The Development and Assessment of Rapid Methods for Fatty Acid Profiling

by

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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

Fatty acid profiling provides information on dietary intakes and an understanding of lipid metabolism. High throughput techniques such as fingertip prick (FTP) sampling has gained popularity in recent years as a simplified method for basic research, and could be further used to assess disease risk in the population, and other similar high-throughput techniques have the potential to assist in the monitoring and labeling of fatty acids in the food supply. With the advancement of high-throughput sample analysis techniques, a more complete understanding of storage stability is required as a larger volume of samples are produced with equal amounts of time to analyze them. Energy-assisted analysis techniques have the potential to help ameliorate some of these issues. Presently, FTP blood, whole blood and salmon storage stability is assessed under various storage conditions, and both microwave-assisted direct transesterification and indirect ultrasound-assisted extraction techniques are assessed. It is determined that storage of FTP blood and whole blood samples at -20°C results in significant and nearly complete highly unsaturated fatty acid (HUFA) degradation compared to all other temperatures examined. This degradation is determined to be the result of hemolysis and subsequent iron release from erythrocytes initiating fatty acid peroxidation reactions. Direct transesterification of FTP blood is reduced from as long as three hours to one minute with microwave-assisted energy and fatty acid extraction from ground flaxseed is reduced to 40 minutes from as long as 24 hours without compromising fatty acid profiles. Results of the current study provides insight into the storage stability of food sample and blood samples collected via high-throughput techniques, and provides support for the utilization of further high-throughput energy-assisted analytical methods that can help to minimize the potentially detrimental effects that long-term storage can have on fatty acid profiles.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid, 20:4n-6
AAPH	2,2'-azobis (2-aminidnopropane) hydrochloride
AI	Adequate intake
ALA	Alpha-linolenic acid, 18:3n-3
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BF_3	Boron trifluoride
BHT	Butylated hydroxytoluene
CE	Cholesteryl sster
CVD	Cardiovascular disease
DFO	Deferoxamine
DHA	Docosahexaenoic acid, 22:6n-3
DPAn-3	N-3 docosapentaenoic acid, 22:5n-3
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid, 20:5n-3
FA	Fatty acid
FAME	Fatty acid methyl ester
Fe	Iron
FTP	Fingertip prick
H_2SO_4	Sulphuric acid
HUFA	Highly unsaturated fatty acid
GC	Gas chromatography

GCMS	Gas chromatography-mass spectrometry
ISO	Internation Organization for Standardization
JELIS	Japanese EPA lipid intervention study
LA	Linoleic acid, 18:2n-6
MDA	Malondialdehyde
MUFA	Monounsaturated fatty acid
NEFA	Non-esterified fatty acids
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
RBC	Red blood cell or Erythrocyte
RDI	Recommended dietary intake
RT	Room temperature
SFA	Saturated fatty acid
TAG	Triacylglycerol
TLE	Total lipid extract
TBARS	Thiobarbituric acid reactive species
WB	Whole blood
VP	Variable power

CHAPTER 1

GENERAL INTRODUCTION

Increased omega-3 polyunsaturated fatty acid (PUFA) intake is shown to reduce the risk of cardiovascular disease (CVD) and mortality (Leaf and Weber, 1988) and supports healthy development of fetal brain and neurological development (Crawford, 1993). Omega-3 intakes can be determined through blood sampling, however, standard sampling methods are tedious and blood fractions analyzed and chemical and analytical processes utilized are variable across research labs. Advancements in high-throughput sample collection such as fingertip prick (FTP) blood sampling results in larger sample numbers and subsequently creates challenges in sample storage and analysis capacities. Currently only two studies have assessed the stability of FTP blood samples (Marangoni, Colombo, and Galli, 2005; Min et al., 2011), leaving a significant void in the literature that the current thesis aims to reconcile. The variability of blood fractions assessed for fatty acid profiling necessitates the need for estimating fatty acid profiles between blood fractions, particularly from FTP samples and practicality of such estimations is assessed. Energy-assisted methods can also help to alleviate the burden on sample analysis created by large sample volumes, and microwave and ultrasound-assisted methods are developed for expediting both FTP and food fatty acid analysis, respectively.

Whole blood may be the simplest blood fraction for omega-3 HUFA profiling, however, plasma and erythrocytes are the most commonly used measures in the literature, although EPA + DHA composition in whole blood has previously been associated with risk of sudden cardiac death (Albert et al., 2002). Fatty acid analysis in many studies requires storage of samples for an extended period of time prior to analysis, and this becomes increasingly difficult with larger sample volumes created by simpler FTP collection methods. Although the accuracy of FTP fatty acid analysis has been verified previously (Armstrong, Metherel, and Stark, 2008; Fratesi et al.,

2009; Marangoni, Colombo, and Galli, 2004; Metherel et al., 2009a), there is currently very little research on the storage stability of FTP blood samples (Marangoni, Colombo, and Galli, 2005; Min et al., 2011) and whole blood samples (Stokol and Nydam, 2005). Briefly, FTP PUFA stability is determined to be less than two weeks at room temperature and between three weeks and three months at 4°C. Whole blood is determined to remain stable for less than 24 hours, 24 hours and longer than one month at 24°C, 4°C and -40°C, respectively. When compared to the more extensive literature coverage of approximately ten storage studies for erythrocytes and plasma blood fractions, it is clear more investigation is required for FTP and whole blood. The variety of blood fractions utilized also makes comparisons between various studies difficult to interpret. Correlations between blood fractions have been determined previously (Bell et al., 2011; Kawabata et al., 2011; Rizzo et al., 2010; Stark, 2008b), however, the development of predictive equations from such analyses could allow for better comparative fatty acid analyses between studies utilizing different blood fractions for analysis.

Sample throughput can be further increased by the implementation of shorter direct transesterification methods as the current FTP method requires one hour of convection heating. Previously, commercial microwaves have been used to quickly transesterify brain phospholipid extracts (Banerjee, Dawson, and Dasgupta, 1992) and FTP blood samples (Armstrong, Metherel, and Stark, 2008), although incomplete transesterification of the triacylglycerol fraction of the blood was shown. The use of a microwave system that allows for manipulation of various parameters such as heat, pressure and power may be beneficial for bypassing such limitations of microwave energy.

Despite the associated health benefits of increasing intake of food high in omega-3 PUFA, the Insititute of Medicine in 2002 was unable to determine EPA and/or DHA adequate

intake (AI) or recommended dietary intake (RDI) values (Institute of Medicine, 2002), although more recently intakes of 500 mg/day EPA + DHA has been suggested (Harris et al., 2009). Such determinations are important for nutritional labeling and the transfer of nutritional information to the general public so that informed healthy dietary choices can be made. Fatty acid analysis in these food samples are subject to similar time and monetary restrictions as discussed in blood. The most common methods of analysis in the food industry include the Association of Official Analytical Chemists (AOAC) method (AOAC, 2005) and the International Organization for Standardization (ISO) method (Luque-Garcia and Luque de Castro, 2004). These techniques can require 4 and 14 hours to complete and cost approximately $3.91 and $2.66 in solvents per sample, respectively (Taha, Metherel, and Stark, 2012). Methods to reduce both overall time and cost of analysis have been attempted with varying degrees of success for both the ISO (Luque-Garcia and Luque de Castro, 2004; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Taha, Metherel, and Stark, 2012) and AOAC (Taha, Metherel, and Stark, 2012) methods, and can be used to vastly improve the bottom line for companies where sample volume is high.

Expediting methods of fatty acid analysis in both blood and food without compromising the results can provide significant economical benefits through reagent and time commitment costs and should garner serious consideration for implementation in field and clinical studies, clinical screening and in large contract laboratories. Methodological improvements can be implemented during collection, storage, extraction, transesterification and data handling steps. The purpose of the current thesis project is to analyze potential improvements in these stages of fatty acid analysis to facilitate the use of high-throughput techniques while maintaining the quality of the results, and minimizing the potentially detrimental effects of long-term sample storage.

CHAPTER 2

BIOCHEMICAL FOUNDATIONS

Fatty Acids

Fatty acids are a group of macronutrients found in all naturally occurring substances and are essential in energy metabolism and storage, cell membrane structure and as substrates for cell signaling pathways. Fatty acids exist as hydrocarbon chains of varying lengths generally between 10 and 24 carbons, but may also exist less abundantly with as little as 4 carbons and as many as 30 carbons. On one end of the hydrocarbon exists a carboxylic acid (-COOH) group with the opposite end having a methyl ($-CH_3$) group. The standard nomenclature for fatty acids is determined by three factors: 1) the number of carbons in the chain, 2) the number of double bonds in the hydrocarbon chain and 3) the position of the first double bond in relation to the methyl terminal group (or the 'omega' end). For example, linoleic acid contains 18 carbons and 2 double bonds with the first double bond located on the 6^{th} carbon from the methyl group, and is numerically denoted as 18:2n-6 (**Figure 1**). Fatty acids also have various numbers of double bonds in the chain, including anywhere from zero in saturated fatty acids (SFA) to one in monounsaturated fatty acids (MUFA) to more than 1 in polyunsaturated fatty acids (PUFA) to more than two in highly unsaturated fatty acids (HUFA). Fatty acids can be found in triacylglycerols or phospholipids, cholesteryl esters, sphingolipids and non-esterified fatty acids. The most common fatty acids are 16:0 (palmitic acid) and 18:0 (stearic acid) as SFA, 18:1n-9 (oleic acid) as MUFA, and 18:2n-6 (linoleic acid) as PUFA. To be considered as a HUFA, a fatty acid must contain at least 20 carbons and a minimum of 3 double bonds in the hydrocarbon chain. The HUFA classification is not universal as they are often classified as long chain PUFA. However, the unique structural and signaling roles of the omega-6 arachidonic acid (20:4n-6,

AA) and the omega-3 EPA and DHA (**Figure 2**) as compared with the 18 carbon PUFA, justifies the unique HUFA classification. These HUFA, particularly the omega-3 HUFA are the focus of extensive research on various health benefits in the prevention and treatment paradigms.

Fatty Acids in Food, Blood and Tissues

The majority of dietary fatty acids are consumed as part of the triacyglycerol (TAG) fraction, although vegetables tend to contain very little TAG. Generally, 18-carbon omega-3 and omega-6 fatty acids (most prominently 18:3n-3 and 18:2n-6) are consumed as part of TAG from plant seeds, as well as in animal fat depots. Omega-3 or omega-6 fatty acids of greater or equal to 20 carbons in length (20:4n-6, 20:5n-3 and 22:6n-3) are more often associated with animal products and can be found in both triacylglycerol and phospholipid fractions (Stark, 2012). However, in Western diets 18:2n-6 intake is much higher than 18:3n-3 (Whelan and Rust, 2006) due to the high consumption of 18:2n-6 enriched oils such as corn and safflower (Denomme, Stark, and Holub, 2005; Fratesi et al., 2009; Stark et al., 2005). Additionally, longer chain omega-6 fatty acid intake is significantly higher in Western diets compared to omega-3, mainly due to a low omega-3 intake from marine sources (Stark, 2012) as 20:4n-6 intake from meat sources remains relatively stable across various human populations (Bang, Dyerberg, and Hjoorne, 1976; Denomme, Stark, and Holub, 2005; Fratesi et al., 2009; Stark et al., 2005).

Following consumption, lipids are broken down and absorbed in the digestive system, and the fatty acids are subsequently repackaged for entry into the blood stream. This repackaging results in the formation of TAG, phosphatidylcholine (PC) and cholesteryl esters (CE), which are subsequently incorporated into chylomicrons that, in turn, are exocytosed into the lymphatic system to bypass the liver into the bloodstream (Gropper, Smith, and Groff, 2005).

Once in the blood, chylomicrons are concentrated in the plasma fraction. Due to the high prevalence of chylomicrons in the plasma, minute changes in plasma fatty acid profile are highly susceptible to dietary intakes, and as such these changes can be monitored as a marker of acute dietary fatty acid consumption. In fasting blood, plasma lipids are 49% TAG, 24% PC and 16% CE (Christie, 1985). Post-prandially, however, the TAG-enriched chylomicrons (82% TAG) in the bloodstream can alter plasma lipid composition as TAG consists primarily of SFA and MUFA, the most abundant dietary fatty acids. Erythrocytes, on the other hand, contain 32% phosphatidylcholine, 21% phosphatidylethanolamine, 30% cholesterol and no TAG (Christie, 1985). Therefore, they are considered as a marker of chronic dietary lipid intake and tissue fatty acid content (Harris and von Schacky, 2004). We have previously shown that individuals taking fish oil supplements demonstrate much slower EPA and DHA incorporation into erythrocytes compared to plasma (Metherel et al., 2009a), and erythrocyte fatty acid composition has previously been shown to have significantly less variation compared to plasma following ingestion of a single meal (Harris and Thomas, 2010).

Upon entry into the blood stream chylomicrons undergo multiple stages of repackaging, thereby causing the fatty acid content of the plasma to be very dynamic. The first stage of repackaging occurs at the adipose tissue where lipoprotein lipase hydrolyzes fatty acids from TAG for storage (Frayn, 2003). The chylomicron remnants are then taken up by the liver via endocytosis, and the resultant lipoproteins continue to deliver fatty acids derived from TAG to multiple extra-hepatic sites such as the muscle (Gropper, Smith, and Groff, 2005). These TAG fatty acids in rat liver consist mostly of 16:0 (27% of TAG fatty acid) most often in the *sn*-1 position, 18:1n-9 (27%) in the *sn*-2 and *sn*-3 positions.18:2n-6 (12%) is also present in the *sn*-2 and *sn*-3 positions (Wood and Harlow, 1969). Adipose tissue fatty acids are almost exclusively

contained in TAG. The TAG-rich adipose is particularly high in 16:0 and 18:1n-9 that is stored until needed for energy purposes in tissues around the body, and TAG contain only trace amounts of DHA and EPA. The majority of skeletal and heart muscle fatty acids are stored in phospholipids for structural purposes in the multiple membranes present in these tissues (Christie 1985). For instance, heart muscle consists of 39% PC and 33% phosphatidylethanolamine (PE), and the *sn*-2 position of both PC and PE are high in AA, EPA and DHA relative to other lipid fractions (Wood and Harlow, 1969), indicating the importance of these fatty acids in heart function, another area of the body that is high in phospholipidsipids.

Hemolysis

Erythrocytes are very high in fatty acid-containing lipids, and in particular phospholipids that account for approximately 59% of all erythrocyte lipids and 86% of all fatty acid-containing lipids (Christie, 1985). The *sn*-2 position of these phospholipids are particularly high in the more peroxidation-susceptible polyunsaturated fatty acids, such as 18:2n-6, AA and DHA comprising nearly 70% of all *sn-2* position fatty acids in PC and PE (Christie, 1985). During hemolysis, the rupturing of the erythrocyte membrane releases its contents, including hemoglobin and lipids, into the surrounding plasma. Abnormal hemolytic rates *in vivo* are associated with numerous diseases that include but are not limited to sickle-cell disease, malaria, thalassemias and numerous forms of anemia (Rother et al., 2005). Hemolysis is also an important factor *ex vivo* during handling and storage of blood samples. Hemolysis *ex* vivo may be caused by factors including preparatory procedures, shear stress, bacterial contamination, membrane defects and deformability, osmotic and pH changes, blood age, leukocyte presence, drugs, irradiation, storage time and temperature (Sowemimo-Coker, 2002).

Blood storage has been shown to significantly alter the erythrocyte membrane integrity and flow properties resulting in an increase in the levels of free hemoglobin in plasma (Arun et al., 1999; Heaton et al., 1984; Weisbach et al., 1999). In addition, markers of the hemolytic process such as extracellular potassium, 2,3-diphosphoglycerate and pH have shown that storage for as little as one day at room temperature results in increased levels of hemolysis (Gulliksson and Van der Meer, 2009), and a reduction in hemolysis is accompanied by a reduction in measures of lipid peroxidation during storage for up to 42 days at 4°C (Arun et al., 1999; Racek et al., 1997). When storage of blood samples for future fatty acid profiling is required, it is generally stored at -80°C; however, when this is not available, -20°C is a common supplementary storage temperature. However, temperatures below freezing result in significant levels of hemolysis, and although the mechanisms are poorly understood they are reviewed elsewhere (Stolzing et al., 2012). Briefly, during freezing, growing extracellular ice forms channels where cells are displaced and the pressure that forms in these channels can cause extensive cell deformation and subsequently rupture or lysis. Intracellular water can also become frozen though gap junction and transmembrane protein ice crystal movement and can cause further mechanical damage to the cell. Hemolysis during this process also has the potential potential to induce lipid peroxidation, which although it has not been reported directly, storage of erythrocytes at -20°C is reported to result in significant PUFA degradation over time (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006; Otto et al., 1997).

The prevention of hemolysis with cryoprotectants is designed to prevent or minimize this freeze-thaw induced lysis. Cryoprotection, however, is not limited to erythrocytes and has been implemented in numerous tissue/sample types including semen (Chakrabarty et al., 2007; Kaeoket et al., 2010; Maldjian et al., 2005), microalgae (Molina Grima et al., 1994), larval cells

(Odintsova et al., 2009) and embryonic stem cells (Li, Tan, and Li, 2010). Examples of cryoprotectants include the smaller cell penetrating agents glycerol, ethylene glycol (McGann, 1978; Vandevoort et al., 2008) and dimethylsulfoxide (Lehle et al., 2005) and larger non-penetrating dextran, hydroxyethyl starch and polyvinyl-pyrrolidon/polyvinylalcohol (Bakaltcheva et al., 2000; Takahashi et al., 1988). Furthermore, a relationship between the polyunsaturated:saturated fatty acid ratio in spermatozoa and susceptibility to cold-shock induced lysis has been demonstrated (Darin-Bennett, Poulos, and White, 1974; Poulos, Darin-Bennett, and White, 1973), and DHA supplementation in boars has been shown to improve spermatozoa plasma membrane integrity following a freeze-thaw cycle (Kaeoket et al., 2010). Interestingly, hemolysis has previously been shown to increase fatty acid peroxidation in erythrocytes (Niki et al., 1988) and the presence of antioxidants has the potential to prevent this fatty acid peroxidation from occurring (Banerjee et al., 2008; Deng et al., 2006; Kunwar et al., 2007; Vosters and Neve, 2002).

Lipid Peroxidation

Polyunsaturated fatty acids, whether bound or unbound to a glycerol or other such molecules, are highly susceptible to peroxidation reactions as a result of the attack of free radicals. Lipid peroxidation occurs when a highly reactive hydrogen atom is removed from the methylene group (–C=C–) of PUFA by a free radical resulting in the formation of a lipid peroxyl radical in its place (Aruoma et al., 1989), which can react further with another PUFA to form the lipid peroxide in a continuous chain of lipid peroxidation reactions (Halliwell and Chirico, 1993) (**Figure 3**). However, this regeneration of lipid peroxidation reactions slows down when the

ratio of proteins to fatty acids becomes high, in which case proteins become more susceptible to free radical attack (Halliwell and Chirico, 1993).

A free radical is any molecule that contains an unpaired electron such as low activity molecules like the superoxide radical ($\cdot O_2$) and highly reactive molecules such as the hydroxyl radical ($\cdot OH$) (Benzie, 1996). The greater the number of double bonds present in a fatty acid, the greater the reactivity of the free radical to the reactive hydrogens on the methylene groups, thus explaining why fatty acids with more double bonds degrade at a faster rate than monounsaturated or saturated fatty acids (Halliwell and Chirico, 1993). Other mechanisms of PUFA peroxidation exist, particularly in erythrocytes that make up nearly half of a whole blood sample. In healthy cells approximately 3% of hemoglobin-iron complex ($Hb\text{-}Fe^{2+}$) is converted to $Hb\text{-}Fe^{3+}$ by O_2, and this conversion results in the production of the $\cdot O_2$ radical (Clemens and Waller, 1987; Hebbel and Eaton, 1989; Misra and Fridovich, 1972) that will subsequently attack PUFA and cause lipid peroxidation. Additionally, Fe^{2+} can accept a proton from H_2O_2 that results in the formation of the more potent $\cdot OH$ radical (Halliwell and Chirico, 1993) (**Figure 4**). The formation Fe^{3+} on its own is able to act directly on PUFA to extract an electron from double bonds and yield a lipid radical (Schaich, 1992). Lipid radicals react with O_2, and the resultant lipid peroxyl radicals formed from PUFA are converted to lipid hydroperoxides by vitamin E for removal; however, if efficient removal does not occur, then the hydroperoxides can decompose to form more free radicals in the presence of iron, thereby, further exacerbating oxidative damage (van den Berg et al., 1992). The iron-mediated pathway is complex with numerous oxidative species becoming recycled within the pathway (Figure 4).

Measures of Lipid Peroxidation

Numerous measures have been identified to assess the extent of lipid peroxidation in tissues and have been reviewed previously (Halliwell and Chirico, 1993). Briefly, the degree of lipid peroxidation can be determined by measuring 1) losses of unsaturated fatty acids, 2) levels of primary oxidation products (i.e. hydroperoxides and conjugated dienes) and 3) levels of secondary oxidation products (hydrocarbons, aldehydes, hydroxyaldehydes and epoxides), and these endpoint measures are highly dependent on the fatty acid composition of the sample and the type of metal compounds (i.e. iron) present, if any. A summary of the breakdown products and the methods used for detection are provided (**Figure 5**). To summarize, PUFA losses are measured by standard GC methods, and the subsequent degradation products lipid hydroperoxides are measured by either the FOX assay (Fe^{3+} levels), chemiluminescence or the iodometric assay (I_2 levels). Lipid hydroperoxides are subsequently broken down into either alkoxyl or peroxyl radicals (Esterbauer, 1995). β-cleavage of alkoxyl radicals yields both alkanes, as measured by gas chromatography (GC), and aldehydes, as measured by thiobarbituric acid reactive species (TBARS), high-performance liquid chromatography, gas chromatography-mass spectrometry (GCMS) or by malondialdehyde (MDA)/HNE (4-hydroxynonenal) adducts (Moore and Roberts, 1998). Peroxyl radicals further breakdown to isoprostanes measured by either GCMS or immunological assays, and mono- and dihydroxylated fatty acids also measured by GCMS (Moore and Roberts, 1998). One of the most common tests for lipid peroxidation is the TBARS test due to its simplicity and low cost. The TBARS test was first identified as a marker for lipid peroxidation in 1948 (Bernheim, Bernheim, and Wilbur, 1948) and it measures MDA, a secondary product of lipid peroxidation. The addition of thiobarbituric acid to a sample results in the formation of a pink chromagen due to the reaction

between thiobarbituric acid and MDA, and its absorbance is measured under UV light. This test is said to work well when applied to defined membrane systems; however, a number of problems may arise when applied to body fluids and tissue extracts. For instance, the presence of other aldehydes in these tissues (Esterbauer, Schaur, and Zollner, 1991) can form alternate aldehyde chromogens (Kosugi, Kato, and Kikugawa, 1987). In addition, the majority of MDA measured in a sample results from a heating step during the assay that breaks down lipid peroxides, and as such the endpoint MDA measures – although qualitatively accurate – may not be an appropriate quantitative measure of MDA levels in a given sample (Gutteridge, 1986). Furthermore, this peroxide decomposition can initiate the peroxidation of other lipid molecules and amplify the response measured (Gutteridge and Quinlan, 1983). MDA is also a product of thromboxane synthesis (McMillan et al., 1978) and changes in platelet activation and subsequent thromboxane production can occur (Moore and Roberts, 1998). However, the TBARS test has been used previously for the lipid peroxidation determinations in both plasma (Brown et al., 1998; Galli et al., 2001; Videla, Sir, and Wolff, 1988) and erythrocytes (Canestrari et al., 1995; Clemens and Remmer, 1982), but it does not appear that the TBARS test has been applied to whole blood, although it appears to be chemically plausible.

Measuring the peroxide value to assess oxidation is another popular method as it measures hydroperoxides, the primary product during lipid peroxidation. Peroxide value measurements limit oxidation assessments to only the early stages of peroxidation, as the lipid peroxides are vulnerable to further degradation and production of secondary peroxidation products (Gray, 1978). Popular peroxide value methods include the iodine liberation and iron oxidation methods. In the iodine liberation method, I^- is oxidized to I_2 by lipid peroxides and measured by titration with thiosulphate; however, hydrogen peroxide (H_2O_2) also oxidizes I^- to I_2

and may overestimate peroxidation levels (Halliwell and Chirico, 1993), while the FOX assay measures the oxidation of Fe^{2+} to Fe^{3+} by lipid peroxides with the latter being determined as ferric thiocyanate content (Gray, 1978).

Finally, assessing the loss of unsaturated fatty acids is an indirect indicator of lipid peroxidation. Although it is indirect, it may be the only way to truly quantitate the total amount of lipid peroxidation that has occurred in a sample, given that there are numerous lipid peroxidation end products (Smith and Anderson, 1987). In addition, by completing fatty acid composition analysis and measuring the loss of individual PUFA, the susceptibility of specific PUFA to lipid peroxidation can also be determined (Anderson, Maude, and Nielsen, 1985). Limitations of the PUFA loss assessment for indirect determination of lipid peroxidation is evident when lipid oxidation is subtle, as measuring small differences in a large PUFA pool may not accurately correlate with lipid peroxidation measures (Smith and Anderson, 1987).

Butylated Hydroxytoluene and Deferoxamine

In 1979, butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-4-methylpheol) was discovered to improve the PUFA recovery in lipids (Stone, Farnsworth, and Dratz, 1979), and is now a frequent additive in fatty acid analysis for the prevention of lipid peroxidation (Metherel et al., 2009a). BHT consists of a toluene ring structure (phenol with a methyl group) with two isobutyl and one hydroxyl group bound to the ring (**Figure 6**). The ring structure of BHT donates a proton to neutralize free radicals thereby preventing them from accepting protons from the methylene group of PUFA, thus preventing PUFA peroxidation during fatty acid analysis and/or during the storage of samples (Pryor, 1994). BHT can react with $\cdot OH$ or $\cdot O_2$ to interrupt peroxidation initiation reactions (Lambert, Black, and Truscott, 1996), and these reactions and

the end products are illustrated (Figure 6). Numerous studies have demonstrated the ability of BHT to prevent PUFA degradation in blood samples during long term storage (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006; Marangoni, Colombo, and Galli, 2004; Min et al., 2011; Otto et al., 1997). BHT has also been shown to prevent Fe-induced PUFA peroxidation (Davies and Goldberg, 1987b; Davies and Goldberg, 1987a; van den Berg et al., 1992). The mechanism of its action occurs at a point after Fe oxidation and prior to PUFA oxidation, as BHT does not prevent the Fe^{2+} oxidation to Fe^{3+} (van den Berg et al., 1992). Therefore, BHT can be utilized in the prevention of PUFA peroxidation during long-term storage of whole blood samples that may be a result from either oxygen or iron-induced mechanisms.

BHT is a universal antioxidant, and as a result its use while effective does not aid in determining if the origin of lipid peroxidation is due to the Fe^{2+}-mediated pathway, a possible mechanism of whole blood degradation due to the presence of iron in hemoglobin. Iron chelators can be administered to individuals undergoing continued blood transfusions when macrophages and hepatocytes can no longer retain all of the surplus iron, and plasma transferrin transport capacity is exceeded (Brittenham, 2011). Consequently, this non-transferrin-bound iron forms a variety of complexes that are mediators of extra-hepatic tissue damage (Breuer, Hershko, and Cabantchik, 2000). Iron chelation therapy, with chelators such as deferoxamine, can circumvent this potential problem by binding to excess iron and removing it from cells (Brittenham, 2011). This same iron chelation method could be used for preventing iron-mediated fatty acid peroxidation reactions. When deferoxamine binds specifically to Fe^{3+} (**Figure 7**), the Fe^{3+} is prevented from reacting with PUFA thereby preventing breakdown of PUFA and formation of lipid peroxidation products (Halliwell and Chirico, 1993). Deferoxamine has previously been used for the prevention of fatty acid peroxidation in

erythrocytes (Knight, Searles, and Clayton, 1996; Otto et al., 1997) and platelets (Knight, Blaylock, and Searles, 1993; Pratico et al., 1999).

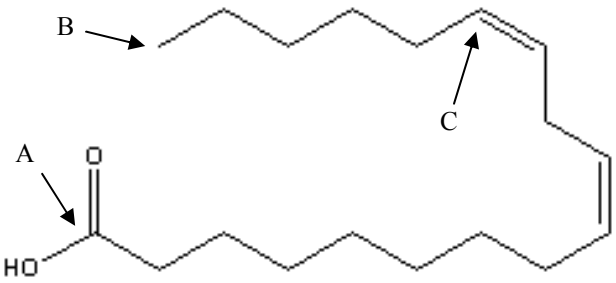


Figure 1 – Linoleic Acid Structure, LA, 18:2n-6. A – carboxylic acid group (-COOH), B – methyl or omega end, C – first double bond at 6th carbon from omega end (n-6)

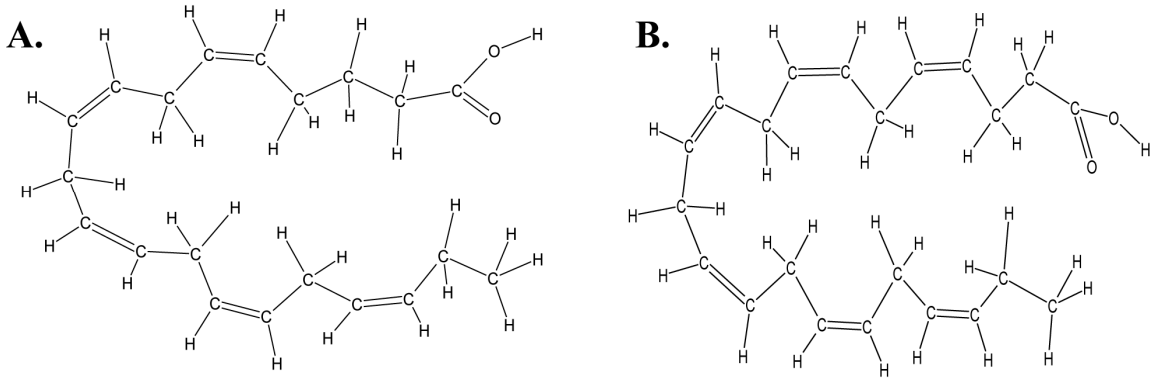


Figure 2 – Omega-3 Highly Unsaturated Fatty Acid Structures. A. Eicosapentaenoic Acid, EPA, 20:5n-3. B. Docosahexaenoic Acid, DHA, 22:6n-3.

