Developing dried milk spot based micro-sampling methods to assess changes to the fat composition of human milk during handling and processing

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Human milk (mother's own milk and pasteurised donor human milk) is the food-of-choice of all infants, especially those born sick and/or prematurely. Premature infants might have to rely on nasogastric tube feeding of expressed and stored human milk rather than being fed directly on the breast before their suckling reflux matures. There has been increasing concern regarding whether the integrity of human milk is preserved after all the necessary storage, handling and processing steps involved in pasteurizing and delivering the milk to infants. Human milk fats are particularly of a concern as more than half of the energy of human milk is derived from milk fats. The endogenous lipases of human milk could breakdown the main fat type triglycerides (TG) and release free fatty acids (FFA), under various conditions. The main goal of the thesis was to develop micro-sampling based dried milk spot (DMS) methods to measure the fat composition of human milk.

Compared to the conventional liquid milk analysis, collecting human milk as DMS reduces the volume required for analysis, requires only ambient temperature storage and transportation, and simplifies the analytical procedures. I first adapted the established dried blood technique for profiling the total fatty acid composition of human milk, using 200 milk samples of mothers from three different countries. The strong correlation and tight variation between human milk samples analysed using the conventional method and the DMS method gave me the confidence to move forward with DMS technique.

The major challenges involved in the process was to develop a DMS method for measuring FFA concentration of human milk, due to the instability of milk fats and the difficulty in separating FFA from TG. I tested several strategies and was able to reduce the contamination from TG to a very low level of 2%, which however was unacceptable as this could result in a falsely inflate the reading of FFA. I then discovered that milk fats collected

as DMS are susceptible to lipolysis due to breast milk lipases. Various attempts were made to inactivate the lipases. The final working system involved collecting human milk on silica gel impregnated paper, followed by microwaving to denature lipases. Milk fats can then be eluted and analysed by gas chromatography using an acid modified column that specifically detects FFA even in the presence of other fats (e.g. TG). The level of FFA measured by the DMS and conventional thin layer chromatography method were highly correlated ($r=0.983$, $P<0.0001$). To test the applicability and sensitivity of my DMS method, I then analysed 256 human milk samples collected in neonatal nursery at Women's and Children's Hospital, from a cohort of 32 mothers who delivered mostly preterm infants.

In conclusion, this thesis is the first report of a DMS technique for reliably measuring the FFA concentration of human milk. This DMS technique can be adapted by human milk banks for monitoring the quality of milk fats throughout processing procedures, it also has the potential to be adapted for measurement of FFA in other biological fluids.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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CHANG GAO

Acknowledgement

Back when I first started my PhD, I was asked to list out three key characteristics of a successful researcher, in a bridging program. My answer at that time was: integrity, curiosity, and good communication skill. If I could just add one more, I would choose one word I learned during my Ph. D journey, perseverance. That word took a lot of tears and sweats to learn, with helps from many people that I wish to express my appreciation and gratitude to.

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Abbreviations

AA	Arachidonic acid
ALA	A-linolenic acid
BHT	Butylated hydroxytoluene
CE	Cholesterol ester
EBM	Expressed breast milk
EDTA	Ethylenediamineteraacetic acid
ELSD	Evaporative light scattering detector
EPA	Eicosapentaenoic acid
DBM	Dried blood spot
DG	Diglyceride
DHA	Docosahexaenoic acid
DMS	Dried milk spot
DMP	Dimethyoxypropane
DPA	Docosapentaenoic acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FFAP	Free fatty acid phase
FID	Flamed ionization detector
FMC	Flinders medical centre
GC	Gas chromatography
HMF	Human milk fortifier
HP	Holder Pasteurisation
HPP	High pressure processing
HPLC	High performance liquid chromatography

HREC	Human Research Ethic Committee
IQR	Interquartile range
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LCMS	Liquid chromatography mass spectrometry
MG	Monoglyceride
MTBE	Methyl tert-butyl ether
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
NEC	Necrotising enterocolitis
NICU	Neonatal intensive care unit
PA	Peak area
PEG	Polyethylene glycol
PL	Phospholipid
PRISMA	Preferred reporting items for systematic reviews and meta-analysis
PUFA	Polyunsaturated fatty acids
PINK	Pregnancy iodine and neurodevelopment in kids
TG	Triglyceride
TLC	Thin layer chromatography
SFA	Saturated fatty acids
SPE	Solid phase extraction
UV	Ultraviolet
WCH	Women's and Children's Hospital
WCHN	Women's and Children's Health Network

List of publications

Peer reviewed publications arising from this thesis:

Published

- Gao, C., Miller, J., McPhee, A.J., Rumbold, A., Gibson, R.A. (2020). Free fatty acid concentration in expressed breast milk used in neonatal intensive care units. *Breastfeeding medicine*, *15*:11.
- Gao, C., Liu, G., McPhee, A.J., Miller, J., Gibson, R.A. (2020). A simple system for measuring the level of free fatty acids in human milk collected as dried milk spot. *Prostaglandins, Leukotrienes and Essential Fatty acids*, *158*: 102035.
- Gao, C., Miller, J., Middleton, P.F., Huang, Y-C., McPhee, A.J., Gibson, R.A. (2019). Changes to breast milk fat composition during storage, handling and processing: a systematic review. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *146*: 1-10.
- Gao, C., Gibson, R.A., McPhee, A.J., Zhou, S.J., Collins, C.T., Makrides, M., Miller, J., Liu, G. (2018). Comparison of breast milk fatty acid composition from mothers of premature infants of three countries using novel dried milk spot technology. *Prostaglandins, Leukotrienes and Essential fatty acids*, *139*: 3-8.

Collaborative publication completed during thesis candidature:

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- Gao, C., Miller, J., Collins, C,T., Rumbold, A. R. (2020). Comparison of different protein concentrations of human milk fortifier for promoting growth and neurological development in preterm infants (Full review). *Cochrane Database of Systematic Reviews*, *11*: CD007090.

- Gao, C., Miller, J., Collins, C.T., Rumbold, A. R. (2008). Comparison of different protein concentrations of human milk fortifier for promoting growth and neurological development in preterm infants (Updated Protocol, published in 2019). *Cochrane Database of Systematic Reviews*, *2*: CD007090.
- Gao, C., Liu, G., Whitfield, K.C., Kroeun, H., Green, T.J., Gibson, R.A., Makrides, M., Zhou, S.J. (2018). Comparison of human milk fatty acid composition of women from Cambodia and Australia, *Journal of Human Lactation*, *34*: 585-591.

List of presentations

Poster presentations with published abstract

- Gao C, Liu G, McPhee A.J et al, Developing a micro-sampling system for analysing breast milk fat composition from dried milk spot, *Journal of Paediatrics and Child Health (2019)*, 55(S1):76.

Chapter 1 Introduction

I have conducted a comprehensive systematic review in the specific subject area which is included in the second chapter of the thesis. This chapter serves as a brief introduction to the overall concept, highlighting the gaps in the knowledge and the overall structure of the thesis.

There is no published work related to Chapter 1.

Human milk is often referred as ‘liquid gold’ by health professionals, care givers and researchers working in the field of neonatal nutrition. The major components of human milk include a range of macronutrients (e.g. fats, proteins, and carbohydrates) and micronutrients (e.g. vitamins and minerals) providing essential nutritional support for normal growth and development. Despite being an optimal source of essential nutrients, human milk also contains unique non-nutritive bioactive components that have anti-infective, anti-inflammatory, and immunomodulating roles that are beneficial for infants’ health [Andreas *et al.* 2015; Ballard & Morrow 2013; Nolan *et al.* 2019]. In addition, human milk contains important digestive enzymes that assist the digestion and absorption of nutrients, which are particularly valuable for infants born with compromised gastrointestinal functions [Ballard & Morrow 2013].

Human milk is the optimal food for all infants but is especially critical for infants born prematurely (<37 weeks’ gestation) who are at increased risk of poor outcomes due to their immaturity. Globally, more than 15 million infants are born preterm each year, and the incidence of preterm birth in Australia is 8.6% [Australian Government Department of Health 2019], with a global incidence estimated at between 5% and 18% [World Health Organisation 2018]. Many of these infants grow up with premature birth related complications (e.g. cerebral palsy, hearing, vision, and dental problems) and require ongoing medical support. In these preterm infants, human milk reduces infant mortality [Abram *et al.* 2014] and has a protective effect against a number of neonatal morbidities including necrotizing enterocolitis (NEC) [Corpeleijn *et al.* 2012; Lucas & Cole 1990; Meinzen-Derr *et al.* 2009; Miller *et al.* 2018; Quigley *et al.* 2019], sepsis [Corpeleijn *et al.* 2012; Furman *et al.* 2003; Petal *et al.* 2013] and retinopathy of prematurity [Bharwani *et al.* 2016; Okamoto *et al.* 2007].

Despite the known benefits of human milk for premature infants, these infants cannot be fed directly by breast due to the immaturity. This occurs as these infants have a range of

feeding difficulties associated with their immaturity, including the inability to suckle from the breast due to underdeveloped suck, swallow, and breathe coordination [Lau *et al.* 2007; Mizuno & Ueda 2003], which only matures between 32 and 34 weeks' gestation [Delaney & Arvedson 2008]. As a result, these preterm infants have to be fed with expressed breast milk (EBM), either mother's own milk or pasteurised donor human milk (PDHM) if mothers' own milk is not available, via nasogastric tube feeding, before they can be fed on breast safely and adequately.

Expressing and collecting milk subjects requires various storage and handling conditions before delivering it to the infants, and the process is further complicated if infants were to receive PDHM. This may include several rounds of storage (home, in the unit, during transportation and distribution), pasteurisation and repeated freezing and thawing, along with the addition of human milk fortifier (HMF), which is usually of bovine origin. These necessary steps for delivering EBM to infants may induce changes to the composition of milk, due to the possibilities of bacterial contaminations and the presence of enzymes that may breakdown the nutrients to smaller molecules during the process.

Human milk fat is especially of a concern as it represents more than 50% of total energy of human milk, and provides the essential fatty acids (e.g. linoleic acid and linolenic acid) as well as long chain polyunsaturated fatty acids (e.g. arachidonic acid and docosahexaenoic acid). It is critical to ensure that the fat content and its composition is maintained to safeguard the normal growth and development of the infant. Lipids of human milk are either esterified (fatty acids bond to another structural component for energy storage) or non-esterified (fatty acids that are not bound to a certain structure and remain circulating freely). The majority (>98%) of the lipids in breast milk exist as esterified lipid in the form of triglyceride (TG), with the remainder made up of other esterified lipids including phospholipids (PL) and cholesterol esters (CE) and non-esterified free fatty acids (FFA). These different lipids are

also collectively referred as ‘lipid classes’. Lipolysis of human milk TG can occur during storage and handlings due to the presence of endogenous lipases (bile-salt stimulated lipase and lipoprotein lipase) that are naturally present in breast milk [Olivecrona & Hernell 1976; Lindquist & Hernell 2010], which would yield FFA and mono- or diglycerides.

There are several ways to report the lipid content of human milk. Total fat is an expression of the total quantity of fat per volume of breast milk. Each lipid class in total fat can be quantified individually following certain separation techniques based on their polarities. The fatty acids attached to each of the structural lipids can be pooled together and expressed as total fatty acid composition. The fatty acid composition of milk is largely determined by maternal dietary intake [Innis 2014], whereas the changes to the proportion of lipid classes is a result of lipase activities and could potentially reflect how well the breast milk has been stored and handled [Bitman *et al.* 1983].

In order to gain a full picture of how lipids of human milk are affected by different storage and handling process, I have conducted a systematic review that is included in **Chapter 2** of the thesis. There have been several reviews in this field of research [Peila *et al.* 2016; Peters *et al.* 2016], which only focused on the effect of a single handling procedure and often only examined its effect on total fat content and/or its fatty acid composition, but not the lipid classes of breast milk, which are considered sensitive parameters reflecting critical changes to human milk fats. Therefore, my review encompassed all possible conditions that EBM may be exposed to such as handling EBM at home, in neonatal nursey as well is in a human milk bank, to understand the collective effects of all different procedures on the fat composition (including total fat, fatty acid composition and lipid classes) of human milk.

Measuring changes to the lipid profile of human milk requires accurate methods. Liquid milk is often used in most of the studies conducted in this field, but cold-chain storage and transportation is a burden for research cost and may introduce changes to the lipid of human

milk. The majority of the research also reported the use of a relatively large amount of human milk for measurement, ranging between 200μL and 10ml, depending on the techniques applied. However, large volumes of breast milk for research purpose are often unavailable, especially when conducting research on preterm populations or collecting colostrum, where the mothers often experience difficulties with establishing feed or have low supply.

Hence, being able to conduct research using smaller volumes of breast milk collected as dried spot would be beneficial for both researchers and participants in terms of collecting multiple samples without compromising infants' need. Therefore the emphasis of my thesis was to develop a dried milk spot (DMS) method for measuring the lipid of human milk, which is covered in **Chapter 3, 4 and 5**. Although the overarching aim was to develop a DMS method, slight differences in focus occurred in each chapter. In Chapter 3 I focus on adapted an established method for profiling the total fatty acid composition of human milk for use in a DMS system, the tested its applicability in a clinical trial. While in Chapters 4 and 5 I focus on developing a DMS method specifically for the quantifying the amount of FFA present in human milk. During the development process, I attempted many different methods, often without success, but detailed experimental planning and discussion have been recorded in Chapter 4. The final successful method is described in Chapter 5 and the clinical study conducted using this method is covered in **Chapter 6**.

To understand the magnitude of changes seen after storage and handling process, I first had to determine the baseline level of FFA in human milk (Chapter 6). There are very limited data reporting the concentration of FFA in fresh EBM, primarily due to the lack of suitable technology. Previous research shows that the fat composition of human milk is highly variable depending on time of collection, lactation stage, maternal diet [Koletzko 2016]. Therefore I planned to collect freshly expressed human milk samples from mothers in the

neonatal units to understand the effect of gestation weeks, lactation stage and short period storage on the FFA concentration of human milk, which will be presented in Chapter 6.

In the final chapter (**Chapter 7**), I discuss emerging areas for future research, including examination of the effects of addition of HMF on the human milk lipid profile and other potential usages of dried spot technique.

Table 1.1. Outline of the thesis and corresponding publications

Chapter	Study aim	Study Design	Conventional thesis or publication
2	To evaluate all evidence regarding the changes to breast milk fat composition after storage, pasteurisation, and other handling processes	Systematic review	Publication *Prostaglandins, Leukotrienes and Essential fatty acids* (2019) 146:1-10
3	To adapt and test the sensitivity and applicability of a dried milk spot method for profiling total fatty acid composition of breast milk from mothers with distinct habitual diets	Analytical method development and validation	Publication *Prostaglandins, Leukotrienes and Essential fatty acids* (2018) 139:3-8
4	To develop a robust dried milk spot method that accurately measures free fatty acids without being contaminated by triglycerides		Conventional thesis
5			Publication *Prostaglandins, Leukotrienes and Essential fatty acids* (2019)
6	To determine the variation of free fatty acid concentration in freshly expressed breast milk collected in neonatal nursery	Cross sectional study	Publication *Breastfeeding medicine* (2020)

7	To discuss the overall findings of the studies in this thesis and the implication for future research	General discussion	Not applicable

Chapter 2 Literature Review - Changes to breast milk fat composition during storage, handling, and processing

This review was extended to include the common practices of mothers returned to work but continue to feed the infants with EBM, practices that are implied in human milk bank to handle donor milk, and new innovative non-thermal pasteurisation techniques to replace the conventional holder pasteurisation, as all these procedures can potentially alter the fat composition of EBM that is fed to a wider population. Although such EBM might not be fed to the preterm population exclusively, the general principles are the same, which is to understand the different procedures involved in handling EBM and how it might affect the fat composition of EBM.

This chapter includes a manuscript in its published form:

Gao, C., Miller, J., Middleton, P.F., Huang, Y-C., McPhee, A.J., Gibson, R.A. (2019). Changes to breast milk fat composition during storage, handling and processing: a systematic review. *Prostaglandins, Leukotrienes and Essential Fatty Acids, 146*: 1-10.

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Principal Author

Name of Principal Author (Candidate)	Chang Gao		
Contribution to the Paper	CG was responsible for designing the study concept, drafting the original study protocol, developing search strategies, conducting the searches, screening title and abstracts, screening full texts, extracting data, assessing the quality of individual study, re-organising and presenting the data. CG drafted the original manuscript with input from RAG and JM, and edited and reviewed it based on comments received from other co-authors.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	01/07/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Robert A Gibson		
Contribution to the Paper	RAG was responsible for co-designing the study concept, re-organising and presenting the data. RAG helped CG with the original draft of the manuscript, and edited and reviewed the manuscript critically.		
Signature		Date	01/07/2020

Name of Co-Author	Jacqueline Miller		
Contribution to the Paper	JM involved in screening title and abstracts, screening full texts, and checking the accuracy of data extraction that was completed by CG, re-organising and presenting the data. JM helped CG with the original draft of the manuscript, and edited reviewed the manuscript critically.		
Signature		Date	

Name of Co-Author	Philippa F Middleton		
Contribution to the Paper	PFM was responsible for developing the original study protocol, providing constructive feedback for search strategies, screening titles and abstracts, double checking data extraction that was completed by CG, re-organising and presenting the final data. PFM reviewed and edited the original draft of the manuscript and provided constructive feedback for the final version.		
Signature		Date	6 / 7 / 20

Name of Co-Author	Yi-Chao Huang		
Contribution to the Paper	YCH involved in double checking the data extraction and quality assessment of studies completed by CG, and reviewed and edited the final draft of the manuscript.		
Signature		Date	01/07/2020

Name of Co-Author	Andrew J McPhee		
Contribution to the Paper	AJM involved in co-designing the study concept, edited and reviewed the original and final draft of the manuscript.		
Signature		Date	2/7/2020.

Please cut and paste additional co-author panels here as required.

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Changes to breast milk fatty acid composition during storage, handling and processing: A systematic review

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ABSTRACT

This review evaluated the effect of various storage and handling conditions on the fat composition of expressed breast milk (EBM). Three databases PubMed, Embase and Scopus were searched in April 2019 with words from the three key components: human milk, handling process (i.e. storage and/or pasteurization), and fatty acid composition. The comparisons were EBM subjected to handling processes versus fresh EBM or versus EBM subjected to another handling processes. Both intervention and observational studies were included, and the outcomes measured included total fat and lipid classes of the EBM. We included 42 studies (43 reports), 41 of which were assessed to be of good quality. Relative changes to the fat composition of EBM subjected to handling processes were calculated based on the data provided in the included studies, and the results were synthesized narratively. The total fat content and total fatty acid composition of EBM was not generally influenced by storage and handling process, with most changes less than 10%, which is likely a result of methodological variation. A reduction in EBM triglyceride concentration and concomitant increase in free fatty acid concentration were seen after exposing to various conditions, probably due to endogenous lipase.

1. Introduction

Breast milk is the gold standard for infant feeding due to the nutrients and other non-nutritive components essential for the growth and development of infants [1]. When not possible to feed directly from the breast, expressed breast milk, either from the mother, or from a donor, is often used and is preferable to formula [2,3]. The use of donor human milk is also becoming more widely used for premature infants. Donated expressed breast milk (EBM) from a milk bank undergoes storage processes, which may involve pasteurization and repeated freeze and thaw cycles, and this may affect the integrity of the EBM.

The impact of various storage and handling processes on EBM has been studied from many perspectives including the preservation of bacteriostatic properties [4–9]; the impact of storage on immunological factors [10], digestive enzymes [11], and the antioxidant capacity of breast milk [12–14]. However, the impact of storage and handling on fat composition of EBM has been less studied.

Fat in breast milk is the major source of energy for infants, contributing more than 50% of the total energy [15]. There are four main lipid classes in breast milk: triglycerides (TG), free fatty acids (FFA), cholesterol esters (CE) and phospholipids (PL). In freshly expressed breast milk, most fats (≥ 98%) are in the form of TG while FFA, CE and PL are only present in small amounts, which makes breast milk energy dense but low in osmotic load. There are lipases that naturally presented in breast milk that can convert TG into FFA, which may assist in the digestion of fats when consumed fresh by infants. However, the effect of lipase activity on the storage of breast milk fats is not clear and can be seen as an indication of milk fat degradation. This degradation in fat could potentially increase the osmolality of breast milk, which may be related to slowed gastric emptying and other feeding intolerance

Abbreviations: CE, cholesterol esters; EBM, expressed breast milk; FFA, free fatty acids; GC, gas chromatography; HP, Holder pasteurization; HPP, high pressure processing; PL, phospholipids; PRISMA, referred Reporting Items for Systematic Reviews and Meta-Analysis; TG, triglycerides; TLC, thin layer chromatography

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events commonly seen in preterm infants [16,17].

While two systematic reviews have been published on the effects of Holder Pasteurization (HP) [18] and short-term refrigeration storage [19] on fat composition, there has been no systematic review encompassing all EBM handling processes conducted to date. This review assesses the impact of handling processes of EBM on the breast milk fat composition, including storage (room temperature, refrigerator and frozen storage), pasteurization (HP and other available techniques), and freeze and thaw methods and repetition of freeze and thaw cycles.

2. Methods

This review has been conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [20] and the PRISMA checklist can be found in **Supplementary** Table 1. The protocol of this systematic review was registered on PROSPERO international prospective register of systematic reviews [21].

2.1. Search strategy

The electronic databases PubMed/Medline, Embase and Scopus were searched during April 2019 using the combination of Mesh/Emtree terms and free words in three main components: breast milk, handling process (i.e. storage and pasteurization), and fat composition (full search strategies are available in **Supplementary** Table 2). References of identified studies were also checked to capture all potential studies. Searches were restricted to humans only, and studies published in English but no date restriction was applied.

2.2. Inclusion and exclusion criteria

Both intervention and observational studies were included in this review. Reviews, case reports and commentaries were excluded but were checked for component studies. Studies that determined the impact of any one, or a combination of storage, pasteurization, and freeze and thaw methods and repeated freeze and thaw cycles were eligible for this review. Outcomes of interest were total fat content (and its total fatty acid composition) and the fat composition (lipid classes: the concentration and composition of TG and FFA). Studies needed to report a change from baseline (fresh EBM) and after being subjected to a handling process or comparison of two different handling process.

Two authors independently screened the studies against the inclusion and exclusion criteria using Covidence [22]. Any conflicts raised during the process were resolved in consultation with the primary author.

2.3. Data extraction and quality appraisal

Data were extracted into two structured tables: general study characteristics and detailed outcomes. Study characteristics included authors, publication year, milk collection method, intervention, methods and outcomes.

Study quality of was assessed by two reviewers independently using the quality appraisal tool retrieved from Academy of Nutrition and Dietetics [23] for primary research papers. Studies were rated positive, neutral or negative based on ten questions related to the validity and four questions related to the rationale and significance of the study.

2.4. Data synthesis

Due to the heterogeneity of both the interventions and outcomes reported, it was not possible to undertake a meta-analysis. Studies were grouped according to the outcome they reported and synthesised narratively. For comparison between studies, the authors calculated the relative percentage change to the fat composition from the baseline to the post-handling procedure, where relevant. Changes were considered minimal if they were less than the expected accuracy or precision (10%) of the techniques used to measure the variable [24].

3. Results

The study selection process is detailed in the PRISMA diagram (Fig. 1). A total of 42 unique studies (with 43 reports), including 40 observational studies, one non-randomized study [25] and two randomized trials [26,27] were included in this review. Characteristics, general information and the quality rating of these studies are available in Table 1. These included studies were conducted in 15 countries, and the sample size ranged between 1 and 90, with most studies having <30 samples. A total of 20 included studies were conducted between 2010 and 2019, and the rest were published prior to 2009 with the earliest records published in 1978 [25,28]. Most included studies were rated as positive quality and reliable, except one rated as neutral due to misreporting of the data [29] and another rated as negative as no clear description of methodology was given [30].

Storage conditions were classified as: room temperature ($\geq 15\,^{\circ}C$), fridge (≤ 0–$7\,^{\circ}C$), domestic freezer (<0 to $-20\,^{\circ}C$) and deep freezer (-70 to $-80\,^{\circ}C$). Changes to breast milk fat composition after exposing to different treatments are classified into two major categories: total fat content and the total fatty acid composition of the total fat, presented in Table 2; and fat composition (lipid classes) including concentration of TGs and FFAs, presented in Table 3.

3.1. Total fat content

A total of 21 studies (22 reports), reported total fat content and are detailed in Table 2. Eight measured fat by gravimetrical method following a modified Folch [31–34] or Rose-Gottlieb [30,35–37] method, four by creamatocrit [38–41], two titration [25,26], two by Gerber butyrometer [42,43] method, and another five a human milk analyzer [27,44–47] and one by lipid test kit [28]. We acknowledge that there are variations within and between these methods [24]. Therefore, studies reporting less than 10% statistically significant changes in total fat content are considered to be within the error of the methodology and will be referred as minimal changes in the following section.

Studies that investigated the impacts of different storage conditions on breast milk total fat content reported changes that were either not statistically significant [30,31,40] or minimal [39,42,45,47], with the exception of Silprasert et al. [39] who found a 18.7% reduction in total fat content of breast milk in samples that were frozen and thawed twice in a domestic freezer for 28 days [39]. Despite a significant reduction in total fat content of breast milk after sterilisation reported by Fidler et al. [35,36] and Legge et al. [28], changes to the total fat content of breast milk after exposing to various other pasteurization technologies (alone or followed by storage) or different freezing and thawing method are either not statistically significant [25–27,32–38,41] or minimal [34,43,44,46].

In conclusion, it appears that storage and pasteurization produce only small changes to breast milk total fat content, most less than 10%, which is potentially a result of methodological variations. The gravimetric method that used in most studies for measuring total fat content of breast milk is particularly likely to produce variations in the results, which may not reflect a true fat loss in the samples. Fat globules can adhere to the surface of containers during storage. Moreover, it is common for the fat of breast milk to separate and float to the surface after defrosting, and accurate measurements of the fat content will not occur unless sufficient homogenisation of the breast milk has been achieved before sampling.

3.1.1. Total fatty acid composition of total fat

Fifteen studies measured total fatty acid composition using gas chromatography (GC) [27,29,33,35–37,48–56] and are detailed in Table 2.

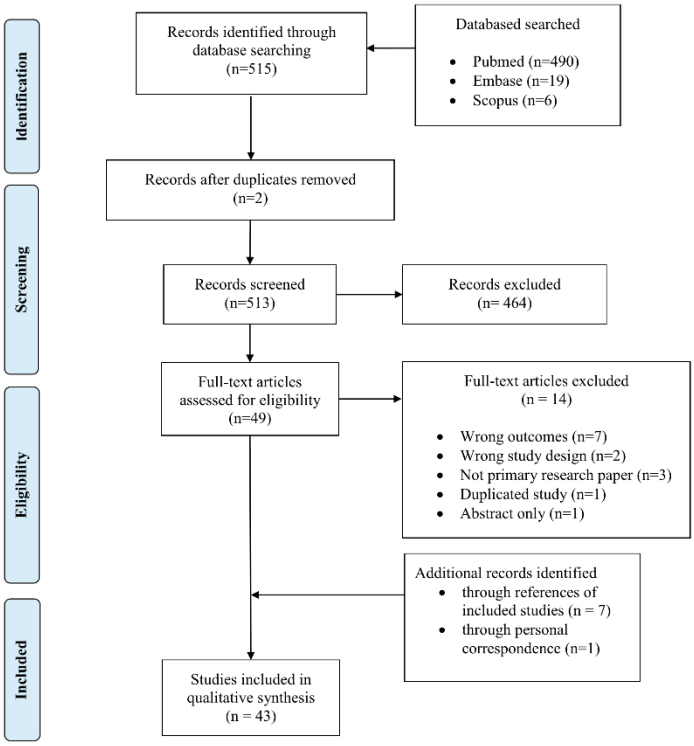

Fig. 1. PRISMA diagram describing the study selection process and reasons for exclusion.

Overall, there were no important changes to the total fatty acid composition of breast milk stored at different temperature regardless of the length of storage with or without HP [29,52,57]. A majority of the studies that determined the effect of HP on total fatty acid composition of breast milk reported no changes after the treatment [33,35,36,48,50,53,55,56], with the exception of a slight reduction in the percentage of C18:0 accompanied by a small increase in the percentage of C22:2 n-6 fatty acid reported by de Oliveira et al. [27]. The total fatty acid composition of breast milk remained stable after being pasteurized by high pressure processing (HPP) [50,56], sterilisation [35,36,53], flash heat treatment [56] or ultraviolet irradiation [49,56], and the concentration of C18:2 n-6 and C18:3 n-3 was not affected by microwave heating [58]. In addition, the combination of HP and freeze storage had minimal effects on the fatty acid composition of breast milk [29]. Nevertheless, Delgado et al. [48] reported a reduction in n-3 PUFA of breast milk after HPP, which correlated with time and pressure of the treatment. The method of freezing (quick vs. slow) did not alter the total fatty acid composition of breast milk [37].

To summarize, we conclude that regardless of any changes in the type of fats in milk (see below), the total fatty acid composition of breast milk is relatively stable and independent of storage and pasteurization conditions.

3.2. Fat composition (lipid classes)

This section reports on the changes in the lipid classes of fat in breast milk, specifically the concentration of TGs and the FFAs in the breast milk fat. The concentration of TG and FFA can vary significantly depending on different storage and handling processes due to the influence of lipases that are naturally present in human breast milk. In general, variations in FFA levels in breast milk are due to lipase activity on TG, but there has been no available data on the variations of FFA level in breast milk from different mothers. Although most of the included studies measured only the concentration of TG or FFA, reduction in TG concentration essentially means a rise in FFA concentration, and vice versa. Caution is required in interpreting FFA data since the concentration of FFA is very low in fresh EBM (≤ 1% of total fat); so that any increase in FFA concentration can result in large proportional changes in the relative percentage increase in FFA levels.

Studies which separated and/or quantified TGs and/or FFAs used several methods including: five by thin layer chromatography (TLC) equipped with scanner [34,59,60] or following a GC analysis [27,61], one by high performance liquid chromatography [62], or gas chromatography-mass spectrometry [63], or mass spectrometry [64] or solid phase extraction [57], or colorimetric [65], or titration [25], or automated TGs analyser [66], three by assay kit [31,32,41] and two by titration method [37,67].

3.2.1. Storage

Ten studies determined the effect of different storage conditions on the concentration of TGs [59,60,62,65,66] and FFAs [31,40,54,57,59,60,62,63,67] in breast milk, and the results are shown in Table 3.

Table 1
Characteristics of included studies.

