Dried Blood Spot Fatty Acid Quantitation: Developing Methods for Determining Unknown Volumes, Identifying Contaminants and Assessing Fatty Acid Stability During Storage

by

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A thesis

presented to the University of Waterloo

in fulfillment of the

thesis requirement for the degree of

Master of Science

in

Kinesiology

Waterloo, Ontario, Canada, 2018

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Omega-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) blood levels are a potential risk factor for coronary heart disease, particularly sudden cardiac death. Venipuncture sampling for fatty acid profiling is invasive, requires highly qualified personnel and requires a multi-step protocol to isolate blood fractions. Alternately, the use of whole blood for fatty acid profiling improves analytical throughput and allows sample collection in field research locations by enabling dried blood spotting (DBS). Dried blood spots are advantageous in comparison to venous blood sampling as they require small blood volumes and is relatively inexpensive to collect. However, FA profiles in DBS are commonly expressed qualitatively (% of total fatty acids) and not quantitatively (µg/mL) as finger-tip prick (FTP) sampling usually results in the collection of an unknown volume of blood. Quantitation can be effected by preexisting fatty acid contaminants on DBS collection materials and oxidative losses of sensitive fatty acids such as n-3 LCPUFA due to the increased surface area of DBS samples. Fatty acid quantitation could detect hypo- and hyper-lipidemia in samples that a qualitative only assessments would miss. To address these issues, the relationship between blood volume and blood spot area on 903 Protein Saver Cards (903 PSC) was examined to determine the blood volume associated with a 6mm hole punch and the Mitra Microsampling Device, a product designed to collect 10 µL of blood regardless of hematocrit, was assessed for FA and lipidomic analyses. To determine if fatty acid contaminants were present, the 903 PSC, the Mitra tips and Whatman chromatography paper (also commonly used to collect blood spots) were examined using gas chromatography and ultra high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). Finally, the stability of the fatty acids in a DBS sample on Mitra and 903 PSC stored at ambient, 4°C, -20°C and -80°C temperatures with and without antioxidant for 3 months was examined. It was determined that the

6mm punch of 903 PSC contained 9.6 µL of blood and that FA profiles determined from the Mitra samples were comparable to FA profiles from wet blood controls. The Mitra tips could also be used to provide similar lipidomic profiles. The 903 PSC, the Mitra tips and the Whatman paper all contained palmitic and stearic acid as free fatty acids (FFA) while the Mitra tips also had palmitoyl and stearoyl lysophosphatidylcholines (LysoPC) present. With DBS storage, the n-3 LCPUFA biomarkers were the most stable with -80°C storage followed by 4°C or ambient room temperatures while samples stored at -20°C storage had the lowest stability in both antioxidant and no antioxidant conditions, which mirrored previous research examining whole blood storage. In conclusion, quantitative fatty acid determinations of DBS samples are possible. Blood volumes can be estimated using a defined hole punch on the commonly used 903 PSC, or defined by using a Mitra sampling device. Analysis of blank sampling devices is recommended to assess the potential impact of fatty acid and fatty acyl contaminants in any DBS collection materials to be used. Finally, storage conditions need to be a consideration with DBS sample collection as preventative steps such as storage temperature and the use of antioxidants can improve sample stability and ensure data integrity.

Acknowledgements

Dr. Ken Stark, thank you for the opportunity and guidance to progress as a researcher, academic and person. I appreciate that you've kept me self-accountable and I appreciate all of the opportunities and projects that you have presented to me. Also, thank you for reminding me to light a fire under my ass at times that I've needed one.

Thank you to my committee members, Dr. Robin Duncan and Dr. Marina Moutrzakis, for your time commitments, guidance and help to progress my work in the right direction. Thank you for being so welcoming and supportive even with your busy schedules.

Juan and Dan, thank you for being such reliable lab mates and friends. I've never had to stress with you two around because I know you both will always have my back. Thank you to all of the past and present members of the Stark lab. Thank you Dr. Richard Smith for sharing your mass spectrometry expertise with me and guiding me towards a better understanding of molecular chemistry. Also, to my friends in Physiology (Duncan and Tupling labs), you're bright and genuinely great people. I thank you whole heartedly for being a part of my graduate experience.

My friends, all of you have individually helped me grow as a person and I thank you. And finally, my family, you've always supported with all of the decisions I've made and I thank you for the continued support.

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List of Abbreviations

% TFA	Percentage of Total Fatty Acids
903 PSC	903 Protein Saver Cards
BHT	Butylated Hydroxytoluene
CVD	Cardiovascular Disease
DBS	Dried Blood Spot
DHA	Docosahexaenoic Acid
EPA	Eicosapentaenoic Acid
FA	Fatty Acid
FFA	Free Fatty Acid
FTP	Finger Tip Prick
GC	Gas Chromatography
HDL	High Density Lipoprotein
HPLC-MS/MS	High Performance Liquid Chromatography coupled with Tandem Mass Spectrometry
HUFA	Highly Unsaturated Fatty Acid
IDL	Intermediate Density Lipoprotein
LDL	Low Density Lipoprotein
TAG	Triacylglycerol
MUFA	Monounsaturated Fatty Acid
N-3	Omega-3
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipid
PS	Phosphatidylserine
PUFA	Polyunsaturated Fatty Acid
RBC	Red Blood Cell
SFA	Saturated Fatty Acid
WBC	White Blood Cell
VLDL	Very Low Density Lipoprotein

Chapter 1

Introduction

The omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are linked to reduced risk of cardiovascular disease (CVD) [1], infant neurodevelopment [2] and health [3]. Omega-3 blood biomarkers such as the omega-3 index (sum of the percentages of EPA and DHA in total fatty acids of erythrocytes), have been characterized in the global population [4] and allowed for the development of blood level targets to optimize cardio-protective effects [5]. Recently, it has been demonstrated that maternal EPA+DHA status during pregnancy is associated with the risk of the infant developing asthma after birth [3]. Thus, screening blood for fatty acid (FA) health biomarkers in various populations has clinical utility in preventing morbidity and reducing health care costs.

One of the challenges with fatty acid profiling of blood samples is the lack of consensus in analytical procedures [4, 6]. This includes different blood fractions, with plasma total lipids, plasma phospholipids and erythrocytes being examined typically. Recently, whole blood sampling has emerged as an option, because it can simplify sample collection as it can be accessed through procedures such as finger-tip prick (FTP) or heel prick to generate dried blood spot (DBS) samples [7]. DBS have logistical and economic advantages in the collection, handling and storage of samples in various clinical and field research settings because they do not require the same amount of infrastructure and highly qualified personnel as compared with venipuncture and blood fractioning methods. DBS can provide informative fatty acid profiles [6] and have been used to assess mothers and newborns [8, 9], Zimbabwean children [10], young Canadians [11] and Tibetan adults [12]. In comparison to whole blood sampling however, DBS samples have limitations as quantitative (ex. µg/mL) results are not possible unless; blood volumes were assessed prior to

spotting, any fatty acid (FA) contaminants present on materials commonly used to spot the blood are determined, and the high risk of PUFA loss during storage is properly managed [13].

Currently in the literature, FA determinations from DBS samples have mainly been expressed qualitatively, which is the percentage of an individual fatty acid in the total fatty acids [10, 14, 15]. This is the most common method of presenting fatty acid data [4] as it highlights the metabolic competition between fatty acids within pools of complex lipids and most blood biomarkers are based on these qualitative measurements. For example, the sum of the weight percent (wt %) of EPA and DHA [16] and the percentage of omega-3 highly unsaturated fatty acids (HUFA) in total HUFA [17] are both qualitative assessments. However, qualitative assessments result in a lack of independence of the data as increases in one fatty acid are reflected by a decrease in another because they are expressed in relative abundance (% of total FA). Qualitative FA profiles also may remain blind to variations in total fat concentration that may be caused by recent food consumption, dietary habits, age, gender and genetic background [18]. Internal fatty acid standards are available and gas chromatography responses are robust, so precise quantitative determinations are possible, but the volume of blood collected by DBS is not usually measured.

This thesis examines quantitation of fatty acid profiles of DBS on two commercially available products. First, a method will be developed to determine unknown volumes of blood on 903 Protein Saver Cards (903 PSC) which are one of the most commonly used DBS materials for clinical blood collections. Second, a novel microsampling device, Mitra® will be validated for FA analysis. The Mitra uses absorptive technology to wick 10 μL of sample regardless of viscosity which can be influenced by hematocrit level in blood.

This thesis will also examine FA contaminants in materials used to collect DBS as they can also impact quantitative efforts. Palmitate (C 16:0) and stearate (C18:0) contamination appears to be common in various chromatography papers used to collect blood samples [19]. Again, the focus will be on the 903 PSC and the Mitra, as these are materials specifically designed to collect DBS. In addition to using gas chromatography (GC) to identify if fatty acids are present, we will also examine the lipid extractions with ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) to determine if the fatty acids are present as free fatty acids or as fatty acyls within a complex lipid. UHPLC-MS/MS will also be used to assess lipidomic profiles in Mitra tips using traditional collection materials.

Finally, given that sample storage can result in losses in PUFA [13] and impact quantitation, the effect of storage of DBS on 903 PSC and Mitra on fatty acid profiles will be determined. Practical fatty acid storage conditions will be examined and include ambient room temperature, 4°C, -20°C and -75°C temperatures. The ambient, 4°C, -20°C may provide information on means of storage when access to the optimal ultra-cold storage (-75°C) is not possible, but when a common household refrigerator and/or regular freezer are available. The use of antioxidants to improve FA stability in DBS on 903 PSC and Mitra will also be assessed given previous reports of improved FA stability of DBS samples on chromatography paper[15, 20].

Chapter 2

Background

2.1 *Blood Fatty Acids as Biomarkers of Diet and Health*

Fatty acids in blood have been used as an assessment tool for determining risk levels for chronic disease, cardiovascular disease, cognitive decline and as markers of dietary fat intake [7]. The omega-3 index, the sum of the percentages of EPA and DHA in erythrocytes is a risk factor for coronary heart disease or sudden cardiac death because of the role the n-3 FAs have in membranes and their interaction with membrane bound proteins [16]. Increased levels of EPA and DHA dietary intake has demonstrated positive health benefits in instances of neurological development in preterm infants [21, 22], rheumatoid arthritis [23], depressive disorder [24], inflammation [25] and cardiovascular disorders [26]. Therefore, some health agencies have recommended increasing n-3 intake through diet or supplementation to reduce disease risk and improve the health of the general population [27, 28]. Dietary recommendations can be made based on blood FA levels as a linear correlation between EPA + DHA intake and n-3 FA blood biomarkers through tightly controlled dietary intake trials and the relationship has been observed in whole blood, RBC and plasma phospholipid pools [29].

2.2 *Blood Fatty Acid Analysis: Blood Fractions and Lipid Pool Considerations*

Fatty acid profiles can either be expressed for whole blood or for common blood fractions of erythrocytes or red blood cells (RBC), plasma, and/or buffy coat. Plasma fatty acid profiles can be presented as the fatty acid composition of total lipids or as the composition of lipid subclasses such as phospholipids, triacylglycerols (TAG), and cholesteryl esters. While the fatty acid

compositions of these different blood pools can predict the composition of the other blood pools [6], each blood/lipid pool can be influenced by diet and metabolism differently.

Plasma fatty acid composition mainly reflects the fatty acyls of lipids in lipoproteins [30] with some contribution from the nonesterified fatty acid pool [18]. The lipoproteins consist of a monolayer of phospholipids that is largely of the phosphatidylcholine species with cholesterol and apolipoproteins. This results in a spherical particle with a nonpolar core of largely TAG and cholesteryl esters. Based on their relative densities dictated by the ratio of triacylglycerol to protein content, plasma lipoproteins can be classified as chylomicrons (CM), very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). However, the lipoprotein classes can further be separated into intermediate-density lipoproteins (IDL) and subclasses of HDL lipoproteins including HDL_1, HDL_2, HDL_3 and so on which have different functions based on their structure [31]. Lipoprotein classification can be attributed to their main metabolic functions; chylomicrons and VLDL transport TAGs from the intestine or liver to peripheral tissues and alternately, HDL removes excess cholesterol and transports it to the liver to be excreted. Thus, lipoproteins play an important role in TAG and cholesterol metabolism. Specifically, the hydrophobic tendencies of TAG molecules require lipoproteins for transport in the aqueous plasma. TAG molecules generally exist in enantiomeric forms and their FA composition represents recent dietary intake from preceding days. The fatty acid composition of TAG tends to be dominated by saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA), specifically C 16:0, C 18:0, and C 18:1 but also contains C 18:2n-6 which is high in the diet of industrialized countries as well its' primary metabolite, C 20:4n-6 [18]. Fatty acid composition data on isolated types of lipoproteins is rare due to tedious separation techniques required, whereas lipid class isolation is more common as it can be achieved with thin layer

chromatography. The nonesterified fatty acid pool is largely maintained by lipolysis of TAG stores within adipose tissue although dietary fatty acids can contribute to the pool post absorption via lipoprotein lipase spillover [18]. In general, of the total fatty acids in plasma, 44-59% are associated with TAG, 24-31% associated with PL, 14-21% associated with CE, and 3-6% as NEFA [18].

Erythrocytes or RBCs are mainly plasma membranes containing hemoglobin. As such, their fatty acids are found predominantly as fatty acyls of phospholipids (PL). As the membrane is a lipid bilayer, it is composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SPH) [18]. Each class of PL can have a characteristic fatty acid composition but they generally have a SFA/MUFA in the sn-1 position and a MUFA/PUFA in the sn-2 position [18]. RBC fatty acid profiles are often promoted as a better marker of long term dietary habits based on the 3-month half-life of erythrocytes. However, it has been shown that the fatty acid profile of RBC membranes can remodel relatively quickly in response to dietary changes [32]. This remodeling may be specific to the outer layer and PC, while inner membrane phospholipids such as PE may take longer to remodel [32]. This has been demonstrated to have an effect on how we interpret omega-3 biomarkers as EPA appears to have a higher remodeling rate compared with DHA [7]. The erythrocyte fatty acid profile can be correlated with plasma fatty acids, although there is considerably less C 18:1n-9 and C 18:2n-6 as these are typically high in the TAG pool absent in RBC [6, 33].