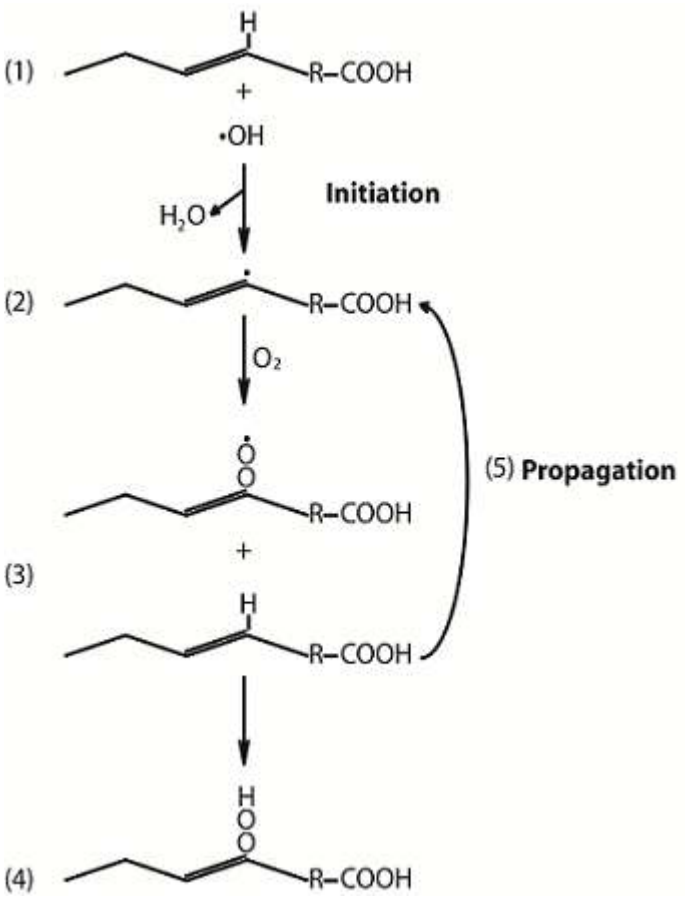


Figure 3 – Mechanism of Free Radical Induced Fatty Acid Peroxidation. (1) Unsaturated fatty acid reacts with hydroxyl radical and yields water to form (2) lipid radical which reacts with oxygen to form (3) lipid peroxyl radical that reacts with a fatty acid to form (4) lipid peroxide. The last reaction forms a new lipid radical that (5) propagates back to step (2) and re-enters the pathway

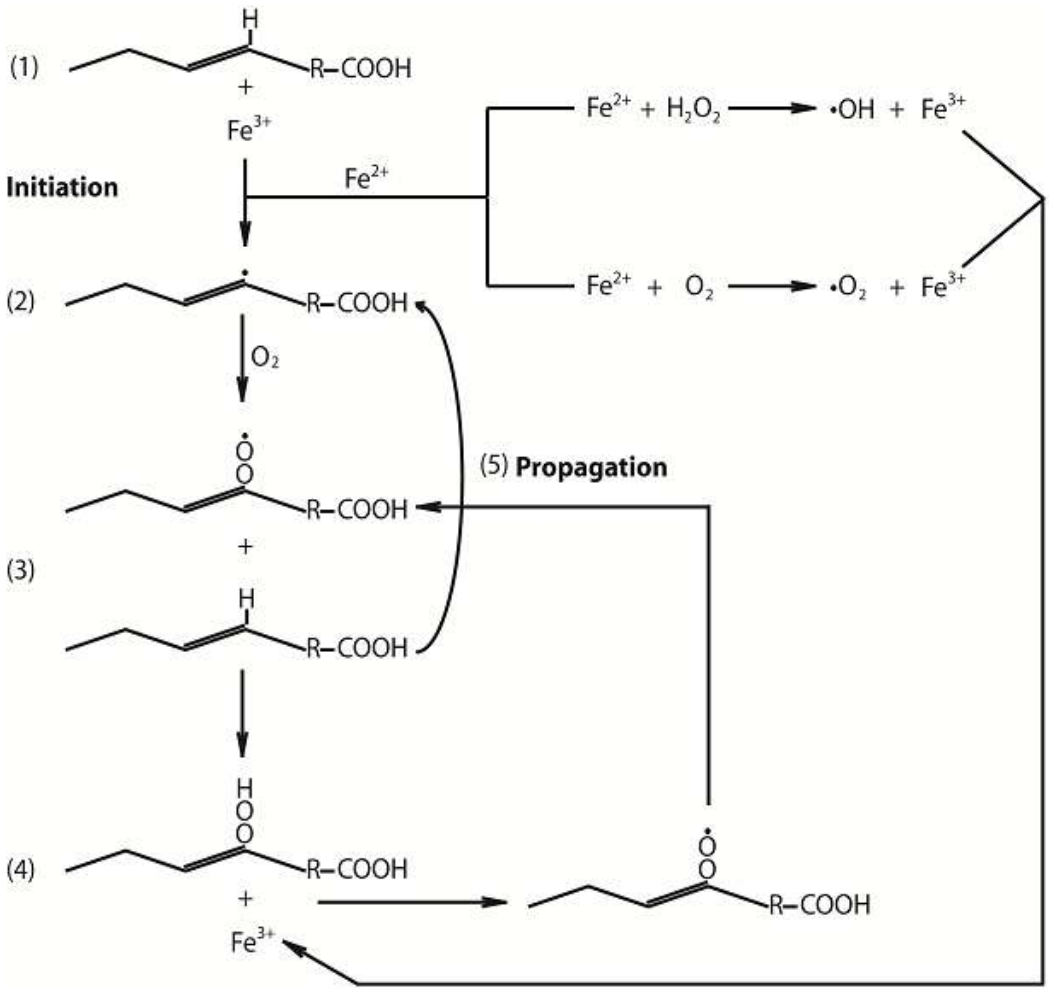


Figure 4 – Mechanism of Iron Induced Lipid Peroxidation. (1) Polyunsaturated fatty acid reacts with Fe^{3+} and yields Fe^{2+} that reacts with either O_2 or H_2O_2 to form hydroxyl or oxygen radicals that can cause further lipid peroxidation and yield Fe^{3+} that can (1) either react again with polyunsaturated fatty acids or (4) react with lipid hydroperoxides to reform lipid peroxyl radicals that (5) propagates back to step (2) and re-enters the reactions.

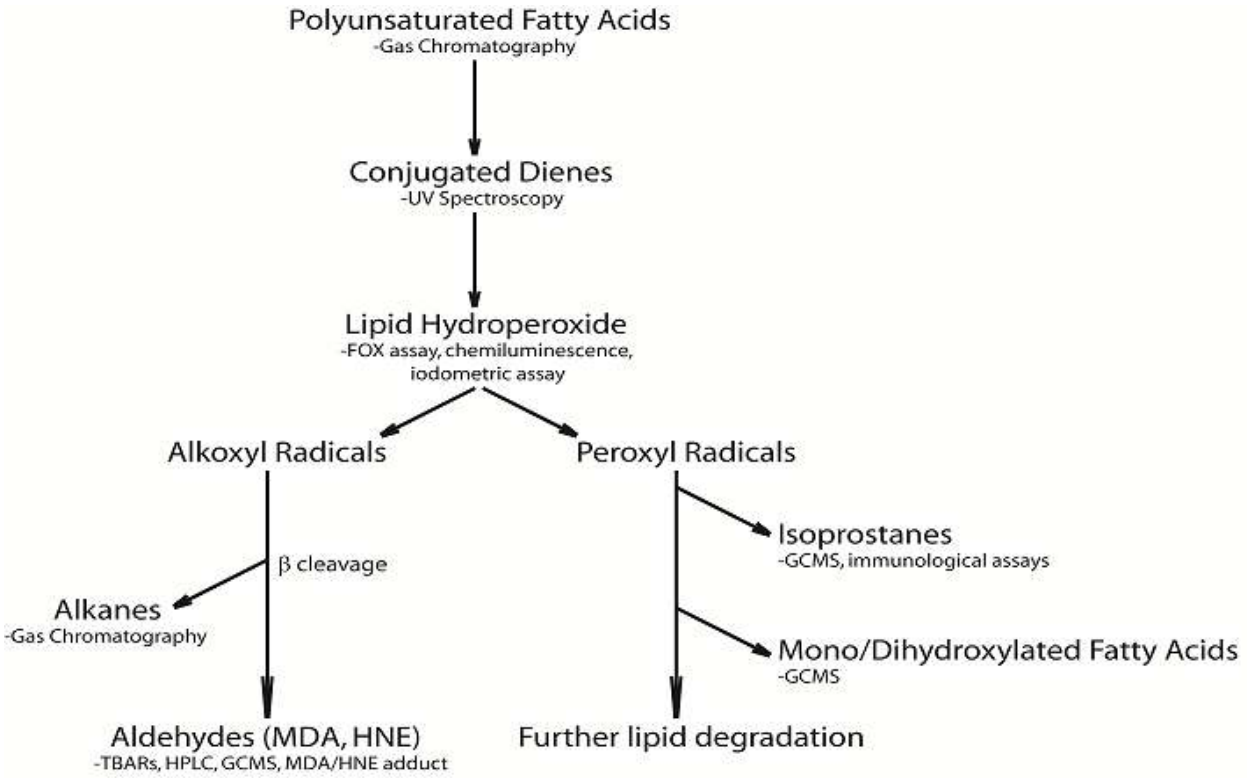


Figure 5 – Lipid Peroxidation Products and Measures of Detection. UV, ultraviolet; FOX, ferrous oxidation of xylenol; GCMS, gas chromatography mass spectrometry; MDA, malondialdehyde; HNE, 4-hydroxynonenal; TBARs, thiobarbituric reactive species; HPLC, high performance liquid chromatography. Modified from Moore and Roberts (1998).

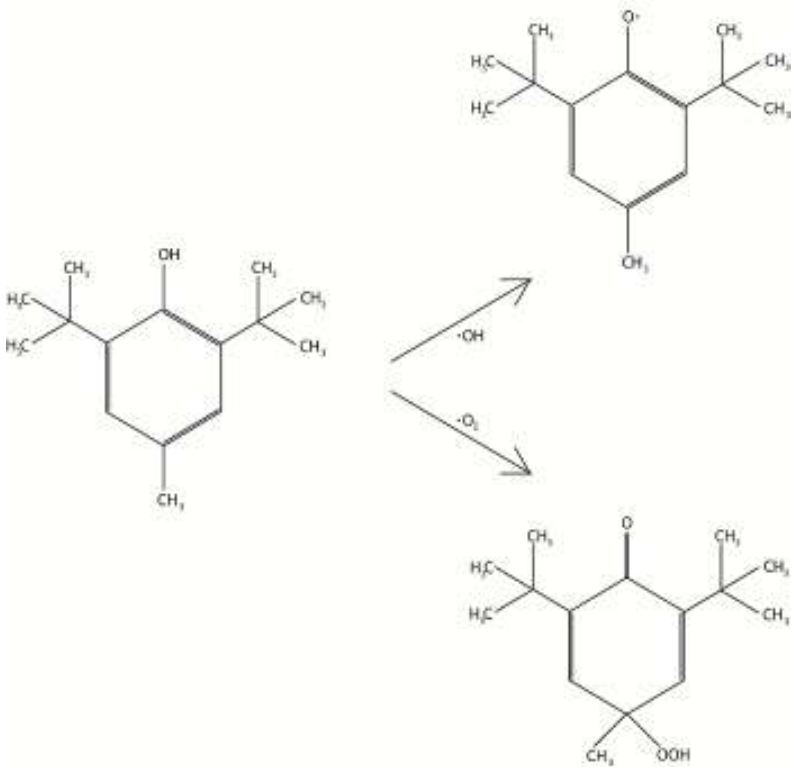


Figure 6 – Butylated Hydroxytoluene and Antioxidant Products

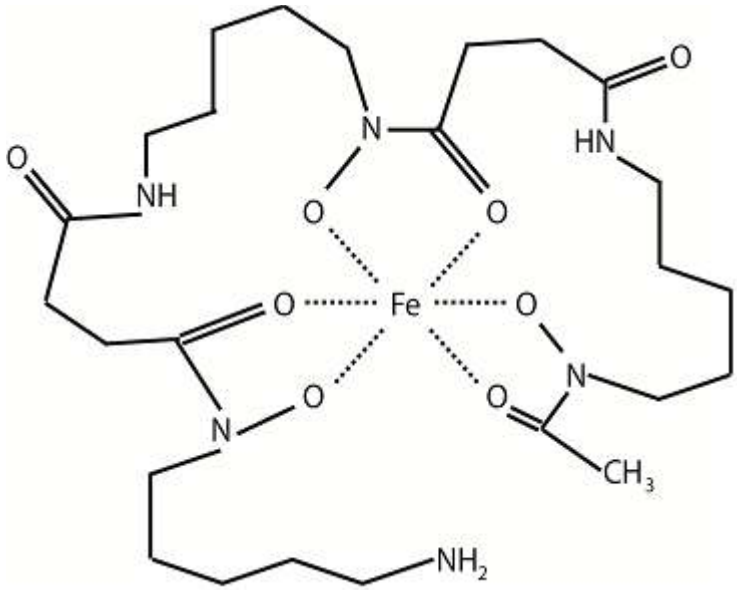


Figure 7 – Iron Chelation by Deferoxamine.

CHAPTER 3

METHODOLOGICAL FOUNDATIONS

Fingertip Prick Blood Fatty Acid Analysis

The use of FTP blood collection for fatty acid composition analysis of plasma was initially proposed in 1990 (Ohta et al., 1990). More recently, validation of FTP blood fatty acid analysis has been performed in human volunteers demonstrating similarities in values between FTP direct transesterification and standard whole fatty acid analysis methods (Armstrong, Metherel, and Stark, 2008; Marangoni, Colombo, and Galli, 2004). FTP analysis has been successfully implemented in a variety of populations, however, is not without its limitations, as quantitation of fatty acids is difficult to determine without knowing the exact weight or volume of the whole blood being measured. Qualitative assessment alone may not provide a complete understanding of the changes occurring in a blood sample. For example, a decrease in a fatty acid in the phospholipid pool may not be detected if the overall phospholipid pool has also decreased, while quantitative analysis would immediately be able to detect such a decrease regardless of changes in the total phospholipid levels (Stark, 2008a). For this reason, qualitative values tend to be more stable with less variability compared to quantitative assessments, particularly for the determination of SFA and MUFA. These fatty acid pools are highly variable in a non-fasted state, and qualitative analysis in these cases may mask significant changes occurring in the TAG pool.

The majority of fatty acid profiling is utilized for the purpose of determining the omega-3 status of an individual. This is due to the numerous positive health implications that increasing omega-3 fatty acid intake has been linked to cardiovascular disease, fetal and infant neurological development, inflammatory diseases, neurological conditions and possibly even some cancers

(Moyad, 2005b; Moyad, 2005a), and may be a good predictor of these disease states. In fact, a more balanced relative proportion of n-3 to n-6 HUFA may provide a more effective primary prevention of CVD than reducing dietary cholesterol intake (Lands, 2008). Although FTP blood fatty acid analysis is limited to qualitative profiling due to unknown quantities of blood, omega-3 blood biomarkers tend to focus solely on qualitative analysis for their measurements, including the sum of weight % erythrocyte EPA + DHA (Harris and von Schacky, 2004) and the percentage of omega-3 HUFA in total HUFA (Stark, 2008b), and for this reason fast omega-3 blood screening methods still prove extremely useful in many cases. Biomarker analysis of the HUFA pool in its simplest form analyzes the competition of fatty acids for the *sn-2* position of phospholipids without the need for isolating these phospholipids, and as such qualitative analysis of HUFA status remains very informative. FTP fatty acid analysis may be particularly useful for on-site field samples, decreasing invasiveness of blood collection in infants and the elderly, rapid screening for omega-3/fish intake, and daily blood sampling. Overcoming stability and storage challenges with FTP blood samples could lead to fatty acid profiling becoming a component of routine clinical health screening.

Blood PUFA Stability During Long-term Storage

Numerous studies have determined the PUFA and fatty acid stability in both erythrocytes and plasma/serum; however, only a few prior studies have been performed on whole blood or finger prick PUFA stability. The latter studies vary widely with regard to their choice of manipulated variables including storage temperature, storage time, storage method and the lipid fraction measured. A summary of these studies can be found in **Table 1**. Although erythrocytes are often noted as the preferential blood fraction for fatty acid analysis, the heme content of

erythrocytes increases the risk of PUFA oxidation compared to the plasma PUFA during storage and handling procedures (Stark, 2008a).

To date, eight different studies have analyzed the storage stability of PUFA in erythrocytes with temperatures ranging from 4°C to -80°C. Of these studies, four examined erythrocyte stability with antioxidants and seven without antioxidants and another two of these studies stored their samples under nitrogen. The duration of these studies ranges from six days to four years. Erythrocyte total lipid extract (TLE) fatty acid composition is stable for up to 24 hours at 4°C (Fernandez et al., 1993) and less than 6 months at -20°C (Di Marino et al., 2000), without the presence of any antioxidant or any other storage condition. Additionally, erythrocyte TLE PUFA are demonstrated to be stable for 4 weeks under similar -20°C storage conditions (Magnusardottir and Skuladottir, 2006) and erythrocyte phospholipid PUFA for less than 4 weeks when stored under nitrogen (Otto et al., 1997) and for up to only two weeks when stored in saline at the same temperature (Ways, 1967). Interestingly, the latter group also determined that the capillary erythrocyte PUFA were more susceptible to degradation as compared with venous samples. They hypothesized that this discrepancy was due to increased hemolysis of erythrocytes, releasing iron and making PUFA more susceptible to iron-induced autooxidation (Chiu, Kuypers, and Lubin, 1989; Gutteridge, 1989). Storage of erythrocyte blood samples in the presence of BHT extends PUFA stability from 4 to 17 weeks at -20°C (Magnusardottir and Skuladottir, 2006). Storage in the presence of BHT or DFO further extends stability of erythrocyte phospholipid PUFA to at least one year at -50°C (Otto et al., 1997), and to at least 2 years with BHT and nitrogen at -80°C (Di Marino et al., 2000). Eryhtrocyte TLE are stable at -70°C for at least one year without the presence of antioxidants. It has been noted that iron can induce the formation of free radicals in erythrocytes resulting in the peroxidation of

PUFA in blood (Dargel, 1992), and as such explains why deferoxamine (DFO), an iron chelator, may protect against PUFA degradation. Another study measured erythrocyte phosphatidylcholine fatty acid composition and found that under no other conditions other than -80°C storage, PUFA stability was maintained for at least 4 years (Hodson et al., 2002).

In addition, eight studies have examined plasma/serum storage stability, with none of them stored with an antioxidant and only one of them stored their samples under nitrogen, for periods ranging between 72 hours and ten years. The plasma fatty acid profiles appear more resistant to degradation during various storage conditions compared to erythrocytes, including storage at 4°C showing no significant changes in profiles for up to a minimum of 6 days in CE, PL, non-esterified fatty acids (NEFA) and TAG (Moilanen and Nikkari, 1981; Stokol and Nydam, 2005), compared to just 24 hours in erythrocytes (Fernandez et al., 1993). At room temperature, two studies indicated that the stability of the plasma/serum fatty acid profiles may only last for a maximum of 24 hours (Stokol and Nydam, 2005), and possibly less (Moilanen and Nikkari, 1981). Further stability has been demonstrated in various plasma/serum lipid fractions at -20°C for a minimum of four weeks with nitrogen (Otto et al., 1997) and a minimum of 1 year without (Hirsch, Slivka, and Gibbons, 1976), at -40°C for one month (Stokol and Nydam, 2005), at -60°C for one year (Moilanen and Nikkari, 1981) and at -80°C for up to ten years or more (Hodson et al., 2002; Matthan et al., 2010).

Fewer storage stability studies have been performed for the whole blood and/or FTP samples. In total, only three studies have examined PUFA stability in these fractions with three of them using an antioxidant and two having a non-antioxidant condition, and only one study utilized a nitrogen storage condition. The study durations ranges from three weeks to four months. The whole blood NEFA fatty acid profiles, without any particular storage condition,

remain stable for less than 24 hours at room temperature, up to 24 hours at 4°C and for at least one month when stored at -40°C (Stokol and Nydam, 2005). This is the only study found to assess the fatty acid stability in whole blood samples during storage. An additional study analyzing the FTP storage have determined the PUFA stability for less than two months upon storage at room temperature in the presence of BHT (Min et al., 2011), and for at least 3 weeks at 4°C in the presence of 50μg of BHT added directly to the chromatography paper prior to blood saturation of the paper (Marangoni, Colombo, and Galli, 2004). Naturally occurring factors in the blood can play a role in enhancing PUFA degradation including iron content (Dargel, 1992), while α-tocopherol in plasma and proportion of omega-3 in erythrocytes of Icelandic women (Thorlaksdottir et al., 2006) and increased vitamin E levels in piglets have been found to help prevent n-3 PUFA peroxidation (Sarkadi-Nagy et al., 2003).

Food PUFA Stability During Storage

PUFA stability in the stored whole food samples have been studied frequently and have most recently been determined in pork (Cardenia et al., 2011), chicken (Narciso-Gaytan et al., 2011), and beef (McArdle et al., 2011) samples under various animal feeding and storage conditions. Briefly, each study found varying degrees of lipid peroxidation during storage of meat samples for 3 days at 8°C, 30 days at 4.4°C and 30 days at 4°C for pork, chicken and beef, respectively. All of these studies used TBARS measurements to estimate the MDA content of food samples as a result of lipid peroxidation. Fish, stored for long periods of time, have the potential for even greater lipid peroxidation due to its increased HUFA content, particularly DHA and EPA. Previously, lipid peroxidation has been shown to occur in rainbow trout that has been stored at either -20°C or -30°C, but not at -80°C for up to 13 months (Baron et al., 2007),

and numerous Mediterranean fish species after 4 days of ice storage (Passi et al., 2005). Atlantic salmon fatty acid fluctuation during storage have also been determined, and lipid peroxidation has been shown to increase during storage for 6 months at -10°C but not at -20°C, and was accompanied by an increase in NEFA content (Refsgaard, Brockhoff, and Jensen, 2000). Lipid peroxidation has further been determined after 72 hours of storage at 15°C (Tuckey, Forster, and Gieseg, 2009), 6 days of at -1°C (Sallam, 2007) and 4°C (Cook et al., 2009) and 12 weeks at -20°C (Cook et al., 2009). To our knowledge, no previous study has compared the PUFA losses in Atlantic salmon during storage at -20°C and -80°C. Such a study would help determine the viability of salmon during home freezer storage before appreciable omega-3 fatty acid degradation occurs.

Lipid Extraction

Although several lipid extraction methods exist, the Folch method (Folch, Lees, and Sloane Stanley, 1957) and the Bligh and Dyer method remain the gold standards in academic research laboratories. The Folch extraction method uses a chloroform:methanol solvent ratio of 2:1 and is generally preferred for use in samples with ≥ 2% fat content, mainly because of its preferred solvent to sample ratio of no lower than 20:1 (Iverson, Lang, and Cooper, 2001). Several variations of the method exist that include introducing chloroform and methanol independently with repeated rounds of homogenization, and although food analysis is generally performed using 1g of sample and 20mL of solvent, the sample and solvent sizes can be scaled down considerably when using more homogeneous samples. Following homogenization, a chloroform/methanol/water ratio of 8:4:3 must be achieved in order to separate organic and aqueous phases, and this ratio is normally achieved by the addition of an aqueous buffer. Methods used for separating the phases generally include filtration, washing and re-extraction.

Although a single extraction of the lower organic phase following homogenization is not ideal, with the presence of an internal surrogate standard any fatty acid losses are assumed to be equal to losses of the standard and therefore negligible.

The Bligh and Dyer (Bligh and Dyer, 1959) method provides a significant advantage over the Folch method in that the solvent to sample ratio is much smaller at only 4:1. This method has been developed for samples that are low in fat (<1%) and high in water content (80% of sample mass) such as muscle tissue (Stark, 2012). When applied to samples with increasing lipid content (>2%), the Bligh and Dyer method results in a significant underestimation of lipid content. Generally, 3mL of 1:2 chloroform/methanol is added to 1g sample to achieve 1:2:0.8 chloroform/methanol/water (water content derived from sample) ratio for homogenization, with the subsequent addition of 1 mL chloroform and 1 mL aqueous buffer to achieve the desired 2:2:1.8 chlorofrom/methanol/water ratio required for extraction (Stark, 2012).

When determining the fatty acid profiles of plant tissues, initial extraction with isopropanol is necessary to deactivate phospholipase D (Nichols, 1963) prior to chloroform:methanol extraction (Christie et al., 1998; Kates, 1960; Nichols, 1963). Modifications to the method have been implemented to expedite the process (Christie et al., 1998). Alternative solvents that may replace 2:1 chloroform:methanol include 3:2 hexane:isopropanol (Hara and Radin, 1978; Radin, 1981) and 1:1 petroleum ether:diethyl ether (AOAC, 2005) that have a significantly milder environmental effect as both solvents are less toxic.

Fatty Acid Transesterification

Fatty acid transesterification is a necessary step in fatty acid analysis, as it optimizes the separation sensitivity and response of fatty acids when introduced to a gas chromatograph. Transesterification of fatty acids for gas chromatography is often accomplished by a reaction in which a methyl group ($-CH_3$) replaces the –H of the carboxylic acid group (-COOH) of the fatty acid, generating a fatty acid methyl ester ($R-COOCH_3$). Additionally, when this reaction occurs in a sample, where fatty acids are already esterified to another moiety such as glycerol in the case of triacylglycerols or phospholipids and cholesterol in the case of cholesteryl esters, the transesterification reaction includes the breaking of these ester bonds prior to esterification of the methyl group. Numerous methods and reagents exist for the transesterification of fatty acids, with these reagents generally being classified as either base- or acid-catalyzed reactions (**Figure 8**).

The majority of fatty acid transesterification reactions involve a base or acid reagent in a solution of methanol, with methanol being the necessary source of the methyl group for the formation of the fatty acid methyl esters. The method used by our laboratory is one of the most common techniques, and it involves the strong acid boron trifluoride in methanol that is heated with the sample for up to one hour (Morrison and Smith, 1964). Other acidic reagents include sulphuric acid (Dugan, Jr., McGinnis, and Vadehra, 1966), hydrochloric acid (Metcalfe and Schmitz, 1961) and acetyl chloride (Lepage and Roy, 1986) in methanol. Base-catalyzed reactions using sodium or potassium methoxide reagents are also popular, but are not efficient catalyzing reagents for non-esterified fatty acids (Christie, 2003). Additionally, diazomethane has also been a popular catalyst in the formation of fatty acid methyl esters for the purpose of gas chromatographic analysis (Schlenk and Gellerman, 1960).

Ultrasound-Assisted Fatty Acid Analyses

Ultrasonication results in cavitation within a liquid solvent medium, in which microscopic bubbles are formed as a result of the longitudinal waves created in the liquid by the mechanical movement of an ultrasonic probe. In liquid, the expansion phase of these waves pull the liquid molecules apart until the negative pressure is enough to create small cavities or bubbles. For this to occur, the negative pressure must be greater than that of the local tensile strength of the liquid. Expansion and contraction of the bubbles continue until the force of expansion within the bubbles (up to 5000°C and 2000 atm.) becomes unstable and collapses. The resultant collapse of these bubbles releases tremendous amounts of heat, pressure and mechanical shear (Chemat et al., 2004). When bubble collapse occurs close to the surface of a solid, the collapse is asymmetrical and results in high-speed liquid jets (400 km/h) that attack the surface of the solid, resulting in significant damage to the impact sites, exposing newly formed and highly reactive sites for solvent penetration and promotes the absorption of cell contents into the solvent (Toma et al., 2001; Vinatoru et al., 1997). Many factors determine the effectiveness of ultrasonication on the extraction of cellular contents into a solvent. These factors include but are not limited to gas and particulate matter in the liquid, external pressure, solvent viscosity, surface tension and vapour pressure, applied frequency, attenuation and temperature (Luque de Castro and Priego-Capote, 2007).

Of particular interest here are the factors or variables that can be easily manipulated such as applied frequency and temperature, as they relate to the solvent viscosity. Attenuation denotes the phenomenon that results in the production of heat due to energy loss as the wave travels from source to sample (Luque de Castro and Priego-Capote, 2007). However, for the ultrasonic setup used previously in our lab (Metherel et al., 2009b), this attenuation would be

negligible due to the close proximity of the ultrasonic source and sample. Temperature does not directly affect cavitation; however, an increase in temperature reduces surface tension and viscosity of the liquid and increases the liquid vapour pressure (Luque de Castro and Priego-Capote, 2007), thereby reducing the effect of ultrasonication on the medium. By maintaining low solvent temperature, a larger amount of energy is required to create the cavitation bubbles during the expansion phase of the ultrasound wave cycle. This results in greater energy storage in the bubble and a subsequently greater energy release during collapse (Luque de Castro and Priego-Capote, 2007). In addition, as temperature decreases it becomes more difficult for bubbles to form due to a decrease in the solvent vapour pressure, resulting in less solvent vapour able to enter the cavity of the bubble. Bubble collapse is cushioned by the vapour present in these bubbles; therefore, lower vapour pressure results in less cushioning of the stored energy and a greater effect on the target medium (Luque de Castro and Priego-Capote, 2007).

Previously, ultrasound-assisted methods have been used to expedite the extraction of fatty acids from various tissues (Cravotto et al., 2008; Luque-Garcia and Luque de Castro, 2004; Metherel et al., 2009b; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Wei et al., 2008). Ultrasonic energy has further been utilized in the extraction of phenols (Ahn et al., 2007), ginsenosides (Wu, Lin, and Chau, 2001), anthraquinones (Hemwimol, Pavasant, and Shotipruk, 2006) and polycyclic aromatic hydrocarbons (Christensen, Ostman, and Westerholm, 2005; Richter et al., 2006). More importantly, ultrasound-assisted extractions from solids have been reported for fatty acid profiling that can reduce extraction times to as little as one hour (Cravotto et al., 2008; Luque-Garcia and Luque de Castro, 2004; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Wei et al., 2008). Ultrasound-assisted extractions have been based largely on replacing the Soxhlet extraction apparatus which is cumbersome and requires large

solvent volumes and sample masses (Luque-Garcia and Luque de Castro, 2004; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Wei et al., 2008). However, these extractions have also been used with small sample and solvent sizes by utilizing a cup horn that holds water for the sample to be immersed into and receive ultrasonic energy though the water medium (Cravotto et al., 2008). In addition, our laboratory has shown that a direct sonication method in which the ultrasonic probe is inserted into a test tube containing the sample and solvent could potentially reduce extraction times to as little as 10 minutes (Metherel et al., 2009b). The cup horn allows for numerous samples to be extracted simultaneously and allows for capping of samples that prevents vapour loss and the need to replenish solvents during the extraction process, and as such the current thesis will test this method of fatty acid extraction on various food sources.

