ELSEVIER

Journal of Chromatography B, 704 (1997) 1–10

JOURNAL OF CHROMATOGRAPHY B

Effect of pasteurization and storage on some components of pooled human milk[1]

Luciano Lepri[a,*], Massimo Del Bubba[a], Rita Maggini[a], Gian Paolo Donzelli[b], Paola Galvan[b]

[a]*Department of Public Health, Epidemiology and Environmental Analytical Chemistry, University of Florence, Via G. Capponi 9, Florence, Italy*

[b]*Chair of Preventive and Social Paediatrics, Department of Paediatrics, University of Florence, Via L. Giordano 13, Florence, Italy*

Received 18 April 1997; received in revised form 11 August 1997; accepted 27 August 1997

Abstract

Pooled human milk was subjected to Holder pasteurization and storage at −20°C up to 90 days and examined for its content of fat and L-lactate and for lipid composition. This treatment reduced fats by 6% and L-lactate by at least 7%. In addition, pasteurization and storage induced triglyceride hydrolysis. The absolute amount of free fatty acids (FFAs) which was 0.5% after collection, doubled after pasteurization and rose even more after storage. Different FFA compositions were found by several authors using the same analytical method even for milk samples subjected to the same treatment. More detailed information on procedures must be given to explain the different results. © 1997 Elsevier Science B.V.

Keywords: Milk; Fatty acids; Lactate

1. Introduction

The human milk bank of Meyer children's hospital (Florence, Italy) in 1995 collected 1756 l of human milk, mostly (≈90%) donated by women from the area of Florence and the rest from other hospitals. The collected milk was heated at 62.5°C for 30 min (Holder pasteurization) to destroy contaminating bacteria and stored at −20°C until use.

It is well known that the conventional heat treatment destroys some vitamins, inactivates a major lipase [1], alters some vitamin binding proteins and causes destruction of most of the lactoferrin [2]. In addition lipolysis of breast milk occurs when milk is stored at temperature greater than −25°C [3–5].

Fat in breast milk plays an important role in feeding premature infants and its alteration may have a notable effect on infant development [6]. In addition the lipid accumulation serves not only for energy storage but also has a structural function in all tissues.

More recently, the effects of storage at different temperatures on the release of individual fatty acids [7,8] were studied owing to the physiological significance of long-chain polyunsaturated fatty acids (LC-PUFA) with chain lengths of 20 and 22 carbon atoms [6].

The purpose of this study was to determine the

*Corresponding author.

[1]Presented at the International Meeting 'The Role of LC-PUFA in Neonatal Nutrition', Florence (Italy), 14–15 November, 1996.

0378-4347/97/$17.00
PII S0378-4347(97)00439-8

influence of Holder pasteurization and storage at −20°C on fatty acid composition and, particularly, on arachidonic and docosahexaenoic acids, fat content, the degree of lipolysis and release of individual free fatty acids. In addition, L-lactate was directly determined in milk samples in order to assess possible alterations of this compound.

2. Experimental

2.1. Reagents and materials

Sodium chloride was heated for 12 h at 450°C to remove any organic matter and used immediately. Anhydrous sodium sulphate was heated for 12 h at 450°C and then kept at 120°C until use. Purified water was obtained by an ELGASTAT-UHQ system supplied from Conacom (Pisa, Italy). Solvents were all for organic trace analysis purchased from Merck (Darmstadt, Germany) and Baker (Deventer, Netherlands); they were bidistilled in our laboratory before use. The free fatty acids and their methyl esters, monoglycerides, 1,2- and 1,3-diglycerides, triglycerides and cholesterol used as standards were supplied by Sigma (St. Louis, MO, USA) and Alltech (Deerfield, IL, USA). The 14% boron trifluoride solution in methanol was purchased from Alltech. Lactate oxidase from the *Pediococcus* species (specific activity 26 IU mg^{-1}) was supplied by Sigma–Aldrich (Milan, Italy). All other reagents were AR grade.

All glass apparatus was cleaned before use by repeatedly washing with hot methanolic potassium hydroxide, chromic and concentrated sulphuric acid hot mixture, purified water and acetone. The Immobilon membrane used for immobilization of enzyme was obtained from Millipore (Milan, Italy). For casting the cellulose acetate membrane, a precision gauge tool was used (Precision Gage and Tool, Dayton, OH, USA).

The hydrogen peroxide sensor was a platinum anode, 0.5 mm in diameter, surrounded with a ring of silver/silver chloride acting as reference and counter electrode. The probe was provided by Instrumentation Laboratory (Milan, Italy).

