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# The Impact of Storage Container Materials on IgA in Human Breast Milk Stored at Two Different Temperatures

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# The Impact of Storage Container Materials on IgA in Human Breast Milk Stored at Two Different Temperatures

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M.S. DEGREE THESIS

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has been examined and approved
by the Thesis Committee as satisfactory
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THE IMPACT OF STORAGE CONTAINERS MATERIALS ON SECRETORY IGA IN HUMAN BREAST MILK STORED AT TWO DIFFERENT TEMPERATURES

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# Dedication

This thesis is dedicated to my parents who have encouraged my higher education and helped me achieved this goal of attaining a Master's Degree. Without their love and support I would not have been able to accomplish this goal. Special thanks go to my Mom who started the idea of researching into the area of human milk and packaging.

# Abstract

This study investigated the relationship between the type of material and the concentration of IgA in human milk stored at two temperatures over time. Twelve breastfeeding mothers participated in the study with samples of milk obtained at locations of the participant's preference and transported to the laboratory in a sterile Snappies PP container stored in a cooler filled with ice. The sample was divided up and pipetted into 12 containers. IgA concentrations were determined by enzyme-linked immunosorbent assay at time zero, 9, 24, 48, 72 and 168 hours. Milk IgA concentration decreased in all containers, with the exception of polycarbonate from 72 hours to 168 hours in -20°C. In 4°C, the results of IgA concentrations showed volatility and did not indicate a stable trend. The data suggested that the concentration of milk IgA was more affected by temperature and the type of material may not have a significant impact on IgA. The data also indicated a maximum storage length of three days in refrigerators to maintain IgA levels.

# Table of Contents

Page

# List of Tables

Page



# List of Figures

Page

# Introduction

In present-day America, women are returning to their jobs earlier after giving birth than previous generations who took longer maternity leaves. Those who choose to breastfeed, find it nearly impossible to feed their infants during their work hours so they must pump then store milk in containers in refrigerators and freezers. Stored milk is then used to feed infants when the women are at work or away from their infants. As a result, it is increasingly difficult for mothers to follow the American Academy of Pediatrics (AAP)'s recently amended recommendation that mothers breastfeed their infants for at least one year. At least 50 percent of women who are employed when they become pregnant return to work by the time their children are three months old, making this study important to analyze the effects of storage time and temperature conditions normally found in refrigerators and freezers (Tyler, 2000). Fortunately, refrigerators and freezers are available to give mothers the option of storing their milk for future feedings to ensure their infants receive milk when breastfeeding is inconvenient or not possible. Ideally, stored milk should be identical to freshly expressed milk in terms of concentration and viability of different biocomponents. It is even more critical for infants in a Neonatal Intensive Care Unit (NICU) to have their mothers pump the optimal milk so they receive the highest quantity of immunologic factors. One in eight babies are born prematurely and placed in the NICU due to the rising number of twins and triplets from assisted reproduction, induced premature deliveries, and Cesarean sections (Maugh, 2008). It is publicized that the colostrum of mothers of preterm infants has higher concentrations of IgA, up to 12 g/L to 0.5-1.0 g/L in mature milk (Butte et al., 1984) or 310.5 mg/g in preterm colostrum compared to 168.2 mg/g in term colostrum (Groer & Walker, 1996; Gross et al., 1981a).

Previous studies in the last 30 years show that human milk loses important components due to a variety of factors, including temperature and adherence of components to the materials of the storage containers. In a groundbreaking study by Goldblum et al. (1981), the concentration of secretory IgA in milk is shown to have no change in glass and polypropylene. However, in a report by Williamson and Murti (1996), there is a loss of IgA in glass with the mean count of IgA in human milk stored in glass decreasing after seven hours. SIgA is stable in rigid polyethylene containers, but is reduced by 60 percent after being stored in flexible polyethylene bags (Goldblum et al., 1981).

IgA has been selected as the milk component for analysis because several lactation consultants and experts recommended IgA due to its important role in the immune system of the nursing infant (Lactnet postings, 11-27-08 to 12-4-08). An additional feature is that IgA is found to be stable at 4°C and -20°C (Evans, Ryley, Neale, Dodge, & Lewarne, 1978, Pardou, Serruys, Mascart-Lemone, Dramaix, & Vis, 1994). Lawrence and Lawrence (2005) recommends up to 48 hours of milk storage in the refrigerator and up to six months in a standard home freezer.

## Purpose of the Thesis

This project will study different storage container materials to find the minimum impact on the adherence of IgA in human milk by quantifying the loss of IgA due to adherence. The goal is to show whether current recommendations proposed by lactation consultants and doctors regarding container types, temperature, and time are still appropriate for optimal levels of IgA.

## IgA and sIgA

IgA antibodies are the first line of defense against a wide berth of pathogens in the human body. They bind to bacteria and toxins, preventing them from invading epithelial cells; particularly in the mucus epithelium of the intestinal and respiratory tracts. By preventing these microbes, no inflammation is induced as other antibodies and phagocytes are not activated (Hale & Hartmann, 2007). IgA also neutralize toxins or bacteria by absorption. There are two forms of IgA found in the human body; the first is monomeric IgA with a molecular weight of 160 kDa and the second is the 390 kDa dimeric type. The dimeric form is two monomeric IgA linked by a J-chain by disulfide bonds (Janeway, Travers, White & Shlomachik, 2001).