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Ahrabi et al. [31] 2016 U.S	N/S	N/S	Electric pump	Fresh	N/S	Freeze storage	40	Gravimetric method (Folch extraction): total fat content Assay kit: concentration of FFA (g/L)	Positive
Andersson et al. [26] 2007 Sweden	Preterm	colostrum	Electric pump	Fresh	Stored at −20°C	HP	5	Titration: total fat content (g/ml)	Positive
Baack et al. [55] 2012 U.S	N/S	N/S	N/S	Frozen	Stored at −20°C	HP	1 pool (31 samples)	GC: FA composition (weight% of total FA)	Positive
Berkow et al. [59] 1984 U.S	N/S	Colostrum and mature	Mechanical pump	Fresh	Stored at +4°C	Freeze/thaw cycle Freeze storage	3	TLC scanner: TG and FFA concentration (% of total fat)	Positive
Bertino et al. [57] 2013 Italy	Preterm	N/S	Electric pump	Fresh	N/S	Fridge storage	3 pools (each with 4, 8 and 5 samples)	GC: FA composition (g/100 g lipid) SPE: FFA concentration (mg/L)	Positive
Bitman et al. [60] 1983 U.S	Preterm & term	N/S	Mechanical pump	Frozen (1st part) Fresh (2nd part)	N/S	Freeze storage + freeze/thaw Freeze storage	25 (1st part) 6 (2nd part)	TLC scanner: lipid classes (% of total fat)	Positive
Borgo et al. [29] 2015 Brazil	N/S	N/S	N/S	N/S	N/S	HP followed by freeze storage	1	GC: FA composition (% mg)	Neutral
Chan et al. [41] 2011 U.S	Preterm NS (milk bank sample)	N/S	N/S	N/S	Stored at −20°C	Thawing methods	17	Creamatocrit: total fat content (%) Assay kit: FFA concentration (nmol/μl)	Positive
Christen et al. [49] 2013 Australia	N/S	N/S	N/S	Frozen	Stored at −20°C	Pasteurisation (UV irradiation)	10	GC: FA composition (log_{10} peak area)	Positive
de Oliveira et al. [27] 2017 France	Preterm	N/S	N/S	Fresh (raw milk group) Frozen (pasteurised milk group)	Stored at +4°C Stored at −20°C	HP	64 (raw milk group) 12 pools (pasteurised milk group)	Human milk analyser: total fat content (g/L) TLC + GC: lipid classes and FFA (%lipolysis); FA composition (weight% of total FA);	Positive
Delgado et al. [48] 2014, Spain	N/S	Mature	N/S	Fresh	Stored in fridge (temperature not specified)	HP Pasteurisation (high pressure processing)	1 pool (6 samples)	GC: FA composition: (weight% of total FA)	Positive
Ezz El Din et al. [30] 2004 Egypt	N/S	Colostrum and mature	Manual expression OR Mechanical pump	Fresh	N/S	Fridge storage Freeze storage	61	Gravimetric method (Roese-Gottlieb method): total fat content (g/L)	Negative
Fidler et al. [35] 1998 Germany and **Fidler et al.** [36] 2001 Germany	Preterm and Term	Colostrum	Electric pump	Fresh	N/S	HP Pasteurisation (sterilisation)	12	Gravimetric method (modified Roese-Gottlieb method): total fat content (g/dl) GC: FA composition (weight% of total FA)	Positive
Friend et al. [37] 1983 U.S	N/S	Mature	Manual expression	Fresh	Stored in fridge	Freezing methods	30 (individual) 20 pools (each pool with 8 − 12 samples)	Gravimetric method (Roese-Gottlieb method): total fat content (g/100 ml) GC: FA composition (weight% of total FA) Titration: FFA (g/100 g Lipid)	Positive
Garcia-Lara et al. [45] 2013 Spain	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Frozen	Stored at −20°C	HP followed by freeze storage	34 (from 28 donors)	Human milk analyser: total fat content (g/dl)	Positive
Garcia-Lara et al. [46] 2012 Spain	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Fresh	Stored at < 5°C	Freeze storage	61 (from 59 donors)	Human milk analyser: total fat content (g/dl)	Positive
Goes et al. [38] 2002 Brazil	N/S	Mature	Manual expression OR Electric/ Mechanical pump	Frozen	N/S	HP followed by freeze storage	15	Creamatocrit method : total fat content (g/L)	Positive

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Table 1 (*continued*)

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Hamosh et al. [67] 1996 U.S	N/S	Mature	Breast milk pump (N/S)	Fresh	N/S	Room temperature storage	11	Titration: FFA concentration (% of total fat)	Positive
Handa et al. [32] 2014 U.S	N/S	N/S	Electric pump	Fresh	N/S	Thawing and warming methods	40	Gravimetric method (Folch extraction) total fat content Assay kit: FFA concentration (% of total fat)	Positive
Henderson et al. [33] 1998 U.S	N/S	Mature	N/S	Frozen	N/S	HP	3 pools	Gravimetric method (Folch extraction): total fat content (g/L) GC: total fat content; FA composition (weight% of total FA)	Positive
Janjindamai et al. [42] **2013 Thailand**	N/S	Mature	Electric pump	Fresh	N/S	Freeze storage	90	Gerber butyrometer: total fat content (g/100 ml)	Positive
Lavine et al. [54] 1987 U.S	N/S	Mature	Electric pump	N/S	N/S	Room temperature and freeze storage	8 (each participants assigned to half treatment)	GC: FFA concentration (mg/ml)	Positive
Legge et al. [28] 1978 New Zealand	N/S	Colostrum	N/S	N/S	N/S	Pasteurisation (sterilisation)	5 pools (each with 2–3 samples)	Lipid test kit: total fat content (g/L)	Positive
Lepri et al. [34] 1997 Italy	N/S	N/S	Manual expression	Fresh	N/S	HP followed by freeze storage	1 pool (16 samples)	Modified Folch extraction: total fat content (mg/mL) TLC scanner/TLC-GC: FFA concentration (weight% of total FA)	Positive
Lev et al. [47] 2014 U.S	Preterm	N/S	Electric pump	Fresh	Stored at −5°C	Freeze storage	60 samples (3 samples from each of 20 mothers)	Human milk analyser: total fat content (g/100 ml)	Positive
Molto-Puigmarti et al. [50] 2011 Spain	N/S	Mature	Mechanical pump	fresh	N/S	HP Pasteurisation (high pressure processing)	10	GC: FA composition (weight% of total fat)	Positive
Morera Pons et al. [62] 1998 Spain	term	Mature	Manual expression	N/S	N/S	Freeze storage Freeze/thaw cycle	30 samples (from 6 mothers)	HPLC: lipid classes	Positive
Ovesen et al. [58] 1996 Demark	N/S	Mature	N/S	Frozen (either pasteurised or unpasteurised)	N/S	Warming methods	1 pool (25 samples)	GC: concentration of LA and ALA (mg/100 g)	Positive
Pitino et al. [56] 2019 Canada	N/S	N/S	N/S	N/S	Stored at −20°C	Pasteurization (HP, HPP, UV irradiation, flash heat)	8	GC: FA composition (weight% of total FA)	Positive
Reynolds et al. [40] 1982 Australia	N/S	Colostrum	Mechanical pump	Fresh	N/S	Freeze storage	20	Creamatocrit: total fat content (%) Copper soap method: FFA concentration (mequiv./L)	Positive
Romeu-Nadal et al. [53] 2008 Spain	N/S	Mature	Mechanical pump	N/S	N/S	HP Pasteurization (sterilization)	10	GC: FA composition (weight% of total FA)	Positive
Romeu-Nadal et al. [52] 2008 Spain	Term	Mature	Mechanical pump	N/S	N/S	Fridge and freeze storage	2 pools from 10 samples	GC: FA composition (weight% of total FA)	Positive
Silprasert et al. [39] 1986 Thailand	N/S	Colostrum	Mechanical pump	N/S	N/S	Room temperature, fridge and freeze storage	72 pools	Creamatocrit: total fat content (%)	Positive
Slutzah et al. [51] 2010 U.S	Term and preterm	N/S	Electric pump	Fresh	N/S	Fridge storage	36	Direct transmethylation of FFA– GC: FFA concentration (g/L)	Positive
Spitzer et al. [64] 2013 Germany	N/S	Mature	Electric/ Mechanical pump	Fresh	N/S	Fridge storage	7 pools from 33 samples	MS: short- to medium-chain FFA composition (μg/kg)	Positive
Tacken et al. [65] 2009 Netherland	N/S	Mature	Electric/ Mechanical pump	Fresh	–	Fridge and freeze storage Warming method	30	Colorimetric test: TG concentration (mmol/L)	Positive

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Table 1 (*continued*)

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Thatrimontrichai et al. [59] 2012 Thailand	Term and preterm	N/S	Electric pump	N/S	Stored at −20°C	Thawing methods	90 samples	Gerber butyrometer: total fat content (g/100 ml)	Positive
Van Zoeren-Grobben et al. [63] 1996 Netherland	N/S	Colostrum and mature	Mechanical pump	Fresh	N/S	Fridge storage	10	GCMS: free LA (μM)	Positive
Vieira et al. [44] 2011 Brazil	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Fresh	N/S	HP Thawing methods	57 samples	Human milk analyser: total fat content (mg%)	Positive
Wardell et al. [61] 1981 U.K	N/S	Colostrum	N/S	N/S	N/S	HP Freeze/thaw cycle	8 samples	TLC-GC: TG composition (weight% of total FA)	Positive
Williamson et al. [25] 1978 U.K	Preterm	Mature	N/S	N/S	N/S	HP Pasteurisation (sterilisation)	7 pools (each pool made of several samples)	Titration: total fat content (g/L) and FFA concentration (%)	Positive
Yuen et al. [66] 2012 China	Term	Colostrum and mature	Manual expression	Fresh	N/S	Fridge storage	25 colostrum samples 11 mature milk samples	Chemistry analyser: TG content (mmol/L)	Positive

Abbreviations: FA: fatty acid; FFA: free fatty acid; TG: triglyceride; TLC: thin layer chromatography; LA: linoleic acid; ALA: α-linolenic acid; HPLC: high performance liquid chromatography; GC: gas chromatography; GCMS: gas chromatography mass spectrometry; SPE: solid phase extraction; MS: mass spectrometry; NS: non-specified; UV: ultraviolet.
The temperature of storage conditions are classified as following: room temperature (≥15°C), fridge (≥0–7°C), domestic (< 0 to −20°C) and deep freezer (−70 to 80°C).
* Quality of these studies were rated using the quality appraisal tool retrieved from Academy of Nutrition and Dietetics for primary research papers.

3.2.1.1. Room temperature. Two studies concur that storing breast milk at room temperature leads to an increase up to 686% in the concentration of FFAs as the result of lipase activity [54,67], and the rates of lipolysis varies slightly across temperatures [67].

3.2.1.2. Refrigeration. Both Tacken et al. [65] and Yuen et al. [66] stored breast milk at 4 °C, for 2 and 3 days, respectively, and detected no changes in the concentration of TGs at either time [64,65]. In contrast, another five studies reported a significant increase in overall [51,54,57] or individual [63,64] FFA of breast milk stored at refrigerator for up to 4 days. Combined, these data may be explained by the fact that small changes in TG levels can result in large changes in FFA level as each TG molecule yield three FFAs.

3.2.1.3. Freezing. The concentration of TG in breast milk stored in a domestic freezer remained stable as reported by Yuen et al. [66] and Tacken et al. [65] for 3 and 28 days, respectively. A reduction in TG concentration was seen in two other studies [59,60] as a result of prolonged storage between 2 and 5 months in domestic freezer [60]. Similarly, one study reported that the FFA concentration after storage of breast milk in a domestic freezer for up to 4 weeks did not result in any changes [40]. However, continuing storage at this condition may lead to increase in the FFA concentration as found by several studies [31,54,59,60,62]. Three studies found the TG concentration of breast milk stored in a deep freezer remained unchanged even after prolonged storage [59,60,62].

3.2.2. Pasteurization

Pasteurization is designed to inactivate pathogens and viruses but can also denature enzymes such as lipases. The effect of HP on the concentration of TGs [61] and FFAs [25,34] was determined by three studies. Lepri et al. [34] reported an 83% increase in the concentration of FFA due to HP, while there was 21% reduction in FFA concentration reported in Williamson et al. [25]. Wardell and colleagues [61] analyzed the fatty acid composition of TG and found only C18:3 n-3 was reduced by 22% [60]. When combined with post pasteurization freeze storage, the TG concentration was found unchanged [62]. In contrast, two studies reported that FFAs concentration in Holder pasteurized and freeze stored breast milk increased significantly through the process [27,34].

3.2.3. Freeze and thaw

Breast milk is often stored frozen in the home and in milk banks. The potential changes in milk lipids as a result of freezing and thawing is therefore of interest.

The concentration of TGs in breast milk samples warmed by microwave remained unchanged [65]. However, the FFA concentration in breast milk increased significantly to different extents (between 35% and 253%) after thawing and warming with either water or waterless method [32]. Chan and colleagues [41] determined changes to the FFA concentration of breast milk that was thawed using different methods. Breast milk that was thawed in a refrigerator at 4°C was used as the reference value, and a reduction was seen in breast milk thawed in a water bath and microwave, but not those thawed at room temperature [41].

Three authors studied [59,61,62] repeated freezing and thawing of breast milk and its impact on the concentration of TGs and/or FFAs. FFAs and other hydrolysis products of TGs were found in breast milk subjected to a single freeze and thaw cycle with domestic freezer storage in Morera Pons et al. [62]. Breast milk that was frozen and thawed three times and then stored in a domestic freezer showed a reduction in overall TG concentration in one study [59], and a reduction selected fatty acid in TG fraction in another study [61]. Significant reduction appeared after the second freeze and thaw cycle, but it was only seen in the relative percentage of C18:3 n-3, and not other fatty acids in TG fraction [61]; and there was a concomitant increase in the FFA

Table 2
Effect of various interventions on the total fat content of breast milk.

Component	Intervention	Effect
Total fat	Room temperature storage	**Reduction** (4.28–7.18%) [39]
	Fridge storage	
	• 24 h	**No statistically significant change** [30]
	• Up to 28 days	**Reduction** (4.48%) [39]
	Domestic freezer storage	
	• Up to 90 days	**Reduction** (up to 18.7%) [39,42,45]
	• Up to 9 months	**No statistically significant change** [30,31,40]
	Deep freezer storage	**Reduction** (9.7%) [47]
	Holder pasteurisation	**Reduction** (3.5–5.5%) [44,46]
		No statistically significant change [25–27,33–36]
	Sterilisation	**Reduction** (13–28%) [28,35–36]
		No statistically significant change [25]
	Holder pasteurisation + domestic freezer storage (up to 6 months)	**Reduction** (5.8–6.2%) [34,46]
		No statistically significant change [38]
	Quick/slow freeze	**No statistically significant change** [37]
	Thawing & Warming	
	• Water bath thawing	**Reduction** (8.5–18%) [41,44,59]
		No statistically significant change [32]
	• Room temperature	**No statistically significant change** [41]
	• Fridge thawing	**Reduction** (7.4%) [43]
	• Microwave	**Reduction** (8.5–31%) [41,44]
Total fatty acid composition	Refrigerator storage	**No statistically significant change** [52,57]
	Domestic freezer storage	**No statistically significant change** [52]
	Deep freezer storage	**No statistically significant change** [52]
	Holder pasteurisation	**Increase in C22:2 n-6 and reduction in C18:0** [27]
		No statistically significant change [33–36,48,50,53,55,56]
	Holder pasteurisation + freeze storage	**No statistically significant change** [29]
	High pressure processing	**Reduction in n-3 PUFA** [48]
		No statistically significant change [50,56]
	Sterilisation	**Reduction in C18:2 n-6 and C20:4 n-6** [35,36]
		No statistically significant change [53]
	Ultraviolet irradiation	**No statistically significant change** [49,56]
	Flash heat treatment	**No statistically significant change** [56]
	Quick/slow freeze	**No statistically significant change** [37]

Table 3
Effect of various interventions on the fat composition (lipid classes) of breast milk.

Component	Intervention	Effect
Triglycerides concentration	Fridge storage	**No statistically significant change** [65,66]
	Domestic freezer storage	
	• Up to 28 days	**No statistically significant change** [65,66]
	• Up to 5 months	**Reduction** (4.5–8.7%) [59,60,62]
	Deep freezer storage	**No statistically significant change** [59,60,62]
	Holder pasteurisation	**Reduction in C18:3 n-3** (22%) [61]
	Pasteurisation + domestic freezer storage for 4 months	**No statistically significant change** [62]
	Warming (microwave)	**No statistically significant change** [65]
	Freeze/thaw cycles + domestic freezer storage	
	• 1 time	**Reduction** [62]
	• 3 times	**Reduction** (13%) [59]
		Reduction in 18:3 n-3 (61%) [61]
	Freeze/thaw cycles + deep freezer storage (1 & 3 times)	**No statistically significant change** [59,62]
Free fatty acids concentration	Room temperature storage	**Increase** (up to 686%) [54,67]
	Fridge storage	**Increase** (up to 502%) [51,54,57,63,64]
	Domestic freezer storage	
	• Up to 4 weeks	**No statistically significant change** [40]
	• Up to 9 months	**Increase** (up to 1002%) [54,59–62]
	Deep freezer storage	**No statistically significant change** [54,59,60,62]
	Holder pasteurisation	**Increase** (83%) [34]
		Reduction (21%) [25]
	Sterilisation	**Reduction** (21%) [25]
	Pasteurisation + domestic freezer storage	**Increase** [27,34]
		No statistically significant change [62]
	Thawing	
	• Water bath thawing	**Reduction** (29%) [41]
	• Room temperature	**No statistically significant change** [41]
	• Microwave	**Reduction (39%)** [41]
	Thawing + warming	**Increase (35–235%)** [32]
	Freeze/thaw cycles + domestic freezer storage (1 & 3 times)	**Increase (353%)** [59,62]
	Freeze/thaw cycles + deep freezer storage (1 & 3 times)	**No statistically significant change** [59,62]

concentration [59,62]. The same authors also found no changes to neither TG nor FFA concentration in breast milk that was freeze-thawed three times but stored at deep freezer [59,62].

In conclusion, inconsistent results are seen in studies reporting the changes to the lipid classes of breast milk subjected to various storage conditions. The concentration of TGs and/or FFAs are affected by both storage temperature and duration. Storing breast milk at lower temperatures suppressed the release of FFAs, however, the concentration increases slowly with time. However, prolonged storage in a deep freezer, but not domestic freezer, seem to preserve the lipid classes of breast milk for at least up to 5 months. It is likely that lipases in breast milk are completely destroyed during pasteurization and therefore no changes to the concentrations of TG or FFAs were seen afterwards during storage. However, during the phase of pasteurization where the temperature is rising, there may be lipolysis of triglyceride. This assumption is in agreement with the findings discovered by Wardell et al. who found C18:3 n-3 in TG fraction decreased significantly while temperature reached 62.5 °C during HP, and no further reduction was seen from this point beyond till the HP process completed [61]. Moreover, there seems to be differences in concentration of FFAs/TGs after various thawing or warming process, which may be due to differing in rate of temperature raise throughout the process.

4. Discussion and conclusion

This systematic attempted to cover all possible procedures involved in EBM storage and handling, including storage under different conditions (room temperature, refrigerator, domestic and deep freezer), various pasteurization methods (HP, HPP, sterilisation and UV irradiation), various freeze and thaw methods (quick and slow freeze, microwave, warm water and refrigerator thawing) and repeated freeze and thaw cycles. The total fat content of breast milk appears to decrease after prolonged storage, pasteurization and thawing. However, we suspect this might be due to poor homogenisation of breast milk samples or other methodological variations as the reduction seen was mostly less than 10%. Total fatty acid composition of breast milk seems likely not be affected by most procedures. Subtle changes to the relative percentage of some fatty acids were only seen after various pasteurization treatments. The TG and FFA concentration of breast milk are sensitive to different conditions and changes are seen after prolonged storage at different temperatures (4.5–8.7% reduction in overall TG concentration) and pasteurization (22% reduction for selected TG fraction). Since FFA are only present in fresh breast milk in trace amounts, small actual changes can result in much larger relative changes in FFA (up to 1000% increase after storage, 83% increase after HP and 35–235% increase after thawing and warming, respectively).

The findings of the current systematic review are in agreement with past reports. Authors from a systematic review of safe management of EBM also reported that short-term refrigeration storage of EBM does not alter the fat content and the total fatty acid composition of such EBM [19]. In another systematic review investigating the impact of HP on various nutritional and immunological component of EBM, the authors concluded that the total fat content and total fatty acid composition is unlikely to be affected, but the FFA concentration increased significantly after HP [18].

This review combined published literature relating to all storage conditions and treatments seen in milk banks, making this the most comprehensive of its kind. However, we were not able to perform meta-analysis due to the high level of heterogeneity of the data, which was a result of different sampling methods, various technologies used to measure the components, different components of fat composition reported with various expressions of results. The fat content of breast milk is highly dynamic, varies between individuals, among the day, before, during and after feeding, and may potentially be different due to collection methods [1,68]. In addition, breast milk samples varied (fresh vs. frozen) at study baseline, which may have already received different treatments beforehand. More than half of the included studies were published dated prior to 2009, and sixteen of which were published before 2000, the technologies used in these studies may be out-dated.

4.1. Implication for practice

The reduction of total fat content seen in some of the studies were likely due to fat adheres to the surface of containers, although methodological errors cannot be ruled out. Therefore, we could expect this to happen clinically as EBM is transferred from container to syringe for delivery to the infant, or transferred between different containers for pasteurization, storage and distribution in milk banks. if we assume that EBM has an energy density of 700 kcal/L [69,70] and fat concentration of 40 g/L, but there is a 20% loss of fat during handling processes, then a 2 kg preterm infant, fed at 150 ml/kg could lose 10% of energy intake. Though the addition of human milk fortifier to EBM may compensate some of the energy loss (mainly derived from proteins), this loss of fat and energy supply from EBM may be clinically significant to preterm infants.

The lipases that are naturally presented in breast milk to assist the digestion and absorption of the fats may be destroyed by HP or other thermal treatments. However, these lipases continue to function in the early part of the process when the temperature is gradually raised. This conversion of milk fat from complex structured molecule TG to the digested form FFA may be beneficial to the infants, and aid fat absorption. However, a study conducted by Andersson et al. [26] revealed a lower coefficient of fat absorption in preterm infant fed with pasteurized milk than those fed with raw unpasteurized milk. In contrast, de Oliveira et al. [27] found pasteurization does not affect the gastric digestion of preterm infants. At this stage, there is not enough evidence to conclude the relationship between the FFA concentration of breast milk and the digestion and absorption in either preterm or term infants, therefore the best practice would be allowing minimal changes to the composition as comparing to the fresh milk.

4.2. Implications for research

Inconsistent results were seen in the studies included in this review are partially due to the techniques used in these studies. The technique for measuring TG are more reliable than the methods available for FFA. Given that the bulk of milk fat is in the form of TG, even a minute (0.1%) conversion of TG to FFA could result in large increase in reported FFA level, whereas a change in TG level could not be detected. TLC was used in most included studies that separated and quantified FFA, which is considered the optimal parameter to measure fat degradation, as it is sensitive to different conditions. However, the use of TLC for separating FFA is very likely to be contaminated by the dominant TG trace (due to tailing of TG on the TLC plate) and this could result in large errors. Development of new technologies for accurately separating and measuring FFA in breast milk fats is required.

While fatty acid metabolites are beyond the scope of this study, new emerging evidence shows that pasteurization increases the concentration of fatty acid metabolites of EBM, but has no effect on fatty acid composition of EBM [56]. Changes to these fatty acids metabolites were also seen under different storage conditions [71]. The effects of various handling processes on the fatty acid metabolites of EBM and its clinical impact on infants receiving these EBM needs to be further studied.

We also recommend that the collection and storage of breast milk, methods for analysis and items and units for reporting breast milk fat composition should be standardised in order to allow direct comparisons between studies and ease of combining data for systematic reviews.

CRediT authorship contribution statement

Chang Gao: Conceptualization, Writing - original draft,

Visualization, Data curation, Writing - review & editing, Validation. **Jacqueline Miller:** Writing - original draft, Visualization, Data curation, Writing - review & editing, Validation. **Philippa F. Middleton:** Writing - original draft, Visualization, Data curation, Writing - review & editing, Validation. **Yi-Chao Huang:** Data curation, Writing - review & editing. **Andrew J. McPhee:** Conceptualization, Writing - review & editing. **Robert A. Gibson:** Conceptualization, Writing - original draft, Writing - review & editing, Validation.

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Supplementary materials

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Chapter 3 Adapt and utilize dried milk spot for profiling fatty acid composition of breast milk

In the previous chapter, it was found that current evidence regarding changes to the fat composition of human milk after handling and processing are largely based on old chemical techniques that are prone to detection error, and thus the results derived from these studies were highly variable and difficult to interpret. Therefore, this chapter and the next two chapters (Chapter 4 and 5) focus on the development of modern DMS method for profiling human milk fats, with different measurements. This chapter describes the work related to adapting a current available dried blood spot method in total fatty acid profiling for human milk analysis, whereas the next two chapters focus on measuring the FFA concentration of human milk.

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Contribution to the Paper	CG co-designed the study protocol, conducted the experiment, performed data analysis and interpretation. CG drafted the original manuscript and was also responsible for reviewing and editing of the manuscript.		
Overall percentage (%)	60%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
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Comparison of breast milk fatty acid composition from mothers of premature infants of three countries using novel dried milk spot technology

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ABSTRACT

Long chain polyunsaturated fatty acid (LCPUFA) intake during infancy has been associated with many health benefits, and the LCPUFA intake of breastfed infants is largely dependent on the composition of breast milk. The conventional method for breast milk fatty acid profiling is complicated by the need for cold-chain transportation and storage, and the newly developed dried milk spot (DMS) technology overcomes these difficulties. This study aimed to determine the accuracy, sensitivity and applicability of the DMS method developed based on the PUFAcoat™ technology. Two hundred breast milk samples were analyzed using the conventional method and compared with the DMS method. In order to evaluate the usefulness of DMS for large scale international studies, we analyzed another 786 breast milk samples collected from mothers of preterm infants who participated in a large clinical trial conducted in Australia, New Zealand and Singapore. Fatty acids were measured using capillary gas chromatography and results were reported as weight percentage of total fatty acids. Strong correlations and tight variation were observed in total saturated, monounsaturated, n-6 and n-3 PUFAs between the conventional and DMS methods. The DMS method proved to be sensitive in differentiating the breast milk fatty acid profiles of women consuming different habitual diets as evidenced by the differences between the breast milk fatty acid composition between Australian and Singaporean population. This study demonstrates that the DMS and the conventional method provide interchangeable results, and the DMS method is a particularly useful tool for large-scale studies.

1. Introduction

The level of LCPUFA intake during infancy and their potential health benefits continue to be studied because of evidence suggesting benefits with respect to visual acuity [1], immune responses [2] and reduced risks of allergic diseases [3,4], that may persist through childhood. Dietary LCPUFA intake of breastfed infants is mainly dependent on breast milk level, which in turn is largely defined by maternal intake. Lactating mothers with differing dietary intakes have distinct breast milk fatty acid profiles, especially the n-6 and n-3 PUFAs [5]. Accurate measurements of breast milk fatty acid composition helps to better define the fatty acid intake of the breastfed infant and to better understand the roles of fatty acids during infancy. The conventional methods for breast milk fatty acid profiling requires liquid breast milk samples and strict temperature control during storage and transportation, which increases the cost of clinical trials and so limits the ability of assessing breast milk fatty acid profile in large multi-centre studies. An easy and inexpensive tool for collecting and analysing the breast milk fatty acid composition would clearly be of value.

One such technique has been developed based on the current dried blood technology [6,7], namely the dried milk spot (DMS) technology, which has been commercialized [8,9]. The method only requires a single drop of breast milk, simplifies the analytical process, and does not require cold-chain transportation and storage. Our group have

Abbreviations: DMS, dried milk spot; LCPUFA, long chain polyunsaturated fatty acid; LA, linoleic acid; EPA, Eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; FAME, fatty acid methyl ester

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developed a dried blood spot collection system (PUFAcoat™) [10] used silica gel impregnated paper in combination with an antioxidant and a chelating agent, which can potentially be used as a DMS method. The stability of fatty acid composition of breast milk spotted on the paper was shown to be stable at room temperature for at least four weeks [10].

Therefore, the aims of the study were to compare the DMS method based on the on the PUFAcoat™ technology against the conventional method for breast milk fatty acid profiling; and to apply this DMS method in a large clinical trial to determine its sensitivity in differentiating the breast milk fatty acid profiles of women with different habitual diets.

2. Materials and methods

2.1. Method comparison

2.1.1. Subjects and sampling

Stored breast milk samples from women who participated in the PINK (Pregnancy Iodine and Neurodevelopment in Kids) study were used for method comparison. The PINK study assessed the relationship between the maternal iodine status during pregnancy and the neurodevelopmental outcomes of infants [11–13]. Briefly, women ($n = 784$) were recruited from Women's and Children's hospital and Flinders Medical Centre, South Australia, between 2011 and 2012. Breast milk samples were collected either at the hospital during a clinic visit or at the participants' home at 3 month postpartum for iodine concentration measurement. Between 5 and 9 am of the collection day, foremilk (10–15 ml) was collected before the first feed using a provided container (70 ml sterilised pot, Southern Cross Scientific Ltd, South Australia), and samples were kept in the home or hospital freezer and transferred to the laboratory and stored at freezer (−80 °C) within an average of 9 days of collection. Two hundred women who consented their surplus samples to be used for further analysis were randomly selected for this current study.

The study was approved by Women's and Children's Hospital Human Research Ethic Committee (HREC) in South Australia. Each breast milk sample was analyzed by both a conventional liquid/liquid extraction method [14] and the DMS method for total fatty acid profiling. The DMS collection cards were prepared in bulk 1–2 months in advance, were packed in foil bags with desiccants and stored at room temperature (~25 °C) prior to use. Milk samples were thawed and then aliquoted onto the collection cards at the same time of initiating the conventional method. The chemical analysis of both methods were conducted at the same time.

2.1.2. DMS system preparation

The procedures of DMS spot card preparation were based on the PUFAcoat™ method described previously [10]. Briefly, Whatman ion exchange papers (Grade SG81, Whatman, Buckingham, UK) were cut to desired size and then coated with antioxidant butylated hydroxytoluene (Sigma–Aldrich, St Louis, MO) at concentration of 2 mg/ml and chelating agent ethylenediaminetetraacetic acid (Chem–supply, Gillman, Australia) at concentration of 5 mg/ml in 70% ethanol; the papers were then dried and packed in pre-manufactured cards.

To prepare DMS samples in the laboratory, 20 μL of thawed breast milk was spotted onto the collection paper, and left to dry at room temperature for at least three hours.

2.2. Application of DMS method

2.2.1. Subject and sampling (international study)

Breast milk samples collected by women, whose very preterm infants (< 29 weeks gestational age at birth) participated in the N3RO (N-3 fatty acids for improvement in Respiratory Outcomes) randomised controlled trial [15,16], were utilised to test the utility of the DMS method in this study. The mother–infant pairs were recruited from 13 centers around Australia, New Zealand and Singapore, where the differences in diet between countries are expected to produce differences in breast milk fatty acid profiles. Details of the recruitment were reported elsewhere [15,16]. Among 1098 mothers involved in the trial, 736 of them provided breast milk for fatty acid analysis and were included in this current study. The study protocol was approved by the relevant HREC (Australia: South Adelaide Clinical HREC; Women's and Children's Health Network HREC; The Royal Women's Hospital HREC; The Southern Health HREC; Mercy Health HREC; Women and Newborn Health HREC; Hunter New England HREC. New Zealand: Northern B Health and Disability HREC. Singapore: SingHealth Centralised Institutional Review Board E).

At the time of discharge from the neonatal unit (36 week of postmenstrual age), each mother spotted breast milk onto a DMS card, which was left to dry at room temperature for at least three hours prior to transporting to a central laboratory. All samples were stored in foil bags containing a desiccant and transported through the Post (Australia) and FedEx (Singapore and New Zealand). Samples were then stored at −20 °C immediately after arrival and were analyzed within 4 weeks of arrival.

3. Methods

3.1. Fatty acids analysis

3.1.1. Conventional method

Breast milk samples were thawed at room temperature in a cardboard box to avoid direct sunlight, and then shaken vigorously before sampling. The breast milk fats were extracted according to a modified Folch method [14]. In brief, 200 μL of breast milk was extracted with chloroform and methanol (2:1 v/v) and the chloroform layer containing all lipids was evaporated under a nitrogen stream. Dried lipids were then redissolved in 300 μL 9:1 chloroform: methanol and 60 μL of this solvent containing the lipid extract was used for fatty acid determination. Fatty acids were transmethylated to fatty acid methyl ester (FAME) using methanol with 1% sulphuric acid as a catalyst for three hours at 70 °C [10,17]. FAME were then extracted into heptane for analysis by gas chromatography.

3.1.2. DMS method

Milk spots were excised using cleaned scissors and tweezers, and were then placed in transmethylation fluid and treated as described above. The resulting FAME were extracted and analyzed by gas chromatography.

3.1.3. Gas chromatography

The analysis of FAME were carried out using a Hewlett–Packard 6890 (Hewlett–Packard, CA, USA) gas chromatograph, which was equipped with a vaporization injector, a flame ionization detector, and a BPX70 capillary column 50 m × 0.32 mm, film thickness 0.25 μm (SGE Pty Ltd., Victoria, Australia). The temperature of the injector and detector were set as 250 °C and 300 °C, respectively. The carrier gas was Helium with a flow rate of 1 ml/min in the column and the inlet split ratio was set at 20:1. The fatty acids in samples were identified based on the retention times and peak area values of the commercial FAME standards (Nu Chek Prep Inc., Elysian, USA).

3.2. Statistical analysis

Fatty acid data are expressed as weight percentage of total fatty acids and presented as median with interquartile range (IQR) since the majority of the data were not normally distributed. The correlation between DMS and conventional method of individual fatty acids results was assessed using Spearman's order–rank correlation. Bland–Altman test, which is defined as the ratio of fatty acid content from two

Table 1
Spearman correlation between conventional and Dried Milk Spot (DMS) method for fatty acid analysis (weight% of total fatty acids) (N = 200).

Fatty acids	Method				Correlation coefficient (r)
	Conventional		DMS		
Total saturates	43.81	(39.85, 47.04)	43.77	(39.96, 46.90)	0.9981
10:0	0.91	(0.78, 1.05)	1.08	(0.89, 1.27)	0.7212
12:0	4.94	(3.93, 5.98)	4.90	(3.84, 5.80)	0.9777
14:0	6.18	(5.24, 7.49)	6.09	(5.14, 7.33)	0.9933
15:0	0.36	(0.29, 0.44)	0.36	(0.29, 0.44)	0.9809
16:0	23.11	(21.22, 24.76)	23.08	(21.22, 24.73)	0.9894
18:0	7.11	(5.91, 8.21)	7.21	(6.06, 8.27)	0.9971
Total trans	1.21	(0.89, 1.52)	1.23	(0.92, 1.51)	0.9589
T16:1	0.09	(0.06, 0.12)	0.09	(0.06, 0.13)	0.5651
T18:1 n-9	0.22	(0.17, 0.26)	0.23	(0.18, 0.27)	0.9348
T18:1 n-7	0.75	(0.51, 0.94)	0.74	(0.52, 0.95)	0.9701
T 18:2	0.14	(0.11, 0.19)	0.14	(0.11, 0.18)	0.8026
Total monounsaturates	40.25	(38.32, 42.69)	40.16	(38.32, 42.59)	0.9958
16:1 n-7	2.31	(1.92, 2.82)	2.30	(1.89, 2.77)	0.9967
18:1 n-9	35.64	(33.38, 38.03)	35.38	(33.51, 37.99)	0.9945
18:1 n-7	1.80	(1.60, 2.01)	1.79	(1.59, 1.99)	0.9387
Total n-9	36.07	(33.86, 38.46)	35.89	(34.00, 38.50)	0.9947
Total n-7	4.20	(3.54, 4.79)	4.14	(3.51, 4.76)	0.9885
Total n-6 PUFA	11.76	(9.87, 14.71)	11.51	(9.70, 14.48)	0.9983
18:2 n-6	10.66	(8.75, 13.55)	10.47	(8.64, 13.34)	0.9978
20:4 n-6	0.38	(0.32, 0.44)	0.38	(0.31, 0.44)	0.9648
Total n-3 PUFA	1.51	(1.26, 1.86)	1.48	(1.23, 1.77)	0.9892
18:3 n-3	0.95	(0.79, 1.23)	0.93	(0.76, 1.18)	0.9842
20:5 n-3	0.08	(0.06, 0.11)	0.08	(0.05, 0.10)	0.8467
22:5 n-3	0.17	(0.14, 0.20)	0.16	(0.14, 0.20)	0.9568
22:6 n-3	0.23	(0.17, 0.34)	0.23	(0.17, 0.33)	0.9953

PUFA: polyunsaturated fatty acids.
Data are presented as median (interquartile range).

methods (DMS vs. conventional) against the average results of the two methods, was carried out to assess the agreement between the DMS and the conventional method. The difference in breast milk fatty acid composition of women from any two of the three countries were assessed using Mann–Whitney U test. Statistical analyzes were performed using the SPSS (Version 23.0, IBM Corp, in Armonk, NY) and GraphPad Prism (Version 6 v008, for Windows, GraphPad software, La Jolla California USA) and the statistical significant level was set to be 0.05.

4. Results

4.1. Method comparison

The Spearman correlation between the results obtained from DMS and conventional method are presented in Table 1. The correlation for most of reported fatty acids were high ($R > 0.95$) with exception of fatty acids C10:0, trans C16:1, trans 18:2 and C20:5 n-3 (eicosapentaenoic acid EPA); the correlations for these four fatty acids were 0.7212, 0.5651, 0.8026 and 0.8467, respectively. The association between breast milk total saturates, monounsaturates, n-6 and n-3 PUFA analyzed using DMS and conventional method are shown in Fig. 1a–d and demonstrated a strong correlation between the two methods (R = 0.9981, 0.9958, 0.9983 and 0.9892, respectively; $p < 0.0001$ for all).

The results of the Bland–Altman test are presented in Fig. 2a–d, the mean ratio (95% limits of agreement) of two methods (DMS vs. Folch) was 1.00 (0.99–1.02) for total saturates, 1.00 (0.98–1.01) for total monounsaturated, 0.99 (0.96–1.01) for total n-6 PUFA and 0.97 (0.91–1.04) for total n-3 PUFA.

4.2. Application of DMS method

The breast milk fatty acid profile of Australian, New Zealand and Singaporean mothers using DMS are presented in Table 2 along with the differences in the profile between any two of the three populations.

Differences in fatty acid profile between Australian and Singaporean women were seen in the majority of the fatty acids except total saturates (p = 0.142), total n-3 PUFA (p = 0.719), EPA (p = 0.052) and n-3 docosapentaenoic acid (p = 0.061). Breast milk of New Zealand women were significantly higher than Australian women in percentage of total saturates (p = 0.001), total monounsaturates, total trans and total n-3 PUFA ($p < 0.0001$), but lower in percentage of total n-6, linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) ($p < 0.0001$). When comparing Australian women with New Zealand women, the breast milk of Australian women was lower in the percentage of total saturates (p = 0.010), total trans ($p < 0.0001$), but higher in the percentage total n-6 PUFA including LA (p = 0.002) and AA (p = 0.003). The breast milk n-3 PUFA levels were similar between the two populations, with the exception of the percentage of DHA being higher in the breast milk from Australian women compared to that of New Zealand women (p = 0.008).