The buffy coat is collected less frequently, but the FA profiles tend to be similar to RBC. This is largely because the buffy coat fatty acids are predominantly from the lipid bilayers of white blood cells and platelets [18]. However, the size of the buffy coat fraction can vary in size within

and between individuals due to various immune conditions and responses. The impact of changes in the buffy coat on fatty acid composition have not be examined in detail.

Blood fractioning into plasma, RBC and WBC requires a venous blood draw, anticoagulant to prevent RBC lysis and a centrifuge which may not be accessible outside of an analytical facility. Thus, whole blood sampling is an attractive alternative for epidemiological studies because it enables microsampling procedures such as finger-tip prick or heel prick DBS collections [7]. The accessibility, low volume collection, simple sample processing and storage options for DBS makes it an attractive option for large screening studies or field related blood collections. DBS samples are, however, a combination of all blood fractions, so the advantages and disadvantages of all the fractions are combined. In being a comprehensive sample, detailed mechanistic insights from the FA composition of DBS can therefore be limited, but the comprehensive approach also means that no fatty acid information of a specific lipid pool has been lost.

2.3 *Dried Blood Spotting and the Growth of Whole Blood Microsampling for Fatty Acid Analyses*

DBS have commonly been used in field research due to the advantages they have in collection, storage and transport in comparison to handling wet blood. DBS requires ~50 μL or less of blood per spot and enables relatively non-invasive and repeatable FTP or heel prick collection procedures. DBS were originally used to test for phenlyketoneuria in neonates in the 1960s and has broadened its scope towards HIV testing and now is commonly used for FA profiling in humans [34]. Advancements in the sensitivity of mass spectrometry and gas chromatography means that very low volumes of blood can be used for meaningful analyses (~10-20 μL).

DBS in comparison to standard venous blood sampling has lower risks due to its non-invasive blood sampling protocol such that venous blood draws may raise ethical concerns in the elderly, infants or other populations. FTP sampling also allows multiple collections within short periods of time which is not possible with venous blood sampling [7]. However, FTP sampling methods collect unpredictable volumes of blood and can only be expressed qualitatively as percent of total fatty acids identified (%TFA). Differences in FAs when expressing data in %TFA can be dictated by increases or decreases in other FAs, which may mask the true quantitative response. This is particularly problematic when considering circumstances that may introduce variation in the total FA pool such as lipid disorders, postprandial responses or other environmental and genetic factors. Alternately, FA profiles can be expressed quantitatively as microgram of fatty acid per milliliter of blood (μg FA/ mL blood) if the volume of sample is known. FA quantitation can give insight into fluctuations in total concentrations and changes in individual FAs are much easier to identify because the variability in the profiles is more visible. Additionally, to truly consider factors that may influence DBS FA quantitation, FA contaminants present on dried blood spotting materials should also be considered.

Palmitate and stearate fatty acid contaminants have been identified on commonly used spotting materials [19]. Contaminants are included in FA determinations as they are methylated with the biological sample during chemical preparations. “Washing” chromatography papers in acetone or a methylating agent has been used to remove FA contaminants on blank spotting materials [35] but certain product designs include inks and other cosmetic features that may saturate into the blood spotting area. Lui et al. recently identified FA contaminants on the Fluka blood collection kit, Hemaspot-80 blood collection paper, Whatman 903 specimen collection card, Whatman 3MM chromatography paper, Whatman ion exchange chromatography paper and

Whatman glass microfiber filter paper [19]. Interestingly, all of the materials were commonly contaminated with palmitate (C 16:0) and stearate (C 18:0). Due to the incorporation of these FA contaminants during the methylation step in DBS analysis, their concentrations should be determined to see if their abundance is high enough to skew FA profiles of DBS samples in common dried blood spotting materials.

The simple blood collection procedure for DBS enables larger field or clinical studies. This leads to challenges with sample processing as larger sample sets require greater storage and/or transportation capacities. Recently, the stability of PUFA during sample storage has been reviewed [32]. In brief, PUFA stability can between 0 and 8 weeks at ambient temperatures, between 21 and 90 days at 4°C, and approximately 2 weeks at -20°C [15, 36-39]. Metherel et al. showed that EPA + DHA levels had the largest decline at -20°C in whole blood when compared to ambient, 4°C and -75°C temperatures [20]. The mechanism for this accelerated loss at -20°C is not well understood but is thought to be attributed to the disruption of the erythrocyte structure with freezing and iron release, since -20°C is not cold enough to prevent the oxidative process. Antioxidants have been used to prevent FA peroxidation as BHT-treated chromatography paper has been shown to reduce PUFA degradation in DBS samples and HUFA levels remains stable in for up to 21 days at ambient temperatures [15].

2.4 *Advances in Fatty Acid and Lipid Analyses*

The use of DBS samples for fatty acid analysis has increased based on advances in fatty acid and lipid analyses in the past 15 years [33]. These advances have increased analytical throughput, decreased costs and enabled larger sample sets to be analyzed. DBS samples are

well suited for high throughput initiatives as they are simple to collect but also have a reduced sample preparation time in comparison with the venous blood draw based sampling.

Until recently, the limiting step in fatty acid analysis was the analysis of derivitized fatty acids by gas chromatography as individual sample run times could take over one hour. The development of miniaturized capillary columns, improved temperature ramping and higher gas flow rates for gas chromatographs allowed for the development and adaptation of “fast” GC protocols. GC run times for a FA profile can now be completed in as little as 10 min [40], which shifted the limiting analytical step towards cumbersome and tedious sample preparations [33]. Traditionally, samples were prepared for fatty acid analysis by extracting the all lipids from the samples, saponifying the complex lipid to release the fatty acyls, and then derivitizing the free fatty acids to methyl esters [33]. These multiple steps are not amenable to automation and robotic handling, therefore the development of one-step chemical reactions [41] was the next critical step in increasing analytical throughput. This also resulted in examining the blood collection process and the use of microwave energy to drive chemical reactions for additional throughput gains [7].

It became clear that fingertip blood sampling and collection of DBS had significant throughput advantages [42, 43]. Because DBS samples are “dry” and there is no water in the sample to interfere with the chemical reactions, DBS samples can be directly transesterified to fatty acid methyl esters (FAMEs) without a lipid extraction step [7]. Samples containing considerable amounts of water typically require lipid extraction to get lipids in anhydrous organic solvents as water can interfere with the rapid boron trifluoride (BF_3) catalyzed reaction (14%) in methanol [44]. The presence of water would result in the production of free fatty acids rather than the methanol being used to derivitize the fatty acyls to methyl esters. Making FAMEs

is required to optimize the resolution of each fatty acid peak by gas chromatography with flame ionization detection [45]. This resolution allows for consistent linear responses across all the individual fatty acids which provides simplified quantitation.

One challenge with GC analysis is that the chemical derivatization required to produce FAMEs results in the loss of information about the native acyl species of complex lipids. While some of this information can be recovered by using additional techniques such as thin layer chromatography to isolate specific lipid classes prior to fatty acid determinations, the actual combinations of fatty acids on the lipids is never precisely known. Advancements in mass spectrometry (MS) and the emergence of the field of lipidomics has resulted in the ability to determine and measure lipids in their native occurring state, including detailed information about their fatty acyl species [46]. MS based analyses can therefore provide more insights on acyl lipid species remodeling events and biological activities that are difficult to observe using GC based FA analysis. This can be very useful for examining lipid metabolism during dietary intervention, pregnancy and metabolic diseases and can potentially reveal novel lipid blood biomarkers for dietary assessment and disease risk. However, at this time MS analysis remains costly, can be time consuming and the identification and quantitation of numerous lipid species remains problematic and difficult.

Chapter 3

Rationale, Objectives and Hypotheses

3.1 *Rationale*

A drop of blood collected by a lancet from a FTP can be done quickly, is relatively simple to perform and requires little infrastructure when compared with venous blood draws. The simplicity and the lower cost per sample collected supports both field research and large clinical studies which can produce a large number of samples that require storage prior to shipment to an analytical facility. FTP procedures typically result in the collection of unknown volumes of blood as DBS which prevents the ability to quantitate FA concentrations and limits data to qualitative units that can be less informative. In addition, the materials used to collect DBS samples can be contaminated with lipids and fatty acids during manufacturing and/or post production handling. Given that DBS samples are a comprehensive mixture of blood fractions and lipid pools, quantitative fatty acid data could be important to control for variability in samples due to conditions that could cause hypo- or hyper-lipidemia [47-49]. This thesis, therefore aims to address limitations in the ability to quantitate FA in DBS samples in 903 PSC, an industry standard, and a novel Mitra Microsampling Device. Methods to determine blood volume in a DBS sample, the determination and impact of FA contaminants on quantitation, and FA stability during different storage conditions will be examined.

3.2 *Objectives*

This thesis involves addressing three limitations in DBS quantitation that include; determining sample blood volumes, assessing FA contaminants on spotting materials, and FA stability during storage (see flow diagram: Figure 1). The initial objective was to develop a method

to determine unknown blood volumes of DBS. For the commonly used 903 PSC, the relationship between blood volume and the area of spotted blood was examined to develop an equation to allow blood volume to be calculated from a hole punch of a defined area. An alternative solution involved the validation of the Mitra Microsampling Device, which collects 10 μL of blood, for FA analysis. The use of Mitra for a lipidomics analyses was also assessed using UHPLC-MS/MS and compared to a wet blood control. Fatty acid contaminants present on commonly used dried spotting materials was examined using blank 903 PSC and the Mitra devices by GC-FID and by UHPLC-MS/MS. Finally, the stability of FA in DBS stored on 903 PSC and the Mitra with and without antioxidants were tested under ambient, 4°C, -20°C and -80°C conditions to determine fatty acid stability over 3 months .

3.3 *Hypotheses*

1. The area blood spotted on 903 PSC will have a strong positive linear correlation ($r > 0.8$) to the volume of blood applied.
2. The linear relationship between area of blood and volume of blood will allow a 6mm hole punch to determine an unknown volume of blood from 903 PSC. The calculated volume will be used to determine FA concentrations through GC-FID which will yield similar FA profiles compared to pipetted wet blood controls.
3. The Mitra Microsampling Device, a novel DBS device that wicks 10uL of blood will provide FA concentrations comparable with wet blood controls through GC-FID analysis.
4. HPLC-MS will be used to determine fatty acyl species of complex lipids on DBS using lipidomic approaches and will be comparable to results from wet blood controls.
5. GC-FID will identify palmitate and stearate fatty acids as contaminants on blank 903 PSC, Mitra tips and Whatman chromatography paper. HPLC-MS will identify palmitic and stearic free fatty acids on all materials.
6. The HUFA status of DBS samples will decrease equally in all storage conditions (ambient, 4°C, -20°C and -80°C) in both the Mitra and 903 PSC.
7. There will be an inverse relationship between temperature of storage and stability of HUFAs where decreasing storage temperatures will increase HUFA stability in DBS and -20°C which will show accelerated losses.
8. The addition of BHT will increase the stability of HUFA in the DBS samples in both materials at all conditions, except at -80°C which will show the most FA stability regardless of the presence of BHT.

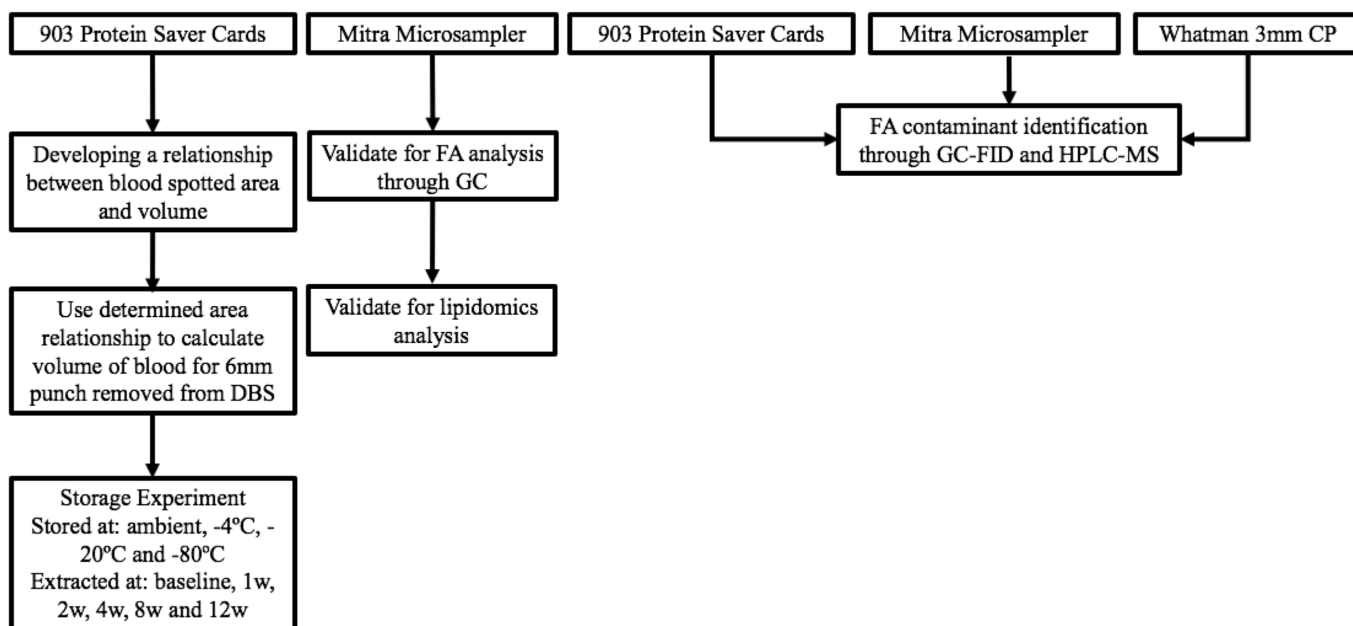


Figure 1. Flow diagram addressing the main experiments in this thesis. The development of an equation to determine unknown amounts of blood on 903 PSC; using the equation to determine volume based on 6mm punch of 903 PSC; Mitra FA analysis validation; Mitra lipidomics validation; blank material FA contaminant identification through GC and UHPLC-MS/MS on 903 PSC, Mitra and Whatman chromatography paper; storage experiment to determine FA stability using the 903 PSC and Mitra.

Chapter 4

Common Methods

Many of the methods in the present thesis were used for different objectives. In this chapter, the common methods are described in detail and can serve as a reference for the individual research chapters. All protocols and procedures have received approval from the Human Research Ethics Committee of the University of Waterloo.