The VA-detector Model 641 (Metrohm, Milan, Italy) was used as a potentiostat. The polarization unit was connected to a Model 868 recorder (Amel, Milan, Italy).

2.2. Milk collection.

Sixteen healthy mothers (age 24–38 years) were recruited for this study after a full explanation of the project and consent was obtained. Samples of human milk were collected by manual expression, after infant feeding, in the same day, stored at +4°C during the transport to the laboratory and pooled. The gestational age ranged from 35 to 42 weeks (mean value 38.6). Milk samples were taken between 08.00 and 13.00 h. The pooled milk was portioned into five homogenous fractions:

1. raw milk (R);
2. pasteurized milk (P);
3. pasteurized milk and stored at −20°C for 35 days (P35);
4. pasteurized milk and stored at −20°C for 70 days (P70);
5. pasteurized milk and stored at −20°C for 90 days (P90).

The lipid extraction from R and P samples was carried out 8 h after the start of the collection.

2.3. Lipid extraction

Several methods were used for lipid extraction from the milk samples. Such methods employed diethyl ether [9], hexane [10] and chloroform–methanol mixtures in the 2:1 [11] or 1:2 [12] ratio, as extractants. In this study total lipids were extracted using a modification of the procedure of Folch et al. [11] as described by Bitman et al. [13]. The method was further modified using more extractions to improve the recovery.

After adding 6 ml 0.7% aqueous sodium chloride and 18 ml chloroform–methanol (2:1, v/v) to a 1-ml aliquot of the homogenized milk, the phases were separated by centrifugation at 800 rpm for 10 min. After removal of the lower phase (first chloroform extract), 6 ml chloroform were added to the remaining aqueous phase and the resulting mixture was centrifuged to obtain a second organic extract.

Finally, the aqueous phase was acidified with 0.1 *M* hydrochloric acid up to pH≅2 and reextracted with an additional 6 ml chloroform (third extract). The organic extracts were dried over anhydrous sodium sulfate.

Recovery tests on standard solutions showed that two extractions were needed to recover 98–99% of total lipids. The volume of the first extract was at best 11.5 ml and allows recovery of about 93%. If a smaller chloroform volume was obtained, a corresponding decrease in the recovery was observed but, in such case, the recovery of the second extraction increased.

2.4. Preparation of fatty acid methyl esters

A 2-ml aliquot of each chloroform extract (containing the free acid $C_{21:0}$ as internal standard) or a 3-ml aliquot of the mixture of the first two chloroform extracts were dry evaporated under a cold gentle nitrogen flow.

The residue was treated with 1 ml of 0.5 *M* methanolic potassium hydroxide solution and heating in boiling water under reflux for 20 min followed by addition of 1.5 ml BF_3–methanol (14:86, w/v) (Alltech) and reheating for 15 min according to the AOAC-IUPAC method [14].

After cooling, 2 ml of a saturated sodium chloride solution were added and the methyl esters were extracted twice with 2 ml hexane. The unified extracts were dried on anhydrous sodium sulfate and analyzed by GC. If the fat content was very low, the hexane extracts were reduced to 0.5 ml under nitrogen flow. Air from methanolic potassium hydroxide solution and from the tube was removed by passing in a stream of nitrogen for a few minutes.

The preparation and extraction of fatty acid methyl esters were effected in the same conical tube (12 ml) with a ground-glass joint.

2.5. HRGC and HRGC–MS analyses

The gas chromatographic analysis was performed by a HRGC-5160 Mega Series (Carlo Erba, Milan, Italy) gas chromatograph equipped with a flame ionization detector. The injection was made using a cold split–splitless liquid injector according to the following temperature program: injection at 40°C, then a rapid increase in temperature to 350°C and splitting after 45 s. The column temperature program is the following: starting period at 40°C for 1 min, linear increase from 40°C to 160°C at 4°C/min, from 160°C to 230°C at 1°C/min, from 230°C to 300°C at 4°C/min and finally a period at 300°C for 10 min.

The methyl esters were separated with a capillary column Supelco PTE-5 (30 m×0.25 mm I.D., 0.25 μm thickness); carrier gas: helium. The chromatographic peaks were analyzed with a Mega 2 computer system (Carlo Erba) with Spectra-Physics software.

Confirmatory HRGC–MS analysis was performed on a Varian 3400 (Palo Alto, CA, USA) gas chromatograph coupled with a Finnigan ion trap detector (ITD). The injection was made by using a septum programmable injector (SPI, Varian) according to the following temperature program: injection at 40°C, then a rapid increase to 300°C and isotherm for 1 min; the column temperature program was identical to that described above. Electron impact mass spectra were obtained at 70 eV of ionization energy.