5. Discussion

The Bland-Altman test showed narrow limits of agreements between the results obtained from the conventional and the DMS method across a range of concentrations for different types of fatty acids. Variations of most samples are within 5% for total saturates, monounsaturates and n-6 PUFA. Slightly greater variation was observed in total n-3 PUFA as these fatty acids only presented in human breast milk in very small quantities, which is more likely to result in greater variations. However, the variation for the vast majority of n-3 PUFAs are within 10%, and only 6 out of 200 samples were greater than 10% but less than 20% variation, which was an acceptable range [18]. Strong correlations between the conventional and DMS methods for n-6 and n-3 PUFA makes the method particularly attractive because of the known clinical importance of these compounds for infants.

The DMS method described in this study allows the measurements of breast milk fatty acid composition more accurately with even less sample required than in the studies published previously [8,9]. The

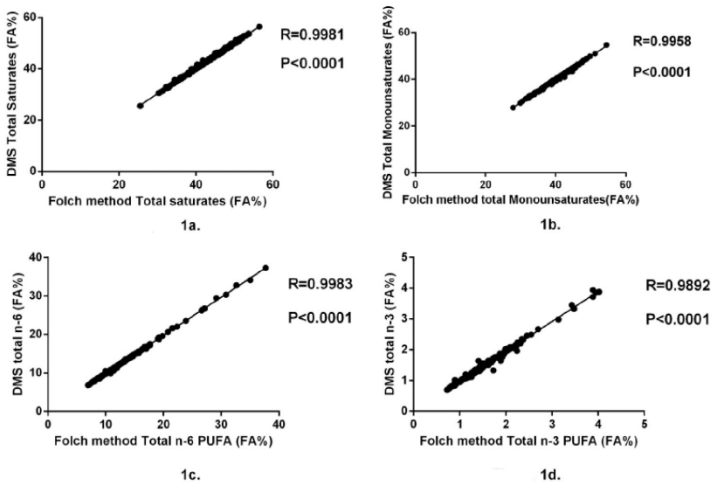

Fig. 1. Correlation of breast milk total saturates (1a.), total monounsaturates (1b.), total n-6 (1c.) and n-3 (1d.) fatty acid analysis between conventional and dried milk spot methods (N = 200). FA%: weight percentage of total fatty acids; PUFA: polyunsaturated fatty acid; DMS: dried milk spot.

current DMS has advantages compared to the methodologies previously published, as it consists of three protecting components that have been proven to be necessary to stabilise LCPUFA [10]. The filter paper used in this study was silica gel impregnated, which allows stronger binding with the lipids than the non-silica coated Ahlstrom (Grade 226) filter paper used in previous studies [8,9], and the addition of chelating agent that used in our PUFAcoat™ system offers more protection from oxidation. The DMS collection card can be prepare in advance of sample collection, as it has been demonstrated to be stable at room temperature for at least two months [19].

A major aim of this study was to utilise DMS method to measure the breast milk fatty acid profiles of populations with known differences in their habitual diets, and hence their breast milk fatty acid profiles. The differences found in breast milk fatty acid profile between Australian and Singaporean women are in line with the existing evidence that the South East Asian populations that eat more fishes that are rich in omega-3 fatty acids [5]. This demonstrated the sensitivity of DMS method in differentiating breast milk fatty acid profiles of women with different dietary habits. Interestingly, the breast milk profiles of Australian and New Zealand women, which are thought to have similar dietary habits, differed in total saturates, total trans fats, n-6 PUFA and DHA content. This might be due to a larger percentage of indigenous participants included in the New Zealand cohort (21% New Zealand vs. 5% in Australia), the diet of whom may be different from Caucasian women in New Zealand or Australia. However, there are no existing data on breast milk profile of New Zealand population and no dietary

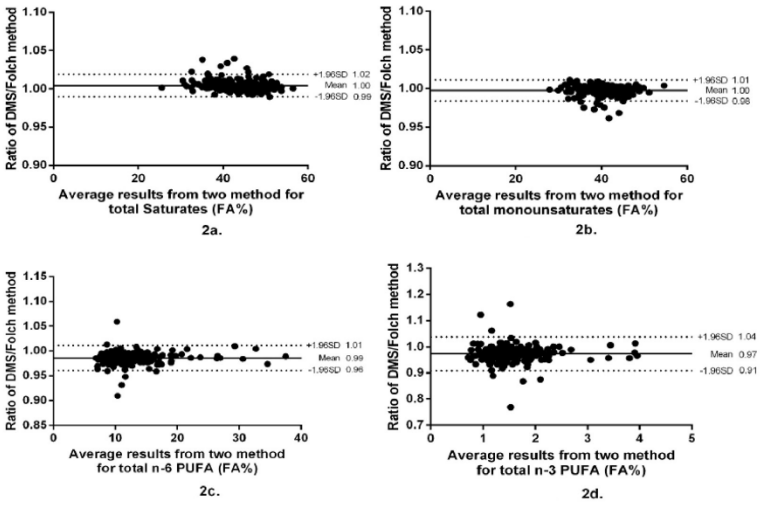

Fig. 2. Bland-Altman plots comparing results of conventional Folch method and Dried Milk spot (DMS) method for total saturated (2a.), total monounsaturated (2b.), total n-6 (2c.) and n-3 (2d.) fatty acid analysis showing the ratio vs mean results from two methods. The solid line represents the mean ratio and the dotted line represents 95% limits of agreement (N = 200). FA%: weight percentage of total fatty acids; PUFA: polyunsaturated fatty acid; DMS: dried milk spot.

Table 2
Breast milk fatty acid composition of women from Australia, New Zealand and Singapore using the Dried Milk Spot (DMS) method (weight% of total fatty acid) (N = 736).

	Australian women (n = 582)		New Zealand women (n = 70)		Singaporean women (n = 84)		p		
							Australia vs. New Zealand	Australia vs. Singapore	New Zealand vs. Singapore
Total saturates	40.99	(37.39–44.43)	42.38	(39.34–45.95)	40.51	(37.33–42.76)	0.010	0.142	0.001
Total monounsaturates	43.35	(40.33–46.01)	43.03	(40.50–45.36)	40.77	(39.16–42.84)	0.348	<0.0001	<0.0001
Total trans	1.15	(0.86–1.49)	1.45	(1.13–1.79)	0.48	(0.27–0.65)	<0.0001	<0.0001	<0.0001
Total n-6 PUFA	12.22	(10.53–14.21)	11.24	(9.59–13.10)	15.82	(14.53–17.98)	0.001	<0.0001	<0.0001
18:2 n-6	10.84	(9.28–12.94)	10.04	(8.42–11.75)	14.48	(13.26–16.76)	0.002	<0.0001	<0.0001
20:4 n-6	0.42	(0.36–0.48)	0.38	(0.32–0.45)	0.46	(0.38–0.51)	0.003	0.005	<0.0001
Total n-3 PUFA	1.75	(1.45–2.10)	1.74	(1.49–1.95)	1.74	(1.43–2.35)	0.439	0.719	0.489
18:3 n-3	1.14	(0.89–1.44)	1.18	(0.93–1.37)	0.84	(0.67–1.21)	0.923	<0.0001	<0.0001
20:5 n-3	0.10	(0.07–0.13)	0.09	(0.07–0.12)	0.08	(0.06–0.12)	0.266	0.052	0.455
22:5 n-3	0.19	(0.16–0.22)	0.19	(0.17–0.23)	0.18	(0.14–0.23)	0.563	0.061	0.080
22:6 n-3	0.26	(0.20–0.37)	0.23	(0.19–0.27)	0.52	(0.39–0.66)	0.008	<0.0001	<0.0001

PUFA: polyunsaturated fatty acids.
Data are presented as median (interquartile range).

information was collected in the N3RO trial to further confirm whether the results are actually a reflection of difference in dietary intake. In addition, the sample size of New Zealand cohort is relatively small, which may not be a true representation of the New Zealand population.

5.1. Strengths and limitations

We established the strong positive linear relationship between the DMS and conventional methods in a large sample (N = 200) over the wide range of fatty acids seen in clinical practices. Thus the DMS method provides robust results compared with conventional liquid/liquid extraction method for breast milk fat and fatty acid profiling. The use of DMS method for breast milk fatty acid analysis also overcomes the difficulties implicit in conventional methods, including the need for temperature regulations during storage and transportation. Thus samples can be transported by standard postal service from the test site to the laboratory for analysis. Because DMS can be transmethylated for GC directly without extraction, the method also simplifies the laboratory procedure, reduces costs and allows increased throughput, making it an attractive option for use in population screening and large clinical trials. However, the current method is limited in the ability to measure the absolute amount of fat in any breast milk sample unless a known volume of breast milk is spotted on the DMS paper. The variations in the absolute fatty acid concentration between DSM and the conventional methods remain to be investigated in further studies. The applicability of method was demonstrated using samples from a multicentre international clinical trials where dietary differences were expected to be both marked (Singapore vs. Australia) and subtle (Australia vs. New Zealand). Though the stability of breast milk fatty acid was not tested in this study, we have previously demonstrated that the fatty acid composition in biological fluids are stable at room temperature for weeks [10].

6. Conclusion

Highly correlated results between DMS and a conventional method for determination of breast milk fatty acids were demonstrated for both accuracy and precision. Using the DMS method, differences were found in fatty acid composition of breast milk between Australian and Singaporean that are consistent with known differences in diet, which support the sensitivity and applicability of the method. This simple, precise and high throughput DMS method is suited for large population studies.

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Author contributions

GL, RAG, AJM, SJZ, CTC, MM and JM designed the concept of the study. CG conducted the experiments and analyzed the data. All authors contributed to the interpretation of the results, drafting of the manuscript and approved the final version to be submitted for publication.

Conflict of interest

RAG and MM have received honoraria for scientific advisory board contributions to Fonterra. All honoraria are paid to the Healthy Mothers, Babies and Children, South Australian Health and Medical Research Institute to support continuing education activities for students and postgraduates. RAG and GL are the inventor of PUFAcoat™ system, the patent of which is owned by the University of Adelaide. The licence for PUFAcoat™ has been granted to Xerion Ltd Melbourne. Other authors declare no conflict of interest.

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Chapter 4 Steps in the journey to develop a micro-sampling system for measuring concentrations of free fatty acid in breast milk collected as dried spot

The DMS method described in the previous chapter fulfilled the needs for an accurate method for profiling the total fatty acid composition of human milk, that was also sensitive and capable of detecting the subtle differences in fatty acid composition in human milk collected from women with different habitual diets. However, it does not fulfill the needs for FFA separation and analysis. Therefore, developing a DMS method that specifically measures the FFA concentration of human milk is the focus for the current and the next chapter of this thesis.

I made many attempts to develop a DMS method for measuring the FFA concentration of human milk, which resulted in varying levels of success. Eventually these attempts led to a successful method that has been tested in a clinical study, and this will be discussed in later chapters. In this chapter, I describe all partially successful experiments, in the hope that these may provide useful evidence for future research in this area.

There is no publication related to this Chapter.

Introduction

- Methods for lipids class separation

The commonly used methods for lipid class separation are the conventional TLC method, and emerging techniques such as the high performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD) and liquid chromatography tandem mass spectrometry (LC-MS/MS). Each of these methods has its own strengths and weakness regarding breast milk lipids separation, which are outlined below.

1) TLC method

The TLC-GC method generally includes three main steps: lipid extraction, separation, and transesterification and analysis. First the lipids are extracted from breast milk (or other biological fluids) using organic solvents. The extracted lipids are then spotted on a silica gel plate and placed in a tank filled with elution solvents, and the separation of lipids is driven by the polarity of individual lipid classes. In the case of breast milk, the four major lipids are separated in the following descending order on a plate: cholesterol ester (CE), triglycerides (TG), free fatty acid (FFA), and phospholipid (PL). After the separation, the TLC plate is taken out of the tank to dry and sprayed on with a thin layer of fluorescent, so that the separated lipids can be visualised under ultraviolet light, which allows the FFA band to be scraped off for further methylation into FAME and analysed and quantified on gas chromatography (GC). This biggest concern with this method is the likelihood of contamination during the chromatographic separation process due to the dominant presence of TG (>98% of fats in breast milk). A tailing of TG band is often seen that leads to unclear separation between TG and FFA, resulting in some TG being scraped off along with FFA causing contamination and inaccurate results. The TLC-GC method is also laborious and hence has low throughput, but it is common choice as it is relatively cheap to run and only requires GC for analysis. It is also important to note that the TLC-GC method not only

provides the quantitative information (e.g. the absolute amount of FFA) but also qualitative information (e.g. the fatty acid composition of FFA).

2) HPLC-ELSD method

There have also been several new methods proposed for separating and identifying lipid classes, and the HPLC-ELSD method for this purpose was reported by Olsson and colleagues (Olsson *et al.* 2012). Extracting lipids from breast milk or other biological fluids is still necessary, and Olsson *et al.* used pure lipids dissolved in chloroform and methanol for injection into the equipment. The system which Olsson and colleagues descried was a normal-phase binary gradient HPLC system using a cyanopropyl column, with various solvents including hexane, pentane, cyclohexane, octane and heptane tested as solvent A, while solvent B was a constant combination of toluene, methanol, acetic acid and triethylamine. The separation of all tested lipid classes was clear with sharp and symmetrical peaks identified on the chromatograph, in the following ascending order according to retention time: TG, diglyceride (DG), monoglyceride (MG), and PL. Although the authors did not use FFA in the experiment, it would be expected to be seen between MG and PL based on polarity. Compared with the TLC-GC method, this HPLC-ELSD is less laborious but requires a higher grade of organic solvents and machines for analysis that increases laboratory costs. In addition, this method would only offer the quantitative data but not the qualitative data.

3) LC-MS/MS

There have been several reports of the use of LC-MSMS in analysing FFA from various sample sources (dried blood spot by Hewawasam *et al.* 2018, marine algae by Scholotterbeck *et al.* 2018, grape skin and seeds by Perez-Navarro *et al.* 2019). Regardless of the status (dried or liquid) of the sample, the first step also involves lipid extraction, which is then injected into the LC-MS/MS for quantification of individual FFA. This method is also less laborious than the TLC method as it encompasses the separation and analysis step which is completed within

the same run of the chromatograph. However, this method requires advanced machines that might not be easily accessed for all laboratories.

In summary, current available methods for separating lipid classes are either prone to detection errors but are less costly (TLC method) or provide accurate result at a much higher cost (HPLC-ELSD and LC-MS/MS). The optimal goal would be to use the least expensive resources to obtain accurate measurements. It is important to keep in mind that though this might not be a concern for other biological fluids, due to the dominance of TG in breast milk fats, even a 1% TG contamination of the sample could mean an over 50% increase in the detected FFA concentration.

- The proposed method for FFA separation

The challenges for new techniques for the analysis of breast milk fats are that they should aim to minimise the volume required for analysis, particularly as EBM is often in short supply for preterm infants. In many of the large clinical trials conducted in recent years, the benefits of using dried spot system and thus eliminating the needs of cold chain transportation and storage has greatly reduced research expenses. Therefore, for the purpose of this thesis, any proposed method was required to have the following characteristics: a filter paper based collection system that 1) requires only micro-amounts of sample; 2) provides accurate and robust measurement of FFA without contamination from TG; 3) is stable at room temperature for long periods. Methods recorded in this chapter were considered unsuccessful if they were not able to meet all the criteria described above.

- Common characteristics of experiments described in this chapter

Two types of collection paper were tested in these experiments: the 903 paper (Whatman 903 specimen collection paper, commonly used for inborn errors of metabolism in newborns) and silica gel impregnated ion exchange paper (Whatman SG81 ion exchange paper), which will be referred as 903 and SG paper in this chapter, respectively. All collection

papers used in these experiments were sized into paper discs with diameter of approximately 1 cm.

Two gas chromatographs with flame ionisation detector (GC-FID) were used in these experiments described blow.

- For fatty acid methyl esters (FAME) analysis: GC (Agilent Technology 7890B, Santa Clara, CA, USA) was equipped with a BPX70 column 50m × 0.32 mm, film thickness of 0.25μm (Trajan Scientific Australia Pty Ltd), the temperature ramp was set to be 140℃ to 220℃, and helium flow was at 2ml/min and a split ratio of 20:1.
- For underivatized FFA analysis: GC (Hewlett-Packard 6890, Palo Alto, CA, USA) was equipped with a BP column cut to 10m × 0.25mm, film thickness of 0.25 μm (Trajan Scientific Australia Pty Ltd) with a temperature ramp form 180℃ to 240℃, helium flow of 3ml/minute and split ratio of 20:1.

The FAME and FFA were identified based on the retention time of a mixed commercial reference standards purchased from NuCheck (Nu-Check Prep, Inc., MN, USA).

As GC-FID was used to analyse FAME derived from fatty acids of different structural lipids, I have used lipids that contain only a single fatty acid (different fatty acids for each lipid class) in order to identify the source of origins of fatty acids. As this was an 18-month progress of method development, during which time I ran out of certain standards and have re-purchased or purchased new ones, the lipid mixture used in the experiments described in this chapter was dependent on the availability at the time and was not entirely the same across different experiments. However, the preparation of lipid mixture always featured TG. Certain lipids like CE sometimes were not present in the lipid mixture because it was not a major

source of contamination, but was in the lipid mixture for a more complete representation of breast milk lipids.

Table 4.1. Detail of the lipid standard (commercial products purchased from chemical companies) used in different experiments.

Lipid standard mixture	Lipid Type	Fatty acid	Amount (concentration and proportion in the mixture)	Source of purchase	Section
A	TG	13:0	9.6 mg/ml, 40%	NuChek Prep Inc	5.1 & 5.2
	FFA	14:0	9.4 mg/ml, 15%	NuChek Prep Inc	
	PL	20:0	11.8 mg/ml, 15%	Sigma-Aldrich	
	CE	17:0	10.1 mg/ml, 15%	NuChek Prep Inc	
	DG	18:1	9.75 mg/ml, 15%	Sigma-Aldrich	
B	TG	13:0	9.87 mg/ml, 40%	NuChek Prep Inc	5.3-part 1
	FFA	14:0	9.4 mg/ml, 20%	NuChek Prep Inc	
	PL	17:0	11.2 mg/ml, 20%	NuChek Prep Inc	
	DG	18:1	12.5 mg/ml, 20%	Sigma-Aldrich	
C	TG	13:0	10.3 mg/ml, 25%	NuChek Prep Inc	5.3 part 2, 5.5, 5.6
		22:6 n-3	10 mg/ml, 25%	NuChek Prep Inc	
	FFA	12:0	11.5 mg/ml, 10%	NuChek Prep Inc	
		20:0	9.4 mg/ml, 10%	NuChek Prep Inc	
	DG	18:1	12.6 mg/ml, 10%	Sigma-Aldrich	
	PL	22:1	10.3 mg/ml, 10%	Sigma-Aldrich	
	CE	17:0	10.1 mg/ml, 10%	NuChek Prep Inc	
D	TG	13:0	4.7 mg/ml, 60%	NuChek Prep Inc	5.4 Part 1
	FFA	14:0	5.4 mg/ml, 25%	NuChek Prep Inc	
	CE	17:0	5.4 mg/ml, 10%	NuChek Prep Inc	
	PL	20:0	4.7 mg/ml, 5%	NuChek Prep Inc	
E	TG	13:0	10.3 mg/ml, 20%	NuChek Prep Inc	5.4-part 2
		22:6 n-3	10 mg/ml, 20%	NuChek Prep Inc	
	FFA	12:0	11.5 mg/ml, 10%	NuChek Prep Inc	
		20:0	9.4 mg/ml, 10%	NuChek Prep Inc	
	DG	18:1	12.5 mg/ml, 20%	Sigma-Aldrich	
	PL	17:0	11.2 mg/ml, 20%	NuChek Prep Inc	

Table 4.2. Summary of all experiments at a glance

Section and Name						
	5.1 Selective methylation of free fatty acids (acid-catalysed methylation)	5.2 Selective methylation of free fatty acids (acid-catalysed methylation)	5.3 Selective transmethylation of esterified lipids (base-catalysed transmethylation)	5.4 Selective extraction of free fatty acids from breast milk collected as dried spot	5.5 Separating free fatty acids with solid phase extraction column material	5.6 Separating free fatty acids by binding free fatty acids with pre-treated collection paper
Rationale	Free fatty acids, because they are not esterified, should theoretically be methylated first due to the simplicity of its structure. By controlling the time, temperature, or acid concentration, it was hoped that free fatty acids would be methylated while other fats remained in intact.	Another acid methylation where the reaction is ceased by addition of pyridine. Same principle as method 5.1.	The base-catalysed transmethylation selectively convert all esterified lipids to fatty acid methyl ester but unable to esterify free fatty acids, therefore free fatty acids can be separated once transmethylation is completed.	Lipids present in breast milk all have different polarities which affect the extraction efficiency of lipids bound to collection paper. Certain solvent may be able to extract only lipids with high or low polarity and leave the others on the paper.	A conventional method for lipid classes separation based on their polarity, similar idea to method 5.4.	Free fatty acids should be able to bind with paper that is treated with anion or basic condition due to its chemical property, and therefore can be separated from other neutral lipids by simple solvent extraction.
Collection paper	903	903	903	903 and SG	Nil	903 and SG

Tested condition (s)	Temperature: fridge, room temperature, domestic and deep freezer Reaction time: 10, 20, 30, 45, 60, 120 mins Acid concentration (0.01%, 0.1%. 1% sulphuric acid in methanol)	Different ratio of all testing solutions (water, pyridine, hydrochloric acid, methanol, dimethoxypropane)	Basic condition with various concentration/ ratio/volume: sodium or potassium methoxide Reaction time: 10, 20, 30 and 60 minutes Number of washes to remove Fatty acid methyl ester from free fatty acids	Various solvents or solvent combination: acetone acetonitrile heptane hexane methanol ethanol propanol cyclohexane	Using solid phase extraction column material for pilot run, with pure standards in liquid status	Various pH condition and combination of salts Stability of breast milk collected on alkaline coated paper
GC analysis	Fatty acid methyl ester	Fatty acid methyl ester	Fatty acid methyl ester	Fatty acid methyl ester	Fatty acid methyl ester	Underivatized free fatty acids

Findings	Highly temperature and time sensitive Contamination of triglycerides	By-products rather than Fatty acid methyl ester formed Incomplete transmethylation of free fatty acids Contamination of triglycerides	Incomplete transmethylation of triglycerides Contamination of triglycerides	No single solute could separate free fatty acids from triglycerides	Difficulties with extraction – not practical Contamination of triglycerides	Success for separation of free fatty acids without triglycerides contamination Hydrolysis of triglycerides due to pH coating of the filter paper Lipases in breast milk remain active even when collected as dried spot

4.1 Selective methylation of free fatty acids in an acid-catalysed condition

4.1.1 Rationale

The lipids present in human breast milk have different structures and levels of complexity. FFA, being unesterified, have the simplest structure and it has been hypothesised to be methylated first relative to other structural lipids present in breast milk [Christie *et al.* 1993; Gros *et al.* 1964; Hartman *et al.* 1965]. The standard method used in our lab for fatty acid methylation and transmethylation takes place in 1% sulphuric acid in methanol solution at 70℃ for three hours [Liu *et al.* 2014]. During the three-hour time frame, fatty acids from all lipids, regardless of their original structure, will be transmethylated or methylated into FAME, which can then be identified by the GC based on retention time of reference standards. If the time, or temperature, or the concentration of acid in the reacting solvent can be controlled so that only FFA are methylated and other lipids remain intact, then only FFA derived FAME will be identified and recognised by GC. My working hypothesis was that by extending the range of conditions tested by others it might be possible to selectively methylate FFA without transmethylating the more complex lipids.

4.1.2 Materials and methods

In order to be able to test the effectiveness of various procedures in separating FFA from other lipid fractions, I have used lipid standard mixture A (containing FFA, TG, CE and PL) listed in Table 4.1.

Twenty microliters of the lipid standard mixture were collected on discs of 903 paper, air-dried and the paper discs were then transferred to 6ml scintillation vials, then 2ml transmethylation solvent (with 1%, 0.1% and 0.01% sulphuric acid in methanol) was added and left at room temperature (at fridge for 1%) for 10, 20, 30, 45 or 60 (120) minutes.

At the time of analysis, the paper discs were removed from the vial and transferred to another set of 6ml scintillation vials containing 2ml of transmethylation solvent and heated to 70℃ for 3 hours to determine lipids that remain bound to the paper. Half of the original transmethylation solvent was transferred to a separate vial, the FAME were extracted with heptane to determine the lipids that had been transmethylated during the time of experimental period. The other half of the transmethylation solvent remained in the vial was heated to 70℃ for 3 hours and resulting FAME were analysed to determine the lipids that had been leached off the paper discs, but yet to be transmethylated. All FAME were analysed by GC-FID and a flow diagram of the experimental process is shown below (**Figure 4.1.1**). All samples were tested in triplicates.

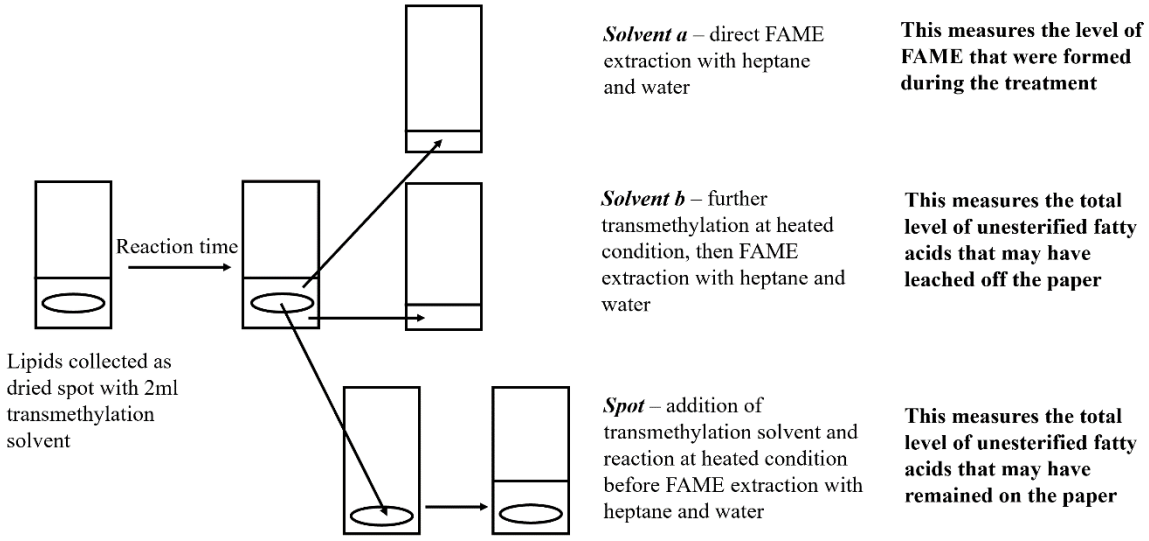

Figure 4.1.1. A flow diagram of the experiment

4.1.3 Results and discussion

All data are expressed as percentage of fatty acids transmethylated/methylated from each lipid class based on their peak area (PA).

$$\%\ of\ lipids\ transmethylated/methylated = \frac{PA\ of\ FAME\ in\ solvent\ a * 2}{PA\ of\ FAME\ in\ solvent\ b * 2 + PA\ of\ FAME\ on\ spots} * 100$$

$$\%\ of\ lipids\ remained\ bound\ to\ the\ collection\ paper = \frac{PA\ of\ FAME\ on\ spots}{PA\ of\ FAME\ in\ solvent\ b * 2 + PA\ of\ FAME\ on\ spots} * 100$$

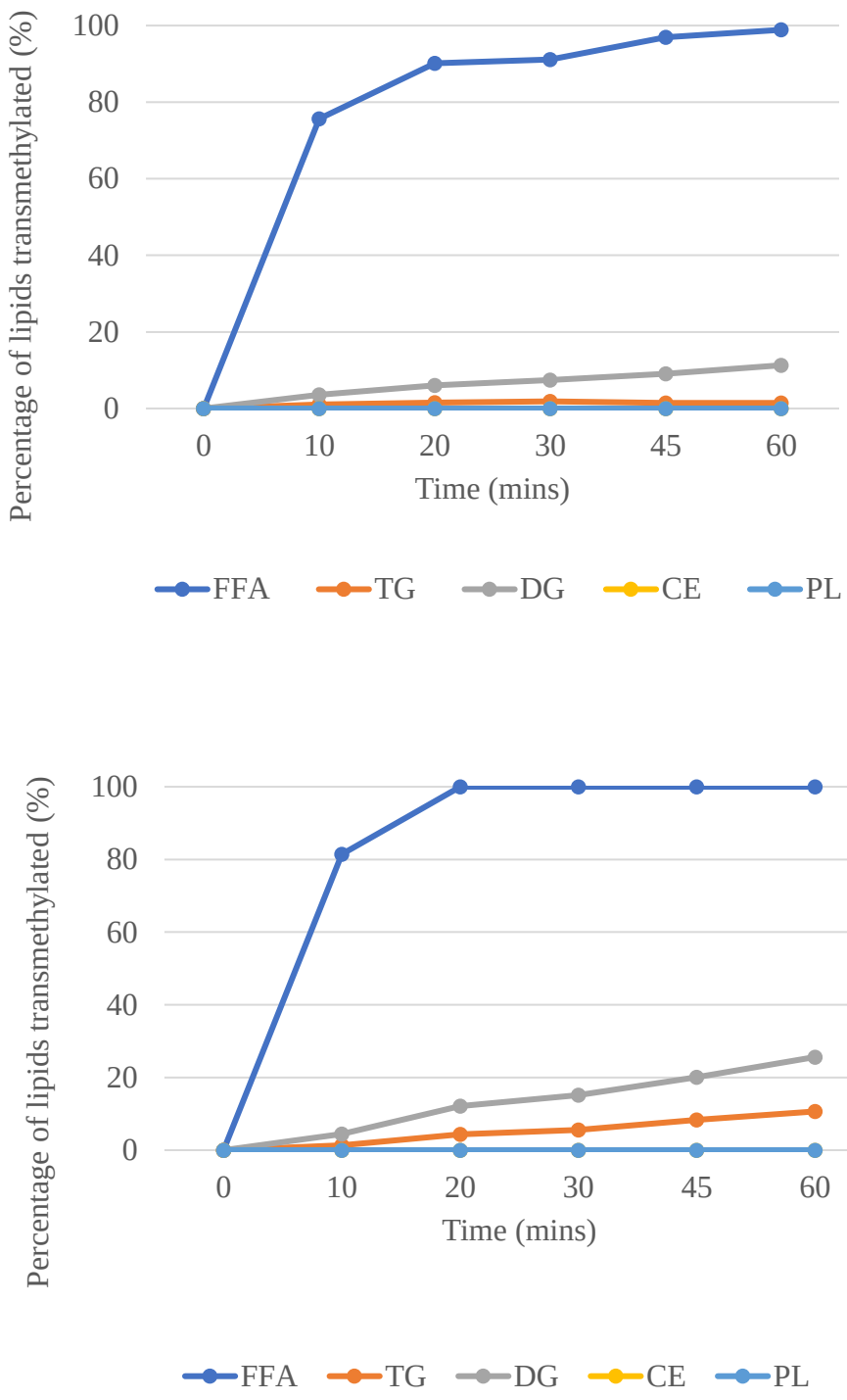

Figure 4.1.2. Completion of transmethylation of a mixture of lipid standard in 1% sulphuric acid in methanol in refrigerator (top) and at room temperature (bottom) over 60 minutes. FFA: free fatty acid (C14:0); TG: triglyceride (C13:0); DG: diglyceride (C18:1); CE: cholesterol ester (C17:0); PL: phospholipid (C20:0).

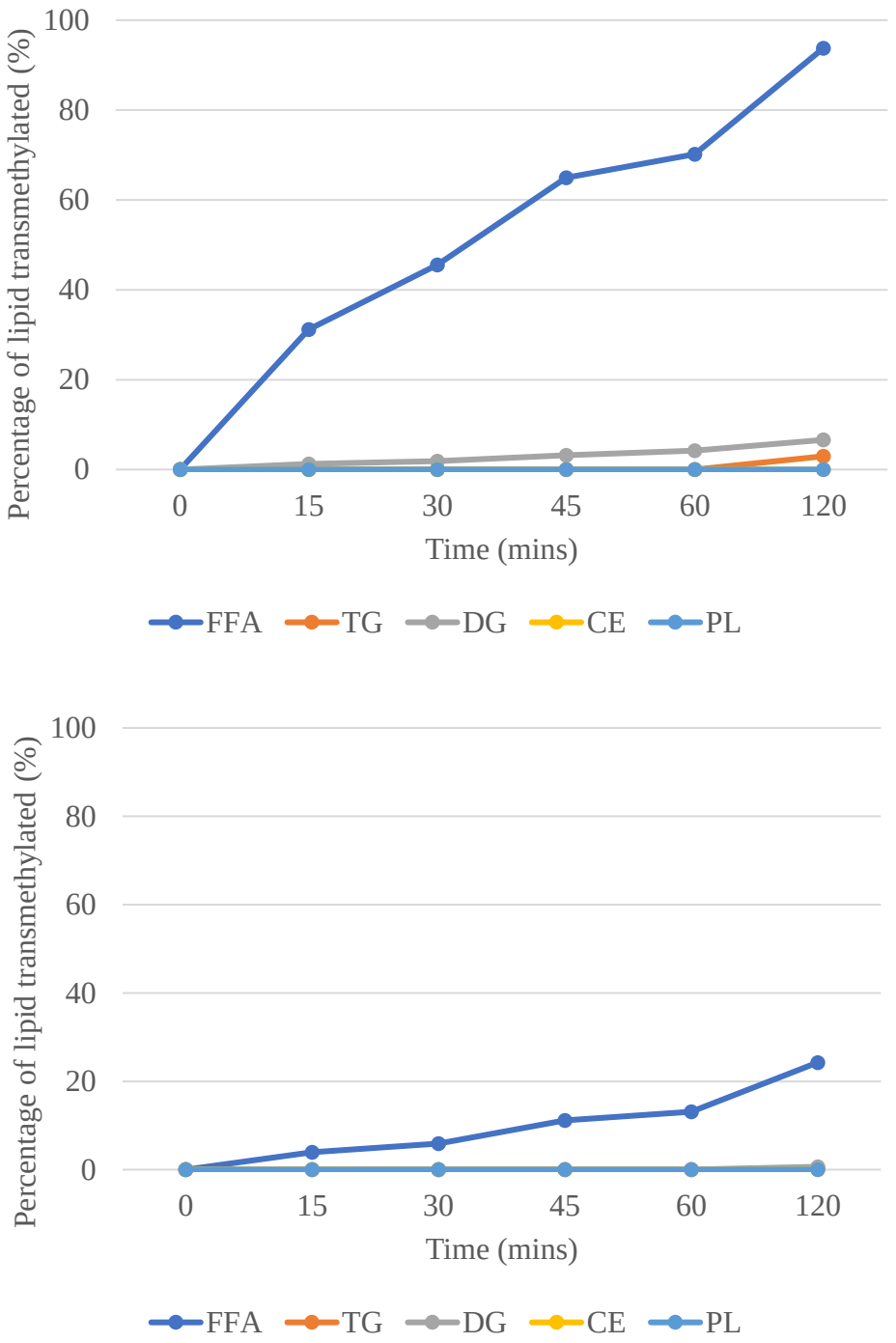

Figure 4.1.3. Completion of transmethylation of a mixture of lipid standard in in 0.1% (top) and 0.01% (bottom) sulphuric acid in methanol at room temperature over 120 minutes. FFA: free fatty acid (C14:0); TG: triglyceride (C13:0); DG: diglyceride (C18:1); CE: cholesterol ester (C17:0); PL: phospholipid (C20:0).