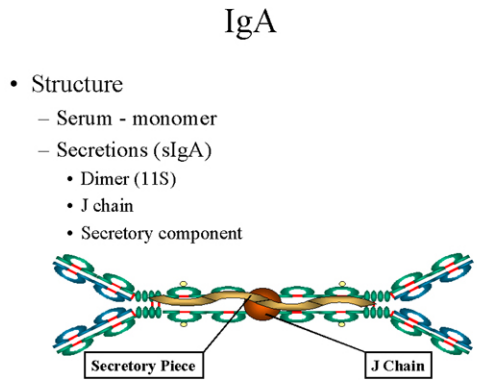



***Figure 1. sIgA with J Chain and Secretory Component (http://www.phasmatodea.org/ap2/immunology2_notes.html)***

IgA in the blood system is mainly monomeric, whereas in the mucosal linings, IgA is usually in the dimeric form, (Janeway et al., 2001). The dimeric form is referred to as *secretory IgA (sIgA)* and is the principal isotype in infants as sIgA can be transported across the epithelium from the mother's mammary glands to the milk. It is the predominant antibody in human milk, making up 80 to 90% of the antibodies in the colostrum and mature milk (Hale & Hartmann, 2007). In the lactating mammary gland, plasma cells synthesize IgA under the basement

membrane with two IgA antibodies binding them to a J chain. A complex of the J-chain and IgA antibodies attaches to a poly-Ig receptor and diffuses across the basement membrane. After being transported through the basement membrane, sIgA enters the mammary gland, which produces human milk and is later lactated to the feeding infant (Janeway et al., 2001).

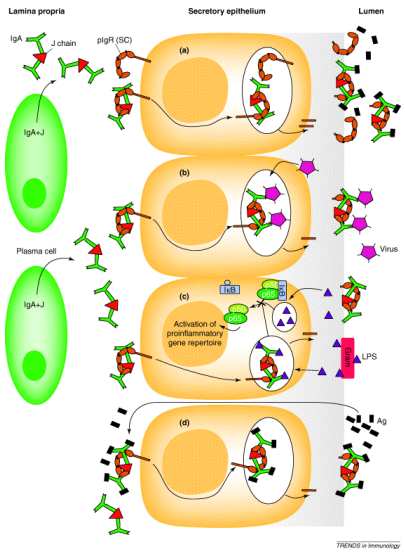



***Figure 2. Transport of sIgA through the secretory epithelium via three methods (http://www.med.uio.no/rh/patologi/liipat/secretory_immunity/pictures/TI-fig1big.htm)***

The role of sIgA is vital to the infant as it plays different functions in the intestinal development of neonates, including promoting intestinal adaptation to extrauterine life and establishment of beneficial microbiota. It also prevents infection and inflammation as well as compensates for the developmental immaturity of the intestine (Donovan, 2006). In infants, sIgA is effective against enterotoxin-producing *E. coli*, *Campylobacter*, *Shigella*, salmonellae, streptococci, staphylococci, and pneumococci (Glass et al., 1983; Ruiz-Palacios et al., 1990; Hayani et al., 1992; Cruz et al., 1983; Waterspiel et al., 1994). SIgA is also shown to be active against a large range of viruses, such as polio virus types 1, 2, 3, coxsackie virus types, ECHO

virus types 6, 9, as well as rotaviruses (Welsh & May, 1979). The infant does not attain adult levels of IgA until two months old.

IgA and sIgA is shown to be unaffected by freezing for four weeks (Tully, Jones & Tully, 2001). Cycles of freezing/thawing do not affect the composition of IgA (Evans, Ryley, Neale, Dodge, & Lewarne, 1978, Pardou, Serruys, Mascart-Lemone, Dramaix, & Vis, 1994). However, it is possible that sIgA proteins may undergo protein denaturalization during thawing after being frozen (Kerner, Quan, Yang et al., 1987). The average concentration of IgA in human milk quantified by the ELISA technique during the first year of lactation is approximately 0.388 to 1.105 grams per liter (g/L) (Weaver, Arthur, Bunn, & Thomas, 1997). The results from O'Connor, Schmidt, Carroll-Pankhurst, and Olness (1998) show a range of 0.32 to 0.49 g/l of sIgA in human milk using the radial immunodiffusion technique.

## Packaging Materials

The following human milk storage containers are chosen for their material composition:

1. Medela Freezing and Storage rigid polypropylene 80 mL bottles- 090607-A part 61095 100
2. Snappies sterile rigid polypropylene 70 mL containers- Lot 071107 Part 10253 Mold #252
3. Ameda rigid polycarbonate milk bottles – Lot 7J058
4. Medela Pump and Save flexible polyethylene (HDPE) storage bags- Lot 39209A 73321
5. Ball canning glass- 14400-80400, 05-604
6. Axifeed rigid polyethylene (HDPE) 100 mL sterile bottles- Lot 121865

## Material Characteristics

There are four different materials being examined in the thesis: polypropylene, polycarbonate, high density polyethylene, and glass. There are two types of plastics. The first type of plastics is flexible yielding materials, such as film, foil, or paper sheeting, which acquire a pliable shape when filled and sealed. The Medela flexible polyethylene bag falls under this category. The second group is rigid packaging, which maintains its shape and dimensions throughout its lifecycle after thermoformed. All rigid plastic containers belong to this category.

Polypropylene (PP), identified by Resin Identification Code #5, is used in food packaging applications, including take-out containers, lids and domes, cups, tubs for yogurt and margarine, ketchup bottles, and medicine bottles. They are also utilized for housewares, media packaging, bottles for cosmetics and household industrial chemicals, plastic syringes, and air/water filtration systems. The advantages of PP are that bottles can be hot-filled and may have a built-in handle. The material has excellent optical clarity in biaxially-oriented films and can be injection molded, thermoformed, and stretch blow molded. They have low moisture vapor transmission, and are inert to acids, alkalis, and most solvents. The density of polypropylene used for bottles, labels, and clear films is low, approximately 0.89 to 0.93 grams/ $cm^3$. Polypropylene's low specific gravity and good stiffness can result in fewer pounds of resin used per thousand packaging units, saving costs to the manufacturer. There are no known safety concerns or worries about consumer applications.