All peaks were identified by using a reference chromatogram and/or comparing their mass spectra with those reported in the National Bureau of Standards (N.B.S.) library and in a second library made in our laboratory. The quantities of lipids were determined by comparing their peak areas with those of the corresponding standards. The detection limit for individual fatty acids was found to be 0.01%.

2.6. TLC experiments

A 4-ml aliquot of the mixture of the three chloroform extracts was cold evaporated under a nitrogen stream; after the addition of 100 μl chloroform, 1–5 μl volumes were spotted on silica gel 60 for HPTLC (10×20 cm plates, Merck) together with standard solutions of cholesterol, phospholipids, mono-, di-, triglycerides and FFA and the plates were eluted in a thermostatic chamber (Desaga, Heidelberg, Germany) at 25°C with a mixture of toluene–ethyl acetate–acetic acid (40:10:0.1, v/v/v). Migration distance was 16 cm.

R_F values: phospholipids (R_F=0.00); monoglycerides (R_F=0.09); FFA (R_F=0.36); cholesterol (R_F=0.43); 1,2-diglycerides (R_F=0.52); 1,3-di-

glycerides (R_F=0.63); triglycerides (R_F=0.97). The compounds were detected by spraying with 10% phosphomolibdic acid in ethanol and heating at 120°C for 15 min.

Quantitative determination of FFA fraction was performed by a CS-9001 PC Shimadzu Scanning densitometer coupled with a 486 IBM compatible PC. Scanning was done in the direction of chromatography with a moving light in a zigzag form over the sample zones.

All functions of the scanner were controlled and the data were processed with TLC-specific software manufactured by Shimadzu. Real time background correction was automatically performed in the zigzag system.

Lipolysis of lipids and content of individual free fatty acids were also determined by scraping off the FFA fraction, corresponding to a 5-μl volume, from the plate. The scraped layer was put in a microcolumn and eluted with 0.5 ml methanol. The eluate was treated with 1.5 ml BF_3–methanol (14:86, w/v), after the addition of free acid $C_{21:0}$ as internal standard, and heated as described previously. The hexane extract of methyl esters was evaporated to 0.5 ml and injected into the gas chromatograph. A blank of the whole procedure was included. The detection limit for individual FFAs was found to be 0.5%.

2.7. Direct determination of L-lactate

The determination of L-lactate in milk was carried out using a biosensor based on immobilized lactate oxidase enzyme [15]. The enzymatic reaction is:

$$\text{L-lactate} \leftrightarrow \text{pyruvate} + H_2O_2 \quad (1)$$

In this study, the sensor response to L-lactate was measured amperometrically by monitoring the oxidation of hydrogen peroxide produced.

L-lactate oxidase was immobilized covalently on the Immobilon membranes. The enzyme (2 mg) was dissolved in 0.1 *M* phosphate buffer pH 7.4 (90 μl). The enzymatic solution was mixed with bovine serum albumin (2 mg) and 2.5% glutaraldehyde (10 μl). The solution was spread directly onto the membrane surface (1 cm^2). After 2 h, the excess of glutaraldehyde and the unbound enzyme were washed with 0.1 *M* glycine solution (pH 10).

A cellulose acetate membrane with a MMCO (molecular mass cut off) of 100 was placed on the platinum surface to eliminate interferences from electroactive compounds, such as ascorbic acid. The cellulose acetate membrane was prepared in our laboratory as described in Ref. [16]. A second membrane with immobilized enzyme was placed on top of the first one and covered with nylon net. The three membranes were held together with a neoprene O-ring.

The working electrode was poised at +650 mV versus silver/silver chloride by the polarization unit. For standard solutions and milk samples, a multiple addition technique was used. Suitable lactate concentrated solutions or milk samples were added to 10 ml of 0.1 *M* phosphate buffer (pH 7.4) in a beaker with stirring at a moderate rate. A steady state current was obtained in 2 min with standard or samples additions. With samples, sandwich additions, standard–sample–standard were performed in order to take into account eventual matrix effects. The lactate concentration in the samples was calculated using the mean value of current obtained from three measurements.

3. Results and discussion

3.1. Fatty acid composition of pooled human milk

The analytical method was checked on ten individual human milk samples taken by manual expression from mothers of term infants (37–40 weeks gestation).

Table 1 shows the percentage of fatty acids in the pooled milk and, for comparison, the concentration range of the same compounds in the above-mentioned ten samples. Mean values of five determinations with standard deviation were reported for all the fatty acids.

