HUMAN MILK STORAGE CONDITIONS IN REGARD TO SAFETY AND OPTIMAL PRESERVATION OF NUTRITIONAL PROPERTIES

BY

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ABSTRACT

This study assessed losses of the linoleic, α-linolenic, arachidonic and docosahexaenoic fatty acids, vitamin B_2 and total vitamin C during storage of human milk, pasteurized and unpasteurized. The volatile compounds' pattern change was monitored. The storage conditions were 8 days at 4°C and 6 months at -20°C and at -80°C, with and without limitation of oxygen.

Fatty acids were analyzed by GC-FID; vitamins - by HPLC; volatile compounds' concentration patterns were obtained using the e-nose machine; ANOVA tests were applied, with the statistical significance assigned to $P<0.05$.

The official recommendations for human milk storage of 5-8 days at 4°C and of 6 or more months at -20°C are appropriate in regard to the analyzed nutrients and can be extended for pasteurized human milk storage. Oxygen limitation and -80°C temperature have no clear benefit for nutrient preservation. The evaluation of the odour cannot be based exclusively on the e-nose analysis.

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DEDICATION

This thesis is dedicated to my family, for their support, patience and belief in me and in all my endeavours, and especially to my beloved husband Daniel, who is my greatest inspiration in everything I do.

LIST OF ABREVIATIONS

AA - L-ascorbic acid

AI - Adequate Intake

ALA – α-linolenic fatty acid

ARA - arachidonic fatty acid

BSSL - bile salt-stimulated lipase

DHA - docosahexaenoic fatty acid

DHAA - dehydroascorbic acid

DI - discrimination index

DTT - DL-dithiothreitol

EDTA - ethylenediaminetetraacetic acid

E-nose - electronic nose

FA - fatty acid

FAD - flavin adenine dinucleotide

FAMEs - fatty acids methyl esters

FFA – free fatty acid

FMN - flavin mononucleotide

GC-FID - gas chromatography coupled with a flame ionization detector

GPx - glutathione peroxidase

HM – human milk

HPLC - high-performance liquid chromatography

LA - linoleic fatty acid

LC-PUFA - long-chain polyunsaturated fatty acid

LPL - lipoprotein lipase

MDA – malon dialdehyde

MOS - metal oxide semiconductor

N/A – not available

NAD - nicotinamide adenine dinucleotide

NICU - Neonatal Intensive Care Unit

NIST - National Institute of Standards & Technology

VCCP - volatile compounds concentration pattern

PCA - principle component analysis

PUFA - polyunsaturated fatty acids

RCFFN - Richardson Centre for Functional Foods and Nutraceuticals

RDA - Recommended Dietary Allowance

SE – standard error of the mean

SRM - Standard Reference Material

WHO - World Health Organization

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CHAPTER 1: LITERATURE REVIEW

1.1. The importance of breastfeeding and of feeding of expressed human milk

Milk is an opaque white liquid produced by the mammary glands of mammals. Human breast milk (HM) refers to the milk produced by a mother to feed her baby.

Diverse and compelling advantages for infants, mothers, families and society from breastfeeding, consuming and use of expressed human milk have been documented by extensive research over the decades. These include health, nutritional, immunologic, developmental, psychological, social, economic and environmental benefits (American Academy of Pediatrics, 1997). Maternal milk has bactericidal capacity, providing defence and protection against infection for newborn infants (Silvestre et al., 2006). Breastfeeding promotes the correct development of jaws, teeth, and speech patterns of the infant, increases cognitive function and bonding with mother (James and Dobson, 2005). Other effects include possible decreased rates of sudden infant death syndrome in the first year of life, reduction in the incidence of insulin-dependent and non-insulin-dependent diabetes mellitus, lymphoma, leukemia, Hodgkin's disease, overweight and obesity, hypercholesterolemia and asthma in children and adults who were breastfed in infancy compared with those who were not breastfed (Armstrong and Reilly, 2002; Bener et al., 2001; Chulada et al., 2003; Davis, 1998; Ford et al., 1993; Grummer-Strawn and Mei, 2004; Owen et al., 2002; Perez-Bravo et al., 1996; Pettitt et al., 1997). Breastfeeding mothers' health also benefits from breastfeeding by decreased postpartum bleeding and more rapid uterine involution attributable to increased concentrations of oxytocin, an earlier return to pre-pregnancy weight, decreased risk of breast cancer and ovarian cancer and possibly decreased risk of osteoporosis in the postmenopausal period (Chua et al,

1994; Dewey et al., 1993; Enger et al., 1998; Newcomb et al., 1994; Paton et al., 2003; Rosenblatt and , 1993). Various economic, family and environmental benefits are also associated with breastfeeding, including the potential for decreased annual health care costs due to the reduction of morbidity, decreased parental employee absenteeism and associated loss of family income, saving of money not spent on formula, the decreased environmental burden for disposal of formula cans and bottles and decreased energy demands for production and transport of artificial feeding products (American Academy of Pediatrics, 2005; Cohen et al., 1995; James and Dobson, 2005).

In a few specific conditions breastfeeding is not recommended for the infant: in cases of classic galactosemia, if the mother has active untreated active tuberculosis, is exposed to radioactive materials or receives certain medications. Adverse effects have been reported on infants of mothers who ingest illicit drugs and/or excessive amounts of alcohol (Aquilina and Winkelman, 2008; American Academy of Pediatrics Committee on Drugs, 2001; Jones et al., 2008; Ressel, 2002; Thompson et al., 2003). HIV-positive mothers are currently recommended by the World Health Organization to breast-feed their infants if replacement feeding, such as infant formula or modified cow's milk, is not "acceptable, feasible, affordable, sustainable and safe". Another two acceptable, though less popular, options for HIV-positive mothers are using an HIV-negative "wet nurse" (another women who can breast-feed the HIV-positive mother's infant) or expressing breast milk and bringing it to a boil before giving it to the baby (WHO et al., 2005). In most cases women infected with HIV, living in developed countries, are recommended to avoid breastfeeding (Jackson et al., 2009). The Canadian Paediatric Society, Dietitians of

Canada and Health Canada (2005) recommend that Canadian mothers who are HIV antibody positive not breastfeed and not use their expressed breast milk.

Breastfeeding is sufficient to support optimal growth and development for approximately the first 6 months of life. Many health organizations recommend exclusive breastfeeding for the first 6 months of life, defined as the consumption of human milk with no supplementation of any type, except for vitamins, minerals and medications (American Academy of Pediatrics, 2005; World Health Organization, 2002). The World Health Organization (WHO) (2002) recommends continuing partial breastfeeding into the second year.

Breast milk is considered an ideal source of nutrients for both term and preterm infants, benefiting host defences, digestion and absorption of nutrients, gastrointestinal function and neurodevelopment (Schanler et al., 1999). Numerous studies have shown that inadequate early nutrition and/or growth has an adverse long-term term influence on development, such as neurodevelopmental impairment, abnormal adipose tissue distribution, insulin resistance, glucose intolerance and higher blood pressure later in life (Ehrenkranz et al., 2006; Hovi et al., 2007; Lapillonne and Jensen, 2009; Uthaya et al., 2005).

1.2. Human milk and premature infants

Special attention must be given to the vulnerable population of preterm babies. A preterm delivery is defined as one that occurs before 37 weeks of gestation. While most newborns are healthy, premature babies are at higher risk of immediate and longer-term health problems, as they miss the last weeks of growth and development in the womb which are crucial to health. Short-term morbidities associated with prematurity include

respiratory distress syndrome (IRDS), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL), necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), sepsis and patent ductus arteriosus (PDA). Long-term morbidities include cerebral palsy, mental retardation, and retinopathy of prematurity (ROP). The risk for these morbidities is directly related to the gestational age and birth weight (Canadian Institute of Health Information, 2004; Goldenberg, 2002).

In developed countries prematurity has a substantial role in all birth-related short- and long-term morbidities and it is the leading cause of neonatal mortality (defined as occurring during the first month of life), accounting for 60–80% of deaths of infants born without congenital anomalies. Approximately 11% of infants are born prematurely in the US and between 5% and 7% in Europe. Despite the advances in obstetric care, the rate of prematurity has not decreased over the past 40 years and even slightly increased in most industrialized countries (Goldenberg, 2002). In part, this is caused by an increasing number of multiple births, which can be a consequence of both maternal aging and Assisted Reproductive Technologies (Health Canada, 2005). Other known risk factors include being younger than 17 or older than 30 years old, previous preterm deliveries, smoking, a low pre-pregnancy weight, very low or high weight gain, black race, single marital status, less than a twelfth grade education level and lower socioeconomic status (Wen et al., 1990).

Though neonatal mortality rates have declined in the recent years, largely because of improved neonatal intensive care and better access to these services, most efforts to prevent preterm labor have not been proven to be effective and most efforts at arresting preterm labor once started have failed (Goldenberg, 2002). Therefore a great effort must

be made after birth to minimize the adverse consequences of prematurity. One area of interest is feeding of human milk. There is a strong evidence that human milk feeding decreases the incidence and/or severity of a wide range of infectious diseases in preterm infants, such as bacterial meningitis, diarrhea, respiratory tract infection, necrotizing enterocolitis (NEC), otitis media, urinary tract infection (UTI) and late-onset sepsis (Blaymore Bier et al., 2002; Duncan et al., 1993; Heinig, 2001; Howie et al., 1990; Hylander et al., 1998; Mårild et al., 2004; Schanler et al., 1999; Takala et al., 1989).

1.3. Composition of human milk

Milks are biological fluids of exceptional complexity containing thousands of compounds, which provide nutritive materials, structural components for cellular membranes and non-nutritive signalling mechanisms, such as immunological systems for host defense (Jensen et al., 1995, p.50). Human milk is a dynamic fluid, changing in composition and providing the infant with the specific nutrients needed at each age (Lawrence, 1994). This makes determination of the exact composition of human milk not feasible. Though infant formula based on cow's milk is modified to match the composition of nutrients in human milk as closely as possible, a perfect match of nutrients cannot be achieved. Certain components' bioavailability, type and quality imitation (e.g. bioavailability of iron) is also challenging. Although the importance of the changes and function of many of the components of human milk is not well understood (e.g. cholesterol, taurine), it is generally believed that they occur for the benefit of the infant (Garza et al., 1993).

Generally, mature human milk contains 3%-5% fat, 0.8%-0.9% protein, 6.9%-7.2% carbohydrate, calculated as lactose, and 0.2% mineral constituents, expressed as ash (Jenness, 1979).

1.3.1. Lipids

The lipids contained in human milk are derived from maternal depot fat stores, endogenous synthesis in the mammary gland and dietary sources (Dotson et al., 1992). Lipids are the key postnatal energy source for growth, metabolism and muscle activity. They are necessary, among others, for the synthesis of cell membrane lipid layers and for the brain and nervous system development. They modulate the activities of membrane bound enzymes and receptors, metabolite exchange, signal transduction and more. Some long-chain fatty acids are precursors of eicosanoids (Jensen, 1999; Koletzko and Rodriguez-Palmero, 1999; Sellmayer and Koletzko, 1999; Uauy et al., 1994). About 98% of the total lipid in human milk are triglycerides (90% of which are fatty acids), cholesterol comprises 0.5%, phospholipids – 0.8% and the rest include traces of other lipids (Jensen, 1992; Jensen, 1999). Human milk fat contains essential nutrients – the lipid soluble vitamins and polyunsaturated fatty acids (PUFA), important in specific biological functions, including the essential linoleic fatty acid (C18:2n-6) and α-linolenic fatty acid (C18:3n-3) (Koletzko and Rodriguez-Palmero, 1999). Over 200 fatty acids (FAs) are known to be present in human milk (Jensen, 1999).

1.3.2. Proteins

Human milk protein content is approximately 1%, which is low compared to other species because of the slower growth rate of humans (Lawrence, 1994). It is more efficiently utilized than the formula milk protein. Human milk protein consists of whey

proteins (enzymes, binding proteins, immunoglobulins) (Lonnerdal and Atkinson, 1995) and casein (curds) in a ratio 3:2. Casein is soft for easier digestion, it allows the milk to have higher levels of calcium and phosphorus (Garza et al., 1993). The predominant whey proteins are α-lactalbumin, which is important for host defense, and the iron-binding protein lactoferrin (Bryan and Zlotkin, 1993; Lawrence, 1994). The essential amino acid pattern of human milk closely resembles that found to be optimal for human infants (Jenness, 1979). Human milk has a low content of phenylalanine and tyrosine, which are difficult to metabolize in the newborn (Lawrence, 1994).

All classes of immunoglobulins are found in human milk, of which the highest concentration is contained in colostrum (Lawrence, 1994). Human milk contains several antimicrobial and antiviral mechanisms. It is rich in secretory IgA, which has antibody activity against bacteria and viruses, and in lactoferrin, which traps iron and thus inhibits growth of iron-requiring bacteria. Bacteriostatic function is attributed also to folic acid, cyanocobalamin, various enzymes (including lactoperoxidase and lysozyme) as well as to some viable cells. The antiviral agents in human milk include phagocytic cells and antiviral IgA antibodies (Ford et al., 1977; Snoeck et al., 2006; Welsh et al., 1978). More than 20 types of enzymes are found in human milk, helping development and digestion, such as protease, lipase and amylase (Lawrence, 1989).

1.3.3. Carbohydrates

The principal sugar of human milk is lactose. Glucose, galactose and more than 30 other oligosaccharides are also present, some of which may function to control the intestinal flora because of their ability to promote growth of certain strains of lactobacilli

(Jenness, 1979). Lactose is important for enhancing calcium absorption, which is low in human milk (Garza et al., 1993).

The prevalence of lactose intolerance is much higher in formula-fed infants compared with breast-fed infants. That may be attributed to the difference in bacterial flora colonization or to the immunity passed on through breast milk (Garza et al., 1993).

1.3.4. Vitamins

Water-soluble vitamins in human milk include ascorbic acid (vitamin C), thiamin (vitamin B_1), riboflavin (vitamin B_2), niacin, pyridoxine (vitamin B_6), folate, pantothenite, biotin and vitamin B_{12} (Picciano, 1995). Human milk is rich in the fat-soluble vitamins A and E (Garza et al., 1993).

Generally, all of the vitamins, except vitamin K, are found in human milk in nutritionally significant concentrations (Jenness, 1979). Vitamin D deficiency may occur in breast-fed infants limitedly exposed to sunlight, as reported levels of vitamin D in human milk are below the minimum amount required to prevent rickets and ensure proper bone mineralization. Vitamin B_{12} is often deficient in milk of vegetarian mothers (Garza et al., 1993; Lammi-Keefe, 1995).

1.3.5. Minerals and trace elements

The principal minerals in human milk are sodium, potassium, calcium, magnesium, phosphorus and chlorine. Iron, copper, and zinc contents vary considerably. A long list of other trace elements has been reported (Jenness, 1979). Though the iron content of human milk is low, it is very well absorbed. Calcium is also of high availability. Iron deficiency is rare before 4 to 6 months in infants who are exclusively breast-fed because

of extensive iron stores in the liver and hemoglobin present at birth (Dallman, 1988; Garza et al., 1993).

Trace elements deficiency is very rare in breast-fed infants (Fransson and Lonnerdal, 1983). Supplementation of the lactating mother is generally not recommended, as excess intake of many elements may be associated with a risk of toxicity to the nursing infant (Institute of Medicine, 1991).

1.3.6. Supplementation

Though breast milk is recognized as the optimal source of nutrition for the full-term neonate, it may not provide adequate calories, protein or minerals to meet the increased demands of premature infants. The nutrient content of human milk of mothers who delivered prematurely provides insufficient quantities of sodium, phosphate, calcium and protein to meet the estimated needs of the infant (Schanler et al., 1999). Premature infants are born with low skeletal stores of calcium and phosphate and have very high requirements for these minerals in order to attain adequate postnatal skeletal growth. Therefore commercially-produced multicomponent fortifiers are available for the supplementation of breast milk for premature infants. These varying in composition mixtures provide additional nutrients in the form of calcium, phosphate and trace minerals along with protein and carbohydrate (Kuschel and Harding, 2004). Human milk fed to preterm infants is routinely supplemented in order to facilitate growth and development (Diehl-Jones and Askin, 2004).

Although there is sufficient evidence demonstrating that fortification of human milk is associated with short-term improvements in weight gain and linear and head growth, no clear effect has been shown on bone mineral content. To date there is no evidence that

these short-term gains in growth lead to any demonstrable long-term benefits, though this may well be related to the absence of follow-up in almost all studies (Kuschel and Harding, 2004).

1.4. Changes in human milk

Human milk is a dynamic fluid, constantly changing in composition (Lawrence, 1994) under the influence of many factors. Nevertheless, according to the Institute of Medicine (1991), with very few exceptions, mothers are able to produce milk of sufficient quantity and quality to support growth and promote health of their infants, even when the mother's supply of nutrients and energy is limited.

1.4.1. Stage of lactation

Iron concentration of human milk is the highest during the first few days after birth and diminishes with the progression of lactation (Casey et al., 1995). Calcium increases markedly during the first few days postpartum and then falls gradually stabilizing for the first 3 months postpartum, then falls gradually and continuously (Atkinson et al., 1995). Its concentration is not affected by manipulations of maternal dietary calcium and vitamin D (Kent et al., 2009). Sodium, potassium and chloride decline significantly during the first month (Atkinson at al, 1986). Zinc and copper concentrations are in significant decline over approximately the first 4 weeks of lactation (Vuori and Kuitunen, 1979). Zinc concentration declines rapidly during the first few months postpartum and more slowly thereafter (Brown et al., 2009). Magnesium falls gradually throughout lactation. Free phosphate and total phosphorus levels vary (Atkinson et al., 1995).

The most dramatic changes in human milk components occur during the transition between the three phases of the milk production: colostrum, transition and mature milk

(Lawrence, 1994). Colostrum presents during approximately 5 days post-partum. Its main functions are to provide substances for rapid growth, to protect the gastrointestinal tract and to assist in increasing the level of bifidobacteria of the gut. In colostrum, the cholesterol, sodium and chloride content, concentration of protein, most immunoglobulins and lactoferrin are the highest among the 3 phases of milk production, but the fat content is lower (2%) (Atkinson et al., 1995; Lawrence, 1994). Transitional milk composition (6-15 days postpartum) varies markedly among mothers. Generally, immunoglobulin levels decrease, fat, lactose and water-soluble vitamins contents increase compared to colostrum (Lawrence, 1994). Mature milk is the most diluted (Lawrence, 1994). While the concentration of many components in mature milk, such as n-3 and n-6 polyunsaturated fatty acids, protein and water-soluble vitamins (except for vitamin B_{12}) declines during the first 3-6 months of lactation reaching a low plateau in late stages, the concentration of some other components, such as the antimicrobial protein lysozyme, increases (Guesnet at al., 1993; Mitoulas et al., 2002; Picciano, 1995; Prentice, 1995).

1.4.2. During a nursing event

The lipid content of human milk consistently rises during a nursing (Jensen, 1995). Foremilk (milk at the beginning of a feeding) is thinner and about 4 times lower in fat than the hindmilk (milk in the middle and the end of a feeding) (Lawrence, 1994). Lactose level decreases, while protein and minerals levels remain constant (Neville et al., 1984).

1.4.3. Within-day variations

The lipid content of human milk varies considerably throughout the day, being influenced by the pattern (degree of emptying of the breast allowing carryover of the

high-fat hindmilk into the next nursing) and by the frequency (interval between nursings) of breast-feedings (Prentice, 1995; Jensen, 1995). Lactose levels remain constant through the day (Garza et al., 1993).

1.4.4. Maternal age

Studies show that the milk of young women has higher concentrations of several constituents, such as protein and fat, compared to the milk of older mothers (Prentice, 1995).

1.4.5. Term milk versus preterm milk

Studies report differences in composition between milk of mothers who delivered prematurely (preterm milk) and mothers who had a term delivery (term milk). Preterm milk contains greater concentration of lipids, vitamins, energy and some minerals and trace elements compared to term milk at similar lactational stages. The causes of the composition differences may be different maternal hormonal profile in the postpartum period, an immature morphology of the mammary gland, various artifacts of perinatal events associated with premature birth or a combination of those factors (Atkinson, 1995).

1.4.6. Maternal diet

Fat, lactose and protein contents of human milk are not affected by the maternal intake (Kent, 2007). Although the total fat content of breast milk is independent of the mother's diet, the proportions of the different fatty acids vary according to the types of fat the mother is consuming (Sanders and Reddy, 1992). For example, the docosahexaenoic fatty acid (DHA) levels in human milk are the lowest in countries with a relatively high meat intake, as in the USA, and the highest in populations where most animal foods are provided as fish, as in the diet of Canadian Inuits (Hamosh and Salem, 1998). Enhanced carbohydrate consumption increases the levels of decanoic acid (C10:0) and lauric acid

(C12:0) in human breast milk (Koletzko et al., 1991). There is a relationship between the content of vitamins A, B_1, B_2, B_5, B_6, B_{12}, D and E, selenium and iodine in human milk and the maternal diet (Kent, 2007).

1.4.7. Study methodology

Reported absolute concentrations of human milk constituents may differ due to the differences in analytical methods, calibration variations and lack of interlaboratory standardization (Prentice, 1995).

1.4.8. Other factors affecting human milk composition

Changes in milk volume and composition may occur as a result of maternal metabolic diseases, such as insulin-dependent diabetes mellitus (Jackson, 1994). Certain medications may also make an impact, as well as infant's birth weight. In developing countries human milk composition is influenced by the season of the year. Fat content of human milk decreases with the increase in parity number (Atkinson, 1995), increases with elevation in mother's adiposity and decreases in malnourished women (Jensen, 1996). Storage of expressed milk and pasteurization also affect milk composition (Atkinson, 1995). Different nutrient concentrations are obtained during different times of the day and within a feeding, there may be even differences in composition between pumped milk and directly nursed milk (Bryan and Zlotkin, 1993).

1.5. Stored human milk

1.5.1. Why human milk is stored

Not all babies are breast fed due to illnesses or abnormalities, separation and other reasons, and therefore expressed milk is fed from a bottle for these babies. Mothers may

express their milk for their own comfort in situations of sore nipples, postpartum breast engorgement, in order to increase milk supply, to leave milk if they are away from their baby and also in situations of adoption or surrogacy (Arnold, 1990; Auerbach and Avery, 1981; Biervliet et al, 2001; Buchko et al., 1994; Chapman et al., 2001; Hills-Bonczyk et al., 1993; Meserve, 1982). Expressed breast milk, often supplemented, is also widely used in hospitals to serve the needs of the premature, small for gestational age and normal term infants who may be ill and cannot suckle (Berkow et al., 1984; Diehl-Jones and Askin, 2004). Expressed breast milk can be donated to milk banks, from where, following pasteurization and screening, it is distributed for the feeding of infants with medical conditions (such as formula intolerance or feeding issues related to prematurity), for adopted infants or for those who are not able to get their own mother's milk (Human Milk Banking Association of North America, 2010). Because breast milk is recommended for exclusive infant feeding up to 6 months of age and for further complementary feeding (World Health Organization, 2001), some breastfeeding mothers working out of the home express their milk, which is later fed to the infant from a bottle (Jensen, 1995; Michalski et al., 2008). According to the mother's choice, breast milk can be expressed by hand or using an electric, battery operated or manual Breast Pump (Public Health, Grey Bruce Health Unit, 2010).

1.5.2. Research and recommendations for storage of expressed human milk

The use of expressed human milk poses specific problems regarding handling procedures, as it immediately becomes susceptible to food degradation processes (Berkow et al., 1984; Olson, 1968). Low-temperature storage may retard microbial growth and delay some changes in the physiochemical character of milk (Jensen, 1995),

therefore various storage conditions protocols, recommended for human milk, have been published. Official recommendations for storage of unpasteurized expressed human milk fed to healthy full term infants can be found in Table 1.1.

Table 1.1: Current official protocols for storage of expressed human milk for healthy full term infants

Institution	Storage Temperature				
	Room temp. (19 - 25°C)	4°C	-15°C	-18°C	-20°C
Winnipeg Public Health – Government of Manitoba (2009)	N/A	8 d	2 w	6 m	12 m
City of Toronto Public Health (2009)	6-8 hr	5 d	2 w	3-6 m	6-12 m
Government of British Columbia (2005)	4 hr	3 d	1 m	6 m	6-12 m
Government of Nova Scotia (2008)	4-6 hr	8 d	2 w	3-4 m	>6 m
La Leche League International (2009)	6 hr	8 d	N/A	12 m	12 m
Academy of Breastfeeding Medicine (2004)	6-8 hr	5 d	2 w	3-6 m	6-12 m
American Dietetic Association (2003)	N/A	48 hr	N/A	3 m	12 m
Children's Hospital Central California (2008)	10 hr	7 d	2-4 w	3-4 m	>6 m
Lucile Packard Foundation for Children's Health (2008)	10 hr	7 d	2-4 w	3-4 m	>6 m
Florida Department of Health: Hernando County Health Department (2009)	5 hr	5 d	N/A	3-4 m	>6 m
Australian Breastfeeding Association (2005)	6-8 hr	3-5 d	2 w	3 m	6-12 m
Government of Western Australia, Department of Health (2006)	4 hr	48 hr	N/A	3 m	12 m

Table 1.1 – Ctd: Current official protocols for storage of expressed human milk for healthy full term infants

Institution	Storage Temperature				
	Room temp. (19-25°C)	**4°C**	**-15°C**	**-18°C**	**-20°C**
South African Paediatric Association (2008)	2 hr	48 hr	N/A	N/A	3 m
Palo Alto Medical Foundation (2009)	27°C: 4 hr 21°C: 10 hr	5 d	2 w	3 m	6 m
Israel Ministry of Health (2005)	19-22°C: 10 hr 22-25°C: 4 hr	8 d	2 w	3-4 m	>6 m

Many studies have been conducted in order to question current recommendations for the expressed human milk storage, concentrating on various factors which may have an influence on shelf life length, such as bacterial growth, bacteriostatic activity preservation, nutrient contents and more. A review of the studies can be found in Appendix 1.

Human milk pasteurized using Holder pasteurization conditions is recommended to be stored at -20°C. There is no agreement regarding the acceptable storage time. Storage for less than 3 months is recommended for milk targeted for feeding of preterm neonates. If the pasteurized HM is refrigerated (4°C), the maximum storage period is 24 hours (Arslanoglu et al., 2010).

1.5.3. Preparation of stored human milk for feeding

The Canadian Paediatric Society (2009) recommends thawing of human milk in the refrigerator, subsequent warming - by placing the container of expressed milk into a container of warm water, followed by shaking to mix any separated layers.

Microwaving should not be used to thaw and warm frozen human milk for feeding because of the danger of creating hot spots which may harm the baby and because of the

destruction of nutrients, such as IgA and lysozyme, due to the excess heating. The growth of E.coli is also increased following microwave heating, probably due to the loss of the anti-infective factors (Centers for Disease Control and Prevention, 2010; Jensen, 1995; Quan et al., 1992).

1.5.4. Reduction of oxygen availability

Oxygen availability has an impact on the lipid oxidation rate occurring in the stored product. Limiting the availability of oxygen using vacuum packaging or nitrogen flushing slows down oxidative reactions (Singh and Cadwallader, 2000). Microbial growth is also reduced when oxygen is limited. However, off-flavours may develop under these conditions due to the accumulation of ethanol, acetaldehyde and other volatiles (Mazza and Jayas, 2001).

1.5.5. Pasteurization of human milk

International best practice requires pasteurization of donor human milk prior to feeding to recipients (Hartmann et al., 2007). The only purpose of pasteurization is to destroy pathogenic microorganisms (Jensen, 1995), as freshly collected breast milk is rarely sterile and normally contains bacteria originating from the maternal skin and nipple duct micro-flora. Generally, the ability of these organisms to infect the suckling infant is uncertain and probably minimal (Bjorksten et al., 1980). However potential pathogens also may be found (Olowe et al., 1995), which produce lipases, proteases and decarboxylases causing damage to antimicrobial proteins or converting free amino-acids into toxic amines (Bjorksten et al., 1980). In general, heavy bacterial contamination at the time of milk collection would be undesirable and should be avoided (Olowe et al., 1995).

Conditions commonly used for pasteurization of human milk are 62.5^{o}C for 30 minutes – a process named "Holder pasteurization" (Tully et al., 2001; Arslanoglu et al., 2010). Holder pasteurization allows a good compromise between microbiological safety and nutritional and biological quality of the milk: is destroys the pathogens in milk, including *M. tuberculosis* and vegetative cells of *Bacillus cereus*, as well as some viruses, such as HIV, HTVL 1-2, Cytomegalovirus, Herpes Simplex and Rubella. These results are not possible to achieve effectively at lower pasteurization temperature, which is currently considered unacceptable for this purpose (Arslanoglu et al., 2010).

Holder pasteurization conditions maintain some bactericidal activity of the milk against *E. coli* as well as key nutritional factors (oligosaccharides, lactose, LC-PUFAs, fatty acids, gangliosides, protein, sulfur amino acids), other biological factors (amylases, EFG) and vitamins A, D, E, B_2, B_{12}, biotin, niacin and pantothenic acid (Arslanoglu et al., 2010; Fidler et al., 1998; Friend et al., 1983; Henderson et al., 1998; Jansen, 1995; Van Zoeren-Grobben et al., 1987). Absorption of nitrogen, calcium, phosphorous and sodium is not affected (Wiliamson et al., 1978).

Despite its indisputable advantages, heat treatment has an adverse effect on some of the milk components. Studies show that pasteurization inactivates milk lipoprotein lipase and BSSL (an enzyme of major importance to the infant, assisting in the hydrolysis and absorption of milk fat in the small intestine), resulting in a reduced absorption of fat in preterm infants (Jensen, 1995; Wiliamson et al., 1978). Levels of vitamin C, folic acid, vitamin B_6 and thiamin decrease (Jensen, 1995; Van Zoeren-Grobben et al., 1987; Wiliamson et al., 1978). Some biologically active, immunologic and anti-infective factors are affected: reports exist about reduction of IgA levels and activities, IgG, lysozyme,

lactoferrin levels and activities, lymphocytes, growth factors, cytokines, lipase level and activity and destruction of IgM (Arslanoglu et al., 2010; Silvestre et al., 2008).

Rapid pasteurization at 72^{o}C for 5 or 15 sec reaches better compromise between microbiological safety and nutritional and biological quality of human milk, but it requires special equipment, which is currently available only at the industrial level (Arslanoglu et al., 2010).

Recently, a home-made high temperature heat treatment method, known as "Flash-Heat treatment method", has been suggested for HIV-positive mothers in developing countries. According to this method, 50 mL of breast milk are poured in an uncovered glass peanut butter jar, which is placed in 450 mL of water in an aluminum pan. The construction is heated over a flame until the water reaches 100°C and is at a rolling boil. The breast milk is then immediately removed from the water. Typically, the milk reaches the temperature of 72.9^{o}C in this process (Israel-Ballard et al., 2006).

1.5.6. Storage containers for expressed human milk

Expressed human milk should be stored in proper containers in order to minimize the potential harm to milk nutrients and to eliminate the possibility of contamination. The modern plastic industry offers a variety of materials for storage containers, some of which are more, and some of which are less suitable for expressed HM storage.

Polyethylene bags should not be used to store expressed HM because of the potential for contamination, loss of cellular components, sIgA specific for E.coli polysaccharides and fat, which adheres to the walls (Goldblum et al., 1981; Hamosh, 1994). Polyethylene bags do not withstand the expansion of milk during freezing and frequently leak upon

thawing. They are fragile, easily punctured and may leak through the seams' seals (Arnold, 1995).

Polypropylene and stainless steel containers may become scratched, accumulating milk in the scratches, thus enhancing potential bacterial contamination possibility (Arnold, 1995). In addition, storage in polypropylene bottles results in some loss of lactoferrin and lysozyme (over 24 hour period), loss of cellular components and some loss of vitamin C (Goldblum et al., 1981; Van Zoeren-Grobben et al., 1987).

Polycarbonate baby bottles have been prohibited by the Government of Canada since March 11, 2010. This plastic material contains bisphenol A, a substance which is suspected to pose a health risk (Government of Canada, 2010).

Glass containers or hard plastic bottles with tight-fitting lids for prevention of leakage and oxidation of components are preferred for storage of expressed human milk. They should be opaque, to prevent photodegradation of nutrients in milk (Arnold, 1995). However there have been reports of greater loss of leukocytes in glass containers, compared to plastic, as a result of cells adhering to the walls of the container (Goldblum et al., 1981).

1.6. Objectives of this study

Nutrients, which concentrations are the objectives of this study, are linoleic fatty acid (18:2n-6, LA), α-linolenic fatty acid (18:3n-3, ALA), arachidonic fatty acid (20:4n-6, ARA), docosahexaenoic fatty acid (22:6n-3, DHA), vitamin C, riboflavin (vitamin B_2) and aroma of expressed human milk, as it may affect milk flavour acceptance and therefore nutrient intake.