Polycarbonate (PC) is identified by Resin Identification Code #7 and is used in medical devices. It is also used for containers, such as reusable/microwaveable housewares and reusable bottles, which include water cooler bottles, sports bottles, and baby bottles. PC is commonly blended with other plastics. Additionally, PC is a durable plastic that can withstand relatively

high temperatures; hence, it is an ideal choice for applications requiring frequent reuse and washing, sterilizing or microwave heating (PlasticsEurope, 2008). The advantages polycarbonate provides are toughness, dimensional stability, optical clarity, high heat resistance, and excellent electrical resistance. PC is also recyclable, and is a high-value resin for recycling due to its relatively high cost per pound of resin (PlasticsEurope, 2008). The cost factors discourage the use of polycarbonate in packaging and other single-use applications. PC is portrayed negatively in the public eye due to a monomer, known as bisphenol A or BPA, from which PC is manufactured. However, the Food and Drug Administration, the EU Scientific Committee on Food, and the Japanese Ministry of the Environment have determined the amount of residual BPA in PC to be typically less than 50 parts-per-million, declaring PC safe for use (FDA, 2008).

High density polyethylene (HDPE) is identified by Resin Identification Code #2; it is known for its low cost in manufacturing. It also has an excellent moisture barrier and does not typically break when dropped. The disadvantages of HDPE are its permeability to oxygen and a high cold-flow rate (Soroka, 2002).

Glass is usually created from combining silica sand mixed with carbonates, such as limestone, soda ash, or alumina in high heat. Advantages of using glass include its inertness to most chemicals. In addition, glass does leach out materials, altering the taste of food. Glass has excellent impermeability to oxygen for long-term storage of products sensitive to oxygen. It is also stable at high temperatures and maintains a rigid shape. The disadvantages of using glass are its brittleness, which makes glass prone to breaking at specific heights. It is also more expensive to manufacture than plastic resins (Soroka, 2002).

## Company's Criteria for Selecting a Material for Their Storage Containers

Companies manufacturing bottles used in the thesis were contacted by e-mail. This e-mail included questions on why the company chose the specific material for their containers. Only two companies, Medela and Snappies, responded.

### *Snappies*

Nancy Fleming, the Commercial Product Manager for the Snappies container at Thermo Fisher Scientific, responded to my e-mail inquiry about the selection of PP in Snappies containers. The company chose polypropylene because most of the company's other products are made from polypropylene. As a result, Thermo Fisher Scientific had a strong relationship with its resin supplier. They also had previous packaging experience when working in the dairy industry prior the company entering the human milk market. Their results showed that milk fat did not stick to polypropylene, leading to more consistent results in testing. From the manufacturing perspective, the suppliers and mold engineers chose polypropylene because human milk needed to be stored in a food-grade plastic container. They also wanted a resin that could flow through the injection-molding equipment and had a low-residual odor.

### *Medela*

Simdon Craig, the Product Introduction Engineer at Medela, responded to my e-mail about their selection of materials in their two containers. Craig mentions their bottles were not regulated medical devices; hence, they did not consider them to be measurement devices. For the Medela flexible polyethylene bags, Craig indicated that the company uses a HDPE inner liner due to its food contact properties and water vapor barrier properties. They chose this resin for their rigid PP containers over other resins, indicating previous studies showing PP as a good material for storing human breast milk. Medela chose polypropylene for its translucent clarity

and its ability to withstand cleaning and sterilization requirements FDA requires. Medela also made a decision ten years ago to not use PC in their containers as the company was aware of the leaching of bisphenol A into liquids.

## Hypothesis

Rigid PP containers will be the optimal storage containers for the least IgA retention at 4˚C and -20˚C. Rigid PP containers will be followed by other materials in order from best to worst performance in least retention of IgA in 4˚C and -20˚C, HDPE, glass, PC, and flexible PP bags.

# Method / Acquirement of Samples

1. The RIT Human Subjects Research Office has approved the participation of human subjects.
2. Lactation consultants, clubs, and organizations targeting new moms, along with word-of-mouth have been used to contact possible candidates.
3. Eligibility for the project has been determined based on voluntary participation and willingness to donate milk 4 to 30 weeks post-delivery. Ideally, the number of participants was 20 to 25 to achieve statistical significance.
4. Participants were briefed on the study goals, risks, and privacy issues before voluntarily agreeing to sign an Informed Consent form prior to participation.
5. Information on the age of mothers and that of their infants were taken to explain possible outliers that may appear in the data analysis.
6. The researcher went to locations of the participant's preference to collect human milk.
7. Sterile Snappies polypropylene containers were used to collect each participant's milk.
8. A single expression of milk from one or both breasts, by breast pump or hand expression, were collected in a sterile Snappies polypropylene tube.
9. Breast milk samples were labeled at the site with a reference number to protect the identity of the participant. The sample was transported to RIT, chilled in a cooler filled with ice.
10. The reference numbers and names were kept in a safe in Professor Deanna Jacobs' office, 70-1313 in the Golisano College of Computing and Information Sciences.
11. The reference number used for each participant was not published in the thesis. Letters (A, B, and C) were used in the published data to protect the identity of the participants.

12. After arriving in the lab, time zero was established when the sample in the Snappies polypropylene bottle was shaken and three 800-microliter aliquots of the sample were placed in separate 1.5 mL centrifuge tubes. The average of the three was the benchmark for IgA levels at time zero.

# Transferring Breast Milk from Snappies Transport Container to the Appropriate Containers

1. 60 mL of milk received from participants were pipetted from the Snappies transport container to the different containers involved in the experiment. Each container had 5 mL of sample.
2. There were 12 containers altogether. One of each type of container was stored in the refrigerator and one in the freezer, which resulted in six containers in the refrigerator and six in the freezer.
3. The containers were placed next to each other in the same area in the freezer/fridge to reduce variability due to location of samples.
4. For the refrigerated samples, three different aliquots were taken from each container and combined to make one sample at 9, 24, 48, and 72 hours. Frozen samples were analyzed at 72 and 168 hours. Each container was warmed under warm running water 45 seconds for refrigerated samples and 130 seconds for frozen samples.
5. Warming of the refrigerated and frozen samples followed the standard procedure provided by lactation consultants on thawing milk and were applied to all containers in this experiment in the previous step.
6. 800 microliters of milk for each container were measured and centrifuged in the tabletop centrifuge (MicroSpin 12, Sorvall Instruments) for 20 minutes. 400 microliters of the bottom whey layer were pipetted and placed into a centrifuge tube. The top layer, compromised of cream, was discarded. Appropriate dilution of 1:2 or 1:4 of each sample combined with sample diluent was performed.