The results show that the method is precise with relative standard deviation (R.S.D.) values of ≈1% in all major components, of 10% in fatty acids with low concentrations (0.1%) and higher than 10% in the compounds present in trace amounts (<0.1%).

This precision was satisfactory for those fatty acids for which baseline separation was achieved but not for substances such as 18:1ω6 (R.S.D.=9.3%, concentration 0.54%) where this was not the case.

Table 1
Fatty acid composition of a mixture of 16 human breast milk (pool)[a] and comparison with lipid concentration range of ten individual milk samples from Tuscan women (A)

Fatty acid	Pool (%, w/w)	A (%, w/w)
8:0	0.21±0.01	0.12–0.26
10:0	1.45±0.03	0.78–1.79
12:0	5.25±0.06	2.19–7.29
14:1ω5	0.21±0.01	0.09–0.40
14:0	6.05±0.06	2.49–8.70
15:0[b]	0.06±0.01	0.02–0.12
15:0[b]	0.11±0.01	0.04–0.21
15:0	0.35±0.01	0.20–0.59
16:1ω7	1.92±0.03	1.60–2.57
16:1*t*ω7	0.06±0.01	0.04–0.11
16:0	22.98±0.18	22.40–28.63
17:0[b]	0.10±0.01	0.05–0.18
17:0[b]	0.18±0.01	0.10–0.27
17:1ω7	0.20±0.01	0.17–0.27
17:0	0.31±0.01	0.23–0.44
18:3ω6	0.10±0.01	0.09–0.13
18:2ω6	11.03±0.10	8.29–14.82
18:1ω9+18:3ω3	38.43±0.38	30.61–44.50
18:1*t*ω9	2.18±0.04	1.75–2.28
18:1ω6	0.54±0.05	0.23–0.89
18:0	5.70±0.06	5.40–7.35
20:4ω6	0.42±0.01	0.26–0.50
20:5ω3	0.05±0.01	0.01–0.08
20:3ω6	0.39±0.01	0.25–0.42
20:2ω6	0.21±0.01	0.16–0.24
20:1ω9	0.41±0.02	0.32–0.47
20:0	0.16±0.01	0.15–0.17
22:6ω3	0.21±0.01	0.11–0.27
22:4ω6	0.08±0.01	0.07–0.10
22:5ω3	0.10±0.01	0.09–0.13
22:3ω6	<0.01	<0.01–0.01
22:2ω6	0.02±0.01	0.01–0.05
22:1ω9	0.07±0.01	0.05–0.08
22:0	0.04±0.01	0.03–0.06
24:1ω9	0.03±0.01	0.02–0.04
24:0	0.03±0.01	0.02–0.04

[a] Mean of five measurements±standard deviation.
[b] Branched.

The distribution of fatty acids in milk was not correlated with the yield or the number of extractions since no change in the composition of the first extract and of the total of the two extracts was observed. Thirty-six compounds were identified under the experimental conditions used but oleic acid (18:1ω9) was not separated, at the high concentrations in which is present in human milk, from α-linolenic acid (18:3ω3). Therefore, the values in Table 1 refer to the sum of the two acids and it should be noted that α-linolenic acid usually accounts for as much as 0.5–1% [17–19].

Instead, both the lower molecular mass fatty acids and those with a higher number of carbon atoms (C_{20} and C_{22}) which are important for nutrition [6], were baseline separated. In particular, the octanoic acid (8:0) identified in this study at 0.21% level was not found by Hartzer et al. [18] and Koletzko et al. [19] but was determined by Gibson and Kneebone [17] in human colostrum (0.04%) and mature breast milk (0.13%) and by Finley et al. [20] in American milk of vegetarians and non-vegetarians at levels of 0.15 and 0.17%, respectively.

Another confirmation regards the presence of some branched saturated fatty acids (iso and ante-iso 15:0 and 17:0), only partially noted by Gibson and Kneebone [17]. Their presence in small quantities together with the two straight-chain fatty acids with an odd number of carbon atoms (15:0 and 17:0) are probably of dietary origin [21–23]. These acids are the ones found in butter and extra virgin olive oil used by Tuscan women.

No acid with an odd carbon number was present at levels ≥0.01%, apart from 15:0 and 17:0 which are generally present in breast milk in significant percentages [17–19].

Two *trans* monoenoic fatty acids (16:1ω7 and 18:1ω9) with a predominance of the latter (2.18%) were identified and these data reflect the importance of the womens' diet. The linoleic acid (18:2ω6) content was 11.03% in accordance with the values previously found for European countries [17,18,24,25]. Nine different long chain polyunsaturated fatty acids were also baseline separated and determined. In spite of their low concentration (≤0.5%) these compounds may well be of physiological importance [6].