1.6.1. Linoleic fatty acid and α-linolenic fatty acid

Linoleic fatty acid (18:2n-6, LA) and α-linolenic fatty acid (18:3n-3, ALA) are essential long-chain polyunsaturated fatty acids (LC-PUFA). They are found in storage lipids, cell membrane phospholipids, intracellular cholesterol esters and plasma lipids (Heird and Lapillonne, 2005) and play an important structural role as membrane constituents. Both have marked perinatal accumulation in membrane-rich tissues such as the brain and the retina (Martinez, 1992). On average, LA comprises approximately 12% of the total fatty acid content of the milk of women consuming a typical western diet and ALA comprises about 1% (Jensen, 1999).

During pregnancy LC-PUFA are transported actively to the fetus from maternal plasma via the placenta (Dutta-Roy, 2000). The last trimester of pregnancy is a period of rapid growth, during which there is a rapid development of the neuronal system, leading to a significant increase in lipid accretion in the developing brain (Koletzko and Rodriguez-Palmero, 1999). The infant who is born early in the third trimester of pregnancy receives less LC-PUFA prior to birth, has very little adipose tissue and decreased reserves of essential fatty acids in addition to having a higher growth rate compared with the infant who is born at term (Salem, 1989).

1.6.2. Docosahexaenoic fatty acid and arachidonic fatty acid

Docosahexaenoic fatty acid (DHA, 22:6n-3) and arachidonic fatty acid (ARA, 20:4n-6) are metabolites of α-linolenic acid and linoleic acid, respectively. Human milk contains small amounts of DHA and ARA (Heird and Lapillonne, 2005). While the ARA content in human milk is nearly constant, averaging about 0.45% of total fatty acids, DHA level varies between 0.1% - 3.8% with the diet (Fleith and Clandinin, 2005). As

DHA and ARA are the major LC-PUFA components in membranes phospholipids of the brain and retina, both fatty acids are considered to be needed for optimal tissue development (Fleith and Clandinin, 2005; Heird and Lapillonne, 2005).

The role of DHA in the infant diet has been emphasized because DHA is selectively enriched in a few particular membrane lipids, which include the glycerophospholipids of the visual elements of the retina and brain grey matter. DHA plays critical roles in the visual and neural systems, including protection from oxidative damage, neurogenesis, neurotransmitter metabolism, membrane protein functions, transmission of the visual signals and regulation of gene expression (Innis, 2007). Decreased DHA status in infancy is associated with poorer retinal development and visual function, as retinal DHA is important for normal photochemical activity of the visual pigment rhodopsin (Birch et al., 1992; Litman and Mitchell, 1996).

ARA is present in all cell membranes and is especially abundant in the central nervous system (Mitmesser and Jensen, 2007). ARA is essential for normal growth and is critically important through its role in cell signalling and as a precursor to series 2 eicosanoids and series 3 leukotrienes, which also play a role in synaptic transmission (Innis, 2003).

Both term and preterm infants can convert LA to ARA and ALA to DHA. However, the amount of the synthesized LC-PUFA is a function of both the pool size of the precursors and the rate of synthesis, which is relatively low in the postnatal period (Heird and Lapillonne, 2005; Uauy et al., 2000). Consequently preterm infants are considered to be particularly vulnerable to LC-PUFA deficiency and expected to have higher postnatal

LC-PUFA requirements, having lower body stores and higher growth rate compared to full term infants (Genzel-Boroviczeny et al., 1997; Salem, 1989).

Due to their importance in infants' development, LC-PUFA have been added to infant formulas, the most important of which are DHA and ARA (Makrides et al., 2005).

1.6.3. Vitamin C

The term vitamin C is used as the generic descriptor for all compounds exhibiting the qualitative biological activity of ascorbic acid (AA). The principal natural compound with vitamin C activity is L-ascorbic acid. In the body, L-ascorbic acid is enzymatically oxidized to dehydroascorbic acid (DHAA) in an easy and reversible reaction. Therefore dehydroascorbic acid also exhibits full biological activity, though ascorbic acid is the principal biologically active form (Ball, 2006).

The functions of AA are based primarily on its properties as a reversible biological antioxidant (Ball, 2006). It plays a role as a redox cofactor and catalyst in a broad array of biochemical reactions and processes and directly moderates oxidative stress by neutralizing free radicals. AA readily scavenges reactive oxygen and nitrogen species, such as superoxide and hydroperoxyl radicals, aqueous peroxyl radicals, singlet oxygen, ozone, peroxynitrite, nitrogen dioxide, nitroxide radicals, and hypochlorous acid. Excesses of such products, which are generated during normal metabolism and rapid growth, have been associated with lipid, DNA, and protein oxidation. Although the pro-oxidant effects of ascorbic acid *in vitro* suggest a possible role for vitamin C in mutagenesis, investigations demonstrate that vitamin C is most often associated with decreased frequency of DNA mutations (Francis et al., 2008; Johnston et al., 2007). AA increases collagen accumulation and alkaline phosphatase activity in osteogenic cells

thereby affecting bone formation. AA is required for the growth and repair of all tissues owing in part to its role as a cofactor for prolyl and lysyl hydroxylase activity, which is essential for collagen formation and wound healing (Johnston et al., 2007). It is known to spare vitamin E, which is another essential nutrient required by infants for healthy development (Bertinato et al., 2007; Francis et al., 2008).

Ascorbic acid is readily soluble in water (Seib, 1985). It is very susceptible to chemical and enzymatic oxidation during the processing, storage, and cooking of food (Ball, 2006). Both AA and DHAA have low stability at mildly acidic and neutral pH (Musulin and King, 1936). Riboflavin acts as a sensitizer for destruction of AA by light, and its removal from milk prevents the light induced oxidation of ascorbic acid (Hartman and, 1965).

Plasma vitamin C concentrations in newborn term infants are ~120 mmol/L, which are approximately 2-3 times higher than in adults (Gopinathan et al., 1994; Block et al., 1999). Ascorbate levels are significantly higher in premature infants at birth in comparison to term babies (Gopinathan et al., 1994).

Human milk from well-nourished US women can be expected to contain, on average, approximately 100 mg/liter of AA (Picciano, 1995), which is enough to prevent scurvy in breast-fed infants (Matano and Kato, 1967). Vitamin C present in human milk plays several biochemical roles linked to the functioning of the immune system. It helps in the maintenance of a natural barrier against infection, stimulates leukocytes for their phagocytic and antimicrobial activity, augments antibody production and complement levels and also enhances synthesis of interferon. Ascorbic acid is not stored in the body,

making regular intake necessary. For growth, development and survival, infants need an optimum supply of ascorbic acid (Francis et al., 2008; Romeu-Nadal et al., 2006).

1.6.4. Riboflavin (vitamin B_2)

Riboflavin (vitamin B_2) is a water soluble vitamin, exerting its biological function through two flavin coenzymes: flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Free riboflavin is naturally present in foods along with protein-bound FMN and FAD. The coenzymes participate in oxidation–reduction reactions in many metabolic pathways. They are necessary for specific reactions in the interconversion of vitamin B_6 vitamers and folate vitamers and in the synthesis of nicotinamide adenine dinucleotide (NAD) from tryptophan (Ball, 2006). As FAD is part of the respiratory chain, riboflavin is central to energy production. Other major functions of riboflavin include drug and steroid metabolism and lipid metabolism (McCormick, 1994). There have been reports indicating that riboflavin deficiency is associated with compromised oxidant defense and furthermore that supplementation of riboflavin and its active analogs improves oxidant status (Miyazawa et al., 1984). A primary deficiency of dietary riboflavin has wide implications for other vitamins, as flavin coenzymes are involved in the metabolism of folic acid, pyridoxine, vitamin K, niacin, and vitamin D (Rivlin, 1994).

Riboflavin is light-sensitive, therefore appreciable amounts may be lost with exposure to UV light, particularly during cooking and processing (Wanner, 1960, Ball, 2006).

Typical values for riboflavin in human milk are 400-600 μg/liter (Picciano, 1995). Breast-milk riboflavin concentrations are fairly sensitive to maternal riboflavin intake,

and can be moderately increased by riboflavin supplementation of the mother when natural intake is low (Nail et al., 1980).

1.6.5. Aroma

Aroma is the mixture of volatiles present in the headspace of a product. It is an important quality for many food products in addition to their nutritional properties. Generally, low molecular weight aldehydes, ketones and fatty acids are responsible for most off-flavours observed in foods and beverages (Ampuero and Bosset, 2003; Xu, 2006).

The unperturbed human milk system delivers energy, nutrients, protective components and metabolic messages to the infant. However, the emulsion is thermodynamically unstable, maintaining its original compartmentation for the few minutes of nursing. As a result, off-flavours may develop during human milk storage to the extent that the infant will not consume stored milk (Jensen, 1999).

The headspace of milk typically presents a complex mixture of organic volatiles (e.g. acetone, hexanal, toluene, limonene, chloroform, heptanal etc.) at varying concentrations (Ampuero and Bosset, 2003). Oxidation off-flavours in milk originate mostly from photo-oxidation, heat, enzymatic activity and bacterial metabolism, which can develop some toxicity (Ampuero and Bosset, 2003; Marsili, 2000). Exposure of milk to light results in a burnt oxidized flavour during the first 2-3 days, probably due to the degradation of sulphur-containing amino acids from the whey. Afterwards, a persistent metallic, cardboard-like off-flavour occurs, attributed to the autoxidation of unsaturated fatty acids by the formation of free radicals induced by the light. Hexanal and pentanal, typical secondary oxidation products which are formed during light-induced oxidation of

unsaturated lipids in milk, are thought to be responsible for the development of light-induced off-flavour in milk and dairy products (Marsili, 1999; Mestdagh et al., 2005). Exposure of milk to heat results in typical boiled off-flavour, probably due to the formation of sulphur compounds (Ampuero and Bosset, 2003).

Odour sensations are induced by the interaction of odorants with specialized receptors in the olfactory epithelium in the top of the nasal cavity. The induced signals are transmitted to the olfactory bulb and ultimately to the brain. In air-breathing animals, including humans, odorants are volatile, hydrophobic compounds that have molecular weights of less than 300 daltons. Odorants vary widely in structure and include many chemical classes including organic acids, alcohols, aldehydes, amides, amines, aromatics, esters, ethers, fixed gases, halogenated hydrocarbons, hydrocarbons, ketones, nitriles, other nitrogen-containing compounds, phenols, and sulfur-containing compounds (Pearce et al., 2003).

The sense of smell is a remarkably sensitive system that responds to very low concentrations of chemicals. Compounds can be detected at concentrations in the low parts-per-billion (ppb) and even low parts-per-trillion (ppt). Most odour sensations are produced by mixtures of hundreds of odorants rather than by a single compound (Pearce et al., 2003).

Humans have several hundred distinct genes that encode olfactory receptor proteins. This extremely broad range of receptor types permits the detection of odour sources comprised of unpredictable mixtures of molecular species (Pearce et al., 2003).

1.6.6. Adequate Intake (AI)

If sufficient or adequate scientific evidence is not available to establish Recommended Dietary Allowance (RDA), Adequate Intake (AI) is usually derived for the nutrient instead. AI is a recommended average daily intake level for a nutrient, based on observed or experimentally determined approximations or estimates of the nutrient intake in a group of apparently healthy people, who are assumed to be maintaining an adequate nutritional state, such as normal growth (Otten et al., 2006).

The average intake by full-term infants born to healthy, well-nourished mothers and exclusively fed human milk, has been adopted as the primary basis for deriving the AI for most nutrients during the first 6 months of life (with the only exception of vitamin D). During the second 6 months of life, solid foods complement the HM feeding. Generally, nutrients needs are considered to remain the same, except for some nutrients such as iron and zinc, for which the requirements are increased (RDA values derived) (Otten et al., 2006).

AI values for pertinent nutrients for infants under 12 months of age, according to the United States Department of Agriculture, National Academy of Sciences, Institute of Medicine, Food and Nutrition Board (2009), are presented in Table 1.2.

Table 1.2: Adequate Intake values for pertinent nutrients for infants younger than 12 months of age

Nutrient	AI for 0-6 months	AI for 7-12 months
n-3 PUFA (α-linolenic acid)	0.5 gr/day	0.5 gr/day
n-6 PUFA (linoleic acid)	4.4 gr/day	4.6 gr/day
Vitamin C	40 mg/day	50 mg/day
Vitamin B_2	0.3 mg/day	0.4 mg/day

1.6.7. The change in the nutrients contents during storage

Immediately after expression human milk becomes susceptible to food degradation processes. During storage, concentrations of many components decline (Olson, 1968). According to the literature, the following changes happen to the LA, ALA, DHA, ARA, vitamin C and vitamin B_2 during the storage of human milk:

Storage of human milk in refrigeration conditions (4^oC):

- The contents of vitamin C (Bank et al., 1985; Buss et al., 2001; Ezz El Din et al., 2004) and vitamin B_2 (Fanelli et al., 1985) decrease in the presence of oxygen (Gubler et al., 1984).
- LC-PUFA concentration decreases due to oxidation (Berkow et al, 1984, Huang et al, 2004). Although according to other sources (Slutzah et al., 2010; Tacken et al, 2009), the fat integrity of fresh HM is not affected by storage at 4^oC.
- The odour profile changes during storage, as human milk system is a thermodynamically unstable emulsion, maintaining its original compartmentation only for the few minutes of nursing. Therefore off-flavours may develop during human milk storage as a result of photo-oxidation, enzymatic activity and bacterial metabolism (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000). The lipolysis of the milk fat also results in the odour profile alteration (Berkow et al, 1984).

Storage of human milk in freezing conditions (at -20^oC):

- Vitamin C levels decrease (Buss et al., 2001; Picciano, 1995, p. 675-688). Controversial reports exist regarding vitamin B_2 stability in frozen food products, generally reporting fairly high retention (Engler and Bowers, 1976).

- Total lipid fatty acid level and relative % of each fatty acid are preserved (Friend et al, 1983; Reynolds et al., 1982).
- The odour profile changes during storage, as explained above (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000).

Storage of human milk in freezing conditions (at -80^{o}C):

- Although -80^{o}C storage temperature is referred to as a gold standard for milk conservation (Arslanoglu et al., 2010), therefore better preservation of nutrients at this temperature might be expected, solubility of gases, especially oxygen, in fat or water decreases by almost 25% for each 10^{o}C rise in temperature (Singh and Cadwallader, 2000), thus the oxidation of nutrients may happen slower at -20^{o}C compared with -80^{o}C.
- The lipolysis of milk fat does not occur (Berkow et al., 1984; Jensen, 1999).
- The odour changes during storage, as explained above (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000).

Holder pasteurization (62.5^{o}C for 30 minutes):

- Vitamin C is affected by the heat treatment (Van Zoeren-Grobben et al, 1987), opposed to the vitamin B_2 levels (Van Zoeren-Grobben et al, 1987).
- The fat content (Fidler et al., 1998) and the fatty acids composition (Henderson et al., 1998; Arslanoglu et al., 2010) are not affected.
- Exposure of milk to heat results in an adverse effect on some of the milk components and in the formation of new compounds (Ampuero and Bosset, 2003; Atkinson, 1995), which lead to alteration of the flavour profile.

Storage with limitation of oxygen:

- Nutrients are better protected from decomposition, as oxygen limitation slows down oxidative reactions (Singh and Cadwallader, 2000).
- The development of off-odours may happen, due to the accumulation of ethanol, acetaldehyde and other volatiles (Mazza and Jayas, 2001).

CHAPTER 2: JUSTIFICATION

The purpose of this study was to address important issues in regard to the storage of human milk.

2.1. Problem statement

Expressed human milk is considered as a regular food product, susceptible to regular food degradation processes, thus its quality decreases with passage of storage regardless of the preservation methods used and the control of storage conditions (Olson, 1968). Many oxidized products of rancidity are considered to be unhealthy and contribute to the development of off-flavours in the milk (Eskin and Przybylski, 2001). Therefore, the use of expressed human milk poses specific problems regarding handling procedures, as human milk storage must preserve its protective, digestive, inductive and nutrient carrier properties (Berkow et al., 1984). On the one hand, in order to prevent the entry of, delay the growth of, or destroy the microorganisms found in human milk, milk is refrigerated, frozen and/or pasteurized (followed by placing for cold storage) and comes in contact with various storage container materials. But on the other hand, these treatments may affect its nutritional properties (Hanna et al., 2004; Jensen, 1995; Van Zoeren-Grobben et al., 1987).

When breast milk extraction and storage are required before ingestion, it is important to establish the conditions that will ensure the least losses in milk quality. Current official recommendations for storage conditions of expressed human milk pose 3 problems: firstly, they recommendations vary, thus it is not clear which protocol should be followed. Secondly, these protocols are aimed at avoiding bacterial growth rather than

preserving nutritional properties, which are lost during storage and handling (Buss et al., 2001). The third problem is that human milk system is a thermodynamically unstable emulsion, maintaining its original compartmentation only for the few minutes of nursing, thus off-flavours may develop during human milk storage to the extent that the infant will not consume stored milk (Jensen, 1999) although its nutritional value is still intact and safety not compromised.

2.2. Objectives

In the light of aforesaid, the objectives of the study were to assess losses of linoleic fatty acid (18:2n-6, LA), α-linolenic fatty acid (18:3n-3, ALA), arachidonic fatty acid (20:4n-6, ARA), docosahexaenoic fatty acid (22:6n-3, DHA), riboflavin (vitamin B_2) and total vitamin C and to monitor volatile compounds pattern changes during human milk storage at various conditions. Beneficial effects of lower storage temperature and oxygen limitation for preservation of the nutrients were questioned.

2.3. Hypotheses

Following the review of the literature, the hypotheses of this study were:

- During storage of unpasteurized human milk at 4^{o}C for 8 days, the vitamins and the fatty acids levels will decrease. The absence of oxygen will slow down decomposition reactions. Holder pasteurization will result in a decrease of the vitamin C content of human milk, but will not affect vitamin B_2 and FAs concentrations; the levels of the nutrients will decrease during storage. The volatile compounds profile will be altered during storage in all milk types.

- During storage of unpasteurized human milk at -20^{o}C for 6 months, the vitamin B_2 and the FAs will remain stable; the vitamin C level will be reduced. With the limitation of oxygen the decomposition of vitamin C will happen slower. The pasteurized HM will follow similar pattern of nutrient stability/decomposition. The volatile compounds profile will be altered during storage in all milk types following nutrients' decomposition, which will result in the formation of new compounds.
- During storage of human milk at -80^{o}C for 6 months, the nutrients and the volatile compounds profile will remain stable in all milk types.

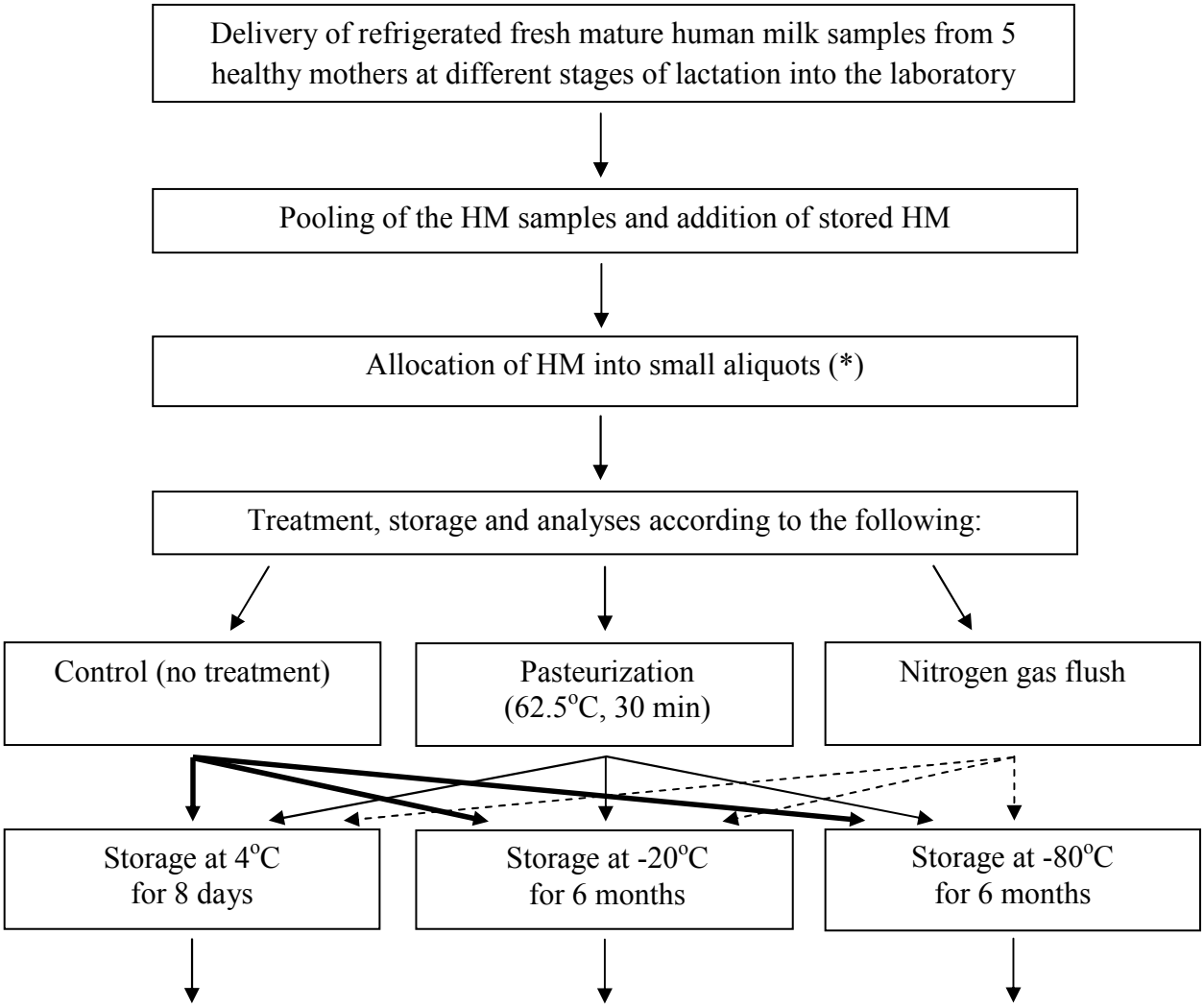
2.4. The importance of the study

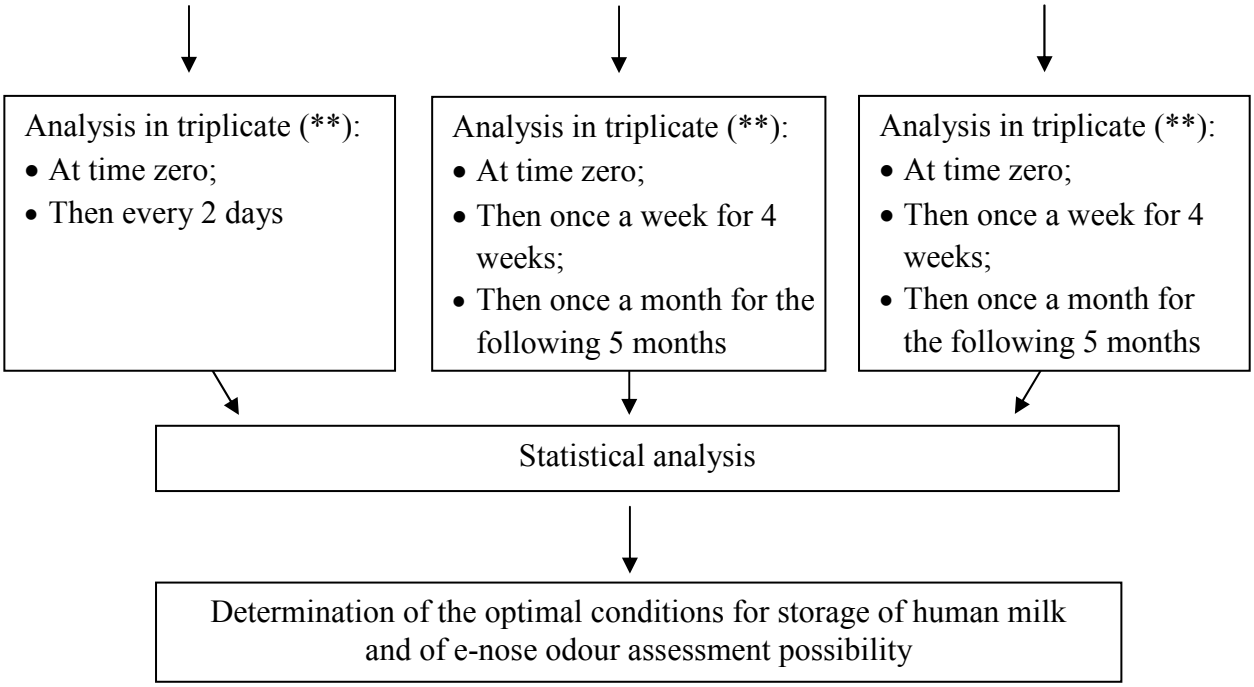
This study's findings, followed by other nutritional properties' studies, will help to review present recommendations for expressed human milk storage in order to establish storage conditions and times which would assure safety of consumed stored human milk along with optimal preservation of its nutritional properties. The findings of this study will monitor the effect of pasteurization on the nutrient content of human milk for human milk banks usage and will show whether the odour acceptability of this stored product can be assessed using a machine, rather than a human sensory panel. Possible improvements of the storage conditions will be evaluated.

CHAPTER 3: METHODOLOGY

In order to determine optimal conditions for the storage of human milk in regard to optimal preservation of nutritional properties, human milk samples, collected and pooled from 5 mothers (with addition of stored HM from a single donor), were stored over a 6 month period at 4°C, -20°C and -80°C. Part of the milk was stored with addition of nitrogen gas and another part was pasteurized prior to storage. At specific time points, the analyses of 4 fatty acids and 2 vitamins contents and monitoring of the volatile compounds profile were performed.

3.1. The study design





(*) Aliquots of 1.2 mL of human milk for HPLC and e-nose analyses; aliquots of 0.3 mL of human milk for GC-FID analyses.

(**) Analyses:

- Vitamin C and vitamin B_2 - by HPLC;
- Linoleic fatty acid, α-linolenic fatty acid, docosahexaenoic fatty acid, arachidonic fatty acid – by GC-FID;
- Volatile compounds' patterns comparison – by electronic nose.

3.2. Experiment parameters choices

3.2.1. Human milk collection

Five mothers of term infants donated their mature milk, expressed by a breast pump or by hand. Milk was delivered into the laboratory on ice. The samples were pooled to obtain a final volume of 300 mL in order to rule out individual alterations in the

nutrients' initial concentrations and to obtain a sample which represented their average nutrients composition. Because the volume of collected fresh HM was not enough for the experiment, aliquots of HM from a single donor, stored at -20^{o}C for 7 months, were thawed in warm water and mixed with fresh HM to obtain a final volume of 700 mL. The milk was warmed to 38^{o}C prior to division into separate aliquots. All preparations and treatments were completed during 2 hours.

Aliquots intended for HPLC and for E-nose analyses contained 1.2 mL of HM.

Aliquots intended for GC-FID analyses contained 0.3 mL of HM.

The study was approved by the Bannatyne Campus Research Ethics Boards, Ethics Reference Number: H2008:217. Date of approval: August 25, 2010; Expiry date: 25 August 2011. The copy of the approval can be found in the Appendix 4.

3.2.2. Storage containers

In accordance with the recommendations regarding containers for storage of expressed human milk (Arnold, 1995), hard, polypropylene containers with tight-fitting lids, covered with aluminum foil, were used (2.0 mL, natural color, Fisher Scientific, USA).

3.2.3. Experiment length

Samples stored at 4^{o}C were analyzed over a period of 8 days, as this is the maximum time for refrigeration storage of human milk derived from official recommendations (see Table 1.1).

This study followed samples stored at -20^{o}C and at -80^{o}C for the period of 6 months, though the official maximum storage time recommendations exceed 6 months (see Table 1.1). Six months is the period recommended for exclusive breastfeeding (WHO, 2001)

and as during this interval of time the infant's nutrient intake depends exclusively on human milk, their preservation is of the greatest importance during this period.

3.2.4. Storage temperatures and treatments – actual conditions imitation

The storage temperatures chosen for this study represented typical storage conditions where human milk is usually stored: at home and in the hospital. A refrigeration temperature of 4^{o}C and freezing temperature of -20^{o}C are accessible both in households and in the hospitals; freezing temperature of -80^{o}C is accessible at the hospitals. Pasteurization is mandatory for human milk distributed from human milk banks, is practiced by some mothers for breast milk intended for their infants' feeding and suggested for feeding of infants of HIV-positive mothers in developing countries (Arslanoglu et al., 2010; Hartmann et al., 2007). Nitrogen gas is accessible at the hospitals.

Refrigerated samples were stored at 4^{o}C in a Kelvinator Scientific refrigerator, model BT30RSFMS-4.1. Samples frozen at -20^{o}C were stored in a Kelvinator Scientific freezer, model BT30CWFMS-2. Samples frozen at -80^{o}C were stored in a Thermo Electron Corporation freezer, model 8693, Mariotta, Ohio, US. Samples intended for storage at -20^{o}C and -80^{o}C were frozen in the upright position to prevent leakage.

3.2.5. Pasteurization of human milk

Conditions commonly used for pasteurization of human milk are 62.5^{o}C for 30 minutes ("Holder pasteurization"). Aliquots of human milk were held in a shaking water bath (VWR, oscillation at 20 rpm) at 62.5^{o}C for 30 min, cooled under tap water at 8^{o}C and then placed for storage (de Oliveira et al., 2009; Tully, 2001; Arslanoglu et al., 2010). The rapid cooling creates a thermic shock for the bacterial flora and minimizes the

growth of spores without altering the immunological components (Arslanoglu et al., 2010).

3.2.6. Reduction of oxygen availability

Nitrogen gas flushing was performed using an Organomation Associates Inc system (Berlin, Ma, US) with an N-EVAP 112 nitrogen evaporator, attached to a PRAXAIR compressed nitrogen gas tank (Mississauga, ON, CA). Containers holding human milk were filled with nitrogen gas for 3 sec and then placed for storage.

3.2.7. The Standard Reference Material

Inter-laboratory studies rely on the availability of reference samples and standards. Standardized fatty acids methyl esters (FAMEs) mixtures are available from the National Institute of Health and several commercial suppliers (Shantha and Napolitano, 1992). For this study, a Standard Reference Material (SRM) 1849 – Infant/Adult Nutritional Formula was purchased from the National Institute of Standards & Technology (NIST, Gaithersburg, MD). The SRM 1849 contained certified concentration values of riboflavin, linoleic acid, α-linolenic acid, arachidonic acid and decosohexaenoic acid and reference concentration value for ascorbic acid, without DHAA. A certified concentration value is the value for which NIST has the highest confidence in its accuracy, while a reference concentration value is noncertified and is the best estimate of the true value based on available data (National Institute of Standards & Technology, 2009).

The SRM was used for method and results validation by comparison of concentrations of the vitamins and fatty acids obtained using the current study methods with the certified concentrations, as obtained by the NIST, the U.S. Department of Agriculture (USDA) and collaborating laboratories.

SRM 1849 powder, stored at -80^{o}C, was diluted by 15 using ddH_2O (Millipore Compact Milli-Q water system, Bedford, MA, USA) in order to obtain concentrations of the analyzed nutrients similar to those, naturally occurring in human milk. Freshly prepared diluted SRM was analyzed at every analysis time point.

According to the deviations of the nutrient analyses results, as declared by NIST, a sufficient degree of the accuracy of a method in this study was assigned to the range of 100.0% ± 10.0%.

3.3. Methods and the rationale behind their choices

3.3.1. Vitamin analysis

3.3.1.1. Literature review for the method development

Vitamins concentrations were analyzed using high-performance liquid chromatography (HPLC) (Agilent 110). HPLC methods are the first choice for determination of several vitamins, including riboflavin and total vitamin C, in foods and dietary supplements, due to higher specificity, increased speed and minimum requirement for sample cleanup in comparison with other methods (Lavigne, 1987; Blake, 2007).

Usually, for determination of the total riboflavin content of food products by HPLC, extraction methods include conversion of bound flavins to free riboflavin. But in the analysis of milk, the extraction method entails only precipitation of the protein (using acidification) and filtration without the conversion procedures, as the predominant naturally occurring flavin present in milk is free or loosely bound riboflavin (Ball, 2006; Lavigne, 1987).

In the analysis of vitamin C content, both ascorbic and dehydroascorbic acid levels must be reported, as ascorbic acid is easily and reversibly oxidized enzymatically to dehydroascorbic acid in the body (Ball, 2006; Romeu-Nadal et al., 2006). Since DHAA has a low absorbance above 210 nm, reduction of DHAA to AA is usually employed before injection into the HPLC (Manoharan and Schwille, 1994; Wechtersbach and Cigic, 2007). The reduction can be performed using sulphydryl compounds, such as homocysteine, dimercaptopropanol, dimercaptoethanol, glutathione, cysteine and dithiothreitol (Deutsch, 2000). More often incubation with DL-dithiothreitol (DTT) is used for liquid chromatography (Brause et al., 2003; Buss et al., 2001; Manoharan and Schwille, 1994; Romeu-Nadal et al., 2006). During preliminary work using stored HM in purpose to determine method parameters, DTT was shown not to co-elute with ascorbic acid. Comparison of HPLC analysis results of stored HM with and without added DTT showed that DTT was effective and necessary for the analysis of total ascorbic acid. Therefore DTT was chosen as an agent for conversion of DHAA to AA prior to the analysis. The required DTT concentration was chosen in accordance with Brause et al. (2003).

