the levels of biotin, niacin, and folic acid. Similarly, the total lipid fatty acid level and relative % of each fatty acid were not significantly different in the frozen samples as compared to the raw samples.

Speculation

Our data suggest that freezing and frozen storage for up to 3 months can be used to preserve mature human milk with minimum loss of its biologic activity. Slow freezing is more preferable because this method requires less effort and equipment but affords the same storage stability as quick freezing.

Human milk, unlike cow's milk or synthetic formulas, possesses a composition uniquely suited to the needs of the human infant. Although it is difficult to assign a precise nutritional or bacteriostatic role to each milk component, the sum total of these components is responsible for the differences in growth, development and health noted for breast-fed *versus* bottle-fed infants (1, 9, 16, 31, 46). The recognition of the superiority of breast milk has led to an increased incidence of breast feeding (7, 28), and a renewed interest in establishing human milk banks to serve the needs of premature infants and full-term infants who are ill or who temporarily cannot be breast-fed (2, 39, 42, 47).

Evidence that pasteurization and other methods of preservation not only eliminate bacterial contaminants but also destroy many beneficial milk components, including the immunoglobulins, lysozyme and *Lactobacillus bifidus* factors (5, 13, 14, 17, 38), has led some to suggest that heating of donor milk samples is unnecessary and unadvisable (2, 8, 44, 47). Recent reports of the transmission of salmonellosis (40) and streptococcal disease (26, 41) through ingestion of infected breast milk necessitate a careful reexamination of milk banking procedures. Determining the effects that processing and storage have on donated milk samples would allow the establishment of procedures which ensure a bacteriologically safe milk with maximum storage stability and minimal loss of nutrients and biologic activity.

This study was initiated to evaluate the effects of several methods of processing and storage on key enzymes, B vitamins, and lipid components of mature human milk. This paper summarizes the average values and ranges of these nutrients in individual raw milk samples and the effects of freezing and frozen storage on these components. A companion paper (15) summarized the effects of freeze-drying, pasteurization, high heat treatment, and storage.

MATERIALS AND METHODS

Collection and pooling of breast milk samples. Individual mature milk samples were obtained from donors 10 days to 6 months postpartum. Samples, ranging from 20–80 ml, were manually expressed by the donor into sterile glass jars, refrigerated, transported to the laboratory, and then analyzed individually as raw samples or pooled (8–12 samples per pool) for freezing studies. Individual samples were refrigerated up to 24 hr after collection, and pooled samples were held up to 48 hr before analysis or freezing.

Freezing studies. Ten pooled samples of mature milk were used for slow freezing studies and ten for quick freezing studies. Each pooled sample was divided aseptically into four equal portions which were placed in sterile containers. One of the four portions (unfrozen control) was analyzed immediately for enzymes, B vitamins, and lipids and the other three portions were frozen either by placing in the freezing compartment of a domestic refrigerator at −25°C (slow freeze) or by immersing in a dry ice/acetone/alcohol bath (quick freeze). Frozen samples were then held at −25°C until analysis for enzyme, B vitamin, and lipid components at 1 wk, 1 month, and 3 months.

Analysis of enzymes, B vitamins, and lipids. Skim milk samples, prepared by centrifugation at 5900 × *g* for 10 min, were used for analysis of lactoperoxidase, lysozyme, and protease. Whole milk was used for remainder of the analyses. Lactoperoxidase (EC 1.11.1.7) activity was measured spectrophotometrically by the method of Gothefors and Marklund (19). The procedure of Parry *et al.* (34), which is based on lysis of *Micrococcus lysodeikticus* cells, was used to quantitate lysozyme (EC 3.2.1.17). Lipase (EC 3.1.1.3) was monitored using a pH stat according to the procedure of Parry *et al.* (35). Protease (EC 3.4.4.) activity was measured as described by Kiermeier and Semper (27).

B vitamins were analyzed using microbiologic methods (20). Biotin was assayed by a spectrophotometric method using *Lactobacillus plantarum* #8014 as the test organism, niacin by a titrimetric method using *L. plantarum* #8014, and pantothenic acid by a titrimetric method using *L. casei* #7469 as the assay organism.