Few studies have tested have tested the derivitization of fatty acids to fatty acid methyl esters (FAME) with the aid of ultrasonic energy. Ultrasonic energy can be a useful tool for the emulsification of immiscible liquids such as those used in transesterification, as the microjets formed from US cavitation bubble collapse can disrupt the phase boundary of the liquids and promote lipid transfer between solvents (Mason and Peters, 2002). Previously, ultrasound energy has been used to assist in base-catalyzed (Hingu, Gogate, and Rathod, 2010; Stavarache et al., 2005; Stavarache, Vinatoru, and Maeda, 2006; Stavarache et al., 2007; Thanh et al., 2010) and acid-catalyzed (Stavarache, Vinatoru, and Maeda, 2006) fatty acid direct transesterification for the production of biodiesel fuel from vegetable oils. Base-catalyzed reactions are the most common due to the low levels of NEFA in vegetable oils, which are inefficiently transesterified using the base-catalyzed methods (Christie, 2003). However, these methods, although faster

than the standard method, require up to 60 minutes to complete; therefore, they would not provide a significant benefit to our labs current BF_3-catalyzed reactions.

The most common methods for acid-catalyzed reactions are boron trifluoride (BF_3) and sulphuric acid (H_2SO_4), although it is shown that H_2SO_4 may provide better results in conjunction with ultrasonic energy when compared to BF_3 (Stavarache, Vinatoru, and Maeda, 2006). Previous H_2SO_4-catalyzed, US-assisted fatty acid transmethylations have been performed on serum (Liu et al., 2010), zebrafish eggs (Li et al., 2009b), plant oil and yeast extracts (Li et al., 2009a) and on haloacetic acids from vegetable oils (Alvarez, Priego, and Luque de Castro, 2008), and as such these methods can be used as a basis for the formation of an US-assisted direct transmethylation procedure in the finger prick whole blood samples, and may significantly reduce the time required for transmethylation.

Microwave-Assisted Fatty Acid Analyses

Microwave radiation consists of electromagnetic waves, so it includes two types of energy fields: electric and magnetic. Microwave energy is a non-ionizing form of energy that results in the migration of ions and rotation of dipoles, while not altering the molecule structure in itself (Luque de Castro M.D. and Luque Garcia J.L., 2002). The nature of the heating effect of electromagnetic waves on a substance refers to the ability of the electric field to exert a force on charged particles. If the charged particles present in the substance of interest are able to move freely, then a current will be induced. Conversely, if the charged particles are bound to a specific region, they will move until an opposite force counters them; the result is dielectric polarization which, along with conduction, causes heating of a sample when subjected to microwave energy (Luque de Castro M.D. and Luque Garcia J.L., 2002). The dielectric

properties of a material are determined by two factors: the dielectric constant and the dielectric loss. The dielectric constant refers to the ability of a molecule to be polarized by the electric field, while the dielectric loss refers to the ability of the material to convert the energy of the microwaves into heat. The dielectric loss reaches a maximum as the dielectric constant decreases (Kingston H.M. and Haswell H.J., 1997), and the way in which these two factors interact is referred to as the dissipation factor (dielectric loss/dielectric constant).

Variables affecting microwave extraction include microwave power and exposure time, temperature and pressure, and the type of solvent used (Luque de Castro M.D. and Luque Garcia J.L., 2002). Microwave power and exposure time are inversely related, and, generally speaking, the maximum power should be used to minimize time required for sample processing (Lamble K.J. and Hill S.J., 1995). However, using very high powers may not be advisable depending on the reagents in use. For example, acids such as H_2SO_4 can react explosively to sudden heating in the presence of organic material (Luque de Castro M.D. and Luque Garcia J.L., 2002). In other cases, high microwave power can decrease extraction efficiency due to the degradation of the analytes of interest (Luque-Garcia et al., 2002). The type of solvent used in a microwave can play a significant role in the efficiency of the process. For example, when extracting non-polar compounds, a non-polar solvent like hexane would be beneficial for dissolving these compounds; however, hexane has a low dielectric constant which lowers the solvents interaction with the microwaves, and thus hexane would require to be mixed with another solvent with a higher dielectric constant (Luque de Castro M.D. and Luque Garcia J.L., 2002). For example, in pilot work our lab has combined methanolic sulphuric acid with toluene to optimize direct transesterifcation of fatty acids in whole blood. However, sulphuric acid can exhibit oxidizing properties, although not at the low concentrations used more commonly (~1%) (Luque de Castro

M.D. and Luque Garcia J.L., 2002). Also affecting microwave heating is sample viscosity and sample size. As viscosity changes from highly viscous to low, the dissipation factor increases as the molecular mobility increases. In addition, as solvent and/or sample size decreases, the amount of energy that can be absorbed within the solvent also decreases and may, therefore, reduce the effectiveness of the microwave energy on the analytical process (Luque de Castro M.D. and Luque Garcia J.L., 2002).

Microwave-assisted extraction techniques have been implemented previously for the extraction of fats from poultry feeds (Mahesar et al., 2008), seeds, meat and bakery products (Virot et al., 2008), acorns (Perez-Serradilla et al., 2007) and avocado pulp (Ortiz et al., 2004). However, for our purposes in fatty acid analysis, we will focus our attention on developing a novel microwave-assisted direct transesterification technique for the analysis of fatty acids in finger prick whole blood samples. Although previous microwave-assisted direct transesterification studies have been reported for biodiesel production (Kim et al., 2011; Patil et al., 2011), brain phospholipids (Banerjee, Dawson, and Dasgupta, 1992) and meat acylglycerides (Tomas et al., 2009), no previous work besides our own (Armstrong, Metherel, and Stark, 2008; Metherel et al., 2009a) has demonstrated the possible benefits of these assisted methods on FTP blood or whole blood.

A microwave-assisted direct transesterification method for finger prick whole blood samples has been tested utilizing BF_3 in methanol as the derivitizing agent (Armstrong, Metherel, and Stark, 2008; Metherel et al., 2009a). It was determined that methods employing a commercially available microwave (like those being used as a kitchen appliance) have some limitations. For instance, direct microwave-assisted transesterification results in significantly higher total SFA and omega-3 PUFA, and lower MUFA and omega-6 PUFA relative to the

standard Folch extraction methods (Armstrong, Metherel, and Stark, 2008). Many of these differences were determined to be specifically a result of inefficient transesterification of TAG fatty acids (Armstrong, Metherel, and Stark, 2008). However, direct transesterification of the FTP blood did not show any significant detriment to the qualitative values, when using the standard convectional 60 minute technique (Armstrong, Metherel, and Stark, 2008; Marangoni, Colombo, and Galli, 2004), and the relative yield of omega-6 and omega-3 PUFA as well as total HUFA remained stable (Metherel et al., 2009a). More recently, pilot work suggests that the microwave-assisted direct transesterification of whole blood with 1% methanolic H_2SO_4 and toluene results in quantitative fatty acid values that are similar or sometimes better compared to a standard whole blood fatty acid analysis method. These improvements were seen both when utilizing a commercially available kitchen-grade microwave and the CEM Discover SP-D microwave specifically designed for laboratory processes (CEM Corporation, Matthews, North Carolina).

Predictive Equations and Fatty Acids

The fatty acid composition in tissues varies greatly based on the proportions of complex lipids including phospholipids, triacylglycerols, and cholesteryl esters. Blood has a mixture of these complex lipids and isolating specific blood fractions such as plasma and erythrocytes can result in different fatty acid compositions or profiles. Predictive equations to enable translation of fatty acid profiles between blood fractions and tissues would be a useful tool in the comparison of data between studies utilizing various collection techniques, and provide a simpler method for predicting the fatty acid content of tissues that demand rigorous analytical procedures. Plasma PL and TAG HUFA composition can be predicted from the intake of 18, 20

and 22 carbon omega-3 and omega-6 fatty acids in the diet (Lands et al., 1992), and reductions in plasma TAG have been predicted from omega-3 fatty acid intake (Musa-Veloso et al., 2010). Despite differences in fatty acid compostions between tissues and tissue lipid fractions, there appear to be defined relationships between fatty acids in one tissue or region with another. Recently, analyses have been performed to determine the correlations between directly transesterified whole blood and erythrocytes for AA/EPA ($R^2 = 0.8675$) and n-6/n-3 ratio ($R^2 = 0.7114$) (Rizzo et al., 2010). Moreover, the predictive value of plasma phospholipid HUFA for the determination of erythrocyte phospholipid HUFA has been determined for AA ($R = 0.364$), EPA ($R = 0.709$) and DHA ($R = 0.653$) (Kawabata et al., 2011). Another study has determined significantly high correlations exist for the omega-3 blood biomarkers % n-3 HUFA in total HUFA, % EPA + DHA and n-6/n-3 ratio in plasma and erythrocytes with those in liver, brain, heart and adipose tissues (Stark, 2008b). Predictive equations from FTP blood to plasma and erythrocytes to whole blood would be very valuable to allow for direct comparisons between past studies, and to improve the understanding and academic acceptance of a simpler sampling technique.

Table 1 – Summary of Previous Blood Storage Studies and Findings

Sample Type	Sample Measure	Storage Temp.	Storage Conditions	PUFA Stability	Study Length	Citation
Erythrocytes	TLE	4°C	None	24 hours	144 hours (6 days)	Fernandez, et al (1993)
		-20°C	None	< 6 months	6 months	Di Marino, et al (2000)
			BHT	≥ 17 weeks	17 weeks	Magnusardottir, Skuladottir (2006)
			No BHT	4 weeks	17 weeks	Magnusardottir, Skuladottir (2006)
		-70°C	None	≥ 1 year	1 year	Stanford, et al (1991)
		-80°C	BHT and N_2	≥ 2 years	2 years	Di Marino, et al (2000)
	PL	-20°C	N_2	< 4 weeks	4 weeks	Otto, et al (1997)
		-25°C	Na_2SO_4	< 20 days	420 days	Broekhuyse (1974)
		-50°C	BHT or Deferoxamine	≥ 1 year	1 year	Otto, et al (1997)
	PL	-20°C	Saline	2 weeks	16 weeks	Ways (1967)
	PC	-80°C	None	≥ 4 years	4 years	Hodson, et al (2002)
Fingertip prick	TLE	Room Temp	BHT	< 2 months	2 months	Min, et al (2011)
		4°C	BHT	≥ 3 weeks	3 weeks	Marangoni, et al (2004)
		4°C	BHT	< 3 months	4 months	Min, et al (2011)
Whole Blood	NEFA	24°C	None	< 24 hours	72 hours	Stokol and Nydam (2005)
		4°C	None	24 hours	72 hours	Stokol and Nydam (2005)
		-40°C	None	≥ 1 month	1 month	Stokol and Nydam (2005)
Plasma/Serum	PL and NEFA	RT	None	< 24 hours	6 days	Moilanen and Nikkari (1981)
	NEFA		None	24 hours	72 hours	Stokol and Nydam (2005)
	CE, PL, NEFA and TAG	4°C	None	≥ 6 days	6 days	Moilanen and Nikkari (1981)
	NEFA		None	≥ 72 hours	72 hours	Stokol and Nydam (2005)
	PL	-20°C	N_2	≥ 4 weeks	4 weeks	Otto, et al (1997)
	TLE		None	≥ 1 year	1 year	Hirsch, et al (1976)
	CE, PL and TAG		None	< 3 years	3 years	Salo, et al (1986)
	NEFA	-40°C	None	≥ 1 month	1 month	Stokol and Nydam (2005)
	CE, PL, NEFA and TAG	-60°C	None	≥ 1 year	1 year	Moilanen and Nikkari (1981)
	TAG	-80°C	None	≥ 4 years	4 years	Hodson, et al (2002)
	CE, PL and TAG		None	≥ 10 years	10 years	Matthan, et al (2010)

BHT, butylated hydroxytoluene; CE, cholesteryl esters; N_2, nitrogen; NEFA, non-esterified fatty acids; PC, phosphatidylcholine; PL, phospholipid; RT, room temperature; TAG, triacylglycerols; TLE, total lipid extract

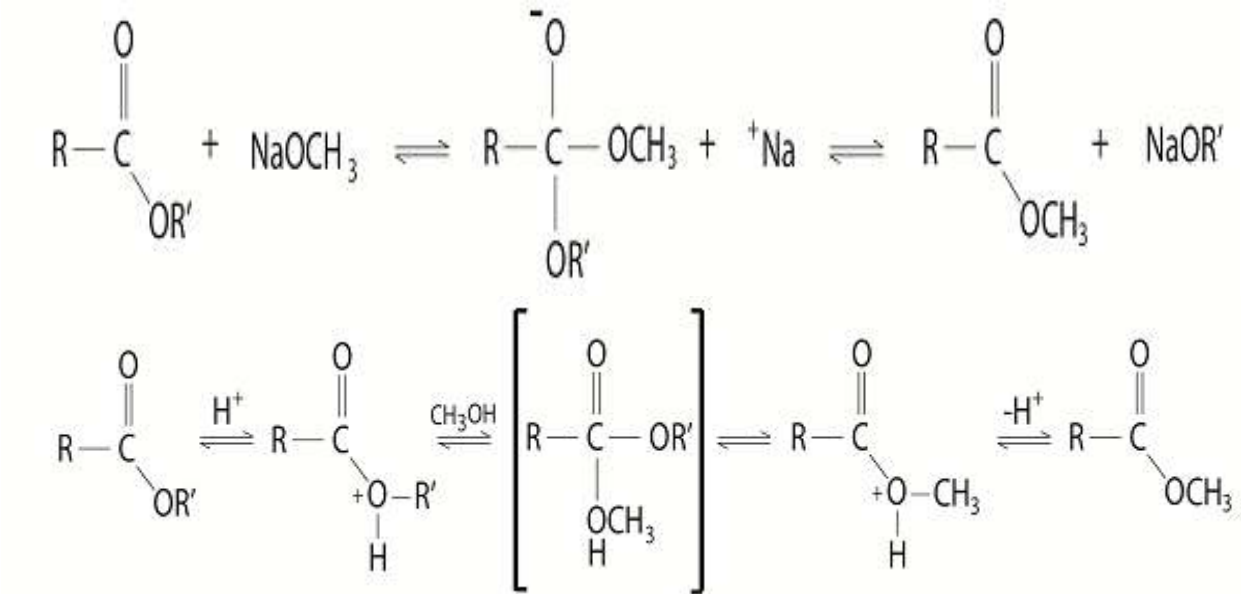


Figure 8 – Mechanisms of Fatty Acid Direct Transesterification. (A) base-catalyzed and (B) acid-catalyzed transesterification reactions for production of fatty acid methyl esters

CHAPTER 4

RATIONALE AND OBJECTIVES

Rationale

Most blood fatty acid analyses have been performed on the erythrocyte or plasma fraction, although a reduction in whole blood EPA and DHA has been associated with the decreased risk of sudden cardiac death in the Physicians' health study (Albert et al., 2002) and, more recently, with coronary artery disease (Moyers et al., 2011) and telomeric aging (Farzaneh-Far et al., 2010) in the Heart and Soul study. Fatty acid analysis in whole blood does not require multiple centrifugation steps, while these steps are required for plasma and erythrocyte analysis. Nonetheless, analysis of whole blood is much the same as plasma fatty acid analysis, which is simpler and faster than erythrocyte analysis (Metherel et al., 2009a). In addition, measuring fatty acid composition in whole blood allows for FTP blood sampling that provides precise and accurate n-3 HUFA status (Armstrong, Metherel, and Stark, 2008; Marangoni, Colombo, and Galli, 2004), and is amenable to high throughput analysis (Stark, 2008a). Fatty acid assessment via FTP blood analysis is gradually gaining popularity among researchers and clinicians, but its applications are still limited (Armstrong, Metherel, and Stark, 2008; Bailey-Hall, Nelson, and Ryan, 2008; Fratesi et al., 2009; Marangoni, Colombo, and Galli, 2004; Marangoni, Colombo, and Galli, 2005; Metherel et al., 2009a), and studies assessing the stability of PUFA in whole blood (Stokol and Nydam, 2005) or finger prick whole blood samples (Marangoni, Colombo, and Galli, 2004; Min et al., 2011) during long-term storage are scarce.

Currently, there is little research on the PUFA stability of the high omega-3 food samples during storage in a domestic freezer. PUFA degradation of the high omega-3 food such as salmon would result in a lower omega-3 intake than a consumer may desire; therefore, the analysis of

PUFA degradation during storage at -20°C is important to be investigated. For this reason, we will also analyze changes in Atlantic salmon fatty acid profiles during storage at -20°C for up to 6 months compared to -80°C storage.

Currently, accepted methods in lipid analysis can be time-consuming and costly, with standardized lipid extraction methods requiring from 4 (AOAC, 2005) to 14 (Luque-Garcia and Luque de Castro, 2004) hours to complete, and in certain instances may require up to 24 hours to complete (Taha et al., 2008; Taha, Metherel, and Stark, 2012). More recently, direct transesterification methods have been adopted that combine extraction and transesterification into one step; however, this may still require an hour for completion when using convectional energy to fuel the process (Fratesi et al., 2009). Alternate methods to expedite this process have been adopted using microwave (Armstrong, Metherel, and Stark, 2008; Banerjee, Dawson, and Dasgupta, 1992; Metherel et al., 2009a) and ultrasonic energy (Alvarez, Priego, and Luque de Castro, 2008; Li et al., 2009b; Li et al., 2009a; Liu et al., 2010), with promising results. Although, to our knowledge no previous studies besides our own have assessed the viability of these energy-assisted methods for expediting fatty acid analysis in whole blood, FTP blood or food samples.

Therefore, the current thesis project is aimed to elucidate PUFA stability in FTP blood, whole blood and salmon samples during storage times of up to six months at temperatures varying from room temperature to -75°C, with or without anti-oxidants, and in low or high omega-3 content blood. A better understanding of the PUFA stability in these samples may allow researchers to use this method of analysis with greater confidence when storage of blood samples is required. In addition, improvements in the time and monetary requirements for analysis will provide even stronger support for the application of the FTP blood samples for fatty acid analysis. For this

reason, my thesis project will also test various conditions of both microwave and ultrasound-assisted direct derivitization methods in the FTP blood fatty acid analysis.

Objectives

1. ***Determine the long-term PUFA stability in fingertip prick blood, whole blood and salmon samples***

a. Store samples at various temperatures and omega-3 status, with and without BHT, for a period of 1 day up to 6 months

b. Remove samples from storage and determine the fatty acid composition and use the losses in PUFA content as an indicator for lipid peroxidation

2. ***Determine the mechanisms involved in the accelerated rate of PUFA degradation during storage of blood samples at -20°C seen in the blood storage study and develop storage methods for the prevention of these losses***

3. ***Develop new energy-assisted methods of direct transesterification of fatty acids in finger prick whole blood and extraction of fatty acids from food samples***

a. Determine the effect of sample temperature, probe amplitude and reaction time on effectiveness of indirect closed-tube ultrasound-assisted fatty acid extraction from flaxseed

b. Determine the effect of reagent composition, applied energy and reaction time on effectiveness of microwave-assisted direct transesterification in fingertip prick blood

4. ***Develop a predictive equation for the estimation of the omega-3 status in erythrocytes and plasma blood fraction from a known composition in fingertip prick whole blood***

Hypotheses

1. Storage temperature will have significant effect on the PUFA stability with lower temperatures delaying the onset of degradation and slowing down lipid peroxidation reactions.

2. The presence of BHT will further delay the PUFA degradation and allowing for longer storage periods.

3. Blood samples with higher omega-3 status will demonstrate increased levels of PUFA degradation due to an increase in unsaturation of stored samples.

4. PUFA degradation during storage at -20°C will be due to iron release from erythrocytes as a result of increased hemolysis and chelation and/or blood drying treatment methods will help prevent fatty acid peroxidation at -20°C.

5. Microwave and ultrasonic energy will expedite the direct transesterification of fatty acids in fingertip prick whole blood samples and extraction of fatty acids from food samples, respectively.

6. We will be able to develop useful predictive equations from fingertip prick whole blood for the estimation of omega-3 status in erythrocytes and plasma total lipid extract and plasma blood lipid fractions.

CHAPTER 5

GENERAL METHODS AND MATERIALS

Blood Collection

Venous whole blood samples for FTP blood, whole blood, erythrocyte or plasma fatty acid analysis were collected by venipuncture into anticoagulant-treated vacutainers by a trained technician. For FTP and whole blood analysis, collected samples were stored appropriately either on chromatography paper (Grade 3MM Chr, Whatman Ltd., Sanford, ME) or in cryovials. For erythrocyte and plasma separation, vacutainers were centrifuged at 3000rpm for 10 minutes, plasma was removed from the top layer and stored at -75°C for further analysis and erythrocyte were washed and centrifuged twice with saline solution (0.9% NaCl). Separated and washed erythrocytes were stored at -75°C for future analysis.

Folch Extraction and Transesterification of Salmon and Flaxseed

All canned salmon extractions and the control flaxseed extraction method are performed using a modified Folch method (Folch, Lees, and Sloane Stanley, 1957). Briefly, 3 mL of 2:1 chloroform:methanol is added to approximately 150 μg of canned pacific red sockeye salmon (GoldSeal, Canadian Fishing Company, Vancouver, BC) homogenate or 25 mg of ground flaxseed (Bob's Red Mill Natural Foods, Milwaukie, OR, USA), and extraction was achieved by vortex for either one minute (salmon) or allowed to sit for 24 hours (flaxseed) due to differences in extraction efficiency. Internal standards of 100 μg 22:3n-3 ethyl ester and 900 μg of 19:0 ethyl ester (Nu Chek Prep Inc., Elysian, MN, USA) were included in extraction solvents. Following extraction, 0.5 mL of 0.2 M sodium phosphate buffer was added, inverted twice and centrifuged at 3000rpm for

five minutes to separate organic and water phases. The bottom organic chloroform layer was pipetted off, dried under a stream of nitrogen and reconstituted in chloroform for storage, and aliquots of these total lipid extracts were subsequently used for transesterification of lipids to fatty acid methyl esters (FAME). Briefly, aliquots were taken and dried under a stream of nitrogen, and 1 mL 14% BF3 in methanol (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 300 mL of hexane (Morrison and Smith, 1964) were added, vortexed briefly and heated for one hour at 95°C on a block heater. After one hour, samples are removed from heat, allowed to cool and 1 mL each of hexane and water are added, vortexed for one minute and centrifuged at 3000rpm for five minutes. The top organic hexane layer (containing FAME) was pipetted off, dried under a stream of nitrogen, reconstituted in 65 μL of hexane and stored in a GC vial for subsequent GC analysis.

Thin Layer Chromatography Analysis

Plasma or whole blood total lipid extracts obtained by the modified Folch method containing internal standards of diheptadecanoin-PC (17:0-PC), triheptadecanoin (17:0-TAG), heptadecanoate-CE (17:0-CE) and non-esterified heptadecanoic acid (17:0-NEFA) were dried under a stream of nitrogen. Lipid fractions phospholipids, triacylglycerols, cholesteryl esters and non-esterified fatty acids were further isolated by thin-layer chromatography (Armstrong, Metherel, and Stark, 2008). Briefly, total lipid extracts of plasma were applied to silica gel plates (250 μm thickness with 60 Å pore size, Whatman International Ltd., Maidstone, UK) and lipids were separated using a mixture of heptane:diethylether:acetic acid (60:40:2 by volume). Samples were visualized with 2,7- dichlorofluorescein (Sigma, St. Louis, MO) under ultraviolet light and the lipid bands were collected for further Folch extraction and transesterification as described above.

Direct Transesterification of Whole Blood and FTP Blood

Whole blood and FTP blood samples (25 μL each) for direct transesterification reactions were added to 1 mL of 14% BF_3 in methanol and 300 μL of hexane (containing 3 μg/mL of the internal standard 22:3n-3 ethyl ester). Samples were then analyzed as described above and FAME were analyzed by GC.

FAME Analysis by Gas Chromatography

Fatty acid methyl esters were analyzed on a Varian 3900 gas chromatograph (GC) equipped with a DB-FFAP 15m × 0.10 mm i.d. × 0.10 μm film thickness, nitroterephthalic acid modified, polyethylene glycol, capillary column (J&W Scientific from Agilent Technologies, Mississauga, ON) with hydrogen as the carrier gas. Samples (2 μl) were introduced by a Varian CP-8400 autosampler into the injector heated to 250°C with a split ratio of 200:1. Initial temperature was 150°C with a 0.25 min hold followed by a 35°C/ min ramp to 200°C, an 8°C/min ramp to 225°C with a 3.2 min hold and then an 80°C/min ramp up to 245°C with a 15 min hold at the end. The flame ionization detector temperature was 300°C with air and nitrogen make-up gas flow rates of 300 and 25 ml/min, respectively, and a sampling frequency of 50 Hz (Metherel et al., 2009b).

Quality Assurance Protocols

Multiple quality assurance protocols are performed daily in our laboratory, and although informal are necessary processes for data reliability. GC column contamination is monitored by the daily 'blank' GC sample analysis of hexane only injections designed to both identify and remove column contamination that may occur during dormant GC analysis periods. Through changes in hexane peak size, the hexane blank can also identify blockages in sample application onto the

column and can suggest the need for GC septum, liner or injection syringe changes. Potential column degradation is monitored by the daily GC analysis of an external standard that contains 28 commonly measured fatty acid methyl esters in equal proportions (GLC-462, Nu Chek Prep Inc., Elysian, MN, USA). Peak areas and retention times are assessed and out-of-range differences are determined. Specifically, peak areas for each of the fatty acids in the external standards are considered acceptable if differences of no more than 5% exist between any and all peaks, and as such an internal standard can be considered as an accurate method for the quantitation of fatty acids in biological or food samples of interest. Peak shapes and peak retention times can shift as GC column ages, and the external standard also allows for monitoring of this effect and subsequent adjustments to the GC software integration method can administered to accommodate these age-related changes. These and other frequent quality control measures allow for suitable troubleshooting, maintenance and quality reassessment analyses to preserve reliability of data and maintain good analytical practices within the laboratory. From this, intra and inter-assay coefficients of variability for whole blood analysis have been determined in our lab at 1.24% and 4.51% for weight percent EPA, 2.40% and 6.36% for weight percent DHA, 3.60% and 5.41% for EPA concentrations and 4.40% and 3.21% for concentration of DHA, respectively, and are considered within acceptable range.

CHAPTER 6

EFFECTS OF STORAGE TEMPERATURE, ANTIOXIDANT/ANTICOAGULANT PRESENCE AND OMEGA-3 STATUS ON FATTY ACID AND N-3 BIOMARKER STABILITY DURING A 6 MONTH STORAGE PERIOD

INTRODUCTION

Fingertip prick (FTP) blood fatty acid analysis is a fast and economical method that does not require a trained phlebotomist for administration, and is less invasive than standard venous blood sampling. FTP fatty acid analysis has been adopted previously in infants (Agostoni et al., 2005; Agostoni et al., 2011) and the elderly (Fratesi et al., 2009) among others (Bailey-Hall, Nelson, and Ryan, 2008; Marangoni, Colombo, and Galli, 2004; Marangoni, Colombo, and Galli, 2005; Metherel et al., 2009a). Similarly, venous whole blood sampling is a simpler blood fraction for fatty acid analysis compared to erythrocyte and plasma analysis as it does not require centrifugation to obtain, but can still be used for more extensive lipid analyses. Although erythrocyte and plasma fatty acid profiling remain the most popular blood fraction analyzed, increasing EPA and DHA in whole blood have been associated with decreased risk of sudden cardiac death (Albert et al., 2002) and coronary heart disease (Moyers et al., 2011). Studies assessing the stability of PUFA in whole blood (Stokol and Nydam, 2005) and FTP blood (Marangoni, Colombo, and Galli, 2004; Min et al., 2011) during long-term storage are limited. Therefore, the goal of the present study is to provide a comprehensive analysis of PUFA stability in whole blood and FTP blood during long-term storage. Storage variables include temperature (room temperature (RT), 4°C, -20°C and -75°C), time (1 day to 6 months), anticoagulant

(heparin or ethylenediaminetetraacetic acid (EDTA)), antioxidant (butylated hydroxytoluene) and omega-3 (n-3) status (low and high).

MATERIALS AND METHODS

Study Design

FTP Blood Storage

One male participant provided two EDTA-lined vacutainers of whole blood (~ 16 mL total) at baseline. Following blood collection, the participant began ingesting fish oil supplements containing approximately 3g per day EPA + DHA (Ocean Nutrition Canada, Dartmouth, NS) for approximately two months at which point his n-3 PUFA whole blood levels had approximately doubled from baseline values, as determined by the % n-3 HUFA in total HUFA (% n-3 HUFA) n-3 biomarker. Following this supplementation period, another two EDTA-lined vacutainers of whole blood were drawn for analysis.

Collected whole blood samples for both low and high n-3 conditions (25 μL) were pipetted onto pre-washed (2:1 chloroform:methanol) chromatography paper strips (Grade 3MM Chr, Whatman Ltd., Sanford, ME) (approximately 1cm x 4cm) that had been pre-treated with either 0 μg or 50 μg of BHT per sample. This approach allows to control the volume of blood applied on the paper thereby providing quantitative fatty acid analyses, but still using the analytical protocol for FTP blood. We have tested this method in long-term storage relative to a standard capillary FTP sampling method, and no differences in the fatty acid profiles between the two methods were detected (data not shown), and this method of collection will henceforth be referred to as FTP blood sampling to differentiate between it and classic venous whole blood. Subsequently, samples were analyzed for fatty acid profiling immediately at baseline or after

storage for 1, 3, 7, 14, 21, 30 days and 2, 3, 4, 5 and 6 months at either RT, 4°C, or -20°C and then analyzed for fatty acid profiles (**Table 2**). Fatty acid analysis for all time points and conditions was performed in triplicate to allow for statistical analyses.