The method for riboflavin and vitamin C content was based on the study of Zafra-Gomez et al (2006), who reported simultaneous determination of several vitamins, including riboflavin and ascorbic acid, by HPLC with high recoveries. Three samples of each HM type were analyzed at every analysis point.

3.3.1.2. Sample preparation

To prepare stored HM samples for analysis of vitamin C and vitamin B_2, aliquots of HM were thawed in warm water (38^{o}C). Thawed HM samples and Standard Reference

Material SRM 1849 samples (diluted 15-fold) were warmed in water (40°C) until the contents reached the temperature of 38°C in order to solubilize the fat which adhered to the walls of the containers (Jensen and Clark, 1984). Then the samples were homogenized by vortexing and sonication in a VWR 250HT sonicator for 10 min at 38°C. One mL of analyzed material was transferred into ultracentrifuge tubes, containing 1.5 mg of DTT (Sigma-Aldrich) and shielded from light using aluminum foil casing. Following vortexing and a 60 min incubation, 0.2 mL of precipitation solution was added. The precipitation solution was prepared by dissolving 9.1 gr Zinc Acetate dihydrate (Sigma-Aldrich), 5.64 gr phosphotungustic acid polyhydrated (Sigma-Aldrich) and 5.8 mL acetic acid (glacial, 99%, Fisher Scientific) in 100 mL of ddH_2O followed by filtration through 0.2 μm, 47 mm membrane (Supor-200, PALL Life Sciences, P/N 60301). The samples were incubated with the precipitation solution for 15 min and then centrifuged for 30 min at 15,000 rpm in IEC Micromax Microcentrifuge (Thermo Electron Corporation). The aqueous layer was isolated, filtered through 0.45 μm, 4mm, PTFE (Acrodisc CR) filters using 1 mL syringes (BD 1 mL, latex free, tuberculin slip tip, sterile, single use, Becton, Dickinson and Company, NJ, USA) into HPLC vials (screw caps, green, amber vial kit, 10071009, Agilent Technologies, US) and injected into the HPLC.

3.3.1.3. HPLC separation parameters

The HPLC system was an Agilent 1100 series (Agilent Technologies, Waldbronn, Germany), with UV and fluorescence detectors. Riboflavin and ascorbic acid were separated on Waters Spherisorb ODS2, C_{18}, 250x4.6 mm I.D. 5 μm analytical column, protected with a Waters Spherisorb ODS2, C_{18}, 10x4.6 mm I.D. 5 μm guard cartridge.

Data acquisition and peaks area calculations were performed using the Agilent ChemStation for LC 3D Rev. B.01.03 [204] system.

The mobile phase A buffer was prepared weekly. It contained 13.6 gr potassium phosphate dibasic (KH_2PO_4, Sigma-Aldrich), 2.2 gr 1-octanesulfonic acid sodium salt (~98%, Sigma-Aldrich), 0.58 gr ethylenediaminetetraacetic acid (EDTA, ~99% purified grade, Sigma Chemical Co), 10 mL triethylamine (≥99%, Sigma-Aldrich), dissolved in 2 L of ddH_2O (Millipore Compact Milli-Q water system, Bedford, MA, USA). Using 85% phosphoric acid (H_3PO_4, ≥85 wt%, Sigma-Aldrich) the pH level was adjusted to 2.95. The buffer was filtered through 0.2 μm, 47 mm membrane (Supor-200, PALL Life Sciences, P/N 60301). The mobile phase B was methanol (Optima grade, Fisher Scientific).

Gradient elution was used for separation. Initial values were 98% A and 2% B; then a decrease of A to 10% over 8 min; hold for 10 min; an increase of A to 98% (returning to initial conditions) and a hold for 6 min for column conditioning and preparation for the next sample injection. The flow rate was 1.0 mL/min during the entire run. The injection volume was 20 μL. The UV detector was set at 245 nm for ascorbic acid; fluorescence detector was set at 400/520 nm (excitation/emission) for riboflavin.

3.3.1.4. Retention times determination and validation of the method and the results

For determination of the retention times for the vitamins of interest and for validation of the method and the results, solutions with known concentrations of vitamin C (3.125 μg/mL – 200 μg/mL), riboflavin (0.078 μg/mL - 5 μg/mL) and DTT (1.5 mg/mL) in 2.4% acetic acid were injected at each analysis time point before HM samples to obtain a

standard curve using GraphPad Prism version 5.00 software (San Diego California, USA). Ascorbic acid powder was purchased from SigmaUltra, A5960, China; riboflavin powder was purchased from Sigma Chemical Co. A linear relationship was observed between concentration and signal at these concentration ranges. The concentrations of vitamins C and B_2 in SRM 1849 powder (freshly prepared at every analysis time point) were interpolated directly from the calibration regression. The results were compared to the concentrations declared by the National Institute of Standards & Technology and the recoveries were calculated.

3.3.2. Fatty acid analysis

3.3.2.1. Literature review for the method development

Linoleic acid, α-linolenic acid, arachidonic acid and decosohexaenoic acid contents were analyzed using gas chromatograph coupled with a flame ionization detector (GC-FID), which is the most often used method for the analysis of fatty acids (Shantha and Napolitano, 1992).

Preparation for the analysis traditionally requires solvent extraction of lipids, purification and derivatization procedures which convert the lipids to their corresponding fatty acid methyl esters (FAMEs) by acid-catalyzed or base-catalyzed esterification. Normally boron fluoride-methanol in various concentrations is used as the methylating agent to convert fatty acids into FAMEs. The 'direct transesterification' method, developed by Lepage and Roy in 1986, circumvents the extraction and isolation of lipids and esterifies fatty acids while still in the sample using acetyl chloride in methanol as methylating agent. The 'direct transesterification' method saves time and chemicals, allowing faster analysis of total lipid in biological samples, particularly in breast milk. In

addition to the advantage of time and chemicals savings, acetyl chloride in methanol, as a methylating agent, opposed to the boron fluoride-methanol, does not produce artifacts and does not cause loss of polyunsaturated fatty acids. The use of acetyl chloride in methanol has been shown to result in a very high lipid recovery. Hexane is preferred over toluene as a solvent in the process because in contrast to toluene, which has to be removed prior to the injection into the GC, the small amount of hexane in this preparation acts as a solubilizer of unpolar lipids. Furthermore, it is used as an extracting solvent, so that FAMEs can be directly injected into the GC without prior evaporation (Clark and Roche, 1990; Kohn et al., 1996; Lepage and Roy, 1986; Shantha and Napolitano, 1992). In their work Lepage and Roy (1986) showed that compared to the 'traditional' method, requiring isolation of lipids prior to transesterification, the 'direct transesterification' method, omitting the isolation step, is more efficient because of its simplicity, rapidity and higher accuracy. Bitman and Wood (1987) supported Lapage and Roy by applying the 'direct transesterification' method to human milk fat. They showed that this method, with the use of acetyl chloride in methanol as the methylating agent, compared well with the 'traditional' method and with other methylating agents for the analysis of fatty acids of interest in this study. Masood and coworkers (2005) facilitated the 'direct transesterification' method of Lepage and Roy (1986), eliminating the methylation reaction neutralization step and using a stock solution of reagents. They demonstrated a quantitative similarity of the developed 'simplified' method to the former method when applied to human plasma.

Five pilot trials were performed in our laboratory using the 'traditional', 'direct' and 'simplified' transesterification methods for preparation of human milk FAMEs (see

results and discussion in Appendix 2). Boron fluoride-methanol in 10% and 14% concentration and acetyl chloride in methanol were used as methylating agents. Different initial volumes of HM and solvents were used. Following qualitative comparison, the 'simplified' one-step transesterification method by Masood and coworkers (2005), slightly modified to allow analysis of human milk, was chosen because of the highest recovery of the fatty acids of interest.

According to Shantha and Napolitano (1992), a linear FID response for long-chain fatty acids can be assumed, allowing quantification of the FAs using an odd-chain saturated fatty acid as an internal standard. An assumption of no discrimination of the different FAs during the processes of derivatization and chromatography was accepted (Shantha and Napolitano, 1992). According to Jensen et al. (1995) the internal standard chosen for this study was heptadecanoic acid (C17:0). Nonadecanoic acid (C19:0) was also examined as a possible internal standard (Shantha and Napolitano, 1992), however, it was shown to undergo incomplete methylation during the process chosen for the analysis, therefore eliminating its possible use (see Appendix 3). Opposed to nonadecanoic acid, heptadecanoic acid has shown sufficient methylation, allowing FAs quantification. The HM samples were shown to contain negligible amounts of the naturally occurring C17:0 fatty acid (see Appendix 3). For these reasons, heptadecanoic acid was chosen as the internal standard for this study. It was added to every sample in the beginning of the procedure in order to undergo all the steps of methylation and extraction, allowing subsequent fatty acids concentrations quantification.

The considerable amount of water in the methylation reagent preparation may lead to an inefficient triglyceride transesterification, but in the case of human milk the

incomplete transesterification of the unpolar lipids has only minor influence on the relative fatty acid composition, although results concerning the absolute amount of specific fatty acids cannot be drawn. Therefore the results of the analyses are usually expressed as relative weight percentage of total fatty acids measured (Kohn et al., 1996). Compounds like free cholesterol, fat-soluble vitamins and pigments principally contribute to the total lipid extract, but not to the total amount of FAMEs (Kohn et al., 1996).

3.3.2.2. Sample preparation

In order to prepare stored HM samples for the analysis of fatty acids by GC-FID, aliquots of HM were thawed in warm water (38^{o}C). Thawed HM samples and SRM 1849 samples (diluted 15-fold) were warmed in water (40^{o}C) until the contents reached the temperature of 38^{o}C. To 100μL of every analyzed material, 2.1 mL of freshly prepared stock solution was added. The stock solution contained 1.7 mL methanol (Optima grade, Fisher Scientific), 100 μL acetyl chloride (Fisher Scientific, Belgium) and 300 μL of 1.5 mg/mL heptadecanoic acid solution in hexane (Nu-Chek Prep. Inc., USA). Samples were capped under nitrogen and, following vortexing, heated at 100^{o}C for 60 min. After cooling, the obtained FAMEs, which appeared in the upper phase, were extracted twice using 2 mL of hexane (Optima grade, Fisher Scientific) and subsequent centrifugation for 5 min at 2500 rpm (Sorvall Legend RT, Thermo Electron Corporation, D-37520 Osterode, Germany). The solvent was evaporated under nitrogen, FAMEs were re-suspended in 500μL of hexane and transferred into GC vials. The samples were stored at -20^{o}C for several days until injection into the GC-FID by the Department Research Technologist.

3.3.2.3. GC-FID analysis parameters

Three samples of each HM type were analyzed at every analysis point by gas chromatograph (Varian 450-GC, Agilent Technologies) with split injection and a flame ionization detector (GC-FID). Selection of the injection and the analysis parameters was based on previous work done in the facility. An Agilent J&W DB-225MS column was used (30m, 250μm I.D., film thickness 0.25μm). Hydrogen was used as carrier gas at a flow rate of 1.3 mL/min with nitrogen as a make-up flow at 25mL/min. Hydrogen (30 mL/min) and air (300 mL/min) were used for combustion.

The initial temperature of the 1 μL injection (10:1 split ratio) was 270^{o}C. The column oven initial temperature was 70^{o}C for 2 min, increased to 180^{o}C at 30^{o}C/min and held for 1 min, increased to 200^{o}C at 10^{o}C/min and held for 2 min, increased to 220^{o}C at 2^{o}C/min and held for 4 min and finally increased to 240^{o}C at 20^{o}C/min and held for 5 min. The detector temperature was 290^{o}C.

FAMEs peaks identification was verified by comparison with GLC Reference Standard 461 (Nu-Chek Prep, Inc. USA). The peak areas were determined by Galaxie Software.

3.3.2.4. Validation of the method

The SRM 1849 served as an indicator for the completeness of the esterification and of the hexane extraction steps. Concentrations of LA, ALA, ARA and DHA in SRM 1849 powder were obtained using the internal standard. The results, as obtained in this analysis, were compared to the concentrations declared by the National Institute of Standards & Technology, and the fatty acids' recoveries were calculated.

3.3.3. Assessment of the volatile compounds pattern change

3.3.3.1. Literature review for the method development

The changes in volatile compounds profile were monitored by electronic nose (e-nose). The e-nose technology is rapid, simple and can be used for overall comparative analysis of volatile compounds (Xu, 2006). These instruments mimic the sense of smell and detect and discriminate among complex odours using a sensor array (Peris and Escuder-Gilabert, 2009). E-noses are mainly used to provide qualitative information (Marsili, 2000).

Electronic noses have many advantages compared to sensory panels. After calibration e-nose can perform on a continuous basis, this technique does not require trained personnel as does a sensory panel, it is not subject to individual breakdown or variation of sensitivity, is not overloaded under normal operation and takes comparatively very little time (Ampuero and Bosset, 2003; Sarig, 2000).

The general principle of e-nose technique is introduction of volatile compounds of a sample into a detection system, which generates a set of signals that contains the information about the volatile composition of the sample (Martí et al., 2004). The sensors used in an e-nose respond to molecules in the gas phase (which are typically volatile organic compounds with different relative molar masses) and convert chemical quantities into electrical signals that are related to the concentration of the specific particles (Pearce et al., 2003; Peris and Escuder-Gilabert, 2009). Each chemical sensor element responds more selectively to a certain group of chemicals, but also shows a broad and overlapping response to other chemical species (cross-selectivity). From these sensors a unique response pattern (a “fingerprint”) is produced. For gas and odour measurements the

chemiresistive metal oxide semiconductor (MOS) sensor system is widely used (Gardner and Hines, 1997). The data processing system of responses generated by each sensor uses pattern recognition (PARC) techniques and unfolds the information contained in the fingerprints (Gardner and Hines, 1997; Peris and Escuder-Gilabert, 2009).

The electronic nose used in this study was an Alpha-Fox 2000 SAS electronic nose (Alpha M.O.S., Toulouse, France), equipped with a headspace autosampler (HS100) and 12 metal oxide sensors. Alpha MOS software package performs principle component analysis (PCA) of the data, in order to visualize the differences and similarities between samples and groups following qualitative analysis (Li et al., 2010). The obtained discrimination index (DI) gives an indication of the separation of the data between groups, indicating how well the e-nose was able to discriminate among the samples. A positive value indicates distinct groups and a negative value indicates overlapping groups. A value between 80 and 100 (the maximum) indicates the most useful separation of the samples.

3.3.3.2. Sample preparation

In order to prepare HM samples for the analysis, aliquots of HM were thawed and heated in water (40^{o}C) until the contents reached a temperature of 38^{o}C. One mL (= 1.0 gr) of each HM sample was transferred into 10 mL e-nose vials (AlphaMOS, London Scientific Ltd), sealed with matching caps (AlphaMOS, London Scientific Ltd) and injected into the e-nose machine.

3.3.3.3. E-nose analysis parameters

The method was adopted from Li et al (2008). The human milk analysis proceeded according to the following parameters:

Headspace generation time: 480 sec

Headspace generation temperature: 80^{o}C

Agitation speed: 500 rpm

Syringe volume: 5 mL

Syringe temperature: 90^{o}C

Fill speed: 0.5 mL/sec

Injected volume: 1 mL

Injected speed: 1 mL/sec

Dry air flow rate: 150 mL/min

Acquisition time: 300 sec

Time between subsequent analyses: 1080 sec

Discrimination index of ≥80 was considered as a good discrimination.

3.3.4. Statistical analysis

The statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Statistical significance was assigned to $P < 0.05$.

In order to find storage periods during which statistically significant changes in a vitamin or a fatty acid concentration occurred for every treatment, one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test was performed for all pairs of data points for each vitamin and each fatty acid.

In order to compare concentrations of vitamins and fatty acids obtained by different treatments at same time points, two-way Repeated Measures ANOVA with Post-hoc Bonferroni Test was performed for all pairs of data points at every time point.

CHAPTER 4: RESULTS

4.1. Changes in the concentrations of vitamin C and vitamin B_2 during storage of human milk

4.1.1. Method validation

Following injection of known concentrations of vitamin C (3.125 μg/mL – 200 μg/mL) and riboflavin (0.078 μg/mL - 5 μg/mL), the contents of the vitamins in SRM 1849 were calculated based on the equation obtained for the linear regression. The regression was derived using GraphPad Prism version 5.00 software. Using the peak areas, calculated by the computer software, vitamins' concentrations in the diluted SRM were derived. Dilution factor of 18 was used in order to determine vitamins' concentrations in the SRM powder (initial dilution 15-fold of SRM powder in water, followed by dilution 1.2-fold during sample preparation). The accuracy of the method for a vitamin analysis was calculated as the ratio between the vitamin content, as obtained by the analysis, and its content, as declared by the NIST.

For example, for the calculation of the accuracy of the method for the analysis of vitamin C in the SRM 1849 at 2 months of storage (mean peak area: 4025.63 mAU*min; standard curve equation: y = 63.38x + 278.45; declared vitamin C concentration in SRM 1849: 1060 μg/mg):

4025.63 = 63.38x + 278.45 ➔ x = vitamin C concentration in diluted SRM = 59.12 μg/mL ➔ vitamin C concentration in SRM powder = 59.12 * 18 = 1064.16 μg/mg

$$\text{The accuracy of the method} = \frac{\text{Vitamin C concentration obtained in the analysis}}{\text{Vitamin C concentration declared by the NIST}} \times 100\%$$

$$= \frac{1064.16\ \mu g/gr}{1060\ \mu g/gr} \times 100\% = 100.4\%$$

The accuracies of the method for the analysis of vitamin C and vitamin B_2 in SRM 1849 are presented in Table 4.1. The trend-line linearity coefficient (R^2) exceeded 0.987 in all analyses.

Table 4.1: The accuracies of the method for the analysis of vitamin C and vitamin B_2 in SRM 1849 at all analysis time points

Analysis time point	R^2 value of the standard curve – vitamin C	R^2 value of the standard curve – vitamin B_2	The accuracy for vitamin C (%)	The accuracy for vitamin B_2 (%)
Time 0	N/A	N/A	N/A	N/A
2 days	0.9980	0.9999	90.90	98.28
4 days	0.9991	0.9999	113.01	51.41
6 days	0.9980	0.9999	88.22	105.52
8 days	0.9876	1	69.96	110.69
1 week	0.9984	1	75.04	96.21
2 weeks	N/A	N/A	N/A	N/A
3 weeks	0.9977	0.9999	112.77	106.90
4 weeks	0.9977	1	86.81	73.45
1 month	0.9977	1	86.81	73.45
2 months	0.9957	0.9998	100.39	109.66
3 months	1	1	44.81	108.62
4 months	0.9993	0.9992	96.62	81.72
5 months	0.9995	0.9999	92.33	100.34
6 months	0.9999	1	92.95	86.90
Average			88.51 ± 5.00	92.55 ± 5.02

4.1.2. Refrigeration storage at 4°C

4.1.2.1. Vitamin C

In this section, the following abbreviations for sample names were used in all the figures (Figures 4.1 – 4.2) and tables (Tables 4.2 – 4.3):

RC - control (untreated) human milk;

RN - human milk stored in nitrogen gas environment;

RP - pasteurized human milk.

The analyses were performed every 2 days during 8 days of storage. The concentrations of the vitamins are expressed as the area of the peaks obtained for the vitamin (mAU*min), mean +/- SE.

The results of the analysis of vitamin C concentration in human milk can be seen in Figure 4.1. No values were obtained for vitamin C concentration at time 0 in control human milk samples and samples stored with nitrogen gas due to technical problems. The results of the analysis of day 8 of the experiment were not included because of analytical uncertainty.

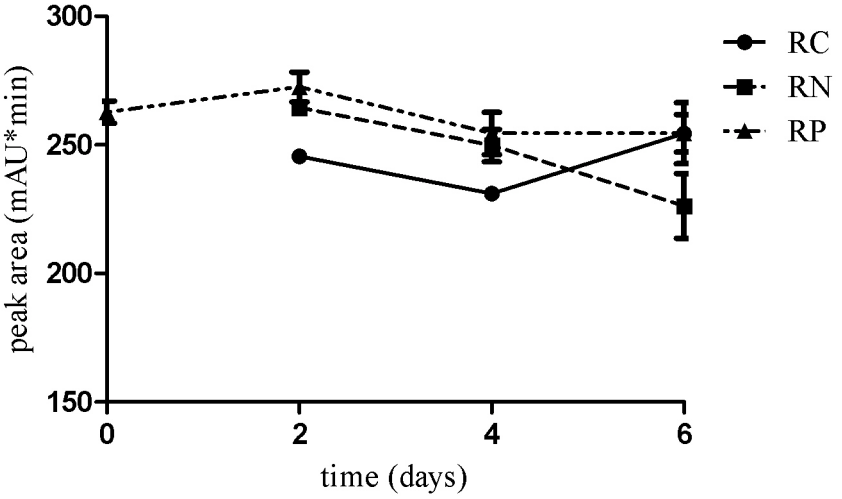


Figure 4.1: Vitamin C concentration during 6 days of storage at 4^{o}C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, as performed for all pairs of data points, can be seen in Table 4.2. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.2: Differences between analysis results at all time points for every treatment - vitamin C

	Time points for comparison (days)					
Treatment	0 vs 2	0 vs 4	0 vs 6	2 vs 4	2 vs 6	4 vs 6
RC	N/A	N/A	N/A	ns	ns	*
RN	N/A	N/A	N/A	ns	*	ns
RP	ns	ns	ns	ns	ns	ns

As can be seen from Figure 4.1 and from Table 4.2, in comparison with the initial conditions (time 0 for pasteurized human milk and day 2 for the control milk and the milk stored in nitrogen gas environment), vitamin C content of the control HM and the pasteurized HM remained stable during 6 days of storage, while the vitamin content of HM stored wit nitrogen gas decreased.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed statistically non-significant differences at $P<0.05$ between all samples.

4.1.2.1. Vitamin B_2

The results of the analysis of vitamin B_2 concentration in human milk can be seen in Figure 4.2.

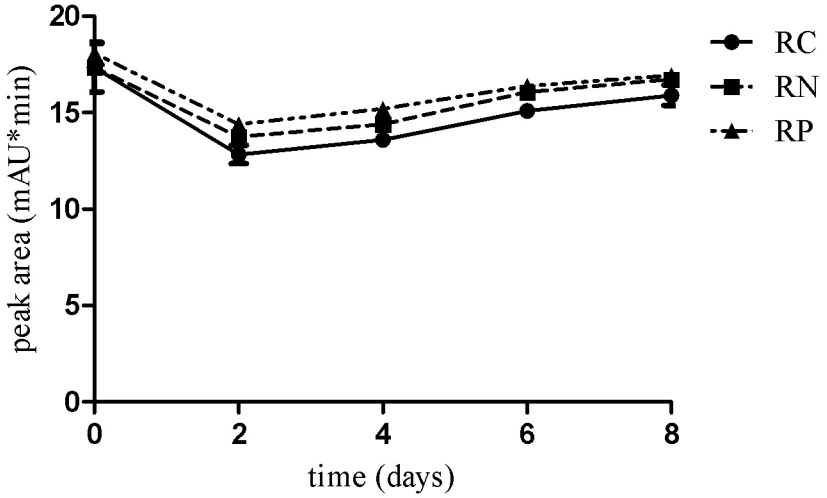


Figure 4.2: Vitamin B_2 concentration during 8 days of storage at 4^oC

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, as was performed for all pairs of data points, can be seen in Table 4.3. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.3: Differences between analyses results at all time points for every treatment – vitamin B_2

	Time points for comparison (days)									
Treat-ment	0 vs 2	0 vs 4	0 vs 6	0 vs 8	2 vs 4	2 vs 6	2 vs 8	4 vs 6	4 vs 8	6 vs 8
RC	*	*	ns	ns	ns	ns	*	ns	ns	ns
RN	*	*	ns	ns	ns	ns	*	ns	ns	ns
RP	*	*	*	ns	ns	*	*	ns	*	ns

As can be seen from Figure 4.2 and from Table 4.3, vitamin B_2 concentration does not differ significantly between the initialization of the experiment and the last day of storage. A decrease in the vitamin concentration was observed during first 4 days of storage for the control sample (RC) and for the sample stored with nitrogen gas (RN) and during first 6 days of storage in the pasteurized sample (RP), followed by further increase in the riboflavin concentration until a level not significantly different from time 0.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test did not reveal statistically significant differences at $P<0.05$ between any of the samples at each analysis point.

4.1.3. Frozen storage

In this section, the following abbreviations for sample names were used in all the figures (Figures 4.3 – 4.6) and tables (Tables 4.4 – 4.7):

FC – control (untreated) human milk stored at -20^{o}C;

FN - human milk stored at -20^{o}C in a nitrogen gas environment;

FP - pasteurized human milk stored at -20^{o}C;

SC – control (untreated) human milk stored at -80^{o}C;

SN - human milk stored at -80^{o}C in a nitrogen gas environment;

SP - pasteurized human milk stored at -80^{o}C.

The concentrations are expressed as the area of the peaks obtained for the vitamin (mAU*min), mean +/- SE.

4.1.3.1. Storage for 4 weeks in the freezer at -20^{o}C and at -80^{o}C

The analyses were performed every week during 4 weeks of storage. No results were obtained at 2 weeks analysis point for any of the samples due to technical problems.

4.1.3.1.1. Vitamin C

The results of the analysis of vitamin C concentration in human milk stored for 4 weeks in the freezer at -20^{o}C and at -80^{o}C can be seen in Figure 4.3. As was mentioned in section 4.1.2.1, no values were obtained for vitamin C concentration at time 0 for control human milk samples and samples stored with nitrogen gas.

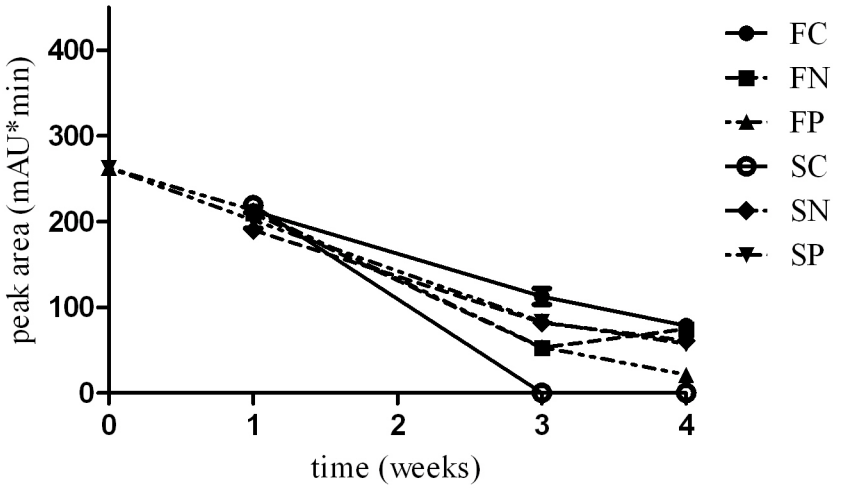


Figure 4.3: Vitamin C concentration during 4 weeks of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, as was performed for all pairs of data points, can be seen in Table 4.4. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.4: Differences between analyses results at all time points for every treatment – vitamin C

	Time points for comparison (weeks)					
Treatment	0 vs 1	0 vs 3	0 vs 4	1 vs 3	1 vs 4	3 vs 4
FC	N/A	N/A	N/A	*	*	*
FN	N/A	N/A	N/A	*	*	ns
FP	*	*	*	*	*	*
SC	N/A	N/A	N/A	ns	ns	ns
SN	N/A	N/A	N/A	*	*	ns
SP	*	*	*	*	*	*

As can be seen from Figure 4.3 and Table 4.4, vitamin C concentration in all samples decreased during 4 weeks of storage at -20°C and at -80°C, dropping significantly every

week. As can be seen in Figure 4.3, control human milk stored at -80°C (SC) lost all its vitamin C during first 2 or 3 weeks.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed statistically significant differences at $P<0.05$ between most of the samples at most of the analysis points. After one week of storage, non-significant differences were detected between all the samples, with 2 exceptions: sample SN results were significantly different from all other samples (but not from sample SP); sample SC results were significantly different from sample SP results. On week 3, non-significant differences were detected between pasteurized samples and samples stored with nitrogen gas at both temperatures (FN and FP; SN and SP). Between other samples the differences were significant. On week 4, non-significant differences were detected only between samples FN and SN and between samples SN and SP.

4.1.3.1.2. Vitamin B_2

The results of the analysis of vitamin B_2 concentration in human milk stored in the freezer at -20°C and at -80°C for 4 weeks can be seen in Figure 4.4.

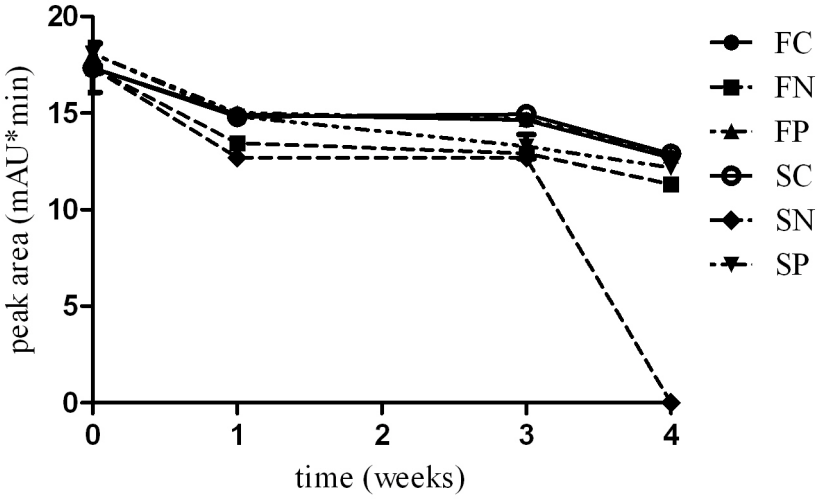


Figure 4.4: Vitamin B_2 concentration during 4 weeks of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, as performed for all pairs of data points, can be seen in Table 4.5. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.5: Differences between analyses results at all time points for every treatment – vitamin B_2

	Time points for comparison (weeks)					
Treatment	0 vs 1	0 vs 3	0 vs 4	1 vs 3	1 vs 4	3 vs 4
FC	ns	ns	*	ns	ns	ns
FN	*	*	*	ns	ns	ns
FP	*	*	*	ns	*	*
SC	ns	ns	*	ns	ns	ns
SN	*	*	*	ns	*	*
SP	*	*	*	ns	*	ns

As can be seen in Figure 4.4 and in Table 4.5, vitamin B_2 content in all samples decreased during 4 weeks of storage. The decrease was slower in the control samples at both temperatures, remaining stable for 3 weeks and decreasing during the last week. As can be seen in Figure 4.4, riboflavin content of HM stored in nitrogen gas environment at -80^{o}C dropped to an undetectable level during the last week of storage.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between most of the samples at most analysis points. Sample SN was different from the samples FC, FP, SC and SP on week 1. Sample SC was different from sample SN on week 3. All the samples were different from the sample SN on week 4.

4.1.3.2. Storage for 6 months in the freezer at -20°C and at -80°C

The analyses were performed every month during 6 months of storage.

4.1.3.2.1. Vitamin C

The results of the analysis of vitamin C concentration in human milk stored in the freezer at -20°C and at -80°C for 6 months can be seen in Figure 4.5. As was mentioned in section 4.1.2.1., no values were obtained for vitamin C concentration at time 0 for control human milk samples and samples stored with nitrogen gas. The results of the analysis of the 3rd month of the experiment were not included because of analytical uncertainty.