## Enzyme-Linked ImmunoSorbent Assay (ELISA) Protocol

1. For the ELISA procedure, the Bethyl Laboratories E80-102 manual was referenced.
2. A change was made at the end of Step B1 in the Bethyl manual. Instead of following Bethyl's recommendation of washing the wells by aspirating each well, the "flick and tap" method was used.
3. The samples were diluted by a factor of 2 or 4 in Step B2. This was done as previous literature showed the average concentration of IgA to be approximately 700 ng/mL, As a result, 1:2 dilutions were performed to ensure the samples fit the standard curve of 7.8 ng/ml to 1000 ng/ml.
4. The substrate's reaction with 3, 3', 5, 5'-tetramethylbenzidine (TMB) wells were read by an at ELISA ELx800 UV reader (Bio-Tek Instruments) at 450 nm.
5. KC Junior (Bio-Tek) was used to convert absorbance readings into IgA concentrations by comparing the samples to the standards provided by Bethyl.

***Table 1. A Sample Set Up of a 96-Well ELISA Plate***

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 1000 ng/mL standard | Blank (sample diluent) | Blank (sample diluent) | Blank (sample diluent) | 1000 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **B** | 500 ng/mL standard | Ameda Bottle Sample | Ameda Bottle Sample | Ameda Bottle Sample | 500 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **C** | 250 ng/mL standard | Snappies Bottle Sample | Snappies Bottle Sample | Snappies Bottle Sample | 250 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **D** | 125 ng/mL standard | Medela PP Bottle Sample | Medela PP Bottle Sample | Medela PP Bottle Sample | 125 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **E** | 62.5 ng/mL standard | Medela HDPE Bottle Sample | Medela HDPE Bottle Sample | Medela HDPE Bottle Sample | 62.5 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **F** | 31.25 ng/mL standard | Axifeed Bottle Sample | Axifeed Bottle Sample | Axifeed Bottle Sample | 31.5 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **G** | 15.625 ng/mL standard | Ball Glass Sample | Ball Glass Sample | Ball Glass Sample | 15.625 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |
| **H** | 7.8 ng/mL standard | Blank (sample diluent) | Blank (sample diluent) | Blank (sample diluent) | 7.8 ng/mL standard | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well | Empty well |

Legend: The table shows the set up of blanks, standards, and samples in a 96- well plate. There are two replicates of each standard and three replicates of each sample.

## Statistical Methods

1. For each subject, the numbers of replicates at time zero were averaged together. The average became the baseline for all replicates at each time point compared.
2. The standard deviation from the baseline was determined for elimination of outliers.
3. Individual replicates for each container were compared to the baseline. If a replicate showed a concentration higher than the standard deviation range, then it was omitted from the results.
4. The remaining replicates were divided by the baseline and converted to a percentage.
5. The percentages were added to represent an average percentage for each container at each time point.
6. The subjects were added together to create the final percent of IgA remaining in milk in each container at every time point.
7. Each container was plotted onto a line graph with the X-axis values representing percent of IgA in milk and the Y-axis values representing hours.

# Results

Twelve subjects participated in the study, with four subjects excluded from the results due to contamination of the samples during the data-gathering process. Contamination occurred during time zero and nine hours, with the samples assumed to be compromised when the blanks had larger readings than the background readings. In a few cases, the blanks were shown to have absorbance values as high as the samples. If the blanks displayed high absorbance values, then the standard curves created from the known standards provided by Bethyl, Inc could not be established. This led to the omission of the samples at that specific time point from the study. The subjects used in the study were labeled as B through L. The range of time from lactation of the milk to the laboratory was anywhere from 15 minutes to one hour and 15 minutes. Most samples had a transportation average time of 30 to 35 minutes. There were six time points (0, 9, 24, 48, 72, and 168 hours) and two temperatures (4 °C and -20 °C) in the data. As seen in Table 2, the mean standard deviation of samples taken at time zero was 338.5 with a high value of 697.6 and a low value of 122.4.

***Table 2. Age of Mother and Infant with Mean IgA Concentration at Time Zero***

| Subject | Age of Subject | Age of Infant | Mean IgA Concentration (ng/ml) | Standard Deviation |
|---|---|---|---|---|
| **B** | 29 years | 4 weeks | 1892.3 | 697.6 |
| **C** | 30 years | 5 weeks | 3116.9 | 122.4 |
| **F** | | | 1889.3 | 312.7 |
| **H** | 30 years | 28 weeks | 3081.5 | 525.3 |
| **I** | 27 years | 9 weeks | 1062.5 | 263.1 |

| Subject | Age of Subject | Age of Infant | Mean IgA Concentration (ng/ml) | Standard Deviation |
|---|---|---|---|---|
| **J** | 33 years | 44 weeks | 3198.0 | 355.0 |
| **K** | 32 years | 4 weeks | 1051.0 | 196.1 |
| **L** | 30 years | 28 weeks | 1231.1 | 235.7 |

Nine-hour values are not included in the results for subjects B, I, and K, In addition, subject F's 48 and 72-hour marks are omitted from the results. Subjects H, J, and L do not have their 24-hour values in their results. Subject H does not include values from 48 hours. The omitted values are ignored because the wells at these time points showed inconsistent results due to contamination of the samples from human error or the researcher's technique.

Table 3 shows how many samples were used to represent the amount of IgA remaining in the human milk over time. The nine-hour mark has an unbalanced number of samples because the Snappies and Axifeed containers have one extra sample. The 24-hour mark has only three samples and the 48-hour mark has five samples. The 72-hour samples and the 168-hour samples have seven subjects. The higher number of subjects in the 72-hour and 168-hour samples compared to the earlier time points is due to reliable standard curves and the low absorbance values from the blanks.