Regardless of the conditions tested, no one method fulfilled the criteria of only methylating FFA while not reacting with other lipid classes. All existing FFA were converted

to FAME in 1% sulphuric acid in methanol within 60 minutes or 20 minutes in the refrigerator or at room temperature, respectively. However, 4.4% and 1.5% of TG, and 11.3% or 12.1% of DG were also transmethylated into FAME at these conditions during the same time frame, respectively **(Figure 4.1.2 & 4.1.3)**. It was clear that changing the temperature from 23℃ to 5℃ had little effect on the rate of transmethylation of the test lipids. Lowering the acid concentration in the transmethylation solvent only slowed the reaction and extended the period of FFA took to be converted to FAME. Lowering the percentage of sulphuric acid from 1% to 0.1% yielded the best result with nearly all FFA (93.8%) being converted to FAME in two hours and only 3.0% of TG being converted to FAME. Decreasing the concentration of sulphuric acid 10-fold further resulted in few lipids being converted. It is possible to further workout a specific condition where only certain percentage of FFA was converted to FAME while none of other lipids are transesterified, and then to estimate the total FFA. However, the concentration of FFA in breast milk could vary significantly depending on the storage and handling procedure that has been experienced [Gao *et al.* 2019; Nessel *et al.* 2019], and it is likely that the rate of transmethylation is concentration dependent and thus less practical to have a universal equation.

In addition, before formally attempting this method of selectively methylating FFA by manipulating the reaction time and temperature, I performed several test trials that resulted in some variations likely due to the temperature. The time taken for completing FFA transmethylation ranged between 20 to 30 minutes and percentage of TG being transmethylated during this time frame ranged between 1.8% and 4.4%. The reaction solvent (1% sulphuric acid in methanol) used in the lab is stored in the fridge to preserve the acid concentration. It was found that the temperature of the transmethylation solvent (whether taken straight out of fridge or being left out at room temperature prior to use) contributed to

the variation observed. This observation suggest that the current method is highly temperature sensitive and therefore less practical in field.

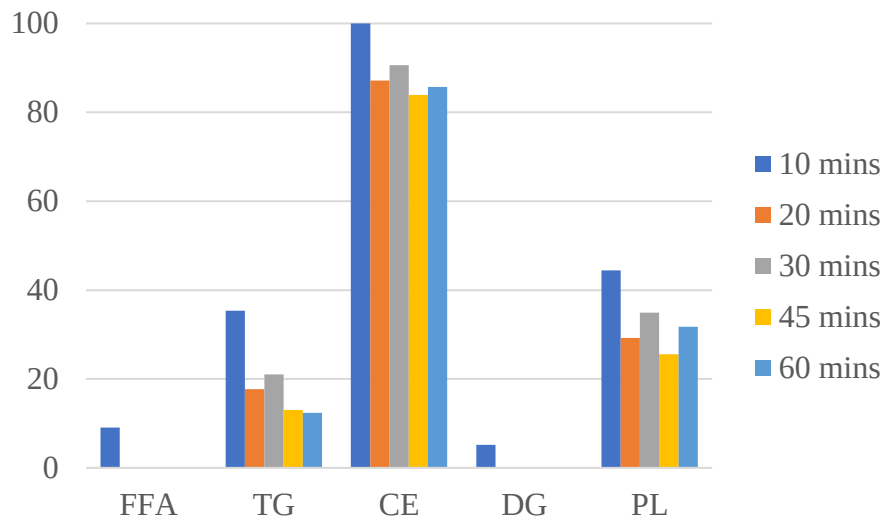

Figure 4.1.4. Distribution of lipid classes on paper disc after being transmethylated in 1% sulphuric acid in methanol at room temperature for 10, 20, 30, 45 and 60 minutes FFA: free fatty acid (C14:0); TG: triglyceride (C13:0); DG: diglyceride (C18:1); CE: cholesterol ester (C17:0); PL: phospholipid (C20:0)..

To understand the distribution of all lipids at different time points during the transmethylation process, whether remaining bound to the collection paper or being eluted into the solvent, the paper discs were transmethylated separately. Most of the lipids were eluted from the collection paper into the solvent following a time-dependent manner for transmethylation (**Figure 4.1.4**). DG and TG acted like FFA, being washed off the collection paper almost immediately except very small portion remained bound to the paper. PL, a polar lipid that were washed off with a polar solvent mixture but were remained in intact under the same condition (Figure 4.1.2 bottom). In contrast, CE, the most non-polar lipids of all in breast milk, tend to remain bound to the collection paper throughout the reaction time, where none of CE derived FAME was formed during the process (Figure 4.1.2 bottom). This offers

the potential of selectively eluting some lipid classes off the collection paper while others remain bound to the paper.

4.1.4 Conclusion

The results of these experiments are in agreement with the literature that lipids in breast milk are being transmethylated at different rates based on their chemical structures. However, the method of altering the time, temperature or acid concentration of transmethylation solvent to selectively convert FFA to FAME has failed to achieve the goal of separating FFA from breast milk without being contaminated by TG and other lipids in breast milk.

4.2 Selective methylation of free fatty acids in an acid-catalysed condition

4.2.1 Rationale

Based on the principle that 'non-esterified FFA should be converted to FAME prior to other more complexed lipids under the same condition' as described in the previous section, Tserng and colleagues [Tserng *et al.* 1981] reported a different acid-catalysed methylation, where all FFA were methylated within 15 minutes at room temperature while there was minimal conversion of all other esterified lipids during the same period. This acid-catalysed method used dimethoxypropane (DMP) and hydrochloric acid (equivalent to 2%) together with liquid plasma and the reaction was terminated by the addition of a small amount of pyridine. Differing from the methylation solvent used in my lab, this methylation method described by Tserng [Tserng *et al.* 1981] appeared to allow direct methylation of liquid biological fluid without a fat extraction/purification step. Unlike the sulphuric acid /methanol system, the presence of water in the DMP/hydrochloric acid system was reported to not interfere with the reaction but generates methanol to promote the reaction. In this system, DMP has three different roles: (a) acting as a water scavenger, (b) to precipitate protein and

(c) to offer methyl groups in the presence of water and acid as shown in the chemical equation below (**Figure 4.2.1**).

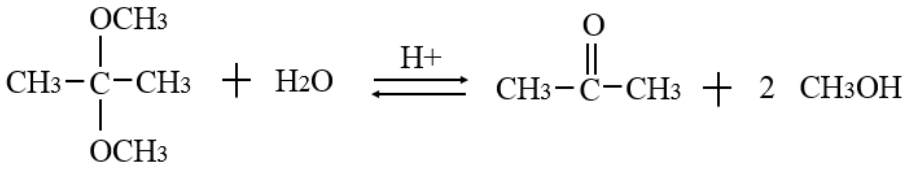

Figure 4.2.1. Reaction between dimethoxypropane and water under an acid-catalysed condition to produce acetone and methanol.

It was hypothesized that this method can be modified and adopted into a DMS based system with small adjustment to the volume of DMP and hydrochloric acid added due to potentially higher concentration of FFA in breast milk compared with plasma, and less DMP may be required due to the absence of water in a DMS system.

4.2.2 Materials and methods

In the original manuscript [Tserng *et al.* 1981], methanol acted as the carrier solvent for internal standards. For the purpose of replicating all details I have decided to add the same volume of methanol. However, considering that the level of fats and therefore FFA should be much higher than that in plasma, the volume of methanol was further increased to assist the methylation process. The volume and ratio of HCl to pyridine was kept constant, as hydrochloric acid acted as a catalyst for the reaction (and only requires a relatively small volume) and the sole purpose of adding pyridine was to terminate the reaction.

As this was an exploratory test and by no mean I could directly measure the completion of transmethylation by the DMP method, therefore a standard transmethylation process described in section 4.1 (1% sulphuric acid in methanol and heated at 70℃ for three hours) was set up as the control group.

Twenty microliters of the lipid standard mixture (A in Table 4.1) were collected on 903 paper discs, air-dried, and then transferred to 6ml scintillation. The solvents were added in the vial in the following order as described in the literature [Tserng *et al.* 1981]: water, methanol, DMP, hydrochloric acid and pyridine, in various volumes and ratios as detailed in **Table 4.2.1 (**for results see **4.2.2)** and **4.2.3 (**for results see **4.2.4)**. Fifteen minutes of reaction time was allowed, counting from the addition of HCl. After the addition of pyridine, resulting FAME was extracted with 500µL of water and 500µL of heptane, which was then analysed on GC-FID. All samples were analysed in triplicates.

4.2.3 Results and discussion

All data are expressed as percentage of fatty acids transmethylated from each lipid class based on their peak area (PA).

$$\%\ of\ lipids\ transmethylated = \frac{PA\ of\ FAME\ formed\ using\ DMP\ method}{PA\ of\ FAME\ formed\ using\ the\ standard\ method} * 100$$

Table 4.2.1. The solvent make-up used in the part 1 experiment to determine the feasibility of the dimethoxypropane (DMP) methylation method to be used in a dried spot system (µL).

Solvent makeup	Experimental group							
	Control	**0**	**1**	**2**	**3**	**4**	**5**	**6**
H_2O	1% H_2SO_4 in methanol	50	50	50	-	50	50	50
Methanol		25	25	-	25	25	200	500
DMP		1000	1000	1000	1000	500	1000	1000
HCl		20	20	20	20	20	20	20
Pyridine		10	10	10	10	10	10	10

Note: Group 0 lipid mixture was spotted directly into a 6ml scintillation vial; Group 1-6, lipids mixture was collected on 903 paper discs prior to reaction. H_2O: water. HCl: hydrochloric acid. H_2SO_4: sulphuric acid.

Table 4.2.2. Completion of transmethylation for each lipid class using the dimethoxypropane (DMP) method (%) compared with the standard transmethylation method, results corresponding to solvents described in Table 4.2.1

Completion of transmethylation (%)	Experimental group						
	0	**1**	**2**	**3**	**4**	**5**	**6**
TG 13:0	0.9	1.2	1.1	-	1.1	1.1	1.5
FFA 14:0	34.0	57.4	48.6	12.6	40.5	49.8	67.5

CE 17:0	-	-	-	-	-	-	-
DG 18:1	-	-	-	-	-	-	-
PL 20:0	-	-	-	-	-	-	-

Note: TG: triglyceride; FFA: free fatty acid; CE: cholesterol ester; DG: diglyceride; PL: phospholipid. Standard transmethylation method was with 1% sulphuric acid in methanol for three hours at 70℃.

Regardless of the experimental conditions tested, none fully converted FFA to FAME but around 1% of TG was transmethylated in each of the treatment groups. The positive result was that other esterified lipids including CE, DG and PL remained intact as there were no FAME derived from these lipids detected and therefore were not a source of contamination in such a system (Table 4.2.2). Comparing results 0 and 1, incorporating a dried spot into the method actually enhanced the methylation for FFA, which demonstrated that this method had a potential to be adapted into a dried spot-based system (Table 4.2.2). Comparing result 1 and 3, with small amount of methanol, the methylation cannot proceed at the absence of water to produce methyl groups (Table 4.2.2). However, with absence of methanol, the methylation could still take place as water reacts with DMP to produce methanol. Addition of methanol could probably increase the proportion of FFA being transmethylated as comparing result 1 and 6 (Table 4.2.2).

There might be two reasons that FFA were not fully transmethylated. First, there might not be enough methyl groups (methanol) for all FFA to be converted to FAME, as the fat content of plasma is far less than that in breast milk. Second, it is suspected that 15 minutes was not enough to allow all FFA in such a lipid standard mixture to be methylated completely. Further experiments were conducted to explore the different ratio and makeup of the reaction solvent to maximize the conversion from FFA to FAME.

In addition to the results presented in the table above, I also noticed that in the gas chromatograph, there was a peak present adjacent to the FFA (C14:0)-FAME peak, which

was suspected to be a by-product formed during the process and may be a by-product of FFA. This by-product was presented in all samples of experimental group with a similar peak area of 60, except for experimental group 4 where only half the volume of DMP was added into the reaction (peak area was 25), thus the by-product may also be dependent on the volume of DMP used. Therefore, further experiments were conducted to investigate the source of the by-product in the hope of eliminating it.

The second part of the experiment was conducted to investigate the cause of the by-product peak observed in part 1 experiment and to further optimize the system for complete methylation of FFA.

Table 4.2.3. The solvent makeup of the part II experiment to investigate source of by-products formed using the dimethoxypropane (DMP) methylation method in a dried spot system (µL).

Solvent makeup	Experimental group									
	Control	**Blank**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
H_2O	1% H_2SO_4 in methanol	50	50	50	100	250	500	50	50	-
Methanol		25	25	25	25	25	25	50	1000	1000
DMP		1000	250	50	1000	1000	1000	1000	1000	1000
HCl		20	20	20	20	20	20	20	20	20
Pyridine		10	10	10	10	10	10	10	10	10

Note: Blank was where no lipid mixture was involved, only solvents were added. Group 1-8, lipids mixture was collected on 903 paper discs prior to reaction. H_2O: water. HCl: hydrochloric acid. H_2SO_4: sulphuric acid.

Table 4.2.4. Completion of transmethylation of each lipid class using the dimethoxypropane (DMP) method (%) as compared with the standard transmethylation method, results corresponding to solvents used as described in Table 4.2.3.

Completion of transmethylation (%)	Experimental group								
	Blank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
TG 13:0	-	-	-	-	-	-	0.8	0.9	1.0
FFA 14:0	-	14.6	3.0	21.6	2.2	-	45.9	50.8	41.5
CE 17:0	-	-	-	-	-	-	-	-	-
DG 18:1	-	-	-	-	-	-	-	-	-
PL 20:0	-	-	-	-	-	-	-	-	-

Note: TG: triglyceride; FFA: free fatty acid; CE: cholesterol ester; DG: diglyceride; PL: phospholipid. Standard transmethylation method was with 1% sulphuric acid in methanol for three hours at 70℃.

Due to the suspected by-product peak observed in the previous experiment, I decided to run a blank sample to further investigate the source of the peak. 'Blank' contained no lipid standard but only reagent solvents were added and the by-product was also found with similar peak area of 52.4, which confirmed that the by-product was formed during the reaction and was independent of any lipids (**Table 4.2.4**). There have been other reports of formation of artifacts in the methylation process using DMP solution, which were eliminated once DMP had been removed from the system [Mason *et al.* 1964; Shimasaki *et al.* 1976; Simmonds *et al.* 1965]

The effect of adjusting the ratio of different solvents in order to maximize the conversion from FFA to FAME was not successful. Where the proportion of DMP was reduced (No 1&2 in Table 4.2.4), there was less FFA derived FAME detected, which was likely due to insufficient methyl groups for methylation and/or the presence of water in the solution likely stopped the reaction. However, increasing the volume of methanol in such system did not enhance the methylation of FFA (67.5% in No. 6 Table 5.2.3 with 500µl methanol versus 50.8% in No. 7 Table 4.2.4 with same other conditions applied). This poorer methylation efficiency may be related to the subtle difference between acid concentration (approximately 1.3% versus 1%).

Combining the results from the two experiments, the highest conversion rate from FAME to FFA (67.5%) was seen where additional methanol (500µl) was added, besides standard amount of DMP (No. 6 in Table 4.2.2). However, under such conditions, 1.5% of TG had also been converted to FAME whilst FFA has not yet completely been methylated. Among all tested conditions, there was no CE, DG or PL derived FAME detected.

There was no further exploration of extending the reaction time, because the FFA concentration in breast milk varies depending on past treatments [Gao *et al.* 2019; Nessel *et al.* 2019]. Trace amounts of FFA are expected in freshly expressed breast milk and higher concentrations of FFA are expected in breast milk that has been stored or pasteurised, therefore it is difficult to define the best time frame for such methylation process. As shown in this experiment, 1.5% of TG was converted to FAME during the time that there were still FFA remaining to be methylated, and thus extending methylation time would also increase the methylation for both lipids and therefore defeating the purpose.

4.2.4 Conclusion

The DMP method for direct and selective transmethylation of FFA failed to work as all FFA were not fully converted to FAME in any of the tested conditions, while at the same time, a small proportion of TG had already been transmethylated. In the context of separating breast milk fats, this method was considered not appropriate as the trace contamination from TG has a significant impact on the reading of FFA concentration due to its dominance in breast milk fats. Nevertheless, this method still has potential relevance for other biological fluids (e.g. plasma sample used in the original manuscript) where the lipid composition is not dominated by TG and the contamination from TG is negligible. There was no mention of the by-product peak observed in the original manuscript [Tserng *et al.* 1981]., which should be further investigated if one was to adapt this method for lipid class separation.

4.3 Selective transmethylation of esterified lipids in basic-catalysed condition

4.3.1 Rationale

Besides the acid methylation/transmethylation method described previously in 4.1, where all lipids (regardless of esterified or non-esterified) can be transmethylated or methylated into FAME. A base-catalysed transmethylation is often reported in the literature [Akoh *et al.* 1988; Glass *et al*. 1971; Hubscher *et al.* 1960; Malko *et al.* 2019] that selectively

transmethylates all esterified lipids (TG and its degraded by-products diglycerides and monoglycerides, as well as CE and PL) while unable to esterify FFA, and this reaction only takes minutes to complete. My working hypothesise was a two-step transmethylation process, where the basic condition offered the ability of selectively transmethylate all esterified lipids into FAME. FFA can then be separated from FAME derived from those esterified lipids, and then be methylated into FAME in an acid-catalysed condition described previously in section 4.1.

4.3.2 Materials and methods

A preliminary test was conducted to test the feasibility of this method in a dried spot system. A lipid standard mixture containing TG, FFA, PL and DG was used (lipid mixture B in table 4.1). This mixture did not contain CE due to the unavailability at the time, and CE only present in human milk in trace amount, which was not considered a major source of contamination.

Twenty microliters of the lipid standard were added to 903 paper discs, dried and then transferred to 6ml scintillation followed by addition of 2ml petroleum spirit (vial 1). Either 100µL of sodium or potassium methoxide (2N, prepared from sodium or potassium hydroxide and methanol) was added into the 6ml vial, and reaction time allowed was 10, 20 or 30 minutes. At the end of reaction, the paper discs were taken out and transferred to another 6ml scintillation vial (vial 2) for transmethylation in 2ml of 1% sulphuric acid in methanol to determine lipids that remain bound to the paper. Then, 250µL of water was added to vial 1 and vortexed. Following phase separation, the upper layer containing fatty acids that were esterified during the treatment was then taken to another 6ml scintillation vial (vial 3). Half of the solvent in vial 3 was transferred to another 6ml scintillation vial (vial 4), 600µL of heptane was added and FAME was extracted for GC analysis, which measures half of the FAME formed during the basic transmethylation process. The remaining solvent in vial 1 and

3 were dried under a steady nitrogen flow and then transmethylated in 2ml of 1% sulphuric acid in methanol at 70℃ for three hours. Vial 1 represented the fatty acids that had been leached off the paper but were not esterified during the basic transmethylation, and vial 3 contained the other half of FAME formed during the basic transmethylation and also unesterified fatty acids that were not merged into the water phase in vial 1.

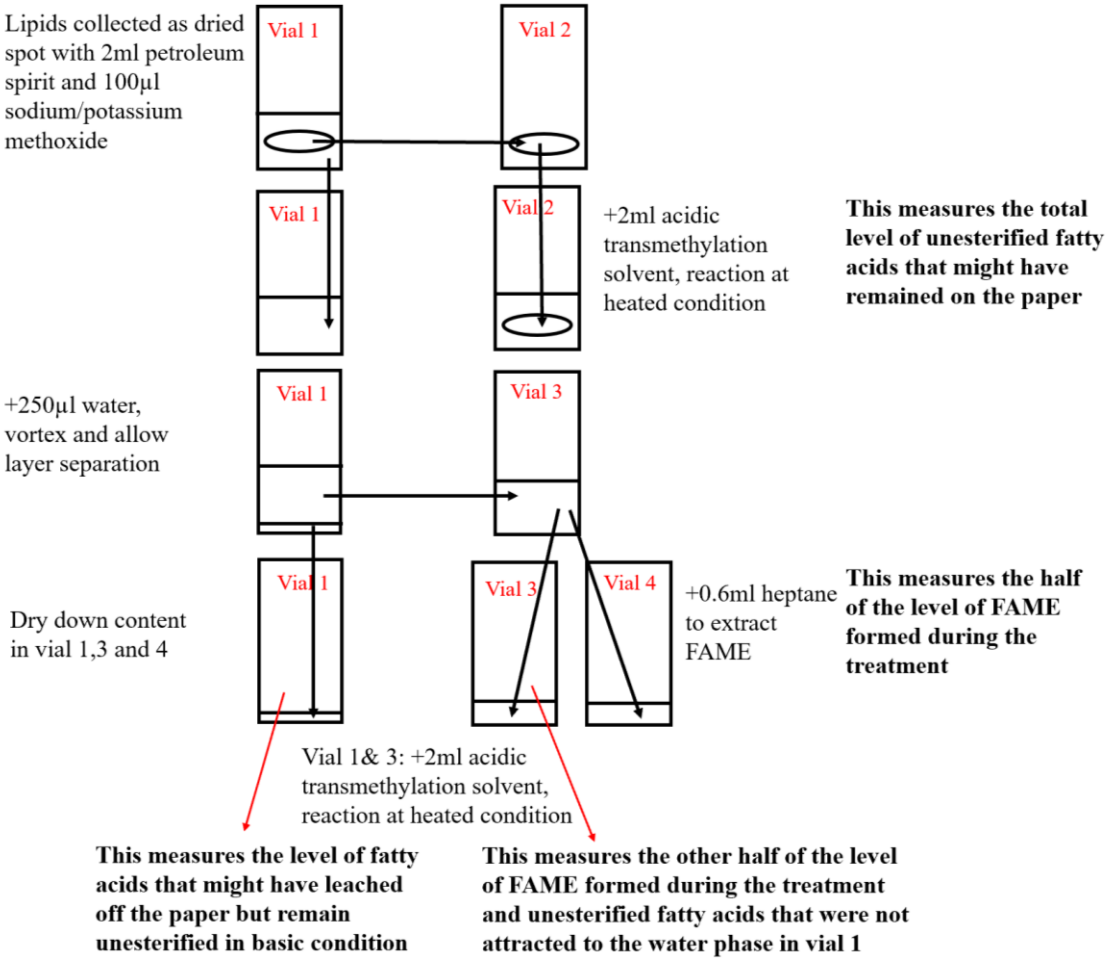

Figure 4.3.1. Preliminary test of the basic transmethylation system.

A further exploration was conducted to reduce the contamination of TG in this system. Twenty microliters of a mixed lipid standard (Lipid mixture C in Table 4.1, contained TG, FFA, PL, CE, and DG) was added to 903 paper discs, which were dried and transferred to 6ml scintillation vials. A basic transmethylation step was conducted in the same way described

above: 2ml of petroleum spirit and 100µL of sodium methoxide (2N) was added into the vial (vial 1), vortexed and allowed 30 minutes for reaction at room temperature. At the end of transmethylation, 500µL of water was added to enforce phase separation and the upper layer that contained FAME formed during the basic transmethylation was transferred to another 6ml scintillation vial (vial 2). An additional 2ml of petroleum spirit was added into vial 1, vortexed and the upper layer that contained any possible left-over FAME from previous step was transferred to another 6ml vial (vial 3). After the content in all three vials has been dried under nitrogen flow, 600µL of heptane was added to vial 2 and 3 to extract the FAME derived from neutral lipids; 2ml of 1% sulphuric acid in methanol was added in vial 1 for transmethylation to measure the concentration of FFA, the FAME derived was extracted with water and heptane and analysed on GC.

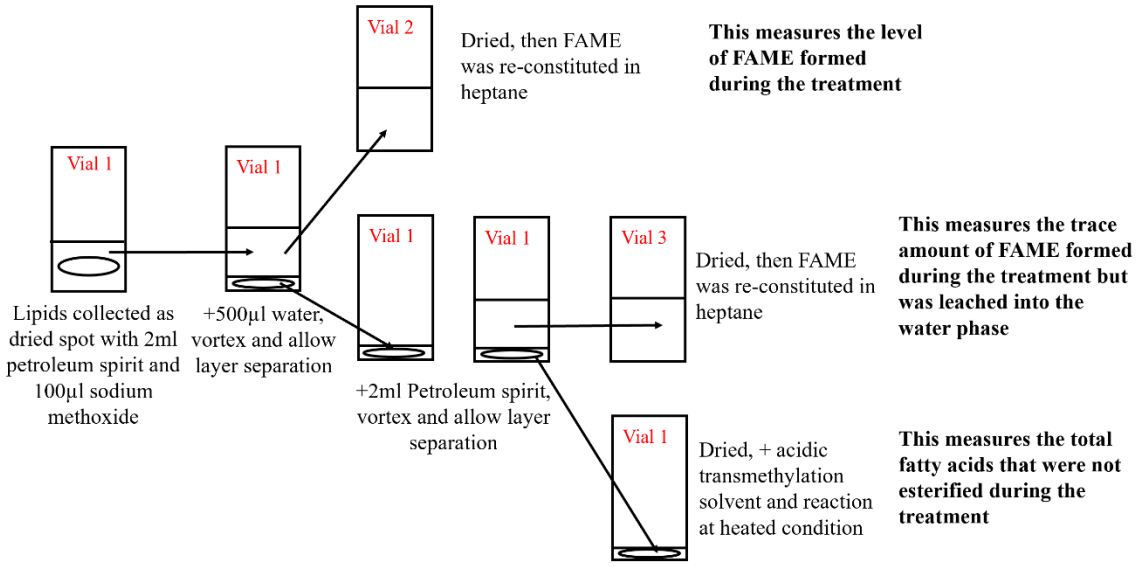

Figure 4.3.2. The flow diagram of additional wash of petroleum spirit to remove excessive triglyceride causing contamination.

4.3.3 Results and discussion

Table 4.3.1. A preliminary experiment of the basic solution transmethylation method based on a single sample (% of lipid classes presented in each vial calculated based on peak area), corresponding to figure 4.3.1.

Reaction time and reagent	Compartment and content	Distribution of lipid classes (%)			
		TG 13:0	FFA 14:0	PL 17:0	DG 18:1
10 minutes Potassium methoxide	Vial 1 – lipids that remained on paper discs	1.3	36.2	10.3	2.3
	Vial 2 – lipids that leached of the paper but not esterified during treatment	0.9	56.6	5.3	5.0
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	48.9	3.6	42.2	46.3
	Vial 4 – lipids that were esterified during the treatment	50.1	0	38.4	42.9
20 minutes Potassium methoxide	Vial 1 – lipids that remained on paper discs	1.0	33.3	5.5	1.4
	Vial 2 – lipids that were not esterified during treatment	2.0	61.8	9.6	9.2
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	48.5	2.4	42.4	44.7
	Vial 4 – lipids that were esterified during the treatment	42.2	0	34.8	37.2
30 minutes Potassium methoxide	Vial 1 – lipids that remained on paper discs	1.1	34.2	4.7	1.4
	Vial 2 – lipids that were not esterified during treatment	1.1	56.1	6.4	5.4
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	48.9	4.9	44.4	46.6
	Vial 4 – lipids that were esterified during the treatment	49.3	0	39.5	42.3
10 minutes Sodium methoxide	Vial 1 – lipids that remained on paper discs	1.1	31.1	10.9	2.7
	Vial 2 – lipids that were not esterified during treatment	0.7	65.1	4.9	1.6
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	49.1	1.9	35.9	41.2
	Vial 4 – lipids that were esterified during the treatment	45.0	0	35.9	41.2
20 minutes Sodium methoxide	Vial 1 – lipids that remained on paper discs	1.0	33.2	8.6	1.6
	Vial 2 – lipids that were not esterified during treatment	0.8	62.2	5.5	2.8
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	49.1	2.3	42.9	47.8
	Vial 4 – lipids that were esterified during the treatment	44.8	0	35.2	39.7

30 minutes Sodium methoxide	Vial 1 – lipids that remained on paper discs	1.1	36.1	5.6	1.5
	Vial 2 – lipids that were not esterified during treatment	1.1	57.9	5.5	3.4
	Vial 3 – lipids that were esterified during treatment plus non-esterified lipids forced into petroleum spirit layer	48.9	3.0	44.5	47.6
	Vial 4 – lipids that were esterified during the treatment	48.7	0	36.8	39.9

Note: TG: triglyceride; FFA: free fatty acid; PL: phospholipid; DG: diglyceride.

Ideally, FFA should be captured in vial 1 and 2 while all esterified lipids derived FAME should be captured in vial 3 and 4. From the results of these tests, it seems likely that transmethylation of the esterified lipids were completed within 10 minutes as the neutral lipids are equivalent in vial 3 and 4, which is consistent with the literature (**Table 4.3.1**). FFA derived FAME was detected in vial 3 but not vial 4, which is consistent with the literature demonstrating that the basic transmethylation method only works for esterified lipids but not for FFA (Table 4.3.1). However, this also showed that small percentage (approximately 4-6%) of FFA was eluted from the paper and released into the petroleum spirit solvent (vial 3), which can be corrected by addition of internal standard as majority of the FFA are either bound to the paper (vial 2) or in the sodium/potassium methoxide layer (vial 1) (Table 4.3.1).

The results of this experiment pointed a direction ahead, as at the end of the reaction, addition of water trapped all FFA in the sodium/potassium and paper discs layer (vial 1+2), whereas FAME derived from all other neutral lipids are extracted to the petroleum spirit layer. However, this method was not considered successful due to contamination of TG derived FAME, where a total of 2% TG derived FAME can still be found on the paper discs and in the sodium or potassium methoxide layer (vial 1+2, Table 4.3.1). Further explorations are to be set to increase the number of washes to further extract TG.

The use of sodium or potassium methoxide generated almost no difference in the results seen in this experiment described above, in term of transmethylation efficiency. However, it

was observed that the layer separation was clearer and more apparent with sodium methoxide than potassium methoxide, therefore the former one was used in the following experiment.

Table 4.3.2. Distribution of lipid classes in each compartment of the reaction reagent (% of total lipid classes based on peak area), results corresponding to the further experiment described in figure 4.3.2

Compartment and content	TG 13:0+22:6	PL 22:1	DG 18:1	CE 17:0
Vial 1 – All unesterified fatty acids	1.8	15	3.6	0
Vial 2 – FAME formed during treatment	96.8	82.6	94.4	83.5
Vial 3 – FAME formed during treatment but leached into water phase	1.3	2.5	83.5	16.5

Note: TG: triglyceride; PL: phospholipid; DG: diglyceride; CE: cholesterol ester

This part of the experiment was designed to further extract any leftover FAME derived from TG with petroleum spirit, a reaction time of 30 minutes was followed to ensure a completed transmethylation.

In previous experiments, it was observed that at the end of transmethylation where FAME derived from esterified lipids had mostly been extracted but small amount of TG (2~3%), DG (5~7%) and PL (~15%) were detected in the layer where ideally only contain FFA (sodium methoxide layer and on the paper discs, Table 4.3.1). It was hypothesized that an additional wash of petroleum spirit at the end of reaction could have fully extracted the left-over TG in the sodium methoxide/paper discs layer. However, although the majority of the esterified lipids were extracted completely by the additional wash of petroleum spirit, there was still approximately less than 2% of TG left on the paper discs and in the water layer along with 3.6% of DG and 15% of PL (**Table 4.3.2,** vial 3). It is likely that fatty acids from the TG fraction were liberated from the structural lipids during the process in preparation for transmethylation, and hence found in the water and sodium methoxide layer. This was

suspected due to the concentration of sodium methoxide, as one step further was taken to increase the concentration of sodium methoxide by using an over-saturated sodium methoxide solution for reaction. However, this offered similar results and did not reduce or eliminate the 2% of TG present in the water/sodium methoxide layer, and hence the results were not shown here. In addition, the numbers of petroleum spirit wash es was increased from two to four (for the same purpose of eliminating TG presented in water/sodium methoxide layer), this did not change the results either and therefore results are not presented here.

4.3.4 Conclusion

The base solution transmethylation method selectively transmethylated all esterified lipids within very short period, and was unable to esterify FFA. However, FFA cannot be separated from the rest of lipids without a small percentage of TG contamination. Though this is another method considered failed for the purpose of separating breast milk lipids due to the TG contamination, it is possibly a promising two-step separation method (first basic transmethylation for all neutral lipids and then acidic transmethylation for FFA) for other biological fluids where trace contamination from TG is negligible.

4.4 Selective extraction of FFA from a dried milk spot

4.4.1 Rationale

In addition to the complexity of structures, lipids in breast milk are also different in terms of polarity, which affects the extraction efficiency of lipids bound to a filter paper. As observed in section 4.1, some lipids remained bound to the collection paper while others had been eluted into the solvent. In this next experiment, the hypothesis was that certain solvents may be able to extract non-polar lipids from the paper and leave others behind, or vice versa; and that a different combination of filter paper and extraction solvent might offer different results and therefore possibly achieve selective elution of FFA.

4.4.2 Materials and methods

Lipid standard mixture D that contained TG, FFA, CE and PL was used for the first part of the experiment. The second part of experiment was conducted under the same principle but was performed at a later date and the lipid mixture used contained TG, FFA, DG and PL (lipid mixture C in table 4.1).

The steps in the experiments were the same but different collection papers were used (903 paper for part 1 and SG paper for part 2). Twenty microliters of the lipid mixture were collected on each paper disc, which were dried and transferred to 20ml (903 paper) or 6ml scintillation vial (SG paper). Solvents tested for elution efficiency included ethanol, methanol, isopropanol, petroleum spirit and heptane, and methyl-tert-butyl ether (MTBE). The testing solvents were added to the scintillation vials containing the paper discs, 2ml for 903 paper or 1ml for SG paper, where the paper discs were left soaking in the solvent for ten minutes. The resulting solvents were then extracted into another set of scintillation vials and this step was repeated once more. A total of 4ml (or 2ml) elution solvents was obtained, which was then dried under a steady nitrogen flow. The lipid extract was then transmethylated in 1% sulphuric acid in methanol at 70℃ for three hours and the FAME obtained were analysed on GC-FID. All analyses were undertaken in triplicates.