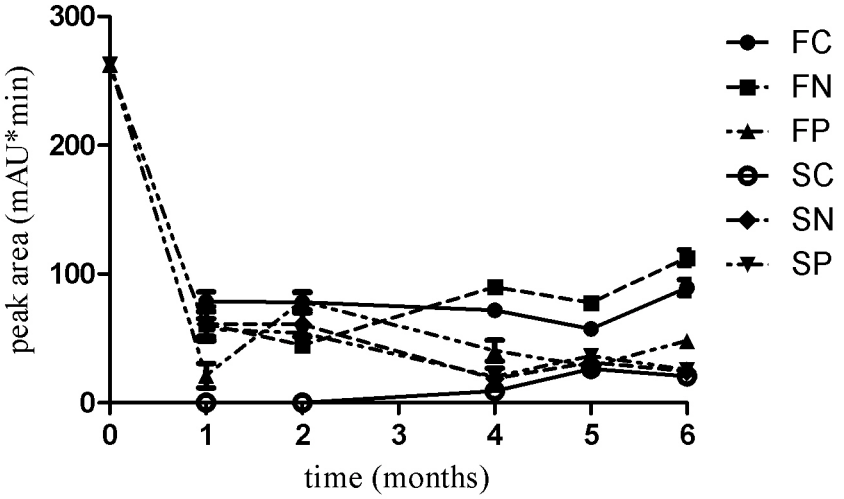


Figure 4.5: Vitamin C concentration during 6 months of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.6. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.6: Differences between analyses results at all time points for every treatment - vitamin C

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 4	1 vs 5	1 vs 6	2 vs 4	2 vs 5
FC	N/A	N/A	N/A	N/A	N/A	ns	ns	ns	ns	ns	ns
FN	N/A	N/A	N/A	N/A	N/A	*	ns	ns	*	*	*
FP	*	*	*	*	*	*	ns	ns	*	*	*
SC	N/A	N/A	N/A	N/A	N/A	ns	*	*	*	*	*
SN	N/A	N/A	N/A	N/A	N/A	ns	*	*	*	*	*
SP	*	*	*	*	*	ns	*	*	*	*	*

	Time points for comparison (months)			
Treat-ment	2 vs 6	4 vs 5	4 vs 6	5 vs 6
FC	ns	ns	ns	*
FN	*	ns	*	*
FP	*	ns	ns	ns
SC	*	*	*	ns
SN	*	*	ns	ns
SP	*	ns	ns	ns

As can be seen from Figure 4.5 and Table 4.6, control samples' vitamin C concentration remained stable from the first until the last month of storage at -20^{o}C. At -80^{o}C vitamin C content dropped to an undetectable level during the first month of storage, remained at this level for one month and then appeared to increase until the 5th month.

In HM stored at -20^{o}C in nitrogen gas environment, vitamin C content remained stable from the first until the 5th month of storage and then appeared to increase during the last month. At -80^{o}C, the vitamin C level decreased during 4 months of storage and appeared to increase further.

In pasteurized HM, vitamin C decreased sharply during the first month of storage at both temperatures. At -20^{o}C the vitamin's level remained stable until the end of the

storage period. At -80^{o}C the vitamin's level decreased until the end of the 4th month of storage and then – appeared to increase.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed:

- At 1st month: statistically non-significant differences at $P<0.05$ between samples FC and FN; samples FN and SN; samples SN and SP.
- At 2nd month: statistically non-significant differences at $P<0.05$ between samples FC and FP; samples FN and SP; samples SN and SP.
- At 4th month: statistically non-significant differences at $P<0.05$ between samples SC and SN; samples SC and SP; samples SN and SP.
- At 5th month: statistically non-significant differences at $P<0.05$ between samples FP and SC; samples FP and SN; samples FP and SP; SC and SN; samples SC and SP; samples SN and SP.
- At 6th month: statistically non-significant differences at $P<0.05$ between samples SC and SN; samples SC and SP; samples SN and SP.

4.1.3.2.2. Vitamin B_2

The results of the analysis of vitamin B_2 concentration in human milk stored in the freezer at -20^{o}C and at -80^{o}C for 6 months can be seen in Figure 4.6.

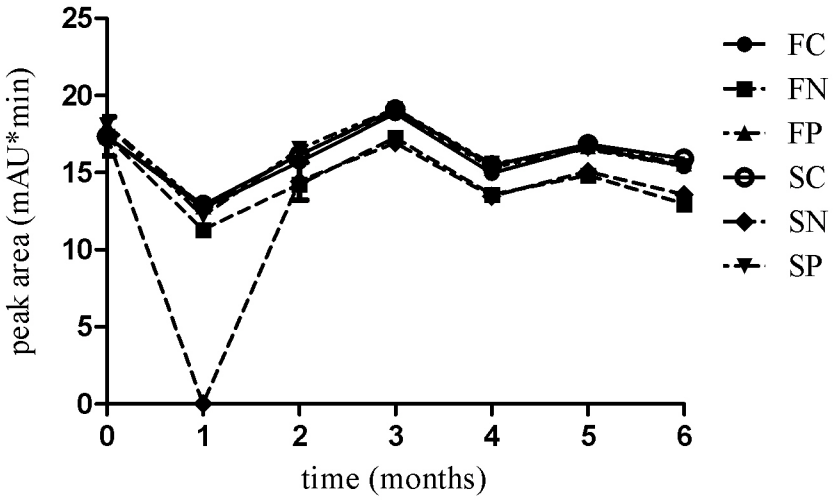


Figure 4.6: Vitamin B_2 concentration during 6 months of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.7. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.7: Differences between analyses results at all time points for every treatment – vitamin B_2

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	*	ns	ns	*	ns	ns	*	*	ns	*	*
FN	*	*	ns	*	*	*	*	*	ns	*	ns
FP	*	*	ns	*	*	*	*	*	*	*	*
SC	*	ns	ns	ns	ns	ns	*	*	*	*	*
SN	*	ns	ns	*	ns	*	*	*	*	*	*
SP	*	*	ns	*	*	*	*	*	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	*	ns	ns	ns	*	ns	*	ns	ns	ns	
FN	*	ns	ns	ns	*	*	*	ns	ns	ns	
FP	*	ns	ns	ns	*	*	*	*	ns	ns	
SC	*	ns	ns	ns	*	ns	*	ns	ns	ns	
SN	ns	ns	ns	ns	*	ns	*	ns	ns	ns	
SP	*	ns	ns	ns	*	*	*	ns	ns	ns	

As can be seen from Figure 4.6 and Table 4.7, in all samples vitamin B_2 level remained stable during the first 3 months of storage. The vitamin levels in the control samples at both temperatures remained stable for 6 months. Vitamin B_2 level decreased in pasteurized samples after 3 months of storage, retaining levels close to those of the control milk. In milk stored with nitrogen gas at both temperatures the vitamin level decreased after 3 months of storage, remaining at lower levels than the other samples.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between riboflavin content of all samples except for:

- At 1st month: Samples SN and every other sample.
- At 2nd month: Samples FN and FP; samples FN and SC; samples FN and SP; samples SN and SP.
- At 3rd month: Samples FC and SN; samples FP and SN; samples SC and SN; samples SN and SP; samples FN and SC; samples FN and SP.
- At 4th month: Samples FN and SC; samples FN and SP; samples SC and SN; samples SN and SP; samples FP and SN.
- At 5th month: Samples FN and SC; samples FC and FN; samples FN and FP.
- At 6th month: samples FN and SN differed from all other samples.

4.2. Changes in the concentration of LA, ALA, ARA and DHA during storage of human milk

4.2.1. Method validation

The contents of the fatty acids in SRM 1849 were calculated based on the peak area obtained for the known concentration of the added internal standard (heptadecanoic acid, C17:0). Using the ratio of the heptadecanoic acid peak area to its concentration and the initial amount of the diluted SRM sample, the total fatty acid content of 15-fold diluted SRM per 1 mL was calculated, using the sum of the peaks areas as obtained by the computer software (equation (1)).

$$(1)\quad \frac{\textbf{FAs content [mg]}}{\frac{\text{Total peak area [}\mu\text{V/sec]}}{\text{1 mL SRM}}} = \frac{\text{C17:0 content [mg]}}{\frac{\text{17:0 peak area [}\mu\text{V/sec]}}{\text{SRM sample quantity [mL]}}}$$

The content of each fatty acid per 1 mL of 15-fold diluted SRM was calculated using the total content of the fatty acids in the diluted SRM sample (obtained from equation (1)) and the area percentage of each FA as obtained by the computer software (equation (2)).

(2) **FA content of 15-fold diluted SRM** = FAs content [mg] x FA peak area percentage

In order to obtain the concentration of the FA in 1 gr of SRM 1849 powder, the FA content, as obtained from equation (2) for 15-fold diluted sample, was multiplied by 15 (equation (3)).

(3) **FA content of SRM powder** = FA content of 15-fold diluted SRM x 15

The accuracy of the method for a fatty acid analysis was calculated as the ratio between the FA content, as obtained by the analysis, and its content, as declared by the NIST (equation (4)).

$$(4)\quad \text{The accuracy of the method} = \frac{\text{FA concentration obtained in the analysis}}{\text{FA concentration declared by the NIST}} \times 100\%$$

For example, for the calculation of the accuracy of the method for the analysis of LA in the first replica of SRM 1849 (total peak area: 5382707μV/sec; C17:0 content: 0.45mg; 17:0 peak area: 1386606.5μV/sec; SRM sample quantity: 0.1mL; LA peak area percentage: 21.30%; declared LA concentration: 60.2 mg/gr):

(1) $$\frac{\frac{\text{FAs content [mg]}}{5382707\ \mu\text{V/sec}}}{1\ \text{mL SRM}} = \frac{\frac{0.45\text{mg}}{1386606.5\ \mu\text{V/sec}}}{0.1\text{mL}} \rightarrow$$

➔ FAs content of 15-fold diluted SRM = 17.47mg/mL

(2) LA content of 15-fold diluted SRM = 17.47mg/mL x 21.30% = 3.72mg/mL

(3) LA content of SRM powder = 3.72mg/mL x 15 = 55.8 mg/gr

(4) The accuracy = $\frac{55.8\ \text{mg/gr}}{60.2\ \text{mg/gr}}$ x 100% = 92.69%

The accuracies of the method for the analysis of LA, ALA, ARA and DHA in SRM 1849 are presented in Table 4.8. The data from the chromatograms which was used for the calculations can be found in Table 4.9.

Table 4.8: The accuracies of the method for the analysis of the fatty acids in SRM 1849

Fatty acid	Accuracy
LA	92.55%
ALA	98.11%
ARA	84.44%
DHA	89.70%

Table 4.9: Data used for the calculation of the accuracies of the fatty acids' analysis method

Area percentage of a fatty acid (%)				C17:0 peak area [μV/sec]	Total peak area [μV/sec]	FAs content of diluted SRM [mg/0.1mL]
LA	ALA	ARA	DHA			
3.721	0.367	0.116	0.040	1386607	5382707	17.47
3.778	0.373	0.118	0.041	1282367	5042228	17.69
3.650	0.361	0.114	0.039	1381293	5283605	17.21

4.2.2. Refrigeration storage at 4°C for 8 days

In this section, the following abbreviations were used in all figures (Figures 4.7 – 4.11) and tables (Tables 4.10 – 4.13):

RC - control (untreated) human milk;

RN - human milk stored in nitrogen gas environment;

RP - pasteurized human milk.

The analyses were performed every 2 days during 8 days of storage. The concentration is expressed as the area percentage of the specific fatty acid of the total peak area, obtained for all fatty acids, mean +/- SE.

4.2.2.1. Linoleic fatty acid

The results of the analysis of LA concentration in human milk stored in the refrigerator at 4°C for 8 days can be seen in Figure 4.7.

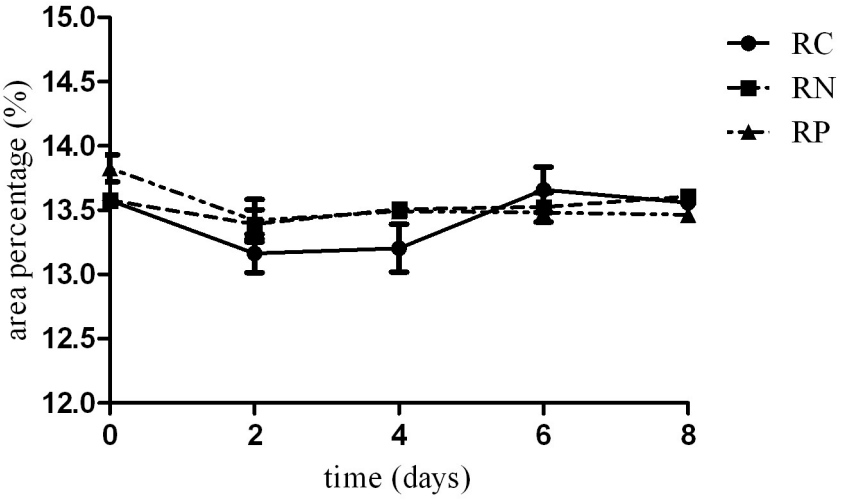


Figure 4.7: Linoleic fatty acid concentration during 8 days of storage at 4°C

The results of the one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.10. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.10: Differences between analyses results at all time points for every treatment – LA

	Time points for comparison (days)									
Treat-ment	0 vs 2	0 vs 4	0 vs 6	0 vs 8	2 vs 4	2 vs 6	2 vs 8	4 vs 6	4 vs 8	6 vs 8
RC	ns	ns	ns	ns	ns	*	ns	*	ns	ns
RN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
RP	*	*	*	*	ns	ns	ns	ns	ns	ns

As can be seen in Table 4.10 and in Figure 4.7, no significant differences in the LA concentrations were observed in unpasteurized human milk during 8 days of storage. The LA content of pasteurized human milk decreased during the first 2 days of storage and then remained stable until the last day.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between all samples, except for samples RC and RN on day 4.

4.2.2.2. α-linolenic fatty acid

The results of the analysis of ALA concentration in human milk stored in the refrigerator at $4^{o}C$ for 8 days can be seen in Figure 4.8.

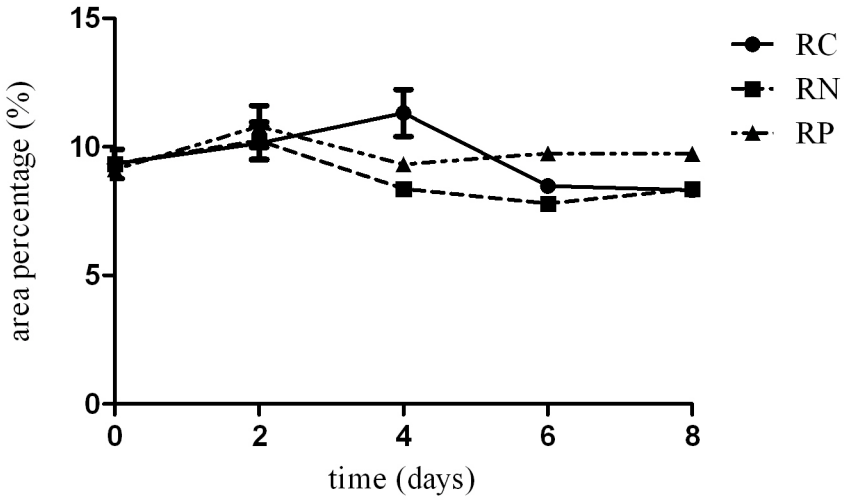


Figure 4.8: α-linolenic fatty acid concentration during 8 days of storage at 4°C

The results of the one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.11. "Ns" represents detection of no significant differences (P>0.05); * represents detection of significant differences at P<0.05.

Table 4.11: Differences between analyses results at all time points for every treatment – ALA

	Time points for comparison (days)									
Treat-ment	0 vs 2	0 vs 4	0 vs 6	0 vs 8	2 vs 4	2 vs 6	2 vs 8	4 vs 6	4 vs 8	6 vs 8
RC	ns	ns	ns	ns	ns	ns	ns	*	*	ns
RN	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
RP	*	ns	ns	ns	*	ns	ns	ns	ns	ns

As can be seen in Table 4.11 and in Figure 4.8, no significant differences in the ALA concentrations were observed in the unpasteurized human milk during 8 days of storage. The ALA content of the pasteurized human milk appeared to increase during first 2 days of storage and then decreased back to the initial level during subsequent 2 days, remaining stable until the last day.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between all samples, except for samples RC and RN on day 4; samples RC and RP on day 4; samples RN and RP on day 6.

4.2.2.3. Docosahexaenoic fatty acid

The results of the analysis of DHA concentration in human milk stored in the refrigerator at 4°C for 8 days can be seen in Figure 4.10.

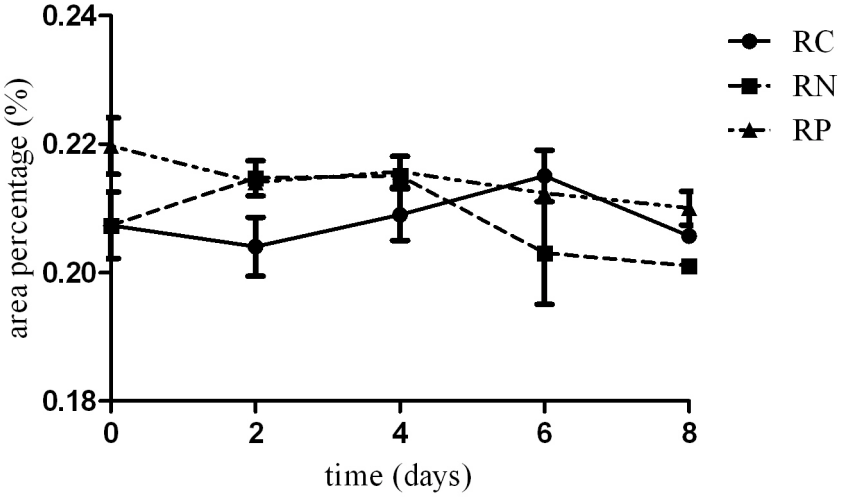


Figure 4.9: Docosahexaenoic fatty acid concentration during 8 days of storage at 4°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.12. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.12: Differences between analyses results at all time points for every treatment – DHA

	Time points for comparison (days)									
Treat-ment	0 vs 2	0 vs 4	0 vs 6	0 vs 8	2 vs 4	2 vs 6	2 vs 8	4 vs 6	4 vs 8	6 vs 8
RC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
RN	ns	ns	ns	*	ns	ns	*	ns	*	ns
RP	ns	*	*	*	ns	ns	ns	ns	ns	ns

As can be seen in Table 4.12 and in Figure 4.9, no significant differences in the DHA concentrations were observed in the unpasteurized human milk during 8 days of storage, in the human milk sample stored in nitrogen gas environment for 6 days of storage and in the pasteurized human milk sample during first 2 days of storage.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between all samples, except for samples RC and RP at time 0; samples RN and RP at time 0.

4.2.2.4. Arachidonic fatty acid

The results of the analysis of ARA concentration in human milk stored in the refrigerator at 4^{o}C for 8 days can be seen in Figure 4.11.

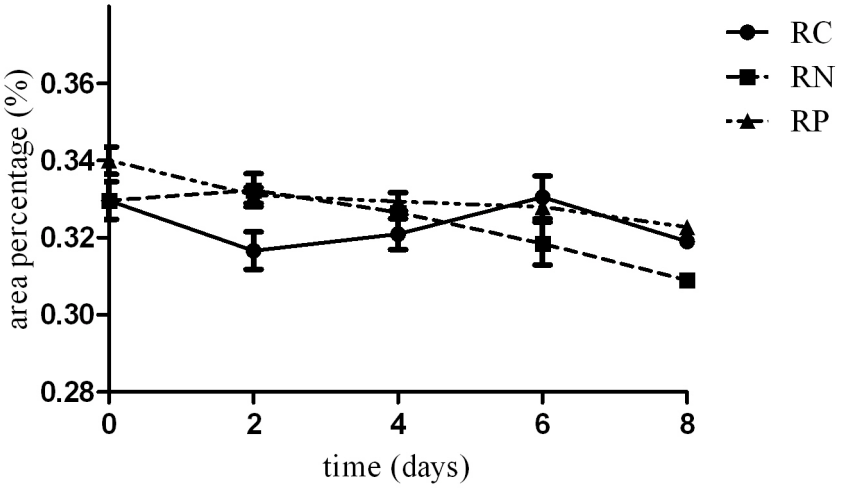


Figure 4.10: Arachidonic fatty acid concentration during 8 days of storage at 4^{o}C

The results of the one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test revealed non-statistically significant differences at $P<0.05$ between all data points for every treatment.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test revealed non-statistically significant differences at $P<0.05$ between all samples, except for samples RC and RN on days 2 and 6; samples RC and RP on day 2; samples RN and RP on day 8.

4.2.2.5. Total peak area

The results of the total peak area assessment, as obtained for all the FAMEs in every analysis for every treatment, can be found in Figure 4.11. The results are presented as the area (μV*sec) +/- SE.

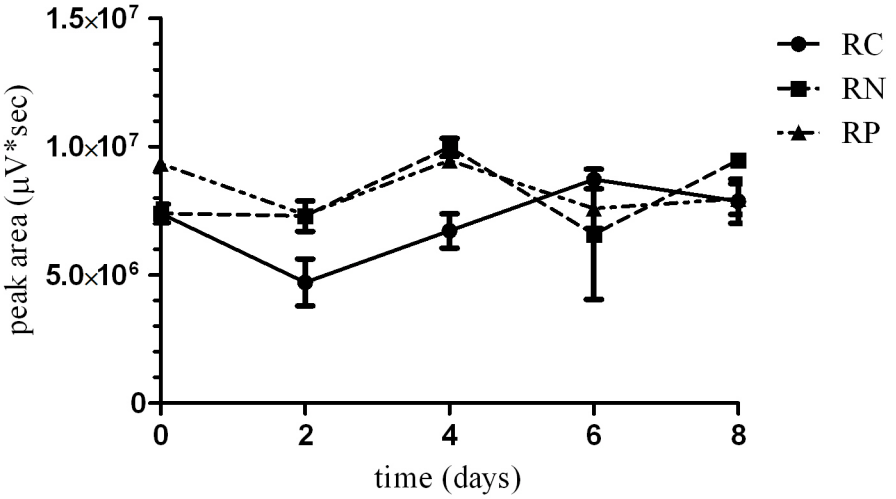


Figure 4.11: Total peak area for all analyses during 8 days of storage at 4°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test can be seen in Table 4.13. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.13: Differences between total peak areas at all time points for every treatment

	Time points for comparison (days)									
Treat-ment	0 vs 2	0 vs 4	0 vs 6	0 vs 8	2 vs 4	2 vs 6	2 vs 8	4 vs 6	4 vs 8	6 vs 8
RC	ns	ns	ns	ns	ns	*	*	ns	ns	ns
RN	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
RP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

4.2.3. Frozen storage

In this section, the following abbreviations for the sample names were used in all figures (Figures 4.3 – 4.6) and tables (Tables 4.4 – 4.7):

FC – control (untreated) human milk stored at -20^{o}C;

FN - human milk stored at -20^{o}C in nitrogen gas environment;

FP - pasteurized human milk stored at -20^{o}C;

SC – control (untreated) human milk stored at -80^{o}C;

SN - human milk stored at -80^{o}C in nitrogen gas environment;

SP - pasteurized human milk stored at -80^{o}C.

The concentrations are expressed as the area percentage of the specific fatty acid of the total peak area, obtained for all fatty acids, mean +/- SE.

4.2.3.1. Storage for 4 weeks in the freezer at -20^{o}C and at -80^{o}C

4.2.3.1.1. Fatty acids

The analyses were performed every week during 4 weeks of storage.

The results of the analysis of LA, ALA, DHA and ARA concentrations in human milk stored in the freezer at -20^{o}C and at -80^{o}C for 4 weeks can be seen in Figures 4.12 – 4.15.

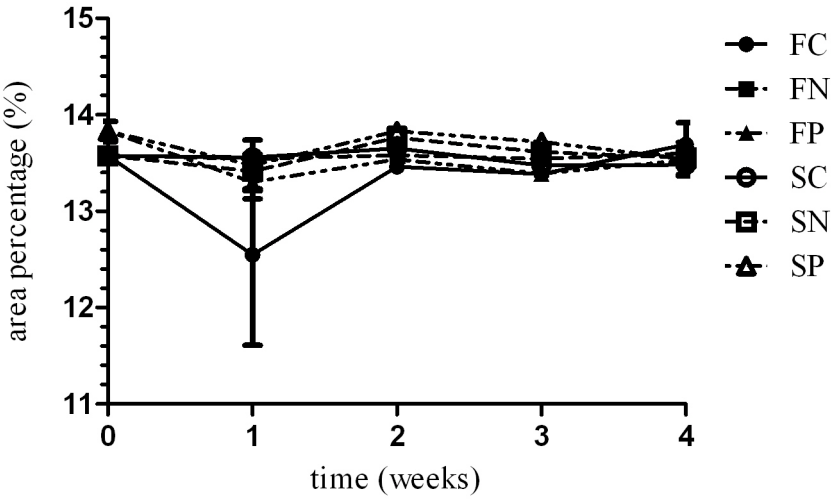


Figure 4.12: Linoleic fatty acid concentration during 4 weeks of storage at -20^{o}C and at -80^{o}C

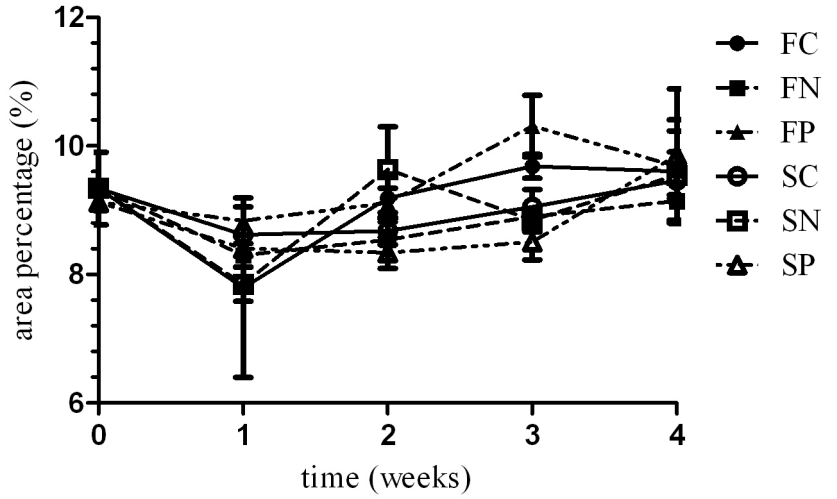


Figure 4.13: α-linolenic fatty acid concentration during 4 weeks of storage at -20^{o}C and at -80^{o}C

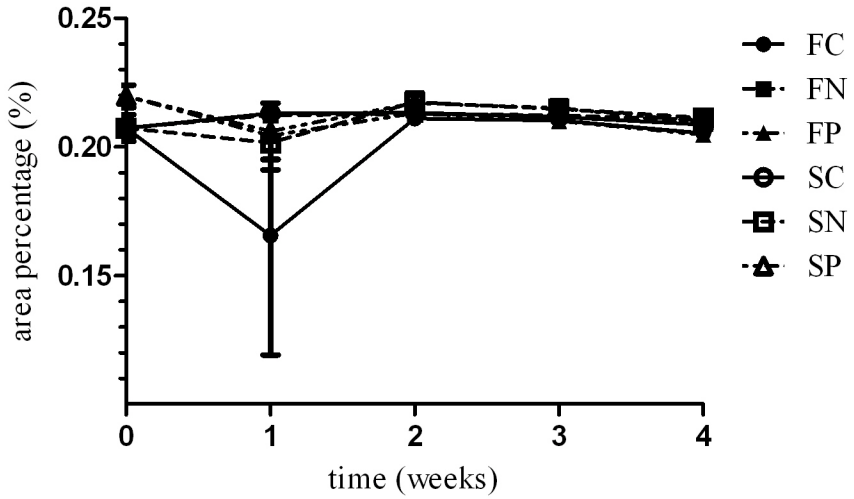


Figure 4.14: Docosahexaenoic fatty acid concentration during 4 weeks of storage at -20°C and at -80°C

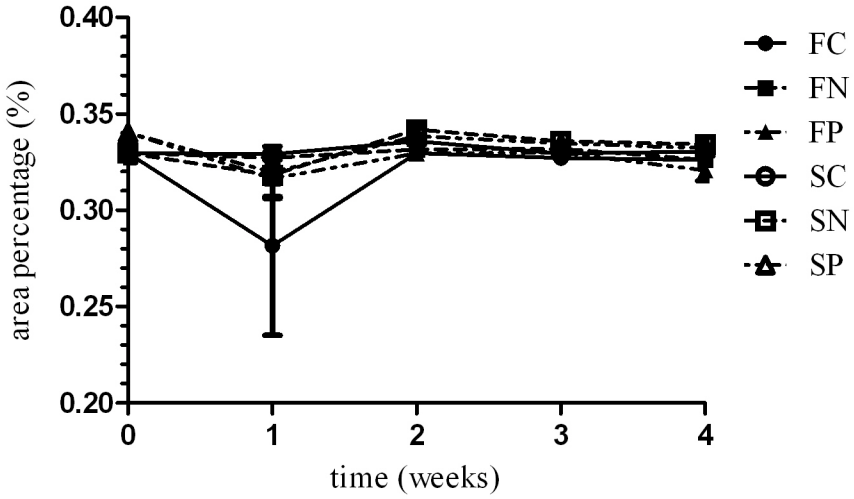


Figure 4.15: Arachidonic fatty acid concentration during 4 weeks of storage at -20°C and at -80°C

One-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test was performed for the results of all pairs of data points for each treatment, for every fatty acid. The results for LA and ARA can be seen in Tables 4.14 and 4.15. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at

P<0.05. In the analyses of ALA and DHA, statistically non-significant differences at P>0.05 were revealed between peak areas for all data points for each treatment.

Table 4.14: Differences between analyses results at all time points for every treatment – LA

	Time points for comparison (weeks)									
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
FC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SC	ns	ns	ns	ns	ns	ns	ns	*	*	ns
SN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 4.15: Differences between analyses results at all time points for every treatment – ARA

	Time points for comparison (weeks)									
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
FC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
FP	*	ns	ns	ns	ns	ns	ns	ns	ns	ns
SC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SN	ns	*	ns	ns	ns	ns	ns	ns	ns	ns
SP	*	ns	ns	ns	ns	ns	ns	ns	ns	ns

As can be seen in Tables 4.14 and 4.15, no significant differences were observed in LA and ARA concentrations of all the samples during 4 weeks storage.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test was performed at each analysis point for the results of all sample types, for each fatty acid. The tests revealed non-statistically significant differences at P<0.05 in all comparisons, except for between sample FC and every other sample on week 1 in the analysis of LA,

DHA and ARA. In the analysis of ALA, statistically significant differences at $P<0.05$ were revealed only between samples FP and SP on week 3.

4.2.3.1.2. Total peak area

The results of the total peak area, as was obtained for all FAMEs at every analysis point for human milk stored in the freezer at -20°C and at -80°C for 4 weeks can be seen in Figure 4.16. The results are presented as the area (μV*sec) +/- SE.

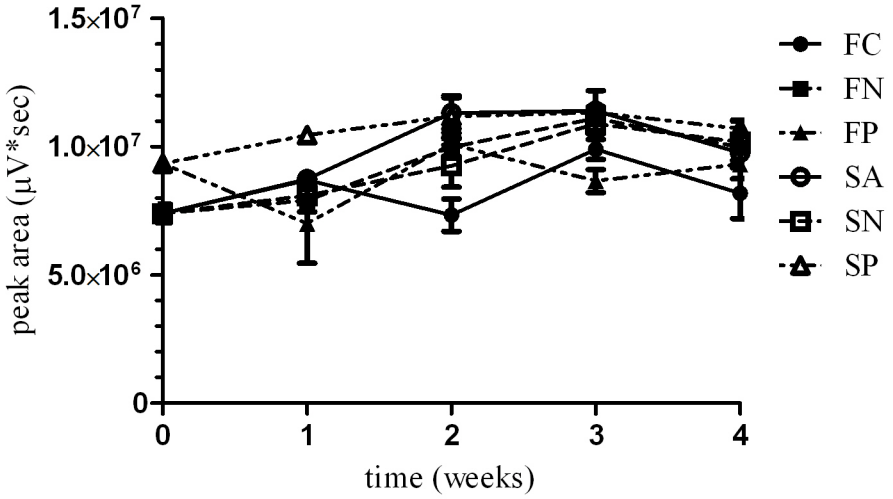


Figure 4.16: Total peak area for all analyses during 4 weeks of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, which was performed for all pairs of data points for each treatment, can be seen in Table 4.16. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.16: Differences between total peak areas at all time points for every treatment

	Time points for comparison (weeks)									
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	1 vs 2	1 vs 3	1 vs 4	2 vs 3	2 vs 4	3 vs 4
FC	ns	ns	*	ns	ns	ns	ns	*	ns	ns
FN	ns	*	*	*	*	*	*	ns	ns	ns
FP	ns	ns	ns	ns	*	ns	ns	ns	ns	ns
SC	ns	*	*	*	*	*	ns	ns	ns	ns
SN	ns	ns	*	*	ns	*	ns	ns	ns	ns
SP	ns	*	*	ns	ns	ns	ns	ns	ns	ns

As can be seen in Table 4.16, the total peak area did not remain stable during 4 weeks of storage.

4.2.3.2. Storage for 6 months in the freezer at -20°C and at -80°C

4.2.3.2.1. Fatty acids

The analyses were performed every month during 6 months of storage.

The results of the analysis of LA, ALA, DHA and ARA concentrations in human milk stored in the freezer at -20°C and at -80°C for 6 months can be seen in Figures 4.17 – 4.20.