The fatty acid composition of pooled milk matches the concentration ranges of the ten individual samples showing that it is representative of the breast milk characteristic of Tuscan women.

3.2. Effect of Holder pasteurization and length of storage on lipid composition

To verify the influence on the fat composition of milk processing performed by the human milk bank at Meyer Hospital, a mixture of sixteen human milks

was analyzed before and after Holder pasteurization and storage up to a maximum of 90 days.

The compounds identified in the various samples are the same as in Table 1 where the 'pool' data refer to raw milk. The mean of the fatty acid fractions points out any eventual difference in the lipid composition as a function of the treatment performed on the milk. The results demonstrated that there are no substantial differences in the fat composition of the various samples: for example the ratio between polyunsaturated and saturated acids (P:S) is constant and equal to 0.29. It must be noted that this value agrees with that found by Koletzko et al. [19] for the German women's milk (0.31%) keeping in mind that 18:3ω3 was not separated from oleic acid and therefore the P:S value is slightly underestimated.

The differences noted in the values of the other lipid fractions are within the experimental error limits but there is a slight decrease for the fractions of LC-PUFAs mostly after storage for 70 and 90 days. This trend concerns both the ω-6 and the ω-3 LC-PUFAs in equal measure, as indicated by their constant ratio value.

3.3. Effect of Holder pasteurization and length of storage on fat content

Usually, total lipids are determined by removing the solvents from the lipid extract and weighing. This procedure is not very accurate and includes all the organic compounds which were extracted with the solvent used. Using the gravimetric method a value of 26±2 mg/ml and a R.S.D. of 7% was obtained for raw milk. The R.S.D. value was too high to show small variations in the lipid content of the five samples of pooled human milk.

For this reason, the fat content was determined by using the total chromatographic peak area concerning the fatty acids identified. This method gives a more accurate and reproducible value even if underestimated with respect to the value obtained by weighing, since cholesterol, phospholipids and any unidentified fatty acids were not included. The results are shown in Table 2. The triglyceride content in raw milk is 24.73±0.58 mg/ml with R.S.D. value of 2.3%. This value agrees with the one determined gravimetrically since the percentage of cholesterol and phospholipids in human milk is less or equal to 1.5% [13]. By the first extraction about 93% of triglycerides was recovered and 5–6% with the second.

Table 2
Fat content (mg/ml) of pooled human milk before and after Holder pasteurization and storage at −20°C

Milk sample	Extraction			Total fat content[a] (mg/ml)
	1st	2nd	3rd	
R	22.98	1.60	0.15	24.73±0.58
P	23.32	1.52	0.24	25.08±0.54
P35	23.20	1.25	0.22	24.67±0.52
P70	18.79	4.22	0.59	23.60±0.58
P90	21.55	1.38	0.39	23.32±0.55

[a] Mean of five measurements±standard deviation.

The only exceptions were the data of the sample stored for 70 days with 79.6% of fat being recovered with the first extraction and 17.8% with the second. This is due to the smaller volume (10.5 ml) of the first chloroform extract with respect to that of the other samples (11.5 ml). In addition the volume of the second extract increased from 6 to 6.5 ml. These findings show that it is not possible to quantitatively determine the lipid content of milk without performing at least two successive extractions. Such a procedure must be used even if the gravimetric method is employed.

The third extraction, effected in strong acid conditions, usually recovers from 0.7 to 1.7% of total fats. The acid medium favors the extraction of low molecular mass free fatty acids which can be produced from lipolysis. In fact, from pasteurized milk (pH 7.6–7.8) 49% of standard free acid C 7:0 was extracted while more than 98% of standard $C_{21:0}$ because of its greater hydrophobicity due to the very high number of carbon atoms. The pK_a of the free fatty acids is between 4.77 and 4.85 and, therefore, at milk pH these compounds are present in neutral form at minimum levels (≈1‰) and their extraction in chloroform is possible owing to the strong hydrophobic properties of the side-chain. Some samples of fresh milks have lower pH values than pasteurized milks, probably due to the carbon dioxide which disappears with pasteurization.

After 35 days, the fat content is slightly lower than that of milk before and after pasteurization but it is well within the R.S.D. value. As storage progresses

(70 and 90 days) the fat content in the milk notably decreases. After 90 days, the decrease is equal to 5.7% with respect to raw milk and this value is above the R.S.D. value. These findings pointed out a real decrease in the concentration of the 36 fatty acids calculated using their chromatographic peak areas.