***Table 3. The Number of Samples in Each Container used in the Data.***

| **4°C** | **Ameda PC** | **Snappies PP** | **Rigid Medela PP bottle** | **Medela Flexible PE Bag** | **Axifeed HDPE** | **Ball Glass** |
|---|---|---|---|---|---|---|
| **9 hours** | 4 | 5 | 4 | 4 | 5 | 4 |
| **24 hours** | 3 | 3 | 3 | 3 | 3 | 3 |
| **48 hours** | 5 | 5 | 5 | 5 | 5 | 5 |
| **72 hours** | 7 | 7 | 7 | 7 | 7 | 7 |
| **-20°C** | Ameda PC | Snappies PP | Rigid Medela PP bottle | Medela Flexible PE Bag | Axifeed HDPE | Ball Glass |
| **72 hours** | 7 | 7 | 7 | 7 | 7 | 7 |
| **168 hours** | 7 | 7 | 7 | 7 | 7 | 7 |

Legend: Each value represents the number of subjects used to represent the data.

Tables 5 and 6 include the average percentage of IgA concentration remaining in each container compared to the baseline of IgA quantified at time zero. The last two rows of the tables show the average of all time points for each container as well as the standard deviation.

*Table 4. Percentages of IgA Concentration Quantified in Human Milk Stored at 4°C*

| Hours | Ameda PC | Snappies PP | Medela PP container | Medela Flexible PE Bag | Axifeed HDPE | Ball Glass |
|---|---|---|---|---|---|---|
| **9** | 76.16 | 71.92 | 72.33 | 72.26 | 71.47 | 69.38 |
| **24** | 76.53 | 85.43 | 83.15 | 71.59 | 79.85 | 78.24 |
| **48** | 73.39 | 74.52 | 71.88 | 78.32 | 76.99 | 73.38 |
| **72** | 82.71 | 74.34 | 76.61 | 76.16 | 78.53 | 81.68 |
| **Average** | 77.20 | 76.55 | 75.99 | 74.58 | 76.71 | 75.67 |
| **Standard Deviation** | 23.54 | 22.12 | 20.67 | 15.98 | 22.88 | 18.69 |

*Table 5. Percentages of IgA Concentration Quantified in Human Milk Stored at -20°C*

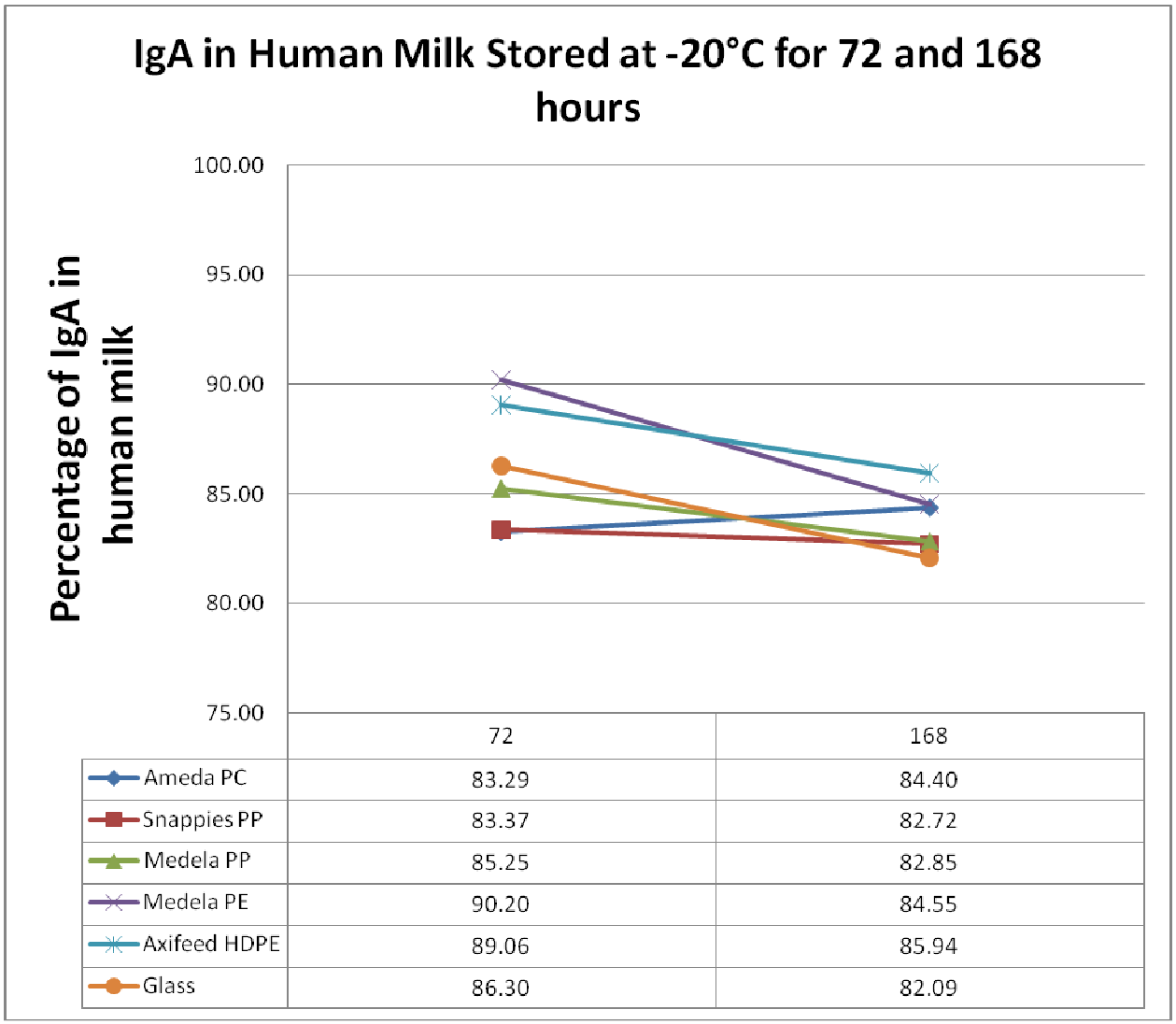
| Hours | Ameda PC | Snappies PP | Medela PP container | Medela Flexible PE Bag | Axifeed HDPE | Ball Glass |
|---|---|---|---|---|---|---|
| **72** | 83.29 | 83.37 | 85.25 | 90.20 | 89.06 | 86.30 |
| **168** | 84.40 | 82.72 | 82.85 | 84.55 | 85.94 | 82.09 |
| **Average** | 83.84 | 83.04 | 84.05 | 87.37 | 87.50 | 84.19 |
| **Standard Deviation** | 20.48 | 18.94 | 16.90 | 15.66 | 15.47 | 21.01 |