Whole Blood Storage

Venous whole blood HUFA stability during multiple storage conditions were measured in the high n-3 status blood (Table 2). Three separate collection methods were used in this case: collection with 1) an EDTA-lined vacutainer, 2) a heparin-lined vacutainer 3) a heparin-lined vacutainer with BHT addition prior to storage. The whole blood samples were separated into 500μL aliquots and stored at RT, 4°C, -20°C and -75°C for up to 6 months in cryovials. In the heparin+BHT condition, 50μg of BHT in methanol (2μg/μL) was added to the aliquot just prior to storage (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006; Otto et al., 1997). This storage condition was designed to provide maximum protection against fatty acid peroxidation with fatty acid peroxidation protection provided by both BHT and heparin (Ross, Long, and Williamson, 1992b; Ross, Long, and Williamson, 1992a).

Salmon Storage and Analysis

To assess the stability of salmon during long-term storage, 12 cans of low sodium salmon (GoldSeal® Pacific Red Sockeye Salmon, Canadian Fishing Company, Vancouver, BC) were hand-mixed and 100g were partitioned into polypropylene freezer bags. Salmon samples were then stored at either -20°C or -75°C for 0, 7, 14, 30, 60, 90 and 180 days. Upon removal from storage, salmon was blended in the presence of water for approximately 30s, or until the mixture was fully homogenized and devoid of salmon chunks. An aliquot of this salmon homogenate

was taken and approximately 150mg of the aliquot was used for extraction in the presence of 22:3n-3 ethyl ester internal standard, as described earlier by a modified Folch method.

Statistical Analyses

Baseline high n-3 whole blood and low and high n-3 FTP comparisons, and the effects of time, antioxidant/anticoagulant treatment and storage temperature were assessed individually as one-way ANOVA. Effects of time a storage temperature for salmon fatty acids were also determined by one-way ANOVA. Post Hoc tests were performed using Tukey's Honestly Significant Difference test and statistical significance was inferred as $P < 0.05$. When assessing HUFA stability as an overall measure of fatty acid degradation during storage, significance was determined by consecutive time point measures of a greater than 10% decline in baseline values and is referred to as clinically significant for the remainder of the study. The first time point of consecutive clinically significant time points are identified as the initial clinically significant time point.

RESULTS

Baseline PUFA Characteristics

Baseline PUFA compositions were determined before and after fish oil supplementation with 3 g/day of EPA (2 g/day) and DHA (1 g/day) for 2 months for both low and high n-3 status conditions (**Table 3)**. Both relative and absolute PUFA profiles demonstrate large changes in individual fatty acids following this supplementation period. Generally, relative changes in n-6 PUFA are more significant than the absolute changes with all except 18:3n-6 and 22:2n-6 displaying significant relative reductions in composition and only absolute concentrations of

22:4n-6 and 22:5n-6 increasing as a result of supplementation. Conversely, each of EPA, DPAn-3, DHA and total n-3 PUFA increase in relative composition by 773, 131, 192 and 217% and in absolute concentrations by 958, 163, 238 and 269%, respectively. In addition, n-3 blood biomarkers % EPA+DHA, % EPA+DPAn-3+DHA and % n-3 HUFA increased by 297, 230 and 215%, respectively, and the % n-6/n-3 ratio decreased by 61% following supplementation.

Omega-3 FTP Blood Biomarkers Changes During 6 Months Storage

Percent of n-3 HUFA in total HUFA

Storage of low n-3 FTP samples without BHT demonstrates minor deviations from baseline for RT and 4°C for % n-3 HUFA (**Figure 9A**), however, these changes do not persist throughout the storage period, and are not present by 180 days of storage. More significant changes in % n-3 HUFA for high n-3 FTP blood are demonstrated for the same storage temperatures (**Figure 9B**), with small decreases occurring for both 0µg and 50µg BHT pre-treatment conditions after 60 days of storage at RT and 4°C. Additionally, % n-3 HUFA in whole blood (**Figure 9C**) at both RT and 4°C storage show minor reductions from baseline throughout the entire storage period. No significant changes in % n-3 HUFA exist after 180 days at -75°C for any n-3 blood conditions.

Low n-3 FTP storage at -20°C without BHT demonstrate significant increases in % n-3 HUFA from baseline (19.0 ± 0.2) after only 14 days (31.1 ± 5.2) of storage and remains elevated until the end of the storage period (42.5 ± 11.9), and these changes disappear when in the presence of BHT. Conversely, high n-3 FTP samples demonstrate significant reductions in % n-3 HUFA at -20°C without BHT from baseline (40.8 ± 0.2) to 14 days (36.1 ± 2.0) of storage. BHT protects against this degradation by delaying degradation until 60 days of storage (37.9 ±

0.3). Whole blood storage at -20°C is more stable than FTP blood with reductions from baseline (40.5 ± 0.2) for EDTA-treated samples reaching a minimum of 36.9 ± 0.6 after 60 days storage before trending back towards baseline until the end of the storage period (38.7 ± 0.1). Heparin and heparin+BHT treated whole blood demonstrate no effects of storage at -20°C for % n-3 HUFA biomarker.

Ratio of omega-6 PUFA to omega-3 PUFA

Small but significant changes in the ratio of n-6/n-3 PUFA (**Figure 10**) occur during storage at -75°C for both low and high n-3 FTP samples. Low n-3 FTP storage with and without BHT at RT does not change the n-6/n-3 ratio significantly after 180 days. High n-3 FTP storage at RT results in a significant increase in the n-6/n-3 ratio that begins after 90 days without BHT and 60 days with BHT, and a similar response is seen at 4°C. There is, however, an immediate rise in low n-3 FTP samples at 4°C after only 1 day of storage (16.1 ± 0.2) compared to baseline (9.4 ± 0.3). Beginning at 3 days of storage, the n-6/n-3 ratio returns to values that are not significantly different than baseline. RT and 4°C storage show no significant changes between baseline and 180 days of storage for EDTA and heparin treated whole blood, and small significant increases in heparin+BHT n-6/n-3 ratio are shown.

Similar to the % n-3 HUFA in total HUFA biomarker measure, the n-6/n-3 ratio demonstrates the most significant changes during storage at -20°C. Storage of the low n-3 FTP at this temperature quickly increases from baseline (9.4 ± 0.3) after only 1 (15.3 ± 0.3) and 3 (15.2 ± 2.2) days before reverting to lower than baseline levels after 90 (4.3 ± 0.5), 120 (4.3 ± 0.8) and 150 (5.5 ± 0.6) days, and BHT stabilizes this response. Without BHT at -20°C, the high n-3 FTP responds oppositely with significant increases from baseline (3.7 ± 0.1) after 14 days

(6.2 ± 0.6) that remain elevated with BHT pretreatment protecting against this increase. Storage of whole blood at -20°C also shows a significant increase after 30 days in the n-6/n-3 ratio when whole blood is treated with EDTA (4.1 ± 0.1) and heparin (4.0 ± 0.3) compared to baseline (3.4 ± 0.1 for both). These values continue to increase and remain elevated after 180 days (5.3 ± 0.2 with EDTA and 5.7 ± 0.2 with heparin). Storage of the whole blood samples with heparin+BHT does not change the n-6/n-3 ratio during storage.

Percent Weight EPA + DHA

Storage at room temperature without BHT yields significant decreases in % EPA+DHA after 1 (1.5 ± 0.5), 60 (1.5 ± 0.5) and 150 days (1.6 ± 0.1) compared to baseline (2.0 ± 0.1) in the low n-3 FTP samples (**Figure 11A**), and BHT provides no additional protection against degradation. Similar reductions occur at RT for the high n-3 FP samples (**Figure 11B**), although degradation from baseline (6.0 ± 0.1) occurs earlier in the storage process beginning after 60 days in the absence (5.2 ± 0.2) and presence of BHT (5.1 ± 0.2). During storage at 4°C, low n-3 FTP storage without BHT shows extensive variability across time points, and as such demonstrates a significant decrease in % EPA+DHA during only 1 day of storage, and BHT protects against degradation. Storage of high n-3 FTP with (5.4 ± 0.1) or without BHT (5.2 ± 0.1) at 4°C after 60 days results in a lower % EPA+DHA that persists until the end of the storage period. When the storage temperature decreases to -20°C, both the low and high n-3 FTP conditions are significantly reduced after 1 (0.8 ± 0.1) and 3 days (5.3 ± 0.3), respectively, and continue to decrease until the 30 day of storage (0.2 ± 0.1 and 1.1 ± 0.1, respectively) where they remain. BHT delays degradation until 120 days (1.7 ± 0.1) in the low and 14 days (5.3 ± 0.1) in the high n-3 FTP samples.

At room temperature, % EPA+DHA for all three whole blood storage conditions (**Figure 11C**) decreases after a minimum of 1 day for EDTA and a maximum of 14 days for heparin, and all remain lower until the end of storage, with similar changes at 4°C. After 180 days of storage, EDTA (2.4 ± 0.1), heparin (3.6 ± 0.1) and heparin+BHT (6.6 ± 0.1) whole blood remains lower than baseline. However, the decrease in heparin+BHT represents a decline of only 6% from baseline, and significantly protects compared to no BHT storage conditions.

N-3 and N-6 PUFA Changes from Baseline During -20°C Storage

Clear effects of FTP blood and whole blood storage at -20°C are seen over a six month period, and these changes appear dependent on the n-3 biomarker measured, and can be explained by analyzing how both n-3 (**Figure 12A**) and n-6 (**Figure 12B**) PUFA changes from baseline. After 7 days at -20°C, absolute (μg/100μL) n-3 PUFA for all blood conditions are different from each other and remain so. Similarly, the percent decreases in concentration from baseline after 7 days are different from each other, however, these differences disappear for the two FTP conditions after 90 days and remain the same thereafter. Specifically, after 7 days of storage n-3 PUFA decreases from baseline by 7.0 ± 1.3, 15.8 ± 1.5 and 11.1 ± 0.1 μg/100μL for whole blood, high n-3 FTP and low n-3 FTP, respectively, and these values correspond with percent decreases of 92.3 ± 0.6 for low n-3 FTP but only 30.7 ± 5.4 for whole blood and 48.3 ± 4.5 for high n-3 FTP. Whole blood and high n-3 FTP continue to decrease in absolute terms by 18.6 ± 0.1 after 90 days and 28.6 ± 0.1 μg/100μL after 30 days, and in relative terms by 81.2 after 90 days and 87.5 ± 0.5 after 30 days, respectively. High n-3 whole blood changes from baseline are significantly less than changes in the high n-3 FTP blood in both absolute and percent changes from baseline.

A similar response is shown for n-6 PUFA in which absolute decreases from baseline are different at all time points between day 7 and day 180 of storage at -20°C, and relative decreases are different until 60 days when whole blood and high FTP n-6 PUFA become equal. After 7 days, absolute decreases of 18.9 ± 1.3 for whole blood, 35.6 ± 4.6 for high n-3 FTP and 102.3 ± 0.1 μg/100μL for low n-3 FTP are shown. These decreases correspond to percent decreases in n-6 PUFA of 90.7 ± 0.1 for low FTP, but only 24.5 ± 1.6 and 30.0 ± 3.9 for whole blood and high n-3 FTP, respectively.

HUFA Stability During 6 Months Storage

To assess the stability of FTP fatty acids profiles during long-term storage qualitative HUFA values have been assessed for both low and high FTP blood (**Table 4**) and whole blood (**Table 5**). Appreciable degradation of the HUFA composition has not been determined as a function of statistical significance, as even small reductions in composition could result in statistical significance. As such, significant HUFA losses as it pertains to the viability of stored FTP samples for accurately assessing fatty acid profiles are assumed at less than 90% of baseline composition. The first time point to reach a value of less than 90% baseline for consecutive time points will be considered unstable under the specified storage condition.

As expected, storage of both the low and high n-3 FTP samples at -75°C protects against any appreciable degradation in HUFA for the entire 6 month storage period. Storage of FTP samples at room temperature demonstrate stability for 4 months in a low n-3 blood condition, however, this stability is shortened to 2 months for high n-3 FTP stability. Pre-treatment of the chromatography paper strips with 50μg of BHT provides no additional protection against HUFA degradation. Storage at 4°C also does not improve HUFA stability for the low n-3 FTP samples,

with HUFA remaining above 90% of baseline values for only 14 days of storage. However, high n-3 FTP samples are stable for 4 months at 4°C. BHT extends low n-3 stability to 3 months and high n-3 stability for the entire 6 month storage period at 4°C. Storage of FTP samples at -20°C represents the least stable of the four storage temperatures. HUFA composition decreases to 51% of baseline after only 1 day of low n-3 FTP storage, and to 66% of baseline after 7 days of storage in high n-3 FTP samples at -20°C. BHT extends the stability of low n-3 samples from 0 days to 3 months and high n-3 samples from 3 days to only 21 days.

Whole blood samples stored at -75°C remain stable for all anticoagulant/antioxidant conditions throughout the 6 month storage period, and the addition of 50μg BHT to the heparin storage condition further stabilizes the HUFA composition for all storage temperatures. Whole blood storage at room temperature is stable for 7 days with EDTA and 1 month with heparin as anticoagulants. Storage at 4°C does not improve stability for EDTA samples compared to room temperature, although heparin sample stability is extended to 6 months at 4°C. Storage at -20°C is stable with EDTA for 14 days, and with heparin for the same 14 day period.

Salmon n-3 HUFA Stability During Long-term Storage

No significant differences exist between -20°C and -75°C storage temperatures for any major n-3 HUFA, including EPA, DPAn-3 and DHA, however, significant changes from baseline are shown at both storage temperatures (**Figure 13**). Percent weight of EPA in total fatty acids begins to decrease from 8.80 ± 0.10 at baseline to 8.00 ± 0.15 after 14 days and to a low of 7.12 ± 0.03 after 180 days of storage at -20°C. Similarly, at -75°C storage, EPA declines significantly from baseline to 8.29 ± 0.07 after only 7 days, and although fluctuations remove significance at 30 and 90 days, with values reaching a low of 7.36 ± 0.03 after 180 days storage.

DHA content decreases from 14.2 ± 0.7 at baseline to 11.4 ± 0.4 after 14 days and 12.8 ± 0.3 after 7 days at -20°C and -75°C, respectively. This decrease is maintained until the end of the 180 days storage at both temperatures reaching 9.95 ± 0.04 and 10.2 ± 0.04 for -20°C and -75°C, respectively. DPAn-3 decreases significantly from baseline (2.03 ± 0.05) at both -20°C and -75°C after 30 days (1.85 ± 0.04 and 1.89 ± 0.01, respectively) and remains until the end of the storage period.

DISCUSSION

Results of the current study indicate that FTP blood samples and whole blood samples are best preserved at -75°C for the purpose of fatty acid profiling regardless of the n-3 status of the blood samples. However, these deep freezer storage conditions are not always available to the academic or clinical researcher, and as such, other storage methods need to be tested, particularly in whole blood and FTP samples as storage stability of these blood samples have not been adequately assessed. Storage of blood samples at -20°C is the least viable temperature assessed, even compared with warmer RT and 4°C storage conditions. In fact, this -20°C degradation phenomenon has been reported previously in erythrocytes (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006; Otto et al., 1997), and although the present study demonstrates this phenomenon in only one subject, the same fatty acid degradation has been viewed in a large multi-subject pool from the Women's Health Initiative study (Pottala et al., 2012). Interestingly, these differences in HUFA stability during storage of whole blood are not seen when storing salmon samples under the same temperature conditions. Both -20°C and -75°C salmon storage reveal no differences between them, although significant reductions in relative amounts of n-3 HUFA do occur for both temperatures over time. In addition, the n-3

profile of the stored blood sample plays a role in the n-3 biomarker stability during storage as the % n-3 HUFA increases during -20°C storage in low n-3 FTP blood and decreases in high n-3 FTP samples, and vice versa for the n-6/n-3 ratio.

The increase in % n-3 HUFA and decrease in n-6/n-3 ratio in the low n-3 FTP samples at -20°C suggests higher relative degradation of n-6 PUFA/HUFA compared to n-3 PUFA/HUFA. This is contrary to what is known about rates of fatty acid degradation, as n-3 fatty acids are reported to peroxidize at a faster rate than n-6 fatty acids due to the generally higher degree of unsaturation in n-3 fatty acids (Halliwell and Chirico, 1993). Peroxidation rates for various PUFA have been determined at 62 $M^{-1}s^{-1}$ for 18:2n-6, 115 $M^{-1}s^{-1}$ for 18:3n-3, 197 $M^{-1}s^{-1}$ for 20:4n-6, 249 $M^{-1}s^{-1}$ for 20:5n-3 and 334 $M^{-1}s^{-1}$ for 22:6n-3 (Howard and Ingold, 1967; Xu, Davis, and Porter, 2009). Upon closer inspection of our n-3 biomarker responses, these peroxidation rates may in fact hold true in the current study. Initially, in low n-3 FTP blood % n-3 HUFA decreases (Figure 9A) after the first day of storage and n-6/n-3 ratio (Figure 10A) increases after 1 and 3 days of storage. Both of these changes indicate an initial and fast peroxidation of n-3 PUFA in the blood samples. After 1 day of storage, the n-3 PUFA have decreased from baseline by more than 67% and n-6 PUFA by only 47%, and after 3 days a reduction of 87% in n-3 PUFA and 79% in n-6 PUFA (Figure 12). At baseline (Table 3), n-3 PUFA represents 10.1% of the total PUFA profile and after 1, 3 and 7 days of degradation, n-3 PUFA represents 6.1, 6.2 and 8.2% of total PUFA, respectively. At subsequent time points between day 14 and 180, n-3 PUFA represents an average of 14.0% of the total PUFA remaining in the sample, and is indicative of earlier and faster n-3 PUFA degradation that over time is matched and surpassed proportionally by n-6 PUFA. This PUFA peroxidation pattern explains

the response of the % n-3 HUFA and n-6/n3 ratio responses during low n-3 FTP storage at -20°C.

The same may not be said for the response of these n-3 biomarkers of high n-3 FTP samples during -20°C as % n-3 HUFA decreases slightly and the n-6/n-3 ratio increases, the opposite of what is shown in low n-3 FTP samples. This response can be attributed to the much higher initial n-3 PUFA at baseline in the high n-3 FTP condition. With storage conditions between the n-3 FTP conditions being equal, the overall capacity for fatty acid peroxidation is unchanged; however, it appears peroxidation has now shifted to the relatively more abundant and more peroxidizable n-3 PUFA. Beginning after only 3 days of storage, n-3 PUFA (-11%) demonstrate larger relative changes from baseline compared to n-6 PUFA (-4%) (Figure 12). Unlike the low n-3 FTP storage response, n-6 PUFA degradation does not reach the same absolute losses, despite similar absolute baseline n-6 PUFA values. This may be attributed to the preferential peroxidation of the more abundant n-3 PUFA before peroxidation shifts to the less susceptible n-6 PUFA, and as a result the 6 month storage period may not be long enough to fully degrade the n-6 PUFA pool. Alternatively, the reduction in n-6 PUFA peroxidation may be attributed to the saturation of the fatty acid peroxidation process, as this process can stop and shift to protein peroxidation as the protein:lipid ratio increases (Halliwell and Chirico, 1993).

High n-3 whole blood samples demonstrate a similar response to high n-3 FTP samples when stored at -20°C. The whole blood response, however, is a slower more linear response compared to the fast high n-3 FTP response that plateaus after only a couple weeks of storage. This is particularly true for the % n-3 HUFA and n-6/n-3 ratio biomarkers (Figures 9C and 10C). This slower response in whole blood is due to the fact that the n-3 PUFA do not demonstrate the same rapid decline relative to n-6 PUFA as shown in the FTP samples. Both n-3 and n-6 PUFA

(Figure 12A and B) decline at a slower rate, although the relative decline from baseline is similar in high n-3 whole blood and FTP as the storage periods nears its end. Whole blood samples stored in our study were stored in 500μL aliquots, and therefore contain 20 times more blood than FTP samples of 25μL. This may explain why peroxidation in the whole blood samples occurs at such a slower rate. The larger volume-to-surface ratio of whole blood compared to FTP indicates that even though whole blood surface exposure may be larger, whole blood still contains significant blood volume that is not in contact with air and may take longer to peroxidize (Pottala et al., 2012). Therefore, although absolute peroxidation levels in whole blood may be higher, relative peroxidation levels would be much smaller as FTP samples are almost entirely surface area with no volume

The stability of fatty acids during storage is important to be aware of and while storage of blood samples at -75°C is ideal, access to such deep freezers may not always be feasible. Studies on the storage stability of whole blood and FTP blood are limited. Previously, only one study has assessed whole blood PUFA stability (Stokol and Nydam, 2005), and determined whole blood non-esterified fatty acid stability of less than 24 hours, 24 hours and at least one month at 24°C, 4°C and -40°C, respectively. Because our samples were assessed in the total lipid extract, the results are difficult to compare to this prior study. However, an attempt was made to determine which lipid fractions were most susceptible to peroxidation at -20°C (data not shown), and the only fraction that did not change significantly after 6 months of storage was the NEFA fraction. FTP stability has been assessed twice previously (Marangoni, Colombo, and Galli, 2004; Min et al., 2011), and in each case BHT was used as an antioxidant for the prevention of fatty acid peroxidation. It has been determined that BHT-treated FTP samples

remain stable for less than two months at RT, and longer than 3 weeks and less than 3 months at 4°C. These determinations are in relative agreement with our findings.

To assess minimum stability of FTP and whole blood fatty acid profiles we assessed the most oxidation-vulnerable fatty acid subclass, HUFA. Due to the high unsaturation level of HUFA they will be the first fatty acid class to peroxide during storage, and decreases of at least 10% from baseline levels for two consecutive weeks has been set as our point of instability. By assessing both low and high n-3 FTP samples we have also been able to account for a range of n-3 blood compositions. Briefly, we have determined the stability of non-BHT treated FTP sample stability to be 2 months, 14 days, 0 days and at least 6 months for RT, 4°C, -20°C and -75°C, respectively. BHT-treated FTP samples are stable for 2 months, 3 months, 3 days and 6 months at RT, 4°C, -20°C and -75°C, respectively. Interestingly, the addition of BHT to FTP samples does not extend HUFA stability at RT. Whole blood samples appear to be considerably more stable when stored in 500μL aliquots. Whole blood stability is determined to be only 7 days at RT, 14 days at 4°C and -20°C when EDTA-treated. Stability is extended to 1 month and 6 months at RT and 4°C, and remains 14 days at -20°C for heparin-treated whole blood. Adding 50μg BHT to heparin-treated samples extends PUFA stability to 6 months for all storage temperatures.

The present study determined further that while baseline composition values are similar between FTP blood and whole blood (Table 3), the average HUFA composition across all time points is higher in the high n-3 status blood samples compared to low n-3 status during storage at 4°C (18.3 ± 1.4 vs. 15.8 ± 1.8), -20°C (7.53 ± 5.81 vs. 3.10 ± 3.87) and -75°C (19.2 ± 0.7 vs. 18.0 ± 0.7). These differences appear to be due to a slower rate of HUFA degradation in the high n-3 status blood samples over time despite a higher unsaturation level with a greater

incorporation of n-3 HUFA into blood. Conventional wisdom would suggest that an increase unsaturation of whole blood samples would result in the same blood sample being more susceptible to lipid peroxidation, however, this is not what our results suggest. In support of these findings, osmotic fragility, a marker of hemolytic susceptibility, has been positively associated with erythrocyte n-6 PUFA content (Ney et al., 2009). In addition, humans (Hagve, Lie, and Gronn, 1993) and rats (Hagve, Johansen, and Christophersen, 1991) receiving diets high in n-3 PUFA content demonstrate a decrease in osmotic fragility. These changes suggest a more stable erythrocyte membrane that is less susceptible to hemolysis as a result of fish oil supplementation, and this membrane instability may provide a possible mechanism for the accelerated HUFA degradation that occurs during -20°C storage as hemolysis can release Fe^{2+} from the cell to initiate peroxidation reactions. Alternatively, the generally high levels of vitamin A, D and E in fish oils(Health Canada, 2012) may also play a significant role in the prevention of HUFA degradation in blood samples following fish oil supplementation. In fact, vitamin E supplementation in pigs (Cardenia et al., 2011) is shown to improve oxidative stability of fatty acids in pork meat during storage.

In addition to whole blood and FTP blood stability, we assessed the stability of salmon stored at -20°C and -75°C over the same 6 month period. Opposite to what is shown in whole blood and FTP blood samples, salmon samples demonstrate a similar HUFA degradation response during storage at -20°C compared to -75°C. These results are in general agreement with previous findings determining that significant lipid peroxidation occurs as determined by the peroxide value in rainbow trout (Baron et al., 2007) at -20°C after 8 months but not 4 months, and by TBARS measurement in Chinook salmon after approximately 3 months (Cook et al., 2009). However, significant increases in peroxide value were not determined when storing

farmed salmon for up to 6 months at -20°C, although significant increases were shown at -10°C in approximately 3 months (Refsgaard, Brockhoff, and Jensen, 2000). These studies are generally more interested in the development of 'off-flavours' that can accompany frozen fish storage over time, and peroxide value is noted as one measure that can best describe this sensory response (Refsgaard, Brockhoff, and Jensen, 2000). As such, none of these studies determined the fatty acid profile changes in the fish samples, and decreases in PUFA content may not be adequately reflected by peroxide value or TBARS analysis.

CONCLUSION

When storing whole blood or FTP samples for up to 6 months, samples should be stored at -75°C whenever possible, and conversely, -20°C storage should be avoided particularly in the absence of an antioxidant such as BHT. In addition, the specific fatty acid or biomarker of interest, and if known the n-3 status of the individual should be accounted for when choosing an appropriate storage condition. For instance, if the entire fatty acid profile is required FTP blood may be stored in a refrigerator for only 14 days, however, if only the n-3 status of the sample is required then storage can occur under the same conditions for up to 6 months without any clinically significant changes in % n-3 HUFA. Although, whole blood samples are generally more stable at 4°C than -20°C, instability can be prevented when stored with BHT as -20°C blood sample storage may be required for the determination of non-lipid analytes. The presence of significantly greater fatty acid degradation at -20°C in blood but not in salmon also suggests a mechanism that is inherent to the blood samples themselves. Evidence presented here suggests an Fe^{2+}-mediated peroxidation mechanism may play a role, however, the present study is limited to a single participant and peroxidation rates may vary between blood samples, and may be

dependent on the presence of naturally occurring pro and antioxidant substances in the blood. Further investigation is needed to elucidate this possibility, and to better understand treatments required to maximize fatty acid stability during long-term storage.