4.1 *Blood Collection*

Venous blood was collected from the antecubital vein by a phlebotomist into sterile vacutainers to which 100 µL of 0.2M ethylenediaminetetaacetic acid (EDTA, Sigma-Aldrich) was added. Blood was aliquoted into smaller vials for application to dried spotting materials and storage. All DBS samples were allowed to dry at ambient temperatures and then stored at -75°C until analysis unless otherwise described.

4.2 *Lipid Extraction*

Total lipids were extracted from the DBS by adding 2:1 chloroform:methanol (v/v) and leaving at ambient temperatures for 24 hours [46]. Samples were then vortexed for 1 minute and 500 µL of a 0.2M sodium phosphate buffer was added to induce separation of the aqueous and organic layers. Samples were inverted once and then centrifuged at 3000 *rpm*. The organic chloroform layer then collected and stored at 4℃ until further analysis.

4.3 *Direct Methlyation and Methylation of Lipid Extracts*

Blood samples, or lipid extracts dried under nitrogen were combined with 300 µL of hexane containing a docosatrienoic acid (C22:3n-3) internal standard, and 1 mL of 14% boron trifluoride in methanol in a 5mL test tube with a silicon lined cap. The samples were then heated for one hour at 100°C on a block heater. Samples were removed and 1 mL each of hexane and water were added to the test tube followed by vortexing for 1 min and centrifugation at 3000 rpm for 5 minutes. The top layer containing the fatty acid methyl esters in hexane was removed, dried under nitrogen, and then reconstituted with 50 µL of heptane. The fatty acid methyl esters were then transferred to GC vials for analysis by gas chromatography.

4.4 *Gas Chromatography – Flame Ionization Detection (GC-FID)*

GC-FID is the most commonly used technology for fatty acid analysis [33]. FAMEs were analyzed on a Varian 3900 gas chromatograph equipped with a nitroterephathalic acid modified polyethylene glycol capillary column with 15 m x 0.10 mm i.d. x 0.10 um film thickness and hydrogen as a carrier gas [20]. Volumes of 1 µL were introduced by a Varian CP-8400 autosampler with a split ratio of 200:1 into an injector heated to 250°C. The initial temperature was held for 0.25min at 150°C followed by a 35°C/min ramp to 200°C, an incline of 8°C/min ramp to 225°C with a hold for 3.2 minutes to a final 80°C/min ramp up to 245°C for a 15-minute hold. The FID was set to 300°C with a nitrogen flow rate of 300mL/min.

4.5 *Ultra-High Performance Liquid Chromatography with Tandem Mass Spectrometry (UHPLC-MS/MS)*

A Thermo Q-Exactive coupled with a Dionex UHPLC system was used to perform UHPLC-MS/MS experiments. Samples were resuspended in a solution of 65:30:5 acetonitrile:isopropanol:water (v/v/v) with 0.1% formic acid. A C18 Ascentis Express column was used (15cm x 2.1mm x 2.0 um) with a binary solvent system. The two solutions for the mobile phase were; 60:40 acetonitrile:water (v/v), 10 mM ammonium formate and 0.1% formic acid (A) and 90:10 isopropanol:acetonitrile (v/v), 10 m M ammonium formate and 0.1% formic acid (B). The multistep gradient consisted of a mixture of the second solvent (B) into the first solvent (A) starting with a 32% ratio ramping up to 95% in 45 minutes [46]. Data was analyzed on the MassLynx 4.0 software. The tandem mass spectrometry (MS/MS) spectra was exported to the NIST 2.0 program for lipid identification using the LipidBlast database.

Chapter 5

Development of a Quantitative Method to Determine Fatty Acid Concentrations on 903 Protein Saver Cards and Validating the Mitra Microsampling Device for Fatty Acid Analysis

5.1 *Introduction*

The fatty acid profiling of DBS samples is increasing due to advantages in simplified sample collection, handling and processing. However, DBS collection has limitations because volume of blood collected on a paper matrix is usually unknown. As a result, FAs cannot be expressed as concentrations and must be represented percent of the total FA (%TFA). Most of the available literature using DBS sampling procedures express their FA data as relative percentage [4] due to the unknown volumes, unless blood is previously quantitated.

In this chapter, the relationship between blood volume pipetted and blood spot area on 903 PSC will be determined. This relationship will then be used to calculate the volume of blood on a 6mm punch of DBS on a 903 PSC. The Mitra Microsampling Device (Figure 1) that is designed to wick 10 μL of blood will also be validated for quantitative FA analysis. This device is designed to wick an accurate volume of blood regardless of hematocrit level and has been used for other analytes including cancer metabolomics and steroids determinations [50, 51] but not fatty acids and lipidomics.

5.2 *Methods*

In the first study, whole blood was collected by venipuncture with EDTA from one fasted healthy male participant as described above in Chapter 4. Five different volumes (15, 20, 25, 30

and 35 μL) were pipetted in triplicate onto 903 PSC (Sigma Aldrich, Oakville, CAN) and left to dry at ambient temperatures. Images were taken by 12 MP camera on a Samsung S7 Edge and saved as jpg files. Image were then analyzed using ImageJ software to determine accurate areas for each blood spot. The mean DBS area and known blood volumes were plotted and the linear equation was determined and assessed by a correlation coefficient.

In the second study, venous whole blood was taken from ten healthy adults attending the University of Waterloo that fasted overnight. Samples were aliquoted as wet whole blood (35 μL), DBS on 903 PSC and DBS on the Mitra (Neoteryx, Torrance, CA). For the 903 PSC, approximately 35 μL was applied, allowed to dry and then a Harris Uni-Core punch (Sigma Aldrich, Oakville, CAN) was used to remove a 6mm hole from the center of each spot, which was transferred to a test tube. For collection on the Mitra, the device was held at the surface of blood at 45° until completely saturated and left to dry at ambient temperatures for an hour. The Mitra tip was then removed and placed in a test tube. Direct transesterification was used to derivitize the fatty acyls and acids to methyl esters (Chapter 4, Section 4.3). In summary, final blood volumes that were used were 35 μL for wet blood, 9.6 μL for 903 PSC (see below for calculations) and 10 μL for the Mitra. The FAMEs were then analyzed by GC (Chapter 4, Section 4.4).

The fatty acid compositions of the wet blood and DBS were expressed as concentrations and relative percentages and compared by one-way ANOVA with Tukey's post hoc testing to determine differences between individual means. Bland-Altman plots were also used to examine the concordance between wet blood and the DBS collection materials. This was completed for the sum of the concentrations of EPA and DHA and the % of n-3 HUFA in total HUFA as these biomarkers of n-3 LCPUFA status are commonly used in the literature.

5.3 ***Results***

In study 1, the area of each blood spot was proportional to the volume of blood applied (Figure 2). After calculating the areas with Image J and plotting against the blood volumes (Figure 3), a linear relationship was determined ($y = 0.029x$, $r = 0.99$, $P < 0.001$). This equation was applied to the 6mm hole punched from the DBS on 903 PSC indicating that the hole punch contained 9.6 µL of blood.

In study 2, the mean fatty acid profiles for the ten individuals using the three different collection methods were relatively similar. Concentration values (µg FA/ mL blood) did not show any significant differences for the sums of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n-6 an n-3 polyunsaturated fatty acids (PUFA), highly unsaturated fatty acids (HUFA), and total fatty acids concentrations (Figure 4). In addition, there were no differences between commonly used biomarkers of omega-3 status (EPA+DHA, N-6/N-3, HUFA score) (Figure 5). However, there were significant differences for a few individual fatty acids (Table 1). These included significantly higher recoveries of C 10:0, C 12:0, C 12:1 and C 22:1n-9 and lower recoveries of C 22:0 for the Mitra and solely higher recoveries of C 22:0 for the 903 PSC in comparison to wet blood controls. When the fatty acids were expressed as relative %, there were no significant differences between any of the individual fatty acids, sums of fatty acid and/or ratios or biomarkers (data not shown). This was explored further by presenting the ranges of classes of fatty acids (Table 2). Within a sample set of 10 heathy individuals, the ranges were much wider when the data was expressed as concentrations. Concordance analysis between wet whole blood and DBS collection materials for EPA + DHA and the % of n-3 HUFA in total HUFA by Bland-Altman plots indicated the 95% confidence

intervals of the bias (average difference between whole blood and spotting method) extended beyond zero.

5.4 *Discussion*

Based on our results, it appears that DBS collected by 903 PSC and Mitra can be used for accurate fatty acid profiling. The blood volume and blood area on 903 PSC showed a strong linear correlation ($r = 0.99$, $P < 0.001$) that allowed for the determination of blood volumes when fatty acid profiles from 903 PSC, Mitra and wet blood were compared. There were some statistical differences between some individual fatty acids, specifically C 10:0 and C 12:0, but these were very low abundant fatty acids (each was < 0.5% of total fatty acids) with little impact to fatty acid subgroups. Concordance testing indicated that both the 903 PSC and Mitra are capable of providing omega-3 status results similar to wet blood analysis. To our knowledge, this is the first report of fatty acid concentrations in DBS. Previous studies reporting DBS fatty acids have presented the relative % of total fatty acids [7, 11, 13, 20, 42, 52]. Blood volume estimates of DBS hole punches have been shown to be linear to blood area on 903 PSC previously for other metabolites (REF 5). For fatty acid analyses, a 6mm DBS punch has been shown to have an average blood volume of 8.7 ± 1.9 µL on PUFAcoat cards, however this material in thinner and less absorbant due to silica gel coating [53]. The 903 PSC and Mitra examined in this chapter have shown to provide similar results to wet blood controls using technical replicates and blood from healthy participants. These preliminary results indicate that quantitative fatty acid determinations are possible from DBS. Further validation should be completed on different populations such as children, aging populations and malnourished individuals, which may have high or low hematocrit.

These observations have limitations. The assessment was completed using technical replicates, therefore the impact of biological variation was not assessed directly. Biological variation was indirectly assessed when the 9.6 µL volume for a 6mm punch was applied to the 903 PSC DBS samples collected from the 10 individuals and compared to wet blood and the Mitra. However, the study sample was restricted to a healthy young population sample that fasted prior to blood sampling. Given field studies often target different populations such as children, the elderly or malnourished, and that postprandial conditions can vary, additional research is needed to validate these DBS collection materials under different conditions. The variability in DBS area to blood volumes is related to blood viscosity that is determined by hematocrit levels, plasma viscosity, and erythrocyte aggregation and deformation. This could result in different linear equations to define the DBS area and blood volume relationship on the 903 PSC. However, using a small area/low volume of blood as we did, should minimize the effects of variation in DBS dispersion. There is evidence in the literature, that a “volcano” effect can occur with blood dispersion where the volume of blood per unit area is decreased around the edges of the DBS. Therefore, removing a hole punch towards the center of the DBS should provide an accurate unit of volume per unit of area.

5.5 *Conclusions*

In conclusion, quantitative FA profiling of DBS from healthy individuals is possible. This can be done by characterizing the blood volume/DBS area dispersion of the 903 PSC or by collecting a known volume of blood using a novel Mitra collection device. Further method validation is required with a larger sample size and especially in populations that may have different DBS blood dispersion on collection materials.

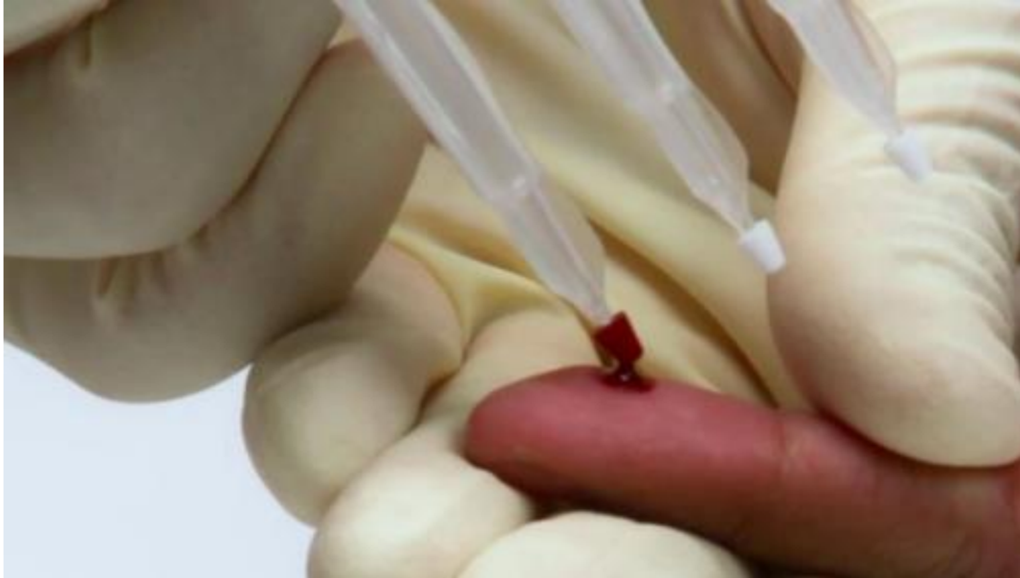


Figure 2. Mitra Microsampling Device (10 μL) with and without blood [54].

Figure 3. Whole blood spotted on 903 Protein Saver Cards. Volumes of 35, 30, 25, 20 and 15 μL were pipetted into the center of each spot and areas were determined using ImageJ.

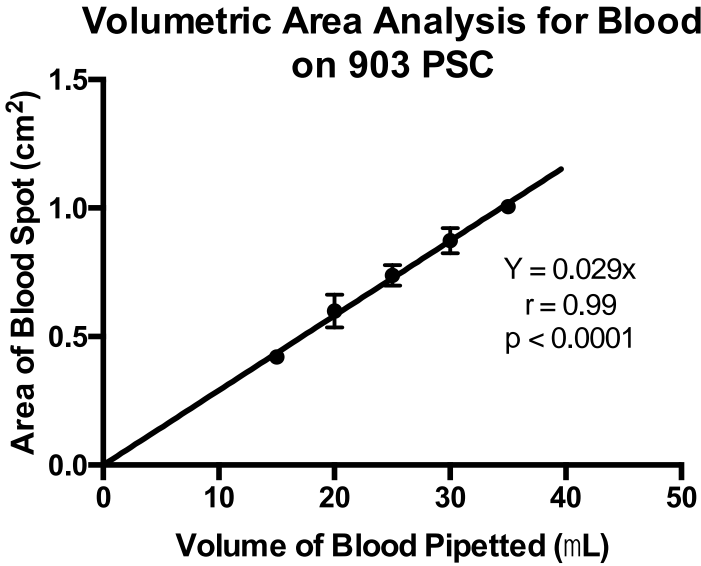


Figure 4. Linear relationship between volume of blood pipetted and saturated are on 903 Protein Saver Cards. The resultant equation was y = 0.029x where the y variable is the area in cm^2 and the x variable is volume in µL. Linear regression and p- values were determined, n = 3 for each volume point.