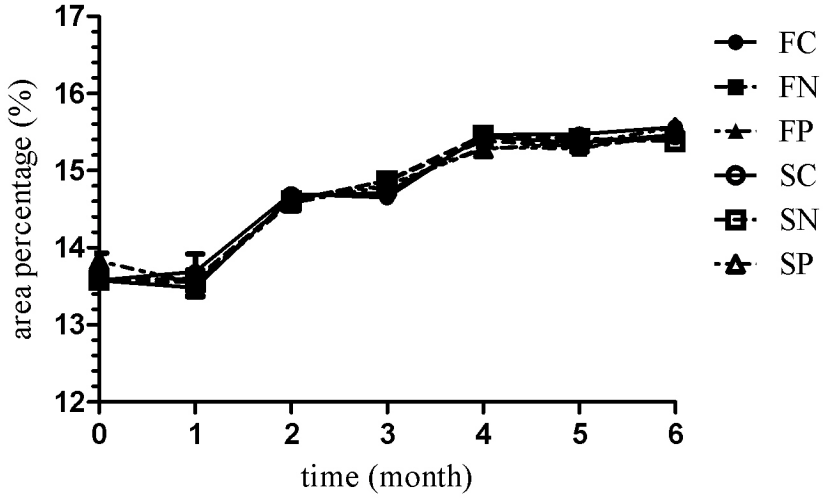


Figure 4.17: Linoleic fatty acid concentration during 6 months of storage at -20°C and at -80°C

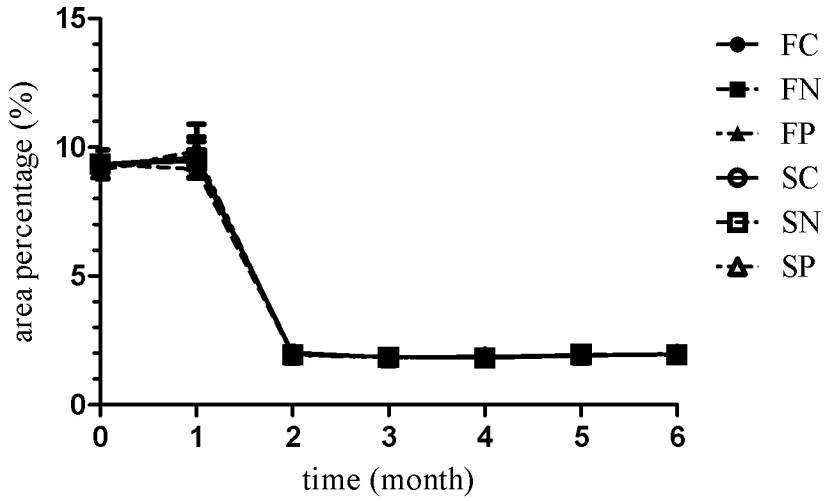


Figure 4.18: α-linolenic fatty acid concentration during 6 months of storage at -20°C and at -80°C

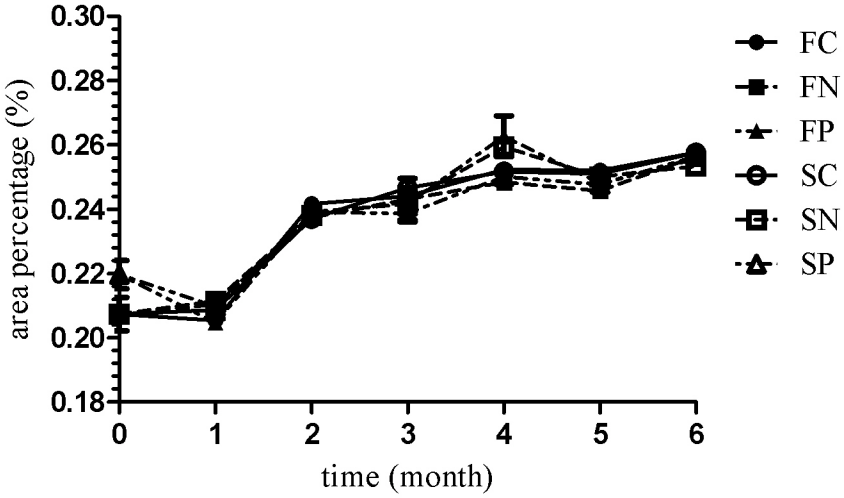


Figure 4.19: Docosahexaenoic fatty acid concentration during 6 months of storage at -20°C and at -80°C

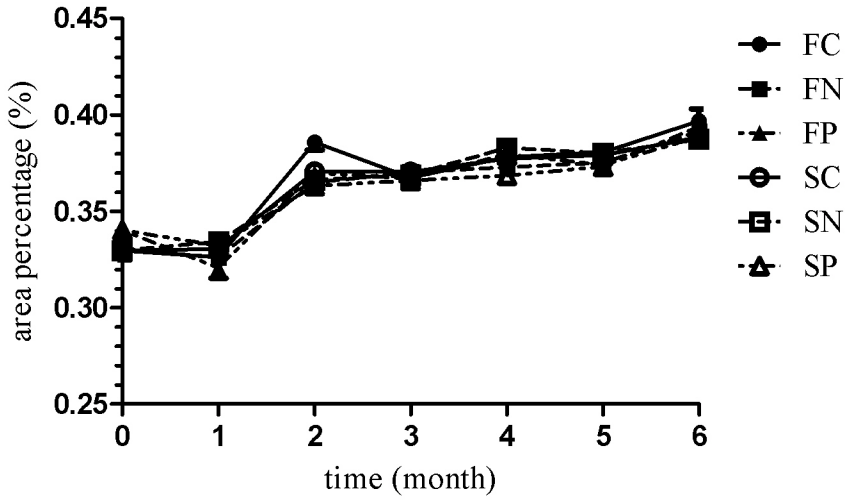


Figure 4.20: Arachidonic fatty acid concentration during 6 months of storage at -20^{o}C and at -80^{o}C

One-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test was performed for the results of all pairs of data points for each treatment, for every fatty acid. The results can be seen in Tables 4.17 - 4.20. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.17: Differences between analyses results at all time points for every treatment – LA

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	ns	*	*	*	*	*	*	*	*	*	*
FN	ns	*	*	*	*	*	*	*	*	*	*
FP	ns	*	*	*	*	*	*	*	*	*	*
SC	ns	*	*	*	*	*	*	*	*	*	*
SN	ns	*	*	*	*	*	*	*	*	*	*
SP	ns	*	*	*	*	*	*	*	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	ns	*	*	*	*	*	*	ns	ns	ns	
FN	ns	*	*	*	*	*	*	ns	ns	ns	
FP	ns	*	*	*	*	*	*	ns	ns	ns	
SC	ns	*	*	*	*	*	*	ns	ns	ns	
SN	ns	*	*	*	*	*	*	ns	ns	ns	
SP	ns	*	*	*	*	*	*	ns	ns	ns	

As can be seen in Figure 4.17 and Table 4.17, in all samples LA content remained stable for 1 month of storage, increasing during subsequent 3 months and from the 4th months – remained stable.

Table 4.18: Differences between analyses results at all time points for every treatment – ALA

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	ns	*	*	*	*	*	*	*	*	*	*
FN	ns	*	*	*	*	*	*	*	*	*	*
FP	ns	*	*	*	*	*	*	*	*	*	*
SC	ns	*	*	*	*	*	*	*	*	*	*
SN	ns	*	*	*	*	*	*	*	*	*	*
SP	ns	*	*	*	*	*	*	*	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
FN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
FP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
SC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
SN	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	
SP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	

As can be seen in Figure 4.18 and Table 4.18, the ALA content of pasteurized and unpasteurized human milk remained stable during the first month of storage, declined fast during the second month and remained stable for the last 4 months of storage.

Table 4.19: Differences between analyses results at all time points for every treatment – DHA

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	ns	*	*	*	*	*	*	*	*	*	*
FN	ns	*	*	*	*	*	*	*	*	*	*
FP	ns	*	*	*	*	*	*	*	*	*	*
SC	ns	*	*	*	*	*	*	*	*	*	*
SN	ns	*	*	*	*	*	*	*	*	*	*
SP	ns	*	*	*	*	*	*	*	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	
FN	ns	ns	ns	*	ns	ns	*	ns	ns	ns	
FP	ns	*	ns	ns	*	ns	ns	ns	ns	ns	
SC	ns	*	*	*	ns	ns	ns	ns	ns	ns	
SN	ns	*	*	*	*	ns	ns	ns	ns	ns	
SP	ns	ns	ns	*	ns	ns	*	ns	ns	ns	

As can be seen in Figure 4.19 and Table 4.19, the DHA content of pasteurized and unpasteurized human milk remained stable during the first month of storage, increased during the next month and remained stable during the 3rd month. During the 4th month of storage the DHA concentration of the pasteurized HM stored at -20^{o}C (FP) and of the unpasteurized HM stored in nitrogen environment at -80^{o}C (SN) appeared to increase, while other samples retained their DHA content. No further change in the FA concentration was observed.

Table 4.20: Differences between analyses results at all time points for every treatment – ARA

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	ns	*	*	*	*	*	*	*	*	*	*
FN	ns	*	*	*	*	*	*	*	*	*	*
FP	*	*	*	*	*	*	*	*	*	*	*
SC	ns	*	*	*	*	*	*	*	*	*	*
SN	ns	*	*	*	*	*	*	*	*	*	*
SP	ns	*	*	*	*	*	*	*	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	
FN	ns	ns	ns	*	ns	ns	*	ns	*	*	
FP	ns	ns	ns	*	ns	ns	*	ns	ns	ns	
SC	ns	ns	ns	*	ns	ns	*	ns	ns	ns	
SN	ns	*	*	*	*	ns	*	ns	ns	ns	
SP	ns	ns	ns	*	ns	ns	*	ns	*	*	

As can be seen in Figure 4.20 and Table 4.20, ARA content remained stable during the first month of storage in all samples, except for sample FP. An apparent increase in both the pasteurized and the unpasteurized human milk was observed during the second month and no further changes were detected, except for slight increase in samples FN and SP during the last month.

Two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test was performed at each analysis point for the results of all sample types, for each fatty acid. The tests revealed non-statistically significant differences at $P<0.05$ in all comparisons in the analysis of LA and ALA. In the analysis of ARA, non-statistically significant differences at $P<0.05$ were revealed between all samples at every time point, except for

sample FC and every other sample at month 2; samples FP and SN at month 1; samples SN and SP at month 4. In the analysis of DHA, statistically significant differences at $P<0.05$ were revealed only between:

- At time 0: Pasteurized sample and control at every temperature;
- At time 0: Pasteurized sample and nitrogen at every temperature;
- At time 0: Pasteurized samples and control at opposing temperature;
- At time 0: Pasteurized samples and nitrogen at opposing temperature;
- At month 4: Sample FP and samples FN, SC, SP; sample FN and SN.

4.2.3.2.2. Total peak area

The results of the total peak area, as was obtained for all FAMEs at every analysis point for human milk stored in the freezer at -20°C and at -80°C for 6 months can be seen in Figure 4.21. The results are presented as the area (μV*sec) +/- SE.

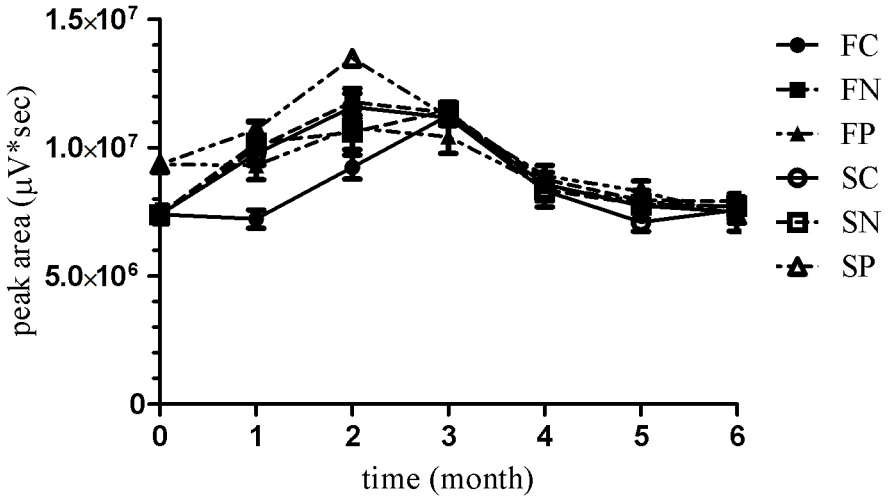


Figure 4.21: Total peak area for all analyses during 6 months of storage at -20°C and at -80°C

The results of one-way ANOVA with Post-Hoc Tukey's Multiple Comparison Test, which was performed for all pairs of data points for each treatment, can be seen in Table 4.21. "Ns" represents detection of no significant differences ($P>0.05$); * represents detection of significant differences at $P<0.05$.

Table 4.21: Differences between total peak areas at all time points for every treatment

	Time points for comparison (months)										
Treat-ment	0 vs 1	0 vs 2	0 vs 3	0 vs 4	0 vs 5	0 vs 6	1 vs 2	1 vs 3	1 vs 4	1 vs 5	1 vs 6
FC	ns	*	*	ns	ns	ns	*	*	*	ns	ns
FN	*	*	*	ns	ns	ns	*	ns	ns	*	*
FP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
SC	*	*	*	ns	ns	ns	*	ns	ns	*	*
SN	*	*	*	ns	ns	ns	ns	ns	ns	ns	ns
SP	ns	*	*	ns	ns	*	*	ns	*	*	*
	Time points for comparison (months)										
Treat-ment	2 vs 3	2 vs 4	2 vs 5	2 vs 6	3 vs 4	3 vs 5	3 vs 6	4 vs 5	4 vs 6	5 vs 6	
FC	*	ns	*	*	*	*	*	ns	ns	ns	
FN	ns	*	*	*	*	*	*	ns	ns	ns	
FP	ns	*	*	*	*	*	*	ns	ns	ns	
SC	ns	*	*	*	*	*	*	ns	ns	ns	
SN	ns	ns	*	*	*	*	*	ns	ns	ns	
SP	*	*	*	*	*	*	*	ns	ns	ns	

As can be seen in Figure 4.21 and Table 4.21, total area of the chromatogram peaks remained stable or apparently increased during the first 3 months of storage in all samples. During the 4th month a decrease to the time 0 values was detected with no changes afterwards.

4.3. E-nose analysis of volatile compounds concentration in stored human milk

4.3.1. Method confirmation

The data acquisition during 300 sec can be found in Figure 4.22. Sensitivity is represented as *[R(t) - R(0)] / R(0)*, where *R(t)* is the sensor's resistance at time *t*.

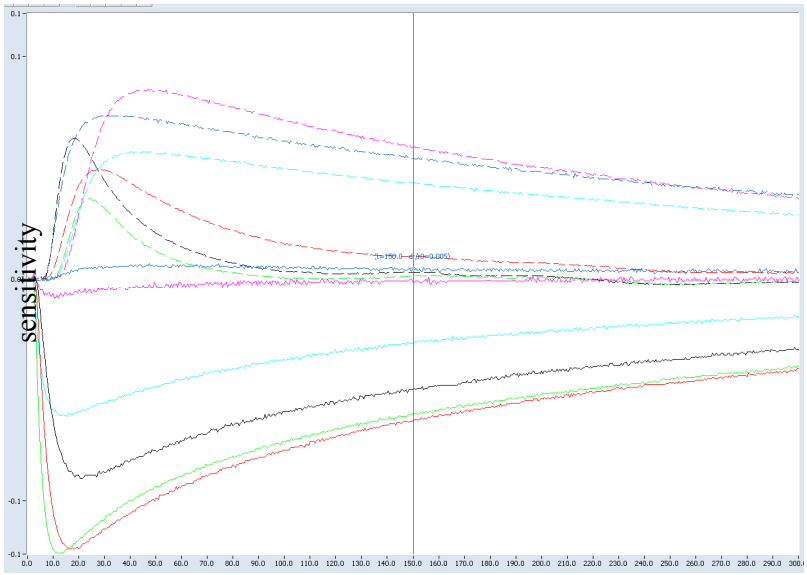


Figure 4.22: Data acquisition

4.3.2. Baseline

No results were obtained during the first 4 days of storage due to equipment unavailability. The first analysis was possible on the 4th day of the refrigeration storage and was referred to as a baseline (time 0). The principle component analysis (PCA) results from data from the first analysis of the volatile compounds concentration pattern (VCCP) in human milk stored in the refrigerator at 4^{o}C not treated (RC), in nitrogen gas environment (RN) or pasteurized prior to storage (RP), can be seen in Figure 4.23. The discrimination index (DI) was 81.

Figure 4.23: PCA of the volatile compounds of stored HM - baseline

4.3.3. Refrigeration storage at 4°C for 8 days

The analyses were performed on day 4, day 6 and day 8.

In this section, the following abbreviations for sample names were used in all the figures (Figures 4.24 – 4.26):

RC - control (untreated) human milk;

RN - human milk stored in nitrogen gas environment;

RP - pasteurized human milk.

The PCA results for each sample type showing the VCCP change from day 4 to day 8 can be seen in Figure 4.24. The discrimination indices were: DI(RC) = 97; DI(RN) = 94; DI(RP) = 87.


Figure 4.24: PCA of the volatile compounds of HM stored at 4°C untreated (left), in nitrogen gas environment (centre), and pasteurized (right), days 4 through 8

A comparison of the VCCP change was performed between control human milk (RC) and HM stored in nitrogen gas environment (RN) and between control human milk (RC) and pasteurized human milk (RP). The PCA results can be seen in Figures 4.25 and 4.26, respectively. The discrimination indices were 93 and 94, respectively.

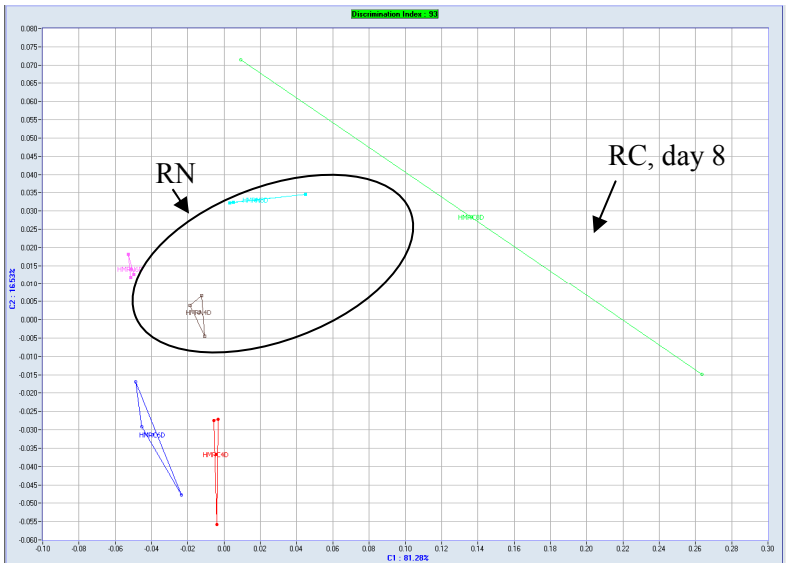


Figure 4.25: PCA of the volatile compounds of HM stored untreated and in nitrogen gas environment at 4°C, days 4 through 8

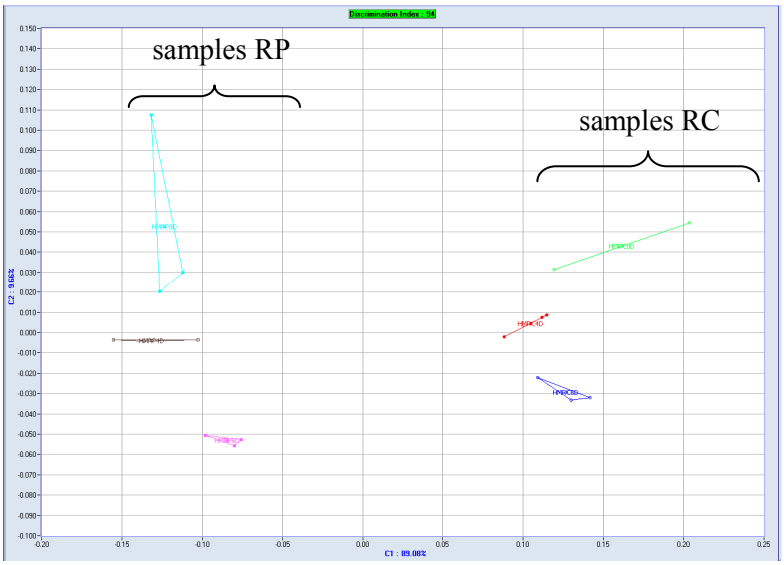


Figure 4.26: PCA of the volatile compounds of HM stored untreated and pasteurized at 4°C, days 4 through 8

4.3.4. Frozen storage

In this section, the following abbreviations for sample names were used in all figures (Figures 4.27 – 4.29) and tables (Tables 4.22 – 4.25):

FC – control (untreated) human milk stored at -20^{o}C;

FN - human milk stored at -20^{o}C in nitrogen gas environment;

FP - pasteurized human milk stored at -20^{o}C;

SC – control (untreated) human milk stored at -80^{o}C;

SN - human milk stored at -80^{o}C in nitrogen gas environment;

SP - pasteurized human milk stored at -80^{o}C.

4.3.4.1. Storage for 4 weeks in the freezer at -20^{o}C and at -80^{o}C

The analyses of the volatile compounds concentration pattern in human milk stored in the freezer at -20^{o}C and at -80^{o}C were performed in the end of each week during 4 weeks of storage.

The discrimination indices calculated from the PCA during the 4 weeks of storage for each sample, can be found in Table 4.22. The PCA of the volatile compounds analysis data of sample SP can be found in Figure 4.27. The PCA of the volatile compounds analysis data of the other samples can be found in Appendix 5 (Figures app.5.1 – app.5.5). As was explained above, the results of the 4th day of the analysis were considered as time 0.

Table 4.22: Discrimination indices calculated from the PCA of the volatile compounds of HM stored at -20^{o}C and at -80^{o}C during 4 weeks

	sample					
	FC	FN	FP	SC	SN	SP
DI	-8	0	0	0	-1	82

Figure 4.27: PCA of the volatile compounds of pasteurized HM stored at -80°C: pattern change during 4 weeks of storage

The discrimination indices calculated from the PCA for the volatile compounds of specific pairs of samples, can be found in Table 4.23. The PCA for other samples' pairs can be found in Appendix 5 (Figures app.5.6 – app. 5.11). As was explained above, the results of the 4th day of the analysis were considered as time 0.

Table 4.23: Discrimination indices calculated from the PCA of the volatile compounds of pairs of HM types stored at -20°C and at -80°C during 4 weeks

	sample					
	FC&SC	FC&FN	FC&FP	FP&SP	SC&SP	SC&SN
DI	-18	-11	-8	0	-10	-24

4.3.4.2. Storage for 5 months in the freezer at -20°C and at -80°C

Analyses of the volatile compounds concentration pattern of human milk stored in the freezer at -20°C and at -80°C were performed in the end of each month during 5 months of storage.

The discrimination indices calculated from the PCA during 5 months of storage for each sample, can be found in Table 4.24. The PCA of the volatile compounds analysis data of the samples FC and FP can be found in Figures 4.28 and 4.29, respectively. The PCA of the analysis data of the other samples can be found in Appendix 5 (Figures app.5.12 – app. 5.15). As was explained above, the results of the 4th day of the analysis were considered as time 0.

Table 4.24: Discrimination indices calculated from the PCA of the volatile compounds of HM stored at -20^{o}C and at -80^{o}C during 5 months

	sample					
	FC	FN	FP	SC	SN	SP
DI	0	91	84	0	-2	0

Figure 4.28: PCA of the volatile compounds of HM stored with nitrogen gas at -20^{o}C (FN): pattern change during 5 months of storage

Figure 4.29: PCA of the volatile compounds of pasteurized HM stored at -20^{o}C: pattern change during 5 months of storage

The discrimination indices derived from the PCA of the different pairs of samples, can be found in Table 4.25. The results for the other samples' pairs can be found in the appendix 5 (Figures app.5.16 – app. 5.21). As was explained above, the results of the 4th day of the analysis were considered as time 0.

Table 4.25: Discrimination indices calculated from the PCA of the volatile compounds of pairs of HM types stored at -20^{o}C and at -80^{o}C during 5 months

	sample					
	FC&SC	FC&FN	FC&FP	FP&SP	SC&SP	SC&SN
DI	-104	-13	0	-102	-10	-144

CHAPTER 5: DISCUSSION

5.1. Changes in the concentrations of vitamin C and vitamin B_2 during storage of human milk

As the aim of the study was to report differences in vitamin C and vitamin B_2 concentration during storage of expressed human milk, the evaluation of the vitamins' concentrations' change was based on the change in the peak areas, as were obtained from the HPLC chromatograms. The calculations of the exact concentrations were not necessary and were not possible to derive due to the very low initial concentration of vitamin C in the fresh human milk in this study, which produced chromatogram peaks of areas outside the standard curve range, although built in agreement with the information from the literature. According to the literature, vitamin C concentration of fresh human milk is approximately 100 mg/liter (Bank et al, 1985; Picciano, 1995). SRM 1849 was diluted 15-fold in order to obtain this concentration and thus allow calculation of the vitamin in the fresh HM obtained from the donors. However the peaks for vitamin C obtained from the SRM were 15-fold larger, than the peaks obtained from HM samples after the earliest storage period (2 days), thus making the standard curve appropriate for the calculations of vitamin C concentration in the SRM, but not in the HM. Further dilution of the vitamin C standards was not possible technically, due to very low concentrations required. However, as was mentioned before, peak areas obtained from the chromatograms allowed the analysis of the changes in the vitamins concentrations during storage. The low vitamin C content of fresh human milk in this study may be attributed to the dilution of the fresh human milk with stored human milk (having a very low vitamin C content) and to the decomposition of the vitamin during collection and

transportation of the HM, as it is very susceptible to chemical and enzymatic oxidation during processing and storage of food (Ball, 2006).

Several results were not included in the data sets due to values clearly out of the range of the results of other replicas or due to unreasonable values. The obtained values could be attributed to various technical errors, such as: loss of the analyzed material during the preparation process; possible adulteration of the analyzed material during pasteurization, preparation for storage or thawing; accidental exposure to light; non-similar temperature of the samples during all preparation procedure steps; some retention of vitamins in the fatty phase of the milk; inefficient injection of the analyzed material to the HPLC machine through the automized injection needle; co-elution of products of decomposition of other water soluble components with the vitamins of interest; imprecise calculation of the peak areas by the computer.

5.1.1. Refrigeration storage at 4^{o}C

5.1.1.1. Vitamin C

No values were obtained for vitamin C concentration at time 0 for control human milk and HM stored with nitrogen gas (which were the same sample at time 0). However, as it is known that pasteurization causes loss of vitamin C in expressed human milk (Jensen, 1995; Silvestre et al., 2008; Romeu-Nadal et al., 2008b; Van Zoeren-Grobben et al., 1987; Wiliamson et al., 1978) and that concentration of the vitamin in non-treated HM declines from its initial content during first 24 hours of storage at 4^{o}C (Bank et al, 1985; Buss et al., 2001, Ezz El Din et al., 2004) and decreases constantly during 4 days of storage according to Romeu-Nadal et al. (2008a), it can be assumed that its initial content

exceeded the concentration found in the pasteurized milk at time 0. Therefore, according to Figure 4.1, we assume that a decrease in the vitamin concentration happened during the first 2 days of storage at 4^{o}C in the control sample (RC) and in the milk stored with nitrogen gas (RN). Although, as the pasteurized sample vitamin C content was no lower than the control's after the 2nd day of storage, it might be suggested that thermal process did not affect the vitamin C content during storage after the 2nd day. These results contradicted expectations of a lower vitamin C content of pasteurized human milk.

The results of the analysis performed on day 8 of the experiment were not included due to analytical uncertainty, as the value of vitamin C which was obtained in SRM was too low (69.96% of the declared value – see Table 4.1).

According to the results, vitamin C concentration remained stable in the control milk from day 2 until day 6 of the experiment and in the pasteurized milk from time 0 until day 6. This result was in disagreement with Romeu-Nadal et al. (2008a), who reported a significant decrease in the vitamin content during 4 days of refrigeration storage.

A detrimental effect was observed for oxygen limitation on vitamin C preservation, as the vitamin concentration in milk stored in nitrogen gas decreased from day 2 until day 6 of storage. These results were in contrast with the expectation of a nutrient preservation effect of oxygen limitation (Singh and Cadwallader, 2000).

The storage period in this study was within the range of many of the official recommendations for HM refrigerated storage, including Winnipeg Public Health – Government of Manitoba, which is 5-8 days (see Table 1.1).

5.1.1.1. Vitamin B_2

Vitamin B_2 concentration did not differ in the beginning and at the end of the storage period in all samples, in contrast with expectations. In a study of photodegradation of several vitamins in cow's milk, Fanelli et al. (1985) reported slow decomposition of riboflavin during 72 hours of storage at 1-3^{o}C in the dark. Although in the present study a decrease in the vitamin concentration was observed in all samples during the first 2 - 4 days of storage, followed by an increase to the initial levels at day 6, this observed trend can be attributed to the low result of the vitamin content in the SRM on day 2 (51.41% of the declared value), thus generating too small peaks. This storage period is within the range of most of the official recommendations for HM refrigerated storage, including Winnipeg Public Health – Government of Manitoba, which is 5-8 days (see Table 1.1).

Vitamin B_2 concentration did not differ at time 0 and through the whole course of the storage at 4^{o}C for 8 days between control human milk and human milk which was stored with nitrogen gas. This suggests no advantage and no detrimental effect of oxygen limitation for riboflavin preservation for 8 days of storage at 4^{o}C.

Vitamin B_2 concentration did not differ at time 0 and through the whole course of the storage at 4^{o}C for 8 days between control human milk and human milk which was pasteurized prior to storage. That was in agreement with Van Zoeren-Grobben et al., (1987), who reported lack of detrimental effects of pasteurization on human milk riboflavin content. Ball (2006) states that riboflavin is generally stable during heat treatments if light is absent, supporting the findings of the present study.

5.1.2. Frozen storage

5.1.2.1. Storage for 4 weeks in the freezer at -20°C and at -80°C

5.1.2.1.1. Vitamin C

No values were obtained for vitamin C concentration at time 0 for control human milk samples and samples stored with nitrogen gas (which were the same samples at time 0). But as it is known that pasteurization causes loss of vitamin C in expressed human milk (Jensen, 1995; Silvestre et al., 2008; Van Zoeren-Grobben et al., 1987; Wiliamson et al., 1978) and that concentration of the vitamin in non-treated HM declines significantly from its initial content during 1 week of frozen storage (as was reported by Buss et al., 2001 for storage at -16°C and by Ezz El Din et al., 2004 for storage at -4-8°C), it can be assumed that its initial content in the untreated sample exceeded the concentration found in the pasteurized sample at time 0 in support of section 5.1.1. A further continuous decline in the vitamin concentration supports this assumption. Therefore, according to Figure 4.3, a significant decrease in the vitamin concentration might have happened during the first week of storage at -20°C and -80°C in the control sample (FC, SC) and maybe also in the milk stored with nitrogen gas (FN, SN).

According to Buss et al. (2001), 2/3 of the initial vitamin C can be preserved in unpasteurized human milk for 1 month of storage at -16°C. As no values were obtained at time 0 in this study, it is difficult to question this statement. Although taking into account the level of the vitamin C in the pasteurized HM at time 0, the insufficient result for the vitamin analysis in the SRM at 4 weeks of storage (86.81% of the declared value) and the higher temperature of storage (-16°C) in the study of Buss et al. (which could contribute

to faster loss of the vitamin), it might be suggested that much less than 2/3 of the initial vitamin C is retained in human milk after 1 month of storage at -20^{o}C.

Vitamin C is very susceptible to chemical and enzymatic oxidation during processing and storage. It is also destroyed by light when riboflavin is present, particularly in human milk (Ball, 2006). This vulnerability is probably the cause of the observations from this study: vitamin C concentration in all samples decreased significantly during each week of 4 weeks of storage at -20^{o}C. These observations are in a general agreement with the study of Picciano (1995), who states that degradation of ascorbic acid during storage at -20^{o}C must be taken into consideration while assessing the vitamin levels in human milk. Buss et al. (2001) also reported a reduction in the total vitamin C levels during frozen storage of HM, analyzing its levels after 1 week and after 1 month at -16^{o}C. In the present study the reduction of vitamin C was observed also in the control milk stored at -80^{o}C (SC), reaching an undetectable level during the 2nd or the 3rd week of storage (no results were obtained at 2 weeks due to technical problems). As oxygen limitation slows down oxidative reactions (Singh and Cadwallader, 2000), the decrease was slower in samples stored with nitrogen gas at both temperatures (FN, SN) during the last week of storage, compared with the other samples. However due to the low result of vitamin C content in the SRM at 4 weeks and the high result at 3 weeks (86.81% and 112.77% of the declared value, respectively), it might be concluded that possibly no significant decrease occurred during the last week of the experiment for all samples.

According to the initial levels of vitamin C in the pasteurized milk and the estimation of the initial level in untreated milk, during 2 weeks of storage at any of the freezing temperatures, the vitamin level drops to ~1/3 of its initial level in the pasteurized milk

and to an even lower proportion of its initial level in the untreated milk. For growth, development and survival, infants need an optimum supply of ascorbic acid (Francis et al., 2008; Romeu-Nadal et al., 2006). Therefore, in support of Romeu-Nadal et al. (2008a) recommendation, if storage for longer than 2 weeks is required in any of these temperatures before feeding, caregivers should be advised to add vitamin C immediately before usage of the stored milk for feeding, due to the low vitamin C levels remaining in the milk. In case of feeding exclusively by stored human milk older than 2 weeks, we recommend supplementation of the infant with vitamin C.