Figures 3 and 4 are a visual representation of Tables 4 and 5, which show the concentrations of IgA varying from temperature to temperature as well as container to container. In the -20°C freezer at 72 hours (Figure 3), all containers performed very close to each other, ranging from 83.3% of IgA in the Ameda PC container to 90.2% of IgA left in human milk in

the Medela PE bag. The concentration of IgA reduces in all containers from 72 hours to 168 hours, with the exception of Ameda, which remains stable. The range varies from 85.9% in Axifeed to 82.1% in glass. None of the containers went below 80% at -20°C in both time points.

Figure 4 shows how each material performs in the least retention of IgA in the 4°C samples. The IgA concentration starts out low at nine hours and steadily increases to 24 hours in all containers, with the exception of Medela PE bags and Ameda PC containers. IgA concentration drops down at 48 hours for all containers, with the exception of Medela PE bags, which increases from 71.6 to78.3%. There are mixed results in IgA concentration in different materials at 72 hours. For all 4°C samples, there is consistency in the performance of all containers throughout the duration of the experiment as the range of IgA concentration goes from 69% to 85% as compared to the original concentration. The worst performing container is the Ball glass with 69.4% of IgA in the human milk at nine hours compared to time zero. The best performing material is the Medela PE bag with 85.4% of IgA in the human milk at 24 hours.

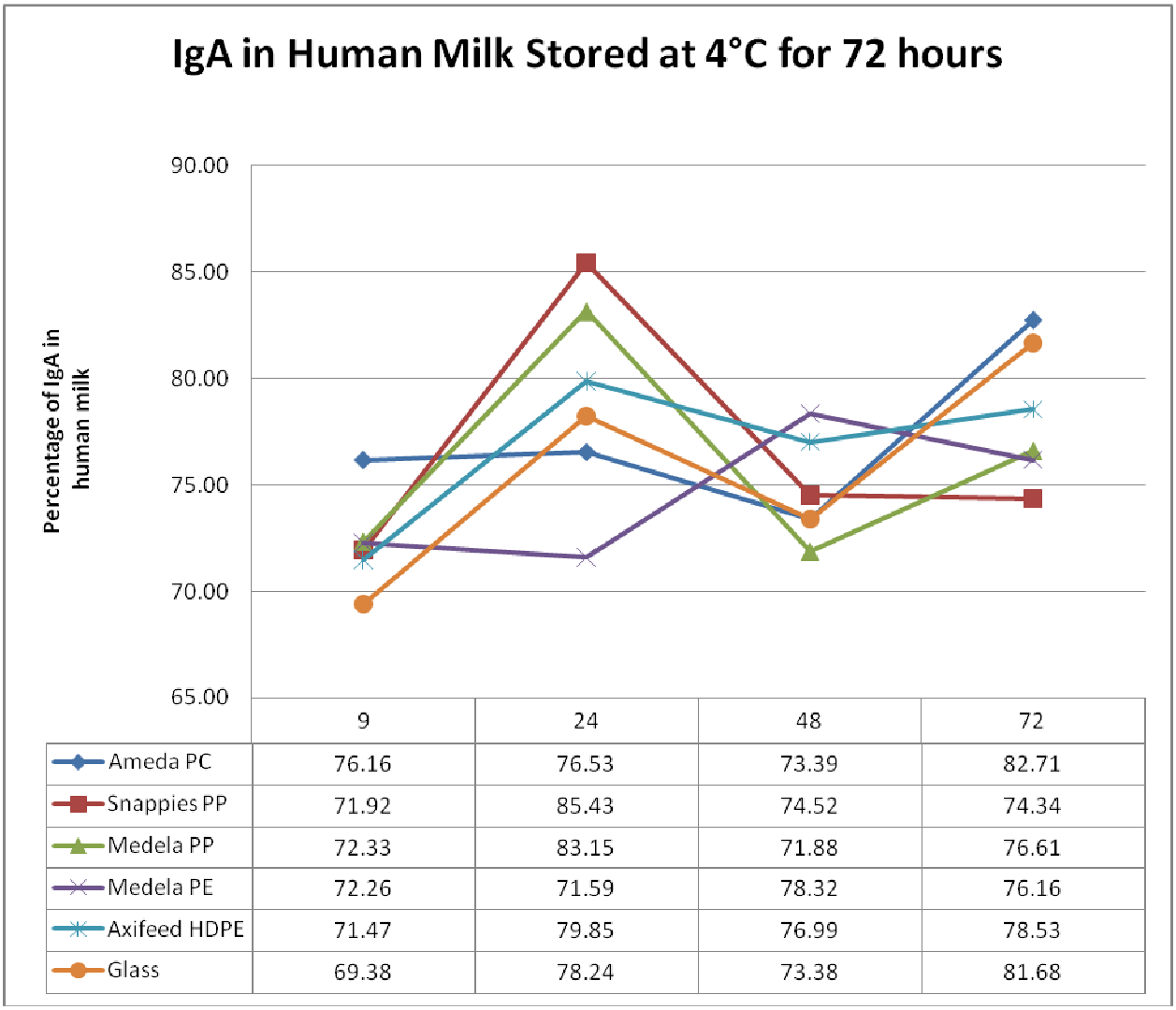
*Figure 3. Percentage of IgA Remaining in Human Milk Stored in the Freezer (-20°C).*




| | 72 | 168 |
|---|---|---|
| Ameda PC | 83.29 | 84.40 |
| Snappies PP | 83.37 | 82.72 |
| Medela PP | 85.25 | 82.85 |
| Medela PE | 90.20 | 84.55 |
| Axifeed HDPE | 89.06 | 85.94 |
| Glass | 86.30 | 82.09 |

Legend: Percentage of IgA in the samples taken from bottles since time zero. Two time points are used to analyze the -20°C (freezer) samples.