3.4. Effect of Holder pasteurization and length of storage on lipolysis of lipids

The lipolysis of triglycerides in the five samples of pooled human milk was determined using densitometry on silica plates and the gas chromatographic technique following extraction of the different lipid fractions on thin-layers (see Section 2.6).

The data in Table 3 show significant FFA concentrations in all the samples. In particular, their levels in raw milk are about 0.5%, much higher than was found in analogous samples collected and immediately analyzed (0.1–0.2%) [3,13,26]. However, the collection procedures, milk pasteurization and transport to the analysis laboratory required almost 8 h, in which time lipolysis can occur. A high increase in lipolytic activity was caused by the pasteurization process which doubles the FFA content. The storage of pasteurized milk at −20°C seems to have less effect even though lipolysis increases with length of storage. The release of FFAs is much lower than the one found by other authors [13,26] for milk samples stored at −20°C without pasteurization, and demonstrates that pasteurization process partially inactivates the lipases. By thin-layer chromatography (TLC) it was also possible to point out the presence, besides FFA, cholesterol and phospholipids, of 1,2-diglycerides which derive from enzymatic hydrolysis of triglycerides. This excludes chemical hydrolysis from FFA formation since in this case there even would be 1,3-diglycerides as the 2-position is more chemically reactive than the other two.

Table 3
Effect of Holder pasteurization and length of storage at −20°C on free fatty acid content of pooled human milk

Milk sample	FFA[a]	
	TLC	GC
R	0.6±0.2	0.39±0.12
P	1.1±0.3	0.81±0.18
P35	1.0±0.2	0.78±0.15
P70	1.1±0.3	0.84±0.16
P90	1.2±0.3	0.94±0.18

[a] Mean of five determinations±standard deviation.

The determination of individual FFAs is, instead, extremely difficult owing to their low concentration. In fact, many acids are present as triglycerides at levels below 0.2% and are therefore below the detection limits of the method when the FFA fraction, which accounts for less than 1% of the total fats, is analyzed.

This is evident also from the gas chromatogram of the second chloroform extract (5–6% of total fats), where the majority of the compounds are not measurable and the concentration of the others increases proportionally (10:0, 12:0, 14:0, 15:0, 16:0, 16:1ω7, 17:0, 18:0, 18:1ω9, 18:1*t*ω9, 18:2ω6 and 20:4ω6). The phenomenon is even more evident if we examine the acid extract (1–2% of total fats), where the above-mentioned fatty acids were determined with the exceptions of 15:0, 17:0 and 20:4ω6, which were present in lower amounts than the others. The fat content in the acid extract is higher than the FFA level and, therefore, the same nine compounds have to be found if the hydrolysis process equally affects the lipids.

Table 4 compares our results with other authors [7,8,26] for human breast milks subjected to various treatments. The FFAs identified in our samples are those previously hypothesized with the exception of 15:0. The analytical procedures used by Chappel et al. [7], Tacconi et al. [26] and ourselves are similar since they are based on a preliminary TLC separation of the different lipid classes before GC analysis. However, in spite of the similar conditions used by the first two authors [7,26], such as storage at −80°C, a different FFA profile was found. The major difference is due to the presence of $C_{13:0}$ among the FFAs reported by Tacconi et al. [26] considering that such compound was present as triglyceride at trace levels in human milk. On the contrary, the free acid $C_{18:1t\omega9}$ was only identified in our study notwithstanding the high percentage of this esterified compound in breast milk [17,19].

Lavine's method which gives a very different FFA profile from all the others, is based on the reextraction, after acidification, of the aqueous phase re-

Table 4
Free fatty acid profile in human milk

Reference	10:0	12:0	13:0	14:0	15:0	16:0	16:1	18:0	18:1	18:1*t*	18:2	18:3	20:4	22:4	22:5
This study[a]	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Tacconi[b]	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−
Lavine[c]	−	+	−	+	−	+	−	+	+	−	+	+	+	+	+
Chappel[d]	+	+	−	+	−	+	−	+	+	−	+	−	−	−	−

[a] Fresh, pasteurized and stored after pasteurization at −20°C.
[b] Fresh, pasteurized and stored without pasteurization at +4°C, −20°C or −80°C [26].
[c] Fresh and stored at +25°C, +4°C, −11°C or −70°C [8].
[d] Fresh, stored at −10°C or −80°C with or without EDTA and warmed to 22°C or 37°C [7].
(+)=detected; (−)=not detected.

sulting from the lipid extraction procedure. It is not completely clear: (1) if the free fatty acids were already extracted, even partially, from the extraction effected at the milk pH; (2) if there are fat residues remaining from such an extraction.