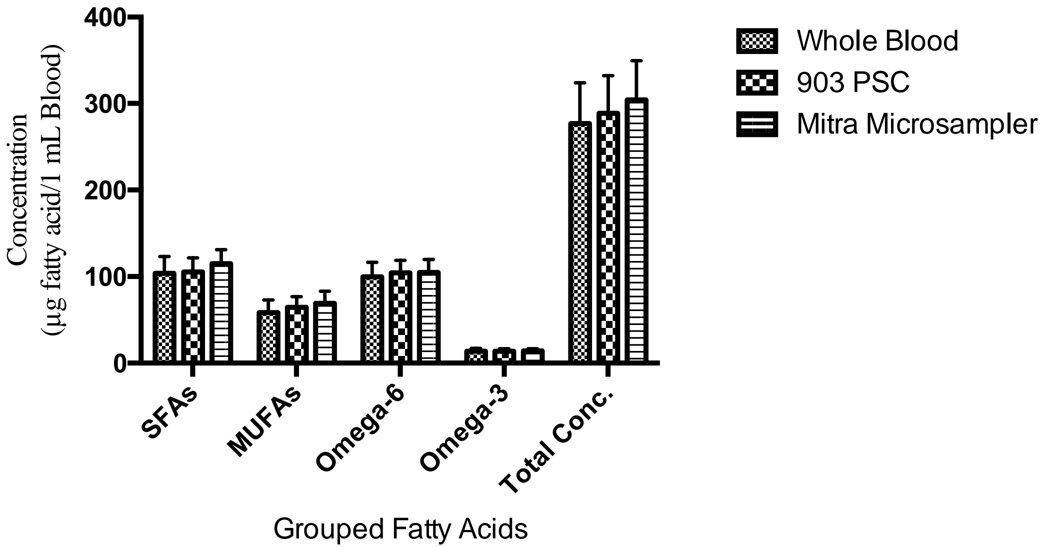


Figure 5. Concentrations (µg FA/ mL of blood) of fatty acid groups in whole blood, 903 Protein Saver Cards and Mitra. SFA, saturated fatty acids; MUFA, monounsaturated fatty acid. Each bar represents means with error bars representing the S.D., n = 10.

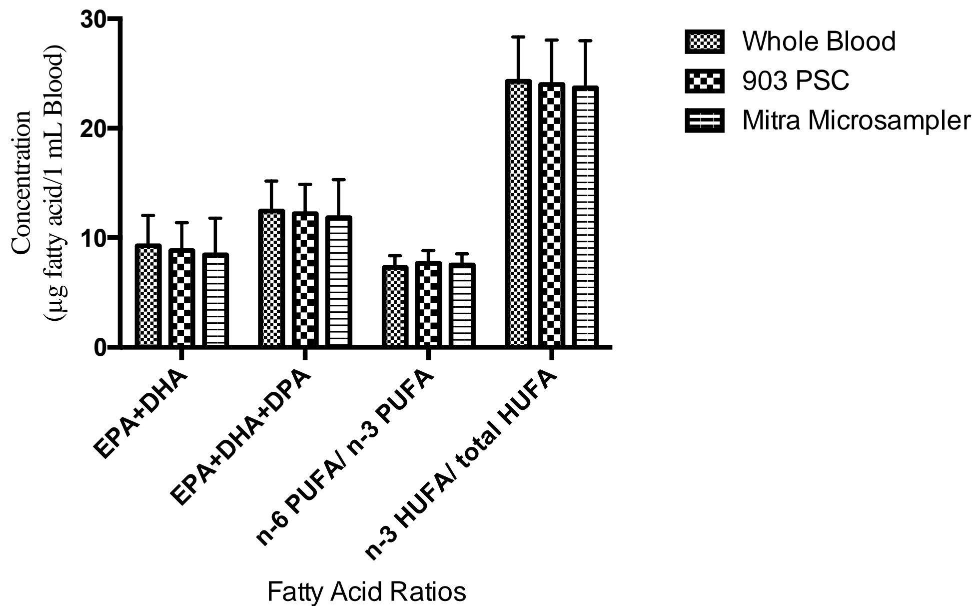


Figure 6. Concentrations (µg FA/ mL of blood) of biomarker status in whole blood, 903 Protein Saver Cards and Mitra. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; n-6, omega-6; n-3, omega-3. Each bar represents means with error bars representing the S.D., n = 10.

Table 1 ***FA compositions of wet blood control, 6mm hole punched 903 PSC and Mitra (µg FA/ mL of blood)***

Fatty Acid	Whole Blood	Dried Blood Spot	Mitra Microsampler
C 10:0	0.1 ± 0.1	0.1 ± 0.1	0.7 ± 0.3*
C 12:0	0.3 ± 0.1	0.5 ± 0.3	0.9 ± 0.3*
C 14:0	2.0 ± 1.0	2.4 ± 0.9	3.3 ± 1.0
C 16:0	58.5 ± 13.6	60.1 ± 13.3	65.3 ± 13.2
C 18:0	32.4 ± 7.5	31.8 ± 3.2	36.4 ± 3.5
C 20:0	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
C 22:0	3.1 ± 0.4	3.7 ± 0.3*	2.5 ± 0.3*
C 23:0	0.7 ± 0.1	0.8 ± 0.2	0.6 ± 0.1
C 24:0	5.9 ± 0.8	5.3 ± 1.1	4.8 ± 0.7
C 12:1	0.1 ± 0.1	0.1 ± 0.1	1.0 ± 0.9*
C 14:1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
C 16:1	2.6 ± 2.0	2.8 ± 1.9	3.1 ± 2.0
C 18:1n-7	4.3 ± 1.0	4.8 ± 1.0	5.0 ± 1.1
C 18:1n-9	44.2 ± 11.2	50.2 ± 9.3	52.4 ± 11.4
C 20:1n-9	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.1
C 22:1n-9	0.2 ± 0.2	0.3 ± 0.2	1.7 ± 0.4*
C 24:1n-9	6.6 ± 0.9	5.5 ± 1.1	5.3 ± 1.0
C 18:2n-6	60.0 ± 9.5	64.5 ± 10.1	64.3 ± 9.9
C 18:3n-6	0.7 ± 0.3	0.8 ± 0.3	0.7 ± 0.3
C 20:2n-6	0.2 ± 0.2	0.4 ± 0.5	0.2 ± 0.2
C 20:3n-6	4.0 ± 1.5	4.0 ± 1.4	4.1 ± 1.4
C 20:4n-6	29.8 ± 5.9	29.4 ± 5.0	29.4 ± 5.4
C 22:2n-6	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.1
C 22:4n-6	3.9 ± 0.9	3.7 ± 0.8	3.7 ± 0.8
C 22:5n-6	1.2 ± 0.5	1.5 ± 0.8	2.2 ± 0.4
C 18:3n-3	1.4 ± 0.6	1.6 ± 0.6	1.5 ± 0.6
C 20:3n-3	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
C 20:5n-3	1.5 ± 0.6	1.5 ± 0.5	1.8 ± 0.5
C 22:5n-3	3.2 ± 0.3	3.3 ± 0.6	3.4 ± 0.6
C 22:6n-3	7.7 ± 2.3	7.3 ± 2.1	7.4 ± 2.1
C 20:3n-9	0.6 ± 0.3	0.7 ± 0.1	0.7 ± 0.1
Total	276.9 ± 47.3	289.0 ± 43.3	304.1 ± 45.4

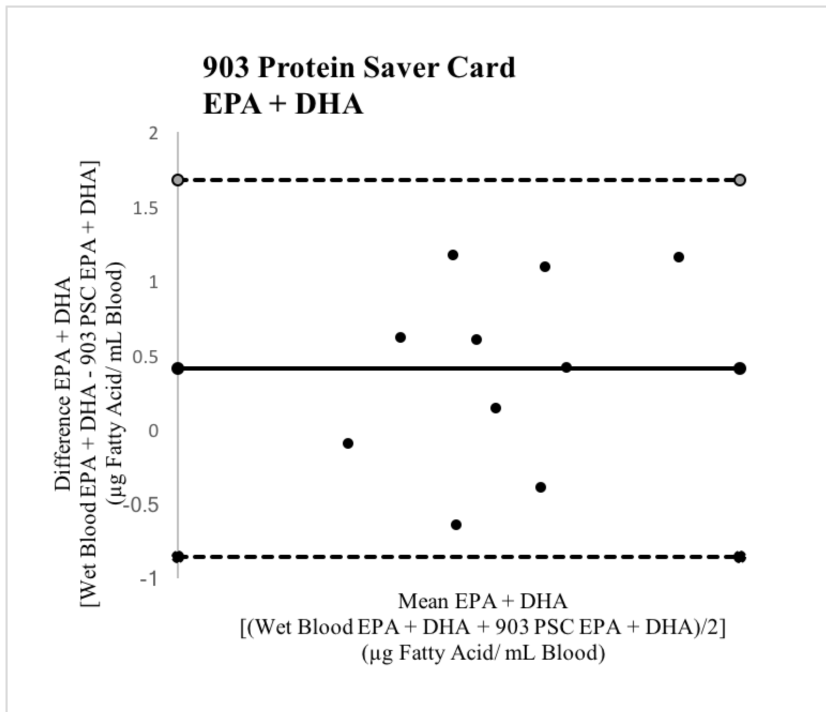
Asterisk used to highlight significantly different values (in comparison to wet blood control) after one-way ANOVA + Tukey HSD post-hoc test with $p < 0.05$. Data shown as mean ± SD.

Table 2 ***FA ranges expressed as relative percentages (%TFA) and concentrations (µg FA/ mL of blood).***

FA Groups	Relative Percent (%TFA)	Concentration (µg FA/ mL of blood)
SFAs	34.0-40.0	79.0-143.4
MUFAs	17.7-24.5	40.6-88.2
N-6	32.1-37.1	81.9-140.6
N-3	3.9-5.4	9.7-21.1
EPA+DHA	2.0-3.8	4.8-14.8

SFA, saturated fatty acids; MUFA, monounsaturated fatty acid, n-6, omega-6; n-3, omega-3; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid. Data is presented as range (min – max), n = 10.

A



B

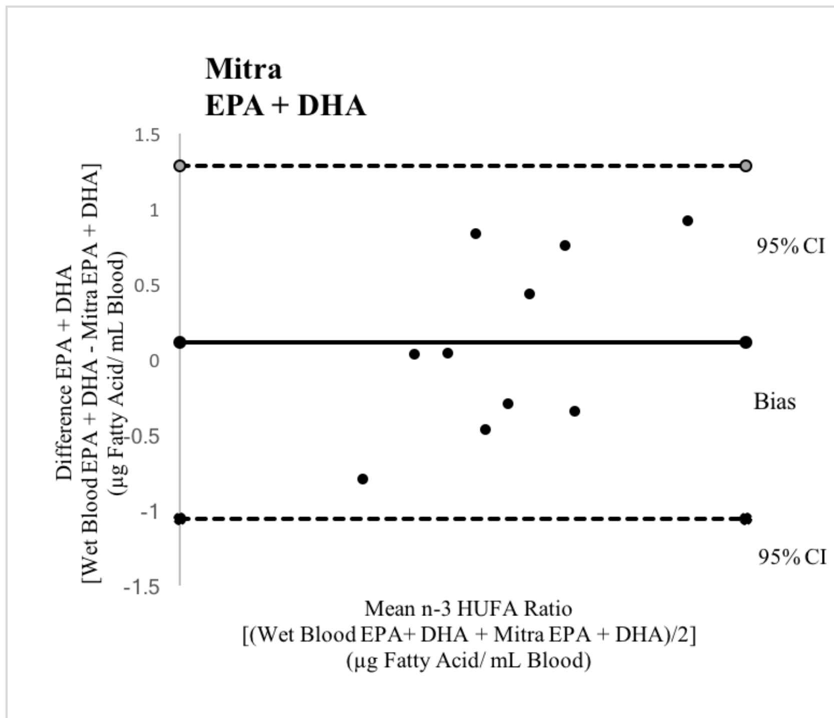
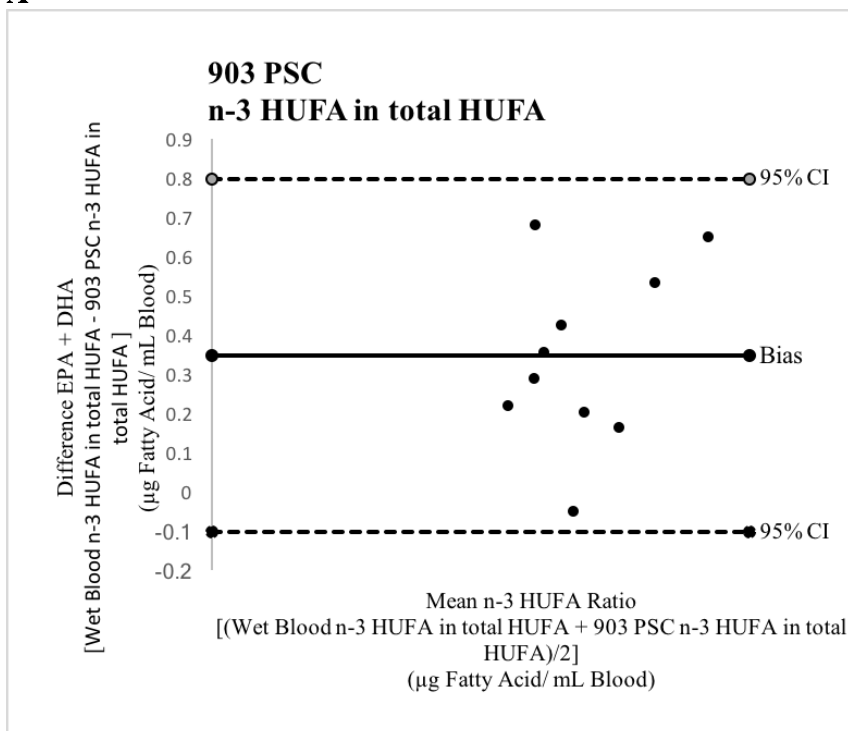


Figure 7. Concordance testing of 903 PSC, Mitra and whole blood for EPA + DHA levels. A. 903 PSC, B. Mitra. Sample tests completed on difference; whole blood, 903 PSC and whole blood, Mitra. (n = 10).