Total lipids were extracted from the milk using the Roese-

Gottlieb method (3). The unesterified fatty acid content was determined by titration using a weighed aliquot of the lipid extract (4). Approximately 150 mg of lipid extract were saponified and then reacted with BF_3-methanol according to the procedure of Metcalfe *et al.* (33). The fatty acid methyl esters were extracted with petroleum ether and then separated on a Varian gas chromatograph, Model 1200, equipped with a 6 ft by ⅛ inch stainless steel column packed with 10% SP-2330 on 100/120 mesh Chromosorb W AW. The column temperature was programmed from 180–230°C at 3°C/min with the nitrogen carrier gas flow-rate at 35 ml/min. The area of each fatty acid peak was determined automatically with a Hewlett Packard Model 5840A GC Terminal Integrator, and results were expressed as relative % of each fatty acid.

RESULTS

In order to establish standard values for the nutrient components with which to compare processed samples, a total of 30 individual raw samples of mature human milk was analyzed (Table 1). There was considerable sample to sample variation as indicated by the large standard deviation and range of values for each component. Nevertheless, the values fell within established ranges for the lactoperoxidase (19), lipase (10), lysozyme (10, 11, 37), protease (22, 43), biotin (12, 23, 30), niacin (12, 30), pantothenic acid (12, 30), folic acid (30) and lipids (6, 18, 24, 25, 32) of mature human milk.

As shown in Table 2, freezing and frozen storage had little effect on the enzymes of mature human milk. Lactoperoxidase activity decreased during frozen storage and at 6 months the activity was significantly lower ($P < 0.05$) as compared to the raw sample. Quick freezing and frozen storage tended to increase lipase activity although the changes were not significant as compared to the raw sample.

Freezing and frozen storage did not significantly affect the levels of biotin, niacin, and pantothenic acid (Table 3). Similarly the total lipid, unesterified fatty acid level, and relative % of each fatty acid were not significantly different in the frozen samples as compared to the raw samples (Table 4).

Table 1. *Enzyme, B vitamin, and lipid content of raw mature breast milk*[1]

Component	Mean ± S.D.	Range
Enzymes		
Lactoperoxidase (ΔOD/min/100 ml)	44 ± 42	(4–155)
Lipase (μmole/min/100 ml)	108 ± 66	(26–311)
Lysozyme (mg/100 ml)	20 ± 20	(2–68)
Protease (μmole/min/100 ml)	0.21 ± 0.15	(0.02–0.56)
B vitamins		
Biotin (μg/100 g)	0.87 ± 0.42	(0.14–1.72)
Niacin (μg/100 g)	211 ± 59	(129–348)
Pantothenic acid (μg/100 g)	297 ± 137	(132–625)
Folic acid (μg/100 g)	2.13 ± 1.59	(0.25–7.88)
Lipids		
Total lipid (g/100 ml)	2.98 ± 1.40	(1.06–6.83)
Unesterified fatty acids (g/100 g lipid)	3.8 ± 15.5	(0.4–14.1)
8:0 (%)	0.24 ± 0.03	(TR[2]–0.7)
10:0 (%)	1.4 ± 1.5	(0.2–7.4)
12:0 (%)	4.2 ± 7.4	(1.6–11.9)
14:0 (%)	5.0 ± 3.8	(2.4–9.7)
16:0 (%)	19.7 ± 18.1	(9.9–25.8)
16:1 (%)	1.4 ± 1.0	(0.2–4.2)
18:0 (%)	9.1 ± 11.8	(3.5–20.9)
18:1 (%)	40.6 ± 46.5	(24.2–53.4)
18:2 (%)	14.7 ± 14.4	(8.6–23.3)
18:3 (%)	1.7 ± 2.8	(0.7–7.4)
P/S[3]	0.41 ± 0.02	(0.18–0.81)

[1] All data represent the average of 30 individual samples.
[2] TR, trace (less than 0.2%).
[3] P/S, polyunsaturate/saturate where P is 18:2 + 18:3.

DISCUSSION

In view of the decreased nutritional value and low antimicrobial activity of pasteurized human milk, it has been suggested that freezing rather than heating be used to preserve breast milk samples (2, 8, 21, 38, 39, 44, 47). As shown in Table 2, frozen storage for up to 3 months after either a slow-freeze or quick-freeze process resulted in a significant decrease ($P < 0.05$) in lactoperoxidase activity and no change in the other enzymes, B vitamins, or lipids. In a similar study, Evans *et al.* (13) also found that lysozyme was unaffected by storage at −20°C for 3 months.