Only the following FFAs (12:0, 14:0, 16:0, 18:0, 18:1 and 18:2) were identified by all the authors in the various milk samples. The differences in the FFA profile found by Tacconi et al. [26], Chappel et al. [7] and ourselves, makes it necessary to completely describe the experimental conditions used and, in particular, the volumes of sample and solvent employed in the various stages of the analytical procedure, the number of extractions and the detection limits and precision of the method.

The gas chromatographic data on the FFA levels in the various milk samples have extremely high (30–40%) R.S.D. values showing the low precision of the method. Under these conditions, the percentages of individual FFAs can only evidence major differences in FFA composition due to pasteurization and storage at −20°C.

For this reason, Table 5 reports the mean of individual FFAs in the five samples of pooled human milk together with the standard deviation. Comparing these findings with the ones in Table 1 for the same esterified compounds, an increase of the FFAs 12:0 and 15:0 and very slight variations of all the others can be noted. This confirms that medium chain saturated fatty acids (C_{10}–C_{15}) are hydrolyzed by lipoprotein lipase in preference to long chain saturated and polyunsaturated species [27].

3.5. Effect of Holder pasteurization and length of storage on lactate concentration

The L-lactate content in the five milk samples is shown in Fig. 1. The trend shows that the L-lactate concentration increases after pasteurization, remains constant after 35 days storage and decreases after 70 and 90 days storage. The increase following the Holder pasteurization (13%) may be due to the release of that part of L-lactate which, interacting with other substances, cannot be determined in raw milk with the technique used. This process is not reversible since the lactate concentration does not decrease after pasteurization and storage at −20°C for 35 days. The significant decrease of this compound after 70 and 90 days may be, therefore, attributed to a real disappearance of L-lactate, probably due to a degradation process or alteration of its optical properties since the electrode is not sensitive to D-lactate.

Table 5
Mean of free fatty acids in the five samples of pooled human milk

FFA (%, w/w)[a]									
10:0	12:0	14:0	15:0	16:0	16:1	18:0	18:1	18:1*t*	18:2
1.8±0.6	8.5±3.3	6.9±2.5	1.9±0.6	21.8±3.6	1.9±1.0	5.1±2.2	38.5±7.5	2.4±1.1	10.8±3.8

[a] Mean±standard deviation.
Detection limit: 0.5%.

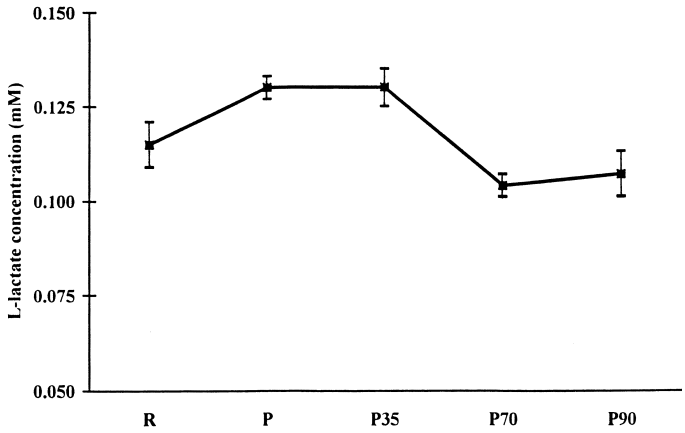

Fig. 1. L-Lactate concentration (m*M*) of the different milk samples; mean of five measurements±standard deviation.

4. Conclusions

The results obtained allows us to draw important conclusions about the effects of collection procedures, Holder pasteurization and storage up to 90 days on the fat content and the L-lactate concentration of pooled human milk. The overlapping of 18:1ω9 and 18:3ω3 peaks is a negative factor and it is probably due to the short column used. On the other hand, the results as a whole are important and valid even though 18:3ω3 was not determined.

In particular, the time lapse from the sample collection to the pasteurization favours hydrolysis of lipids up to 0.5%, much higher than the values normally found in human breast milk (0.1–0.2%) [3,13,26].

Holder pasteurization results in an increase in lipolytic activity and the artefacts increase up to ≈0.9%. This fat alteration is added to the ones pointed out for other compounds [1,2]. The storage at −20°C of pasteurized milk causes, over long periods, an additional hydrolysis of the fats and a slightly decrease in L-lactate and triglyceride content. The treatment reduces the fat by 6% and L-lactate by 7% and 18% with respect to raw and pasteurized milk, respectively. The entire procedure does not seem to substantially change the percentage composition of fatty acids. These data agree with the different composition of FFAs in the five milk samples, even if with dissimilar percentages, since both the free and esterified fatty acids were extracted at the milk pH. In fact, only the low molecular mass FFAs, such as 12:0, 10:0 and, above all, 8:0, are partially extracted by chloroform but the latter is present in raw milk at percentages too low (0.21%) to point out any hydrolysis process.