A



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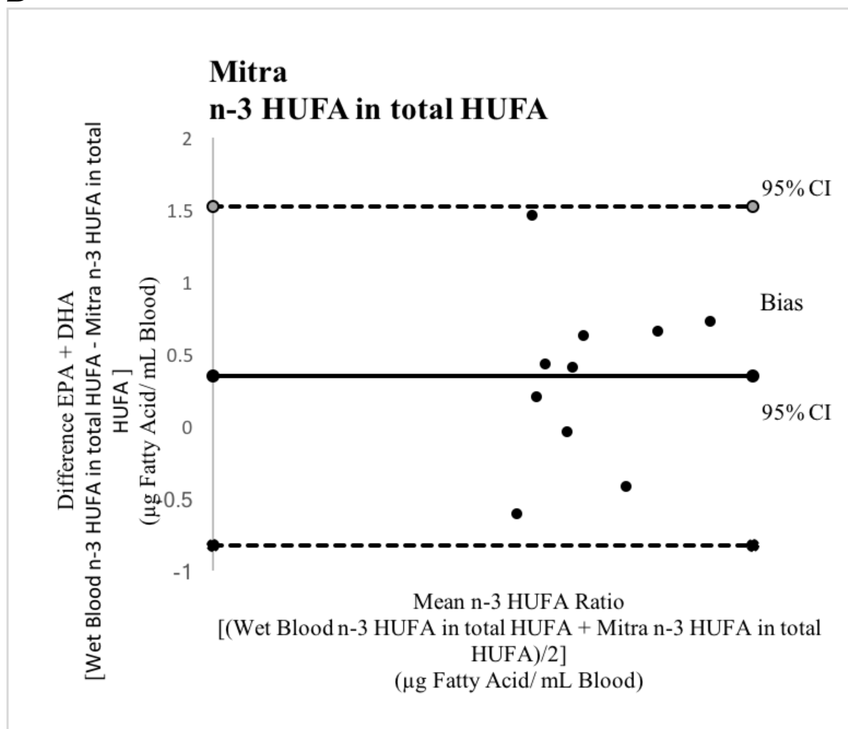

Figure 8. Concordance testing of 903 PSC, Mitra and whole blood n-3 HUFA in total HUFA. A. 903 PSC, B. Mitra. Sample tests completed on difference; whole blood, 903 PSC and whole blood, Mitra. (n = 10).

Chapter 6

Identification of Fatty Acid and Lipid Contaminants Commonly found in Blank Dried Blood Spotting Materials

6.1 *Introduction*

DBS collections use materials that may be contaminated with lipids. Using gas chromatography methods, lipids are either extracted or the fatty acyls on lipids are directly transesterified prior to fatty acid composition analysis. However, due to this procedure, any lipid and fatty acid contaminants on the DBS collection materials can be incorporated into the biological sample which potentially reduces the accuracy and precision of the FA determinations.

In the past, measures have been taken to reduce the prevalence of contaminants by soaking the materials in a methylating agent for 1h, 3h and 10h at ambient temperatures and 1h, 2h and 3h at 70°C. The study showed a 60% reduction in contaminants after 2h and 80% reduction after 3h at 70°C but the texture of the cards was destroyed and unusable [19]. Alternatively, others have used a 2:1 choloroform:methanol wash (v/v) to remove nearly 80% of the contaminants for most collection papers in 3h [19]. Although methods exist to treat collection materials to remove contaminants, certain materials with cosmetic features such as ink cannot be washed. Saturated FA, specifically palmitate (C 16:0) and stearate (C 18:0) are the commonly found contaminants on tested dried spotting materials [19].

Understanding the concentration and type of contamination in commonly used spotting materials can be used to see how FA profiles in biological samples may be affected. The experiments in this chapter will examine contaminants on 903 Protein Saver Cards, Whatman chromatography paper and the Mitra through GC analysis. These three materials were chosen due to the popularity in 903 PSC in FA literature, the prevalence of Whatman chromatography strips

in our current lab use and no information on the current blank FA profile on the Mitra. UHPLC-MS/MS will also be used to determine the actual lipid source of the fatty acid contaminants.

6.2 *Methods*

Blank Whatman chromatograph paper, 903 PSC and Mitra tips without blood samples were used and all materials were handled using nitrile gloves. In triplicate, hole punches (6mm) were taken from the paper materials, and tips were removed from the Mitra, and then placed in clean glass test tubes. Total lipids were extracted from tips using a modified Folch protocol [55]. In brief, the samples were submerged in 3 mL of 2:1 chloroform:methanol (v/v) and incubated overnight. The organic chloroform layer was then separated using a sodium phosphate buffer (Na_2PO_4) and separated into two aliquots.

The first aliquot was used in study 1 for GC analysis. The organic chloroform layer was dried under nitrogen and FAMEs were prepared using 14% boron trifluoride in methanol. The FAMEs were then analyzed by GC as described in Chapter 4 (4.3, 4.4).

The second aliquot of chloroform was used in study 2 for mass spectrometry analysis. The sample was dried under nitrogen and then re-suspended in 65:35:5 acetonitrile:isopropanol:water + 0.1% formic acid and analyzed using a reverse phase UHPLC multi-step binary protocol as described in section 4.5. Positive ESI mode was used to identify all lipid species but could not identify FFA species. Therefore, negative ESI mode was used to detect FFA because of the chemical properties of the compound, specifically the deprotonated form of the carboxylic acid ($R\text{-}COO^-$). A spray voltage of -3.0kV was used to ionize and identify the FFA species. The mass spectra was exported to the NIST 2.0 program for lipid identification using the LipidBlast database.

6.3 *Results*

In the first study, 16:0 and stearic 18:0 fatty acids were identified in all three DBS collection materials through GC-FID analysis and no other fatty acids were detected. Specifically, Whatman chromatography paper had the highest level of contaminants with 0.97±0.08 μg of 16:0 and 1.43±0.16 μg of 18:0 per 6mm punch, the 903 PSC had 0.64±0.05 μg of 16:0 and 0.88±0.04 18:0 per 6mm punch and the Mitra had 0.80±0.08 μg 16:0 and 0.98±0.10 μg 18:0 per tip.

The second study used the GC results for the UHPLC-MS/MS analysis to perform a targeted search for lipids containing stearate and palmitate. Negative ionization mode identified palmitic acid and stearic acids in all materials as free fatty acids with the [M-H]- mass-to-charge ratios of 255.23 and 283.26 respectively. Additionally, positive mode analysis revealed that the Mitra tips also contained palmitoyl and stearoyl lysophosphatidylcholines (LysoPC) with [M+H]+ m/z ratios of 496.34 and 524.37, respectively.

6.4 *Discussion*

This study suggests that Whatman chromatography paper, 903 PSC and the Mitra are all contaminated with palmitate and stearate containing lipids. The presence of these contaminants can skew FA profiles due to the inclusive methylation protocol through GC-FID analysis. Pre-treatment of chromatography papers can be used to reduce concentration amounts on certain collection materials. However, FA species and abundances should be determined in circumstances where washing procedures cannot be applied. Whatman chromatography paper showed the highest amount of C 16:0 and C 18:0 contaminants followed by Mitra and then 903 PSC. The amount of contaminants when compared to C 16:0 and C 18:0 in the blood of the healthy subjects is

minuscule and well below the typical variation observed with intra-individual analysis. Given the prevalence of FA contaminants is below 1 µg, it does not impact the total concentrations by much, as we estimate the contaminants are 1% of the total C 16:0 and 3% of C 18:0 in blood. Contaminants including C 20:0 and C 22:0 have been identified on 903 PSC previously, but at extremely concentrations [19]. We did not detect C 20:0 and C 22:0 by either GC-FID or UHPLC-MS. We analyzed blank 6mm (0.28cm^2) punches of 903PSC with minimal handling. The previous study examined a 1.5 X 1.5 cm blank (2.25cm^2) that was treated with an internal standard [19]. Therefore, the C 20:0 and C 22:0 could have been below the limits of detection for our analyses, or they were introduced onto the blanks during handling in the previous study. Further testing should be completed on glassware, solvents and plastics (such as pipette tips) and different steps of sample processing during FA analysis to identify potential sources of contamination.

The fact that the Mitra was the only material to have palmitoyl and stearoyl lysophosphatidylcholines was interesting. While lysophosphatidylcholines exist naturally in various sources, their presence in the absence of phosphatidylcholines suggests they have been added to the material intentionally. Lysophosphatidylcholines or lysolecithin has industrial applications as wetting and/or emulsification agents [56]. Therefore, the lysoPC could be intentionally added to contribute to the wicking abilities of the Mitra because of its water soluble and fat soluble properties (also seen in emulsifiers). If lipidomics analysis is used, these lysoPC species can be selectively excluded as contaminants from the data extraction or acquisition and the remaining complex lipids can still be characterized fully.

This study shows that FA contaminant exist, but that they have low impact on blood FA profiles in DBS. Steps can be taken to “wash” materials to remove the SFA contaminants;

however, this can effect ink on the 903PSC and potentially impact the wicking capabilities of the Mitra.

6.5 *Conclusions, Limitations and Future Directions*

In conclusion, palmitate and stearate FAs were found on Whatman chromatography paper, 903 PSC and the Mitra. The m/z ratios of these FAs were used to identify their encompassing lipids using mass spectrometry. Palmitic and stearic FFAs were identified in all materials and palmitoyl and stearoyl LysoPCs were identified solely in the Mitra. A more comprehensive determination of contaminants should be completed by examining different batches of each material to test the consistency of the contaminants present.

Chapter 7

Lipidomic Profiling of Human Whole Blood using the 10 μL Mitra Microsampling Device

7.1 *Introduction*

Blood fatty acid profiling has shown a link between lipid metabolism, dietary intake and disease risk. FA analysis is commonly gas chromatography based and require a derivatization reaction to prepare FAMEs for analysis and as a result, there is no available information on the fatty acyl parent lipid species. Techniques such as thin-layer chromatography (TLC) can isolate lipid classes before FAs are determined but the acyl species of the original lipids remain unknown [57]. The advances in UHPLC-MS/MS has enabled the identification of complex lipid species as they exist in biological samples. A recent study has determined lipidomic profiles on Whatman cellulose paper, the first of its kind to apply lipidomics to DBS samples [46]. The development of a high-throughput UHPLC-MS/MS method identified a vast number of lipid species that occurred in DBS samples and also addressed the complexity in studying whole blood lipids due to the polar and non-polar lipid mixtures and range of abundances as well as the retention of specific lipids on the collection materials. The untargeted lipidomics profiling method characterized the main lipid species in blood including phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), triacylglycerol (TAG) and cholesterol esters (CE). The other two recorded studies on DBS lipidomics used Ahlstrom 226 collection cards to determine lipid species in infant heel prick collections [58, 59]. This chapter examines the ability to use the Mitra sampling device for lipidomic assessments of DBS. A semi-quantitative, untargeted UHPLC-MS/MS method is used

and the lipid species identified in the Mitra was compared to those identified in wet blood controls.

7.2 *Methods*

Whole blood was collected by venipuncture with EDTA from one fasted healthy male participant as described above in Chapter 4 (Section 4.1). Samples were aliquoted as wet whole blood (35 μL) into clean test tubes and the Mitra tips were held at 45° until completely saturated, left to dry at ambient temperatures and placed into clean test tubes. Total lipids were extracted from tips using a modified Folch protocol [55]. In brief, the samples were submerged in 3 mL of 2:1 chloroform:methanol (v/v) and incubated overnight. The organic chloroform layer was then separated using the lipid extraction protocol discussed in Section 4.2 dried under nitrogen. Samples were then re-suspended in 65:35:5 acetonitrile:isopropanol:water + 0.1% formic acid and analyzed using a reverse phase UHPLC multi-step binary protocol as described in section 4.5. The lipids were presented as arbitrary units which is the qualitative ratio between amount of substance compared to a predetermined reference measurement, in this case a 17:0/17:0 PC internal standard (Avanti, Alabaser, AL). The mass spectra were exported to the NIST 2.0 program for lipid identification using the LipidBlast database.

7.3 *Results*

There were significant differences between the lipidomics profiles generated from the Mitra DBS and the wet whole blood control. The Mitra DBS resulted in significantly lower amounts of 16:0/18:2 PC, 16:0/20:4 PC, 18:0/22:6 PS, 16:0 lyso PC and 18:0/20:4 PS. However, a pattern in the other PC species that were not significantly different was also observed in that all the PC compounds where roughly 10% lower when determined from Mitra DBS. For

PE, 16:0/18:2 PE was also roughly 10% lower (not significantly different) but 16:0/22:6 PE from Mitra was very similar. Determinations of PS species were particularly low from the Mitra (approximately 55% lower than control) as well as 16:0 lysoPC (32% lower than control). In contrast, the Mitra lipidomics profile showed consistent higher but not significant recoveries of the identified TAG (10-15% higher) and CE (29% higher) species; 16:0/18:1/18:2 TAG, 16:0/18:1/18:1 TAG, 18:1/18:1/18:2 TAG and 18:2 CE (Table 3).

7.4 *Discussion*

The generally lower recoveries of phospholipids (~10%) and higher recoveries of TAG/CE species is likely due to the polarity of the compounds and interactions with the Mitra tip and the ability to extract lipids from the Mitra tip. A recent study examining extraction protocols for lipidomics from DBS on chromatography paper showed similar profiles to wet blood could be obtained with 24h exposure to extraction solvents, homogenization of the collection material, and acid treatment to assist in recovering PS species [46]. The PS recovery required acid extraction to disrupt the hydrogen bonding between the carboxylic acid group of PS with the cellulose fibers in the paper [46].