Table 3. *Effect of freezing and frozen storage on B vitamins of mature breast milk*

Process and storage time	No. of pooled samples	Biotin (μg/100 g)	Niacin (μg/100 g)	Pantothenic acid (μg/100 g)
Slow Freeze	8			
Unfrozen		0.50	217	332
Frozen/stored 1 wk		0.63	209	312
Frozen/stored 1 mo		0.52	208	276
Frozen/stored 3 mo		0.51	212	286
Quick Freeze	7			
Unfrozen		0.47	209	273
Frozen/stored 1 wk		0.51	208	286
Frozen/stored 1 mo		0.45	214	277
Frozen/stored 3 mo		0.50	213	319

Table 2. *Effect of freezing and frozen storage on enzymes of mature breast milk*

Process and storage time	No. of pooled samples[1]	Lactoperoxidase (ΔOD/min/100 ml)	Lipase (μmole/min/100 ml)	Lysozyme (mg/100 ml)	Protease (μmole/min/100 ml)
Slow Freeze	10				
Unfrozen		36	95	32	0.18
Frozen/stored 1 wk		38	98	23	0.24
Frozen/stored 1 mo		26	92	32	0.24
Frozen/stored 3 mo		17[2]	133	37	0.19
Quick Freeze	10				
Unfrozen		93	88	32	0.18
Frozen/stored 1 wk		58	102	24	0.14
Frozen/stored 1 mo		50	111	21	0.15
Frozen/stored 3 mo		14[2]	111	18	0.12

[1] Each pool consisted of 8–12 individual samples.
[2] Significantly different ($P < 0.05$) from unfrozen control as determined by a paired sample *t* test.

Table 4. *Effect of freezing and frozen storage on the lipids of mature breast milk*

Process and storage time	Total lipid (g/100 ml)	Unesterified fatty acids (g/100 g lipid)	Fatty acid composition (relative%)										
			8:0	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	P/S[3]
Slow Freeze (10)[1]													
Unfrozen	2.49	7.7	TR[2]	0.6	3.6	5.6	25.4	1.4	9.2	38.6	4.3	1.2	0.36
Frozen/stored 1 wk	2.38	7.2	TR	0.8	4.1	6.0	23.4	1.4	9.4	38.1	14.9	1.0	0.39
Frozen/stored 1 mo	2.57	8.9	TR	0.6	3.6	5.7	23.4	1.2	10.0	40.1	14.4	0.8	0.36
Frozen/stored 3 mo	2.53	8.0	TR	0.7	4.1	6.2	24.9	1.6	9.3	38.3	13.9	0.5	0.33
Quick Freeze (10)[1]													
Unfrozen	2.75	6.8	TR	0.7	4.2	5.7	20.9	1.4	9.1	41.0	16.1	0.7	0.42
Frozen/stored 1 wk	2.53	10.9	TR	0.6	3.9	5.3	23.1	1.2	9.5	40.0	15.4	0.9	0.36
Frozen/stored 1 mo	2.73	7.1	TR	0.7	4.1	5.9	24.7	1.2	9.1	38.1	15.1	0.9	0.39
Frozen/stored 3 mo	2.51	11.5	TR	0.6	4.3	5.8	23.9	1.2	8.9	39.1	15.8	0.5	0.38

[1] Number of pooled samples analyzed.

[2] TR, trace (less than 0.2%).

[3] P/S, polyunsaturate/saturate where P is 18:2 + 18:3.

During storage, there appeared to be a progressive increase in the activity of lipase (Table 2) and elevation in the level of unesterified fatty acids (Table 4), indicating that spontaneous lipolysis was occurring in the milk. The evidence of lipolysis was most pronounced in the quick-frozen samples stored 3 months. Tarassuk *et al.* (45) noted that human milk lipase may be activated at low temperatures resulting in significant lipolysis in refrigerated samples of raw breast milk. It is possible a similar process was occurring in the frozen samples.

Several authors (8, 13, 17, 29, 36, 44, 48) have reported that the breast milk leucocytes and immunoglobulins decrease with freezing, resulting in a gradual loss of the milk's antibacterial activity during prolonged storage (17, 21); however, the effects of frozen storage are insignificant compared to those caused by pasteurization or sterilization (5, 13, 14, 17, 38). Our data also suggest that slow freezing offers the same storage stability as quick freezing with much less effort and equipment and thus would be the method of choice for preserving the milk. A comprehensive program of donor screening, proper aseptic collection, immediate freezing of samples and routine quality control must also be established and enforced in order to ensure microbiologic safety in any unheated samples (13, 39, 42, 44, 47).

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