Table 2 –Summary of Fingertip Prick and Whole Blood Storage Conditions

			Storage Time										
N-3 Status	**BHT (µg)**	**Storage Temp**	**0 d**	**1 d**	**3 d**	**7 d**	**14 d**	**21 d**	**28 d**	**2 m**	**3 m**	**4 m**	**5 m**	**6 m**
Low	0	20°C (RT)	X	X	X	X	X	X	X	X	X	X	X	X
		4°C (fridge)		X	X	X	X	X	X	X	X	X	X	X
		-20°C (freezer)		X	X	X	X	X	X	X	X	X	X	X
		-80°C (deep freezer)				X	X		X	X	X	X	X	X
	50	20°C (RT)	X	X	X	X	X	X	X	X	X	X	X	X
		4°C (fridge)		X	X	X	X	X	X	X	X	X	X	X
		-20°C (freezer)		X	X	X	X	X	X	X	X	X	X	X
		-80°C (deep freezer)				X	X		X	X	X			X
High	0	20°C (RT)	X	X	X	X	X	X	X	X	X	X	X	X
		4°C (fridge)		X	X	X	X	X	X	X	X	X	X	X
		-20°C (freezer)		X	X	X	X	X	X	X	X	X	X	X
		-80°C (deep freezer)				X	X		X	X	X	X	X	X
	50	20°C (RT)	X	X	X	X	X	X	X	X	X	X	X	X
		4°C (fridge)		X	X	X	X	X	X	X	X	X	X	X
		-20°C (freezer)		X	X	X	X	X	X	X	X	X	X	X
		-80°C (deep freezer)				X	X		X	X	X			X

Whole Blood PUFA Stability			Storage Time										
N-3 Status	**Anti-coagulant/oxidant**	**Storage Temp**	**0 d**	**1 d**	**3 d**	**7 d**	**14 d**	**21 d**	**28 d**	**2 m**	**3 m**	**4 m**	**5 m**	**6 m**
High	EDTA	20°C (RT)	X	X	X	X	X	X	X	X				
		4°C (fridge)		X	X	X	X	X	X	X	X			X
		-20°C (freezer)				X	X		X	X	X			X
		-80°C (deep freezer)				X	X		X	X	X			X
	Heparin	20°C (RT)	X	X	X	X	X	X	X	X				
		4°C (fridge)		X	X	X	X	X	X	X	X			X
		-20°C (freezer)				X	X		X	X	X			X
		-80°C (deep freezer)				X	X		X	X	X			X
	Heparin + BHT	20°C (RT)	X	X	X	X	X	X	X	X				
		4°C (fridge)		X	X	X	X	X	X	X	X			X
		-20°C (freezer)				X	X		X	X	X			X
		-80°C (deep freezer)				X	X		X	X	X			X

EDTA, ethylenediaminetetraacetic acid; RT, room temperature; d, days

Table 3 – Fatty Acid Profiles in Fingertip Prick Whole Blood Pre- and Post-Supplementation of Omega-3 Fish Oil Capsules

	% weight fatty acid in total fatty acids (%)			Concentration (μg/100μL)		
Fatty Acid	**Low N-3 FTP**	**High N-3 FTP**	**High N-3 WB**	**Low N-3 FTP**	**High N-3 FTP**	**High N-3 WB**
C 14:0	0.43 ± 0.04^{a}	0.76 ± 0.01^{b}	0.55 ± 0.04^{c}	1.36 ± 0.18^{a}	2.99 ± 0.12^{b}	1.32 ± 0.09^{a}
C 16:0	17.9 ± 0.3^{a}	20.4 ± 0.2^{b}	20.4 ± 0.4^{b}	56.6 ± 5.3^{a}	80.8 ± 4.0^{b}	49.1 ± 1.3^{a}
C 17:0	0.26 ± 0.02^{a}	0.35 ± 0.01^{b}	0.32 ± 0.01^{b}	0.83 ± 0.11^{a}	1.37 ± 0.03^{b}	0.78 ± 0.02^{a}
C 18:0	11.4 ± 0.2	12.1 ± 0.7	11.3 ± 0.4	36.1 ± 3.8^{a}	47.8 ± 2.0^{b}	27.2 ± 0.4^{c}
C 20:0	0.43 ± 0.12	0.36 ± 0.02	0.25 ± 0.02	1.35 ± 0.37^{a}	1.42 ± 0.10^{a}	0.61 ± 0.03^{b}
C 22:0	1.06 ± 0.06^{a}	1.00 ± 0.05^{a}	0.70 ± 0.06^{b}	3.35 ± 0.14^{a}	3.96 ± 0.36^{b}	1.69 ± 0.09^{c}
C 23:0	0.26 ± 0.05	0.28 ± 0.03	0.20 ± 0.02	0.82 ± 0.08^{a}	1.09 ± 0.08^{b}	0.48 ± 0.04^{c}
C 24:0	2.19 ± 0.12^{a}	1.99 ± 0.06^{a}	1.54 ± 0.17^{b}	6.92 ± 0.43^{a}	7.88 ± 0.55^{a}	3.71 ± 0.28^{b}
SFA	**37.0 ± 0.3**	**38.6 ± 0.7**	**38.2 ± 1.2**	**117 ± 11**^{a}	**152 ± 5**^{b}	**91.9 ± 1.6**^{c}
C 16:1	0.57 ± 0.02^{a}	0.71 ± 0.01^{b}	0.55 ± 0.04^{a}	1.79 ± 0.21^{a}	2.81 ± 0.15^{b}	1.32 ± 0.16^{c}
C 18:1n-7	1.70 ± 0.06^{a}	1.51 ± 0.01^{b}	1.59 ± 0.02^{b}	5.39 ± 0.57^{a}	5.98 ± 0.20^{a}	3.82 ± 0.17^{b}
C 18:1n-9	15.2 ± 0.3^{a}	15.0 ± 0.1^{a}	13.9 ± 0.4^{b}	48.1 ± 4.4^{a}	59.2 ± 2.6^{b}	33.5 ± 2.3^{c}
C 20:1n-9	0.22 ± 0.01^{a}	0.19 ± 0.01^{b}	0.21 ± 0.01^{ab}	0.71 ± 0.04^{a}	0.75 ± 0.07^{a}	0.50 ± 0.02^{b}
C 22:1n-9	0.21 ± 0.02^{a}	0.43 ± 0.02^{b}	0.17 ± 0.05^{a}	0.66 ± 0.06^{a}	1.68 ± 0.01^{b}	0.41 ± 0.12^{c}
C 24:1n-9	2.25 ± 0.09^{a}	2.11 ± 0.09^{a}	1.58 ± 0.18^{b}	7.11 ± 0.42^{a}	8.33 ± 0.67^{b}	3.80 ± 0.28^{c}
MUFA	**20.7 ± 0.4**^{a}	**20.1 ± 0.2**^{a}	**18.5 ± 0.2**^{b}	**65.5 ± 5.6**^{a}	**79.6 ± 3.7**^{b}	**44.5 ± 2.3**^{c}
C 18:2n-6	20.5 ± 0.4^{a}	18.2 ± 0.3^{b}	18.2 ± 1.2^{b}	64.8 ± 4.3	71.9 ± 3.7^{a}	43.9 ± 4.6^{b}
C 18:3n-6	0.17 ± 0.10	0.22 ± 0.01	0.15 ± 0.02	0.52 ± 0.30^{ab}	0.88 ± 0.04^{a}	0.36 ± 0.06^{b}
C 20:2n-6	0.26 ± 0.02^{a}	0.19 ± 0.01^{b}	0.21 ± 0.01^{b}	0.81 ± 0.06^{a}	0.75 ± 0.04^{a}	0.51 ± 0.01^{b}
C 20:3n-6	1.57 ± 0.05^{a}	1.17 ± 0.02^{b}	1.30 ± 0.03^{c}	4.97 ± 0.5^{a}	4.62 ± 0.15^{a}	3.13 ± 0.10^{b}
C 20:4n-6	11.0 ± 0.3^{a}	8.99 ± 0.17^{b}	10.6 ± 0.1^{a}	34.8 ± 3.1^{a}	35.6 ± 2.2^{a}	25.6 ± 0.8^{b}
C 22:4n-6	1.70 ± 0.10^{a}	1.01 ± 0.02^{b}	1.28 ± 0.04^{c}	5.38 ± 0.58^{a}	4.00 ± 0.19^{b}	3.06 ± 0.03^{c}
C 22:5n-6	0.37 ± 0.03^{a}	0.23 ± 0.02^{b}	0.26 ± 0.01^{b}	1.15 ± 0.01^{a}	0.89 ± 0.10^{b}	0.63 ± 0.03^{c}
N-6 PUFA	**35.6 ± 0.9**^{a}	**30.1 ± 0.4**^{b}	**32.1 ± 1.0**^{b}	**113 ± 8**^{a}	**119 ± 6**^{a}	**77.4 ± 5.6**^{b}
C 18:3n-3	0.37 ± 0.02^{ab}	0.41 ± 0.01^{a}	0.34 ± 0.02^{b}	1.18 ± 0.05^{a}	1.60 ± 0.10^{b}	0.82 ± 0.08^{c}
C 20:3n-3	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.14 ± 0.04	0.21 ± 0.06	0.11 ± 0.01
C 20:5n-3	0.37 ± 0.02^{a}	2.86 ± 0.06^{b}	3.07 ± 0.07^{c}	1.18 ± 0.11^{a}	11.3 ± 0.6^{b}	7.40 ± 0.47^{c}
C 22:5n-3	1.37 ± 0.05^{a}	1.79 ± 0.02^{b}	2.13 ± 0.05^{c}	4.34 ± 0.25^{a}	7.07 ± 0.38^{b}	5.13 ± 0.12^{c}
C 22:6n-3	1.64 ± 0.11^{a}	3.15 ± 0.03^{b}	3.92 ± 0.09^{c}	5.22 ± 0.65^{a}	12.4 ± 0.6^{b}	9.44 ± 0.23^{c}
N-3 PUFA	**3.81 ± 0.17**^{a}	**8.25 ± 0.13**^{b}	**9.51 ± 0.05**^{c}	**12.1 ± 1.1**^{a}	**32.6 ± 1.8**^{b}	**22.9 ± 0.8**^{c}
PUFA	**39.4 ± 1.0**^{ab}	**38.3 ± 0.5**^{a}	**41.6 ± 1.0**^{b}	**125 ± 9**^{a}	**152 ± 8**^{b}	**100 ± 6**^{c}
HUFA	**18.1 ± 0.7**^{a}	**19.2 ± 0.3**^{b}	**22.6 ± 0.2**^{c}	**57.2 ± 5.2**^{a}	**76.1 ± 4.2**^{b}	**54.5 ± 1.7**^{a}
EPA+DHA	**2.02 ± 0.13**^{a}	**6.00 ± 0.09**^{b}	**6.99 ± 0.02**^{c}	**6.40 ± 0.75**^{a}	**23.7 ± 1.3**^{b}	**16.8 ± 0.6**^{c}
N-6/N-3	**9.35 ± 0.25**^{a}	**3.65 ± 0.01**^{b}	**3.38 ± 0.12**^{b}	**9.35 ± 0.25**^{a}	**3.65 ± 0.01**^{b}	**3.38 ± 0.12**^{b}
% n-3 HUFA	**19.0 ± 0.2**^{a}	**40.8 ± 0.2**^{b}	**40.5 ± 0.2**^{b}	**19.0 ± 0.2**^{a}	**40.8 ± 0.2** ^{b}	**40.5 ± 0.2**^{b}
Total	**97.0 ± 1.5**	**97.1 ± 0.1**	**98.2 ± 0.1**	**307 ± 25**^{a}	**384 ± 16**^{b}	**237 ± 10**^{c}

Different letters within fatty acid values indicates significant differences between blood fractions as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid

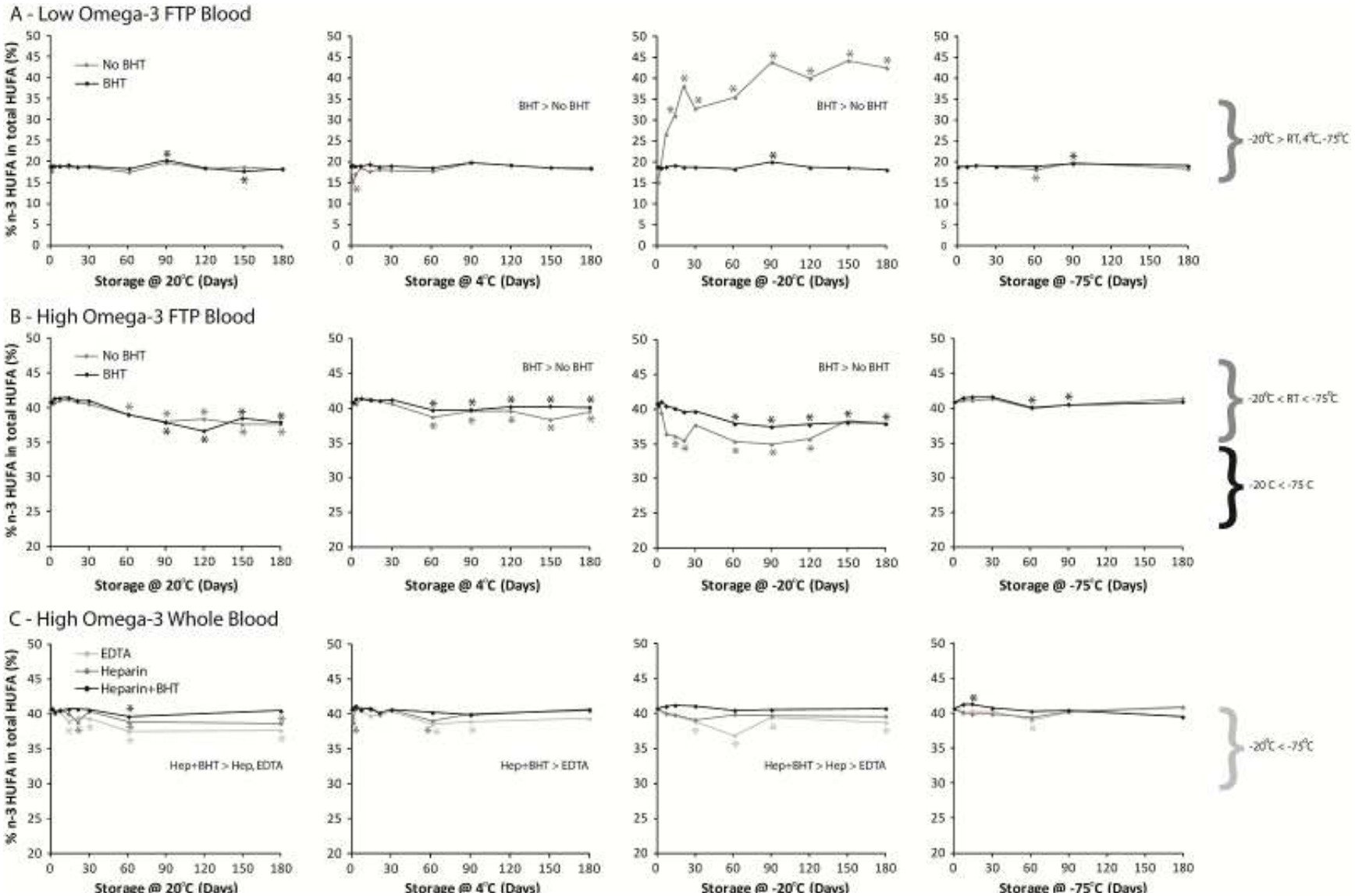


Figure 9 – Percent n-3 HUFA in total HUFA response during long-term blood storage. A,B – fingertip prick blood and, C – whole blood. *Indicates qualitative fatty acid values significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Effect of antioxidant/anticoagulant and storage temperature is also indicated under the same statistical analyses model. Data presented as means. HUFA, highly unsaturated fatty acid; FTP, fingertip prick; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; RT, room temperature.

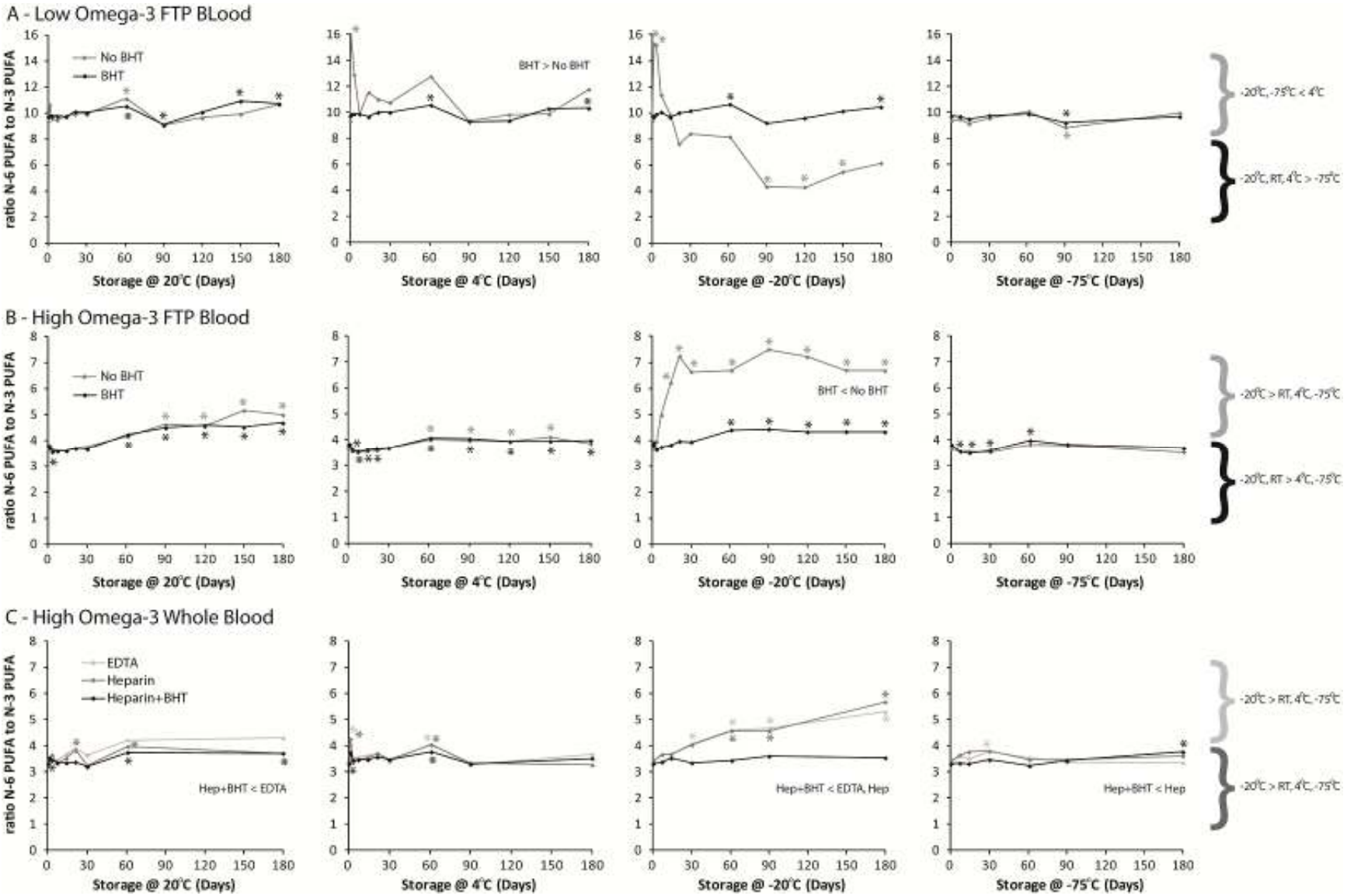


Figure 10 – Ratio of n-6 PUFA to n-3 PUFA response during long-term blood storage. A,B – fingertip prick blood and, C – whole blood. *Indicates qualitative fatty acid values significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Effect of antioxidant/anticoagulant and storage temperature is also indicated under the same statistical analyses model. Data presented as means. PUFA, polyunsaturated fatty acid; FTP, fingertip prick; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; RT, room temperature.

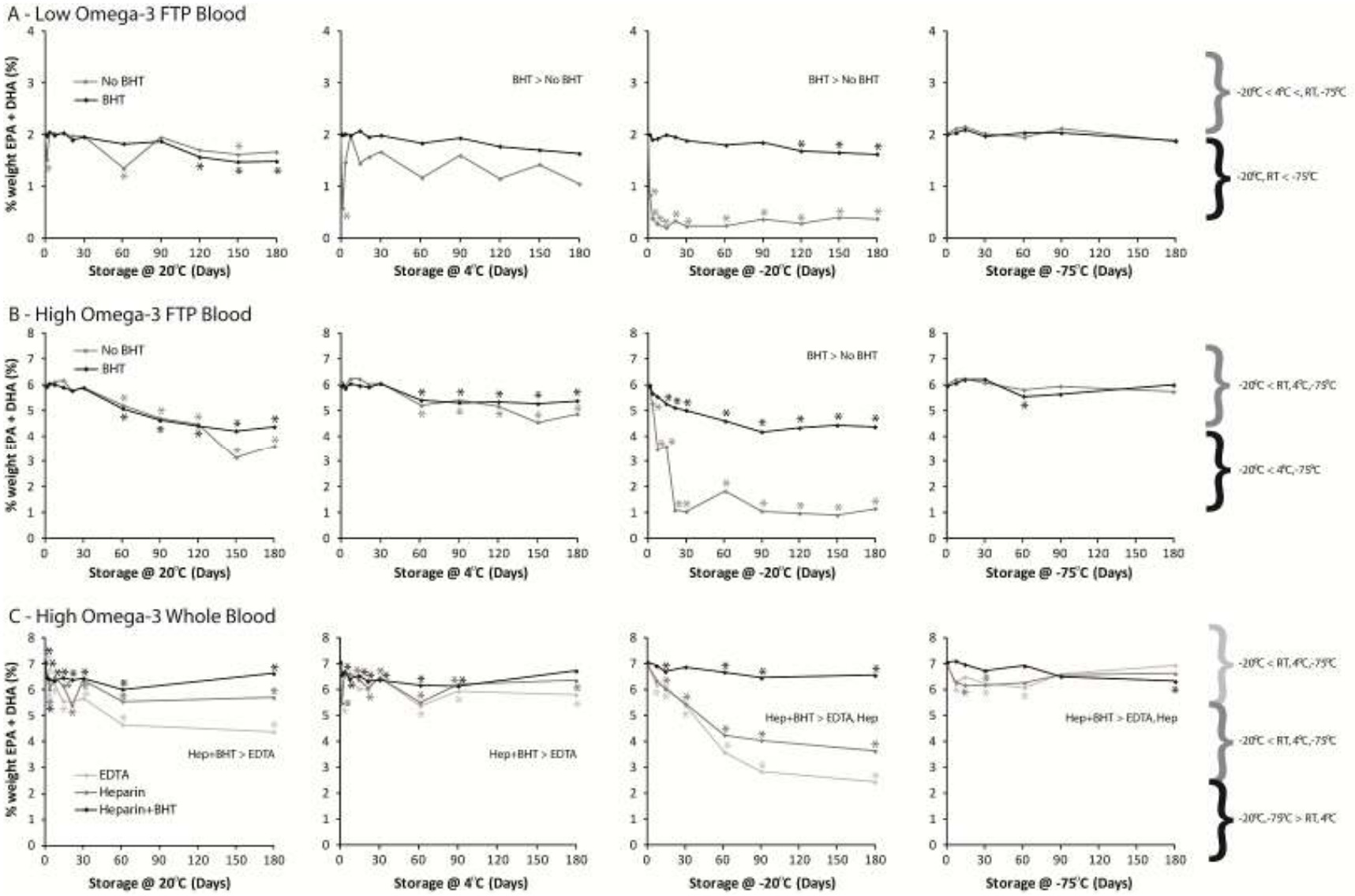


Figure 11 – Percent weight EPA + DHA response during long-term blood storage. A,B – fingertip prick blood and, C – whole blood. *Indicates qualitative fatty acid values significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Effect of antioxidant/anticoagulant and storage temperature is also indicated under the same statistical analyses model. Data presented as means. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; FTP, fingertip prick; EDTA, ethylenediaminetetraacetic acid; BHT, butylated hydroxytoluene; RT, room temperature.

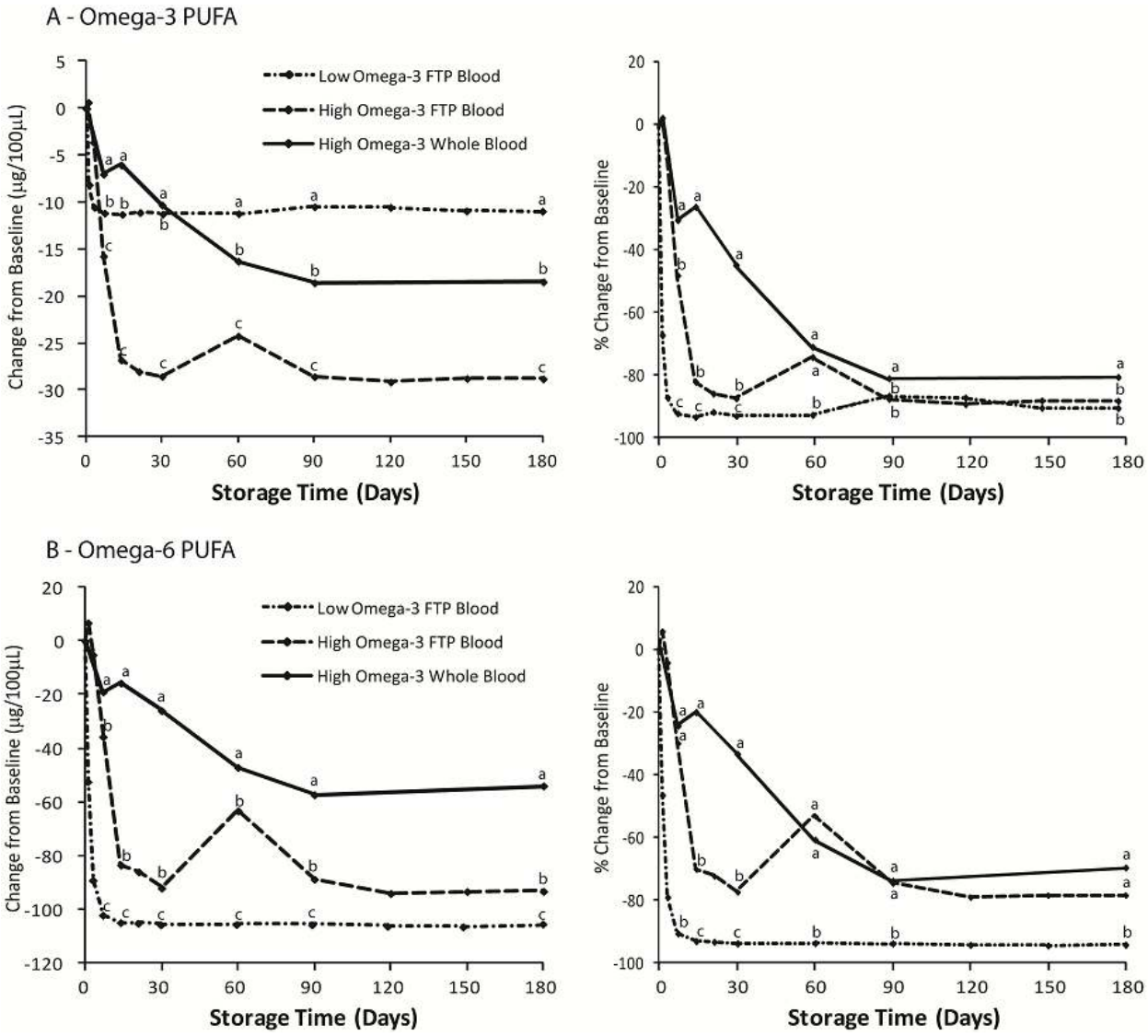


Figure 12 – N-3 and n-6 PUFA changes from baseline during long-term blood storage at -20°C. A – omega-3 and B – omega-6 PUFA. Different letters within a single time point indicates fatty acid values different between blood fraction as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. PUFA, polyunsaturated fatty acids; FTP, fingertip prick.

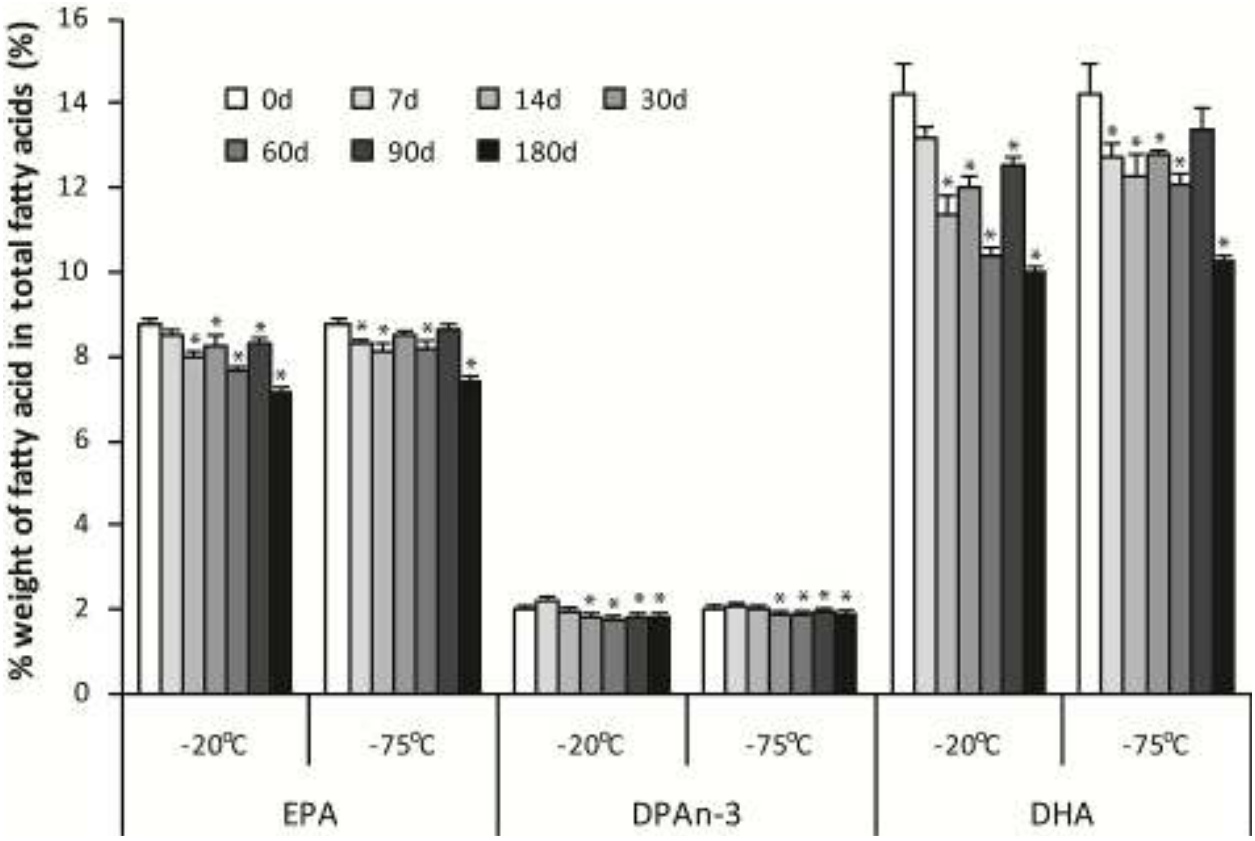


Figure 13 – N-3 HUFA changes in canned salmon during long-term storage. *Indicates qualitative fatty acid values significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. EPA, eicosapentaenoic acid; DPAn-3, omega-3 docosapentaenoic acid.

Table 4 – Stability of HUFA During Storage of FTP Blood at Various Temperatures for 6 Months

Low Omega-3 FTP Blood (weight % HUFA in total FA)													
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
None	20°C	18.1 ± 0.7	16.7 ± 0.6	18.6 ± 0.1	18.5 ± 0.8	18.4 ± 0.1	18.1 ± 0.1	17.7 ± 0.1	17.3 ± 0.1	17.1 ± 0.1	16.6 ± 0.1	15.2 ± 0.3*	15.9 ± 0.2*
	4°C		-	18.2 ± 0.1	18.0 ± 0.4	16.4 ± 0.1	14.7 ± 2.5*	15.7 ± 0.6*	15.8 ± 0.3*	14.8 ± 0.1*	14.2 ± 0.1*	13.6 ± 0.5*	14.0 ± 0.2*
	-20°C		9.2 ± 0.2*	3.9 ± 0.4*	1.9 ± 0.1*	1.4 ± 0.2*	1.4 ± 0.2*	1.1 ± 0.2*	1.1 ± 0.1*	1.5 ± 0.1*	1.4 ± 0.2*	1.3 ± 0.1*	1.1 ± 0.2*
	-75°C		-	-	18.7 ± 0.3	19.0 ± 0.4	-	17.9 ± 0.2	17.8 ± 0.2	18.1 ± 0.2	-	-	17.3 ± 0.3
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
50µg BHT	20°C	17.9 ± 0.8	17.7 ± 0.2	18.3 ± 0.1	17.8 ± 0.3	18.1 ± 0.4	17.3 ± 0.7	17.6 ± 0.1	17.0 ± 0.3	16.2 ± 0.5	14.8 ± 1.4*	14.6 ± 0.7*	14.0 ± 0.2*
	4°C		17.6 ± 0.4	18.0 ± 0.6	17.5 ± 0.1	18.2 ± 0.1	17.6 ± 1.0	17.7 ± 0.3	16.9 ± 0.1	17.1 ± 0.7	16.0 ± 0.7*	16.0 ± 0.8*	15.3 ± 0.8*
	-20°C		17.9 ± 0.4	17.4 ± 0.8	17.4 ± 0.5	17.7 ± 0.5	17.8 ± 0.1	17.2 ± 0.2	16.8 ± 0.3	16.3 ± 0.3	15.9 ± 0.5*	15.7 ± 0.5*	15.6 ± 0.4*
	-75°C		-	-	18.2 ± 0.2	18.6 ± 0.3	-	17.8 ± 0.2	18.1 ± 0.4	17.5 ± 0.4	-	-	16.6 ± 0.2
High Omega-3 FTP Blood (weight % HUFA in total FA)													
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
None	20°C	19.2 ± 0.3	19.1 ± 0.4	19.2 ± 0.2	19.6 ± 0.3	19.7 ± 0.5	18.5 ± 0.3	19.0 ± 0.6	17.3 ± 0.3	16.1 ± 0.5*	15.5 ± 0.4*	11.4 ± 0.8*	12.8 ± 1.7*
	4°C		19.4 ± 0.2	19.0 ± 0.3	19.7 ± 0.2	19.8 ± 0.2	19.1 ± 0.3	19.5 ± 0.2	17.6 ± 0.3	17.7 ± 0.4	17.3 ± 0.8	16.0 ± 0.3*	16.3 ± 1.1*
	-20°C		19.0 ± 0.3	17.5 ± 0.7	12.6 ± 0.4*	4.6 ± 0.4*	3.9 ± 0.4*	3.8 ± 0.2*	6.6 ± 0.7*	3.8 ± 0.3*	3.6 ± 0.2*	3.4 ± 0.2*	3.9 ± 0.8*
	-75°C		-	-	19.7 ± 0.3	19.9 ± 0.1	-	19.5 ± 0.3	18.7 ± 0.3	19.1 ± 0.2	-	-	18.3 ± 1.6
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
50µg BHT	20°C	18.9 ± 0.4	18.9 ± 0.3	18.9 ± 0.2	19.1 ± 0.2	18.6 ± 0.3	18.6 ± 0.4	18.9 ± 0.6	16.9 ± 0.4*	16.0 ± 0.5*	15.7 ± 0.7*	14.8 ± 0.1*	15.3 ± 0.4*
	4°C		19.0 ± 0.2	18.5 ± 0.3	19.2 ± 0.2	19.0 ± 0.1	18.7 ± 0.4	19.1 ± 0.3	17.6 ± 0.4	17.4 ± 0.2	17.3 ± 0.2	17.4 ± 0.2	17.5 ± 0.3
	-20°C		19.1 ± 0.1	18.1 ± 0.5	18.0 ± 0.4	17.6 ± 0.1	17.3 ± 0.7	17.2 ± 0.5*	16.2 ± 0.1*	15.2 ± 0.9*	15.6 ± 0.1*	16.1 ± 0.7*	15.7 ± 0.3*
	-75°C		-	-	19.2 ± 0.5	19.7 ± 0.5	-	19.5 ± 0.5	17.9 ± 0.2	18.2 ± 0.7	-	-	19.4 ± 0.3

*Indicates qualitative fatty acid values that have decreased to less than 90% of baseline values. Data presented as mean ± SD. HUFA, highly unsaturated fatty acids; FA, fatty acids; BHT, butylated hydroxytoluene; FTP, fingertip prick; FA, fatty acid; d, days of storage.