The decrease of L-lactate suggests a possible hypothesis of the lowering of triglyceride content which may happen through the FFA degradation owing to their higher solubility. This process may consist in *cis–trans* transformations and/or more profound alterations in their structures. In these cases the effective lipolysis would be higher than that found after 90 days (≈1.1%).

In order to limit the alterations of some important compounds of human breast milk, it is useful to reduce the period between collection and pasteurization and to use the pasteurized and stored milk within five weeks of collection.

References

[1] O. Hernell, T. Olivercrona, Biochem. Biophys. Acta 369 (1974) 234.
[2] J.E. Ford, B.A. Low, V.M.E. Marshall, B. Reiter, J. Pediatr. 90 (1977) 29.
[3] J. Bitman, D.L. Wood, N.R. Mehte, P. Hamosh, M. Hamosh, J. Pediatr. Gastroenterol. Nutr. 2 (1983) 521.
[4] B.A. Friend, K.M. Shahani, C.A. Long, E.N. Agel, J. Food Protect. 46 (1983) 330.
[5] C.W. Dill, C.T. Chen, E.S. Alford, R.L. Edwards, R.L. Richter, C. Garza, J. Food Protect. 47 (1984) 690.

[6] C. Galli, A.P. Simopoulos (Eds.), Dietary ω3 and ω6 Fatty Acids: Biological Effects and Nutritional Essentiality, NATO ASI Series, Vol. 171, Plenum Press, New York, 1989.
[7] J. Chappel, M.T. Clandinin, M.A. Mc Vey, Lipids 20 (1985) 216.
[8] M. Lavine, R.M. Clark, J. Pediatr. Gastroenterol. Nutr. 6 (1987) 769.
[9] K. Helrich (Ed.), Official Methods of Analysis of the Association of Official Analytical Chemists; Association of Official Chemists, Arlington, VA, 15th ed., 1990, p. 811.
[10] W.B. Emery III, N.L. Canolty, J.M. Aitchison, W.L. Dunkley, Am. J. Clin. Nutr. 31 (1978) 1127.
[11] J. Folch, M. Lees, G.H. Sloane-Stanley, J. Biol. Chem. 226 (1957) 497.
[12] E.G. Bligh, W.J. Dyer, Can. J. Biochem. Physiol. 37 (1959) 911.
[13] J. Bitman, D. Wood, M. Hamosh, P. Hamosh, R.N. Mehte, Am. J. Clin. Nutr. 38 (1983) 300.
[14] K. Helrich (Ed.), Official Methods of Analysis of the Association of Official Analytical Chemists; Association of Official Chemists, Arlington, VA, 15th edn., 1990, p. 963.
[15] P. Skladal, M. Mascini, C. Salvadori, G. Zannoni, Enz. Microb. Technol. 15 (1993) 108.
[16] A. Amine, G.J. Patriarche, G. Marrazza, M. Mascini, Anal. Chim. Acta 242 (1991) 91.
[17] R.A. Gibson, G.M. Kneebone, Am. J. Clin. Nutr. 34 (1981) 252.
[18] G. Hartzer, M. Haug, I. Dieterich, P.R. Gentner, Am. J. Clin. Nutr. 37 (1983) 612.
[19] B. Koletzko, M. Mrotzek, B. Eng, H.J. Bremer, Am. J. Clin. Nutr. 47 (1988) 954.
[20] D.A. Finley, B. Lonnerdal, K.G. Dewey, L.E. Grivetti, Am. J. Clin. Nutr. 41 (1985) 787.
[21] G. Harzer, M. Haug, Z. Ernahrungswiss 23 (1984) 113.
[22] R.P. Mensink, M.B. Katan, Lancet 1 (1987) 122.
[23] A.C. Frazer, Nature 194 (1962) 908.
[24] W. Droese, E. Pape, H. Stolley, Eur. J. Pediatr. 122 (1976) 57.
[25] H.J. Sternowsky, G. von Shutz, Monatsschr Kinderheilkd 131 (1983) 269.
[26] M.T. Tacconi, M. Salmona, G. Moro, I. Minoli, presented at the International Meeting: The Role of LC-PUFA in Neonatal Nutrition, Florence 14–15 November 1996.
[27] C.S. Wang, A. Kuksis, F. Marganaro, Lipids 17 (1982) 278.