4.4.3 Result and discussion

The distribution of the lipids in each compartment was calculated based on the peak area using the following formula:

$$\%\ of\ lipids\ eluted = \frac{PA\ of\ FAME\ eluted\ in\ the\ solvent}{PA\ of\ FAME\ in\ the\ solvent + PA\ of\ FAME\ left\ on\ the\ spot} * 100$$

Table 4.4.1. Distribution of lipids (%) collected on 903 paper present in different compartments after two washes with various solvents.

Solvent	Compartment	TG 13:0	FFA 14:0	PL 22:1	CE 17:0
Ethanol	Solvent	99±0.1	98.4±3	94.3±0.2	98.4±0.2
	Paper discs	1±0.1	1.7±2.9	5.7±0.2	1.6±02
Methanol	Solvent	98.7±0.1	100	96.4±-0.3	86.1±1.4
	Paper discs	1.3±0.1	-	3.6±0.3	13.9±1.4
Isopropanol	Solvent	97.4±0.8	98.3±2.9	57.8±7.1	97±1.2
	Paper discs	2.6±0.8	1.7±2.9	42.4±7.1	3±1.2
Petroleum Spirit	Solvent	98.1±0.1	77.2±1	-	97.6±0.2
	Paper discs	1.9±0.1	22.8±1	100	2.4±0.2
Heptane	Solvent	97.9±1.6	79.3±1	-	97.9±0.3
	Paper discs	2.1±1.6	20.7±1	100	2.1±0.3

Note: TG: triglycerides; FFA: free fatty acids; PL: phospholipids; CE: cholesterol ester.

Table 4.4.2. Distribution of lipids (%) collected on SG paper present in different compartments after two washes with various solvents.

Solvent	Compartment	FFA 12:0	TG 13:0	PL 17:0	DG 18:1	FFA 20:0	TG 22:6
Ethanol	Solvent	95.5 ±2.0	95.7 ±2.1	28.3 ±1.0	95.2 ±2.2	89.4 ±1.8	95.4 ±1.8
	Paper discs	4.5 ±2.0	4.3 ±2.1	71.7 ±1.0	4.8 ±2.2	10.6 ±1.8	4.6 ±1.8
MTBE	Solvent	96.1 ±0.4	100	0	99.6 ±0	96.8 ±0	100
	Paper discs	3.9 ±0.4	-	100	0.4 ±0	3.2 ±0	-
Petroleum Spirit	Solvent	13.1 ±1.5	15.2 ±1.2	-	5.3 ±2.2	16.1 ±1.2	6.2 ±0.7
	Paper discs	86.9 ±1.5	84.8 ±1.2	100	94.7 ±2.2	83.9 ±1.2	93.8 ±0.7
Heptane	Solvent	12.3 ±1.2	19.5 ±1.6	-	4.6 ±0.4	16.6 ±1.5	8.4 ±1.0
	Paper discs	87. 7±1.2	80.5 ±1.6	100	95.4 ±0.4	83.4 ±1.5	91.6 ±1.0

Note: TG: triglycerides; DG: diglyceride; FFA: free fatty acids; PL: phospholipids; MTBE: methyl-tert-butyl ether

There were some discrepancies regarding the methodologies used in these experiments as they were conducted quite some time apart. One of the most obvious differences between the two parts of experiment was the lipid mixture used. Part one of the experiment in this section was one of the earliest experiments I conducted, and at the time I considered it was only essential for the lipid mixture to contain the four major lipids present in human breast milk, that is, TG, FFA, PL and CE. I have later learned that the by-product of TG degradation

(DG) could potentially be another major source of contamination for FFA, which was then added to the lipid mixture used in later experiments, including part two in this section. In addition, there were more than one TG and FFA standards included in the lipid mixture for part two, as it was suspected that the elution efficiency may also differ due to the length of the carbon chain and the saturation of fatty acids, therefore I used two TG (C 13:0 and C 22:6) and FFA (C14:0 and C 20:0) standards with different chain lengths to cover the two ends of the spectrum. Second, the volume of solvent used for elution was reduced from 2ml (part 1) to 1ml (part 2), which was partly due to change in the size of the scintillation vial used (20ml vs. 6ml), and also the 903 paper is approximately twice thicker than the SG paper. Finally, different solvents were used, where methanol and isopropanol were replaced with MTBE in part two. Alcohols generally perform relatively similarly in these conditions, therefore only one was tested for the ability of elution at later time with SG paper.

Although data were not presented, the numbers of washes required were tested, including one, two, three and four times. It was shown that two washes were sufficient to extract all possible lipids, and additional washes beyond two washes did not provide any further extraction.

The elution efficiency of all three alcohols tested was relatively similar for TG and FFA in the 903 paper system (**Table 4.4.1**), where only a small percentage (or none) of lipids remain bound to the collection paper after two rounds of elution. Less polar solvents like petroleum spirit and heptane had a better elution efficiency with TG (>98%) as compared to with FFA (<80%) (Table 4.4.1). However, there was no tested solvents that could separate TG from FFA purely based on polarity. It is also noted that these less polar solvents cannot possibly wash off PL, the most polar lipids in breast milk, from the collection paper (Table 4.4.1).

Since there were no major differences between the alcohols tested in previous experiment, ethanol seemingly had the best elution efficiency for all lipids as compared to methanol and isopropanol and was therefore chosen in the second experiment. It was noted that the elution efficiency was affected by the chain length of fatty acids as 96% of FFA C12:0 compared with 90% of FFA C20:0 had been eluted (**Table 4.4.2**). MTBE was brought into the test as my lab group was developing a new method for lipid extraction and was hunting for less harmful solvents to replace chloroform that is often used. It was observed that MTBE was capable of eluting off lipids while leaving PL bound to the SG paper (Table 4.4.2). Compared with the results shown in Table 4.4.1, heptane and petroleum spirit performed worse in terms of eluting all lipids tested where majority of these lipids remain bound to the SG paper. This is likely due to the silica gel impregnated layer of the SG paper that offered additional binding strength, less polar solvent would not be able to extract the lipids from such a system.

Overall, regardless of the collection paper used, none of the tested solvents were able to separate TG from FFA, these two lipids were often eluted off the collection paper together.

4.4.4 Conclusion

The separation of FFA from TG based on polarity of solvents was unsuccessful as TG and FFA tended to remain bound to the paper or being eluted into the solvent at a similar rate. The highlight from this experiment was that the combination of MTBE plus SG collection paper was able to retain PL while releasing all other lipids from the paper, this offers great potential for future studies that would like to selectively determine the concentration of PL in any given biological fluids; further tests would be required to refine this.

4.5 Separating FFA with solid phase extraction column material

4.5.1 Rationale

Conventional methods for lipids separation are mostly based on their polarity. Solid phase extraction (SPE, Ruiz-Gutierrez *et al.* 2000), is an alternative to TLC method and based on a similar principle. With a different combination of column material and extraction solvent, the lipids are either retained in the column or washed through the column at a different rate.

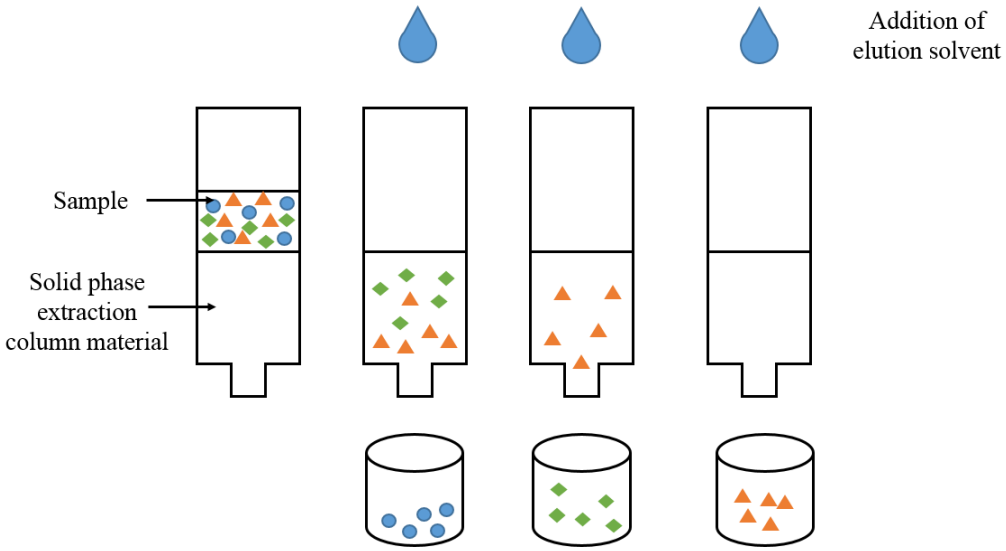

Figure 4.5.1. Principle of solid phase extraction.

SPE has several advantages over TLC: 1) fewer steps, and therefore less labour work; 2) does not involve fluorescence and ultraviolet light for visualisation of lipid separation; and 3) is less wasteful of solvents. However, the SPE method might be less practical than the TLC method, as the column has to maintain a certain level of moisture to prevent dry out, which could lead to poor performance of separation. The SPE method for breast milk lipid separation shares a common disadvantage with TLC, which is the contamination of TG due to its dominance in milk fats. The idea of this experiment was to use a column material of SPE in a powdered form rather than a cartridge. Instead of performing the separation in a SPE

system, the column material can be added into a scintillation vial where extracted lipids have been collected. The extracted lipids simultaneously bound to the binding material. It was hypothesised that the separation of lipid classes can be achieved by two steps of extraction, where the less polar neutral lipids (CE, TG and DG) can be extracted with chloroform, and the FFA can then be extracted with an acidified solvent or a more polar solvent (but not polar enough to extract PL), and leaving the PL bound to the column material.

4.5.2 Materials and methods

Lipid standard mixture C (containing TG, FFA, PL, DG and CE) listed in Table 4.1 was used to test the effectiveness of the system.

Twenty microliters of lipid mixture were added into a 6ml scintillation vial followed by the addition of 500 µL of chloroform and 0.1g amino propyl powder, which was then vortexed for 10 seconds. After the phase separation, the upper layer was extracted and transferred to another 6ml scintillation vial. This process was repeated once further with chloroform and then repeated another time but with 2% acetic acid in diethyl ether. The resulting solvent from each elution was dried under a steady nitrogen flow, and the lipids extract was transmethylated with 1% sulphuric in methanol for three hours at 70℃, and the FAME formed was analysed on GC-FID. Results were based on a single sample.

4.5.3 Result and discussion

Table 4.5.1. Peak area of the mixed lipid standard in different washes (wash 1&2 with chloroform, wash 3 with 2% acetic acid in diethyl ether).

Compartment	FFA 12:0	TG 13:0	CE 17:0	DG 18:1	FFA 20:0	PL 22:1	TG 22:6
Wash 1	-	54.7	11.6	30.1	-	-	64.2
Wash 2	-	32	6.7	18.6	-	-	36.7
Wash 3	20.8	10.8	2.2	7	28.3	-	-

This was only a pilot run and the results were not presented as proportion of lipids in each wash (%) since the lipids that remained bound to the amino propyl were not measured, results were simply presented as peak area.

Ideally, all neutral lipids (except the most polar PL) should be released from the amino propyl within the first two washes with chloroform, and the third wash of acidified diethyl ether was used to separate FFA from PL. Based on the peak area, the majority of TG (regardless of chain length), CE and DG were extracted with the first two washes of chloroform, where FFA and PL remained bound to amino propyl (**Table 4.5.1**). However, in the third wash with acidified diethyl ether, not only FFA, but also TG that had not been released from previous elution was washed off (Table 4.5.1). If assuming all TG had been released from the amino propyl after the third wash, this is roughly equivalent to 10% contamination from TG. SPE method could be seen as TLC method in a different display and this contamination might represent the tailing of TG on TLC plate. Further exploration could be made by increasing the number of washes to get rid of the effect of TG tailing; however, it is less likely this method would have much potential considering the difficulties and contaminations of separation experienced in this pilot trial.

4.5.4 Conclusion

This simple adopted SPE method resulted in approximately 10% contamination of TG during separation, and therefore failed for the purpose of developing a separation method of FFA that is not interfered by TG.

4.6 Separating FFA by binding FFA to filter paper

4.6.1 Rationale

From a basic chemistry point of view, a FFA molecule, when disassociated, is a cation that carries negative charge, which theoretically should be able to bind to collection paper that is treated with alkaline solution or positively charged ions. The hypothesis was that once FFA is bound to the paper with either basic or anion molecules, separation of FFA from other esterified lipids can be achieved using an extracting solvent described in section 4.4. The remaining FFA can be eluted with further extraction using acidified extracting solvent and methylated in acid-catalysed condition for identification on gas chromatography. A similar idea was proposed by Hornstein [Hornstein *et al*. 1960] and further improved by Needs [Needs *et al.* 1983], where FFA were bound to a basic anion-charged resin column and allowed other fats to be eluted through the column to achieve separation.

4.6.2 Materials and methods

Lipid standard mixture C described in Table 4.1 (containing TG, FFA, CE, PL, and DG) was used to establish and define the optimal pH of the collection paper, and the concentration of acidified acetone for further elution of FFA.

1) Define the optimal pH of the collection paper

Calcium chloride (CaCl2) was prepared at a concentration of 1M, the pH of which was approximately 6.5. The same calcium chloride was then mixed with sodium hydroxide (0.1M) in 1:1 ratio to obtain a solution with pH 10. Sodium bicarbonate (NaHCO3 0.5M, pH 8.5), sodium carbonate (Na2CO3 0.5M pH 11) was mixed at a ratio of 1:1, 2:1 and 5:1, the pH of such mixed solution was 10.5, 10 and 9.5 respectively. The collection paper was then treated by spotting 50µL of each solution, which were left at room temperature for 1 hour before use.

Twenty microliters of the lipid mixture were collected on the alkaline treated SG paper, which was dried and then transferred into 6ml scintillation vials, 1ml of acetone was added and the paper discs were left soaking in the solvent for ten minutes. The resulting solvent was transferred to another 6ml vial, this step was repeated one more time and a total of 2ml of solvent was collected and dried under nitrogen flow. The lipid extract was then transmethylated in 2ml 1% sulphuric acid in methanol at 70℃ for three hours, and resulting FAME was extracted with heptane and analysed on GC-FID. All samples were conducted in triplicate.

2) Define the concentration of acidified solvent for FFA extraction

To extract the FFA that were bound to the alkaline treated SG paper, the following tests were conducted. Twenty microliters of lipid mixture were collected on alkaline treated paper discs (5:1 sodium bicarbonate: sodium carbonate, 0.5M each) as described above. The lipids extraction step was conducted as described above. The paper discs were transferred to a set of 6ml scintillation vials followed by acetone washes for two times to collect the neutral lipids. Following the neutral lipids extraction, 1ml of the acidified acetone (acetic acid in acetone, 1%, 5% and 10%, v/v) was added to the 6ml vial, where the paper discs were left soaking in for 10 minutes before transferring the resulting solvents to another set of 6ml scintillation vial. This step was repeated once more to extract all FFA. The acidified acetone containing extracted FFA was dried, and 2ml of 1% sulphuric acid in methanol was then added to the vial for transmethylation at 70 for three hours. Resulting FAME was extracted and analysed on GC the same way described above.

To further confirm that this system works even when the FFA concentration of EBM is abnormally high, I prepared another two sets of standards, named high FFA and high TG lipid mixture, representing an abnormally high proportion of FFA (60%) and a relatively low proportion of FFA (20%). Twenty microliters of lipid mixture were collected on the same

alkaline paper discs used above with a pH of 9.5. The paper discs were transferred to a 6ml scintillation vial for lipid extraction as described above. Two acetone washes (1ml each time) were used to extract neutral lipids including TG (and degraded products MG and DG) as well as CE. Further two acidified acetone wash (0.01%, 0.1% and 1%) were used to separate FFA from PL. The resulting acetone and acidified acetone containing extracted lipids were dried and transmethylated in 1% sulphuric acid in methanol for three hours at 70℃.

3) Reconstitution of FFA after extraction

Breast milk (20µL) was collected on SG paper discs treated with NaHCO3 and Na2CO3 (0.5M each, 5:1 ratio), air-dried for 3 hours prior to analysis. The paper discs were transferred to 6ml scintillation vial, where lipids were extracted with two 1ml washes of acetone and two 1ml washes of 1% acetic acid in acetone. The acidified acetone was then dried under a steady nitrogen flow, and the eluted FFA was re-solubilised with 600µL of heptane, or acetone, or 50µL water and 600µL of heptane. The underivatized FFA was then analysed on the GC-FID with a polyethylene glycol column.

To determine the sensitivity and precision of the system for measuring the concentration of FFA in EBM, a series of mixed breast milk samples with different concentration of FFA was prepared. A single breast milk sample was left at room temperature to deliberately increase the FFA concentration (aged breast milk), which was then mixed with another freshly thawed breast milk sample (fresh breast milk). The proportion of the aged breast milk accounted for 0, 25, 50, 75 and 100% of the mixed breast milk sample. These breast milk samples were collected on the alkaline treated SG paper discs, and the FFA was extracted as described above with acidified acetone after removal of neutral lipids with acetone. The extracted FFA was then re-solubilised in 50µL water and 600µL of heptane and analysed with GC-FID for underivatized FFA. Results were based on triplicates.

4) Stability of breast milk collected as dried spot on alkaline treated collection paper

A frozen breast milk sample from a single milk donor was thawed for this part of the experiment. The thawed breast milk was either left not treated (raw) or heated treated (holder pasteurised or boiled). As described above, twenty microliters of breast milk were collected on plain SG paper (without alkaline coating), or pre-treated SG paper discs (pH 9.5), or on plain SG paper discs treated with alkaline solution at the time of analysis (post-treated). Baseline analysis was carried out three hours post sample collection after the paper discs had been dried completely and the rest of DMS samples were packed in a cellophane bag and kept at room temperature till analysis at day seven post collection. The analysis method was the same as described above, using acetone and acidified acetone to separate neutral lipids and FFA, which were then transmethylated into FAME for analysis on GC (neutral lipids) or injected into GC straight away for underivatized FFA analysis.

5) Stability of TG standard collected on alkaline treated collection paper

A mixture of triglyceride standards (C13:0 and C22:6 n-3) was collected on SG papers treated with solutions of varying levels of pH, ranging from 7 to 11. Plain SG paper without any coating was macerated in water, the pH of which was approximately 7 as measured by several pH testing papers (Riedel-de Haen pH 1~11; Whatman-BDH pH 8~10; Whatman-BDH pH 6~8) for cross validation. Solutions with different pH was prepared by diluting an original stock solution of Na2CO3 (1M) with a pH of 11.

The stability test was conducted similar to the method described above. Paper discs were left air dried for three hours after collecting twenty microliters of the TG mix. Baseline analysis was conducted immediately after the paper discs had been dried and the rest of samples were packed in cellophane bags and stored at room temperature for day 7 and 12 post collection analysis. At the time of analysis, the samples underwent the same elution process

(firstly acetone then acidified acetone) as described above, the resulting solvents were dried under nitrogen and extracted lipids were transmethylated in 1% sulphuric acid in methanol. The FAME formed was analysed by GC-FID. The acetone wash contained TG that remained intact and the acidified acetone contained TG that had been degraded into FFA due to hydrolysis. All results were based on triplicates.

4.6.3 Results and discussion

Results were calculated based on the peak area of individual lipids in different compartments.

$$\%\ of\ lipids\ eluted = \frac{PA\ of\ FAME\ eluted\ in\ the\ solvent}{PA\ of\ FAME\ in\ the\ solvent + PA\ of\ FAME\ left\ on\ the\ spot} * 100$$

1) Define the optimal pH of the collection paper

Table 4.6.1. Distribution of lipids eluted into the solvent and left on silica gel paper treated with various solutions (% of lipids).

Solution	Ratio	pH	Compartment	FFA 12:0	TG 13:0	CE 17:0	DG 18:1	FFA 20:0	PL 22:1	TG 22:6
CaCl2 (1M)	-	6.5	Paper discs	5	-	-	-	59.4	100	-
			Acetone wash *2	95	100	100	100	30.6	-	100
NaOH (1M)	-	11	Paper discs	99.6 ±0.7	0.9 ±0.1	-	1.8 ±0.1	100	100	1.0 ±0.5
			Acetone wash *2	0.4 ±0.7	99.1 ±0.1	100	98.2 ±0.1	-	-	99.0 ±0.5
CaCl2 (1M): NaOH (0.1M)	1:1	10	Paper discs	92.7 ±1.9	0.2 ±0	0.5 ±0	0.8 ±0.1	97.5 ±0.3	100	-
			Acetone wash *2	7.3 ±1.9	99.9 ±0.2	99.5 ±0	99.2 ±0.1	2.5 ±0.3	-	100
NaHCO3 (0.5M): Na2CO3 (0.5M)	1:1	10.5	Paper discs	98.5 ±0.1	0.8 ±0	0.6 ±0.2	1.5 ±0	99.0 ±0.2	99.8 ±0.2	1.0 ±0.2
			Acetone wash *2	1.5 ±0.1	99.2 ±0	99.4 ±0.2	98.5 ±0	1.0 ±0.2	0.2 ±0.2	99.0 ±0.2
	2:1	10	Paper discs	98.3 ±0.2	1.1 ±0	0.6 ±0.2	1.7 ±0.1	99.2 ±0.2	99.6 ±0.2	1.3 ±0.1
			Acetone wash *2	1.7 ±0.2	98.9 ±0	99.4 ±0.2	98.3 ±0.1	0.8 ±0.2	0.4 ±0.2	98.7 ±0.1

	5:1	9.5	Paper discs	98.5 ±0.2	1.3 ±0.2	0.5± 0.1	2.1 ±0.1	99.4 ±0	99.5 ±0.2	1.6 ±0.1
			Acetone wash *2	1.5 ±0.2	98.7 ±0.2	99.5 ±0.1	97.9 ±0.1	0.6 ±0	0.5 ±0.2	98.4 ±0.1
Na2CO3 (0.1M)	-	9.5	Paper discs	98.2 ±0.3	1.1 ±0	0.5 ±0.1	2.1 ±0.2	98.9 ±0.2	99.7 ±0.2	1.6 ±0.1
			Acetone wash *2	1.8 ±0.3	98.9 ±0	99.5 ±0.1	97.9 ±0.2	1.1 ±0.2	0.3 ±0.2	98.4 ±0.1
NaHCO3 (0.5M)	-	8.5	Paper discs	98.5 ±0.1	3.4 ±0.3	0.9 ±0.1	4.1 ±0.3	99.3 ±0.1	99.3 ±0.1	4.9 ±0.6
			Acetone wash *2	1.5 ±0.1	96.6 ±0.3	99.1 ±0.1	95.9 ±0.3	0.7 ±0.1	0.7 ±0.1	95.1 ±0.6

Results are based on triplicates except for calcium chloride.

There was no binding property related to $CaCl_2$ treated paper (**Table 4.6.1**), however whether this was due to a slight acidic pH value of the solution (approximately 6.5 due to the water used to prepare the solution) or the binding force between calcium ion and the FFA were not strong enough needed to be further investigated. I then continued to explore the potential binding property offered by positively charged calcium ion, but to mix it with alkaline solution to adjust its pH level. It was shown that the binding effect observed was dependent on the pH of the SG paper but had little to do with the calcium ions. A range of different pH was therefore tested to determine the optimal level of pH that achieve a binding effect.

It was apparent that the binding effect observed was independent of the calcium ion, or the hydroxyl group, but dependent on the pH level. There was very little difference between the binding effects across different levels of pH. The 5:1 NaHCO3 and Na2CO3 solution with pH of 9.5 retained FFA equally as well as other solutions with higher pH, and retained the least level of TG than the pH 8.5 NaHCO3 solution, and therefore was chosen to be the final pH of the paper. However, an increased pH level of the paper could lead to chemical hydrolysis of the TG itself, therefore the lowest possible pH was the preferred option.

2) Define the concentration of acidified solvent for FFA extraction

After all neutral lipids being washed off the filter paper, I was then set to look for a solvent to further extract the FFA that bound to the alkaline filter paper. After elution of neutral lipids, there were only FFA and PL left on the filter paper, the goal was to extract FFA only without contamination from PL, therefore a mild acid, acetic acid was chosen for this part of experiment.

Table 4.6.2. Exploring the optimal concentration of acid in acetone for extracting FFA from alkaline treated SG paper.

	Compartment	FFA 12:0	TG 13:0	CE 17:0	DG 18:1	FFA 20:0	PL 22:1	TG 22:6
1% H+/acetone	Paper discs	0.6	0	0	1.5	0	99.2	0
	Acetone wash*2	2.2	98.8	100	95.9	1	0	98.4
	H+/acetone wash*2	97.2	1.2	0	2.6	99	0.8	1.6
5% H+/acetone	Paper discs	0	0	0	1.2	0	84	0
	Acetone wash*2	2	98.6	100	96.1	0.7	0	97.9
	H+/acetone wash*2	98	1.4	0	2.7	99.3	16	2.1
10% H+/acetone	Paper discs	0	0	0	1.1	0	64.9	0
	Acetone wash*2	2.2	98.2	100	96.8	1.5	0	98.8
	H+/acetone wash*2	97.8	1.8	0	2.1	98.5	35.1	1.2

Note: the pH of the filter paper was 9.5. Acidified acetone was prepared with acetic acid in acetone (v/v). Results are based on a single sample.

There was no difference between the three different concentrations of acidified acetone used in terms of the ability to elute FFA from the alkaline filter paper (>97%). However, the proportion of PL being washed off the filter paper increased with increasing the acidity of the acetone solvent, which defeated the purpose of purifying the FFA by leaving PL bound to the paper. It seems that 1% would be considered the highest possible concentration of acid in acetone for extracting FFA from the alkaline treated paper; further exploration around lowering the acid content of acetone was then needed.

In my previous research conducted during my honours degree, I have observed that the FFA concentration of aged breast milk (collected between 2012-12, stored at -80℃ until analysis in 2016) could be as high as 30% to 40% of breast milk fats. As a result, for this next

part of experiment, I specifically prepared a lipid mixture containing 60% FFA to simulates this abnormal fat composition.

Table 4.6.3. Extraction efficiency of lipids (% of total lipids) spotted on silica gel paper with various solvents.

Lipid mix	Compartment	**MG 12:0**	**TG 13:0**	**FFA 14:0**	**CE 17:0**	**DG 18:1**	**FFA 20:0**	**PL 22:1**	**TG 22:6**
High FFA mix	Paper discs	1.0 ±0	0.1 ±0.1	0.5 ±0.1	0.1 ±0.1	0.6 ±0	0.5 ±0.4	94.4 ±0.4	-
	Acetone wash*2	97.8 ±0.2	99.6 ±0	0.6 ±0.1	99.6 ±0.2	97.5 ±0.3	0.20 ±0	0.5 ±0.1	99.9 ±0.2
	1% H+/acetone wash*2	1.2 ±0.2	0.3 ±0.1	98.9 ±0.2	0.3 ±0	1.9 ±0.2	99.2 ±0.4	5.1 ±0.4	0.1 ±0.2
	Paper discs	1.3 ±0	0.2 ±0	33.6 ±1.6	0.2 ±0	0.8 0	72.4 ±3.9	98.7 ±0	-
	Acetone wash*2	97.7 ±0	99.4 ±0	0.5 ±0.1	99.6 ±0.1	97.3 ±0.1	0.2 ±0	0.5 ±0.3	99.5 ±0.2
	0.1% H+/acetone wash*2	1.0 ±0	0.4 ±0.1	65.9 ±1.7	0.1 ±0.1	1.8 ±0.1	27.4 ±4.0	0.5 ±0.3	0.5 ±0.2
	Paper discs	1.9 ±0.1	0.6 ±0	91.8 ±0.4	0.3 ±0	1.3 ±0.1	97.1 ±0.1	99.3 ±0.3	0.6 ±0.1
	Acetone wash*2	97.1 ±0.3	98.9 ±0	0.5 ±0	99.6 ±0.1	96.9 ±0.1	0.2 ±0	0.5 ±0.2	98.8 ±0.1
	0.01% H+/acetone wash*2	1.0 ±0.3	0.5 ±0	7.6 ±0.4	0.2 ±0.1	1.8 ±0	2.7 ±0.1	0.2 ±0.2	0.6 ±0.2
High TG mix	Paper discs	0.9 ±0.1	0.1 ±0	1.6 ±0.6	-	0.4 ±0.1	2.7 ±1.5	92.7 ±1.6	-
	Acetone wash*2	96.4 ±0.4	96.6 0.2	2.9 ±0.1	97.5 ±0.1	95.7 ±0.5	0.9 ±0.1	1.0 ±0.3	98.9 ±0.2
	1% H+/acetone wash*2	2.7 ±0.3	3.3 ±0.2	95.5 ±0.6	2.5 ±0.1	3.9 ±0.4	96.4 ±1.6	6.3 ±1.4	1.1 ±0.2

Note: The pH of the filter paper was 9.5. Acidified acetone was prepared with acetic acid in acetone (v/v). Results are based on triplicates.

It was revealed that 1% acidified acetone is considered the optimal concentration to release FFA from the alkaline collection paper, in both high FFA lipid mixture and the high TG lipid mixture (**Table 4.6.3**). Lowering the acid concentration of acetone weakened its ability to elute FFA from the collection paper (Table 4.6.3). The loss of a small percentage of FFA in the acetone wash and left on the paper discs occurred but can be corrected with addition of an internal standard, and therefore is not a major concern.

3) Reconstitution of FFA after extraction

After lipid class separation using the method described above, all TG (and its degraded product DG and MG) and CE were collected in the first two acetone washes while FFA was collected in the latter acidified acetone wash and PL was still bound to the collection paper. The plan was that all neutral lipids should then undergo the standard transmethylation process, from which I can calculate the amount of these neutral lipids with an internal standard. Then the underivatized FFA collected in the acidified acetone wash would then be dried, reconstituted and injected straight into a polyethylene glycol column designed specifically for separation and identification of FFA. This experiment was therefore conducted to determine the best way to reconstitute FFA after lipid extraction and separation.

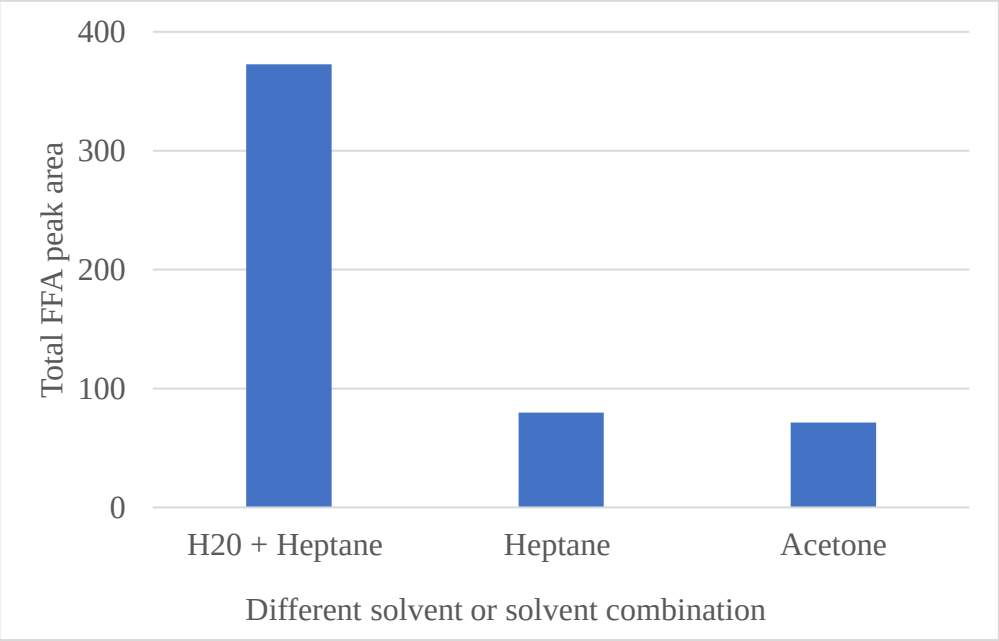

Figure 3.6.1. Re-solubilisation of free fatty acid with various solvent after being eluted from pre-treated silica gel paper (pH of 9.5) with acidified acetone (1% acetic acid in acetone, v/v).

It was observed that the peak area of total FFA recorded was the highest for those re-solublised in water plus a heptane system, rather than in heptane or acetone alone (**Figure 3.6.1**), however this number did not reveal whether or not all FFA had been counted. In this experiment I tested these two solvents only as they are the most used carrier solvents for GC system, any

other more polar solvents are likely to create back flush in the system and lead to contaminations between samples and possible damage to the column itself. It was suspected that the FFA eluted from the SG paper was protonated (due to the acidified acetone) and therefore could not be re-solubilised simply into a solvent. The protonated FFA molecules must be neutralised in ordered to be re-solubilised properly. Water is the preferred option over an alkaline solution that might in turn combine with FFA to create salt and therefore precipitate FFA and defeat the purpose of re-solubilisation. In addition, the idea of reconstitute FFA with water and heptane was inspired by our routine practice in the laboratory, where FAME derived from all lipids were extracted with water and heptane after transmethylation. The ratio between water and heptane is important, as increases in the volume of water would result in an increased volume of emulsified layer between heptane and water, This would not only lead to difficulties with heptane extraction, but also may trap some FFA in the emulsion layer and result in incomplete extraction. It was determined that the best ratio between water and heptane was 1:12, which was 50µL of water with 600µL of heptane that gives almost no emulsion layer and a relatively clear separation between the two layers.

It was observed that reconstituted FFA with water and heptane resulted in the highest total peak area when compared with the reconstituted FFA with heptane or acetone alone. However, there was no confirmation that all FFA had been reconstituted. In order to address this concern, I then undertook a series of EBM samples containing varying concentration of FFA to test the feasibility of this system.