After 1 week of storage HM stored with nitrogen gas at -80^{o}C (SN) retained a significantly lower concentration of vitamin C compared with other samples, except for the pasteurized samples at the same temperature. Because the vitamin concentration in sample SC dropped to zero after 1 week of storage (monthly analyses results confirmed the absence of a technical mistake – see Figure 4.5), it can be concluded that this temperature is detrimental to vitamin C in HM. However, if HM is required to be stored at this temperature, it is beneficial to limit oxygen availability in the storage container. The data from this study supported the proposed preservation effect of oxygen limitation on nutrients levels (Singh and Cadwallader, 2000).

Vitamin C concentration in the control HM stored at -20^{o}C did not benefit from the limitation of oxygen over the course of the 4 weeks storage, contrary to the expectations of oxygen limitation preservation effect, as implied by Singh and Cadwallader (2000).

At 1 week of frozen storage at both temperatures, vitamin C content of pasteurized HM sample did not differ from the other samples' contents. After the first week it decreased in a similar to the other samples pattern, in accordance with the expectations.

Overall, for pasteurized milk, lower freezing temperature helped to retain more vitamin than the higher freezing temperature.

5.1.2.1.2. Vitamin B_2

While untreated human milk retained its vitamin B_2 content during 3 weeks of storage both at -20^{o}C and at -80^{o}C with no difference between them, pasteurized samples and samples stored in nitrogen gas showed a significant decrease during this period. The decrease in vitamin B_2 content while oxygen was limited contradicted the expectations of these conditions' preservation effect (Singh and Cadwallader, 2000). Though during the 4th week of storage a decrease in the riboflavin content occurred in all samples according to Figure 4.4 and Table 4.5, from this part of the study it is unclear whether the decrease indeed occurred due to a low result of the vitamin content in the SRM at 4 weeks (73.45% of the declared value). During the 4th week of storage vitamin B_2 concentrations dropped to an undetectable level in the SN sample. However, as further monthly analyses detected vitamin B_2 presence in the SN sample type (see Figure 4.6), the drop to an undetectable level may be attributed to a technical error. Furthermore, as monthly analyses showed insignificant differences in riboflavin concentration during 2 months of storage in samples FC, SC and SN (see Table 4.7), it is evident that there was no decrease during the last week of storage in the control samples stored at both temperatures (FC, SC) and in the sample SN. The presence or the absence of the decrease in riboflavin content in pasteurized samples at both temperatures and in sample FN during the 4th week still remains uncertain due to the low result of the vitamin content in the SRM, as mentioned above.

Samples stored with nitrogen gas at -80^{o}C did not achieve significance in the difference in the content of riboflavin at every analysis time point through the course of 4 weeks compared with the control milk stored at the same temperature. This data suggests that these conditions are not beneficial for frozen storage of human milk. Oxygen limitation did not give an advantage for the riboflavin preservation also at -20^{o}C.

According to the expectations based on the literature (Van Zoeren-Grobben et al, 1987), pasteurized samples were not different in riboflavin content than untreated samples initially. However the vitamin levels decreased during storage to ~70% of the initial level by the end of the 4th week. Lower storage temperature of pasteurized milk did not contribute to the vitamin stability during 4 weeks of storage.

5.1.2.2. Storage for 6 months in the freezer at -20^{o}C and at -80^{o}C

5.1.2.2.1. Vitamin C

The results of the analysis performed at 3 months of the experiment were not included due to analytical uncertainty, as the value of vitamin C which was obtained in SRM was too low (44.81% of the declared value – see Table 4.1). As during the first 3 weeks of storage the vitamin content dropped to a low level, further storage can only maintain or result in its further decrease. Thus results showing other than a decrease may be attributed to experiment errors and/or to the limitations of the samples' preparation method to allow consistent accuracy of extraction of such low contents of vitamin C from human milk.

In this study vitamin C content dropped to an undetectable level during the first month of storage of the control sample at -80^{o}C, opposed to the control milk stored at -20^{o}C, which retained some of the vitamin (see section 5.1.2.1.1). During the apparent

increase of vitamin C content after 2 month of storage at -80^{o}C, its level remained significantly lower than that of HM stored at -20^{o}C. Therefore it may be concluded that -20^{o}C temperature is preferred for storage of unpasteurized human milk, opposed to the hypothesis. If HM is required to be stored at -80^{o}C, limitation of oxygen would help to retain some vitamin C.

Although no results were obtained at time 0 for the vitamin C content in unpasteurized human milk, it is reasonable to assume that its initial content in unpasteurized milk exceeded the content found in the pasteurized HM at time 0, therefore pointing at the vitamin decomposition during the first month of storage (see section 5.1.2.1.1). The control sample stored at -20^{o}C retained some level of the vitamin after the drop during the first month. That was in agreement with the study of Bank et al. (1985), who reported a significantly lower vitamin C content in the milk of mothers to term infants after 3 months of storage at -20^{o}C, compared with the initial levels. Buss et al. (2001) analyzed the total vitamin C level during storage of HM at -16^{o}C after 1 month and after 2 months and also reported significant reductions. Ezz El Din et al. (2004) analyzed vitamin C after 1 week of storage at -4-8^{o}C and also reported a decrease. These results can be compared with the results of the present study, in spite of the different temperatures applied, because of the freezing conditions.

After 4 months of storage the control sample stored at -20^{o}C (FC) and the sample stored in nitrogen gas environment at -20^{o}C (FN) were shown to contain levels of vitamin C similar to those obtained at the 1 month analysis, implying that after 1 month of storage at these conditions HM maintains low, but stable, vitamin C levels. The increase in the

vitamin content in these 2 samples during the last month of storage was probably caused by a process or a computer calculation error.

After 4 months of storage, the control sample stored at -20^{o}C (FC) and the sample stored in nitrogen gas environment at -20^{o}C (FN) retained significantly higher content of vitamin C compared with all other samples, FN retaining higher concentration of the vitamin than the control FC. No significant differences were detected between these samples after 1 month of storage. Therefore it might be concluded that nitrogen gas environment benefits vitamin C stability in human milk stored longer than 4 months at -20^{o}C, that in accordance with the expectations (Singh and Cadwallader, 2000). During storage for 6 months unpasteurized human milk stored without or with nitrogen gas retained some vitamin C, in accordance with the period recommended by the official recommendations for HM frozen storage at -20^{o}C, including Winnipeg Public Health – Government of Manitoba, which is at least 6 months (see Table 1.1).

According to the expectation, vitamin C content of pasteurized human milk declined during storage for 6 months. The decline was sharp during the 1st month of storage. By the 4th month it contained significantly lower levels of vitamin C than the unpasteurized milk stored at -20^{o}C. Lower storage temperature presented no clear benefit for the vitamin preservation.

These observations support the previously made recommendation of addition of vitamin C to human milk immediately before feeding, if was stored for longer than 2 weeks at -20^{o}C or at -80^{o}C.

5.1.2.2.2. Vitamin B_2

Vitamin B_2 content measurements at 1 month and at 4 months of storage are not very reliable, due to the low result of the vitamin content in the SRM (73.45% and 81.72% of the declared content, respectively). The vitamin level in the SN sample was undetectable after the first month of storage, but that can be attributed to an experimental error, as its concentration increases sharply afterwards, retaining its level.

The results show that untreated human milk can be stored at -20^{o}C and at -80^{o}C for 3 months, retaining its initial vitamin B_2 content. The vitamin stability at -20^{o}C is in agreement with the literature: Engler and Bowers (1976) claim generally high retention of riboflavin in stored frozen meat. The literature lacks information about storage at -80^{o}C. The lower freezing temperature did not have an advantage over higher freezing temperature in the present study.

Samples stored in a limited oxygen environment at both temperatures also retained initial vitamin B_2 contents for 3 months, but further the modified atmosphere had a slight negative effect, in contrast to the expected preservation effect of oxygen limitation as implied by Singh and Cadwallader (2000).

Vitamin B_2 content remained stable at both storage temperatures in the pasteurized samples during 3 months of storage. Afterwards the vitamin levels decreased, remaining at levels close to those of the control milk. These observations suggest similar trend of the vitamin decomposition in the pasteurized and unpasteurized HM.

Control samples retained their vitamin B_2 content for 6 months of storage at both temperatures, with no benefit for either temperature in accordance with the expected stability of the vitamin during storage, as was described for frozen meat (Engler and

Bowers, 1976). This period is within the range of the official recommendations for HM frozen storage at -20^{o}C, including Winnipeg Public Health – Government of Manitoba, which is at least 6 months (see Table 1.1). The nitrogen gas environment was not beneficial for riboflavin preservation for any of the storage temperatures, and even detrimental, in contrast with the expected preservation effect of oxygen limitation (Singh and Cadwallader, 2000). In the pasteurized samples, vitamin B_2 levels decreased after 3 months at both temperatures, with no benefit of either temperature, but still retained appreciable levels.

At the end of the storage period of 6 months, all samples retained appreciable levels of vitamin B_2. These results are in accordance with the expected stability of the vitamin during storage (Engler and Bowers, 1976). As the fresh human milk obtained from the donors for this research was mixed with thawed human milk which was stored for 7 months prior to this study initialization, resulting in approximately 2-fold dilution, it can be inferred that vitamin B_2 is stable for 6 months, and probably even for 12 months of storage in the conditions of this study.

5.2. Changes in the concentrations of LA, ALA, ARA and DHA during storage of human milk

As the aim of the study was to report differences in the fatty acids' concentrations during storage of expressed human milk, evaluation of the FAs concentrations' change was based on the presence or the absence of a decrease in the peak area percentages obtained from the GC-FID chromatogram. These conclusions could be drawn in case that the total peak area for the particular sample over the course of storage period remained stable. An observed increase in

a fatty acid area percentage indicates that this fatty acid maintains stability or degrades slower, compared with other FAs in the same sample.

Several results were excluded from the data sets due to values clearly out of the range of the results of other replicas or due to unreasonable values. The obtained values could be attributed to various technical errors, such as: loss of the analyzed material during the FAMEs preparation process; more or less efficient technical performance of the extraction steps; incomplete methylation of fatty acids due to temperature fluctuations of the heating block; co-elution of products of decomposition of other lipids with the fatty acids of interest; contamination of the analyzed material with residue of lipids from previous analyses as a result of insufficiently cleaned test-tubes; inefficient injection of the analyzed material to the GC-FID machine through the automized injection needle; possible adulteration of the analyzed material during pasteurization, preparation for storage or thawing; imprecise calculation of the peak areas by the computer.

Holder pasteurization did not affect LA, ALA, ARA and DHA, in support of Fidler et al. (1998), Friend et al. (1983), Henderson et al. (1998), Jansen (1995).

The fatty acid content of fresh human milk was in the range of the values stated in the literature for milk of Canadian women (Jensen, 1999): 13.58 +/- 0.05% for LA (expected: 5.79% – 21.35%); 2.03 +/- 0.02% for ALA (expected: 0.58% – 1.90%); 0.21 +/- 0.005% for DHA (expected: up to 0.53%); 0.33 +/- 0.005% for ARA (expected: 0.05% – 0.69%).

5.2.1. Refrigeration storage at 4°C for 8 days

As no difference was observed for the total peak areas at each analysis for each fatty acid during 8 days of storage, comparison between fatty acids relative concentrations can be made based on their peak areas.

The linoleic fatty acid content of the unpasteurized human milk samples (RC, RN) remained stable during storage for 8 days at 4°C. Storage in nitrogen gas environment had no advantage for LA preservation. The LA level of the pasteurized human milk (RP) did not differ from the unpasteurized milk level (RC, RN) at time 0, decreased during first 2 days of storage and then remained stable until the 8th day. Starting from day 2, it was not different from the 2 other samples' LA concentration at any of the analysis time points. These results bring the conclusion of no detrimental effects of Holder pasteurization on LA content of human milk.

The α-linolenic fatty acid content of both unpasteurized human milk and the pasteurized human milk did not differ at time 0 and remained stable during the 8 days of storage, suggesting no detrimental effects of Holder pasteurization on ALA content of human milk. The increase and the subsequent decrease to the initial level in the ALA content in the pasteurized sample (RP) can be attributed to a technical error. Storage in nitrogen gas environment had no advantage for ALA preservation. Although according to the two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test, differences in the FA content were observed between a few samples on day 4 and on day 6, due to the trend of the ALA stability in all samples, these differences can be attributed to technical errors and therefore disregarded.

The DHA content of the control sample (RC) remained stable for the 8 days of storage. The DHA content of human milk stored with nitrogen gas (RN) remained stable for the first 6 days of storage, slightly decreasing during the subsequent 2 days. Though the DHA content of the pasteurized milk (RP) and the unpasteurized milks (RC, RN) differed at time 0, further results suggested no difference between the fatty acid concentrations in these milk types. Therefore the differences in the initial FA content between sample RP and samples RC/RN can be disregarded.

No difference in the arachidonic fatty acid content was observed during the course of 8 days storage at 4^{o}C for any of the samples, including time 0, thus suggesting no detrimental effects of Holder pasteurization of ARA content of human milk. Storage in nitrogen gas environment had no advantage for DHA preservation. Differences in the FA content between samples at each analysis point, as revealed by the two-way Repeated Measures ANOVA with Post-Hoc Bonferroni Test, can be attributed to technical errors and therefore disregarded.

Overall, the 4 fatty acids remained stable during 8 days of storage at 4^{o}C. That was in agreement with Slutzah et al. (2010, storage for 4 days) and Tacken et al (2009, storage for 2 days), who state that the fat integrity of fresh HM is not affected by storage at this temperature. In accordance with the hypothesis, pasteurized HM did not differ from unpasteurized HM during the course of the 8 days storage. This storage period is within the range of the official recommendations for HM refrigeration storage at 4^{o}C, which is 5-8 days, including Winnipeg Public Health – Government of Manitoba (see Table 1.1).

5.2.2. Frozen storage

5.2.2.1. Storage for 4 weeks in the freezer at -20°C and at -80°C

No decrease in the total peak area was detected in the weekly analyses (see Figure 4.16 and Table 4.16). Therefore an estimation of relative changes in fatty acids contents, based on area percentages, can be made.

A decrease in the four FAs contents in the control sample stored at -20°C can be seen after 1 week of storage, but its subsequent increase to their initial levels suggests a technical error.

As it was mentioned in section 4.2.1., Holder pasteurization does not affect LA, ALA, DHA and ARA levels in human milk during the thermal treatment.

The concentration of LA did not change through the course of 4 weeks storage at -20°C and at -80°C in all samples, suggesting no benefit of a lower temperature storage, no benefit of nitrogen gas environment for FA preservation and also no detrimental effect of pasteurization on LA content during storage.

The concentration of ALA did not change through the course of 4 week storage at -20°C and at -80°C in all samples, suggesting no benefit of lower temperature storage, no benefit of a nitrogen gas environment for the FAs preservation and also no detrimental effect of pasteurization on ALA content during storage. Differences between the FA concentration in the pasteurized samples after 3 weeks of storage can be disregarded and attributed to a technical error, as no differences were observed further.

The concentration of DHA did not change through the course of 4 weeks storage at -20°C and at -80°C in all samples, suggesting no benefit of lower temperature storage, no

benefit of nitrogen gas environment for the FAs preservation and also no detrimental effect of pasteurization on DHA content during storage.

The concentration of ARA did not change through the course of 4 weeks storage at -20^{o}C and at -80^{o}C in all samples, suggesting no benefit of lower temperature storage, no benefit of nitrogen gas environment for the FAs preservation and also no detrimental effect of pasteurization on ARA content during storage. The changes in ARA concentration in various samples during the first 2 weeks of storage can be disregarded due to the subsequent increase to the initial levels, suggesting a technical error.

Overall, the 4 fatty acids remained stable during 4 weeks of storage at -20^{o}C and at -80^{o}C. These results were in accordance with the expectations based on the literature, which report a stability of HM fat at these conditions. Ezz El Din et al (2004) reported similar levels of the fat content after 1 week of storage at –4 to –8^{o}C. Tacken et al. (2009) showed that mature human milk can be stored safely at -18^{o}C for 28 days without a loss of fat. Friend et al. (1983) reported stability of total FA level and relative % of each FA in the frozen samples stored for 3 months at -25^{o}C. Reynolds et al. (1982) concluded that storage of HM at -20^{o}C for 1 month does not change the concentrations of fatty acids. Berkow at al. (1984) reported no hydrolysis of the HM fat during storage for 5 months at -70^{o}C.

In accordance with the hypothesis, pasteurized HM did not differ from unpasteurized HM during the course of the 4 weeks storage.

5.2.2.2. Storage for 6 months in the freezer at -20^{o}C and at -80^{o}C

During the first 3 months of storage the total peak area changed, but not decreased, in all samples, except for the sample FP, as can be seen in Figure 5.21 and Table 5.21.

During the 4th month of storage the peak area decreased to its initial values with no change afterwards. The technical process, such as more effective extraction of lipids, may be suggested as a reason for the increase during the first months.

All 4 fatty acids showed relative stability during 6 months of storage in accordance with the literature, as was discussed in the section 5.2.2.1. As the fresh human milk obtained from the donors for this research was mixed with thawed human milk which was stored for 7 months prior to this study initiation, resulting in approximately 2-fold dilution, it can be implied that the 4 FAs are stable for 6 months, and probably even for 12 months of storage in the conditions of this study.

As was discussed in section 4.2.1, Holder pasteurization did not affect LA, ALA, DHA and ARA levels in human milk during the thermal treatment.

An increase in the LA content of pasteurized an unpasteurized human milk was observed after the first month of storage, following a period of stability. That may be attributed to a beginning of a decomposition process of other fatty acids in HM and the relative stability, or much slower rate of decomposition, of LA. No differences were detected between the LA content of any of the samples at any analysis point, suggesting no benefit of nitrogen gas environment or lower storage temperature for the LA preservation and no detrimental effect of pasteurization on LA content during storage for 6 months.

A rapid decline in the ALA content of pasteurized and unpasteurized human milk was detected during the second month following a period of stability. Afterwards the low level remained stable for the last 4 months of storage. However, during the analyses performed until the 2nd month, the nonadecanoic acid fatty acid (C19:0) was added to the

HM samples as an internal standard, which did not result in a peak on the chromatogram, therefore being substituted with the heptadecanoic acid (C17:0) afterwards, which did result in a peak. At the same time of the substitution (2^{nd} month) the ALA content dropped significantly to the level which is in accordance with the literature (from ~10% to ~2%). Furthermore, according to the literature, ALA content does not change during storage in the conditions of this study. Therefore it was assumed, and confirmed further, that the C19:0 co-eluted with the ALA while being added to the HM samples, and that the ALA content did remain stable around the 2% level during the storage period.

No differences were detected between the ALA content of any of the samples at any analysis point, suggesting no benefit of nitrogen gas environment or lower storage temperature for the ALA preservation and no additional detrimental effect of pasteurization on ALA content during storage for 6 months.

Overall, after one month of stability during the first month of storage, there was a constant increase in DHA content during the next 2 to 3 months of storage, suggesting the beginning of a decomposition process of other fatty acids in HM and the relative stability, or much slower rate of decomposition, of DHA during this period. The observed increase in DHA content of HM stored in nitrogen gas environment at -80^{o}C (SN) during the 4^{th} month suggests that during the 4^{th} month of storage, -80^{o}C temperature combined with nitrogen gas environment has some advantage for the storage of unpasteurized HM, though it is not reflected in further advantage. For the storage of pasteurized HM, the -20^{o}C temperature is better than -80^{o}C after 3 months, as during the 4^{th} month its DHA content apparently increased significantly (implying higher stability), resulting in a significantly higher DHA content at the 4^{th} month analysis compared with the pasteurized

milk stored at -80^{o}C (SP). Although no further advantage for the -20^{o}C temperature was detected: no change in the DHA level in the FP sample during the 5th and the 6th months of storage occurred and no difference compared with the FA concentration in other samples was detected after 6 months of storage. As no differences were detected between the DHA content of any of the samples at any analysis point during the 6 months of storage, the data indicates that there is no benefit of nitrogen gas environment or for lower storage temperature for the DHA preservation and no detrimental effect of pasteurization on DHA content during this storage period.

An increase in ARA concentration after 1 month period of stability suggests a beginning of a decomposition process of other fatty acids in HM and the relative stability, or much slower rate of decomposition, of ARA. Overall, no differences were detected between the FA content in any of the samples at any analysis point, suggesting no benefit of nitrogen gas environment or lower storage temperature for the ARA preservation and no detrimental effect of pasteurization on its content during storage for 6 months. It might be suggested that after 3 months of storage -80^{o}C temperature combined with nitrogen gas environment can be somewhat beneficial for untreated HM, although no further observed advantage rules out this recommendation.

Overall, the 4 fatty acids remained stable during 6 months of storage at -20^{o}C and at -80^{o}C, suggesting a redundancy of oxygen restriction and lower storage temperature for FA preservation. That was in agreement with the official recommendations, including Winnipeg Public Health – Government of Manitoba, of at least 6 months of frozen storage at -20^{o}C and in accordance with the expectations for high human milk fat stability during storage at -20^{o}C and at -80^{o}C based on the literature (Berkow et al., 1984; Friend

et al, 1983; Reynolds et al., 1982). According to the hypotheses, pasteurized HM did not differ from unpasteurized HM during the course of the 6 months storage. The stability of the fatty acids is not a surprise: although polyunsaturated fatty acids are sensitive to light, oxygen exposure and temperature, in milk they are incorporated into fat globules. As long as the milk is carefully handled (no centrifugation or homogenization), intact milk fat globule membranes protect fatty acids. In this study, we exposed milk to various temperatures but not to light or pressure, which resulted in preservation of milk fatty acid composition (Fidler et al., 1998).

5.3. E-nose analysis of volatile compounds concentration in stored human milk

The majority of the PCAs of the volatile compounds analysis data are presented in Appendix 5, as will be mentioned during the progression of this section. As can be seen in Figures 4.25 – 4.29 and Figures app.5.1 - app.5.21, the volatile compounds concentration patterns of the samples appear as triangles or lines, as formed by the results of a triplicate or a duplicate analysis of each sample. Several results were not included in the figures, if located in a distance from the other points of the same sample and if represent a middle of the progress of the experiment (rather than the beginning or the end of the storage period). The obtained edge values could be attributed to various technical errors, such as a contamination of those particular milks with volatile compounds during the preparation for the analyses.

Initially, the experiment was planned to be carried out during 6 months. However the analysis in the end of the 6^{th} month was not possible due to equipment problems.

5.3.1. Method confirmation

The data acquisition during 300 sec presented in Figure 4.22 shows that all the sensors approach the middle baseline, consequently returning to the initial conditions by the end of each run cycle, thus not causing a drag of a signal from one sample to another.

5.3.2. Baseline

The DI for the baseline of 3 types of human milk - control (untreated), stored with nitrogen gas and pasteurized prior to storage, was 81, revealing appreciable differences in the volatile compounds concentration pattern (VCCP) of the 3 types of the samples. Before initiation of storage, the control milk (RC) and the HM stored in the nitrogen gas (RN) are the same milk, as the effect of the limitation of oxygen can manifest only during storage. The separation between these samples, as was detected by the e-nose machine, is a result of the baseline being the 4^{th} day of storage at $4^{o}C$, when the differences can already be sensed by the sensors.

It can be seen from Figure 4.23 that the PCA results of the pasteurized milk samples are grouped aside of the results of the unpasteurized milks. These results are in agreement with the expectations, as a heat treatment has an adverse effect on some of the milk components (Atkinson, 1995), resulting in the formation of new compounds, such as sulphur compounds (Ampuero and Bosset, 2003; Atkinson, 1995) and other volatiles, which alter the volatile compounds pattern. The correlation with the study results for nutrient stability was not conclusive during the first 4 days of storage. The FAs and the riboflavin content remained stable during the heat treatment and this storage period, while vitamin C content remained similar from day 2 to day 4 in the pasteurized and in

the unpasteurized HM. However the lack of the time 0 vitamin C result in the control milk does not allow an estimate of the similarity of the 2 milk type VCCPs to be made following pasteurization and during the first 2 days of storage.

The RC and RN samples PCA results are located close to each other at 4 days of storage, in accordance with the vitamins and fatty acids analyses for the first 4 days: during this time, a same pattern for vitamins C and B_2 stability/decomposition was monitored in these samples, as well as no decomposition of the LA, ALA, DHA and ARA fatty acids was detected.

5.3.3. Refrigeration storage at 4^{o}C for 8 days

The positive and higher than 80 discrimination index values of the individual human milk types (DI(RC) = 97; DI(RN) = 94; DI(RP) = 87) reflect that all the HM types underwent appreciable changes in the VCCP during the storage at 4^{o}C for 8 days. That was in agreement with the literature, reporting a change in odour as detected by a human and by a machine during storage of milk and milk products. Jensen (1999) reports that off-flavours develop in stored human milk to the extent that the infant may refuse to consume it. Ampuero and Bosset (2003) describe the evidence of correct classification of groups of cow milk and cow milk products of different ages by the e-nose, based on the difference in the volatile compounds. Marsili (2000) showed that a technique based on solid-phase microextraction, mass spectrometry and multivariate analysis (SPME-MS-MVA), as an electronic-nose system, can function as an accurate technique for prediction of the shelf life of reduced-fat milk and chocolate milk, stored at ~7^{o}C. The results obtained in this part of the study are in a partial agreement with the results of the nutrient

content analysis part: vitamin C and DHA contents decreased in HM stored with limited oxygen, while in the control and in the pasteurized milks all the nutrients remained stable. Probably decomposition of other components, which were not objectives of this study, resulted in a creation of volatiles which changed the VCCP.

Figure 4.25 and the DI value of 93 of this PCA reflect appreciable differences in the VCCP between HM stored at 4^{o}C with and without nitrogen gas from day 4 to day 8. These results were in accordance with the results of the nutrient analysis of this study: limitation of oxygen resulted in a higher decomposition of vitamin C after 4 days of storage compared with the control milk. These conditions also resulted in a decrease in the DHA level after 6 days of storage, in contrast with the stability of this FA in other human milk types. The literature reports a development of off-odours due to accumulation of ethanol, acetaldehyde and other volatiles during storage when oxygen is limited (Mazza and Jayas, 2001), in support of the study results.

Figure 4.26 and the DI value of 94 of this PCA reflect appreciable difference in the VCCP between pasteurized and control human milk during the 4 days of storage at 4^{o}C. This was not reflected in the results of this study, which showed similar vitamin and fatty acid stability/decomposition pattern in the pasteurized HM and the control HM during this period. However that was in agreement with the literature: heat treatment has an adverse effect on some of the milk components (Atkinson, 1995) and results in the formation of new compounds, some of which are volatile, such as sulphur compounds (Ampuero and Bosset, 2003; Atkinson, 1995), therefore differences in the VCCP are expected to happen during the storage. The PCA results allow one to deduce that the change in odour which is reported by the literature does not originate from the

decomposition of vitamin C at 4°C, but rather from the decomposition of other components, which are not objectives of this study. Another possible cause for the observed difference in volatile compounds concentration can be an accidental exposure to light of sample RC or sample RP during the preparation of the samples for the analysis by the e-nose machine, which could generate volatile products of light oxidation (Marsili, 1999; Mestdagh et al., 2005). Possible contamination of samples with volatiles from pipette tips or the vial/cap during preparation is also possible. Another possible reason can be the big spread of the results for the volatile compounds of the sample RC on day 8, which may suggest different DI outcome.

5.3.4. Frozen storage

5.3.4.1. Storage for 4 weeks in the freezer at -20°C and at -80°C

Only the PCA of the volatile compounds analysis data of pasteurized human milk stored at -80°C for 4 weeks is presented in this section (Figure 4.27), as being of an interest (DI > 80). The rest of the analyses, showing that no differences occurred in the VCCP during the storage (DI ≤ 0), can be found in Appendix 5 (Figures app.5.1 – app.5.5).

Figure 4.27 shows the change in the VCCP of the pasteurized human milk during 4 weeks of storage at -80°C. The most significant change happened during the first week of storage, locating its PCA results aside of the results of the other analysis times. This is supported by the results of this study, showing a decrease in the vitamin C and vitamin B_2 content in this type of milk on the early stages of storage. However, in contrast with the expectation of a similar outcome, no difference in the VCCP was detected in the pasteurized HM stored at -20°C. That might be explained by the increase in the solubility

of gases, especially oxygen, with a decrease in the temperature (Singh and Cadwallader, 2000), which could lead to the relatively increased oxidation processes of other components in the SP sample, compared with the sample FP, despite the very low temperature of storage. The big spread of the results for the volatile compounds of the sample SP on week 3 does not suggest very different DI outcome, as the rest of the results clearly show a difference in the VCCP of this sample between time zero and other analysis points.

Each sample other than SP showed no change in the VCCP during storage at -20^{o}C and at -80^{o}C for 4 weeks (DI $\leq$ 0). That was in contrast with the expectations based on this study results: vitamin C concentration decreased notably during the first 3 weeks of storage in all samples and the vitamin was completely lost in the control milk stored at -80^{o}C during the first week of storage. Limitation of oxygen failed to prevent the decomposition processes. That was expected to lead to appreciable changes in the volatile compounds concentration patterns. The changes were also expected according to the literature: Jensen (1999) mentions possible development of soapy and oxidized flavours in frozen human milk. The obtained results can be attributed to the difference between the volatile compounds concentration analysis provided by a machine and the odour perception as provided by human nose, which is not in direct relation to the concentration of volatiles. The sense of smell is a remarkably sensitive system which involves the specialized receptors in the olfactory epithelium in the top of the nasal cavity that respond to very different concentrations of chemicals and to mixtures of hundreds of odorants. The receptors induce signals which are transmitted to the olfactory bulb and ultimately to the brain. In this study, the machine, on the contrary, provided only the numerical values

for concentration and used a statistical analysis, without the "translation" into the sense of smell, not necessarily corresponding with the actual perception of the odour by a human nose (Pearce et al., 2003). Therefore a change in the concentration of volatile compounds, which was considered by the machine as insignificant, could be significant to the odour perception of a human being.

In order to question the effect of pasteurization and possible beneficial effects of lower storage temperature and oxygen limitation for preservation of the nutrients, comparison of VCCP between samples FC&SC (storage temperature effect), samples FC&FN (oxygen limitation effect at -20^{o}C storage), samples FC&FP (pasteurization effect at -20^{o}C storage), samples FP&SP (storage temperature effect for pasteurized HM), samples SC&SP (pasteurization effect at -80^{o}C storage) and samples SC&SN (oxygen limitation effect at -80^{o}C storage) were performed. All obtained DI values for these pairs reflected no difference in the VCCP between them during the 4 weeks of storage (DI $\leq$ 0). These results were in accordance with the findings of this study: vitamin C notably decreased in the control HM at -20^{o}C and at -80^{o}C; limitation of oxygen was not beneficial nor detrimental for vitamin C content; in pasteurized samples the vitamin content reached similar levels to the unpasteurized milk's levels at both temperatures. No different trends were observed also for the vitamin B_2 content and for the fatty acids content between these pairs on analyzed material. Therefore no changes in the VCCP were expected for these pairs of samples. Although based on the literature, changes in the odour profile were supposed to occur (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000), the lack of the expected changes, as obtained by the electronic nose machine, can

be attributed to the difference in the machine's and human's "perception of smell", as was explained above.

5.3.4.2. Storage for 5 months in the freezer at -20°C and at -80°C

Only the PCA of the volatile compounds of the pasteurized human milk and HM stored with nitrogen gas at -20°C for 5 months (samples FP and FN) are presented in this section, as being of interest (Figures 4.28 and 4.29; DI = 84 and DI = 91, respectively). Other results, showing that no differences occurred in the VCCP during the storage of each of the other samples (DI < 0), can be found in Appendix 5 (Figures app.5.12 – app. 5.15).

The significant change in the VCCP of the pasteurized HM (FP) and HM stored with nitrogen gas (FN) at -20°C were in accordance with the expectations based on the results of this study: vitamin C content of these types of milk (and other types) decreased sharply during the first month of storage, maintaining on low levels during subsequent storage, probably producing products of decomposition which are also volatile compounds. The changes were also expected according to the literature (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000).

Each sample other than FN and FP showed no change in the VCCP during storage at -20°C and at -80°C for 5 months (DI ≤ 0). This contradicted the expectations based on the literature (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000) and on the results of this study: vitamin C content dropped in all samples during the first month of storage and remained almost stable levels of the vitamin during the subsequent storage. That was expected to be reflected in differences in the VCCP for every sample. The lack of the expected changes, as obtained by the electronic nose machine, can be attributed to the difference in the machine's and human's "perception of smell", as was explained above.