Table 5 – Stability of HUFA During Whole Blood Storage for 6 Months

High Omega-3 Whole Blood												
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
EDTA	20°C	22.6 ± 0.2	21.1 ± 0.2	20.5 ± 0.8	20.6 ± 0.4	19.0 ± 0.3*	18.4 ± 0.4*	19.0 ± 0.2*	16.6 ± 0.2*	-	-	-	15.4 ± 0.1*
	4°C		19.3 ± 0.7	21.0 ± 0.2	20.8 ± 0.4	20.1 ± 0.3*	19.8 ± 1.6*	21.1 ± 0.3*	18.6 ± 0.2*	20.4 ± 0.4*	-	-	19.7 ± 0.7*
	-20°C		20.4 ± 1.0	-	-	20.3 ± 0.5	-	18.4 ± 0.4*	12.4 ± 0.1*	8.9 ± 0.2*	-	-	7.7 ± 0.2*
	-75°C		20.6 ± 0.8	-	-	21.2 ± 0.2	-	20.3 ± 0.6	20.6 ± 0.5	21.4 ± 0.4	-	-	22.3 ± 0.3
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
Heparin	20°C	22.5 ± 0.2	21.0 ± 0.1	19.7 ± 0.3	21.1 ± 0.2	20.5 ± 0.8	18.4 ± 0.9	20.9 ± 0.2	18.8 ± 0.4*	-	-	-	19.4 ± 0.3*
	4°C		18.4 ± 0.1	21.5 ± 0.3	21.2 ± 0.2	20.6 ± 0.3	19.8 ± 1.0	20.9 ± 0.5	18.6 ± 0.2	20.5 ± 0.4	-	-	20.9 ± 0.5
	-20°C		20.8 ± 0.7	-	-	20.0 ± 0.3	-	18.3 ± 1.2*	13.4 ± 0.3*	12.5 ± 0.1*	-	-	10.9 ± 0.3*
	-75°C		20.7 ± 0.3	-	-	20.5 ± 1.1	-	20.3 ± 0.8	21.1 ± 0.8	21.2 ± 0.7	-	-	21.0 ± 0.9
Treatment	**Temp**	**0d**	**1d**	**3d**	**7d**	**14d**	**21d**	**30d**	**60d**	**90d**	**120d**	**150d**	**180d**
Heparin+BHT	20°C	22.7 ± 0.3	20.5 ± 0.3	20.7 ± 0.3	20.8 ± 0.2	20.8 ± 0.1	20.5 ± 0.1	21.0 ± 0.3	19.9 ± 0.3	-	-	-	21.3 ± 0.1
	4°C		20.8 ± 0.1	21.3 ± 0.4	20.9 ± 0.4	20.9 ± 0.4	20.6 ± 0.5	20.6 ± 0.4	19.9 ± 0.2	20.4 ± 0.1	-	-	21.6 ± 0.1
	-20°C		22.1 ± 0.5	-	-	21.3 ± 0.3	-	21.9 ± 0.4	21.6 ± 0.1	20.6 ± 0.6	-	-	21.1 ± 0.2
	-75°C		22.4 ± 0.4	-	-	22.2 ± 0.2	-	21.5 ± 0.1	22.6 ± 0.2	21.1 ± 0.1	-	-	20.8 ± 1.6

*Indicates qualitative fatty acid values that have decreased to less than 90% of baseline values. Data presented as mean ± SD. BHT, butylated hydroxytoluene; EDTA, ethylenediaminetetraacetic acid; d, days of storage; HUFA, highly unsaturated fatty acids.

CHAPTER 7

DETERMINING THE MECHANISMS OF ACCELERATED HIGHLY UNSATURATED FATTY ACID DEGRADATION DURING BLOOD STORAGE AT -20°C

INTRODUCTION

FTP blood sampling for fatty acid profiling is a relatively new method of analysis that is gaining popularity as an inexpensive and quick method of blood collection. Storage of FTP samples and whole blood at various temperatures has demonstrated that storage at -20ºC for any period of time results in HUFA degradation that occurs much sooner than at -75ºC, 4ºC or even at RT. This is an unexpected finding as plasma and serum storage at -20ºC has demonstrated fatty acid stability during storage for between 1 (Hirsch, Slivka, and Gibbons, 1976) and 3 years (Salo, Gey, and Nikkari, 1986). However, previous studies have demonstrated that storage of erythrocytes at -20ºC results in significant PUFA degradation after as little as 4 weeks with no other treatments prior to storage (Magnusardottir and Skuladottir, 2006), and with the presence of nitrogen providing no additional protection in a separate study (Otto et al., 1997). The marked difference in PUFA stability between plasma/serum and erythrocytes suggests that the mechanism of degradation may be attributable to an erythrocyte-related factor.

It is well understood that freeze-thawing of erythrocytes results in significant and complete lysis of erythrocytes that results in the release of the iron-containing heme group. A review of numerous factors that affect hemolysis including temperature is available (Sowemimo-Coker, 2002). In addition to free radicals, free iron (Fe^{2+}) is highly capable of accepting hydrogen ions from H_2O_2, producing the ·OH radical that is highly reactive with polyunsaturated fatty acids to initiate fatty acid peroxidation reactions (Halliwell and Chirico, 1993). Presently, it is hypothesized that the enhanced HUFA degradation demonstrated at -20ºC in whole blood and

FTP blood is a result of hemolysis that releases iron for the subsequent attack on polyunsaturated fatty acids. To test this hypothesis blood samples are treated with glycerol, a cryoprotectant, and subsequently stored at -20ºC. In addition, blood treatment methods for the prevention of hemolysis-induced fatty acid peroxidation are tested, and include iron chelation and sample drying.

METHODS AND MATERIALS

HUFA Standard Storage

To determine the effect of -20°C storage conditions on HUFA independent of blood factors, 1μg of the three HUFA standards (Nu-Chek Prep, Inc., Elysian, MN, USA) arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) were applied to pre-washed chromatography strips and stored at -20°C for up to 14 days to mimic FTP storage conditions. After 1, 3, 7 and 14 days HUFA chromatography strips were removed from storage and analyzed in triplicate by direct transesterification with 14% BF_3 in methanol as described earlier.

Cryoprotection of Erythrocytes and Whole blood

To determine if HUFA degradation of blood stored at -20°C could be attributed to iron release during erythrocyte lysis, erythrocytes were separated from whole blood obtained by venipuncture from a single participant as previously described and glycerolized prior to freezing at -20°C for up to one month. The glycerolization process was performed according to previously published methods (Chaplin Jr. et al., 1954; Valeri et al., 2001). Briefly, separated erythrocytes were mixed (1:1 v:v) with 40% glycerol in saline (w:v) and allowed to equilibrate

for approximately 45min. Glycerolized erythrocyte samples were then aliquoted to cryovials in 200μL portions and stored at -20°C for between 3 days to one month. On analysis days, samples were removed from storage and 50μL aliquots were taken for analysis and directly transesterified in 14% BF_3 in methanol and hexane (Armstrong, Metherel, and Stark, 2008) in triplicate. An internal standard of 3μg 22:3n-3 ethyl ester was included in the hexane. A saline only condition was included as a control to the glycerolized condition.

The above method was adapted from erythrocytes and applied to whole blood samples for the application of glycerolized whole blood to chromatography strips prior to storage at -20°C. Briefly, the hematocrit value (0.47) of an individual participant was determined by venipuncture blood collection into a graduated vacutainer. The same 40% glycerol in saline solution was added to the whole blood sample in a 1:1 (v:v) ratio of the calculated erythrocyte content of the whole blood. For example, if 3mL of whole blood was used then 47% (0.47 hematocrit) or 1.41mL of the whole blood is erythrocytes. Therefore, 1.41mL of 40% glycerol in saline was added to whole blood and allowed to equilibrate for 45 minutes. Glycerolized whole blood was then applied to chromatography strips so that 25μL of whole blood was included in the sample. Chromatography strips were previously treated with either 1000μg of deferoxamine mesylate salt (DFO, Sigma Chemicals, St. Louis, MO, USA), 50μg of BHT (Thermo Fisher Scientific Inc., Rockford, IL, USA) or not treated at all (control). A 100% saline solution was used as a control. Samples were then stored at -20°C for between 3 days and one month. On analysis days, samples were removed from storage and directly transesterified in 14% BF_3 in methanol and hexane containing 3μg 22:3n-3 ethyl ester internal standard.

Iron Chelation and Storage

The iron chelator, deferoxamine (DFO) was pre-treated to chromatography strips as a potential method for the prevention of iron-induced HUFA degradation during whole blood storage at -20°C. Whole blood samples were applied to chromatography strips that were pre-treated with 0, 50, 200, 1000 or 5000μg of DFO diluted in 25μL of deionized water. Each DFO condition was subsequently stored together in a capped 20mL test tube at -20°C for between 3 days and one month. On analysis days samples were removed from storage and directly transesterified as described above.

Sample Drying and Storage

Drying of blood drops on chromatography paper was also tested as a method for the prevention of erythrocyte lysis during -20°C storage. Chromatography strips were pretreated with either 50μg of BHT, 1000μg of DFO or not treated at all (control). Whole blood samples applied to chromatography strips were placed in a 20mL test tube and dried under a stream of nitrogen for approximately 10 minutes until blood was sufficiently dried. Following drying the test tube for each condition was capped and stored for between 3 days and one month at -20°C. On analysis days samples were removed from storage and directly transesterified as described above.

Erythrocyte Staining

To demonstrate the presence or absence of erythrocyte lysis during storage at -20°C, the Giemsa stain (Sigma-Alrdich Canada Ltd., Oakville, ON) (Garcia, 2006) was utilized for the staining of phosphate groups located in phospholipids of erythrocyte cell membranes (Shapiro

and Mandy, 2007). The Giemsa stain was applied to the following whole blood conditions: 1) fresh whole blood, 2) whole blood stored overnight at -20°C, 3) saline-control whole blood stored at 4°C stored overnight, 4) glycerolized whole blood stored at -20°C overnight, 5) wet whole blood frozen on a microslide overnight at -20°C and 6) dried whole blood frozen on a microslide overnight at -20°C. Briefly, whole blood samples were allowed to dry on the microslide and are subsequently fixed in 95% methanol in a Coplin jar for 4 minutes. Following removal from methanol, samples were allowed to air dry for 30 minutes, and stained in a Coplin jar with a Giemsa stain in saline solution (1:15, v:v) for 20 minutes. Microslides were then removed from the stain solution, rinsed with deionized water and allowed to air dry. Stained erythrocytes were then viewed under a Nikon Eclipse 50*i* microscope (Nikon Instruments Inc., Melville, NY, USA) at 50X magnification using PixeLINK Capture OEM image capturing software (PixeLINK, Ottawa, ON).

RESULTS

FTP Blood Storage at -20ºC and -75ºC

It has been determined from the previous chapter that storage of FTP samples at -20ºC results in significant and nearly complete HUFA degradation during a one month storage period and that storage at -75ºC shows no significant changes during the same storage period (**Table 6**). HUFA (μg/100μL whole blood) decrease significantly after 3 days by 58%, 7 days by 72%, 14 days by 84% and finally by 93% after 30 days. Significant decreases in HUFA concentration when stored with 50μg BHT at -20ºC was delayed until 30 days of storage and represents a 13% decline from baseline. Whole blood staining reveals that complete erythrocyte lysis occurs during overnight storage at both -20ºC and -75ºC, but not at 4ºC (**Figure 14**).

Storage of HUFA Standards at RT and -20ºC

To verify that HUFA degradation during -20ºC is a result of an internal blood-borne factor, three HUFA standards were applied to chromatography paper strips, capped and stored at -20ºC for 14 days, with controls stored in an open test tube at room temperature (**Table 7**). The standards AA, EPA and DHA demonstrated no significant declines from baseline during storage at either -20ºC or -75ºC throughout the entire two-week storage period. When stored in an open test tube at room temperature, however, AA decreased by 17, 61 and 81%, EPA by 20, 64 and 87%, and DHA by 29, 69 and 91% after 3, 7 and 14 days, respectively.

Effect of the Cryoprotectant, Glycerol, on HUFA Degradation

Initial tests on the effectiveness of glycerol treatment on erythrocyte HUFA degradation was assessed (**Table 8**). Glycerolized erythrocytes stored at -20ºC without antioxidant demonstrates that under these conditions, glycerol prevents HUFA degradation for up to 30 days of storage compared to a saline-control condition. Under the saline-control storage condition HUFA decreased by 11% after 3 days, and continued to decrease throughout the storage period and at 30 days represents a decline of 85% from baseline levels. This erythrocyte glycerolization treatment method for the purpose of preventing cell lysis was altered and applied to whole blood samples that were then stored on chromatography strips in the presence of BHT, DFO or no antioxidant for 30 days (**Figure 15**). Saline-controlled FTP blood samples demonstrate significant declines in total HUFA from baseline (45.6 ± 1.0, mean ± SD) after 7 (28.6 ± 2.9), 14 (14.7 ± 0.8) and 30 (6.8 ± 0.2) days of storage. Glycerolized FTP samples show no changes from baseline values across all antioxidant conditions for the entire 30 day period. In addition,

the three glycerolized FTP blood conditions do not differ from each other at any time point, with the saline-control being significantly lower at 7, 14 and 30 days. This protection against HUFA degradation during -20 ºC is witnessed in conjunction with an absence of cell lysis (**Figure 16**).

Effects of Iron Chelation on FTP Whole Blood

DFO, an iron chelating agent, was applied to chromatography paper strips as a pretreatment method for the protection against iron-induced HUFA degradation as iron is released from lysed erythrocytes. Chromatography strips were pretreated with either 0, 50, 200,1000 or 5000μg of DFO and stored at -20ºC for up to 30 days before determining HUFA content (**Figure 17**). At baseline, HUFA in all DFO storage conditions were equal and ranged from 54.3 ± 1.0 to 61.9 ± 2.0 μg/100μL. At all time points, HUFA in the DFO conditions were significantly higher compared to the non-treatment condition. HUFA in the 50DFO and 200DFO conditions are equal throughout storage at each time point, as were HUFA in the 1000DFO and 5000DFO conditions. However, both the 1000 and 5000DFO are significantly higher in HUFA levels compared to all others after 14 and 30 storage days. DFO storage conditions become significantly lower than baseline values after 3 days for 50DFO (46.5 ± 3.9) and 200DFO (51.8 ± 0.6), 7 days for 1000DFO (45.2 ± 5.4) and 14 days for 5000DFO (40.5 ± 4.5) conditions.

Effect of Drying FTP Blood Prior to -20ºC Storage

Chromatography strips were pretreated with BHT, DFO or no antioxidant and whole blood was applied and then dried under a stream of nitrogen. Storage at -20ºC was from 3 to 30 days. A wet FTP blood sample was used as a control (**Figure 18**). As discussed previously, the

wet sample shows significant decreases in HUFA blood levels beginning after 7 days of storage and remaining throughout storage. HUFA in wet samples are significantly lower than all three dried storage conditions beginning after 7 days of storage compared to all dried conditions. In addition, drying + BHT yields higher HUFA levels after 14 days (52.5 ± 2.6) compared to drying alone (44.1 ± 1.0), and higher HUFA after 30 days (50.8 ± 1.5) compared to drying alone (40.7 ± 2.9) and drying + DFO (45.7 ± 1.2) with drying + DFO being higher than drying alone. Neither of the drying + antioxidant conditions demonstrate significant reductions in HUFA content throughout the 30 day storage period. Whole blood staining indicates that drying of whole blood prior to storage prevents significant amounts of erythrocyte lysis from occurring (**Figure 19**), although erythrocyte conformation appears different than glycerol treated blood samples (Figure 16).

DISCUSSION

Presently, the treatment of both erythrocytes and FTP blood with glycerol prevents any significant HUFA losses during one month of storage at -20ºC compared to controls. In addition, pretreatment of chromatography strips with the iron chelator, DFO, partially inhibits HUFA losses during -20ºC storage in a concentration dependent manner between 50 and 5000μg of DFO pretreatment concentration. This DFO pretreatment, however, does not fully protect against HUFA losses. Finally, drying of FTP samples prior to -20ºC storage significantly protects against HUFA losses, although not completely, and the pretreatment of chromatography strips with either DFO or BHT provides additional protection against HUFA losses, with greater and complete protection shown with BHT pretreatment.

Similar rapid degradations in HUFA compositions have been determined during storage at -20ºC for erythrocyte TLE (Di Marino et al., 2000; Magnusardottir and Skuladottir, 2006) and phospholipids (Otto et al., 1997; Ways, 1967) but not plasma (Hirsch, Slivka, and Gibbons, 1976; Otto et al., 1997; Salo, Gey, and Nikkari, 1986). However, no previous study found in the literature has attempted to determine the effects of erythrocyte or whole blood cryopreservation on HUFA stability during -20ºC storage conditions. In fact, very few studies on cell cryoprotection and the effects on fatty acid composition have been performed, whether using glycerol or another cryoprotectant. One such study, however, examined the effects of freezing microalgae with and without cryopreservation with glycerol (Molina Grima et al., 1994). Briefly, they determined that there was no effect of freezing at -20ºC on the fatty acid profile, although minor decreases in PUFA do occur. In addition, microalgae frozen at -20ºC with 10% v/v glycerol does not provide significant protection against PUFA degradation. The inability of glycerol to protect against PUFA degradation may be due to the lysis of these cells not releasing a fatty acid peroxidation trigger, such as iron in the case of erythrocytes.

AAPH (2,2'-azobis (2-aminidnopropane) hydrochloride)-induced hemolysis has previously been shown to increase fatty acid peroxidation in erythrocyte membranes (Niki et al., 1988), and various antioxidant/free radical scavengers such as curcumin (Banerjee et al., 2008; Deng et al., 2006), thiol-containing drugs (Vosters and Neve, 2002) and 3,3'-diselenodipropionic acid (Kunwar et al., 2007) can reduce or prevent peroxidation in these hemolyzed erythrocytes. However, the mechanism for AAPH-induced hemolysis is believed to be initiated by peroxyl radicals generated by the decomposition of AAPH in the presence of oxygen (Niki et al., 1988; Niki, 1990), indicating that lipid peroxidation may be occurring in conjunction with or prior to hemolysis. Our results suggest that freeze-thaw induced hemolysis is occurring prior to fatty

acid peroxidation as prevention of hemolysis protects fully against HUFA degradation. In addition, erythrocytes stored at 4ºC for extended periods of time may undergo hemolysis that in turn causes lipid peroxidation (Arun et al., 1999; Racek et al., 1997). Conversely, allyl alcohol-induced hemolysis in mice *in vivo* increases fatty acid peroxidation as measured by malondialdehyde content and reductions in AA and DHA content (Ferrali, Ciccoli, and Comporti, 1989). These effects were removed when treated with the antioxidant Trolox C or the iron chelator DFO, indicating the role of iron in hemolysis and peroxidation reactions.

Fe^{2+} can induce lipid peroxidation through the Fenton reaction, in which it reacts with H_2O_2 to form Fe^{3+} and the highly reactive ·OH radical, as well as ^{-}OH (Halliwell and Chirico, 1993). This reaction can be prevented though iron chelators that bind Fe^{3+} and essentially removes the free reactive iron from reacting with PUFA. In the present study, DFO reduces HUFA losses to only a 22% reduction from baseline values compared to a 92% reduction in control FTP whole blood samples after 1 month of storage at -20ºC. This protection against HUFA degradation with DFO provides clear evidence for an iron-mediated peroxidation mechanism, however, DFO is unable to fully prevent losses in HUFA and indicates that at least one other mechanism must be involved. Erythrocytes stored at -50°C in the presence of DFO has been shown to prevent HUFA degradation for up to one year (Otto et al., 1997), however, a control storage condition was not included, and as such it is unclear whether fatty acid degradation prevention is due to the lower storage temperature or the presence of DFO. As erythrocyte and whole blood storage at -80ºC provides extended protection, it would be expected that storage at -50ºC without antioxidant would in and of itself provide additional protection against HUFA losses compared to -20ºC. Additionally, studies have noted the protective ability

of deferoxamine against lipid peroxidation in erythrocytes (Knight, Searles, and Clayton, 1996) stored at 4ºC, and during platelet storage (Knight, Blaylock, and Searles, 1993).

Iron chelation or antioxidant treatment may not be the only method for preventing lipid peroxidation during long-term FTP whole blood storage. The mechanism behind freeze/thaw-induced hemolysis may be directly due to the presence of water and its subsequent freezing in a whole blood or erythrocyte sample. The mechanisms for this are poorly understood and have been reviewed recently (Stolzing et al., 2012). Presently, it was hypothesized that drying of FTP whole blood samples prior to -20°C storage may prevent some of the mechanical stresses placed on the cell due to ice crystal formation. Indeed, we determined that drying FTP whole blood samples under a stream of nitrogen prior to storage provided significant protection from HUFA losses during at -20°C for up to one month compared to wet samples. Dried FTP samples without any oxidation prevention treatment resulted in only a slight loss of HUFA content. This loss was prevented by pretreatment of chromatography paper with DFO and to a greater extent BHT. This suggests that the sample drying process alone provides significant protection against iron-mediated HUFA losses, although the drying process itself may also cause some amount of hemolysis. Strong flows of nitrogen are applied to FTP blood samples, and the air turbulence of this action may cause appreciable damage to the erythrocyte bilayer and as a result release iron into the blood sample. Although no studies on the effect of turbulent nitrogen/air flow on hemolysis could be found, it is generally accepted that excessive shaking or agitation of blood can result in significant amounts of hemolysis (Sowemimo-Coker, 2002), and nitrogen drying at increased pressure could theoretically cause hemolysis in a similar manner.

To combat the potential problem of excessive flow, air drying of FTP sample may reduce the turbulent flow experienced by erythrocytes, and thereby reduce drying-induced hemolysis. A

longer drying time in direct contact to air may increase oxygen-mediated lipid peroxidation. However, we have demonstrated in our lab that greater than one day under these conditions would be required before significant HUFA degradation occurs. In addition, interaction of erythrocytes with solid surfaces can also cause damage resulting in cell lysis (Leverett et al., 1972), and as such, whole blood interaction with chromatography paper may provide an alternate explanation for the observed results. BHT pretreatment of the chromatography strips provides further protection against HUFA degradation, and in support of our DFO treatment experiment suggests alternative peroxidation mechanisms in addition to iron-mediated pathways during storage at -20°C.

Hemolysis will occur during storage at both -20°C and -75°C, however, studies have demonstrated that storage of erythrocytes without antioxidant protection at no warmer than -70°C (Hodson et al., 2002; Stanford, King, and Kristal, 1991) maintains fatty acid profiles for at least 1 to 4 years and possibly longer. Such results suggest that while lipid peroxidation reactions are still highly active at temperatures as low as -20°C, storage at lower temperatures such as -75°C are likely cold enough to slow down rates of peroxidation to a rate that is not measurable in the fatty acid profile for many years.

CONCLUSION

HUFA degradation observed in previous literature, and presently, in whole blood and erythrocyte storage at -20°C appears to be attributed to mechanisms related to hemolysis, and subsequent lipid peroxidation as a result of iron-mediated pathways. Storage of whole blood or erythrocytes in the presence of the cryopreservant glycerol can prevent this degradation and allow for storage of samples at -20°C. Enabling blood storage at -20°C may benefit field studies

that do not have access to deep freezers, and require frozen preservation of blood samples for analysis of analytes of interest that are in addition to fatty acids. Furthermore, pretreatment of chromatography paper with DFO or BHT in conjunction with nitrogen drying of FTP whole blood samples can also protect against HUFA degradation during storage periods of up to one month, although hemolysis in this case is likely not prevented. To conclude, HUFA degradation in whole blood and erythrocytes can be abolished through glycerol cryopreservation that prevents hemolysis, and pretreatment with DFO or BHT and drying of FTP whole blood samples can reduce or prevent the deleterious effects of hemolysis on blood HUFA composition.

Table 6 – Effect of Temperature and Antioxidant on HUFA Stability

	Storage (days)				
Storage Condition	**0**	**3**	**7**	**14**	**30**
-20°C	61.9 ± 1.6	26.3 ± 2.1*	17.1 ± 2.8*	9.95 ± 2.18*	4.64 ± 0.36*
-20°C + 50μg BHT	60.5 ± 5.2	55.0 ± 0.2	57.7 ± 1.9	55.1 ± 1.1	52.4 ± 1.0*
-75°C	61.9 ± 1.6	58.4 ± 2.6	56.8 ± 0.9	58.0 ± 2.1	57.6 ± 0.6
-75°C + 50μg BHT	60.5 ± 5.2	61.5 ± 2.8	53.4 ± 0.5	55.1 ± 4.1	58.6 ± 0.7

*Indicates fatty acid concentrations (μg/100μL) significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. BHT, butyated hydroxytoluene; HUFA, highly unsaturated fatty acid.

Table 7 – Effect of -20°C Storage on AA, EPA and DHA Standard Stability

			Storage (days)			
Fatty Acid	**Storage**	**0**	**1**	**3**	**7**	**14**
Arachidonic Acid	**Open Air**	1.07 ± 0.04	-	0.89 ± 0.05*	0.42 ± 0.04*	0.20 ± 0.02*
	-20°C		1.02 ± 0.01	1.06 ± 0.02	1.09 ± 0.03	1.01 ± 0.03
	-75°C		0.96 ± 0.10	1.07 ± 0.03	1.07 ± 0.03	1.04 ± 0.01
Eicosapaentaenoic Acid	**Open Air**	1.04 ± 0.04	-	0.83 ± 0.04*	0.37 ± 0.03*	0.14 ± 0.01*
	-20°C		1.02 ± 0.00	1.05 ± 0.01	1.09 ± 0.01	1.01 ± 0.02
	-75°C		0.97 ± 0.12	1.08 ± 0.03	1.08 ± 0.04	1.04 ± 0.01
Docosahexaenoic Acid	**Open Air**	1.08 ± 0.03	-	0.77 ± 0.07*	0.33 ± 0.05*	0.10 ± 0.01*
	-20°C		1.02 ± 0.01	1.05 ± 0.01	1.10 ± 0.03	1.01 ± 0.03
	-75°C		0.97 ± 0.11	1.06 ± 0.03	1.07 ± 0.04	1.03 ± 0.00

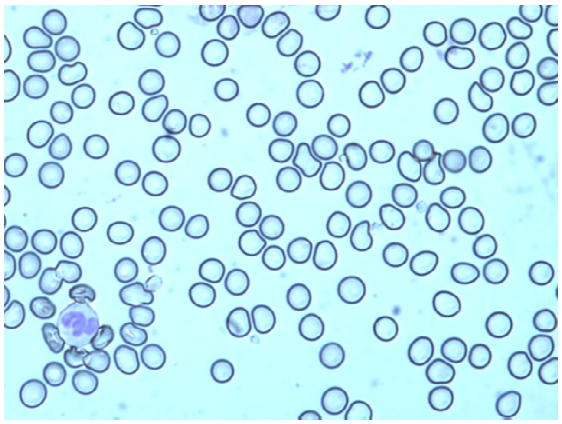
*Indicates fatty acid (μg) significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. HUFA, highly unsaturated fatty acid.

Table 8 – Protection of HUFA Degradation in Erythrocytes by Glycerol at -20°C Storage

	Storage (days)				
Cryoprotectant	**0**	**3**	**7**	**14**	**30**
Control	45.9 ± 1.0	41.0 ± 3.1*	28.6 ± 2.9*	14.7 ± 0.8*	6.78 ± 0.17*
Glycerol	45.0 ± 0.4	46.7 ± 0.8	42.9 ± 3.3	46.5 ± 0.8	46.8 ± 0.6

*Indicates fatty acid concentration (μg/100μL) significantly lower than baseline values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. HUFA, highly unsaturated fatty acid.