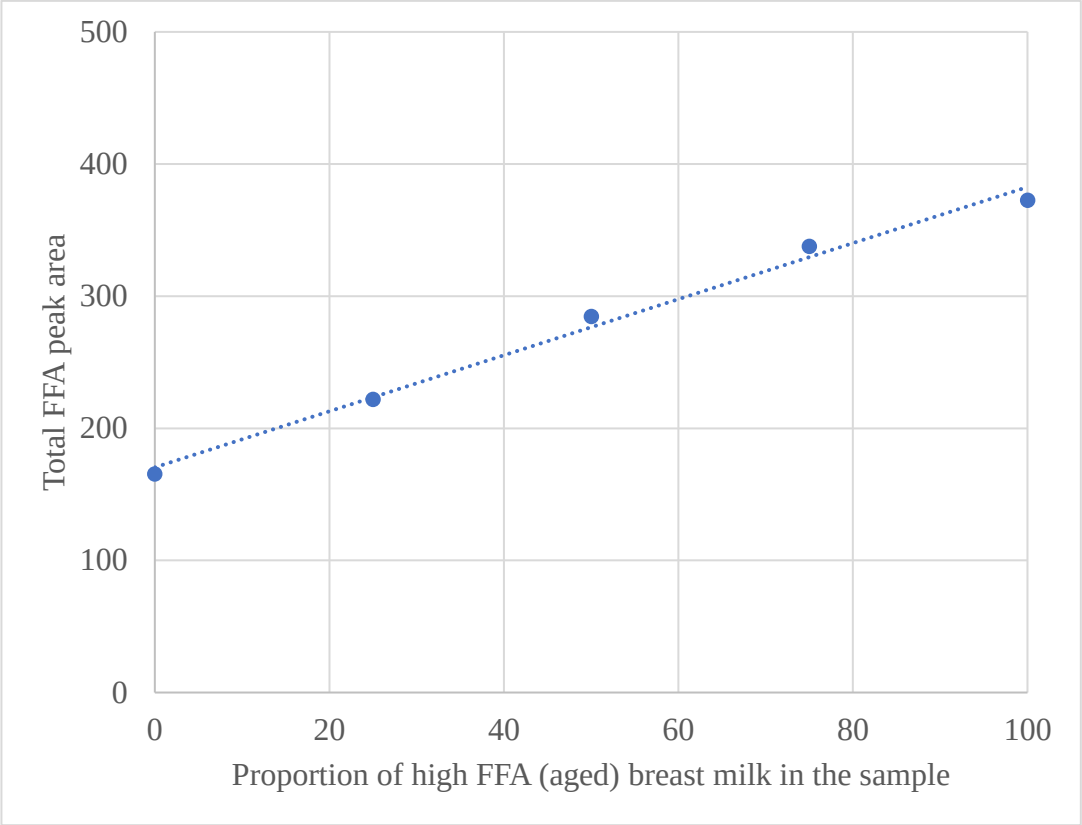

Figure 4.6.2. Relationship between the proportion of aged breast milk (v/v, %) in the sample and the free fatty acid peak area measured by GC-FID with polyethylene glycol column.

There was an almost linear relationship between the total FFA peak area of breast milk and the proportion of aged breast milk in the sample. It revealed that this system was precise enough to identify the difference in the concentration of FFA between different milk samples. However, this was still not adequate to answer the question of whether all FFA had been reconstituted.

Given all the data presented above, I believed that this system was a functioning system for separating and identifying FFA, however, there were two major concerns to be examined before being used further. The first was a test of the stability of the system at room temperature, and the second was to measure the accuracy of this system by lipid standards and cross validation with another method.

4) Stability of breast milk collected as dried spot on alkaline treated collection paper

While I continued to work on investigating how to determine whether all FFA was reconstituted after elution, I proceeded to tackle the next problem, the stability of DMS at room temperature. Before attempting this experiment, I had simply collected some human milk samples as dried spot and left it at room temperature and -20℃ freezer for two weeks to monitor the changes to its FFA concentration. To my surprise, the FFA concentration of samples stored at room temperature, but not in freezer, was elevated significantly. This was observed in both samples collected on alkaline treated paper with pH of 9.5, also samples collected on plain SG paper (as a control/reference). It was suspected that the hydrolysis of TG in human milk was due to the high pH of the paper which resulted in chemical breakdown. It was also possible that there was bacterial contamination of this donated milk sample which caused biological breakdown of TG. Cold storage in both cases could have supressed the rate of hydrolysis and therefore such elevation of FFA concentration was not seen in samples stored at -20℃ freezer. This was tested repeatedly, and I found that the most apparent changes to the FFA concentration of DMS occurred within the first week of storage in any testing condition, which seemed reached a plateau thereafter. Hence, in order to save time and speed up the progress, a 7-day storage period at room temperature was considered sufficient for determining whether there were any changes occur to the fat composition of human milk.

To assess whether the increase in FFA concentration of samples collected as DMS was due to chemical hydrolysis or bacterial contamination, the following experiment was planned. Three types of human milk (raw, holder pasteurised and boiled milk) was prepared from a single donor milk, any potential bacterial contamination could be destroyed by boiling or holder pasteurisation. These three types of milk were collected on SG paper that were plain (not coated with alkaline solution), coated with alkaline solution prior to collection or after

storage but prior to analysis. It was hoped that this combination of filter paper and different milk samples could reveal the source of TG breakdown.

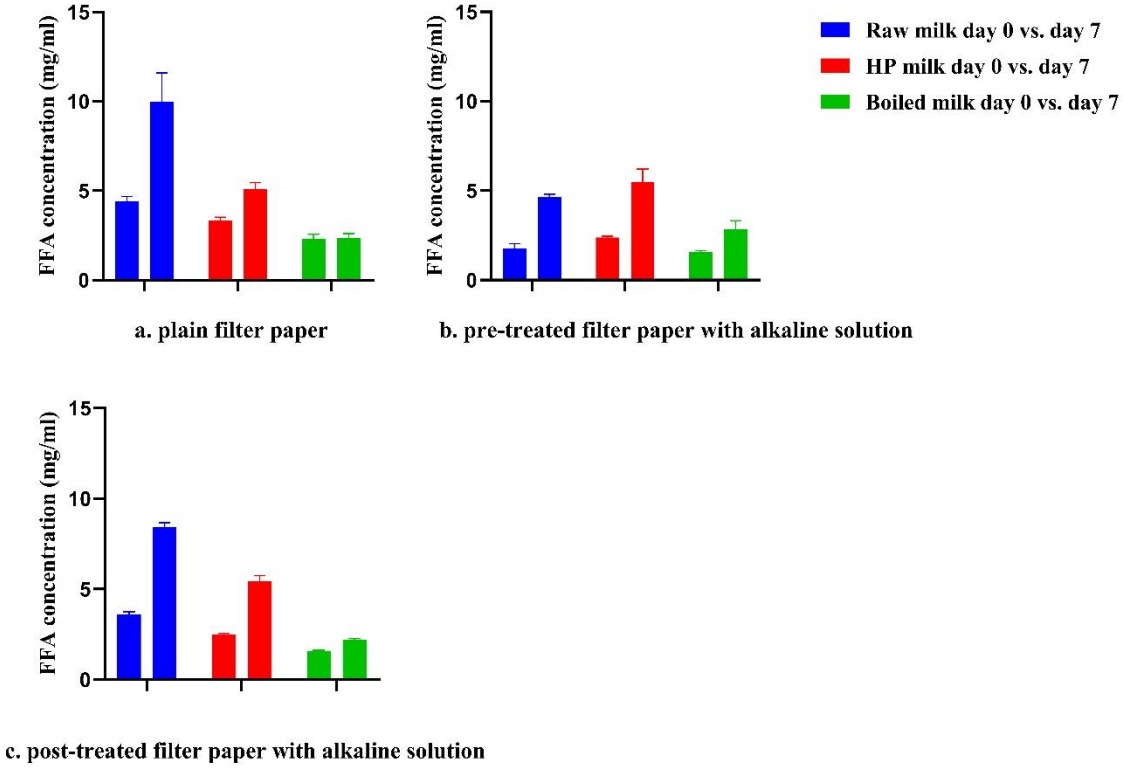

Figure 4.6.3. Changes to the free fatty acid concentration of raw, holder pasteurised and boiled breast milk collected on a. pre-treated alkaline paper with pH 9.5, or b. post-treated alkaline paper with pH 9.5, or c. on plain filter paper at baseline and 7 days after storage at room temperature. HP: holder pasteurised.

The concentration of FFA in raw breast milk collected on plain SG paper doubled after 7 days of storage at room temperature. In contrast, that of holder pasteurised milk and boiled milk increased slightly and remained unchanged, respectively. For milk samples collected on pre-treated SG paper, the FFA concentration of all breast milk, regardless of whether it was raw, holder pasteurised or boiled, all increased between baseline and 7 days but to a different extent. Notably, the FFA concentration in raw and holder pasteurised milk was slightly higher than the concentration in boiled milk at 7 days. An elevated concentration of FFA was observed in raw and pasteurised breast milk collected on post-treated alkaline paper, but not

in boiled milk collected on the same collection paper. There were two likely reasons for degradation of TG and hence an increase in the FFA concentration observed at 7 days. On plain SG paper, there was no chemical exposure, therefore, any changes to the FFA concentration of breast milk collected are likely due to biological breakdown as a result of lipase activity (raw milk) or bacterial contamination. Holder pasteurisation and boiling reduced or eliminated the potential sources of biological breakdown seen in raw milk, resulted in small or no increases in the FFA concentration. Whereas, the FFA concentration of boiled milk collected on pre-treated filter paper, which supposedly inactivates all lipases and eliminated all bacteria, also increased from 1.5 mg/ml to 2.8mg/ml, which was likely a result of chemical breakdown due to pH of the paper. No change was observed in the FFA concentration of boiled milk collected on post-treated alkaline paper, which might indicate that the chemical breakdown only happens when the sample has been stored for prolonged periods. Increasing in the FFA concentration of all types of milk collected on post-treated SG paper was likely due to biological breakdown of TG.

It was suspected that the pH of the paper was too high and hence resulted in chemical breakdown of TG. Therefore, the pH of the paper was further lowered to 8.5 which is the minimum level that achieves binding effect of FFA. However, in term of stability test, FFA concentration in boiled breast milk collected on SG paper with pH of 8.5 was increased significantly at 7-day post collection. Therefore, the stability of a mix of TG standard collected on SG paper varying in pH value was conducted at room temperature to determine whether the pH was responsible for breakdown of TG.

5) Stability of TG standard collected on alkaline treated collection paper

In previous section I explained how I discovered there was a ‘window of most apparent changes’ to human milk FFA concentration, which happened within the first seven days of collection. This part of experiment of stability of pure TG standards collected as dried spot at

room temperature went along before that discovery and was originally set for a 14-day storage. However, upon realising that changes should have occurred within the first seven days, I decided to terminate this stability test earlier than its due date, which resulted in a 12-day storage period at room temperature.

$$\%\, TG\ being\ liberated = \frac{Peak\ area\ of\ FAME\ formed\ in\ the\ acidified\ acetone\ wash}{Peak\ area\ of\ FAME\ formed\ in\ the\ acetone\ wash + in\ acidified\ acetone\ wash} * 100$$

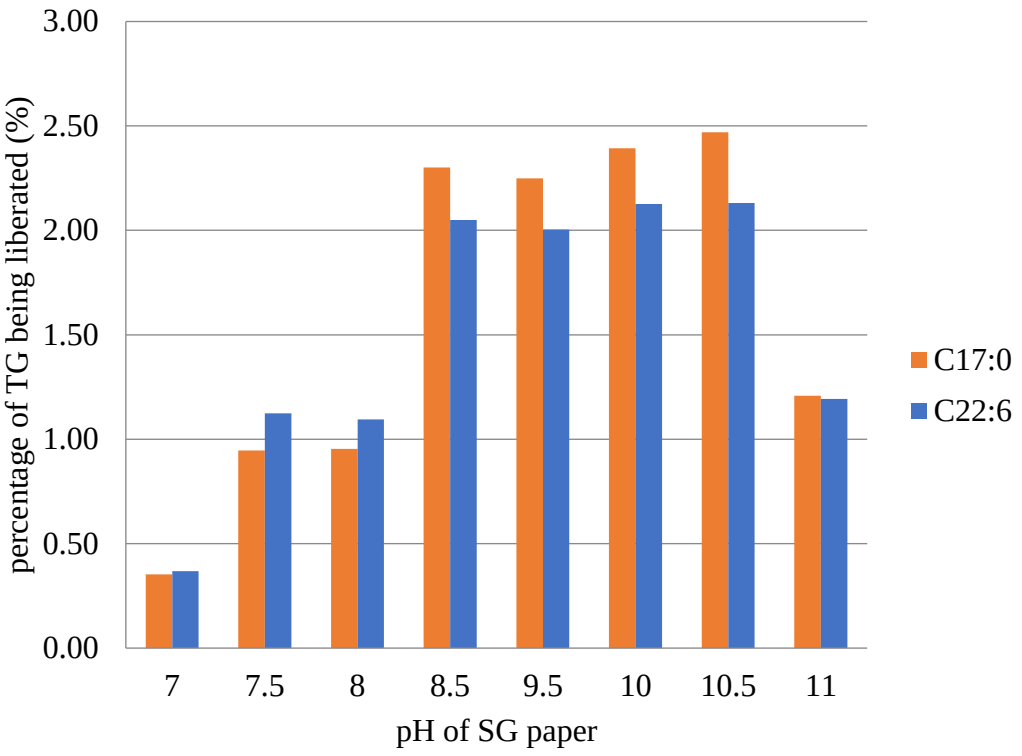

Figure 4.6.4. Percentage of triglyceride mix standard (C17:0 and C22:6, 5mg/ml each) collected on SG paper with various pH level being liberated during storage at room temperature for 12 days.

It was found that, using a mixed TG standard (C17:0 and C22:6 n-3, both in 5mg/ml), detection of FFA varied according to the pH level of the filter paper at the end of a 12-day storage. The results suggest that the breakdown of TG and liberation of its corresponding FFA

may be correlated with the pH of the paper. The release of FFA on the filter paper that had a neutral pH level could have been due to spontaneous hydrolysis at room temperature rather than due to the effect of pH.

4.6.4 Conclusion

This method was very close to success as it gets rid of the tailing of TG by binding FFA to the paper and therefore selective elution of TG was achieved; GC equipped with the polyethylene glycol column further purified the results as only FFA can be recognised by the machine. However, the chemically treated paper itself hydrolysed TG and resulted in instability of the milk fats collected on such paper which cannot be stabilised at room temperature for long periods of time. In addition, it was found that breast milk itself when collected as dried spot is still susceptible to TG breakdown due to the presence of milk lipases and possibly bacterial contamination. The final working system was further developed based on data gathered from these experiments, and by addressing unresolved questions regarding whether all FFA were resolubilised or can be resolubilised, and the optimal way to denature breast milk lipases or eliminate bacteria. This will be discussed in the next chapter.

Discussion and conclusion

To summarise the three essential characteristics of to support widespread use of this proposed filter paper-based method for measuring the free fatty acid concentration of human milk are: 1) requires a small volume of sample; 2) provides an accurate FFA measurement that is not contaminated by TG and 3) is stable at room temperature.

All the experiments described in this chapter were tested repeatedly, however for brevity, only results from a single set of experiment are presented. The majority of the methods described in this chapter failed to separate FFA from TG without contamination, except the very last method described in 4.6, which involved binding the FFA with alkaline treated filter paper and analysed on a polyethylene glycol column that only recognised underivatized FFA

and was not interrupted by the TG fraction. Therefore, only the method described in 4.6 progressed to stability testing. The use of polyethylene glycol column cannot be applied in any other methods described in section 4.1-4.5 because the FFA were methylated into FAME. Method described in section 4.4 eventually evolved into the successful method that will be described in the next chapter.

For most of the experiments presented in this chapter, the peak area was used as the parameter for various calculations (e.g. for the completion of transmethylation or elution), with the exception of the stability test of method 4.6, where an internal standard was used. Using peak area for calculation was the best measurement at the time to test the feasibility of each method. Nevertheless, it is acknowledged there were very small variations in peak area arising due to the instrument itself. Internal standards would have been a better option, however, most of the methods described in this chapter failed in the early stage of feasibility testing. Because of this and the need to run many trials for each method tested, the use of the peak area of individual lipids was a convenient and appropriate choice, that was sufficient for the purpose.

At the beginning, only 903 paper was used as the collection tool because it is the most used filter paper for collection in the field for inborn error screening in newborns. There have been several reports of using SG paper in dried blood or milk spot technique for the measurement of long chain polyunsaturated fatty acids [Gao *et al.* 2019; Liu *et al.* 2014;] and its metabolic product oxylipins [Hewawasam *et al.* 2019]. The silica gel coating of the filter paper offered additional binding strength with the polar lipid, PL in human milk. At the early stage of my research, PL was not considered the major source of contamination and therefore the use of SG seemed unnecessary. However, in later experiments, it was discovered that the SG paper used in combination with acetone as an elution solvent is capable of trapping PL and further purify the lipid extracts obtained from the DMS. SG paper was therefore the preferred

collection assay used in section 4.6. It was also later found that this combination resulted in the highest recovery rate of FFA from the DMS, which will be discussed in the next chapter.

From the stability tests conducted in the FFA binding method in 4.6, it was clear that there were two different possible reasons behind the TG breakdown, the chemical hydrolysis due to high pH of the filter paper and the lipolysis caused by bacteria and/or lipases. Chemical hydrolysis was associated with the alkaline coating (the fundamental component of this FFA binding method), which could have caused chemical breakdown of the TG collected on paper due to its high pH level. It is also possible that the milk was bacterially contaminated and hence the breakdown of TG could have been due to bacteria. Alternatively, the lipases that are present in breast milk were not stabilised by the fact that the collection of milk in a DMS format and may have still actively broke down TG. I looked further into investigating the source of TG degradation and potential means to prevent it, which will be discussed in the next chapter.

In conclusion, there were a number of challenges in developing this micro-sampling DMS system, notably, contamination by TG fats and the instability of milk fats caused by either bacterial contamination or endogenous lipases. All the unsuccessful methodologies described in this chapter ultimately shaped the final DMS system that will be described in **Chapter 5**. It is important to note that though methods recorded in this chapter failed to separate FFA from other lipids of breast milk without contamination from TG, these separation methods remain possible for other biological fluids where trace contamination from TG is negligible.

Chapter 5 A simple system for measuring the level of free fatty acid in human milk collected as dried milk spot

It has been repeatedly shown in the previous chapter that there were two major challenges involved in developing a dried milk spot method for measuring the FFA concentration of breast milk. These were 1) eliminate the contamination from TG for an accurate FFA measurement and 2) identify and eliminate the causes of lipolysis (likely due to lipases) to preserve the fat composition. Therefore, this chapter will describe how these two major issues were tackled and lead to a successful sensitive and precise DMS method, specifically for determining the FFA concentration of human milk, even at a tiny volume. This method has achieved long term stability even at room temperature by denaturing the lipases in human milk.

This chapter includes a manuscript in its published form:

Gao, C., Liu, G., McPhee, A.J., Miller, J., Gibson, R.A. (2020). A simple system for measuring the level of free fatty acids in human milk collected as dried milk spot. *Prostaglandins, Leukotrienes and Essential Fatty acids, 158*: 102035.

Statement of Authorship

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ii. permission is granted for the candidate in include the publication in the thesis; and
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Original research article

A simple system for measuring the level of free fatty acids in human milk collected as dried milk spot

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ABSTRACT

Breast milk dried on filter paper is a useful collection device for the study of breast milk because it avoids the costs associated with cold-chain storage and transportation. Although the fatty acid profile of breast milks as dried spots is stable, changes to the composition of lipid classes of breast milk due to lipase activity have been reported and are best reflected by its free fatty acid (FFA) concentration. This study aimed to develop a robust dried milk spot (DMS) system where fats in the breast milk are stable at room temperature, and the FFA concentration of the milk can be accurately measured without interference by the high level of triglyceride, which normally constitutes around 98% of the fats in fresh milk. Our system involves applying a small amount breast milk (20 μL) on silica gel impregnated filter paper and microwaving at high power to denature lipases. At the time of analysis, the milk fats are eluted with acetone, re-constituted in heptane and injected directly into a gas chromatograph equipped with an acid modified polyethylene glycol column. This DMS method was validated against the conventional TLC method across a range of FFA concentrations. The breast milk fats collected using this DMS system are stable at room temperature for at least eight weeks which allows for transportation by post and has the potential for use in multi-centred international clinical trials.

1. Summary

Current dried milk spot (DMS) technology is limited to measuring the fatty acid composition of the total fats in breast milk, but not its free fatty acid (FFA) concentration. This study describes a new DMS system whereby milk fats can be stabilised at room temperature for long periods and the level of FFA can be accurately measured in the presence of other fats. Breast milk fats were stabilised by inactivating endogenous lipases via microwaving; the fats were then eluted, re-constituted and injected into gas chromatography equipped with an acid modified polyethylene glycol column that specifically detects the FFA without interference from the triglycerides. This DMS method enables faster throughput and more precise measurement of breast milk FFA concentration than via the conventional thin layer chromatography method, and is an efficient tool for clinical research and commercial uses.

2. Introduction

With the increased use of donor human milk through milk banks and neonatal units in recent years, changes to the composition of breast milk during storage, processing and transportation have received increased attention with several systematic reviews dedicated to the topic [1–6]. Breast milk fats represent 50% of the energy of breast milk and are of particular interest as they are essential for the optimal growth and development of infants [7,8]; however, they appear to be particularly sensitive to different storage and handling processes [5,6].

Breast milk fats are in four major forms: triglyceride (TG), which constitutes around 98% of the fats, while the remainder exist as free fatty acid (FFA), phospholipids and cholesterol esters [8]. Breast milk contains a number of lipases that aid infant digestion [9,10], but which may break down breast milk TG and liberate FFA during various processing steps and storage conditions [5,11,12]. FFA levels are usually very low in fresh breast milk; therefore their elevated presence is

Abbreviations: DMS, dried milk spot; FAME, fatty acid methyl ester; FFA, free fatty acid; FFAP, free fatty acid phase; GC-FID, gas chromatography with flame ionization detector; MTBE, methyl-tert-butyl ether; PEG, polyethylene glycol; TG, triglyceride; TLC, thin layer chromatography

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considered a sensitive indicator of changes to the breast milk fats. Breakdown of a single TG molecule results in liberation of four molecules (one glycerol backbone with three fatty acids) and hence may increase the osmolality of breast milk, which may have clinically significant impact on preterm infants fed with such milk.

To date, thin-layer chromatography (TLC) is still the most commonly used method for separation and quantification of FFA in breast milk and other biological fluids, though high performance liquid chromatography [13–15] and enzymatic kits are available. However, the limitation of the TLC method is that the dominant TG fraction can contaminate the FFA fraction due to tailing on the TLC plate during separation, especially when the TLC plate is overloaded. We set the goal of developing a tool for measuring the FFA in breast milk accurately after collection as in a dried milk spot (DMS). The use of dried spot technology in large clinical trials in recent years has shown many benefits in term of ease of sample collection, less expense required for transportation and less space required for storage [16,17]. However, the biological breakdown of TG during storage at different conditions has been reported, either due to enzymatic reaction of lipases or bacterial contamination; hence any DMS system needs to be able to stabilise the fats for long periods to allow transportation to central laboratories.

The aim of this study was to develop a robust DMS system that was stable at room temperature for long periods and allowed the accurate detection of FFA in breast milk without interference from TG.

3. Materials and methods

3.1. Subjects

A single breast milk sample (50 ml) from a single donor was used to establish the system and test its stability. The sample was previously frozen at −15 °C in a home freezer, and was thawed and apportioned into 40 parts and stored at −20 °C on arrival at the laboratory. Another 20 breast milk samples were sourced from the Australian Red Cross Blood Service Milk Bank for the purpose of method validation; each breast milk sample was divided and stored at −20 °C prior to analysis.

Ethical approval for developing and validating novel methods for measuring nutrients in biological fluids was obtained from the University of Adelaide Human Research Ethic Committee (H-2016-088), and relevant approval from the Milk Bank was granted by the Australian Red Cross Blood Service (18-09SA-13).

3.2. Development of method for separating FFA in DMS system

3.2.1. Separation and identification of FFA on polyethylene glycol (PEG) column

Twelve FFA standard between C12:0 and C22:6 n-3 (1.5 mg/ml, Nu-Check Prep, Inc., MN, USA) commonly found in breast milk were separated on an acid modified PEG column (BP21 (Free fatty acid phase, FFAP), Trajan Scientific Australia Pty Ltd) and readily detected by gas chromatograph equipped with flame ionization detector (GC-FID) (Fig. 1a). This column was specifically designed for FFA separation, therefore the TG molecule (C13:0, 1.65 mg/ml, Nu-Check Prep, Inc., MN, USA) is not detected by the FID (Fig. 1b). The presence of TG in the lipid mixture of FFA and TG (1:1.5 v/v) did not interfere the separation of FFA (Fig. 1c).

3.2.2. Selection of paper

Two collection papers were tested: the most commonly used Whatman 903 specimen collection paper for dried spot test in the clinical field (903), and the silica gel (SG) impregnated ion exchange paper used for dried blood spot and DMS analysis published previously (Whatman Grade SG81, Whatman, Buckingham, UK) [16–18].

3.2.3. Fats elution efficiency from different filter papers

A mixture of FFA standard or breast milk samples were collected on 903 and SG paper and were eluted with various solvents to determine the best combination for extracting fats from filter papers.

Briefly, 20 μL of the FFA standard mix or breast milk was collected on the paper spots, and air-dried for three hours prior to elution. The FFA or milk fats were eluted twice with 1 ml washes of solvents (acetone, heptane, chloroform, methyl-tert-butyl ether, methanol, petroleum spirit, and propanol), by soaking the paper spots in the solvent for 10 min. The two solvent washes were then combined and dried under a steady nitrogen flow, and the FFA or milk fats were reconstituted with 100 μL heptane. All samples were analysed in triplicate with internal standards.

3.2.4. Linearity

A mixture of FFA standards were prepared at 0.5, 1, 1.5, 2 and 3 mg/ml, which were either directly injected into the column or applied on SG paper and then eluted, dried and re-constituted as described above. A series of breast milk samples with a range of FFA concentrations were prepared by mixing an aged breast milk (high FFA concentration) that was thawed and refrigerated at 5 °C for 3 days with a freshly thawed breast milk. The proportion of the aged breast milk accounted for 0, 25%, 50%, 75% and 100% of the mixed samples (v/v, %). Then, 20 μL of breast milk was collected on SG paper from each of these prepared samples, air-dried, and the milk fats eluted with acetone and re-constituted with heptane as described above. All samples were analysed in triplicate with internal standard.

3.3. Stability of the system

Various approaches were attempted to inactivate lipase or bacteria in order to stop biological breakdown of TG and achieve long-term stability of the DMS system.

3.3.1. Temperature treatments

For control, a triheptadecanoin standard (TG C17:0, 3.54 mg/ml, Nu-Check Prep, Inc., MN, USA) was collected on its own on SG paper with/without the heat treatment at 100 °C for 15 min. The TG (C17:0) was also collected on SG paper mixed with either raw or boiled breast milk, or mixed with raw breast milk followed by conventional heating at 100 °C for 15 min.

3.3.2. Antibiotics

Breast milk was collected on SG paper that was impregnated with antibiotics (Ibilex 500, Cephalexin Capsules, Alphapharm Pty Ltd.) at 1.25 mg/ml and 25 mg/ml in methanol.

3.3.3. Salt and alcohol

Breast milk was collected on SG paper followed by immediately over-spotting 20 μL of saturated ammonium sulphate, acetone, methanol, or propanol.

3.3.4. Microwave

Breast milk was collected on SG paper and then microwaved at high power for one, two or three minutes (NN-S235WF, 5.4A, 50 Hz, 800 W, Panasonic Home Appliance Co., Ltd.).

To understand the changes to lipid classes and its effect on the total fatty acid composition of DMS, milk fats of those DMS microwaved at high power for three minutes were re-constituted milk and collected after FFA analysis. The fats were then transmethylated in 2 ml of 1% sulphuric acid in methanol for three hours, with the resulting fatty acid methyl esters (FAME) extracted with heptane and water. A total of 11 fatty acids between C12:0 and C22:6 n-3 were reported as weight% of total fatty acids.

For all stability tests, baseline analysis was carried out after the filter papers had air-dried completely, which was either 3 h after the

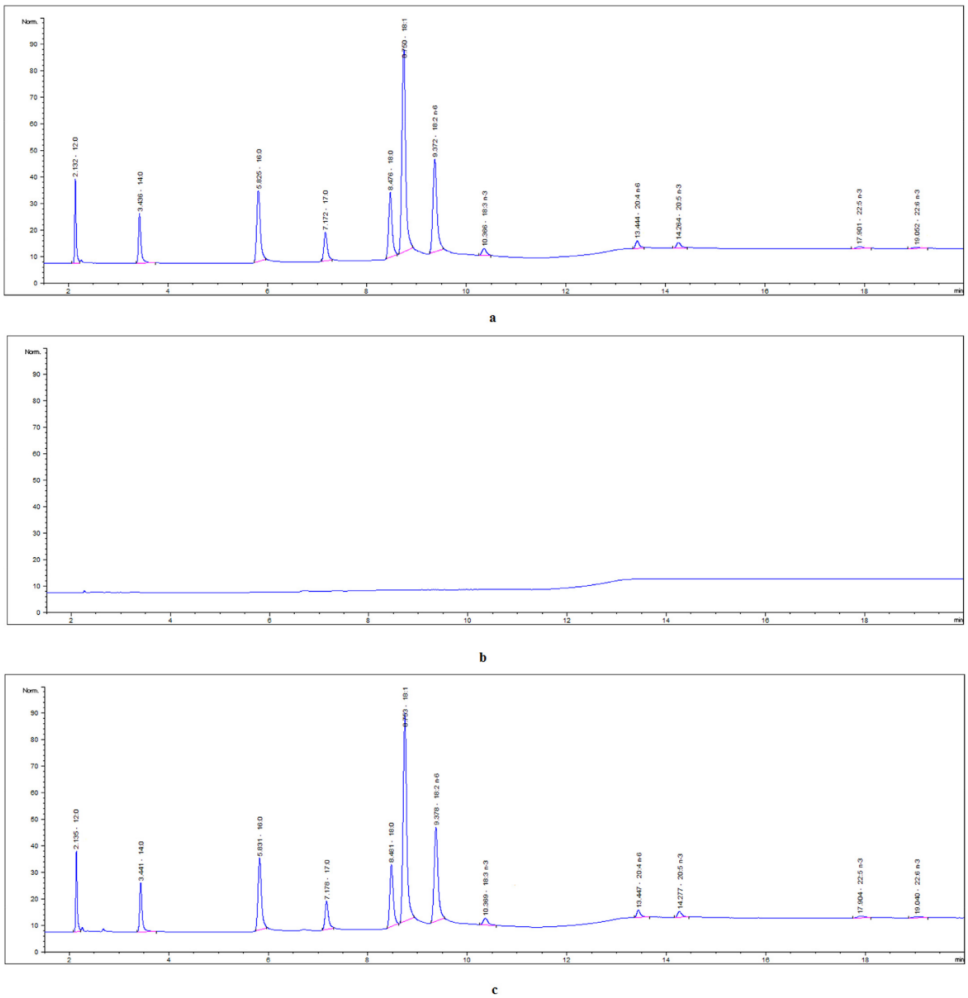

Fig. 1. The gas chromatograph of twelve free fatty acid (FFA) standards between C12:0 and C22:6 n-3 (a); a single TG standard C13:0 (b); and a mix of the FFA standards and the single TG standard that has a matched the concentration in a&b (c).

breast milk was collected on the papers or after heat or microwave treatment was completed. The rest of the samples were packed in cellophane bags and then kept in an aluminium bag with desiccant at either room temperature or −20 °C freezer for subsequent analysis after 1 and 2 week(s), and/or 4 and 8 weeks. All experimental groups were analysed in triplicate with internal standard.

3.4. Laboratory method validation against thin layer chromatography

The new system for using DMS method to measure FFA concentration was validated against conventional TLC separation using 30 breast milk samples that varied in FFA concertation (low, medium and high, $n = 10$ for each group). Preparation of these breast milk samples were as following. All 20 breast milk samples from the Milk Bank were divided into two, one half ($n = 10$) was thawed and left at room temperature for four days to deliberately increase its FFA concentration (high FFA), and the other half ($n = 10$) remained frozen until analysis (low FFA). Samples from the two groups were then mixed with its counterparts in a 1:1 ratio to create a group of samples with intermediate FFA concentration (medium FFA $n = 10$).

For the conventional TLC method, fat was extracted from 200 μL of breast milk according to a modified Folch method [19] with methanol

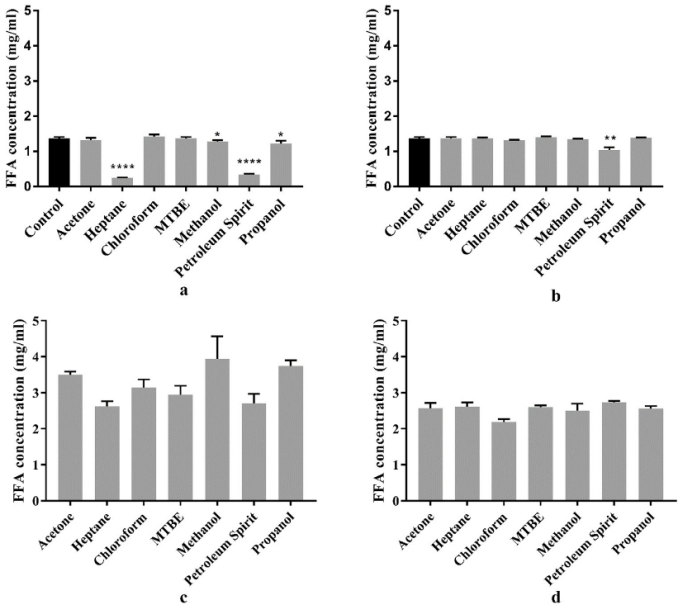

Fig. 2. Elution of mixture of free fatty acid (FFA) standards collected on a. silica gel paper and b. 903 paper and elution of FFA of breast milk collected on c. silica gel paper and d. 903 paper. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$ as compared to the control. MTBE: methyl-tert-butyl ether.

and chloroform (1:2 ratio, v/v) with internal standard. The extracted fats were dried and re-constitute in 1 ml of 9:1 chloroform: methanol and 100 μL was spotted on TLC plate, the lipid classes separation was achieved with the petroleum spirit, diethyl ether and acetic acid (180:30:2 v/v). The FFA band was visualized by UV lamp, and scrapped off and transmethylated in 1% sulphuric acid in methanol for 3 h at 70 °C. The resulting FAME were extracted with water and heptane.

For the DMS method, 20 μL of breast milk was collected on a pre-made collection card, which was then microwaved at high power for 3 min. The paper spot was excised with clean scissors and placed into 6 ml scintillation vial with internal standard, the fats were then eluted and re-constituted as described above.

3.5. Gas chromatography

FAME and un-derivatised FFA were separated and identified using GC-FID (Agilent Technology 7890B, Santa Clara, CA, USA; Hewlett-Packard 6890, Palo Alto, CA, USA, respectively). The GC-FID for FFA analysis was equipped with a BP21 (FFAP) PEG column 10 m × 0.25 mm, film thickness of 0.25 μm (Trajan Scientific Australia Pty Ltd), with a temperature ramp form 180 °C to 240 °C, helium flow of 3 ml/minute and split ratio of 20:1. A BPX70 column was used for GC-FID for FAME analysis, which was 30 m × 0.25 mm, film thickness of 0.25 μm (Trajan Scientific Australia Pty Ltd). The temperature ramp was set to be 140–240 °C, and helium flow was at 2 ml/min and split ratio of 20:1. The FFA and fatty acids in samples were identified based on the retention time and peak area values of commercial FFA and FAME standards (Nu-Check Prep, Inc., MN, USA).