While the Mitra tip composition is proprietary, it appears to share some of the properties of chromatography paper in regard to the retention of lipids. The three dimensional shape (cone) of the Mitra appears to result in less contact exposure to the extraction solvents (chloroform:methanol, v/v) during the overnight soak in compared with the relatively two dimensional shape of DBS on chromatography paper resulting in potentially poorer extraction efficiencies. The decreased extraction efficiencies of the polar lipids presently observed strongly suggests that the Mitra tips are made of a polar material that is retaining polar compounds, particularly acidic compounds The higher recoveries of TAG/CE species we observed with the

Mitra tip suggests extraction efficiencies for non-polar compounds are relatively high, and is further evidence that the Mitra materials are nonpolar. The slightly higher recoveries of the nonpolar lipids from the Mitra tip relative to whole blood is likely due to the use of a polar lipid internal standard (17:0/17:0 PC) to calibrate responses in the mass spectrometer. Lower recoveries of the internal standard likely resulted in an overestimation in the amounts of the polar lipids.

7.5 *Conclusions, Limitations and Future Directions*

In conclusion, the Mitra was not validated for semi-quantitative lipidomics assessment. However, it appears that additional steps in the extraction of the lipids from the Mitra could enable validation in the future. Understanding the materials and specific chemical composition of the Mitra tip would facilitate validation but at this time this information remains unknown. It would appear that many of the steps defined by Henao et al. [46] could be applied to improve lipidomic profiling from DBS collected by Mitra tips.

Table 3 ***Lipidomic examination of the Mitra and a wet blood control***

Lipid	Ion	m/z	Mitra Mean	WB Mean	% of WB
16:0/18:2 PC*	[M+H]+	758.57	10.6 ± 0.80	12.1 ± 0.24	88
16:0/20:4 PC*	[M+H]+	782.57	7.94 ± 0.16	8.46 ± 0.27	94
16:0/18:1 PC	[M+H]+	760.59	3.66 ± 0.27	3.98 ± 0.11	92
18:0/18:2 PC	[M+H]+	786.6	3.27 ± 0.16	3.54 ± 0.08	92
16:0/18:1/18:2 TAG	[M+NH4]+	874.79	3.42 ± 0.28	3.11 ± 0.11	110
16:0/18:1/18:1 TAG	[M+NH4]+	876.81	3.20 ± 0.26	2.79 ± 0.10	115
18:2 CE	[M+NH4]+	666.62	1.32 ± 0.32	1.03 ± 0.03	129
18:1/18:1/18:2 TAG	[M+NH4]+	900.81	1.09 ± 0.08	0.98 ± 0.03	111
18:0/20:4 PC	[M+H]+	810.6	0.91 ± 0.05	1.01 ± 0.05	90
18:0/18:1 PC	[M+H]+	788.62	0.82 ± 0.06	0.90 ± 0.02	91
16:0/22:6 PC	[M+H]+	806.57	0.78 ± 0.04	0.85 ± 0.02	91
18:0/22:6 PS*	[M+H]+	836.54	0.43 ± 0.34	0.93 ± 0.03	46
16:0 LPC*	[M+H]+	496.34	0.46 ± 0.20	0.67 ± 0.03	68
16:0/22:6 PE	[M+H]+	764.52	0.58 ± 0.02	0.56 ± 0.01	103
18:0/20:4 PS*	[M+H]+	812.54	0.27 ± 0.11	0.61 ± 0.03	45
18:0/22:6 PC*	[M+H]+	834.6	0.21 ± 0.01	0.24 ± 0.01	91
16:0/18:2 PE	[M+H]+	716.52	0.07 ± 0.01	0.08 ± 0.01	90

Arbitrary units (AU), Mass-to-charge ratio (*m/z*); Phosphatidylcholine (PC); Phosphatidylethanolamine (PE); Phosphatidylserine (PS); Triacylglycerol (TAG); Cholesteryl ester (CE); Highly unsaturated fatty acid (HUFA). Asterisk used to highlight significantly different values after one-way ANOVA + Tukey HSD post-hoc test with $p < 0.05$. (n = 5).

Chapter 8

Fatty Acid Changes in Dried Blood Spots when Stored at Various Temperatures

8.1 *Introduction*

The proper storage and transport of biological samples are integral to maintain FA stability in DBS. Field studies with limited access to ultra-cold storage or analytical laboratories should consider their best option for storage and use anti-oxidants to preserve FA profiles. Currently, only a few studies have assessed the stability of FTP profiles stored on chromatography paper [7, 11, 14, 15, 20]. Findings have shown PUFA stability to be between 0 and 8 weeks at ambient temperatures, between 21 and 90 days at 4°C, two weeks at -20°C and longer than 30 days at -40°C [15, 37-39, 43]. Although -20°C is often considered the second best storage option compared to -80°C, Metherel et al. [14] showed that -20°C storage has the highest instance of FA loss in comparison with 4°C and ambient temperatures and its use should be avoided if possible. In addition to using ultra-cold storage conditions, the antioxidant butylated hydroxytoluene (BHT) has been shown to improve PUFA stability between 3 weeks and 3 months at 4°C and less than 2 months at room temperature [15]. This study also showed that using a concentration of 5.0 mg/mL of BHT in blood can prevent HUFA degradation and EPA + DHA degradation from baseline for up to 28 days and PUFA degradation for up to 8 weeks at ambient temperatures.

Currently, there is there no literature on FA stability for the 903 PSC and Mitra for different storage conditions. Determining FA behavior can aid with the understanding of sample collection, transport and storage prior to analysis by clinical, medical and academic researchers. Blood from 10 participants were spotted onto 903 PSC and Mitra and stored at ambient, 4°C, -

20°C and -80°C temperatures and wet blood was stored at -80°C to act as controls for each extraction point. The conclusions made from this chapter can be used to develop a deeper understanding of FA stability which can be used to guide academic, research, clinical and medical sample collection and storage.

8.2 *Methods*

Venous blood was collected from the antecubital vein into two vacutainers, one with BHT and one without BHT, for each of the ten fasted, healthy, adult participants as described in Chapter 4, Section 4.1. Samples were aliquoted as wet whole blood in a cryovial, DBS on 903 PSC and DBS on the Mitra. For the 903 PSC, approximately 35 μL was applied to the center of each spot and allowed to dry at ambient temperatures. A Harris Uni-Core punch (Sigma Aldrich, Oakville, CAN) was used to remove a 6mm hole from the center of each spot and then transferred to a clean test tube. For blood collection on the Mitra, the device was held at the surface of blood at 45° until completely saturated (10 μL) and left to dry at ambient temperatures for an hour. The Mitra tip was then removed and placed in a test tube. Samples were then stored at ambient, 4°C, -20°C and -80°C conditions and lipids extracted at baseline, 1 week, 2 week, 4 week, 8 week and 12 week time points. For wet 35 μL of blood was used for lipid extraction. FAMEs were prepared using 14% boron trifluoride in methanol and were analyzed through GC as described in Chapter 4, Section 4.3, 4.4.

Individual fatty acids were identified by comparison to an external reference standard and data was expressed as the relative weight percentage of total fatty acids and as concentrations per volume of blood. Common biomarkers such as the relative percentage of EPA + DHA (omega-3 index) and percentage of n-3 HUFA in total HUFA were also calculated. The effect of storage

temperatures, time, blood collection materials and anti-oxidant use on the percentage of EPA+DHA were assessed using a three-way ANOVA with repeated measures as it is the most popular biomarker, and it has been shown to be sensitive to oxidative losses [14]. In addition, repeated measure ANOVAs were used for each condition to examine the effect of time on the percentage and concentration of EPA + DHA, the percentage of n-3 HUFA in total HUFA and individual fatty acids. After a significant F-value as determined for a repeated measures ANOVA, post hoc tests were performed using a Dunnett's test with baseline data as control with statistical significance inferred when $p < 0.05$.

8.3 *Results*

The interaction between time × collection material × storage temperature × antioxidant use was not significant. Time and storage temperature were involved in several significant interactions, while the use of antioxidant largely required interaction with time for a significant F-value. Collection material tended to be involved in significant effects except for an interaction with time and antioxidant use. To simplify, the relative percentage for EPA + DHA are presented according to the repeated measures ANOVA for each condition with post hoc comparisons to baseline levels (Table 4). The 903 PSC without BHT had no differences for up to 8 weeks compared to baseline at ambient and 4°C storage conditions and significant losses as soon as 1 week at -20°C. With BHT, there were similarly no significant differences from 4 to 8 weeks at ambient and 4°C however it did increase the stability of the ratio from 4 8 weeks at -20°C. The Mitra presented similar results to the 903 PSC. In brief, the Mitra without BHT had FA stability for 4 to 8 weeks at baseline and 4°C, but was significantly lower as soon as 1 week at -20°C. With the addition of BHT to the Mitra, the EPA + DHA ratio was still stable for 4 to 8

weeks at ambient and 4°C and up to 2 weeks at -20°C. Over the 12 week storage period, both the 903 PSC and Mitra did not show any significant losses at -80°C.

The n-3 HUFA in total HUFA ratio was determined in relative abundance (%TFA) at all conditions for both the 903 PSC and the Mitra (Table 5). The 903 PSC without BHT did not show any significant differences from baseline for the n-3 HUFA in total HUFA ratio for up to 8 weeks at both ambient and 4°C storage conditions. The -20°C condition showed significant differences as soon as week 2 without BHT but increased from 4 to 8 weeks with BHT. With the addition of BHT, the n-3 HUFA to total HUFA ratio was not significantly different from baseline for 4 to 8 weeks at ambient and 4°C storage conditions. As expected, the -80°C storage condition showed no significant differences from baseline for up to 12 weeks. The Mitra without BHT showed similar results to the 903 PSC. Significant differences were observed from baseline at 8 weeks with no BHT at ambient temperatures and 4°C conditions and 2 week at -20°C. The addition of BHT had no differences from baseline for 4 to 8 weeks at ambient, 4°C and -20°C. Similar to the 903 PSC, the -80°C condition showed no significant differences from baseline for up to 12 weeks.

To determine actual losses of EPA + DHA rather than relative changes, concentrations were determined in ambient, 4°C, -20°C and -80°C conditions over 12 weeks for 903 PSC (Figure 8). From baseline at ambient and 4°C temperatures, EPA + DHA concentrations were not significantly different for up to 8 weeks with and without the addition of BHT. At -20°C, EPA + DHA concentrations were significantly different from baseline as soon as 1 week without BHT and 2 weeks with BHT. The -80°C remained stable up to 12 weeks and showed no significant differences from baseline. The Mitra showed a similar pattern to the 903 PSC (Figure 9). In brief, EPA + DHA concentrations were significantly different for 4 to 8 weeks with or

without BHT at ambient and 4°C temperatures, 1 week without and 2 weeks with BHT at -20°C and remained stable through 12 weeks both with and without BHT at -80°C.

The 903 PSC showed highest FA stability over 12 weeks when stored at -80°C in comparison to baseline values and only showed significant differences in the total FAs category (Table 6). The relationship between time, the presence or absence of BHT and temperature showed significant differences at the -20°C conditions. The highest differences from baseline were seen at the -20°C condition without BHT (Table 6). There were significantly lower concentrations for most FAs in comparison to baseline; the total saturated fatty acids (SFAs), HUFA, PUFA, total n-6, total n-3, and total concentrations were all significantly lower in comparison to baseline values. Similarly, the Mitra FA profiles showed the most significant differences at -20°C for most individuals FAs and biomarkers including total SFAs, HUFAs, PUFAs, total n-6, and total n-3 (Table 7).

8.4 *Discussion*

The fatty acids in the samples were the most stable at -80°C, followed by 4°C and ambient temperature, and the least stable at -20°C in both BHT and no BHT conditions. The stability of the EPA + DHA and n-3 HUFA in total HUFA biomarker ratios were increased with the use of BHT in all conditions. The reason for the accelerated degradation at -20°C is because of the hemolysis and iron-initiated peroxidation in RBCs [52] as the hemoglobin-iron complex (HB-Fe^{2+}) can be converted into (HB-Fe^{3+}) by O_2 resulting in O_2 free radical and Fe^{3+} formation which can interact with PUFAs and cause peroxidation and FA loss. This study supports the conclusions by Metherel et al. [52] stating that ambient and 4°C storage conditions should be used if there is no access to -80°C, if solely using BHT as a preservation agent. We also

confirmed that expressed omega-3 status data as the percentage of n-3 HUFA in total HUFA results in an omega-3 status that is more resistant to changes due to oxidation as the HUFA pool tends to degrade at a similar rate in comparison with data expresses relative to total fatty acids where the saturated and monounsaturated pools are much more stable [14].

The interaction effect involving collection material and time and antioxidant on the percentage of EPA+DHA was not anticipated. This appears be driven in part by an increase in EPA + DHA measured in the Mitra after 1 week of storage with no BHT. This seems to be a spurious result as an increase above baseline is unlikely. In contrast, the EPA + DHA in 903 PSC does not appear to change at week 1 with and without BHT while there is a slight decrease in Mitra with BHT.

This study contributed to the literature by determining FA stability quantitatively on both 903 PSC and the Mitra. Quantitative data is overall rather low in comparison to qualitative data for DBS due to the collection of unknown volumes so most FA stability information is presented in relative percentage. Iron chelators to prevent iron initiated oxidation, glucose to prevent cell lysis and BHT combined could improve FA stability at -20°C [52]. However, using BHT alone shows biomarker stability from 4 to 8 weeks at both ambient and 4°C conditions. Ultra-cold storage should be used whenever available as studies have shown biomarker stability for at least 6 months at -75 °C [14].

8.5 *Conclusions, Limitations and Future Directions*

In conclusion, storage of DBS on 903 PSC and Mitra should be at ultra-cold temperatures such as -80°C whenever possible. If ultra-cold storage is not possible, ambient and 4°C should be considered, while storage at -20°C should be avoided. In addition, efforts should be made to add BHT to DBS samples to also prevent losses of PUFA to oxidation. In the present study,

BHT was added to blood during collection, but this may not be possible for samples collected from capillary beds such as fingertip or heel prick. Further research is needed to determine if both 903 PSC and Mitra can be pretreated with BHT prior to blood collection. Both EPA + DHA and n-3 HUFA in total HUFA displayed similar behavior when considering stability over time. Similarly, to have reliable biomarker data, samples should be stored at -80°C followed by 4°C or ambient temperatures. The conditions reviewed in this study are applicable to field research and the potential storage limitations that may accompany it. Ultra-cold storage is generally limited to analytical facilities and therefore ambient, 4°C and -20°C were examined as alternatives.