In order to question the effect of pasteurization and possible beneficial effects of lower storage temperature and oxygen limitation for preservation of the nutrients, comparison of VCCP between samples FC&SC (storage temperature effect), samples FC&FN (oxygen limitation effect at -20^{o}C storage), samples FC&FP (pasteurization effect at -20^{o}C storage), samples FP&SP (storage temperature effect for pasteurized HM), samples SC&SP (pasteurization effect at -80^{o}C storage) and samples SC&SN (oxygen limitation effect at -80^{o}C storage) were performed. All obtained DI values reflected no difference in the VCCP between all pairs of samples ($DI \leq 0$). These results were in general agreement with the findings of this study: vitamin C notably decreased in the control HM at both temperatures, therefore no discrimination was expected between sample FC and sample SC. Although limitation of oxygen had some beneficial effect for vitamin C preservation, but the vitamin concentrations were already at low levels, possibly not influencing the VCCP. In the pasteurized samples the vitamin content reached lower levels to the unpasteurized milk's levels at both temperatures, but the vitamin concentrations were already at low levels, possibly not influencing the VCCP. No different trends were observed also for the vitamin B_2 content (a slight decrease was observed in the pasteurized milk) and for the fatty acids content between these pairs on analyzed material, therefore no changes in the VCCP were expected. Although based on the literature, changes in the volatiles' profile were supposed to occur (Ampuero and Bosset, 2003; Jensen, 1999; Marsili, 2000), the lack of the expected changes, as obtained by the electronic nose machine, can be attributed to the difference in the machine's and human's "perception of smell", as was explained above.

CHAPTER 6: CONCLUSIONS

This study's objective was the evaluation of the shelf life of expressed human milk under different storage conditions. Shelf-life of a food can be defined as the period for which it will retain an acceptable level of eating quality, from a safety and sensory point of view. There are four critical factors in this endeavor: formulation, processing, packaging and storage conditions (Singh and Cadwallade, 2000), three of which - processing, packaging and storage conditions – were controlled during this study and two of them – processing and storage conditions – were altered. As an understanding of the interplay between these factors is the key to shelf-life estimation and testing (Singh and Cadwallade, 2000), this study's objective was to monitor this interplay in order to establish the optimal shelf life of processed and stored human milk.

Section 6.1 presents the overall conclusions of this study. Section 6.2 presents the conclusions of every stage of this study, from which the overall conclusions were drawn.

6.1. Conclusions of this study

The results of this study regarding the change in the content of 6 nutrients during storage and/or processing of human milk showed that the nutrient that limits the shelf life of stored human milk is vitamin C, being the most unstable. The 4 fatty acids and vitamin B_2 maintain a general stability during refrigeration, frozen storage and Holder pasteurization.

The results of this study were in a general agreement with the literature. The exceptions were the expected lower vitamin C content of pasteurized human milk compared with the untreated milk (the levels didn't differ), the expected decrease in

vitamin B_2 during storage at 4°C (the level remained stable) and the expected preservation effect of oxygen limitation and -80°C temperature (had only occasional beneficial effect). The volatile compounds concentration pattern did not always follow the expectations.

The results of this study confirmed the appropriateness of the official storage recommendations, which are based on avoiding bacterial growth, for preservation of the 6 nutrients. Therefore the infants are fed both safe and nutritious human milk, if stored accordingly. The results also showed that these recommendations, as were made for untreated human milk, can be extended for storage of human milk pasteurized by Holder pasteurization.

The results of this study implied a lack of a clear advantage of freezing temperature of -80°C over -20°C for pasteurized and unpasteurized human milk and a lack of a clear benefit of oxygen limitation for the nutrients stability.

As vitamin C is necessary for growth and development, if an infant is fed pasteurized or unpasteurized human milk which was stored for longer than 2 weeks, vitamin C addition to the milk immediately before feeding should be practiced, in support of the literature. If this milk is fed exclusively, daily supplementation of the infant with vitamin C is recommended.

This study results showed that evaluation of the odour of human milk cannot be based on the analysis provided by the e-nose machine exclusively, requiring a sensory panel, though it is very difficult to implement due to the target population for consumption of this product – young infants.

6.2. Conclusions from different stages of the study:

6.2.1. Changes in the concentrations of vitamin C and vitamin B_2 during storage of human milk

6.2.1.1. Refrigeration storage at 4^oC

- Vitamin C concentration in unpasteurized human milk decreases during first 2 days of storage at 4^oC and then remains stable for additional 4 days, in disagreement with the expectations of a decrease based on the literature.
- Limitation of oxygen availability using nitrogen gas causes a decrease in vitamin C content. These observations contradicted the expectations of the effect of nutrient preservation which is attributed to oxygen limitation.
- The vitamin C concentration in pasteurized human milk remains stable from the end of the heat treatment process until the 6^{th} day of storage. Pasteurized HM does not differ in the vitamin content from the non-pasteurized milk at these conditions. This data is in contrast to expectations of a lower vitamin C content of pasteurized human milk.
- Vitamin B_2 concentration in expressed human milk stored untreated, pasteurized or stored in nitrogen gas environment remains stable for 8 days at 4^oC. That is in contrast with the expectations of a riboflavin decrease during storage under these conditions.
- In agreement with other reports, riboflavin is not affected by Holder pasteurization during heat treatment and during subsequent storage.
- **In conclusion,** 1) expressed human milk, pasteurized and non-pasteurized, stored at 4^oC for 8 days, preserves appreciable concentrations of vitamin C and vitamin B_2.

The obtained data supports the majority of the official recommendations which consider safety, including Winnipeg Public Health – Government of Manitoba. 2) Oxygen limitation does not benefit vitamin C and B_2 stability. 3) Pasteurization process affects vitamin C levels, but not vitamin B_2 levels. The decrease of both vitamins during storage is similar to that of unpasteurized HM.

6.2.1.2. Frozen storage

6.2.1.2.1. Storage for 4 weeks in the freezer at -20°C and at -80°C

- Vitamin C concentration in expressed human milk stored untreated, pasteurized or stored in nitrogen gas decreases notably during the first 3 weeks of storage both at -20°C and at -80°C, remaining stable for another week. That is in agreement with expectations of vitamin decomposition during storage at -20°C, based on the literature. After the first week of storage, HM quickly looses its vitamin C, retaining ~1/3 of the initial level in the pasteurized milk and less in the untreated milk by the 2nd week of storage.
- Infants need an optimum supply of ascorbic acid for growth, development and survival. Therefore, for pasteurized or unpasteurized HM which was stored for longer than 2 weeks at any of the temperatures and is intended to be used for infant feeding, addition of vitamin C immediately before the feeding is recommended. If this milk is fed exclusively, daily supplementation of the infant with vitamin C is recommended.
- In disagreement with the hypothesis, storage of unpasteurized human milk at -80°C does not slow vitamin C decomposition during the first week of storage. After the first week this temperature is detrimental. If HM storage at -80°C for longer than 1 week is required, oxygen availability should be limited for preservation of some of

the vitamin C. The latter finding supports the proposed nutrient preservation effect by oxygen limitation.

- Limitation of oxygen is not beneficial for vitamin C content preservation in case of storage of expressed human milk at -20^{o}C, contrary to the expectations.
- Pasteurized human milk reaches similar vitamin C level to non-pasteurized milk after 1 week of storage at -20^{o}C and at -80^{o}C, further decreasing in a similar pattern. During further storage, -80^{o}C temperature is beneficial.
- Untreated human milk can be stored at -20^{o}C and at -80^{o}C for 4 weeks with no loss of vitamin B_2; a lower temperature does not benefit vitamin B_2 preservation.
- Oxygen limitation using nitrogen gas is not beneficial for vitamin B_2 preservation during storage of HM at any of the freezing temperatures, contradicting expectations.
- During frozen storage of pasteurized HM for 4 weeks, vitamin B_2 content decreases to ~70% of the initial level. Lower temperature does not benefit vitamin B_2 preservation in pasteurized human milk.
- **In conclusion,** 1) For storage for 4 weeks, expressed unpasteurized human milk should be stored at -20^{o}C, and not at -80^{o}C, in order to maintain vitamin B_2 and some vitamin C. 2) If pasteurized or non-pasteurized human milk which was stored for longer than 2 weeks is fed to the infant, addition of vitamin C immediately before the feeding should be considered. In case of exclusive feeding, daily supplementation of the infant with vitamin C is recommended. 3) Limitation of oxygen availability is not beneficial for vitamin C and B_2 preservation, but is recommended in case that HM is required to be stored at -80^{o}C. 4) Vitamin C and B_2 contents of pasteurized HM do

not differ from unpasteurized milk and also decrease during storage for 4 weeks. A lower temperature helps to retain higher levels of the vitamins in pasteurized HM.

6.2.1.2.2. Storage for 6 months in the freezer at -20^{o}C and at -80^{o}C

- After a sharp decline during the first month of storage, vitamin C content remains at low levels until the 6th month of storage in untreated, pasteurized and unpasteurized HM which is stored with the limitation of oxygen.
- For storage periods longer than 1 month, a storage temperature of -80^{o}C is not recommended, as vitamin C content drops to an undetectable level during the first month. Therefore during storage for 6 months, frozen human milk should be stored at -20^{o}C for preservation of some vitamin C in HM, in agreement with expectations. This is in agreement with most of the official recommendations, including Winnipeg Public Health – Government of Manitoba, which are at least 6 months of storage at -20^{o}C.
- According to the conclusions made from weekly analyses, if an infant is fed exclusively HM which was stored for longer than 2 weeks at any of the chosen temperatures, supplementation of vitamin C is recommended due to the low levels of the vitamin which remain in the milk.
- Limitation of oxygen benefits vitamin C preservation at -20^{o}C in accordance with expectations. If HM is required to be stored at -80^{o}C, limitation of oxygen helps to maintain some vitamin C in the milk.
- In pasteurized HM, the vitamin C content decreases during storage for 6 month, as expected. During the first month the decrease is sharp and the concentration is

maintained at lower level compared with the unpasteurized milk. A lower freezing temperature does not benefit the vitamin preservation.

- Vitamin B_2 level does not change in the control samples during storage at -20^{o}C and at -80^{o}C for 6 months, in agreement with the reports of HM storage at -20^{o}C. As fresh human milk obtained from the donors for this experiment was diluted approximately 2-fold with 7-month-old human milk, a longer period of riboflavin stability can be reasonably assumed. The storage period is in agreement with most of the official recommendations for human milk storage, which are at least 6 months, including Winnipeg Public Health – Government of Manitoba.
- The -80^{o}C freezing temperature does not have a benefit for vitamin B_2 preservation.
- Limitation of oxygen is not beneficial for vitamin B_2 preservation at any of the storage temperatures and may be detrimental, in contrast to the expected preservation effect.
- Vitamin B_2 content in pasteurized samples does not differ from the controls at the same temperatures at 3 months, but opposed to the controls, it decreases during the next 3 months of storage, with no benefit from either storage temperature.
- **In conclusion:** 1) A temperature of -20^{o}C, but not of -80^{o}C, is recommended for storage of unpasteurized human milk for 6 months. This period is in agreement with most of the official recommendations considering safety, of at least 6 months, including Winnipeg Public Health – Government of Manitoba. 2) Limitation of oxygen is beneficial during -20^{o}C storage. 3) In pasteurized HM the vitamins levels also decrease during 6 months, with no benefit of either of the storage temperatures. 4) Due to vitamin C importance for growth, development and survival, addition of

vitamin C immediately before infant feeding should be considered if the infant is fed breast-milk, pasteurized or not, stored for longer than 2 weeks. Daily supplementation of the infant with vitamin C is recommended, if fed exclusively this milk.

6.2.2. Changes in the concentrations of LA, ALA, ARA and DHA during storage of human milk

6.2.2.1. Refrigeration storage at 4^{o}C for 8 days

- Both pasteurized and unpasteurized human milk can be stored at 4^{o}C for 8 days preserving initial levels of the linoleic fatty acid, α-linolenic fatty acid and arachidonic fatty acid. Oxygen limitation is unnecessary. Pasteurization does not affect LA, ALA and ARA content, as expected based on the literature review.
- The unpasteurized human milk retains its initial DHA content during 8 days of storage at 4^{o}C. Oxygen limitation shortens this period to 6 days. Pasteurization process does not affect the initial DHA content during the heat treatment and during subsequent storage.
- The FAs stability is in agreement with the literature, which generally state that the fat integrity of fresh HM is not affected by storage in these conditions.
- **In conclusion,** 1) during storage for 8 days at 4^{o}C, there doesn't appear to be a difference in the LA, ALA, DHA and ARA contents in unpasteurized human milk. That supports the official recommendations considering safety, including Winnipeg Public Health – Government of Manitoba, of 5 - 8 days of refrigeration storage. 2) Oxygen limitation in unnecessary for the FAs preservation and it does not have a considerable detrimental effect. 3) Holder pasteurization does not result in a decrease

in the FAs contents during the process and allows the maintenance of levels similar to the untreated human milk during storage for 8 days at 4°C.

6.2.2.2. Frozen storage

6.2.2.2.1. Storage for 4 weeks in the freezer at -20°C and at -80°C

- The LA, ALA, DHA and ARA concentration of pasteurized and unpasteurized HM remains stable during 4 weeks of storage at -20°C and at -80°C, as expected from other reports. Lower temperature and oxygen limitation do not have a benefit for the four FAs preservation.
- **In conclusion,** 1) pasteurized and unpasteurized human milk can be safely stored at -20°C for 4 weeks without decomposition of LA, ALA, DHA and ARA. 2) Lower temperature storage is redundant. 3) Oxygen limitation is unnecessary and does not have a detrimental effect. 4) Holder pasteurization does not result in a decrease in the FAs contents during the process or during the storage for 4 weeks at -20°C and at -80°C, maintaining similar levels to the untreated human milk.

6.2.2.2.2. Storage for 6 months in the freezer at -20°C and at -80°C

- The LA, ALA, DHA and ARA concentration of pasteurized and unpasteurized human milk remains stable during the first month of storage at -20°C and at -80°C, and possibly afterwards. After the first month, a slow decrease is possible until the end of 6 months of storage at both temperatures.
- Lower temperature and oxygen limitation do not have a benefit or a detrimental effect on the FAs preservation.
- Pasteurization has no detrimental effect on the FAs content during storage for 6 months.

- The FAs stability is in agreement with the literature, which generally states that the fat integrity of fresh HM is not affected by storage in these conditions.
- **In conclusion,** 1) during storage for 6 months at -20^{o}C and at -80^{o}C, there is no appreciable decrease in the LA, ALA, DHA and ARA contents in unpasteurized human milk. As the fresh human milk obtained from the donors for this experiment was diluted approximately 2-fold with 7-month-old human milk, an even longer period of stability of these FAs can be reasonably assumed. That supports the official recommendations considering safety, including Winnipeg Public Health – Government of Manitoba, of at least 6 months of storage at -20^{o}C. 2) Lower storage temperature and oxygen limitation are redundant and don't have a detrimental effect. 3) Human milk can be pasteurized using Holder pasteurization without a loss of LA, ALA, DHA and ARA during the process and during storage for 6 months at -20^{o}C, with no benefit of lower storage temperature.

6.2.3. Volatile compounds concentration in stored human milk

6.2.3.1. Refrigeration storage at 4^{o}C for 8 days

- Pasteurized human milk differs in the volatile compounds concentration pattern from the control milk and milk stored with limited oxygen on the early stages of storage, in agreement with the expectations based on the literature.
- The volatile compounds patterns of control HM and HM stored with limitation of oxygen are close, in accordance with the stability of the 6 nutrients in this study on the same stages of the storage.

- The VCCPs of all human milk types changed during 8 days of storage at 4^{o}C, in accordance with the expectations based on the literature and in partial agreement with the stability of the nutrients as was monitored in this study.
- Appreciable differences in the VCCP between human milk stored with and without nitrogen gas at 4^{o}C were detected, in agreement with the results of this study for nutrients stability and with the expectations based on the literature.
- Appreciable differences in the VCCP between pasteurized and control human milk stored at 4^{o}C were detected in agreement with the literature, but in contrast with the expectations based on this study results for nutrients preservation. Probably the decomposition of other compounds in human milk, other than the 6 nutrients, is the cause of the reported changes in odour which is described in the literature. Process errors are also possible causes.

6.2.3.2. Frozen storage

- In pasteurized human milk stored at -80^{o}C, a change in the VCCP was monitored during storage for 4 weeks with the biggest change occurring during the first week of the storage. That was supported by this study results for concentration of the nutrients in this milk.
- Pasteurized human milk stored at -20^{o}C for 4 weeks did not undergo changes in the VCCP, probably due to the decreased oxidation processes as a result of lower oxygen solubility, compared with milk stored at -80^{o}C.
- All human milk types, with the exception of pasteurized milk stored at -80^{o}C, did not undergo a change in the VCCP during 4 weeks of frozen storage, in contrast with the expectations based on this study results for the nutrients stability and in contrast with the

expectations based on the literature. That could be attributed to the differences in the "odour perception" of a machine and of a human nose.

- While questioning the effect of pasteurization and possible beneficial effects of lower storage temperature and oxygen limitation for preservation of the nutrients during 4 weeks period, no differences in the VCCP were detected between relevant pairs of human milk samples. That was in agreement with this study findings regarding the stability of the 6 nutrients, but in contrast with the expectations based on the literature, which can be attributed to the differences in the "odour perception" between the e-nose machine and a human nose.
- During frozen storage for 5 months, all human milk types, with the exception of the pasteurized HM and HM stored with nitrogen gas at -20^{o}C, did not present a change in the VCCP during storage, in contrast with the expectations based on this study results for the nutrients stability and in contrast with the expectations based on the literature. That could be attributed to the differences in the "odour perception" of a machine and of a human nose.
- Only the pasteurized HM and HM stored with nitrogen gas at -20^{o}C presented a change in the VCCP during 5 months of frozen storage, in accordance with the expectations based on the results of the nutrients stability in this study and on the literature.
- While questioning the effect of pasteurization and possible beneficial effects of lower storage temperature and oxygen limitation for preservation of the nutrients during 5 month period, no differences in the VCCP were detected between relevant pairs of human milk samples. That was in agreement with this study findings regarding the

stability of the 6 nutrients, but in contrast with the expectations based on the literature, which can be attributed to the differences in the "odour perception" between the e-nose machine and a human nose.

- **In conclusion,** not always the results of the volatile compounds concentration pattern analyses of human milk, as were interpreted by the E-nose, were in accordance with the expectations based on the literature review and/or the results of this study regarding the 6 nutrients concentrations change during storage and/or processing of human milk. Nevertheless, the majority of these contradictions could be explained by the different "odour perception" of the machine and the human nose: in this study the machine provided a strictly numerical concentration of the volatile compounds and used a statistical analysis, therefore the obtained results did not necessarily correspond with the actual perception of the odour by a human nose. The human nose odour perception is not in a direct relation to the concentration of volatiles and considers also the different combinations of volatiles, producing a perception of a particular smell. Therefore the evaluation of the odour of human milk cannot be performed using only the e-nose machine. A sensory panel is necessary, though it is very difficult to implement for the study of human milk, as the target population for consumption of this product is young infants.

6.3. Limitations of this study

The limitations of the study were:

- Human milk storage volumes were non-representative due to the limited access on such early stages of lactation (first month). Actual reactions might happen at a different rate in the environment with larger quantities of particles;
- Storage containers which are recommended as the most appropriate for storage of expressed human milk were used in this study. However, due to the large variability of the containers used for this purpose, the loss of the nutrients might happen at a different rate in conditions in which different containers are used;
- As early milk has higher fat and vitamins concentrations, shelf life of more mature milk may differ from that recommended on the basis of this study results, as the spoilage processes occur at different rate in more dilute/concentrated environment;
- This study was able to consider only the odour component of flavour, without assessment of the taste component, which plays an equal role in the formation of flavour;
- No sensory panel could be involved in the odour study, as the target population for consumption of human milk is young infants;
- This study was conducted in the temperature of the environment which is typical to North America. In regions with higher or lower ambient temperatures the rate of the reactions may be delayed or accelerated, even though the storage temperatures are kept the same.

CHAPTER 7: IMPLICATIONS AND LONG-RANGE OUTCOMES

This study has great importance for infants who are fed expressed human milk: healthy infants, sick infants in the Neonatal Intensive Care Units (NICU) and the infants who receive human milk from Human Milk Banks.

7.1. The impact of this study

Feeding of human milk to infants has numerous advantages for them and their mothers, and therefore also for families and society. Breast milk is considered an ideal source of nutrients for both term and preterm infants, but not all babies who are fed human milk are fed directly from the breast. Expressed HM is fed from a bottle for various health and social reasons and also because today many mothers of very young infants work out of home. Expressed breast milk is often fed to hospitalized infants, expressed and pasteurized HM is distributed from human milk banks. Before feeding, expressed milk is often stored, which has an impact on its nutritional integrity and flavour, therefore the storage conditions have major importance for the milk composition at the time of feeding.

The results of this study show that human milk stored at $4^{o}C$ for 8 days and at $-20^{o}C$ for 6 months, as recommended by Winnipeg Public Health – Government of Manitoba and by other institutions (Canadian and international), preserves not only its microbiological safety, on which these protocols are based. These conditions preserve also some of the milk nutritionally important compounds which are of major importance for infants: vitamin C, vitamin B_2 and linoleic, α-linolenic, docosahexaenoic and arachidonic fatty acids. However, in support of the literature, we recommend vitamin C

supplementation, if the pasteurized or unpasteurized HM is stored for longer than 2 weeks before feeding, due to the vitamin degradation and its great importance. The results of this study imply that HM which is fed after storage according to these recommendations reaches the healthy and also the ill and weak hospitalized babies while still both safe and nutritious. The long-chain fatty acids, which are generally prone to oxidation, don't add to the oxidative load of infants when these PUFAs reach them through stored breast milk.

This study shows that the exclusion of oxygen and the freezing temperature of -80^{o}C, which are widely acceptable means of shelf life extension of stored food products, are redundant in the case of storage of human milk and can be even detrimental. That implies that additional means and expensive equipment for better preservation of expressed human milk in the hospital or at home are not required, saving appreciable financial resources.

This study shows that the recommendations for the storage of untreated expressed human milk may be extended for pasteurized milk, confirming that infants fed by pasteurized HM from human milk banks receive milk of no lower nutritional quality than infants fed unpasteurized human milk. An important conclusion from this study is also that vitamin C should be supplemented to HM distributed from milk banks.

This study shows that the one-step method of Masood and coworkers (2005) for converting the lipids to their corresponding fatty acid methyl esters without the need of the isolation step, as developed for human plasma, can be successfully applied to human milk, allowing relative simplicity, rapidity and high accuracy of HM lipid analysis. An

HPLC method was developed for vitamin B_2 and total vitamin C analysis in human milk, based on the work of Zafra-Gomez et al (2006).

This study results show that evaluation of the odour of human milk, which is a very important nutritional attribute, cannot be based on the analysis provided by the e-nose machine alone. A sensory panel is necessary, though it is very difficult to implement due to the target population for consumption of this product, which is young infants.

7.2. Future research

- Studies of other nutritional and functional properties of unpasteurized and pasteurized stored human milk, such as other vitamins and fatty acids, oligosaccharides, proteins, mineral absorption, hormonal and enzymatic components, anti-infective and growth factors, are needed to accompany this study's findings. After an optimization in regard to the levels and the relative importance of these properties, a review of the present recommendations for expressed human milk storage will be possible. Storage conditions and times will be established, which would assure safety of consumed stored human milk along with optimal preservation of its nutritional properties.
- Studies of other nutritional and functional properties of expressed human milk stored at -80^{o}C and in an environment with oxygen availability limitation are required. These conditions may have a different influence on the compounds, other than the objectives of this study, resulting in different general recommendations after optimization.
- Further studies are needed in order to evaluate when the compounds' concentrations fall below the AI, thus establishing the shelf life of the stored human milk and the requirement of the supplementation of the infant.

- Studies are needed to establish how much vitamin C should be added to human milk stored for longer than 2 weeks.
- Research of human milk pasteurized by Holder pasteurization should be broadened in order to establish designated storage protocols, especially as HM banks are gaining popularity and as pasteurized breast-milk is one of the suggested solutions for feeding of infants of HIV-positive mothers.
- The change in the flavour of human milk during storage should be studied, in order to study the flavour acceptance of stored milk by the infants.

CHAPTER 8: BIBLIOGRAPHY

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CHAPTER 9: APPENDICES

APPENDIX 1: Storage conditions studies for expressed human milk - literature review

Storage Temp.	Recommended storage time	Compo-nent studied	Findings
25°C	4 hr	bacterial growth	Bacterial growth was restricted mainly to non-pathogens, was minimal at 15°C throughout the 24 hours of storage, was low at 25°C for the first 4 to 8 hours, and was considerably higher at 38°C even during the relatively short period of 4 hours (Hamosh , 1996).
4°C	72 hr	bacterial growth	Breast milk contaminated with various bacteria can be safely stored at 4°C and at 6°C for at least 72 hours. Even longer storage periods at 6-8°C pose a minimal risk for growth of potential pathogens (Bjorksten , 1980).
4°C	24 hr	bacterial growth	Mean bacterial count (no heavy contamination) at any time during the 24 h was not significantly differrent from that at the beginning of the storage in the refrigerator (Olowe et al., 1987).
4°C	8 d	bacterial growth	Refrigeration has a significant inhibitory effect on bacterial growth, while freezing does not (Pardou et al., 1994).
-20°C	1 m	bacterio-static activity, nutrients contents	Loss of bacteriostatic activity was detected. There was no change in levels of IgA, IgM, IgF, lactoferrin, lysozyme, concentrations of amino acids and fatty acids (Reynolds et al., 1982).
4-6°C	48 hr	bacteri-cidal activity	Degree of bacteriolysis was measured. Bactericidal activity persisted after refrigeration for 48 hours and after freezing for 10 days (Silvestre et al., 2006).
-20°C	10 d		
-20°C	3 m	bacteri-cidal activity	Up to two-thirds of the original bactericidal activity level was maintained. Bactericidal activities of refrigerated samples diminished rapidly during 24 hours storage, but compensated for by enhanced bacteria sequestration activity (greatly enhanced during the first few days, whereas in frozen samples - gradually lost) (Ogundele , 2002).

Appendix 1 – Ctd: Storage conditions studies for expressed human milk - literature review

Storage Temp.	Recommended storage time	Compo-nent studied	Findings
4-6^{o}C	48 hr	bacteri-cidal activity	Refrigeration for 48 hours did not cause significant modifications, whereas storage beyond 72 hours significantly lowered the degree of bacteriolysis versus fresh milk (Martínez-Costa et al., 2007).
4-6^{o}C	24 hr	bioavai-lable vitamin C	During storage for 1 month in a freezer or for less than 24 hr in a refrigerator, 2/3 of the initial vitamin C can be preserved (Buss et al., 2001).
-16^{o}C	1 m		
4-6^{o}C	24 hr	vitamins A, C, E, total protein, fat, lactose, zinc	Refrigeration (4^{o}C) caused a statistically significant decline in levels of vitamins C and A during 24 hours of storage. Freezing (-4 to -8^{o}C) for 1 week resulted in a significant decline in the vitamins A, C and E levels, however, the mean and the range of values remained within the international reference ranges. Other nutrients showed a statistically non-significant decline at the mentioned storage conditions (Ezz El Din et al., 2004).
-4-8^{o}C	1 w		
4^{o}C	48 hr	antioxi-dant activity	Freezing of HM is not recommended, as it resulted in a greater decrease in antioxidant activity compared with refrigeration (Hanna et al., 2004).
room	6 hr	bacterial growth	Streptococcus colony count increased more rapidly at 0 - 4^{o}C compared to storage at -19^{o}C (Hegde and Vikyath, 2007).
-19^{o}C	14 d		
< 38^{o}C	24 hr	digestive enzymes (amylase and lipases)	The digestive enzymes of human milk were not affected during storage for 24 h at temperatures up to 38°C, even though pH decreased sharply (Hamosh et al., 1997).
-20^{o}C	5 m	FFA levels, lipoprotein lipase (LPL) and bile salt-stimulated lipase (BSSL) activity	Lipase activity levels were unaffected by rapid freeze-thawing followed by storage for 1 month at -20°C or -70°C. LPL and BSSL remained fully active during frozen storage. Milk fat was hydrolyzed at -20°C but not at -70°C (Berkow et al., 1984).
-70^{o}C			

Appendix 1 – Ctd: Storage conditions studies for expressed human milk - literature review

Storage Temp.	Recommended storage time	Compo-nent studied	Findings
15°C	24hr	pH, proteo-lysis, lipolysis, bacterial growth	Human milk pH decreased 2 units by 24 hours of storage at all temperatures tested. Proteolysis was minimal during milk storage. Lipolysis was rapid, starting in the first hours of storage. Bacterial growth was restricted mainly to non-pathogens, was minimal at 15°C throughout the 24 hours of storage, was low at 25°C for the first 4 to 8 hours, and was considerably higher at 38°C during 4 hours. Milk should not be stored at 38°C (Hamosh et al., 1996).
25°C	4 hr		
-20°C	N/A	glutathi-one pero-xidase (GPx), malondi-aldehyde (MDA) concent-ration	Refrigeration storage during 48 hours and freezing storage during 10 days leaded to a significant decrease in GPx enzymatic activity. MDA concentration significantly increased during 48 hours of refrigeration, but the increase was non-significant during frozen storage for 10 days. Frozen storage is preferred to refrigeration for preservation of mother's milk quality (Miranda et al., 2004).
4°C	48 hr	macro-phages, B- and T-lym-phocytes, neutron-phils	Storage in glass containers for 48 hours at 4°C resulted in a significant decrease in cell viability and macrophage and neutrophil concentration, but not lymphocytes. Freezing of human milk had only minimal effect on its antibody content, but altered cellular stability. Refrigeration for short periods of time offers an effective means of supporting milk cells in storage (Pittard and Bill, 1981).
4°C	96 hr	bacterial colony co-unts, white blood cell counts, os-molality, pH, sIgA, lactoferrin, protein, total fat, FFA	Declines in pH, white cell counts, total protein, gram-positive colony counts and a rise in free fatty acid concentrations were detected. No significant changes in osmolality and concentration of sIgA, lactoferrin, total fat and total and gram-negative colony counts were observed. Despite the decline in white blood cell counts, more cells remain after storage up to 96 hours than after freezing or pasteurization. Thus integrity of fresh HM is not affected by 5 days storage in the refrigerator (Slutzah et al., 2010).

Appendix 1 – Ctd: Storage conditions studies for expressed human milk - literature review

Storage Temp.	Recommended storage time	Compo-nent studied	Findings
4°C	48 hr	triglyce-rides, caroteno-ids	Triglyceride and carotenoid concentrations remained stable, with the exception of lutein concentration which decreased during storage at 4°C and during freezing (Tacken et al, 2009)
-18°C	28 days		
-20°C	60 days	MDA, GPx activity	MDA content of HM remained stable for 30 days during storage at both temperatures. This stability was lost during storage for another 30 days. GPx content remained stable for 30 days at -80°C, but dropped during storage for an additional 30 days. GPx content of HM stored at -20°C decreased during storage for 60 days. The advisable storage temperature is -80°C for a maximum duration of 30 days (Silvestre et al., 2010).
-80°C			
0°C - 38°C	72 hr	microor-ganisms	Storage duration for expressed HM should not exceed 24 hours at 4°C-10°C, 8 hours at 15°C-27°C and 4 hours at 30°C-38°C. Although 0°C-4°C seemed safest for HM storage, it is not recommended due to the hazards of the thawing process (Igumbor et al., 2000).

APPENDIX 2: The choice of the best FAMEs preparation method – preliminary work

In order to establish FAMEs preparation method for this study, human milk of 2 donors was analyzed for the fatty acid profile. FAMEs were prepared from the same human milk sample of each donor using 5 different protocols. Methods which require extraction prior to methylation of the HM fat (methods A, B, E, F) as well as a method in which FAs are methylated while still in the sample (method D) were applied.

Both HM types showed a similar pattern of differences between the applied methods. The summary of the preliminary work for one type of the donor milks is presented in the table app.2.1.

Table app.2.1 – Methods used for HM FAMEs preparation – preliminary work

	Extraction method	Methylation method	HM initial volume	Methylation agent
A	HE[a]	Masood[b]	1 mL	Acetyl chloride in methanol
B	HE	Dr. Suh[c]	4 mL	Boron fluoride-methanol (10%)
C	Masood		100 μL	Acetyl chloride in methanol
D	RCFFN[d]	RCFFN	1 mL	Boron fluoride-methanol (14%)
E	HE	RCFFN	1 mL	Boron fluoride-methanol (14%)

[a] – Protocol used in the Human Ecology Faculty of University of Manitoba.

[b] – Method developed by Masood and coworkers (2005).

[c] – Protocol used by Dr. M. Suh group, Food Science Department, University of Manitoba.

[d] – Protocol used by Dr. P. Jones group, Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba.

Following injection into the GC-FID machine, the chromatograms were compared qualitatively in order to choose the FAMEs preparation method for this study.

As can be seen in the figures app.2.1 and app.2.2, compared with method B, method A generates picks of larger area for almost all FAs of interest, thus allowing more precise evaluation of the FAs concentration (890.9 vs 59.5[pA*s] for C17:0; 196.2 vs 583.5[pA*s] for LA; 1290.2 vs 63.2[pA*s] for ALA; 310.2 vs 29.2[pA*s] for ARA; 170.6 vs 14.1[pA*s] for DHA). In addition, several samples were burnt during a heating step in the method B procedure, making it less convenient.