A



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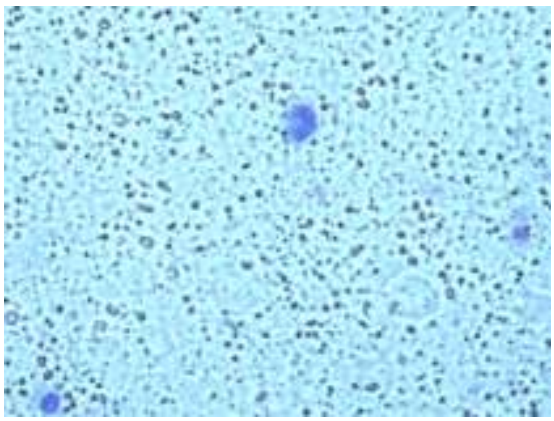

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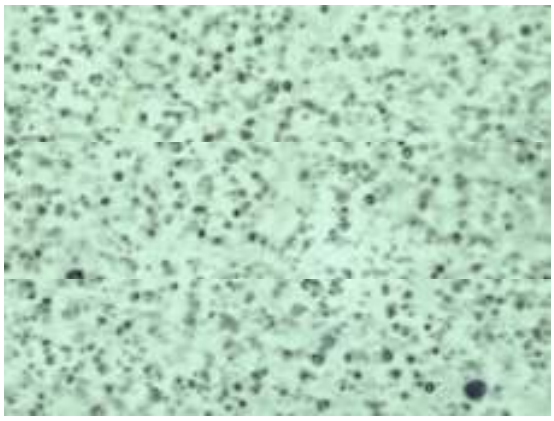


Figure 14 – Microslide of erythrocyte lysis after overnight storage of whole blood. A) whole blood stored at 4°C, B) whole blood stored at -20°C and C) whole blood stored at -75°C overnight.

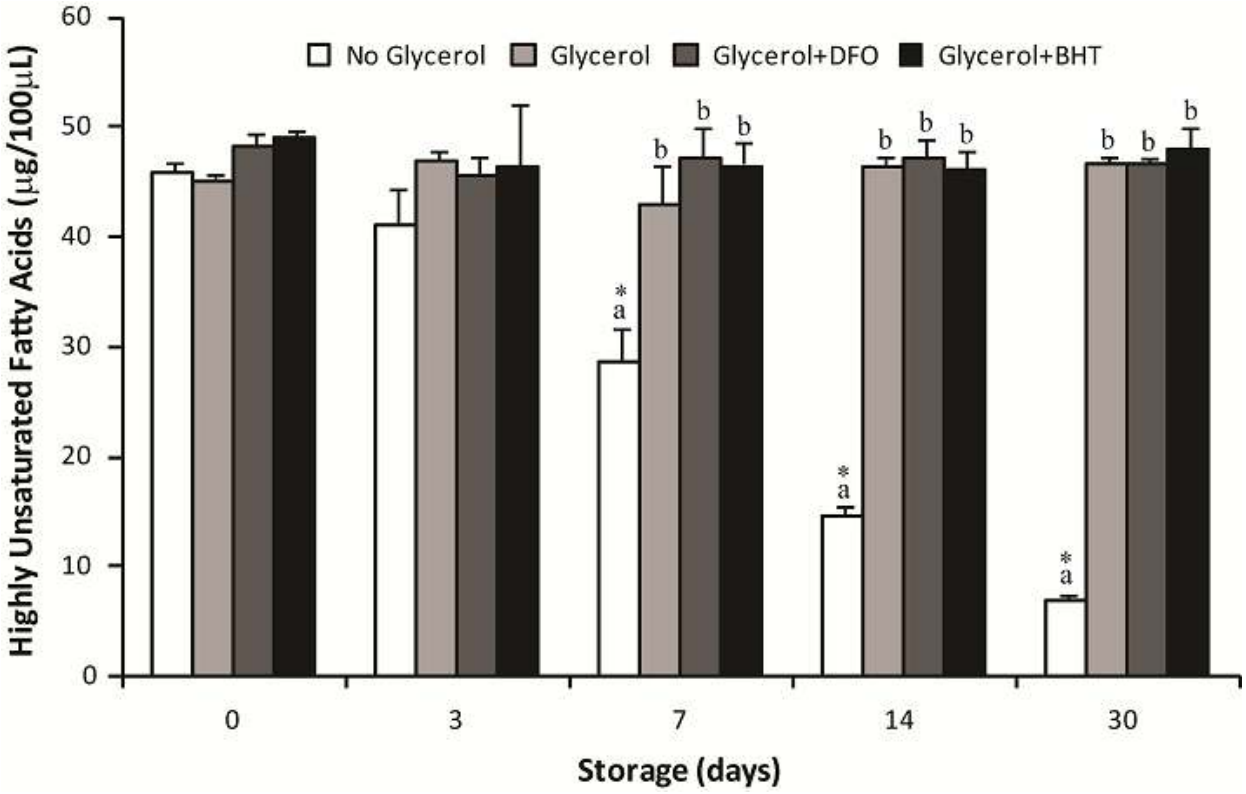
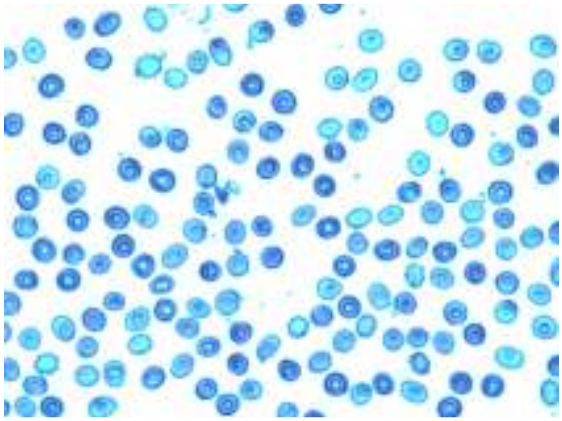


Figure 15 – Effect of glycerolization on FTP blood HUFA degradation during -20°C storage. *Indicates fatty acid concentration (μg/100μL) significantly lower than baseline values, and letters different within a time point indicates differences between treatments as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. BHT, butylated hydroxytoluene; DFO, deferoxamine; HUFA, highly unsaturated fatty acid.

A



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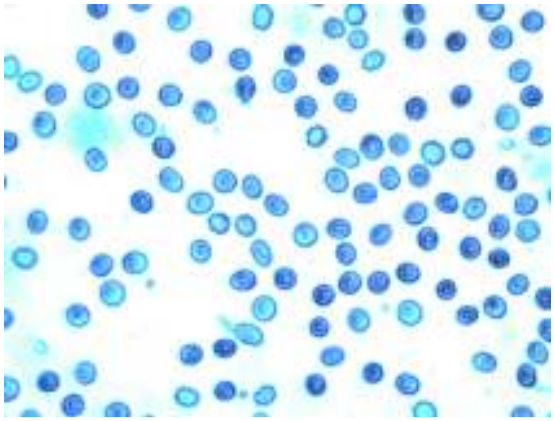

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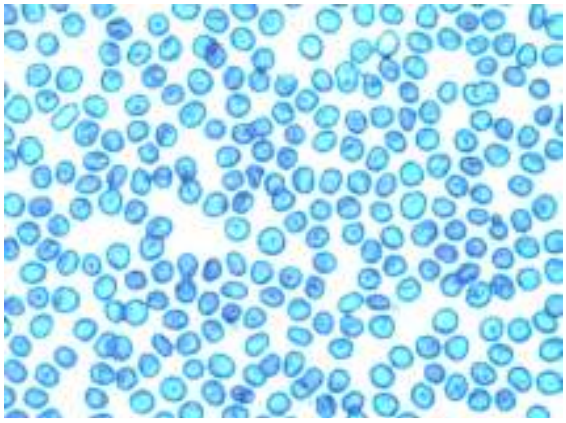


Figure 16 – Microslide of erythrocyte lysis following whole blood glycerolization. A) fresh whole blood and B) saline-control whole blood each stored at 4°C overnight and C) glycerol-treated whole blood stored at -20°C overnight.

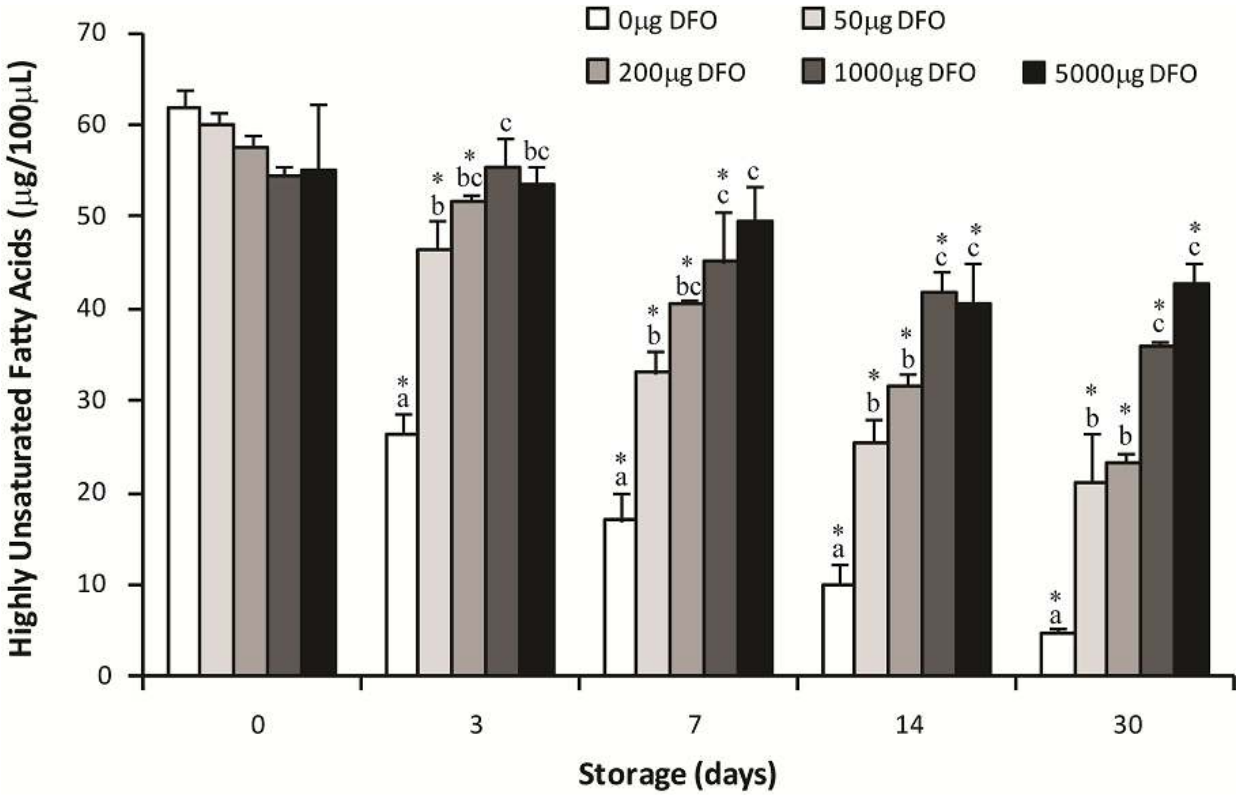


Figure 17 – Effect of DFO on FTP blood HUFA degradation during -20°C storage. *Indicates fatty acid concentration (μg/100μL) significantly lower than baseline values, and letters different within a time point indicates differences between treatments as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. DFO, deferoxamine; FTP, fingertip prick; HUFA, highly unsaturated fatty acid.

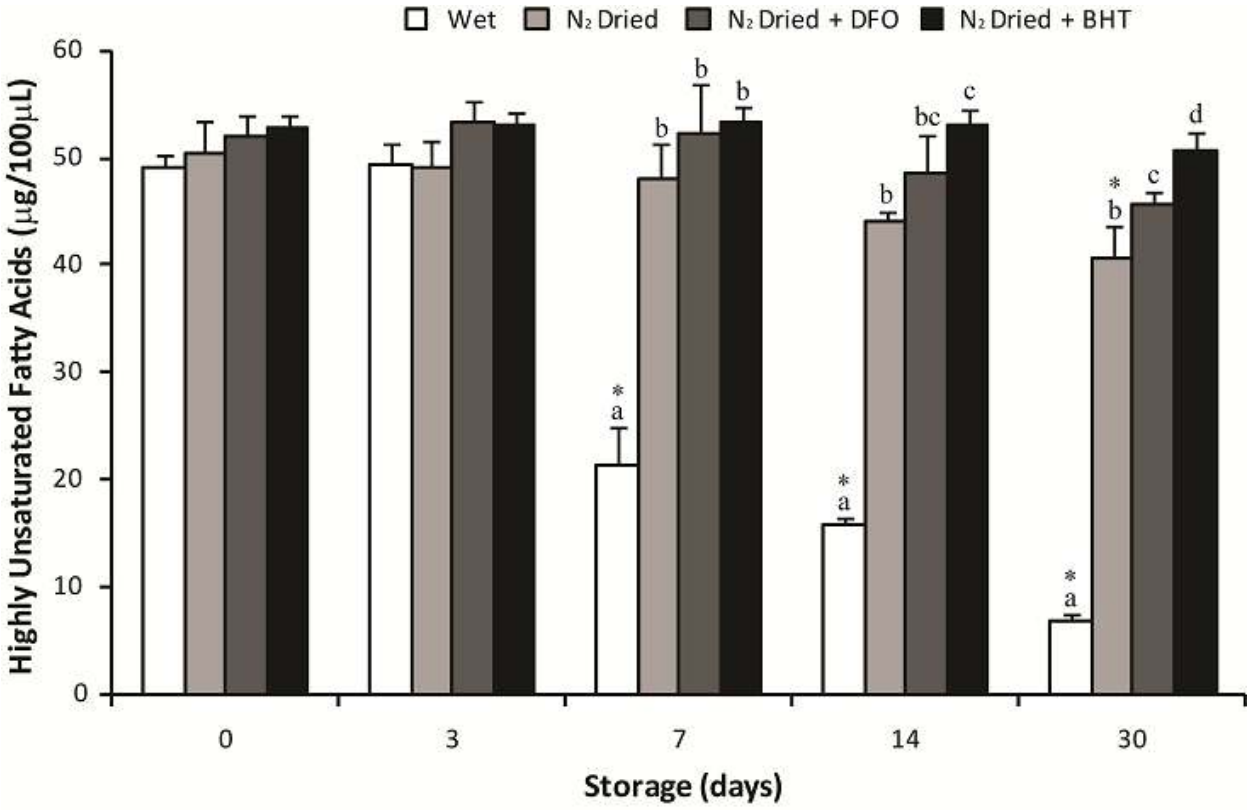
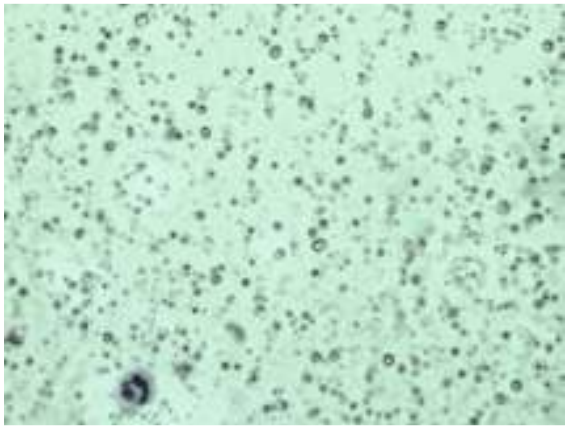


Figure 18 – Effect of sample drying and antioxidant on FTP blood HUFA degradation during -20°C storage. *Indicates fatty acid concentration (µg/100µL) significantly lower than baseline values, and letters different within a time point indicates differences between treatments as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD.
BHT, butylated hydroxytoluene; DFO, deferoxamine; FTP, fingertip prick; HUFA, highly unsaturated fatty acid.

A



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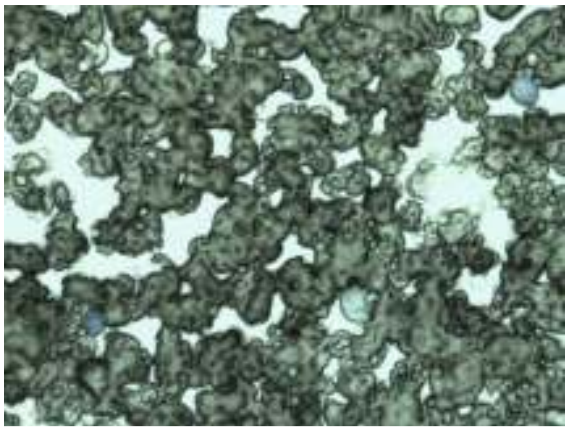

Figure 19 – Microslide of erythrocyte lysis following drying and freezing of whole blood. A) wet whole blood stored at -20°C on microslides overnight and B) dried whole blood stored on microslide at -20°C overnight.

CHAPTER 8

NOVEL MICROWAVE-ASSISTED DIRECT TRANSESTERIFCATION OF FINGERTIP PRICK BLOOD AND ULTRASOUND-ASSISTED EXTRACTION METHODS OF FLAXSEED

INTRODUCTION

Omega-3 fatty acid consumption has been linked to numerous benefits in health and has been reviewed extensively elsewhere (Moyad, 2005b; Moyad, 2005a). Although erythrocytes and plasma may be the most commonly used blood fractions for fatty acid analysis, whole blood is simpler and amenable to high-throughput FTP omega-3 profiling. FTP blood omega-3 biomarker screening has the potential to be used by health professionals for the rapid assessment of omega-3 status with subsequent dietary recommendations for improvement of blood values. FTP blood analysis can be transesterified directly and does not require a separate extraction step (Marangoni, Colombo, and Galli, 2004), however, this process still requires a minimum of one hour to complete. Previously, microwave-assisted direct transesterification has been used for brain phospholipids (Banerjee, Dawson, and Dasgupta, 1992), and microwave-assisted direct transesterification of FTP blood reports are limited and used a commercial kitchen microwave (Armstrong, Metherel, and Stark, 2008; Metherel et al., 2009a). These studies indicated that microwave methods failed to fully transesterify triacylglycerols when using 14% boron trifluoride (BF_3) in methanol (Armstrong, Metherel, and Stark, 2008). The present study will determine the applicability of an industrial scientific microwave with H_2SO_4 in methanol for fatty acid profiling.

In addition to blood analyses, fatty acids in the food supply are routinely determined for government mandated food labeling. Currently accepted methods for the extraction and

subsequent determination of lipids from foods are time-consuming, costly, and require large solvent and sample sizes (AOAC, 2005; Luque-Garcia and Luque de Castro, 2004). Ultrasound-assisted methods have also been used for the rapid extraction of lipids from various tissues (Cravotto et al., 2008; Luque-Garcia and Luque de Castro, 2004; Metherel et al., 2009b; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Wei et al., 2008) and are well suited for solid sample matrices common with foods due to various disruptive forces. The use of ultrasound can reduce extraction times to as little as one hour, and a novel direct ultrasound-assisted extraction technique yielded acceptable results (Metherel et al., 2009b), but is limited in application as the ultrasound probe induced solvent evaporation that requires monitoring and only a single sample could be prepared at a time. Presently, an indirect multi-sample ultrasound-assisted extraction method requiring small sample masses and solvent volumes is tested on the extraction of fatty acids from flaxseed with 3:2 hexane:isopropanol under various temperature, time and probe amplitude conditions.

METHODS AND MATERIALS

Study Design

Microwave-Assisted Fingertip Prick Direct Transesterification

Two vacutainers of approximately 8mL each of venipuncture whole blood were collected from a single participant, and 500μL aliquots were stored at -75°C until time of analysis. It is shown (Chapter 6) that storage of the whole blood samples under these conditions will not significantly affect the fatty acid profile. For analysis, whole blood samples were removed from storage and allowed to thaw on ice. After thawing, 25μL of whole blood was aliquoted to pre-washed chromatography paper strips and allowed to fully saturate into the paper prior to

microwave analysis. Microwave-assisted (CEM Discover® SP, CEM Corporation, Mathews, NC, USA) direct transesterification reactions were compared to standard direct transesterification reactions using 1% H_2SO_4 in methanol (Dugan, Jr., McGinnis, and Vadehra, 1966), and 14% BF_3 in methanol (Morrison and Smith, 1964) using convectional block heating for 3 hours and 1 hour, respectively. The utilization of 1% H_2SO_4 for direct transesterication of whole blood has been validated in pilot work and 14% BF_3 validation has been published previously (Armstrong, Metherel, and Stark, 2008).

Baseline microwave-assisted direct transesterification reactions were performed using 1% H_2SO_4 in methanol using a pilot microwave method that was previously developed for whole blood direct transesterification. The settings for this method were 5min at 30W power with a maximum temperature setting of 165°C. This maximum temperature setting prevents the microwave system from exceeding the internal microwave maximum pressure limit of 300 PSI, at which point the microwave will automatically stop. These microwave settings of 30W and 165°C were also tested during 4, 3 and 2min reaction times. Further methodological adjustments were made by maintaining the same temperature limit 165°C and allowing the software program to determine the optimal power output that would reach the temperature limit in the shortest amount of time while not exceeding set pressure limits. The software program uses variable power output in an attempt to reach the 165°C temperature setting . This 165°C variable power method was determined to be superior to the 30W method and was used under various additional time (1 – 3min) and reagent (1 – 10% H_2SO_4) conditions.

Ultrasound-Assisted Fatty Acid Extraction

In all analyses conditions, 25 mg of ground flaxseed (Bob's Red Mill Natural Foods, Inc., Milwaukie, OR, USA) were extracted in quadruplicates. Previously, we have determined that 3:2 hexane:isopropanol (Hara and Radin, 1978) is the optimal extraction solvent in conjunction with a direct (open tube) ultrasound-assisted technique for the extraction of lipids from flaxseed (Metherel et al., 2009b). Presently, the use of 3:2 hexane:isopropanol combined with a novel indirect (closed tube) ultrasound-assisted technique that utilizes a water bath as the medium between the ultrasonic source and the test tube containing the solvent and flaxseed sample is tested (**Figure 20**). Ultrasonic-assisted extraction variables that were manipulated and tested included water bath temperature (20°C or 40°C), time exposed to sonication (20, 40 or 60min) and amplitude of ultrasonic probe (144μm or 240μm, representing 60% and 100% of maximum probe amplitude, respectively). Potential variables that were held constant included the sample position relative to the probe and the extraction solvent. Manipulated and constant variable settings were selected based on a previous study determining optimal extraction conditions in a direct (open tube) ultrasound-assisted method utilizing 3:2 hexane:isopropanol for 10min at 60% of maximum amplitude. A longer extraction time was used in the present study as it was expected that the increased distance between probe and sample and the presence of water between probe and solvent would diminish strength of the cavitation.

Statistical Analyses

Qualitative and quantitative values for individual fatty acids were determined and are expressed as means ± SD. All statistical analyses were performed with the SPSS System (SPSS Inc., Chicago, IL). Various ultrasound-assisted extraction protocols and the microwave-assisted

direct transesterification methods were examined by one-way ANOVA with individual means compared to standard analysis techniques following a significant F-value by the Tukey's Honestly Significant Different post hoc procedure. In addition, a three-way ANOVA was performed to compare the effects of changing amplitude, time and temperature setting for ultrasound-assisted extraction. Significance was inferred at $p < 0.05$.

RESULTS

Quantitative Microwave-Assisted FTP Blood Direct Transesterification

Multiple microwave settings were tested for the direct transesterification of fingertip prick (FTP) whole blood samples, including time and power, and major fatty acid subclasses are reported for both quantitative (**Table 9**) and qualitative (**Table 10**) results. Initial methods test a constant 30W power application to the sample until after approximately 2min at which point the maximum pressure of 300 PSI is reached. After which, 30W is continuously cycled on and off to maintain a pressure of approximately 300 PSI. For example, the 4min at 30W represents a constant 30W for 2min and an additional 2min of cycling 30W on and off. Briefly, application of 30W of power for 5min yields significant concentration increases in SFA, MUFA, n-6 PUFA, PUFA and total fatty acids of 17%, 18%, 11%, 10% and 15%, respectively, compared to a 3h standard 1% H_2SO_4 direct tranesterification reaction. These differences appear to be driven primarily by the very-long chain SFA and MUFA 22:0, 23:0, 24:0 and 24:1n-9 (**Figure 21**). These four fatty acids increase by 111%, 100%, 135% and 137%, respectively, during this 5min microwave direct transesterification method compared to the 3h standard method. Shortening the time of the microwave reaction subsequently lessens the absolute increase in these fatty acids to the point where fatty acid concentrations for 22:0, 24:0 and 24:1n-9 is lower for the 2min

reaction compared to control. Similar, but non-significant increases are also shown for the 4min reaction time; however, significance is reached for MUFA. Conversely, 2min reaction time yields significantly lower values for all fatty acid subclasses of 25% - 35% below control values. No differences for any fatty acid subclass were shown for 3min of microwave-assisted direct transesterification.

The variable power method described earlier is compared to this 30W constant power method. With the variable power method, the software program automatically determines the power output required to increase the reaction temperature at a fast but safe rate while maintaining pressure below the 300 PSI limit. This variable power method was determined to yield significantly higher fatty acid concentrations compared to 30W constant power method (data not shown) for each of the SFA, MUFA, n-6 PUFA, n-3 PUFA, PUFA , HUFA and total fatty acids as determined by independent t-test between each method for both 2 and 3min reaction times.

The variable power method direct transesterification (Table 9) with 1% H_2SO_4 for 2min yields lower values for all fatty acid subclasses (13% – 23%) and total fatty acids (18%) compared to control. Similarly, 10% H_2SO_4 yields lower values compared to control for n-6 PUFA (18%), n-3 PUFA (24%), PUFA (19%) and HUFA (22%) with no differences in SFA, MUFA and total fatty acids. Both 2% and 5% H_2SO_4 show no significant differences compared to control values for all fatty acid subclasses, and as such 5% H_2SO_4 is used in an attempt to decrease reaction times further. Using 5% H_2SO_4 in methanol for either one and a half or 2min yields no significant differences across all fatty acid subclasses. Increasing the reaction time to 3min results in 14% and 13% lower values compared to control for n-3 PUFA and HUFA,

respectively. In addition, decreasing the reaction time to 1min yields significantly lower values of approximately 17% lower than control values for all subclasses except SFA and MUFA.

Qualitative Microwave-Assisted FTP Blood Direct Transesterification

Qualititative assessment of the major fatty acid subclasses demonstrates less variation between microwave direct transesterification methods (Table 10). The 30W constant power microwave method yields no significant qualitative differences during both three and 4min of reaction time for all fatty acid subclasses. The 5min reaction time results in a decrease in HUFA by 5% but in no other subclass. The 2min reaction reveals 7% higher SFA and 4%, 6% and 6% lower MUFA, n-6 PUFA and PUFA, respectively. Utilizing the variable power method for 2min qualitative differences are demonstrated for all H_2SO_4 concentrations compared to the standard 3h method. The largest differences are shown in decreases of 7%, 26%, 31%, 27% and 29% for MUFA, n-6 PUFA, n-3 PUFA, PUFA and HUFA, respectively with 10% H_2SO_4 in methanol. In addition, n-3 PUFA are lower in all four H_2SO_4 concentrations and MUFA are higher in the 2% and 5% concentrations. Testing the effect of time during the variable power method with 5% H_2SO_4 yields 6%, 7%, 6% and 11% lower values during 3min for n-6 PUFA, n-3 PUFA, PUFA and HUFA, respectively. 2min reaction time increases MUFA and decreases n-3 PUFA and HUFA, and 3min decreases both n-3 PUFA and HUFA. Interestingly, the shortest reaction time of 1min yields no significant differences for any of the fatty acid subclasses compared to control.

Microwave-Assisted N-3 Blood Biomarker Direct Transesterification

N-3 biomarkers % EPA+DHA, n-6/n-3 ratio and % HUFA in total HUFA determinations were are further compared between all microwave methods tested and compared to the standard H_2SO_4 and BF_3 direct transesterification methods (**Figure 22**). Briefly, % EPA+DHA yields

lower values compared to the standard H_2SO_4 (4.33 ± 0.09) as a result of the 1.5min (3.93 ± 0.07) and 3min (3.84 ± 0.11) 5% H_2SO_4 variable power methods. In addition, 1min (4.59 ± 0.27) 10% H_2SO_4 variable power method yields higher and the 2min (3.00 ± 0.16) 10% H_2SO_4 variable power method yields lower values than the standard BF_3 method (4.07 ± 0.09). For the n-6/n-3 ratio the 2min variable power methods for both 5% (5.49 ± 0.05) and 10% (5.59 ± 0.12) yields higher values compared to H_2SO_4 control (5.17 ± 0.07), and the 1% + 30W + 2min method (4.94 ± 0.06) and the 1min variable power with 10% H_2SO_4 (4.95 ± 0.23) is lower than the BF_3 standard method (5.33 ± 0.08). The % n-3 HUFA in total HUFA biomarker only yields a lower value for the 2min 10% H_2SO_4 variable power method (31.14 ± 0.32) compared to the standard H_2SO_4 method (32.13 ± 0.35). No other differences exist for the % n-3 HUFA in total HUFA biomarker compared to standard methods.

Quantitative Indirect Ultrasound-Assisted Flaxseed Extraction

Indirect (closed test tube) ultrasound-assisted fatty acid extraction methods were performed using 3mL of 3:2 hexane:isopropanol under various temperature (20°C and 40°C), probe amplitude (60% and 100%) and time (20, 40 and 60min) conditions. Both quantitative (**Table 11**) and qualitative (**Table 12**) differences in fatty acids between each ultrasound-assisted extraction method and the standard 24 hr Folch method were examined. Six of the twelve tested ultrasound-assisted methods revealed no significant differences in quantitative values (mg/25mg flaxseed) between any of the fatty major fatty acids in flaxseed including 16:0, 18:0, 18:1n-9, 18:2n-6 and the most abundant fatty acid in flaxseed, ALA. These six ultrasound-assisted methods include all four of the 60min methods and each of the 40min + 60% amplitude methods. The highest yield of any of the methods are the 60% amplitude + 40min methods at both 20°C

and 40°C resulting in ALA yields of 97% and 94% at 20°C and 40°C, respectively. The lowest ALA yield of 73% was obtained from the 100% amplitude + 20min extraction at 20°C.

An interaction between temperature and time was determined for both 18:0 and total SFA. Briefly, at 20°C extraction for 20min, both 18:0 and total SFA are lower than at 40C, and after 60min extraction no differences exist in concentrations. The remaining fatty acids measured including 16:0, 18:1n-9, 18:2n-6, ALA, total PUFA and total fatty acids resulted in higher yields with increasing time of extraction and higher yields with the lower 60% probe amplitude. ALA is higher during extraction at 60% (4.60 ± 0.27) compared to 100% (4.23 ± 0.42) probe amplitude and ALA yields after 40min (4.53 ± 0.34) and 60min (4.62 ± 0.22) are significantly higher than after 20min (4.09 ± 0.40) of extraction time. This same pattern is viewed for all other fatty acid measures except for 18:0 where the yields after 60min were also significantly higher than values after 40 minutes of extraction. No effects of temperature on fatty acid concentrations were determined.