Table 4 ***Relative percentage (% of total fatty acids) of EPA + DHA in 903 PSC and the Mitra during storage over 12 weeks.***

Material	Condition	Temperature	Storage Day					
			Baseline	1w	2w	4w	8w	12w
903 PSC	No BHT	Ambient	3.04 ± 0.65	3.01 ± 0.55	3.10 ± 0.42	3.05 ± 0.59	2.72 ± 0.44*	2.45 ± 0.51*
		4ºC		3.12 ± 0.50	3.20 ± 0.39	2.99 ± 0.55	2.62 ± 0.32*	2.35 ± 0.41*
		-20ºC		2.31 ± 0.44*	2.05 ± 0.40*	1.55 ± 0.38*	1.23 ± 0.44 *	1.01 ± 0.39*
		-80ºC		3.01 ± 0.51	3.11 ± 0.34	3.05 ± 0.37	2.98 ± 0.41	2.99 ± 0.49
	BHT	Ambient	3.01 ± 0.51	3.00 ± 0.45	3.11 ± 0.41	3.06 ± 0.39	2.55 ± 0.43*	2.41 ± 0.40*
		4ºC		3.11 ± 0.42	3.07 ± 0.34	3.01 ± 0.32	2.67 ± 0.42*	2.60 ± 0.30*
		-20ºC		2.87 ± 0.36	2.57 ± 0.29*	2.44 ± 0.35*	2.11 ± 0.23*	2.01 ± 0.25*
		-80ºC		3.12 ± 0.44	3.05 ± 0.34	2.99 ± 0.25	2.82 ± 0.42	2.85 ± 0.38
Mitra	No BHT	Ambient	3.14 ± 0.35	3.31 ± 0.41	3.11 ± 0.34	2.99 ± 0.62	2.65 ± 0.44*	2.35 ± 0.41*
		4ºC		3.22 ± 0.40	3.11 ± 0.33	3.01 ± 0.45	2.52 ± 0.31*	2.27 ± 0.31*
		-20ºC		2.33 ± 0.34*	1.99 ± 0.37*	1.44 ± 0.39*	1.22 ± 0.42*	1.04 ± 0.32*
		-80ºC		3.11 ± 0.52	3.01 ± 0.32	3.15 ± 0.40	2.97 ± 0.42	2.99 ± 0.51
	BHT	Ambient	3.10 ± 0.33	3.05 ± 0.39	3.15 ± 0.30	3.18 ± 0.29	2.65 ± 0.37*	2.48 ± 0.44*
		4ºC		3.15 ± 0.32	3.01 ± 0.28	3.17 ± 0.26	2.52 ± 0.40*	2.41 ± 0.42*
		-20ºC		2.91 ± 0.31	2.59 ± 0.33*	2.51 ± 0.35*	2.19 ± 0.39*	2.05 ± 0.45*
		-80ºC		3.15 ± 0.38	3.08 ± 0.24	3.07 ± 0.29	3.17 ± 0.35	3.13 ± 0.34

903 Protein Saver Cards (903 PSC); butylated hydroxytoluene (BHT); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA). Asterisk used to highlight significantly different values from baseline after one-way ANOVA + Dunnett's test with $p < 0.05$. (n = 10).

Table 5 ***Relative percentage (% of total fatty acids) of omega-3 HUFA in total HUFA in 903 Protein Saver Cards and the Mitra during storage over 12 weeks.***

Material	Condition	Temperature	Storage Day					
			Baseline	1w	2w	4w	8w	12w
903 PSC	No BHT	Ambient	25.72 ± 1.58	25.06 ± 3.33	25.55 ± 2.15	25.45 ± 2.37	23.74 ± 2.09*	23.15 ± 1.38*
		4°C		25.26 ± 3.11	25.45 ± 2.11	25.65 ± 2.12	23.77 ± 2.19*	25.28 ± 1.55
		-20°C		25.84 ± 3.04	22.75 ± 1.93*	20.23 ± 1.82*	20.54 ± 2.45*	20.01 ± 1.67*
		-80°C		25.46 ± 3.12	25.56 ± 2.14	26.55 ± 3.27	26.16 ± 3.23	25.96 ± 3.11
	BHT	Ambient	24.70 ± 2.13	25.02 ± 2.14	24.96 ± 2.11	24.88 ± 2.21	23.04 ± 2.32*	22.99 ± 1.01*
		4°C		25.16 ± 3.01	24.51 ± 2.12	24.22 ± 2.13	22.99 ± 2.11*	22.91 ± 1.65*
		-20°C		24.64 ± 3.01	24.46 ± 2.14	24.51 ± 1.45	22.45 ± 2.23*	22.56 ± 1.25*
		-80°C		24.57 ± 2.01	24.45 ± 2.21	25.02 ± 3.12	24.77 ± 3.01	24.12 ± 3.23
Mitra	No BHT	Ambient	24.59 ± 2.90	24.16 ± 3.11	24.44 ± 2.21	24.71 ± 1.19	23.44 ± 2.46*	23.56 ± 1.57*
		4°C		24.56 ± 2.34	24.65 ± 2.14	24.55 ± 2.01	23.04 ± 2.54*	22.99 ± 1.26*
		-20°C		24.33 ± 2.22	21.45 ± 1.59*	20.33 ± 1.57*	20.04 ± 2.10*	19.98 ± 1.47*
		-80°C		24.61 ± 2.12	24.55 ± 2.11	24.81 ± 2.10	24.13 ± 2.45	25.12 ± 1.99
	BHT	Ambient	24.89 ± 2.11	24.88 ± 2.50	24.69 ± 2.01	24.66 ± 1.76	23.04 ± 2.10*	23.05 ± 1.05*
		4°C		24.06 ± 3.11	24.44 ± 2.14	24.90 ± 1.89	23.01 ± 1.55*	23.10± 1.35*
		-20°C		24.14 ± 2.11	24.54 ± 1.58	24.18 ± 1.04	22.13 ± 2.39*	22.59 ± 1.59*
		-80°C		24.57 ± 2.14	24.59 ± 2.19	24.35 ± 1.66	25.01 ± 2.90	25.61 ± 3.21

903 Protein Saver Cards (903 PSC); butylated hydroxytoluene (BHT); highly unsaturated fatty acid (HUFA)
Asterisk used to highlight significantly different values from baseline after one-way ANOVA + Dunnett's test with $p < 0.05$. (n = 10).

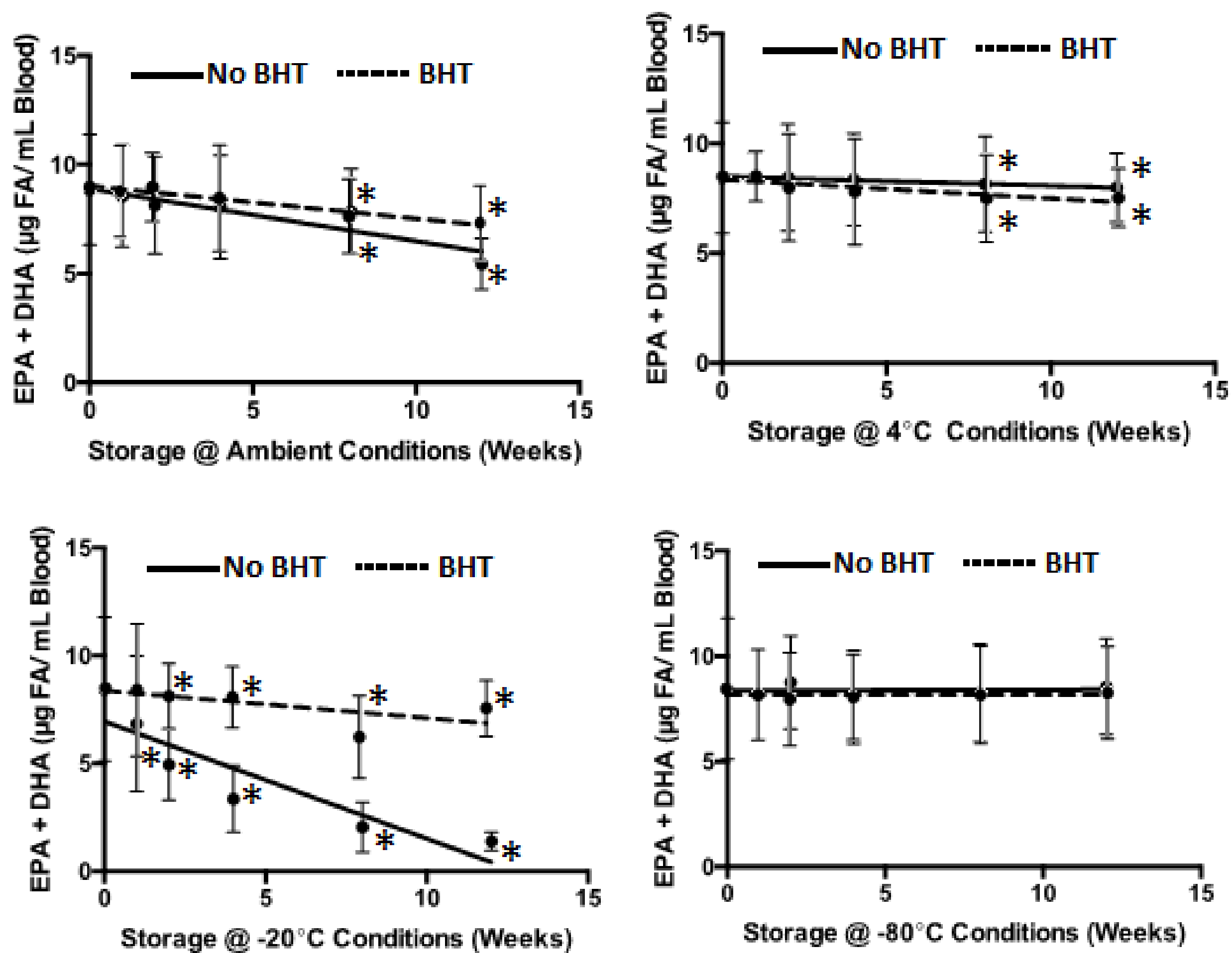

Figure 9. The effects of BHT on concentrations of EPA + DHA in blood spotted 903 PSC stored at ambient, 4°C, -20°C and -80°C storage temperatures over 12 weeks. Data presented in concentration (µg FA/ mL blood). *Indicates EPA + DHA concentration significantly lower than baseline (day 0) as determined by Dunnett's test following a significant F-value by one-way ANOVA. Data presented as means ± SD. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; BHT, butylated hydroxytoluene.

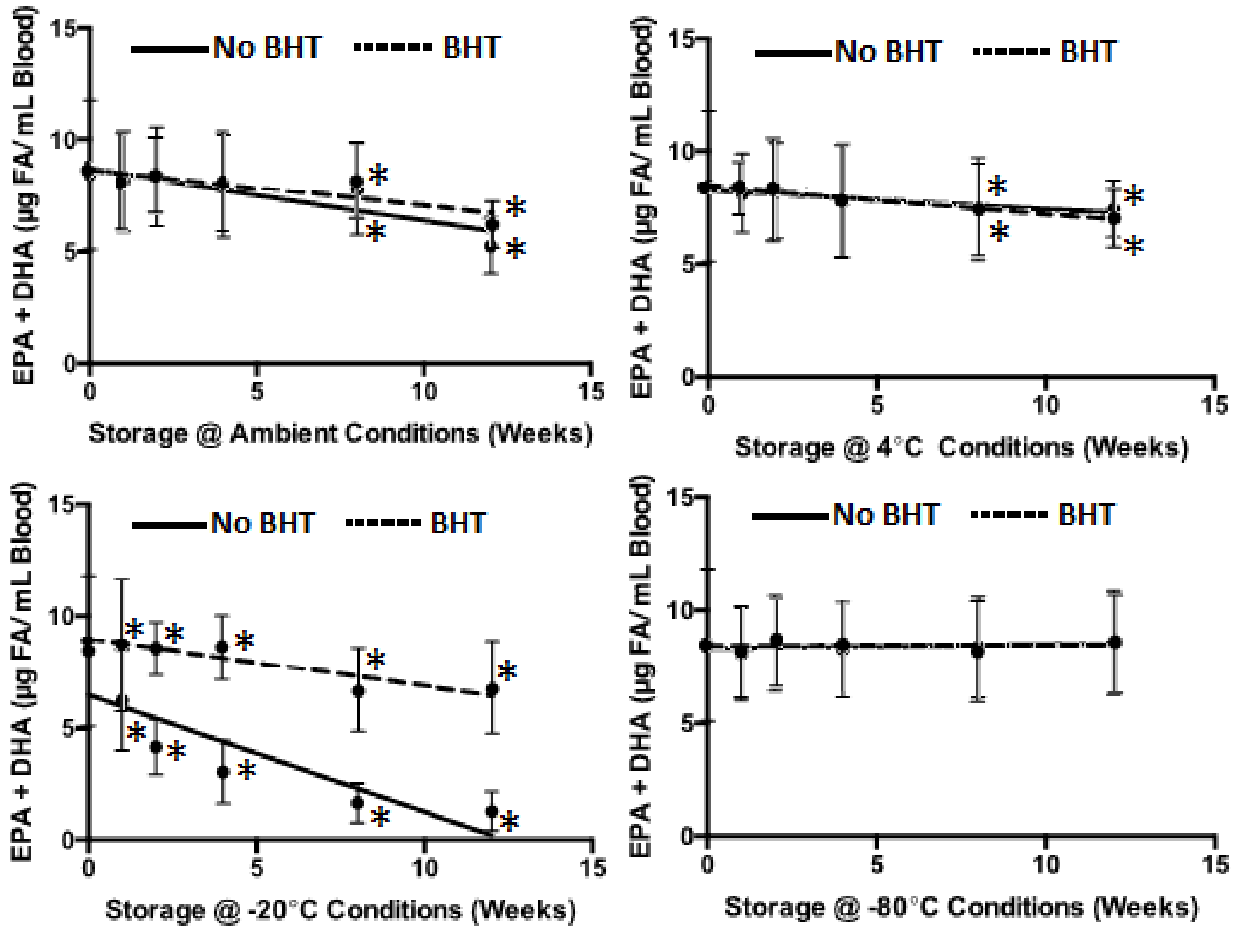

Figure 10. The effects of BHT on concentrations of EPA + DHA in the Mitra stored at ambient, 4°C, -20°C and -80°C storage temperatures over 12 weeks. Data presented in concentration (µg FA/ mL blood). *Indicates EPA + DHA concentration significantly lower than baseline (day 0) as determined by Dunnett's test following a significant F-value by one-way ANOVA. Data presented as means ± SD. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; BHT, butylated hydroxytoluene.