3.6. Statistical analysis

FFA concentration and its composition was expressed as mg/ml and weight percentage of total FFA with mean and standard deviation, respectively. Comparison of the elution efficiency between control and various solvents was conducted using *t*-test. The correlation between DMS and TLC method for FFA profiling was assessed using Pearson's correlation. The statistical analyses were conducted using GraphPad Prism (version 7.02, GraphPad Software, San Diego, CA, USA) and SPSS (version 24.0, IBM Corporation, New York, USA) with significance threshold set at 0.05.

4. Results and discussion

4.1. Elution efficiency

Overall, it was found that SG paper had higher elution efficiency with less fats of breast milk retained on the paper spot as compared to 903 paper. The solvent acetone performed consistently well for eluting both FFA standards and breast milk fats collected on SG paper with relatively small standard deviation compared to other solvents (Fig. 2) and hence was the solvent of choice.

4.2. Linearity

There was a close relationship between levels of FFA detected, regardless of whether the samples were directly injected into the GC or applied to a dried spot prior to extraction and injection ($r = 0.999$ for both, $P < 0.0001$, Fig. 3a). This further proved that SG paper has minimal matrix effect as little or none fats were retained on the spots after elution. The relationship between the proportion of aged breast

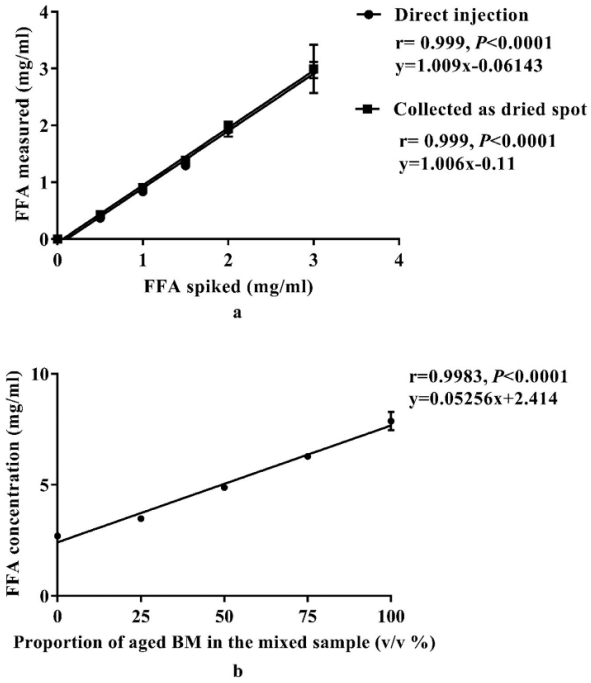

Fig. 3. Relationship between the free fatty acid (FFA) mix standard spiked and the FFA concentration measured by the system for both direct injection or applied to dried spot prior to elution and reconstitution (a.); relationship between the proportion of aged breast milk (BM) in the mixed sample and its FFA concentration measured by dried milk spot method (b.).

milk (containing higher levels of FFA) in the mixed sample and the FFA concentration measured using DMS method was highly correlated (r = 0.994 and $P < 0.0001$; Fig. 3b). This method of applying breast milk on to a filter paper, and eluting and reconstituting the milk fats before injecting into an acid modified PEG column is capable of accurately measuring various levels of FFA in breast milk.

4.3. Stability

It proved difficult to stabilise the fats of breast milk collected as DMS to avoid biological breakdown of TG to FFA in breast milk for longer-term storage at room temperature (Fig. 4)

4.3.1. Temperature treatment

The FFA concentration of boiled milk was lower than that of raw milk at baseline at the end of 3-h air-drying (Fig. 4a). Increases in FFA concentration were observed in raw breast milk collected on SG paper from 2.1 ± 0.2 mg/ml to 4.9 ± 0.06 mg/ml, and raw breast milk collected on SG paper and heated treated at 100 °C for 15 min from 1.9 ± 0.15 mg/ml to 3.3 ± 0.39 mg/ml, at 14 days post-collection (Fig. 4a). In contrast, there was no changes to the FFA concentration of boiled milk stored at room temperature or raw milk stored at −20 °C for 14 days (Fig. 4a).

A TG standard (C17:0) was spiked on SG paper with breast milk to determine whether the rise in FFA concentration was due to endogenous factors of the breast milk rather the contaminations during experiments. There was no C17:0 FFA derived from TG standard detected at baseline or during storage at room temperature for 14 days when TG standard was collected on its own or with boiled breast milk stored at room temperature, regardless of the heat treatment following collection (Fig. 4b). This showed that there was no spontaneous breakdown of TG collected on SG paper or that due to the presence of boiled breast milk. Although TG standard mixed with raw breast milk was heat treated at 100 °C after collection, there was still apparent increase to the C17:0 FFA concentration after 14-day room temperature storage (Fig. 4b). Low level of C17:0 FFA was found in TG samples spotted with raw milk at baseline, the concentration did not increase when the sample was stored at −20 °C freezer, but the concentration was increased from 0.06 ± 0.01 mg/ml to 0.37 ± 0.03 mg/ml when stored at room temperature (Fig. 4b). This indicated that the breakdown of TG might have initiated early in the collection stage and progressed during storage. Overall, it seems that the rise in levels of FFA seen after storage is likely due to endogenous factors of breast milk rather than contamination during experiment, whether it was enzymatic or bacterial contamination; both are supressed by low temperature storage or boiling.

We obtained inconsistent results when we attempted to negate lipase activity by simply placing the milk spots in an oven at 100 °C for 15 min. This was despite the complete absence of FFA accumulation when milk samples had been boiled. This anomaly may have been due to the poor heat conductibility of paper/air, which may only partially denature the lipase present in DMS when heated in oven. This is in line

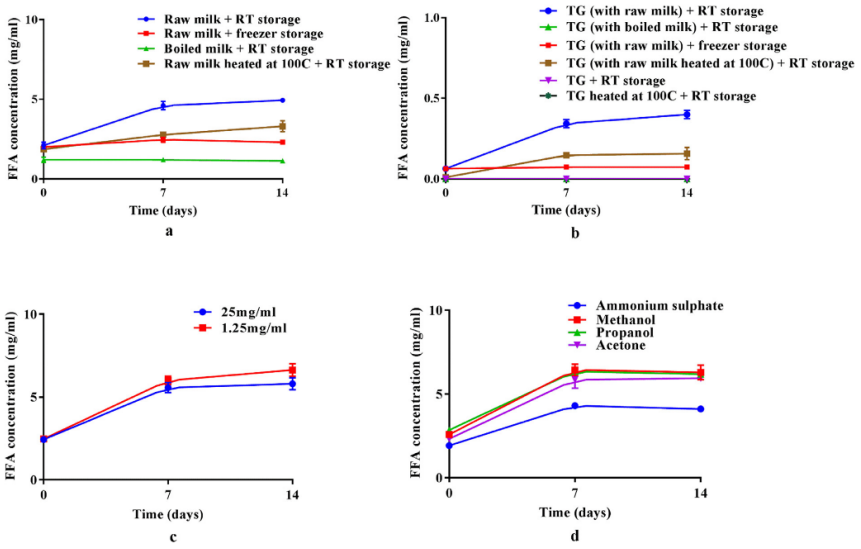

Fig. 4. Changes to the concentration of free fatty acids (FFA, mg/ml) of a. boiled or raw breast milk collected on silica gel paper and followed by a heat treatment at 100 °C for 15 min and stored at room temperature or −20 °C freezer; b. derived from triglyceride C17:0 standard collected on silica gel paper alone or with raw or boiled milk and stored at room temperature or −20 freezer; c. breast milk collected on SG paper impregnated with antibiotics at two different concentrations; d. breast milk collected on SG paper followed by addition of various salt or alcohols. RT: room temperature; TG: triglyceride.

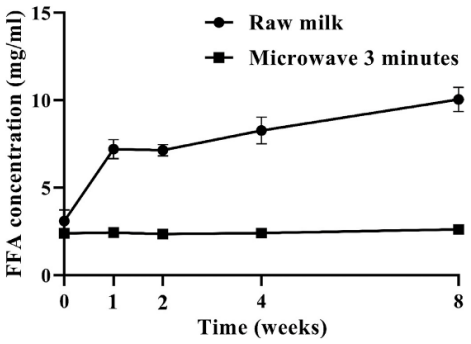

Fig. 5. Changes to the concentration of free fatty acid (FFA) of breast milk collected as dried spot and microwaved at higher power for 3 min and stored at room temperature for 8 weeks.

with a study reported air heated Holder Pasteurisation (HP) process is not as efficient as water-heated HP due to poor homogenous heat flow with air [20]. Given that heating in air for long periods increased the likelihood of oxidation of polyunsaturated long chain fatty acids, we decided not to proceed with oven heating as a method for stabilising milk lipids.

4.3.2. Antibiotics and salt/alcohol

In an further attempts to precipitate lipases or to supress potential bacterial activity, the levels of FFA of breast milk collected on SG paper treated with antibiotics and various salt, alcohols and acetone increased significantly after 14 days of storage at room temperature from approximately 2.5 mg/ml to 6–7 mg/ml (Fig. 4c and d). Although salts and alcohols had little effect on FFA accumulation, these results gave credence to the hypothesis that accumulation of FFA was not due to bacterial contamination, and we concluded that endogenous lipase acting on TGs were more likely the causal agents of FFA accumulation.

4.3.3. Microwave

We then further tested the effect of microwaving DMS samples in order to deactivate lipases. Increases in FFA concentration were observed in breast milk collected on SG paper and microwaved at high power for one and two minutes, but not for three minutes and then stored at room temperature for 7 days (Supplementary Figure 1.). The FFA concentration of breast milk collected as dried spot and microwaved at high power for three minutes remained stable during storage for 8 weeks at room temperature (Fig. 5).

An increase in the FFA concentration in raw milk collected as dried spot did not change the total fatty acid composition of the milk (Table 1) This is in agreement with previous reports suggesting that total fatty acid composition of breast milk remains unchanged through various storage and pasteurisation procedures while its FFA [5] and FFA metabolites [21,22] concentration increased significantly.

4.4. Comparison of new DMS system vs. TLC method

There was a significant correlation ($r = 0.983$) between the FFA concentration measured by the DMS method and FFA measured by the TLC method over a range between 0.3 mg/ml and 10.6 mg/ml, $P < 0.0001$ (Fig. 6). The levels of FFA measured by TLC were consistently higher than the new DMS method, which is likely a result of tailing of TG on the TLC plate. FFA composition measured by the DMS method and TLC method displayed strong correlations among most of

Table 1
The free fatty acid concentration (mg/ml) and total fatty acid composition (% of total fatty acid) of breast milk collected on silica gel paper and stored at room temperature for 8 weeks.

	Baseline		1 week		2 week		4 week		8 week	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD
FFA concentration (mg/ml)	3.09	0.64	7.21	0.54	7.15	0.33	8.27	0.77	10.04	0.69
Total SFA	42.82	0.10	42.45	0.30	42.64	0.06	41.85	0.11	42.05	0.19
12:0	7.84	0.23	7.45	0.04	7.45	0.11	7.13	0.23	7.53	0.43
14:0	5.30	0.05	5.21	0.04	5.24	0.02	5.17	0.03	5.23	0.01
16:0	23.97	0.10	23.92	0.07	24.04	0.05	23.72	0.12	23.42	0.19
18:0	5.70	0.08	5.87	0.30	5.91	0.07	5.83	0.03	5.88	0.08
Total MUFA	35.08	0.19	35.52	0.13	35.79	0.04	36.06	0.10	35.76	0.18
18:1 n-9	35.08	0.19	35.52	0.13	35.79	0.04	36.06	0.10	35.76	0.18
Total n-6 PUFA	19.37	0.06	19.33	0.14	19.16	0.03	19.55	0.02	19.44	0.13
18:2 n-6	18.88	0.06	18.88	0.11	18.72	0.03	19.09	0.02	18.99	0.14
20:4 n-6	0.49	0.00	0.45	0.04	0.43	0.00	0.46	0.01	0.46	0.01
Total n-3 PUFA	2.74	0.04	2.70	0.02	2.41	0.01	2.55	0.01	2.76	0.06
18:3 n-3	1.85	0.02	1.84	0.01	1.78	0.00	1.83	0.00	1.82	0.05
20:5 n-3	0.13	0.01	0.14	0.01	0.13	0.01	0.13	0.01	0.12	0.02
22:5 n-3	0.23	0.01	0.22	0.01	0.16	0.00	0.19	0.00	0.28	0.03
22:6 n-3	0.51	0.01	0.50	0.00	0.34	0.01	0.40	0.01	0.52	0.03

FFA: free fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid: PUFA: polyunsaturated fatty acid.
Data are presented with mean and standard deviation.

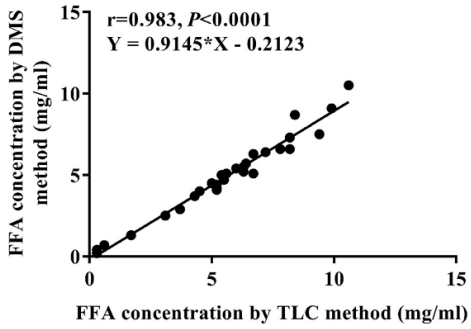

Fig. 6. Correlation between the free fatty acid (FFA) concentrations of breast milk measured by DMS method vs. TLC method. DMS: dried milk spot; TLC: thin-layer chromatography.

the FFA with correlation coefficient greater than 0.8, except for C12:0, C16:0 and C18:0, where the correlation coefficient was 0.718, 0.764 and 0.646, respectively (Table 2).

4.5. Significance and future research

Previously reported DMS technology has been limited to the ability of measuring the total fatty acid composition of breast milk, and it was assumed there was no change to the milk fats from the activity of endogenous lipases. Clearly, our results indicated that TG in breast milk are liberated into FFA, presumably via endogenous lipases, but this activity can be eliminated. To the best of our knowledge, this is the first report on a DMS system that stabilises milk fats at room temperature for long periods and separates FFA from TG without contamination; this extends the usefulness of DMS technology.

There is a general acknowledgement that mothers own breast milk is the food-of-choice for all infants. For the preterm infant who cannot suckle, mothers need to express their milk for delivery by gavage to their infants. This often involves expression at home, transporting to the neonatal intensive care units, storage in refrigerator and subsequent warming and delivery to infants from syringe pumps over a 2–4 h period. The FFA concentration and osmolality of such milk may have

Table 2
Correlation between the free fatty acid profile (weight% total FFA) by TLC and DMS method (N = 30).

	TLC method	DMS method	Correlation coefficient (r)	P
	Mean	STD	Mean	STD
Total SFA	25.0 ± 7.9	23.7 ± 6.8	0.865	< 0.0001
12:0	9.7 ± 2.4	8.1 ± 2.4	0.718	< 0.0001
14:0	4.1 ± 1.7	4.1 ± 1.2	0.932	< 0.0001
16:0	8.4 ± 3.8	7.7 ± 3.0	0.764	< 0.0001
18:0	2.9 ± 1.3	3.8 ± 3.1	0.646	< 0.0001
Total MUFA	49.0 ± 4	53.2 ± 3.2	0.817	< 0.0001
18:1 n-9	49.0 ± 4	53.2 ± 3.2	0.817	< 0.0001
Total n-6 PUFA	22.4 ± 5.3	20.3 ± 5.3	0.941	< 0.0001
18:2 n-6	21.4 ± 5.1	19.5 ± 5.2	0.943	< 0.0001
20:4 n-6	1.0 ± 0.6	0.8 ± 0.4	0.934	< 0.0001
Total n-3 PUFA	3.6 ± 1.4	2.8 ± 1.2	0.953	< 0.0001
18:3 n-3	2.6 ± 1.1	2.3 ± 1.0	0.954	< 0.0001
20:5 n-3	0.2 ± 0.1	0.2 ± 0.1	0.858	< 0.0001
22:5 n-3	0.4 ± 0.2	0.2 ± 0.1	0.891	< 0.0001
22:6 n-3	0.4 ± 0.2	0.2 ± 0.1	0.803	< 0.0001

DMS: dried milk spot; TLC: thin layer chromatography; FFA: free fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid: PUFA: polyunsaturated fatty acid.

increased as a result of lipase activity, and studies that determine the impact of such milk on infant feeding are warranted. This newly reported DMS technology offers an easy tracking system to capture changes to the milk fats in this particular setting. Most importantly, this DMS methodology requires only a small volume (20 μL), so that multiple samples can be taken without compromising the milk supply to the infant. The processing of pasteurised donor human milk is further complicated by HP and multiple freeze and thaw cycles, so this DMS technology may be useful for Milk Banks or other related entities for 'real time' monitoring the FFA levels in breast milk.

4.6. Strength and limitation

The DMS strategy described in this method has several advantages over the conventional TLC method for separating and measuring FFA in breast milk, including high precision with smaller sample volumes, higher throughput and lower cost. Most importantly, our system stabilised breast milk fats at room temperature. The use of microwave to

denature lipase in breast milk is feasible in most clinical settings and can be replicated easily.

In conclusion, we established a simple system for stabilising the fats and measuring FFA from breast milk collected as dried spots using GC equipped with an acid modified PEG (BP21) column. As with liquid breast milk, fats in milk collected as dried spots can be subject to changes during storage due to the activity of endogenous lipases. The fats of DMS can be stabilised by microwaving and then be stored at room temperature for at least 8 weeks without any changes to its FFA concentration. Results from this study further strengthen the body of evidence that the total fatty acid composition of breast milk is independent of its lipid classes and not interfered by lipases activity. Increased FFA concentration and osmolality of breast milk as a result of lipase action may be clinically important to preterm infants, which requires further investigation. This DMS technology requires small volumes of sample, is stable at room temperature for long periods may be useful in the clinical field for monitoring the quality of milk fats in donor milk from milk bank or other clinical studies.

CRediT authorship contribution statement

Chang Gao: Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Validation. **Ge Liu:** Conceptualization, Data curation, Writing - review & editing, Validation. **Andrew J. McPhee:** Conceptualization, Data curation, Writing - review & editing. **Jaqueline Miller:** Conceptualization, Data curation, Writing - review & editing. **Robert A. Gibson:** Conceptualization, Data curation, Writing - review & editing, Validation.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2019.102035.

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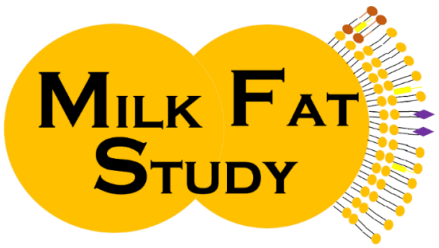
Chapter 6 Free fatty acid concentration of expressed breast milk used in neonatal nursery

Elevated FFA concentration has been associated with prolonged storage and handling process due to the presence of lipases in human milk. However, there is lack of data on the endogenous concentration of FFA in human milk prior to any storage and handling to define the magnitude of changes that may have occurred, primarily due to lack of suitable technique. With the dried milk spot method described in previous chapter, I was able to collect a wide range of samples from the neonatal units of one of the local hospital to describe the common levels and variations of FFA in human milk that were freshly expressed and collected at cot side, and that of human milk collected at home and brought into the unit for use.



(The study logo used in the neonatal units)

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Contribution to the Paper	CG was responsible for trial designing, sample collection and analysis, data analysis and interpretation, drafting the original manuscript and critically reviewing the manuscript for final publication.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

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Free Fatty Acid Concentration in Expressed Breast Milk Used in Neonatal Intensive Care Units

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Abstract

Preterm and sick term infants are commonly fed with expressed breast milk (EBM) that has been subjected to various storage and handling conditions before feeding that may cause lipase-mediated elevation of free fatty acids (FFA). This study was designed to describe the variation, between mothers' and within the same mother over time, in the concentration of FFA in EBM used in an Australian neonatal unit. A total of 256 EBM samples, 149 freshly expressed in the unit cot-side and 87 expressed at home and brought in to the unit, were collected from 32 mothers with an infant admitted to the neonatal intensive and/or special care units at the Women's and Children's Hospital, Adelaide. Among the fresh EBM samples collected cot-side, the average total fat content was 29.78 ± 9.28 mg/mL, and the FFA concentration was 1.70% of total fats (interquartile range [IQR]: 1.17–2.37%). Among the 10 mothers who provided fresh EBM at different stages of lactation, the concentration of FFA remained low overall, with some day-to-day variation (min 0.58% and max 5.0% of total fats within the same mother). The average total fat content of home collected EBM was similar to the cot-side collected samples, at 27.37 ± 8.23 mg/mL, and the FFA concentration was slightly higher at 2.49% of total fats (IQR: 1.74–3.29%). Overall, the FFA concentration of breast milk in the neonatal unit before and even after a short period of cold storage and handling is universally low.

Keywords: expressed breast milk, free fatty acids, preterm infants, neonatal intensive care unit, dried milk spot

Introduction

HUMAN MILK, FROM an infant's own mother or, if unavailable, milk from a donor, is the preferred food for infants.[1] Human milk is especially critical for infants born preterm (<37 weeks' gestation). A recent meta-analysis found that an exclusive human milk diet for preterm infants reduces the risk of necrotizing enterocolitis and may also be beneficial for late-onset sepsis and retinopathy of prematurity, compared with an exclusive preterm formula diet.[2] Preterm infants who are too immature to suckle and near-term to term infants who are too sick to feed from the breast must be fed expressed breast milk (EBM) using a tube. This EBM may have been subjected to various handling and storage processes, including expression, fridge and freezer storage, transport to the neonatal unit, and then thawing. These processes may alter the composition of milk, particularly the fat composition, due to the presence of endogenous lipases that are capable of breaking down milk triglycerides (TG) and releasing free fatty acids (FFA) even at low temperature.[3]

Previous studies[4–6] indicate that only small amounts of FFA (under 2%) are present in fresh EBM, which is similar to levels in fresh bovine milk. There is debate regarding the significance of elevated concentrations of FFA in breast milk in infants' health: whether it is beneficial by providing a predigested readily available source of energy[6] or a cause of concern as it can increase osmolality of the milk, which can cause adverse gastrointestinal symptoms.[7] These questions are particularly relevant to the sickest, most vulnerable preterm infants who start life in the neonatal unit.

The fat composition of breast milk is known to be affected by many factors including, but not limited to maternal diet, lactation stages, gestation of infants at birth, and timing of expression.[8] To better understand the role of FFA, further research that accurately measures the endogenous concentration

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of FFA in fresh breast milk is required, with exploration of possible variation within and between individuals and across different stages of lactation. Earlier studies have suffered from methodological issues, including small sample sizes and suboptimal handling technique that may lead to enhanced degradation of TG during the sampling and processing,[5] resulting in an inflated FFA concentration. In addition, conventional thin layer chromatography, the predominant method used in earlier studies, can be prone to contamination from TG tailing during FFA separation.

We have recently described a dried milk spot (DMS) system whereby these two major concerns were eliminated using a conventional microwave oven to denature lipases and a specific analytical column that detects FFA without interference from TG.[9] Using the DMS method, we aimed to profile naturally occurring variations in fresh EBM that was sampled immediately after expression of milk at the cot-side. We also aimed to measure the effect of stage of lactation on the concentration of FFA in fresh EBM within the same mother and to measure the variations in FFA concentration of breast milk that was expressed at home and brought into the neonatal unit.

Materials and Methods

Participants

Eligible women included all mothers with an infant or infants admitted to the neonatal unit (intensive and special care units) at the Women's and Children's Hospital, North Adelaide, who were supplying EBM and had a surplus of milk beyond what their infant or infants' required for growth.

Breast milk sample collection

Two types of EBM were collected in this study: (1) fresh EBM that was expressed at the cot-side during a visit in the neonatal unit and (2) home collected EBM that was subsequently transported to the unit. Participating mothers EBM primarily using hospital grade electric pumps, and hand expression might be used to assist the expression to maximize the quantity of milk obtained. At the end of a single episode of expression, EBM from both breasts were pooled together into a sterile container. Fresh EBM was sampled immediately after completion of expression at the cot-side, and home collected EBM was sampled immediately upon arrival in the unit. All home collected EBM in the study was stored in the home fridge before delivery to the hospital. All mothers followed the hospital guidelines and transported EBM in an insulated bag or a hard-walled cool box with ice bricks or equivalent.

As the goal of the study was to examine the FFA concentration overall and then investigate the effect of stage of lactation, we aimed to collect EBM from each participant twice a week, during the first week postpartum, second week postpartum, and third week postpartum and beyond until hospital discharge. However, in the context of a busy neonatal unit and to minimize impost on families, we took a pragmatic and opportunistic approach to sampling, which meant that not all women provided samples at each of these time points, and women could provide either fresh EBM samples or home collected EBM (or both). Furthermore, as we did not collect samples postdischarge, mothers of longer stay infants tended to contribute the most samples.

Ethical approval for the study was granted by the Women's and Children's Health Network Human Research Ethics Committee (Approval number HREC/19/WCHN/19).

Laboratory analysis

For each collection, 20 μL of EBM was collected on each of two filter paper discs in a premanufactured card and allowed to dry. The DMS card was then microwaved (NN-S235WF, 5.4A, 50Hz, 800W; Panasonic Home Appliance Co., Ltd) at high power for 3 minutes as described in a previous report.[9]

The total fatty acids (equivalent to total fats) and FFA analyses were conducted using methods detailed previously.[9] Briefly, each DMS spot was excised with cleaned scissors and tweezers and then transferred into 6 mL vials. Twenty microliters of internal standard (triheptanoic acid C13:0) were added on the spots. For FFA analysis, the elution of milk fats from one of the spots was achieved with two washes of acetone (1 mL each), and the resulting acetone was dried under nitrogen and then reconstituted in 100 μL heptane. The other DMS spot was used for total fatty acid analysis, which was achieved by transmethylating into fatty acid methyl esters (FAME) in 2 mL 1% sulfuric acid in methanol at 70°C for 3 hours. The FAME was then extracted with heptane.

The nonesterified FFA and FAME were then analyzed by gas chromatography equipped with flame ionized detector (GC-FID) as described previously.[9] For FFA separation we used a GC-FID (Agilent Technology 7890B, Santa Clara, CA) equipped with a BP21 column (free fatty acid phase, 10 m $\times$ 0.25 mm, film thickness of 0.25 μm; Trajan Scientific Australia Pty Ltd); the temperature ramp was set between 180°C and 240°C with a helium flow of 3 mL/min. For FAME separation, GC-FID (Hewlett-Packard 6890, Palo Alto, CA) equipped with a BPX70 column (30 m $\times$ 0.25 mm, film thickness of 0.25 μm, Trajan Scientific Australia Pty Ltd) was used, with a temperature ramp set between 140°C and 240°C and helium flow of 2 mL/min.

Statistical analysis

Breast milk total fat content was expressed as mg/mL, and FFA concentration was reported as percentage of total fats. Results were presented as either a mean and standard deviation or median and interquartile range (IQR; based on the distribution of data). Data were analyzed using SPSS (Version 24; IBM Crop, Armonk, NY) and plotted using GraphPad Prism 8 (GraphPad Software, LA Jolla, CA). The possible association between storage time and the fat composition of home collected EBM was examined by calculating the Pearson's correlation coefficient. No further statistical analysis was performed, as this exploratory observational study was designed to explore the possible variations occurring in EBM samples, but was not powered to define the levels of FFA in EBM.

Results

Thirty-four mothers were enrolled in the study: one withdrew due to low milk supply after an episode of mastitis and another withdrew as she returned to work. EBM samples were collected from the remaining 32 mothers, who are described in Table 1. The majority (28/32) gave birth to preterm

infants, including 21 who gave birth to an infant or infants' born <34 week's gestation, considered early preterm.

A total of 256 EBM samples were collected, including 149 freshly expressed in the unit and 87 expressed at home and brought into the unit. The total fat data of all were normally distributed, while the FFA concentration data were negatively skewed.

Fresh EBM

Overall, the total fat content in the fresh EBM samples was 29.78 ± 9.28 mg/mL, with FFA accounting for 1.70% (IQR: 1.17–2.37%) of the total fats. The concentration of FFA and total fats of fresh EBM based on different stages of lactation (first, second, and third week postpartum and beyond) and by gestation are presented in Table 2. There was a trend toward increased total fat concentration as lactation progressed from ~21 mg/mL in the first week postpartum to 31 mg/mL in the third week postpartum and beyond, but the concentration of FFA remained relatively stable, accounting for ~2% of total fats across each stage. There was a possible trend that the FFA concentration of fresh EBM from mothers of late preterm infants tended to increase over time, whereas the EBM from mothers of early preterm infants declined slightly (Table 2).

Ten mothers were able to provide EBM samples at each stage of lactation, and the FFA concentration of the EBM at each collection point is shown in Figure 1. For most mothers, the FFA concentration in EBM was below 5% of total fats across all stages, and there was no clear trend. There was some variation within the same mother across different lactation stages (e.g., minimum of 0.58% and maximum of 5.08%, Fig. 1).

Home collected EBM

In comparison, the total fat content of home collected EBM was 27.37 ± 8.23 mg/mL, with FFA accounting for 2.49% (IQR: 1.74–3.29%) of the total fats. Similar to fresh EBM collected at the cot-side, there was also a trend toward gradual increase in total fat content as lactation progressed (Table 3). However, the FFA concentration of EBM brought from home was slightly but constantly higher compared with EBM freshly collected at the cot-side, across all different stages of lactation (Table 3).

For home collected EBM, the fat composition was plotted against the time from expression at home to collection at arrival of the unit (Fig. 2). There was a significant correlation

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF MOTHERS (N=32)

Characteristics	
Maternal age, years	30.5 ± 5.7
Gestation at birth	31.0 ± 4.7
Early preterm birth (<34 weeks)	21 (65.6%)
Late preterm birth (34–36^{+6} weeks)	7 (21.9%)
Term birth (≥37 weeks)	4 (12.5%)
Multiple pregnancy	9 (28%)
Sex of newborn—female	17 (53%)

Data are either presented as mean ± SD or *n* (%).
Superscript refers to number of days of a complete week.

TABLE 2. THE TOTAL FAT CONTENT (MG/ML) AND CONCENTRATION OF FREE FATTY ACIDS (% OF TOTAL FATS) OF ALL FRESHLY EXPRESSED BREAST MILK AT THE COT-SIDE ACCORDING TO GESTATION AT BIRTH AND STAGES OF LACTATION (N=256)

	First week postpartum			*Second week postpartum*			*Third week postpartum and beyond*		
Stage of lactation	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*
All	37	1.89 (1.45, 2.88)	20.67 ± 10.09	33	1.91 (1.23, 2.31)	31.45 ± 8.53	79	1.43 (1.10, 2.40)	31.01 ± 8.70
By gestation									
Term	7	1.50 (1.27, 2.09)	23.68 ± 3.76	1	2.14	24.70	1	3.47	19.15
Late preterm	6	1.65 (1.56, 2.68)	16.20 ± 1.75	9	1.91 (1.52, 2.71)	31.56 ± 2.60	17	2.87 (1.36, 2.94)	27.88 ± 2.08
Early preterm	24	2.13 (1.54, 3.39)	28.61 ± 2.00	23	1.64 (1.19, 2.32)	31.70 ± 1.89	61	1.24 (1.04, 2.28)	32.08 ± 1.09

Data are either presented as median (interquartile range) or mean ± SD.
FFA, free fatty acids.

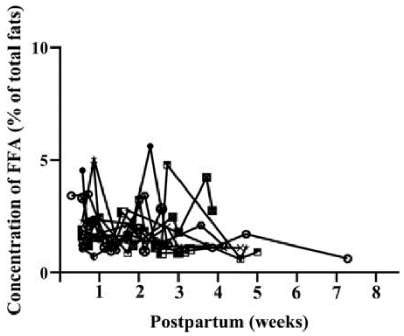

FIG. 1. FFA concentration (as % of total fats) of fresh breast milk from 10 mothers who provided samples at each stage of lactation. FFA, free fatty acids.

between this time and the concentration of FFA ($r=0.6043$, $p<0.0001$, Fig. 2a). There were two apparent outliers with elevated FFA concentration observed in EBM that was collected 27 and 58 hours postexpression with a FFA concentration of 7.9% and 9.7% of total fats, respectively (Fig. 2a). The FFA concentration of home collected EBM was mostly less than 5% of total fats when sampled within 20 hours postexpression (Fig. 2a). In contrast, the total fat content of home collected EBM was independent of the time from collection to arrival in the unit ($r=-0.1402$, $p=0.1951$, Fig. 2b).

Discussion

This is the first study that has used a DMS method specifically developed to measure the concentration of FFA in breast milk by eliminating the potential errors that may occur during sample processing as a result of lipolysis or from TG contamination. With this methodology, we report the average concentration of FFA in breast milk from a sample of Australian mothers who had mostly given birth to premature infants. It was found that FFA accounted for only a small percentage of fats in breast milk, regardless of the stage of lactation, or gestation at birth of the infant, although it tended to increase modestly over time with storage. Despite some variation within and between individuals, the concentration of FFA in fresh EBM was mostly below 5%, with a median of 1.7% of total fats.

Due to the technological difficulties associated with the need to eliminate the impact of lipases and preserve the endogenous FFA concentration, few studies have been able to measure the endogenous concentration of FFA in EBM.[5,6] Previous research has done this by immediately adding lipase inhibitor[6] or organic solvent[5] following milk collection to prevent the simultaneous breakdown of TG and the release of FFA, but both methods are less practical in a clinical setting and require greater volumes of milk. In addition, potential errors could also be generated when separating FFA from TG. Nevertheless, our findings are broadly consistent with previous studies. For example, Chappell et al.[6] reported that the average concentration of FFA in EBM from mothers delivered term and preterm was 1.9% and 1.5% of total fats, respectively, based on EBM donated by 15 mothers using a TLC separation method.[6] The same authors also claimed that there was a significant increase in the concentration of FFA in EBM with lactation stages from 0.5% to 1.5% of total fats of colostrum and EBM collected on day 7 postpartum, respectively.[6] This trend was observed in both mothers who delivered early preterm infants and term infants.[6] Although not directly comparable, due to differences in the study population and collection and analytical method, in the present study there appeared to be an increase in the FFA concentration of EBM of mothers who delivered late preterm and term infants, from first week postpartum until 3–4 weeks after birth. In contrast, the FFA concentration in the breast milk of mothers who delivered early preterm appeared to decline over time. The reasons for this are unclear and require confirmation in further studies that are appropriately powered to examine the effects of gestation at birth. In addition, further research is required to determine whether these small differences have implications for infant developmental outcomes.

We did not find any other changes in FFA concentration by stage of lactation. Among the subset of 10 mothers who provided repeat samples at each stage, all of whom delivered preterm, there was some day-to-day variation within individuals, where the FFA concentration of fresh EBM from the same person could be as low as 0.5% but as high as just over 5% of total fats. However, overall, the FFA concentration remained universally low at each stage.