***Figure 4. Percentage of IgA Remaining in Human Milk Stored in the Refrigerator (4°C).***




| | 9 | 24 | 48 | 72 |
|---|---|---|---|---|
| Ameda PC | 76.16 | 76.53 | 73.39 | 82.71 |
| Snappies PP | 71.92 | 85.43 | 74.52 | 74.34 |
| Medela PP | 72.33 | 83.15 | 71.88 | 76.61 |
| Medela PE | 72.26 | 71.59 | 78.32 | 76.16 |
| Axifeed HDPE | 71.47 | 79.85 | 76.99 | 78.53 |
| Glass | 69.38 | 78.24 | 73.38 | 81.68 |

Legend: Percentage of IgA in the samples taken from bottles since time zero. Four time points are used to analyze the 4°C (refrigerator) samples.

Further statistical analysis was performed on the 72 hour mark to compare the influence of the material on IgA stored at 4°C and -20°C. It was the only time point when both the refrigerator and freezer samples were present. Eight subjects were used to compare both temperatures. These subjects were B, C, F, H, I, J, K, and L.

Table 6 shows the results of the concentration of IgA at 72 hours.

***Table 6. Concentration of IgA (ng/ml) in Each Container Stored at 4°C and -20°C for 72 Hours.***

| | | Mean | Standard Deviation | Minimum | Median | Maximum |
|---|---|---|---|---|---|---|
| **Ameda PC** | -20°C | 1887 | 1071 | 841 | 1323 | 3942 |
| | 4°C | 1792 | 883 | 846 | 1429 | 4892 |
| **Snappies PP** | -20°C | 1751 | 942 | 841 | 1337 | 4054 |
| | 4°C | 1533 | 501 | 736 | 1679 | 2449 |
| **Medela Rigid PP** | -20°C | 1761 | 887 | 773 | 1477 | 3205 |
| | 4°C | 1710 | 871 | 609 | 1393 | 3882 |
| **Medela Flexible PE** | -20°C | 1944 | 1065 | 737 | 1394 | 4156 |
| | 4°C | 1522 | 488 | 699 | 1456 | 2365 |
| **Axifeed HDPE** | -20°C | 2170 | 1421 | 829 | 1697 | 5615 |
| | 4°C | 1542 | 517 | 845 | 1451 | 2648 |
| **Glass** | -20°C | 1971 | 1303 | 345 | 1360 | 5862 |
| | 4°C | 1586 | 494 | 716 | 1571 | 2321 |

Legend: Three observations were made for each container. Design was unbalanced as Subject K in the Snappies PP and Subject I in the Medela Flexible PE at 4°C had only two observations, so the third observation came from the mean of the first two observations.

The first significant analysis demonstrated that each container had a higher mean in the -20°C as compared to 4°C; however, the median was lower in -20°C for all containers. The Axifeed HDPE and the Medela PP containers had higher means and median in the freezer. The standard deviation was higher in the freezer for all containers.

The next significant observation was the interaction of three variables: temperature, subject, and container (see Table 7). When the subjects were random with the container and temperature values being fixed, the temperature ($p=0.277$) and subject ($p =0.050$) were more significant on influencing the concentration of IgA than the container ($p=0.521$). There was a

strong interaction between the subjects and temperatures (p=0.00) when compared against the interaction of containers and temperatures (p=0.168).

***Table 7. Interaction of Different Subjects, Containers, and Temperatures at 72 Hours.***

| **Source** | **P-value** | **Source** | **P-value** |
|---|---|---|---|
| **SubjectA_72** | 0.050 | SubjectA_72 | 0.050 |
| **Container_72** | 0.521 | Container_72 | 0.514 |
| **Temp_72** | 0.277 | Temp_72 | 0.277 |
| **SubjectA_72*Temp_72** | 0.000 | SubjectA_72*Temp_72 | 0.000 |
| | | Container_72*Temp_72 | 0.168 |

# Discussion

The transportation method was derived from the study by Goldblum et al. (1981). The method varied a little as their study mentioned the collection of milk usually took ten minutes with transportation in 4°C in an ice chest to the laboratory. The participant contributed two times for the study; one sample was stored in a Pyrex glass cylinder while the other sample was stored in a polyethylene container. In this study, only one contribution was taken from each participant with the average transportation time taking 30 minutes. The sample was stored in a polypropylene container rather than a Pyrex glass cylinder and a polyethylene container. The study by Friend, Shahani, Long, and Vaughn (1983) was also referenced as the researchers collected milk at participants' houses and traveled to the laboratory. The decision of starting time zero in this study was based on the Friend et al study, where their experiment started at the point they arrived at their laboratory.

Different subjects had different concentrations of IgA ranging from 1051.0 to 3198.0 ng/ml (Table 2). Previous literature noted different variables may affect the concentration of IgA in different women, including environmental stresses, the health of mother and infant, and the week of gestational age of the infant (Weaver et al., 1998, & Goldman et al., 1982, & Prentice, Prentice, & Cole, 1984, & Hennart, Brasseur, Delgone-Desnoeck et al., 1991). The standard deviation of the subjects at time zero (338.5 ng/ml) was shown to be less than the published standard deviation of 1360 ng/ml (1.360 g/l) in the paper of Koenig, Diniz, Barbosa, and Vaz (2005). The smaller standard deviation was encouraging; indicating that the data obtained in this experiment may have been more consistent than previous studies.

The technique used in the experiment may have led to the inconsistency of the results from the samples. It is strongly believed the inconsistency of samples in the shorter time points was due to lack of concentration or drowsiness when working in the lab nine to twelve hours in the first 24 hours of experiment for each subject. The 48, 72, and 168 hours may have come out better due to more time apart between experiments.