Table 6. ***Fatty acid concentrations for 903 PSC without BHT at baseline and -20 ℃ and -80 ℃ at 12 weeks (µg FA/ mL of blood)***

	Baseline	-20°C @ 12w	-80°C @ 12w
10:0	0.12 ± 0.10	0.10 ± 0.10	0.11 ± 0.09
12:0	0.51 ± 0.26	0.19 ± 0.25*	0.48 ± 0.24
14:0	2.38 ± 0.89	1.31 ± 0.87*	2.29 ± 0.82
16:0	60.17 ± 13.26	42.88 ± 12.96*	57.82 ± 12.23*
18:0	31.82 ± 3.21	16.61 ± 3.14*	30.63 ± 2.97
20:0	1.11 ± 0.08	0.68 ± 0.08*	1.07 ± 0.08
22:0	3.68 ± 0.27	3.16 ± 0.26	3.54 ± 0.25
23:0	0.82 ± 0.17	0.76 ± 0.17	0.79 ± 0.16
24:0	5.29 ± 1.07	3.43 ± 1.05*	5.11 ± 0.99
SFAs	105.59 ± 16.17	71.29 ± 15.81*	101.63 ± 14.92
12:1	0.07 ± 0.04	0.14 ± 0.04	0.07 ± 0.04
14:1	0.13 ± 0.15	0.21 ± 0.15	0.13 ± 0.14
16:1	2.82 ± 1.86	2.92 ± 1.82	2.71 ± 1.72
18:1n-7	4.76 ± 1.01	4.95 ± 0.98	4.58 ± 0.93
18:1n-9	50.15 ± 9.29	55.95 ± 9.09*	48.27 ± 8.58
20:1n-9	0.71 ± 0.15	0.68 ± 0.14	0.69 ± 0.13
22:1n-9	0.34 ± 0.15	0.29 ± 0.15	0.33 ± 0.14
24:1n-9	5.45 ± 1.10	3.17 ± 1.08*	5.27 ± 1.02
MUFAs	64.42 ± 12.36	67.09 ± 12.08	62.01 ± 11.40
18:2n-6	64.53 ± 10.13	61.09 ± 9.90	62.12 ± 9.35
18:3n-6	0.77 ± 0.34	0.49 ± 0.33*	0.74 ± 0.31
20:2n-6	0.36 ± 0.51	1.53 ± 0.50*	0.34 ± 0.47
20:3n-6	3.99 ± 1.38	2.88 ± 1.35*	3.86 ± 1.27
20:4n-6	29.36 ± 4.98	18.91 ± 4.87*	28.26 ± 4.60
22:2n-6	0.22 ± 0.21	2.42 ± 0.20*	0.21 ± 0.19
22:4n-6	3.71 ± 0.84	2.09 ± 0.82*	3.57 ± 0.78
22:5n-6	1.47 ± 0.84	1.43 ± 0.82	1.42 ± 0.77
N-6	104.28 ± 14.66	86.32 ± 14.34*	100.37 ± 13.53
18:3n-3	1.56 ± 0.56	1.21 ± 0.55	1.50 ± 0.52
20:3n-3	0.15 ± 0.09	0.12 ± 0.09	0.14 ± 0.08
20:5n-3	1.53 ± 0.53	1.05 ± 0.52*	1.48 ± 0.49
22:5n-3	3.33 ± 0.60	1.69 ± 0.58*	3.22 ± 0.55
22:6n-3	7.32 ± 2.14	2.43 ± 2.09*	7.04 ± 1.98
N-3	13.88 ± 2.92	7.66 ± 2.86*	13.36 ± 2.70
20:3n-9	0.67 ± 0.12	0.17 ± 0.12*	0.65 ± 0.11
PUFAs	118.39 ± 16.89	93.05 ± 16.52*	113.96 ± 15.59
HUFAs	48.24 ± 14.35	28.76 ± 14.03*	46.44 ± 13.24
Total	288.97 ± 43.31	222.83 ± 42.36*	278.13 ± 39.98

Data presented in concentration (µg FA/ mL of blood) means ± SD. FA, saturated fatty acids; MUFA, monounsaturated fatty acid, EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; n-3H/TH, n-3 HUFA in total HUFA, n = 10. Asterisk used to highlight significantly different values (in comparison to baseline) after one-way ANOVA + Dunnett's test with $p < 0.05$.

Table 7. ***Fatty acid concentrations for Mitra without BHT at baseline and -20 °C and -80 °C at 12 weeks (µg FA/ mL of blood)***

	Mitra Baseline	Mitra -20°C @ 12w	Mitra -80°C @ 12w
10:0	0.65 ± 0.32	0.54 ± 0.29	0.61 ± 0.29
12:0	0.91 ± 0.27	0.36 ± 0.25*	0.82 ± 0.25
14:0	3.31 ± 0.95	1.86 ± 0.88*	3.12 ± 0.86
16:0	65.26 ± 13.18	47.70 ± 12.25*	61.53 ± 11.98
18:0	36.36 ± 3.45	19.44 ± 3.21*	34.30 ± 3.14
20:0	0.99 ± 0.09	0.62 ± 0.09*	0.93 ± 0.09
22:0	2.53 ± 0.29	2.22 ± 0.27*	2.39 ± 0.27
23:0	0.57 ± 0.12	0.52 ± 0.11	0.53 ± 0.11
24:0	4.84 ± 0.70	3.21 ± 0.65*	4.55 ± 0.63
SFAs	115.27 ± 16.03	79.67 ± 14.90*	108.72 ± 14.58
12:1	1.04 ± 0.88	2.31 ± 0.82*	0.98 ± 0.80
14:1	0.11 ± 0.08	0.18 ± 0.08*	0.13 ± 0.08
16:1	3.09 ± 2.02	3.24 ± 1.88	2.89 ± 1.84
18:1n-7	5.01 ± 1.10	5.32 ± 1.02	4.723± 1.00
18:1n-9	52.35 ± 11.37	59.82 ± 10.57*	49.38 ± 10.34
20:1n-9	0.71 ± 0.10	0.74 ± 0.10	0.72 ± 0.09
22:1n-9	1.66 ± 0.37	1.11 ± 0.35*	1.54 ± 0.34
24:1n-9	5.33 ± 0.95	3.12 ± 0.88*	5.04 ± 0.87
MUFAs	69.23 ± 13.81	71.93 ± 12.84	65.39 ± 12.56
18:2n-6	64.34 ± 9.85	62.27 ± 9.16	60.69 ± 8.96*
18:3n-6	0.72 ± 0.32	0.44 ± 0.30*	0.65 ± 0.29
20:2n-6	0.17 ± 0.18	0.73 ± 0.17*	0.16 ± 0.17
20:3n-6	4.05 ± 1.38	2.97 ± 1.28*	3.82 ± 1.25
20:4n-6	29.40 ± 5.44	19.40 ± 5.06*	27.73 ± 4.95
22:2n-6	0.13 ± 0.09	1.32 ± 0.08*	0.12 ± 0.08
22:4n-6	3.72 ± 0.78	2.11 ± 0.73*	3.51 ± 0.71
22:5n-6	2.21 ± 0.44	1.92 ± 0.41*	2.10 ± 0.40
N-6	104.71 ± 15.19	88.75 ± 14.12*	98.77 ± 13.81
18:3n-3	1.48 ± 0.57	1.27 ± 0.53*	1.38 ± 0.52
20:3n-3	0.17 ± 0.13	0.11 ± 0.12*	0.17 ± 0.11
20:5n-3	1.76 ± 0.54	1.22 ± 0.50*	1.67 ± 0.49
22:5n-3	3.40 ± 0.61	1.74 ± 0.56*	3.20 ± 0.55
22:6n-3	7.38 ± 2.13	2.05 ± 1.98*	6.95 ± 1.94
N-3	14.16 ± 2.72	8.01 ± 2.53*	13.36 ± 2.47
20:3n-9	0.67 ± 0.14	0.20 ± 0.13*	0.65 ± 0.13
PUFAs	119.10 ± 17.36	95.85 ± 16.14*	112.31 ± 15.79
HUFAs	52.46 ± 8.99	32.03 ± 8.36*	49.47 ± 8.17
Total	304.11 ± 45.36	240.16 ± 42.18*	286.86 ± 41.25*

SFA, saturated fatty acids; MUFA, monounsaturated fatty acid, n-6, omega-6; n-3, omega-3; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; n-3H/TH, n-3 HUFA in total HUFA, n = 10. Asterisk used to highlight significantly different values (in comparison to baseline) after one-way ANOVA + Dunnett's test with $p < 0.05$. Data presented in concentration (µg FA/ mL of blood) means ± SD.

Chapter 9

General Discussion and Conclusion

9.1 *General Discussion*

Dried blood spot methods have many advantages when considering the tedious nature of handling wet blood and its related analytical procedures for fractioning especially for sample collection in field research circumstances. Most DBS FA profiles have been expressed in relative abundance or %TFA and FA concentrations are not possible due to the inability to quantitate the volume of blood collected. This thesis presented a method to quantitate blood and FA concentrations on commonly used 903 Protein Saver cards by determining a relationship between area and volume to create a predictive linear equation. As an alternative for blood quantitation on traditional DBS papers, FA analysis was performed on the Mitra. To supplement accurate quantitation, factors that that may influence quantitation, such as contaminants on blood collection material were identified and FA stability was determined at various storage temperatures with and without the addition of antioxidant.

In line with the stated hypothesis 1 (Chapter 3), a linear relationship was determined between the area of blood on the 903 PSC and volume applied which was then validated using the blood from ten individuals. The equation determined a blood volume of 9.6 µL when a 6mm punch was removed from a 903 PSC which was then used to confirm that accurate fatty acid concentrations could be determined from DBS on 903 PSC (hypothesis 2). Limitations may arise when considering how hematology can affect the blood and volume relationship on collection materials, therefore in the future, the DBS area/blood volume relationship on 903 PSC using blood samples with high or low hematocrit should be examined. As an alternative to dried blood spots, the 10 µL Mitra Microsampling Device that can collect accurate collections of volume

regardless of viscosity [60] was assessed and validated for the determination of fatty acid concentrations (hypothesis 3). While the fatty acid analyses of the Mitra were similar to wet blood controls, the lipidomics profile generally had lower recoveries in phospholipids and higher TAG and CE recoveries indicating that hypothesis 4 must be rejected at this time. However, the lipidomic profiles from the Mitra resembled a pattern similar initial attempts to perform lipidomics on DBS on other collection materials [46]. As such, the cause appears to be due to differences in the retention of polar and nonpolar lipids on the Mitra material. The Mitra material, the three dimensional geometry of the Mitra tip and the wicking agents may be contributing to these differences in retention. It is possible that Mitra derived lipidomic profiling could be improved using a tailored extraction procedure such as physical disruption of the Mitra and/or an acidified extraction protocol.

Contaminants on commonly used dried spotting materials were identified to determine their influence on FA quantitation. In line with hypothesis 5, palmitate and stearate were found as lipid contaminants. Using UHPLC-MS/MS the form of the palmitate and stearate was as FFA in 903 PSC, Whatman chromatography paper and the Mitra, while the Mitra also had16:0 lysoPC and 18:0 lysoPC. LysoPC or "lysolecithin" is used as an industrial emulsifying agent and could play a role in the Mitra's wicking abilities. FA and lipid contaminants on collection materials can be reduced through "washing" procedures. In the case of the Mitra, washing could potential impact the wicking ability. Overall, the impact of 16:0 and 18:0 contaminants would be small as the concentration of these FA contaminants on the materials was very low (0.97 ± 0.08 µg of 16:0 and 1.43 ± 0.16 µg of 18:0 per 6mm punch of Whatman CP, 0.64 ± 0.05 µg of 16:0 and 0.88 ± 0.04 18:0 per 6mm punch of 903 PSC and 0.80 ± 0.08 µg 16:0 and 0.98 ± 0.10 µg 18:0 per

Mitra tip) in comparison to their amounts of 16:0 and 18:0 in total lipids of whole blood samples (58.54±13.61 and 32.37±7.47 respectively, n=10).

Optimal storage conditions for FA stability using 903 PSC and the Mitra were also examined. The storage experiment results were generally in line with the stated hypotheses (Chapter 3). In short, the HUFA status of DBS samples decreased in the Mitra and 903 PSC during storage (hypothesis 6), HUFA in DBS were the most stable when stored at -80°C and the least stable at -20°C (hypothesis 7), and the addition of BHT increased the stability of HUFA in all the DBS samples (hypothesis 8). These results confirm previous reports in the literature for the storage of DBS on chromatography paper [14, 15].

Steps can be taken to improve the studies discussed in this thesis. The 903 PSC and Mitra should be tested on other populations including children, elderly, malnourished individuals and individuals in postprandial states as further validation is required with high and low hematocrit blood. Tailoring the extraction procedure for the Mitra could provide higher overall lipid recoveries and as lipidomics is an emerging field of research, alternatives to wet blood should be considered and further tested. The presence of SFAs in low abundance does not have a large impact in comparison to the SFAs present in blood samples. To further assess the interferences of FA contaminants on quantitation, all materials and solvents involved (gloves, glassware, hexane, water, BF_3, etc.) should be tested for contaminants as well. The FA stability of samples at -20°C can be improved using iron chelators, glycerol, vacuum sealing and antioxidants. However, BHT alone can provide biomarker stability from 4 to 8 weeks at both ambient and 4°C.

9.1 *Conclusion*

This thesis set out to address three main limitations in DBS which were blood and FA quantitation, the presence of spotting material contaminants and storage/handling conditions

which may lead to sample oxidation. Dried blood quantitation on 903 PSC and the Mitra were used to provide means to collect a quantifiable volume of blood which could be used in field research circumstances. Biological variates were used to confirm these methods but all blood was collected from healthy adults attending the University of Waterloo with relatively normal hematocrit content. The Mitra was validated FA analysis but lipidomics analysis still requires additional research to determine an ideal extraction procedure. Additionally, other factors that may interfere with DBS FA quantitation such as contaminants and FA stability in various storage conditions were identified. Cumulatively, these findings indicate that the limitations associated with fatty acid analysis of DBS can be improved and validated in the future and enable low cost and high throughput analyses in laboratory and field settings.

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