TABLE 3. THE TOTAL FAT CONTENT (MG/ML) AND CONCENTRATION OF FREE FATTY ACIDS (% OF TOTAL FATS) OF HOME EXPRESSED BREAST MILK ACCORDING TO GESTATION AT BIRTH AND STAGES OF LACTATION ($N=87$)

	First week postpartum			*Second week postpartum*			*Third week postpartum and beyond*		
Stage of lactation	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*	n	*FFA concentration (% of total fats)*	*Total fat content (mg/mL)*
All	23	2.02 (1.75, 3.12)	22.83±7.86	20	2.55 (2.03, 3.17)	25.99±1.08	44	2.62 (1.62, 3.67)	30.37±7.91
By gestation									
Term	10	1.87 (1.75, 2.06)	27.41±3.43	7	2.23 (1.73, 3.06)	29.11±6.86	—		
Late preterm	1	1.50	32.05	2	2.34	22.90±0.29	5	2.13 (1.76, 3.56)	24.06±8.91
Early preterm	12	2.86 (1.87, 3.65)	18.25±8.01	11	2.59 (2.19, 4.24)	24.57±6.91	39	2.62 (1.54, 3.68)	31.18±7.52

Data are either presented as median (interquartile range) or mean±SD.
FFA, free fatty acids.

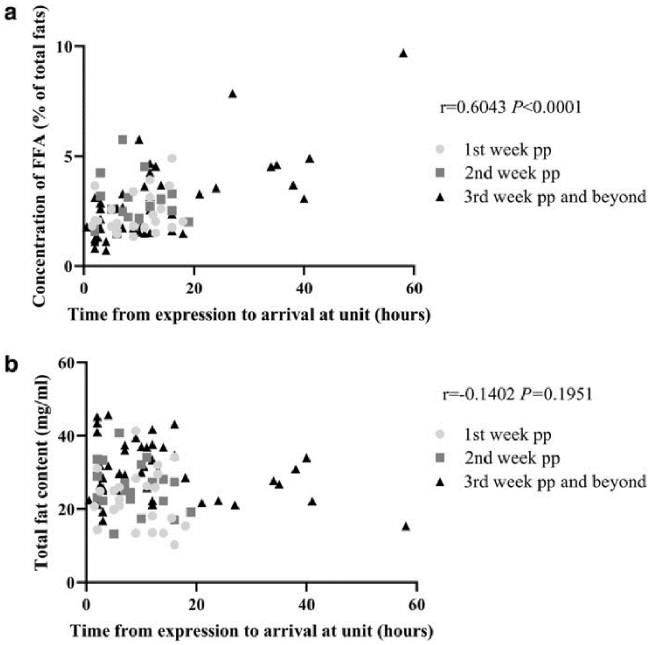

FIG. 2. The concentration of FFA (% of total fats, **[a]**) and the total fat content (mg/mL, **[b]**) of home expressed breast milk transported to neonatal unit (n=87). FFA, free fatty acids.

In this study, all home collected EBM was refrigerated before delivery to the unit. As the majority of the mothers visited their infants in the unit on a daily basis, the EBM was only temporarily stored in a fridge before transportation to the hospital and collection was completed mostly within 20 hours postexpression. However, as lactation progresses some mothers may accumulate surplus milk and bring the ''oldest'' EBM for feeding, exposed to the longest storage time. Many in vitro studies have reported an almost linear increase in the concentration of FFA in EBM when the milk is stored at fridge temperatures for prolonged periods,[10] and some claimed a three-fold increase in the FFA concentration from $\sim$1% to 4%[11,12] of total fats (based on a content of milk fats of 30mg/L). Our study observed a small but significant correlation between the storage/transportation time and the concentration of FFA measured in EBM; however, most of the values observed were still less than 5% of total fats and fell within the range of daily variations seen in the fresh EBM samples. These results highlight the stability of EBM under these conditions and therefore, may provide some reassurance to mothers who need to express milk to feed, as well as reinforcing current practices in the neonatal units to promote human milk feeding. Our findings also emphasize the need for caution when interpreting any research related to the concentration of FFA in breast milk as any changes observed may still fall within the variation displayed between individuals, and also the daily variation within the same individual.

A major strength of this study is that we used a DMS method that provides an accurate measurement of FFA concentration, even at very low levels, which eliminated potential errors of detection. The limitations of the study are that the sampling schedule (time of the day) was not prespecified, and not all women contributed EBM samples at each lactation stage. This occurred as we took a pragmatic approach to minimize impost on families in the neonatal unit and timed collection of EBM with their planned visits to the unit. In addition, we had only small numbers of women in some subgroups based on gestational age. However, the purpose of this study was to explore the variation that naturally occurred between mothers; it was not designed to define the FFA concentration of EBM in mothers of preterm and term infants.

Conclusion

The present study reports the median concentration of FFA in fresh and stored EBM collected from Australian mothers of mostly premature infants. We found that the FFA concentration of EBM is universally low, irrespective of gestation at birth and lactation stage, and remain relatively low even after a short period of cold storage postexpression. Future studies are warranted to confirm these findings and to explore the factors that might influence the FFA concentration of EBM.

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Chapter 7 General discussion

With the expansion of human milk banking in Australia and globally, there is a need to develop a simple and robust system for assessing the stability of human milk fats during collection, processing, storage, and distribution. Understanding the magnitude of changes that may occur to milk fats during this journey is one critical step to safeguard the nutrition of vulnerable preterm infants. The most notable change likely to occur to breast milk fat composition during storage and/or handling processes is the liberation of FFA from the main fat type in breast milk, TG (**Chapter 2**). Because there was no modern accurate tool for its measurement, the investigations I conducted during my candidature were directed towards developing a dried milk spot (DMS) system to accurately and precisely determine the fat composition of human milk (**Chapter 3** for total fatty acid composition, **Chapter 4** and **5** for FFA concentration). Collecting human milk as a DMS reduces the volume of samples required for analysis, eliminates the needs for cold-chain storage and transportation, and bypasses the liquid-liquid extraction step and allows direct measurements of the fat composition. Following development of a stable method, the clinical applicability and sensitivity of the DMS method was tested using freshly collected human milk samples from one local hospital (**Chapter 6**).

This is the first report of a DMS method for measuring the FFA concentration in human milk, which also stabilises the fat composition at room temperature for a minimum of eight weeks. Conventional TLC-GC methods for measuring the FFA concentration of human milk are time-consuming, requires lipid extraction and purification prior to separation and esterification. Some are also prone to falsely FFA measurement due to contamination from the TG fraction (due to tailing of TG on TLC plate). Even though there have been previous

reports of selective esterification of FFA by first adsorbing it with an anion pre-treated resin filled column [Hornstein *et al.* 1960; Needs *et al.* 1983], separation of FFA from other lipids required several rounds of elution with various solvents. Such a method is solvent-wasteful and time-consuming and could potentially result in uneven recovery of fatty acids, which limits its applicability in practice. In contrast, collecting human milk as a dried spot bypasses the extraction and purification step that is required in many conventional methods for FFA analysis. The final system described in Chapter 5 used a special polyethylene glycol column in the gas chromatograph that allows the separation of underivatized FFA even in presence of other esterified lipids, such as TG. Using this DMS system eliminates the needs for esterification, improves the accuracy of measurement and further simplifies the analytical process.

The successful development of the DMS for FFA measurement did not come without setbacks. The major challenge that I faced was due to the unique lipid composition of human milk. Unlike other biological fluids (e.g. plasma), human milk fats are overwhelmingly dominated by one type of fat, TG (>98%). Although it would be deemed unimportant and negligible in research of other bodily fluids, any trace contamination from TG is not acceptable for the analysis of human milk fats. Although I was able to reduce the contamination from TG to approximately 2% in some of the methods I have attempted (Chapter 4), I was still not satisfied as even a 1% contamination from TG could mean a 50% apparent increase in FFA and therefore a falsely high FFA reading. To my surprise, it was found that lipases of human milk remained active even when collected in the form of dried spot (Chapter 4 and 5). Identifying a method to effectively inactive these lipases that would not interrupt the fatty acid composition of human milk, and would be suitable in a range of clinical and other settings was the next problem to tackle. The two commonly used methods, conventional oven heating and protein precipitation generated inconsistent results and/or did

not completely stop the lipase from breaking down TG and liberating FFA during storage. The only effective tool to denature lipases to achieve long term stability at room temperature was microwaving at high power for three minutes (Chapter 5).

The usefulness of the DMS method for FFA measurement was demonstrated via a small clinical project (Chapter 6). In a three-month study, I collected over 250 expressed human milk samples from 32 mothers with an infant or infants admitted to the neonatal unit at the Women's and Children's Hospital. A recent report revealed that the main barrier for human milk collection in neonatal units is low milk supply by the mothers, followed by discharge prior to collection and staff unavailability [Galante *et al.* 2019]. The fact that I was able to collect hundreds of samples within a relatively short period mostly on my own, was largely due to the utility of the DMS technique. It required only a minute amount of sample, was easy to manage in practice and was well accepted by the participants. This study was also one of the few studies that were able to measure the endogenous FFA concentration of human milk prior to any storage and handling [Bitman *et al.* 1983; Chappell *et al.* 1985]. The other studies achieved this by immediately adding lipase inhibitor or organic solvent following milk collection, however, this is not feasible to be undertaken outside of a laboratory (e.g. in a neonatal nursery) due to safety concerns. In contrast, the use of a microwave for deactivating lipases in the DMS system is safe and more practical for clinical settings.

This thesis would have been more complete with one more component, to determine the effect of fortification on the fat composition of human milk during storage and feeding. Fortification with human milk fortifiers (containing hydrolysed bovine or human milk protein and minerals) is one last preparation step prior to the delivery of expressed milk to infants that are born with a birth weight less than 1800 grams. To the best of my knowledge, there has only been three previous studies to examine the effects of fortification [Donovan *et al.* 2017; Jocson *et al.* 1997; Schlotterer *et al*. 2019].

While conducting research in the neonatal unit, I have observed that there were some notable differences between fortified mother's own milk (MOM) and fortified pasteurised donor human milk (PDHM), in term of smell, colour and consistency. In our local neonatal unit, fortified human milk (both MOM and PDHM) is prepared once every 24 hours and loaded into several syringes (one syringe per feed), which are kept in a refrigerator until use. Each feeding lasts two hours at room temperature, therefore the longest storage duration that any feed may be exposed to is 24 hours in the refrigerator plus two hours at room temperature. Further research is needed to investigate how fortification affects the stability of fats in human milk during storage and over time, and whether addition of human milk fortifier would further accelerate the rate of FFA liberation compared to non-fortified milk. Other research questions to be addressed include whether there are any differences between MOM and PDHM in terms of the degree of FFA liberation, and the variations in concentration of FFA in fortified human milk at its end-of-life (after feeding has been completed), which represents the actual human milk infants may receive (detailed experimental plan see Appendix 2.).

One other interesting observation was that the FFA concentration of DMS rapidly increased during the first seven days after collection at room temperature, which then reached a plateau. During this process, the FFA concentration increased to approximately 5~7mg/ml, which is equivalent to 16~23% of total fats, assuming a fat content of 30mg/ml (Chapter 4 and 5). A similar pattern of change in FFA concentration in liquid human milk was also observed in some other smaller experiments I have conducted, where a plateau was seen after 24 hours storage at room temperature and 48 hours in the refrigerator, respectively (unpublished data). Whether this was due to a lack of substrate or that the activity of lipases naturally decayed over time was not further investigated. However, in some other human milk samples I measured during my Honours degree, the FFA concentration of human milk after

prolonged storage at -80℃ (collected at 2012-13 and analysed in 2016), had reached 30~40% of total fat. It may suggest that the number of lipases may have been reduced but their activity remained. However, it is noted that not all TG were converted to FFA even after prolonged storage. It was also found that the FFA composition changed with storage time, with an increase in the proportion of total n-6 polyunsaturates and correspondent reduction of total saturates and total n-3 polyunsaturates (unpublished data). Whether the liberation of FFA is related to the position of the fatty acid on the glycerol backbone [Qi *et al.*] needs further investigation.

Researchers have delved into studying the total fat content and fatty acid composition (pooled from both esterified and non-esterified lipids) of human milk but have paid very little attention to the FFA of human milk (e.g. its role and function, and determinants of its concentration in human milk). This is surprising considering the first step of fat digestion is to liberate fatty acids from their esterified structure, to then be freely circulated and readily absorbed. FFA, without being bound to a pre-existing esterified structure, are considered the primary source of usage, the role of which in infant health needs to be further explored. Previous research with preterm infants has shown that intestinal fat absorption may be impaired as a result of pasteurisation of human milk when compared with raw milk [Andersson *et al.* 2007]. This is likely due to the absence of lipases and to a lesser extent lipolysis during cold storage, leading to a lower concentration of FFA [Vincent *et al.* 2020]. However, this has received limited attention in the research literature. Future studies are required to clarify the consequences of FFA in human milk for health and developmental outcomes (fat absorption, growth, incidence of NEC etc.). This includes observational studies to define the relationship between FFA and clinical outcomes, and the ultimately clinical trials that examine whether deliberately increasing the FFA concentration of human milk (e.g. through repeated freeze and thaw or prolonged storage) would be favourable for infants'

growth and development. A DMS method that is reliable and requires only a small amount of sample for analysis provides the optimal tool to monitor FFA in future studies.

In the current DMS system I developed, accurate quantification of FFA relies on precise control of the volume of milk collected on the paper. At the time of analysis, a single underivatized odd chain fatty acid at a known concentration (internal standard) is added into the DMS sample as the standard reference to calculate the total amount of FFA in the milk sample. This could be improved by using an endogenous component of human milk that occurs at a constant concentration as the standard instead (e.g. lactose). Without having to add the standard manually, this endogenous standard could be extracted along with the fat of milk and injected into a high performance liquid chromatography (HPLC) or liquid chromatography mass spectrometry (LCMS) machine for separation and quantification. Using such a parameter as standard, collecting human milk at an exact volume would no longer be required for this DMS method. This is a more practical option as it allows the participants to be directly involved and engaged in the collection procedure, collecting milk themselves and faster milk collection, which would enable multiple samples to be collected per day. This should be a priority for future research, as it would enable more accurate examination of the maternal factors and diurnal factors that may affect the FFA concentration of human milk.

The development of new advanced technologies in lipidomic analysis has seen the discovery of oxylipins, the metabolic products of FFA, which are smaller molecules that are thought to mediate the biological effects of fatty acids. Although the role of oxylipins in human health is still being clarified, previous research has shown that blood FFA and oxylipin profile [Suganuma *et al.* 2020] can influence the growth trajectory of preterm infants during hospital stay [Alexandre-Gouabau *et al.* 2018], irrespective of whether the lipid source is human milk or parenteral nutrition. Whether this relationship occurs for long term growth remains to be investigated.

Not only does the fat composition of human milk change after handling and storage (Chapter 2), recent reports also suggest that the oxylipins of human milk are also thermal sensitive, being affected by both pasteurisation [Pitino *et al.* 2019] and storage conditions [Wu *et al.* 2016]. Although oxylipins were not the focus of this thesis, a fellow student had developed a method for determining the oxylipins in blood collected as a dried spot [Hewawasam *et al.* 2019]. There is great potential to further extend the applicability of my DMS method to include oxylipin analysis. One potential improvement is to use a hole punch rather than the whole DMS paper disc for analysis, which would mean it is possible to measure both FFA and oxylipins from a single collection. This final system is likely to be of great interest to human milk banks, as it could be adopted as a simple quality assurance step to measure the integrity of milk pre- and post-pasteurisation.

Further research is warranted to understand the optimal techniques to safely process donor human milk. The current gold standard, the holder pasteurisation method, though with proven abilities to eliminate transmissible viruses that may be present in donor milk, also destroys the valuable components of human milk that are thermally sensitive. We have seen many attempts made by scientists to develop non-thermal technologies (e.g. high pressure processing [Delgado *et al.* 2014; Molto-Pugmarti *et al.* 2011; Pitino *et al.* 2019] and ultra-violet radiation [Christen *et al.* 2013; Pitino *et al.* 2019]) for pasteurising human milk in the hope that these techniques preserve the pivotal components of breast milk. None have so far been introduced to human milk processing on an industrial scale. There needs to be a continuous effort in searching for alternative non-thermal technologies to replace holder pasteurisation. This could not happen without a reliable and practical tool to identify changes in the thermal sensitive components of breast milk from different processing techniques. A DMS method with minimal usage of human milk is the perfect tool to undertake this. Another fruitful area for further research is to explore the feasibility of using DMS as a tool for

measuring other nutritive components of human milk such as oligosaccharides, as well as chemical contaminants such as phthalates [Main *et al.* 2005]. The process of which would require machines such as HPLC and LCMS, but the extraction and analysis could all be completed in one 96-well plate setting.

In addition to the huge potential applicability of DMS to human milk banking, as well as the dairy and oil industry, where FFA concentration is often an indicator of rancidity of the products. This dried spot technique could be extended to determine the level of FFA in other biological fluids, such as plasma and serum. Serum and plasma FFA concentration have been associated with a range of adult chronic disease including metabolic dysfunction (obesity and type-2 diabetes [Schrieks *et al.* 2018; Steffen *et al.* 2015]) coronary artery disease [Jin *et al.* 2019; Pilz *et al.* 2006; Schrieks *et al.* 2018] and hypertension [Tabara *et al.* 2014]). However, the exact role of FFA in the increased risk of these diseases is unclear, including the relative importance of the total concentration of FFA versus specific FFA types. The current standard method for measuring FFA concentration in serum and plasma in clinical settings is limited to enzymatic assays, which only provides quantitative data (e.g. the amount) but not qualitative data about the types of fatty acid. Addition, there is concern that the results may only be valid within a narrow range of FFA concentration [Song *et al.* 2019]. As such, the current FFA detection methods in serum and plasma are not reliable enough to be used as a screening to assess disease risk. The DMS method described in this thesis has huge potential to be adapted to serum and plasma FFA measurement. This would provide a cheaper alternative tool that is highly accurate and precise, which provides both quantitative and qualitative results for screening potentially clinical diagnosis and prognosis of chronic disease. This is an exciting area for future research.

In conclusion, I have developed a micro-sampling method for measuring the fat composition of human milk collected as dried spot, which is stabilised by microwaving, and

can be stored at room temperature for a minimum of eight weeks without deteriorating. In a small clinical project conducted in the neonatal nursery, I demonstrated that the DMS method is easy to manage and well accepted by the breastfeeding mothers due to that small amount of sample that is required. This is the first report of a reliable and accurate DMS method for detecting changes in human milk fat composition that could be suitable for implementation not only large scale clinical studies, but also the human milk banking industry for monitoring the quality of donor human milk throughout the processing

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Appendix 1.

Supplementary material for the systematic review included in Chapter 2

Additional records for the systematic review

Since the publication of the systematic review in May 2019, there have been eight new studies published, and two were missed out during the initial search for the published manuscript, the characteristics of these studies are detailed in **Table 2.1** and the results are shown in **Table 2.2**.

Six [Adhisivam *et al.* 2019; Chang *et al.* 2020; Kim *et al.* 2019; Orbach *et al.* 2019; Paulaviciene *et al.* 2020; Tanriverdi *et al.* 2019] of the ten additional studies reported changes to the total fat content of human milk after various storage and handling procedures, while only one study [Wesolowska *et al.* 2019] determined its effect on the total fatty acid composition. Three [Chappell *et al.* 1984; Hung *et al.* 2018; Wesolowska *et al.* 2019] of the included studies measured the changes to free fatty acid concentration/proportion, and one [Capriati *et al.* 2019] reported for triglyceride concentration/proportion.

Overall, results from the additional records captured are in line with the observation and conclusion of the published systematic review. The total fat content and total fatty acid composition of human milk is less likely affected by different storage and handling process, where changes were seen in term of total fat content, it was likely due to poor sample processing technique and may also be due to methodological errors. For instance, the total fat content of human milk samples measured after various treatment varied significantly in Kim *et al.* 2019 (e.g. 21% increase after microwave heating while 20% reduction after storage), and this is likely due to a poor homogenisation of samples prior to analysis.

Similar to the findings of the published systematic review, changes to the fat composition (lipid classes), the concentration or proportion of triglycerides and free fatty acids were observed after various storage and handling processes. Capriati and colleagues [Capriati *et al.* 2019] reported approximately 20% reduction in triglyceride concentration after holder pasteurisation (HP) or modified HP, the results are in agreement with previous observation, which fit in with our hypothesis that the lipases remained functioning during the time frame where temperature was gradually increased and result in further lipolysis. However, Welsolowska *et al.* determined the effect of HP and high-pressure processing on the free fatty acid concentrating of human milk using a conventional titration method and concluded that both method resulted in reduction in FFA concentration, for up to 50%. For the effect of storage, the concentration of FFA in human milk continuously to rise, following a time-dependent manner, in both room temperature and domestic freezer storage setting [Chappell *et al.* 1984; Hung *et al.* 2018]. Whereas, no changes to the FFA concentration was observed if the samples were stored in deep freezer [Chappell *et al.* 1984]. Although the data were not formally reported, Chappell *et al.* also reported that the FFA concentration of human milk was further increased after two rounds of freeze-thaw cycle, but not any more after the third cycle.

In conclusion, current storage and handling practices of human milk is less likely to affect its total fat and total fatty acid composition, unlike the fat composition (or lipid classes), which is altered by storage, pasteurisation and repeated freeze and thaw cycles.

Table 2.1. Characteristics of records in addition to existing systematic review

Study, Country/Region	Preterm/ term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Treatment/intervention	Sample Size	Analytical method	Quality*
Adhisivam *et al* (2019, India)	N/S	Transitional and Mature	Hospital Grade pump (N/S)	N/S	Frozen	- HP	30 pooled samples from 90 mothers	Human milk analyser – total fat content (g/L)	Positive
Capriati *et al* (2019, Italy)	N/S	N/S	N/S	N/S	N/S	- HP - Modified HP	27 samples from 25 donors	Automated chemistry system – TG concentration (mg%)	Neutral
Chang *et al* (2020, China)	N/S	Transitional and Mature	N/S	Frozen	Frozen	- HP - HP with frozen storage	100 samples from 100 donors	Infrared analyser – total fat content (g/dL)	Positive
Chappell *et al* (1984, Canada)	N/S	Mature	Mechanical/ electrical pump	Fresh	N/A	- Frozen storage	15 samples from 5 mothers	TLC-GC: FFA concentration (%w/w of total fatty acids)	Positive
Hung et al (2018, China)	Term	N/S	N/S	Refrigerated	Refrigerated	- Frozen storage	10 mothers	Gravimetric method (modified Roese-Gottlieb method) GC: after basic and then acidic transmethylation – FFA concentration (mg/g of fat)	Positive

Kim *et al* (2019, Korea)	N/S	Mature	N/S	N/S	N/S	- RT, fridge and frozen storage - Warming methods (RT, bottle warmer, microwave)	119	Infrared analyser – total fat content (g/dL)	Negative
Orbach *et al* (2019, Israel)	Term and preterm	N/S	Electric pump	Refrigerated	Refrigerated	- Frozen storage	137 samples from 25 donors	Human milk analyser – total fat content (g/dL)	Positive
Paulavicine *et al* (2020, Lithuania)	Term and preterm	Mature	Electric and mechanical pump	Fresh	Refrigerated	- Holder pasteurisation	42 mothers	Human milk analyser – total fat content (g/dL)	Positive
Tanriverdi *et al* (2019, Turkey)	Term	Colostrum to Mature	Hand expression	N/S	N/S	- Frozen storage	43 donors	Human milk analyser – total fat content (g/dL)	Positive
Weslowska *et al* (2019, Poland)	N/S	Transitional/Mature	Mechanical/ electrical pump	Refrigerated	Refrigerated	- HP - HHP	6 pools of sample from 3-4 donors	Titration – free fatty acid concentration (reported as % loss as compared to control) GC: FA composition	Neutral

Abbreviations: HP: holder pasteurisation; HHP: high pressure processing; NS: non-specified; TG: triglycerides; FA: fatty acids; FFA: free fatty acid; GC: gas chromatography; FID: flame ionization detector; RT: room temperature; HPLC: high performance liquid chromatography.

Table 2.2. Effect of different intervention on the fat composition of breast milk as published in the additional records

Component	Intervention	Effect
Total fat	Room temperature storage	
	• 1 week	**Reduction (20.6%)** [Kim 2019]
	Refrigerator storage	
	• 1 week	**No statistically significant change** [Kim 2019]
	Domestic freezer storage	
	• 1 week	**Reduction (11.6%)** [Kim 2019]
	• 1 month	**Reduction (14.2%)** [Kim 2019]
	• 2 months	**Reduction (11.3%)** [Kim 2019]
	• Up to 6 months	**Reduction (21.4%)** [Tanriverdi 2019] **No statistically significant change** [Orbach 2019]
	Deep freezer storage	
	• Up to 6 months	**No statistically significant change** [Orbach 2019]
	Holder Pasteurisation	**No statistically significant change** [Paulaviciene 2020] **Reduction (up to 25%)** [Adhisivam 2019; Chang 2020]
	Thawing	

	• RT	**Reduction (12.2%)** [Kim 2019]
	• Bottle warmer	**Reduction (11.6%)** [Kim 2019]
	• Microwave	**No statistically significant change** [Kim 2019]
	Warming	
	• Microwave	**Increase (24.1%)** [Kim 2019]
Total fatty acid composition	Holder pasteurisation	**No statistically significant change** [Wesolowska 2019]
	High pressure processing	**No statistically significant change** [Wesolowska 2019]
Triglyceride concentration/proportion	Holder pasteurisation	**Reduction (18.3%)** [Capriati 2019]
	Modified Holder pasteurisation	**Reduction (21.1%)** [Capriati 2019]
Free fatty acid concentration/proportion	Domestic freezer storage	
	• 1 week	**Increase (78%)** [Hung 2018]
	• 1 month	**Increase (250%)** [Hung 2018]
	• Unspecified timeframe	**Increase (585%)** [Chappell 1984]
	Deep freezer storage	
	• Unspecified timeframe	**No statistically significant change** [Chappell 1984]

	Holder pasteurisation	**Reduction (50.2%)** [Wesolowska 2019]
	High pressure processing	**Reduction (up to 19.2%)** [Wesolowska 2019]

References for these additional records:

Adhisivam, B., Vishnu Bhat, B., Rao, J., Kingsley, S.M., Plakkal, N., Palanivel, C. (2019). Effect of holder pasteurisation on macronutrients and immunoglobin profile of pooled donor human milk. *Journal of maternal, fetal and neonatal medicine*, *32*: 3016-3019.

Capriati, T., Goffredo, B.M., Argentieri, M., De Vivo, L., Bernaschi, P., Cairoli, S., Laureti, F., Reposi, M.P., Marino, D., Benedetti, S., Diamanti, A. (2019). A modified holder pasteurisation method for donor human milk: preliminary data. *Nutrients*, *11*:1139.

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Hung, Y.H., Hsu, Y.Y., Su, P.F., Chang, Y.J. (2018). Variations in the rancid-flavor compounds of human breast milk under general frozen-storage conditions. *BMC Pediatrics*, *18*: 94.

Kim, M.H., Shim, K.S., Yi, D.Y., Lim, I.S., Chae, S.A., Yun, S.W., Lee, N.M., Kim, S.Y., Kim, S. (2019). Macronutrient analysis of human milk according to storage and processing in Korean mother. *Pediatric Gastroenterology, Hepatology & Nutrition*, *22*: 262-269.

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Wesolowska, A., Brys, J., Varvarsja, O., Strom, K., Szymanska-Majchrazk, J., Karzel, K., Pawlikowska, E., Zielinska, M.A., Hamulka, J., Oledzka, G. (2019). Lipid profile, lipase bioactivity, and lipophilic antioxidant content in high pressure processed donor human milk. *Nutrients*, 11:1972.

Supplemental Table 1. The PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	4
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	6

Section/topic	#	Checklist item	Reported on page #
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	6
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5-6
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	Table 1
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7-13

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	7
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	Table 2-3
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	13-14
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	13
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	14-15
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	15

Supplementary Table 2. Search strategies used for individual database

Database	Search strategies
PubMed	((((((Human breast milk[tw] OR Human milk[tw] OR Milk, human[mh] OR Milk bank[tw] OR Milk banks[mh] OR Breastfeeding[mh]))) AND (Food preservation[mh] OR Preservation, biological/methods[mh] OR Food storage[mh] OR Food storage/methods[mh] OR Time factors[mh] OR Refrigeration[mh] OR Freezing[mh] OR Freezing/adverse effects[mh] OR Refrigeration/adverse effects[mh] OR Temperature[mh] OR Cold temperature[mh] OR Hot temperature[mh] OR Hot temperature/adverse effects[mh] OR Heating/adverse effects[mh] OR Microwaves[mh] OR Microwaves/adverse effects[mh] OR Pasteurization[mh] OR Pasteurization/methods[mh] OR Sterilization[mh] OR Thawing[tw] OR Thaw[tw] OR Freeze/thaw[tw] OR Fortifier[tw] OR Milk fortification[tw] OR Enteral nutrition/adverse effects[mh] OR Enteral nutrition[mh] OR Food handling[mh] OR Food handling/methods[mh])) AND (Milk, Human/analysis[mh] OR Fats/analysis[mh] OR Fatty acids[tw] OR Fatty acids[mh] OR Fatty acids/analysis[mh] OR Fatty acids, Nonesterified/analysis[mh] OR Lipids[tw] OR Lipids[mh] OR Lipids/analysis[mh] OR Lipolysis[tw] OR Lipolysis[mh] OR Dietary fat[tw] OR Dietary fats[mh] OR Dietary fats/analysis[mh] OR Triglycerides/analysis[mh] OR Hydrolysis[mh] OR Diglycerides/analysis[mh] OR Docosahexaenoic acid/analysis[mh] OR Linoleic acid/analysis[mh] OR Linolenic acid/analysis[mh] OR Arachidonic acid/analysis[mh]))) NOT ((animal[mh] NOT human[mh])) AND (Humans[Mesh] AND English[lang] AND Female[MeSH Terms])
Embase	(('breast milk':de,ti,ab OR 'milk bank':de,ti,ab) AND ('food handling':exp OR 'food storage':exp OR 'storage temperature':exp OR 'low temperature':exp OR 'refrigeration':de,ti,ab OR 'food freezing':exp OR 'cryopreservation':exp OR 'pasteurization':de,ti,ab OR 'high temperature':exp OR 'instrument sterilization':exp OR 'temperature dependence':exp OR 'heating':de,ti,ab OR 'thawing':de,ti,ab OR 'freeze thawing':de,ti,ab OR 'time factor':exp OR 'food irradiation':exp OR 'microwave irradiation':de,ti,ab OR 'milk fortification':de,ti,ab OR 'enteric feeding':exp) AND ('lipid':de,ti,ab OR 'lipid analysis':exp OR 'lipolysis':exp OR 'lipid hydrolysis':exp OR 'fatty acid':de,ti,ab OR 'fatty acid analysis':exp OR 'triglycerol':exp OR 'diglycerol':exp OR 'monoglycerol':exp OR 'arachidonic acid':exp OR 'linoleic acid':exp OR 'docosahexaenoic acid':exp)) NOT [medline]/lim

Scopus	(TITLE-ABS-KEY (*"human breast milk"*) AND TITLE-ABS-KEY (*"Storage temperature"* OR *"refrigeration"* OR *"pasteurization"* OR *"freezing"* OR *"sterilization"* OR *"heating"* OR *"thawing"* OR *"freeze-thaw"* OR *"microwave"* OR *"milk-fortification"* OR *"enteral nutrition"* OR *"enteral feeding"*) AND TITLE-ABS-KEY (*"lipid"* OR *"fatty acid"* OR *"lipolysis"* OR *"free fatty acid"* OR *"lipid hydrolysis"*)) AND NOT INDEX (*medline*)

Appendix 2.

Flow diagram of the planned experiment regarding fortification of human milk mentioned in Chapter 7

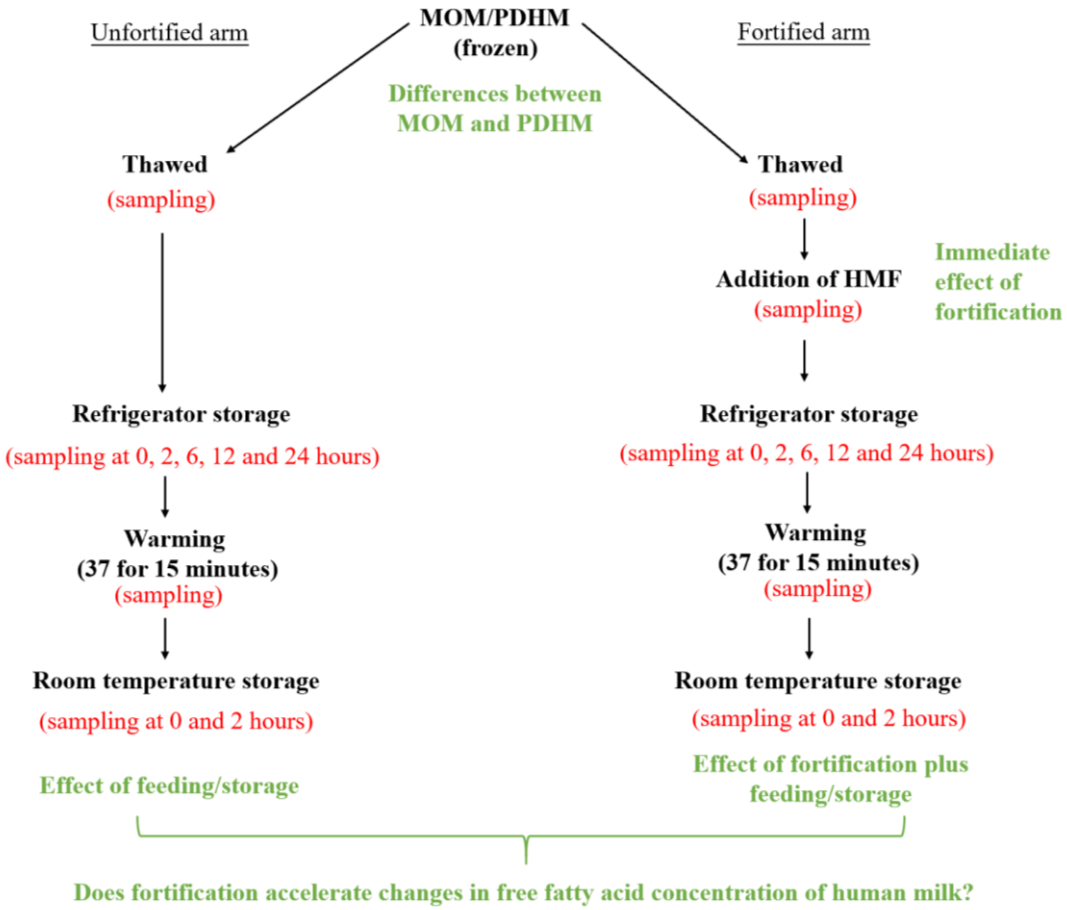

Figure 7.1. Flow diagram of planned in vitro experiment. Black text represents the steps or procedures, red highlights the sampling points and green represents the research questions to be answered. MOM: mothers' own milk. PDHM: pasteurised donor human milk.