The sample size in the study is very small, creating a higher volatility in the data as compared to larger sample sizes. For instance, only three out of eight subjects were used in the 4°C samples at the 24-hour mark, while the nine-hour and 48-hour marks included four or five subjects. This could explain why most of the containers increased at least 5% from the nine-hour mark to 24-hour mark while the percentage decreased at least 5% at the 48 hour mark. Even with a small sample size and a large range of high and low values in each container, the average of all percentages for each container showed each container performed very consistently throughout the duration of the experiment. When all the containers were compared to each other, they essentially performed very closely, with a range of 74.6 to 77.2% in the 4°C samples and 83.0 to 87.5% in the -20°C samples.

It is exciting to show a material did not retain more than 20% of the original concentration in the -20°C samples. Further, it was encouraging to know that loss of concentration was not necessarily directly related to the retention of the IgA by the material, but rather attributed to the protein denaturalization that may occur during the thawing process as noted in the study by Kerner et al. (1987). When the 20°C samples were compared to the 4°C samples, there was less loss of IgA in the human milk, supporting Lawrence and Lawrence (2005)'s recommendation that frozen samples may be left for six months.

Further research, however, must be taken to study to determine whether the length of duration is still appropriate for maintaining IgA levels. This could mean the lower temperature may be more optimal in terms of IgA remaining inside the milk. A lower temperature may be credited with less movement of IgA; thus, reducing the material and IgA interaction. Alternatively, a lower temperature may prevent the degradation of IgA as microbes reproduce at a faster rate in 4°C as compared to -20°C.

A surprising finding indicated the concentration of IgA found in PE bags in the freezer and refrigerator is drastically higher than reported in earlier studies, which showed PE losing up to 60% of sIgA antibodies specific for *E.coli* during storage (Arnold, 1995). This may be attributed to Medela adding a HDPE inner lining to the PE bag. It is also possible that the performance of the PE bag has improved over 20 years with the addition of HDPE linings.

The 72-hour statistical analysis comparing the refrigerator and freezer samples indicated the null hypothesis demonstrated a strong correlation of temperature and subject interaction, with little, if any, influence from the containers. Figures 2 and 3 as well as the statistical analysis showed less IgA loss in the freezer. The p-values showed no specific type of material made a significant difference on the retention of IgA, disproving the hypothesis that loss of IgA was attributed to the retention of IgA by the material in the container.

# Conclusion

The Rigid PP containers were not the optimal storage containers in both temperatures, which disproved the hypothesis. HDPE (Axifeed) performed the best while Rigid PP (Snappies and Medela) fared the worst in -20°C. At 4°C, there was no clear evidence to confirm the best performing material.

The decrease of IgA from 72 hours to 169 hours may be attributed to IgA degradation as the 168 hour samples went through two thawing cycles as compared to one thawing cycle for the 72 hour samples. As a result, the impact of the IgA concentration may be from the extra freezing/thawing cycles; not from time.

The current recommendations of the lactation consultants and doctors are still practical after seeing these results. An important issue is to prove is whether temperature is a greater influence on IgA levels. Women do not need to worry about the container they use to achieve the best results in terms of protecting one biocomponent of human milk studied in this thesis. Further, mothers do not need to concern themselves with the selection of the optimal container in terms of preserving IgA levels. Rather, mothers are encouraged to focus more important factors, including the ease of the container for the infant and mother; the shelf space available in the refrigerator and freezer; and the compatibility of the container with the breast pump. Results indicate that three days is the maximum length of time for storing breast milk in refrigerators. If mothers do not expect to use their milk within three days, then they should place the milk in the freezer.

# Limiting Factors

The small number of samples used in the experiment is the limiting factor, discouraging the significance of the results. More samples are needed to prove whether the results from the experiment maintain the same trend for a larger population. However, results presented from this research is a good indication that storage container material may not be the primary factor causing decreased levels of IgA, but rather degradation may be attributed to temperature. With the data seen in this experiment, the next step is to study a larger collection of human milk to support or disprove the results found in this study.

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# Appendix A - Consent Form

Matthew Jenkins
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Thank you for agreeing to participate in this study which will take place from 3/10/08 to 6/20/08. This form outlines the purposes of the study and provides a description of your involvement and rights as a participant.

The purposes of this project are:

1.) To gain insight into the topic of the influence of different milk containers on the retention of an immune protein called Immunoglobulin A in breast milk stored several days in the refrigerator and freezer after pumping.

The methods used to collect information are explained below:

A. 20 mothers who are feeding their infants with breast milk 4 to 30 weeks after delivery will voluntarily give breast milk once or twice during the study.

B. A single pump from both breasts, either by breast pump or hand, will be placed in a sterile container provided by the researcher.

C. That is all that is required from you, the milk will be taken to a biology laboratory at RIT where it will be analyzed for the amount of IgA.

You are encouraged to ask any questions anytime about the nature and goals of this study and the methods that I am using. Your suggestions and concerns are very important to me so please contact me at any time at the email address listed at the top of the paper.

I will use the data from your milk sample along with other samples to write about the influence of storage container materials, including glass and different plastics, IgA. I will send a copy of my final thesis if you want to see it.

Your personal information will be kept confidential.

This copy is to be signed by you and for the researcher to keep:

I guarantee that the following conditions will be met:

1.) Your real name will not be used at any point of information collection after the donation of your milk. The written records, data, and final thesis will only use letters to represent you.

2.) Your participation in this research is voluntary; you have the right to withdraw at any point of the study, for any reason, and without any prejudice, and the information collected from you will be omitted from the study.

3.) You may ask for a copy of the published thesis for you to keep.

Do you grant permission to donate your breast milk and have it analyzed by me, Matthew Jenkins?

Yes_____ No _____

By signing this, you are agreeing to the terms written above:

Respondent _____

Date _____

Researcher_____

Date _____

# Appendix B - Background Information Form

Participant's Name:

_____

Age:

_____

How many weeks or months have passed since your delivery?

_____