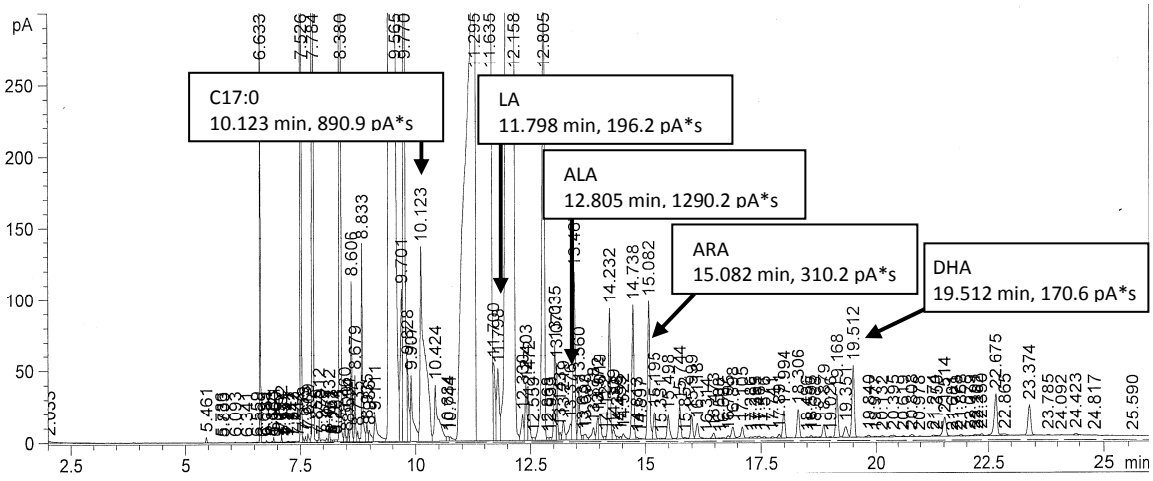

Figure app.2.1: Human milk fatty acids GC-FID chromatogram, generated by method A

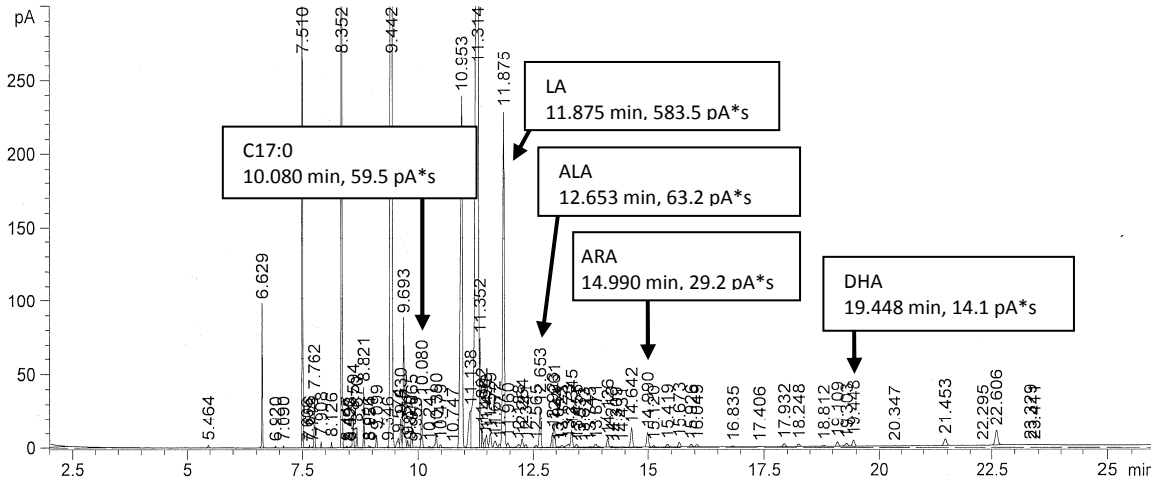

Figure app.2.2: Human milk fatty acids GC-FID chromatogram, generated by method B

As can be seen in the figure app.2.3, generally, method C generates picks of fatty acids of interest of smaller area compared with method A (863.7 vs 890.9[pA*s] for C17:0; 980.0 vs 196.2[pA*s] for LA; 115.3 vs 1290.2[pA*s] for ALA; 49.8 vs 310.2[pA*s] for ARA; 33.3 vs 170.6[pA*s] for DHA). Although being a 10-fold more diluted sample (10-fold smaller amount of human milk is used for the C procedure, while the injection volume is identical) it allows more precise evaluation of the FAs concentration. Method C procedure is also simpler and more rapid, as it does not require fat extraction step, making it the method of choice for this study.

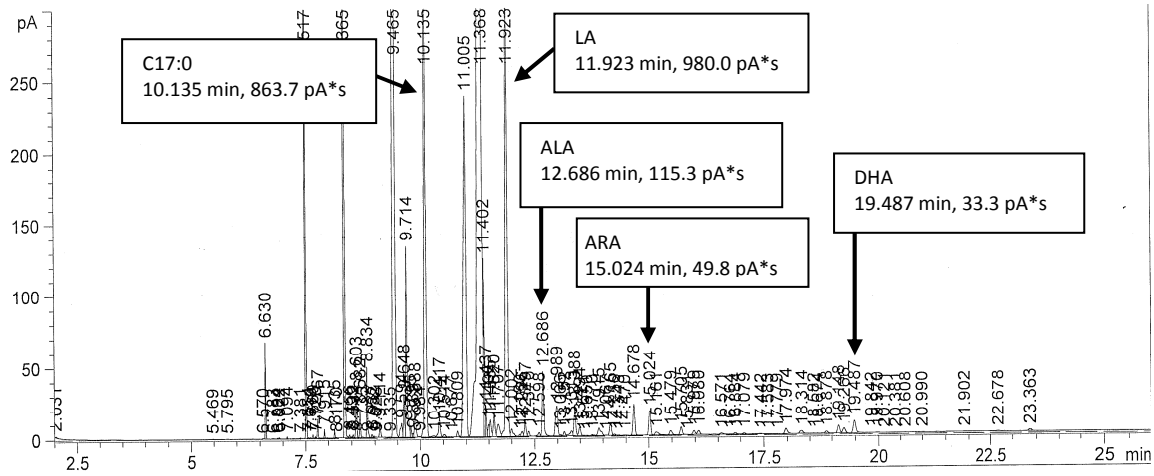

Figure app.2.3: Human milk fatty acids GC-FID chromatogram, generated by method C

As can be seen in the figure app.2.4, method D does not generate a chromatogram of sufficient quality.

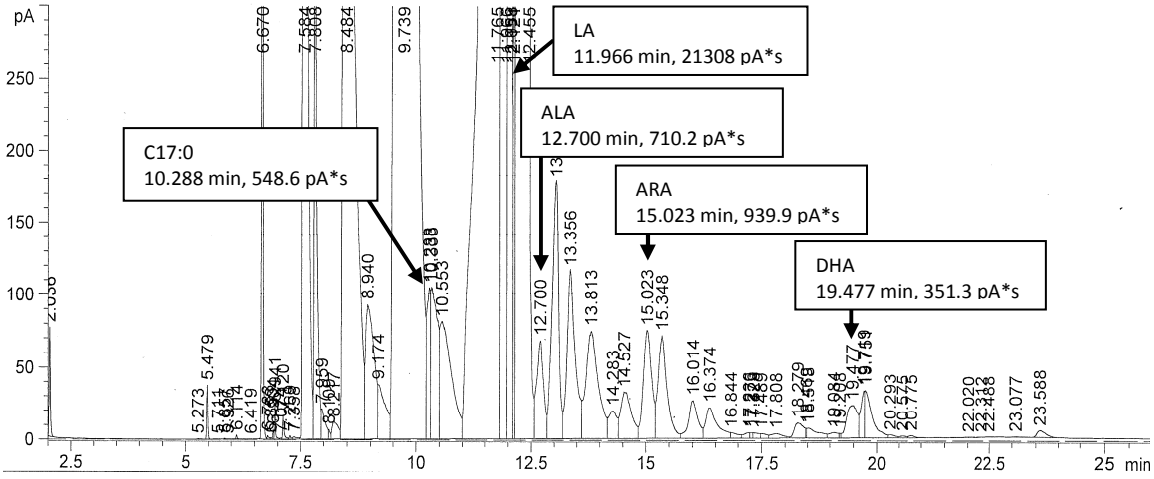

Figure app.2.4: Human milk fatty acids GC-FID chromatogram, generated by method D

As can be seen in the figure app.2.5, method E does not generate picks after 23 minutes of run time, thus making detection of DHA not possible, as DHA retention time is expected at 25 min.

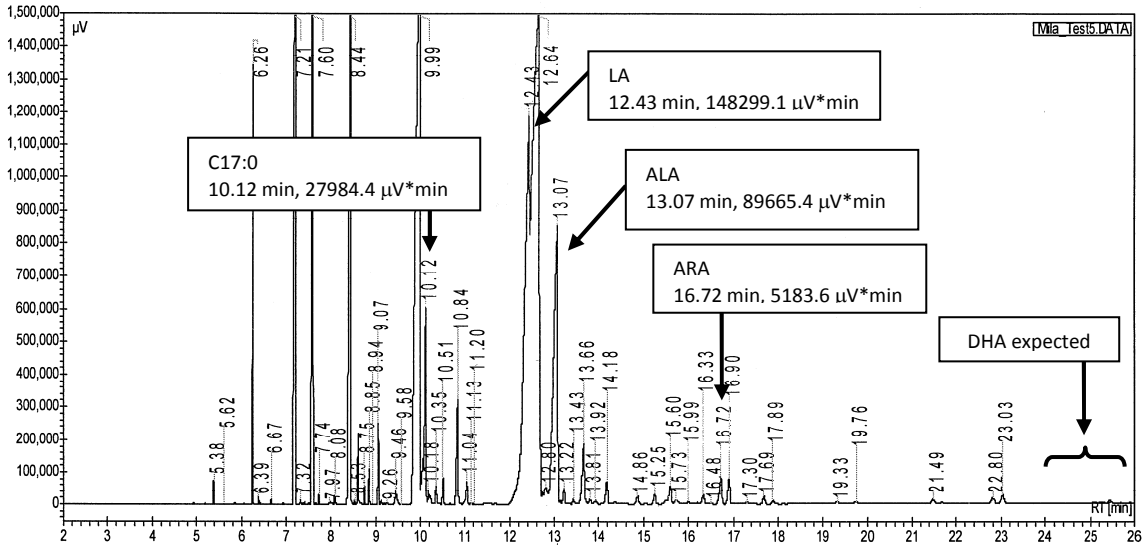

Figure app.2.5: Human milk fatty acids GC-FID chromatogram, generated by method E

In light of the aforesaid, method C, developed by Masood and coworkers (2005), was chosen for this study. It was further validated using SRM 1849, as described in "Results" section.

APPENDIX 3: Incomplete methylation of nonadecanoic acid

In this experiment nonadecanoic acid (C19:0) was added to a HM sample as an internal standard. Heptadecanoic acid (C17:0) was not added. As can be seen in figure app.3.1., nonadecanoic acid was not suitable as an internal standard for preparation of FAMEs using the method of choice for this study, as it underwent uncompleted methylation, not resulting in a peak (expected retention time: 12.07 min). Heptadecanoic acid was detected at a very low level (0.26%), thus, if used as an internal standard, allowing to use its whole peak area for FA concentrations calculations in the study, as its naturally occurring amount can be disregarded.

The chromatogram of a GC-FID analysis using nonadecanoic acid as an internal standard can be seen in the figure app.3.1.

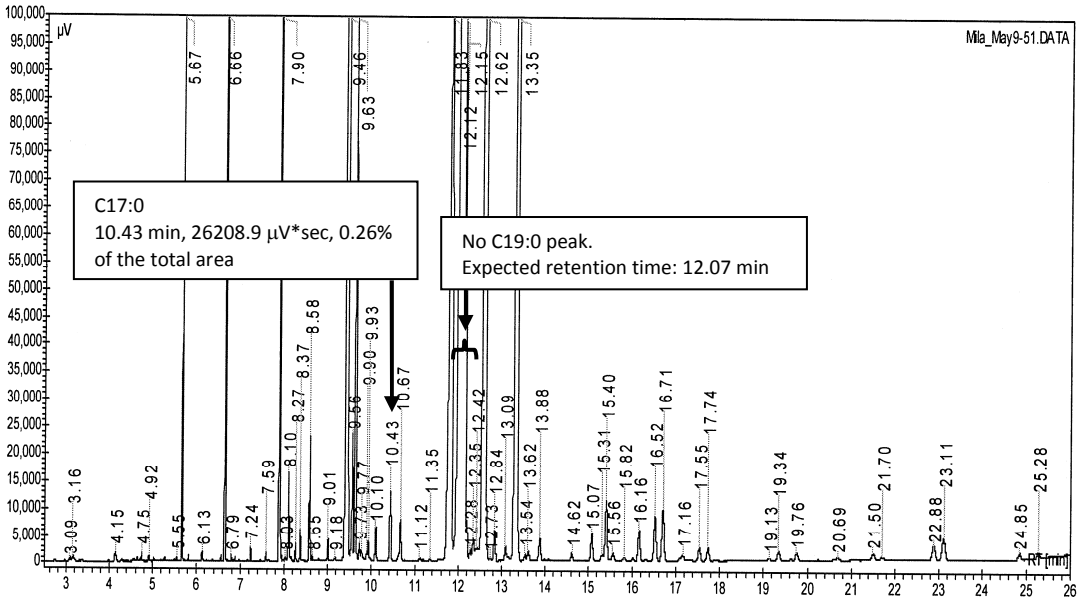

Figure app.3.1: Human milk fatty acids GC-FID chromatogram, with nonadecanoic acid as the internal standard

APPENDIX 4: Ethics committee approval

APPENDIX 5: The PCA of the volatile compounds analysis data of samples and samples' pairs, which were not presented in the section 4.3.

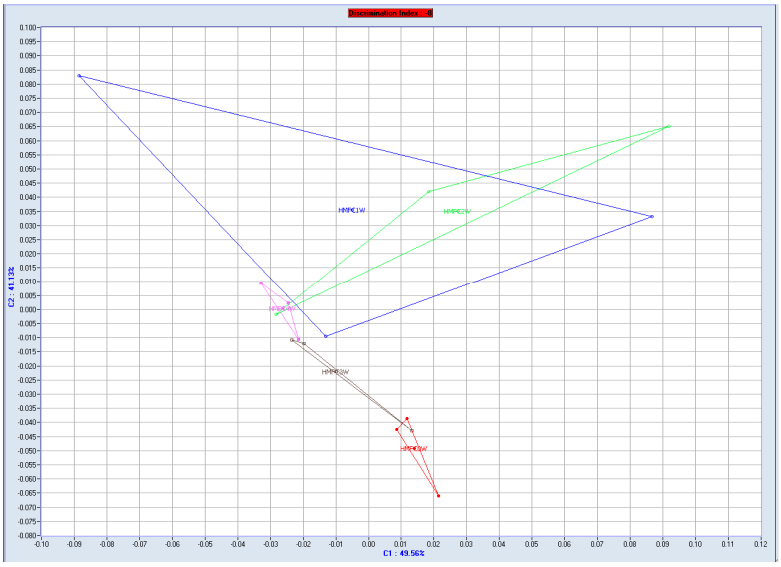

Figure app.5.1: PCA of the volatile compounds of untreated HM stored at -20^{o}C (FC): pattern change during 4 weeks of storage

Figure app.5.2: Principle component analysis of the volatile compounds of HM stored with nitrogen gas at -20^{o}C (FN): pattern change during 4 weeks of storage

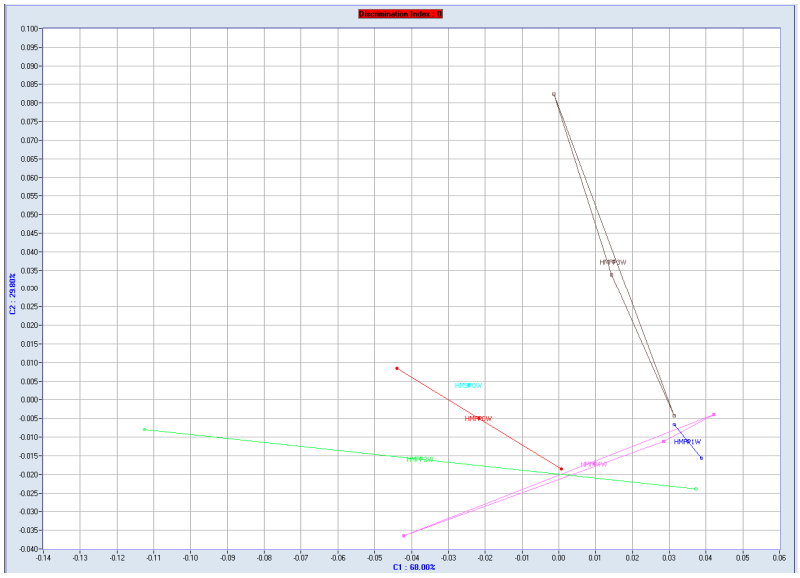

Figure app.5.3: PCA of the volatile compounds of pasteurized HM stored at -20°C (FP): pattern change during 4 weeks of storage

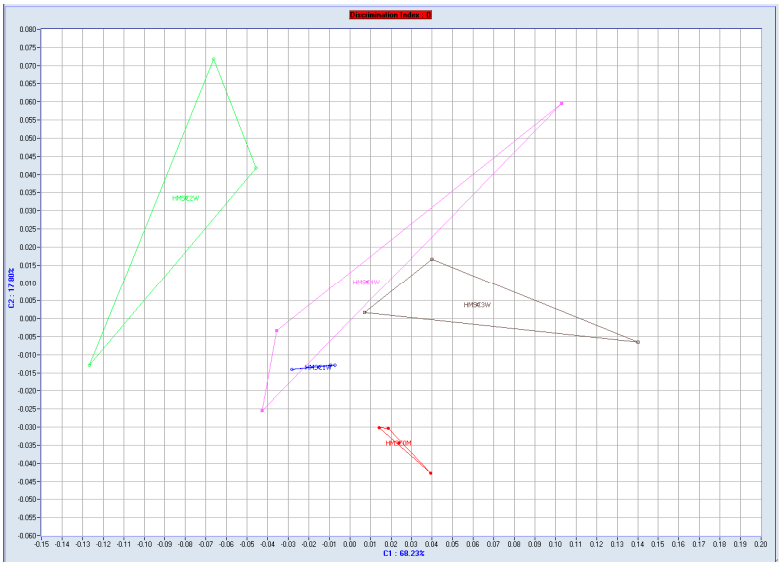

Figure app.5.4: PCA of the volatile compounds of untreated HM stored at -80°C (SC): pattern change during 4 weeks of storage

Figure app.5.5: PCA of the volatile compounds of HM stored with nitrogen gas at -80°C (SN): pattern change during 4 weeks of storage

Figure app.5.6: PCA of the volatile compounds of untreated HM (FC) and of HM stored with nitrogen gas (FN) at -20°C: pattern change during 4 weeks of storage

Figure app.5.7: PCA of the volatile compounds of untreated HM stored at -20°C (FC) and untreated HM stored at -80°C (SC): pattern change during 4 weeks of storage

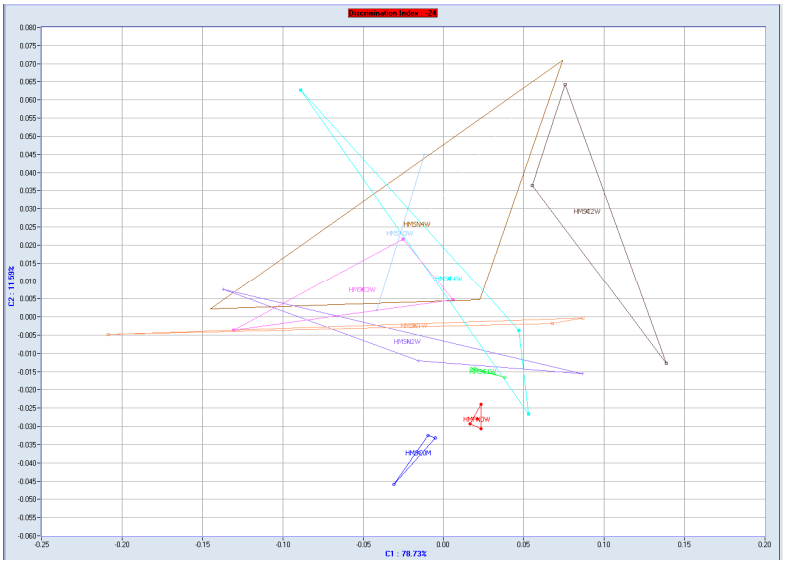

Figure app.5.8: PCA of the volatile compounds of untreated HM (SC) and of HM stored with nitrogen gas (SN) at -80°C: pattern change during 4 weeks of storage

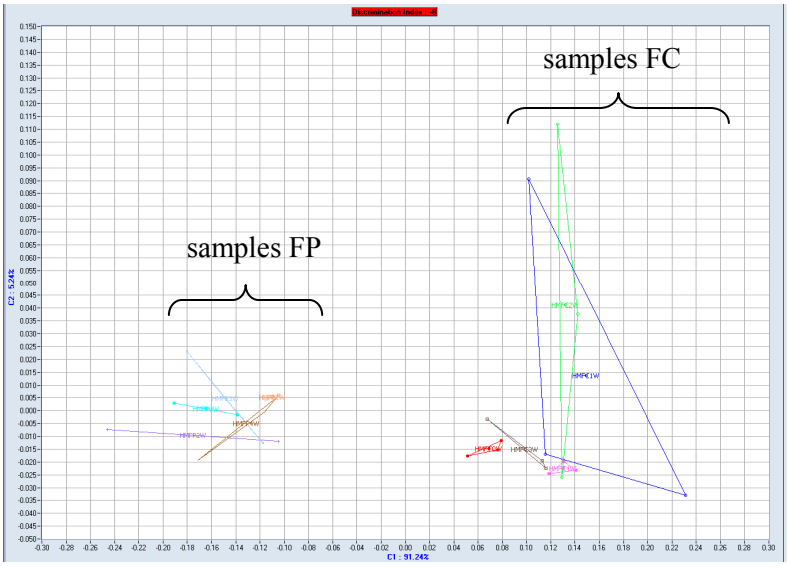

Figure app.5.9: PCA of the volatile compounds of untreated (FC) and of pasteurized HM (FP) stored at -20°C: pattern change during 4 weeks of storage

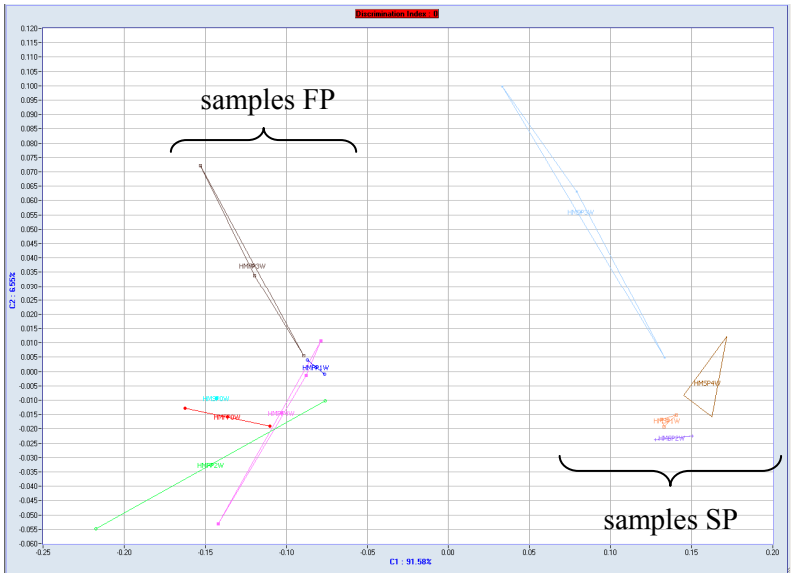

Figure app.5.10: PCA of the volatile compounds of pasteurized HM stored at -20°C (FP) and at -80°C (SP): pattern change during 4 weeks of storage

Figure app.5.11: PCA of the volatile compounds of untreated HM (SC) and of pasteurized HM (SP) stored at -80°C: pattern change during 4 weeks of storage

app.5.12: PCA of the volatile compounds of untreated HM stored at -20°C: pattern change during 5 months of storage

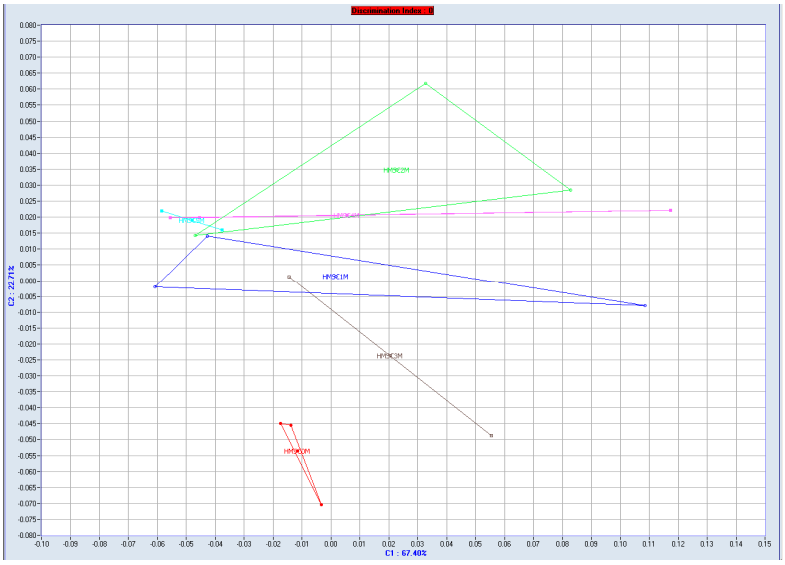

Figure app.5.13: PCA of the volatile compounds of untreated HM stored at -80°C (SC): pattern change during 5 months of storage

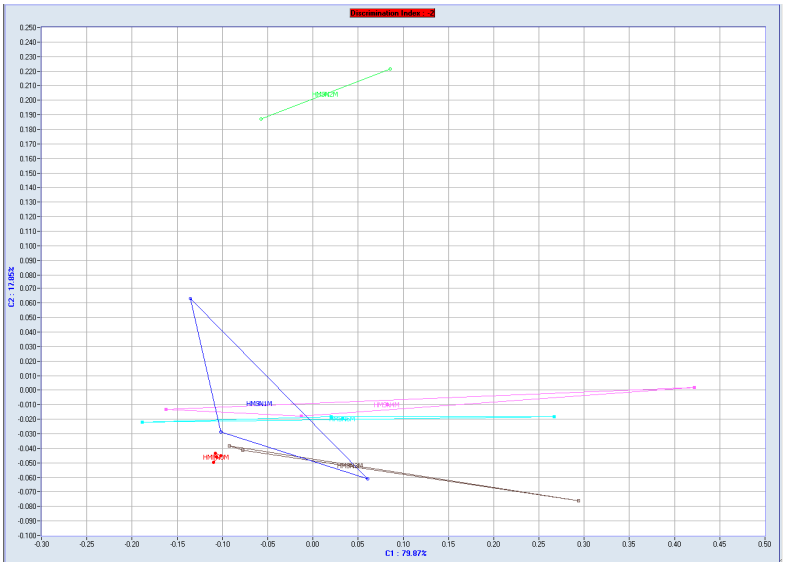

Figure app.5.14: PCA of the volatile compounds of HM stored with nitrogen gas at -80°C (SN): pattern change during 5 months of storage

Figure app.5.15: PCA of the volatile compounds of pasteurized HM stored at -80°C (SP): pattern change during 5 months of storage

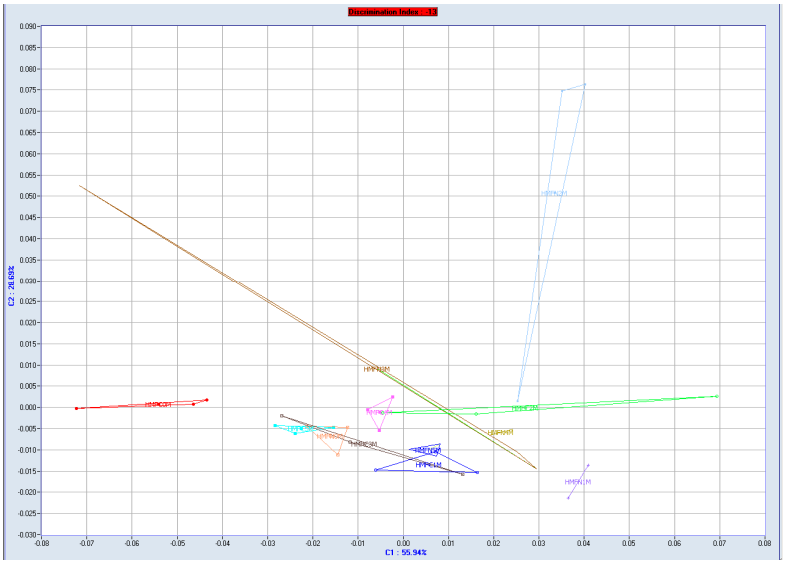

Figure app.5.16: PCA of the volatile compounds of untreated HM (FC) and of HM stored with nitrogen gas (FN) at -20°C: pattern change during 5 months of storage

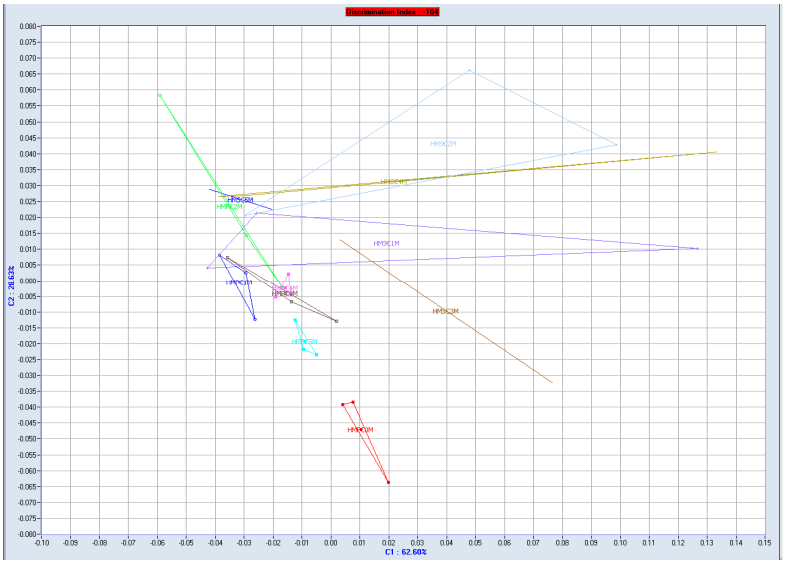

Figure app.5.17: PCA of the volatile compounds of untreated HM stored at -20°C (FC) and untreated HM stored at -80°C (SC): pattern change during 5 months of storage

Figure app.5.18: PCA of the volatile compounds of untreated HM (SC) and of HM stored with nitrogen gas (SN) at -80°C: pattern change during 5 months of storage

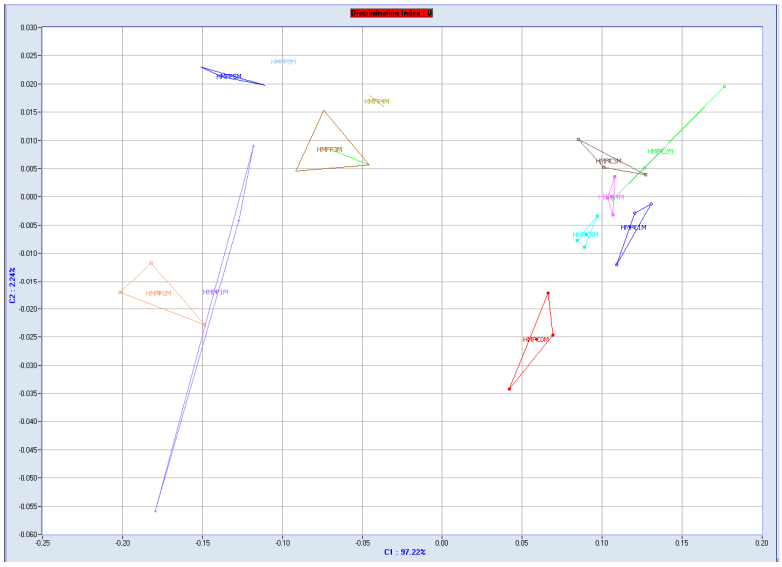

Figure app.5.19: PCA of the volatile compounds of untreated (FC) and of pasteurized HM (FP) stored at -20°C: pattern change during 5 months of storage

Figure app.5.20: PCA of the volatile compounds of pasteurized HM stored at -20°C (FP) and at -80°C (SP): pattern change during 5 months of storage

Figure app.5.21: PCA of the volatile compounds of untreated HM (SC) and of pasteurized HM (SP) stored at -80°C: pattern change during 5 months of storage