Qualitative Indirect Ultrasound-Assisted Flaxseed Extraction

Qualitatively (% weight fatty acid in total fatty acids) 18:0 and total SFA with ultrasound were significantly higher than Folch extraction values at 20°C and 100% amplitude after 40min and 60min extractions and at 40°C and 60% amplitude for 20min and 60min and 100% amplitude for 20min. Only 18:0 is higher during 20°C and 100% amplitude extraction for 20min. There were no other differences.

DISCUSSION

Presently, microwave-assisted direct transesterification of FTP blood at 30W with 1% H_2SO_4 in methanol for 5min or less is demonstrated to be a viable replacement method to the standard three hour block heater method, and microwave transesterification has the potential to significantly reduce time and monetary commitment for analysis. Briefly, microwave-assisted direct transesterification with 1% H_2SO_4 in methanol yields higher concentrations of total SFA, MUFA and n-6 PUFA, PUFA and total fatty acid values during 5min reaction time, and generally non-significantly higher values at 4min. The 3min reaction time yields no differences across fatty acid subclasses and 2min yields lower values for all subclasses.

Interestingly, very-long chain SFA and MUFA appear to be driving the increase in fatty acid yields during the four and 5min reaction times with 30W + 1% H_2SO_4 reactions. The four fatty acids, 22:0, 23:0, 24:0 and 24:1n-9 represent less than 5% of all SFA and MUFA in these FTP blood samples, however, these same fatty acids are responsible for approximately 32% of the observed increase in SFA and MUFA during the 5min reaction method. Moreover, SFA and MUFA changes represent 71% of the observed total fatty acid increases during microwave direct transesterification. These very-long chain SFA and MUFA are located abundantly in sphingolipids, with fatty acid compositions represented by 14% 22:0, 9% 23:0, 24% 24:0 and 13% 24:1n-9 in rat liver (Fex, 1971). In addition, 16:0 (22%) and 18:0 (10%) are also higher in sphingolipids. Approximately 5% of whole blood samples are comprised of the sphingomyelin lipid fraction (Christie, 1985). Therefore, the improvement in fatty acid yield, particularly in SFA and MUFA, with microwave-assisted direct transesterification techniques may be due to a more effective transesterification of the sphingolipid fraction in whole blood. Pilot work has confirmed that both sphingolipids separated from whole blood and pure shpingolipid standards

are more readily transesterified by microwave-assisted direct transesterification compared to block heating for three hours with 1% H_2SO_4 in methanol (unpublished data).

Shingolipid-derived fatty acids are bound by an amide (C – N) bond as opposed to an ester bond (C – O – C) in glycerol-bound fatty acids. Sphingolipids are demonstrated to be more difficult to tranesterify (Morrison and Smith, 1964) which may be due to the need to break the more stable amide bond. This increased amide bond stability compared to ester bonds is due to resonance stabilization in which the double bond between the carbon and oxygen atoms bond is shared between the oxygen and nitrogen (C = O ←→ C = N) (Martin, 2001). It is suspected that in the present study, the microwave energy applied to FTP samples for transesterification is sufficiently greater than convectional block heater energy and results in the breakage of the amide bond and complete transesterification of these sphingolipid-derived fatty acids.

Using the variable power microwave method improves fatty acid yield compared to the 30W constant power method as the variable power method reaches maximum temperature and pressure settings sooner. Increasing the H_2SO_4 concentrations using 1%, 2%, 5%, and 10% H_2SO_4 in methanol generally increases the fatty acid yield up to 5% H_2SO_4, after which 10% H_2SO_4 yields significantly lower total n-6 PUFA, n-3 PUFA, PUFA and HUFA. A similar decrease in n-3 PUFA and HUFA yield is also demonstrated using the same variable power method with 5% H_2SO_4 in methanol and extending the reaction time to 3min from 2min. The losses in fatty acid yields appears to occur only in PUFA and HUFA and suggests that balancing time and H_2SO_4 composition used in microwave analysis is crucial for the prevention of fatty acid peroxidation during transesterification. Dilute sulphuric acid, such as the 1% and 2% H_2SO_4 in methanol used here, exhibit no oxidizing properties (Luque de Castro and Luque Garcia, 2002). However, in concentrated form it is capable of oxidizing many substances, and

under the conditions in the present study H_2SO_4 concentrations of 5% and 10% in methanol appear capable of oxidizing PUFA in as little as three and 2min, respectively.

This appears to be the first report demonstrating that microwave transesterification can increase yield through increased sphingolipid fatty acid transesterification, and decrease yield with higher H_2SO_4 compositions and/or longer reaction times through fatty acid degradation. The current system, however, is limited by the microwave apparatus itself as only one sample at a time can be analyzed, and is further limited by internally set pressure limits that reduce the amount of power and heat that the sample may be exposed to.

In the second part of the present study, indirect ultrasound-assisted lipid extraction from flaxseed in a closed test tube system is optimized for quantitative assessment at settings of 60% of maximum probe amplitude for 40min at either 20°C or 40°C. The previously tested direct extraction method (Metherel et al., 2009b) has drawbacks that are avoided under the present ultrasound-assisted extraction design. The present indirect method prevents solvent evaporation that can change the ratio of extraction solvents and negatively affect extraction rates. In addition, the water surrounding the sample/solvent tube can be regulated to maintain lower temperatures that increase ultrasonic cavitation (Luque de Castro and Priego-Capote, 2007) on solids such as flaxseed and improve extraction yields. However, no difference between 20°C and 40°C on lipid extraction from flaxseed were observed presently. It is possible that both of these temperatures are low enough to create significant strength of cavitation and any further increases in temperature could reduce cavitation strength. The current ultrasonic setup is limited by the temperature of the tap water supplying the system, and as such 20°C – 40°C was the temperature range examined, and methods for accommodating a wider temperature range during extraction requires investigation.

The lower 60% probe amplitude provides less energy to the system and yields significantly higher fatty acid values compared to 100% amplitude. This is an expected result as longer probe amplitudes produce more cavitation bubbles and these bubbles tend to interfere with one another and reduce the interaction of the bubbles with the surface of the solid (Luque de Castro and Priego-Capote, 2007). The indirect ultrasound-assisted system also enables up to 13 samples to be processed at one time thereby providing further gains in throughput. Previous ultrasound-assisted methods have been based on adaptations to the Soxhlet apparatus that requires large solvent volumes and sample masses, as well as being suitable for only single sample analysis per probe (Luque-Garcia and Luque de Castro, 2004; Ruiz-Jimenez, Priego-Capote, and Luque de Castro, 2004; Wei et al., 2008). There is however, one study that also examined indirect ultrasound-assisted energy in combination with microwave-assisted energy for fatty acid extraction, although this study also used larger solvent and sample volumes and extraction of a single sample (Cravotto et al., 2008).

The current multi-sample indirect method of ultrasound-assisted extraction appears to be novel and has potential for application for the extraction of lipids from various food sources. Standard accepted methods for this purpose include the methods of the Association of Official Analytical Chemists (AOAC, 2005), International Organization for Standardization (Luque-Garcia and Luque de Castro, 2004) and the Folch method (Folch, Lees, and Sloane Stanley, 1957). A comparison of these methods for the extraction of lipids from flaxseed has been performed recently (Taha, Metherel, and Stark, 2012). These methods can require between 4 and 24 hours for completion of a single extraction protocol, and use up to 100mL of solvent and 10g of sample, as compared with the currently tested method of 40min, 3mL of solvent and 25mg of sample.

The primary goal of the indirect ultrasound-assisted methods tested was to determine the potential application of such a method for the expedited simultaneous extraction of lipids from flaxseed, and this potential is clearly demonstrated. The applicability of this method to other food matrices also requires further investigation, although a hard seed was selected in the present study due to their relative difficulty in extraction efficiency, and as such applicability to other foods is inferred. In addition, further refinement of the methods are required to determine optimal probe amplitude and the potential for combining other assisted-extraction techniques to further expedite the determined improvements in analysis shown here compared to standard methods of analysis. Such additional techniques may include convectional block heating, heated water baths or microwave-assisted lipid extractions.

CONCLUSION

In the present study, methods for the microwave-assisted direct transesterification of FTP whole blood and indirect ultrasound-assisted extraction of total lipids from flaxseed were developed and compared to conventional techniques. Reaction times are reduced significantly from 3h to 1.5min for microwave-assisted direct transesterification with H_2SO_4 and from 24 hours to 40min for ultrasound-assisted fatty acid extraction without any significant changes in either quantitative or qualitative fatty acid profiles. Shorter FTP analysis makes omega-3 health screening tests a more realistic pursuit as the cost and time of analysis may continue to drop allowing for high-throughput omega-3 fatty acid profiling for the purpose of disease detection and risk assessment. In addition, rapid analysis of foods also reduces time and monetary commitments for the determination of fatty acid content and subsequent government mandated nutritional labeling.

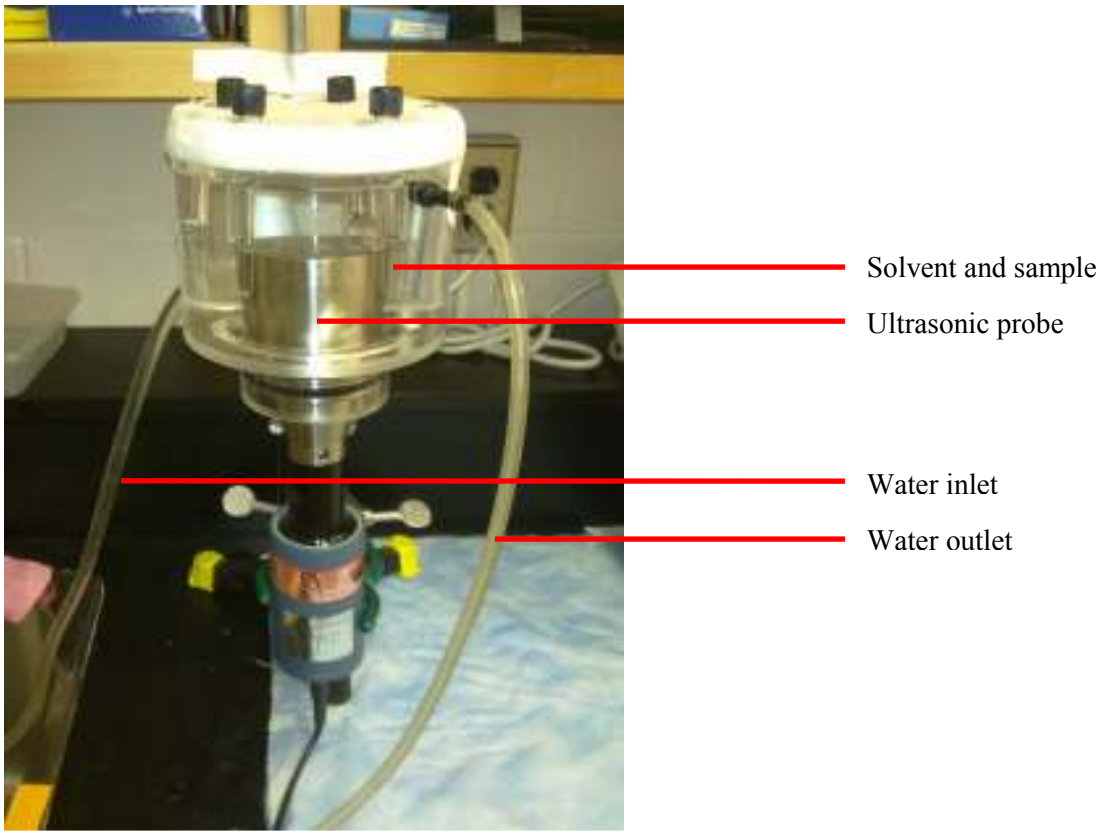

Figure 20 – Novel indirect ultrasound-assisted lipid extraction apparatus.

Table 9 – Fatty Acid Concentrations as Determined by Microwave-Assisted Direct Transesterification of FTP Blood

	1% H_2SO_4 Transesterification				
	Convection	Microwave with 30W			
Name	**3hr**	**5min**	**4min**	**3min**	**2min**
SFA	103 ± 4	121 ± 7#	115 ± 10	99.1 ± 5.5	77.6 ± 5.5*
MUFA	45.9 ± 2.1	54.0 ± 2.1#	52.0 ± 3.4#	43.5 ± 1.6	30.7 ± 1.1*
N-6 PUFA	85.1 ± 3.8	94.5 ± 3.6#	91.7 ± 5.8	78.2 ± 3.2	55.7 ± 1.8*
N-3 PUFA	16.5 ± 0.6	17.8 ± 0.9	17.5 ± 1.1	15.0 ± 1.1	11.3 ± 0.4*
PUFA	102 ± 4	112 ± 4#	109 ± 7	93.2 ± 4.3	67.0 ± 2.2*
HUFA	48.4 ± 2.2	52.5 ± 2.1	51.7 ± 3.2	44.8 ± 2.5	33.2 ± 1.0*
Total	251 ± 9	288 ± 11#	277 ± 20	237 ± 10	175 ± 8*
		Variable Power @ 160°C Max for 2min with H_2SO_4			
Name		**1%**	**2%**	**5%**	**10%**
SFA	103 ± 4	90.1 ± 11.1*	103 ± 8	106 ± 3	108 ± 6
MUFA	45.9 ± 2.1	38.2 ± 3.5*	48.0 ± 3.3	50.9 ± 1.6	46.8 ± 2.6
N-6 PUFA	85.1 ± 3.8	66.1 ± 2.5*	82.1 ± 4.6	84.3 ± 3.0	69.5 ± 2.3*
N-3 PUFA	16.5 ± 0.6	12.6 ± 0.6*	15.4 ± 0.8	15.4 ± 0.6	12.4 ± 0.6*
PUFA	102 ± 4	78.7 ± 3.1*	97.5 ± 5.4	99.6 ± 3.7	81.9 ± 2.9*
HUFA	48.4 ± 2.2	37.6 ± 1.5*	45.6 ± 2.4	45.8 ± 1.7	37.7 ± 1.4*
Total	251 ± 9	207 ± 17*	248 ± 17	256 ± 7	237 ± 11
		Variable Power @ 160°C Max with 5% H_2SO_4			
Name		**3min**	**2min**	**1.5min**	**1min**
SFA	103 ± 4	104 ± 9	106 ± 3	114 ± 5	92.3 ± 6.6
MUFA	45.9 ± 2.1	46.1 ± 2.1	50.9 ± 1.6	50.2 ± 3.3	39.8 ± 4.6
N-6 PUFA	85.1 ± 3.8	77.3 ± 3.3	84.3 ± 3.0	86.9 ± 5.0	70.8 ± 7.5*
N-3 PUFA	16.5 ± 0.6	14.1 ± 0.3*	15.4 ± 0.6	16.0 ± 0.9	13.6 ± 1.4*
PUFA	102 ± 4	91.5 ± 3.7	99.6 ± 3.7	103 ± 5.9	84.3 ± 8.8*
HUFA	48.4 ± 2.2	42.1 ± 1.3*	45.8 ± 1.7	47.7 ± 2.7	40.1 ± 4.2*
Total	251 ± 9	242 ± 13	256 ± 7	267 ± 14	216 ± 18*

*Indicates fatty acid values significantly lower and # indicates values significantly higher than 3 hr control values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data is presented as μg fatty acid per 100μL blood, mean ± SD. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid.

Table 10 – Qualitative Fatty Acid Profiles as Determined by Microwave-Assisted Direct Transesterification of FTP Blood

	1% H_2SO_4 Transesterification				
	Convection	Microwave with 30W			
Name	**3hr**	**5min**	**4min**	**3min**	**2min**
SFA	40.5 ± 0.9	41.4 ± 1.3	40.9 ± 0.8	41.4 ± 1.2	43.3 ± 1.2#
MUFA	17.9 ± 0.3	18.4 ± 0.5	18.5 ± 0.2	18.2 ± 0.3	17.1 ± 0.4*
N-6 PUFA	33.3 ± 0.7	32.3 ± 0.7	32.6 ± 0.6	32.6 ± 0.8	31.1 ± 0.7*
N-3 PUFA	6.44 ± 0.12	6.06 ± 0.21	6.22 ± 0.08	6.27 ± 0.24	6.30 ± 0.17
PUFA	39.7 ± 0.8	38.3 ± 0.9	38.8 ± 0.7	38.9 ± 0.9	37.4 ± 0.9*
HUFA	18.9 ± 0.4	17.9 ± 0.5*	18.4 ± 0.3	18.7 ± 0.5	18.6 ± 0.5
		Variable Power @ 160C Max for 2min with H_2SO_4			
Name		**1%**	**2%**	**5%**	**10%**
SFA	40.5 ± 0.9	42.4 ± 1.7	40.4 ± 0.4	40.2 ± 0.2	38.4 ± 1.2
MUFA	17.9 ± 0.34	18.0 ± 0.7	18.8 ± 0.2#	19.3 ± 0.2#	16.6 ± 0.3*
N-6 PUFA	33.3 ± 0.7	31.2 ± 1.6*	32.3 ± 0.6	32.0 ± 0.6	24.7 ± 0.8*
N-3 PUFA	6.44 ± 0.12	5.97 ± 0.24*	6.03 ± 0.14*	5.84 ± 0.10*	4.42 ± 0.2*
PUFA	39.7 ± 0.8	37.2 ± 1.8*	38.3 ± 0.7	37.9 ± 0.7	29.1 ± 0.9*
HUFA	18.9 ± 0.4	17.8 ± 0.9	17.9 ± 0.4	17.4 ± 0.3*	13.4 ± 0.6*
		Variable Power @ 160C Max with 5% H_2SO_4			
Name		**3min**	**2min**	**1.5min**	**1min**
SFA	40.5 ± 0.9	41.5 ± 1.9	40.2 ± 0.2	41.8 ± 0.6	41.8 ± 2.2
MUFA	17.9 ± 0.3	18.4 ± 0.6	19.3 ± 0.2#	18.3 ± 0.2	18.0 ± 0.8
N-6 PUFA	33.3 ± 0.7	30.8 ± 1.0*	32.0 ± 0. 6	31.8 ± 0.5	32.0 ± 1.2
N-3 PUFA	6.44 ± 0.12	5.64 ± 0.21*	5.84 ± 0.1*	5.83 ± 0.06*	6.13 ± 0.26
PUFA	39.7 ± 0.8	36.4 ± 1.1*	37.9 ± 0.7	37.6 ± 0.6	38.1 ± 1.5
HUFA	18.9 ± 0.4	16.8 ± 0.6*	17.4 ± 0.3*	17.5 ± 0.3*	18.1 ± 0.8

*Indicates fatty acid values significantly lower and # indicates values significantly higher than 3 hr control values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data is presented as % weight of fatty acid in total fatty acids, mean ± SD. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; H_2SO_4, sulphuric acid in methanol.

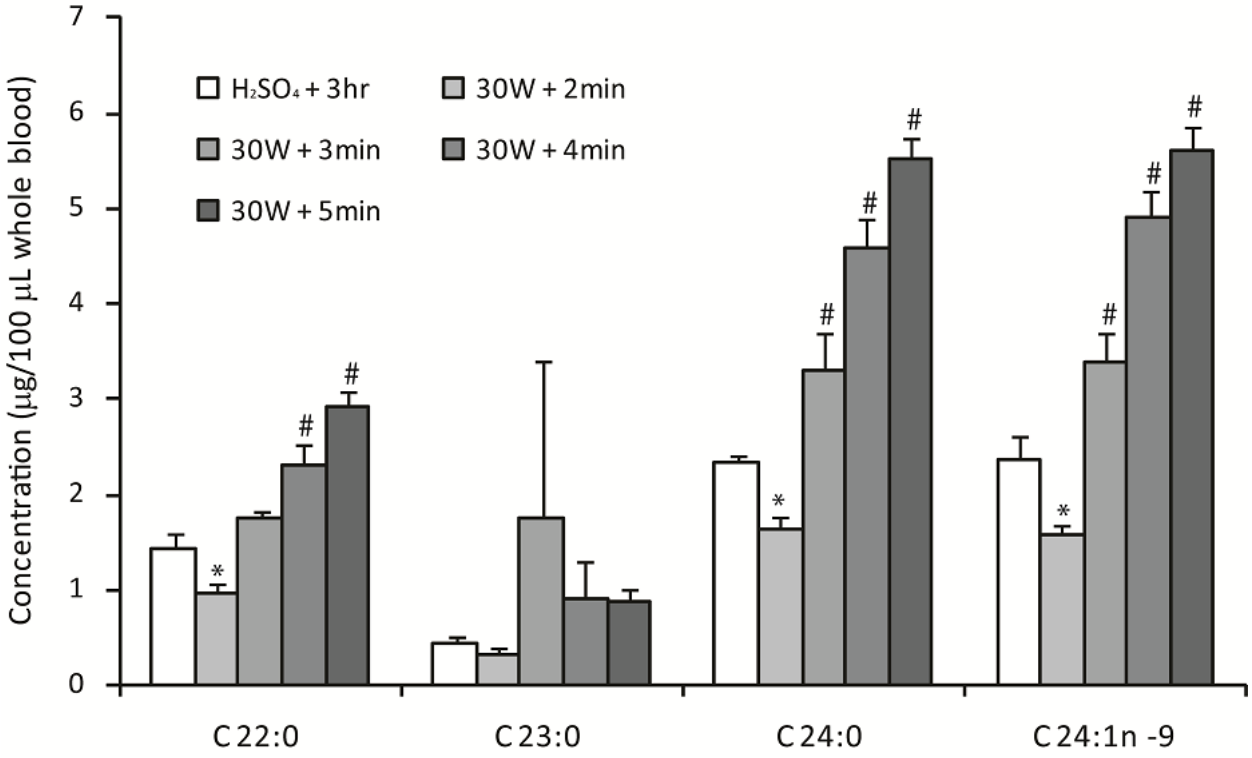

Figure 21 – Very long-chain SFA and MUFA yield in FTP blood following microwave-assisted direct transesterification. *Indicates fatty acid values significantly lower and # indicates values significantly higher than 3 hr control values as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data is presented as µg fatty acid per 100µL blood, mean ± SD. H_2SO_4; 1% sulphuric acid in methanol.

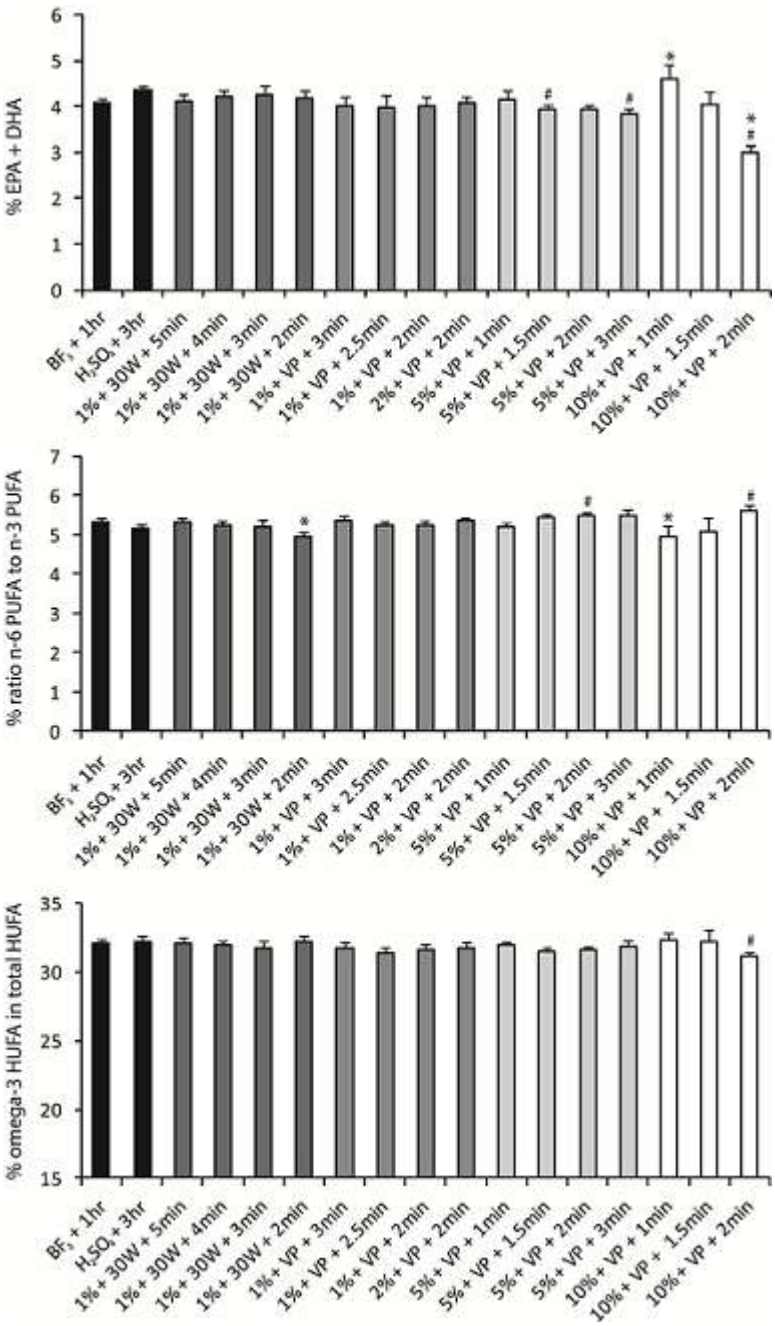

Figure 22 – FTP blood n-3 biomarkers as determined by microwave-assisted direct transesterification. *Indicates omega-3 biomarker significantly different than conventional BF_3 method and # indicates values significantly different than conventional H_2SO_4 method as determined by Tukey's HSD post hoc test following a significant F-value by Oneway ANOVA. Data presented as mean ± SD. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; VP, variable power method; BF_3, boron trifluoride in methanol; H_2SO_4, sulphuric acid in methanol.

Table 11 – Fatty Acid Concentration Yields Following Ultrasound-Assisted Lipid Extraction from Flaxseed

		20°C					
		60% Amplitude			100% Amplitude		
Name	**Folch**	**20min**	**40min**	**60min**	**20min**	**40min**	**60min**
C 16:0 [1,2]	0.42 ± 0.01	0.35 ± 0.02*	0.39 ± 0.01	0.38 ± 0.03	0.30 ± 0.04*	0.37 ± 0.03	0.39 ± 0.04
C 18:0 [3]	0.29 ± 0.01	0.26 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.24 ± 0.02*	0.28 ± 0.02	0.29 ± 0.02
C 18:1n-9 [1,2]	1.50 ± 0.07	1.29 ± 0.08	1.49 ± 0.09	1.43 ± 0.08	1.11 ± 0.19*	1.33 ± 0.16	1.42 ± 0.12
C 18:2n-6 [1,2]	1.38 ± 0.02	1.19 ± 0.05*	1.35 ± 0.01	1.28 ± 0.06	1.01 ± 0.16*	1.18 ± 0.11*	1.25 ± 0.10
C 18:3n-3 [1,2]	5.07 ± 0.17	4.39 ± 0.17*	4.91 ± 0.07	4.66 ± 0.18	3.68 ± 0.57*	4.29 ± 0.32*	4.57 ± 0.35
SFA [3]	0.75 ± 0.02	0.65 ± 0.03	0.73 ± 0.02	0.71 ± 0.03	0.58 ± 0.07*	0.70 ± 0.05	0.73 ± 0.06
PUFA [1,2]	6.47 ± 0.19	5.60 ± 0.22*	6.28 ± 0.08	5.96 ± 0.25	4.71 ± 0.73*	5.49 ± 0.43*	5.84 ± 0.45
Total [1,2]	8.79 ± 0.27	7.60 ± 0.32*	8.56 ± 0.11	8.17 ± 0.33	6.45 ± 0.99*	7.58 ± 0.63*	8.05 ± 0.62
		40°C					
		60% Amplitude			**100% Amplitude**		
Name		**20min**	**40min**	**60min**	**20min**	**40min**	**60min**
C 16:0 [1,2]		0.35 ± 0.01*	0.41 ± 0.01	0.38 ± 0.02	0.34 ± 0.02*	0.35 ± 0.03*	0.38 ± 0.02
C 18:0 [3]		0.27 ± 0.01	0.31 ± 0.01	0.27 ± 0.01	0.26 ± 0.02	0.26 ± 0.02	0.28 ± 0.01
C 18:1n-9 [1,2]		1.25 ± 0.05	1.51 ± 0.07	1.42 ± 0.09	1.22 ± 0.09*	1.32 ± 0.12	1.33 ± 0.04
C 18:2n-6 [1,2]		1.15 ± 0.05*	1.31 ± 0.02	1.28 ± 0.05	1.10 ± 0.04*	1.18 ± 0.09*	1.23 ± 0.04
C 18:3n-3 [1,2]		4.23 ± 0.22*	4.77 ± 0.07	4.62 ± 0.18	4.04 ± 0.17*	4.32 ± 0.30*	4.47 ± 0.09
SFA [3]		0.67 ± 0.01	0.76 ± 0.02	0.70 ± 0.02	0.64 ± 0.05*	0.65 ± 0.06	0.70 ± 0.03
PUFA [1,2]		5.40 ± 0.26*	6.09 ± 0.09	5.91 ± 0.23	5.16 ± 0.21*	5.51 ± 0.38*	5.71 ± 0.12
Total [1,2]		7.37 ± 0.31*	8.43 ± 0.17	8.10 ± 0.34	7.07 ± 0.32*	7.56 ± 0.56*	7.81 ± 0.18

*Indicates fatty acid values significantly lower than 24 hr standard Folch extraction as determined by Tukey's HSD post hoc test following a significant F-value by one-way ANOVA. [1], indicates a significant amplitude effect; [2], indicates a significant time effect; [3], indicates a significant temperature x time effect as determined by three-way ANOVA. Data is presented as mg fatty acid per 25mg flaxseed, mean ± SD. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid

Table 12 – Qualitative Fatty Acid Profiles Following Ultrasound-Assisted Lipid Extraction from Flaxseed

		20°C					
		60% Amplitude			100% Amplitude		
Name	**Folch**	**20min**	**40min**	**60min**	**20min**	**40min**	**60min**
C 16:0 [5]	4.71 ± 0.04	4.53 ± 0.06	4.56 ± 0.12	4.64 ± 0.13	4.69 ± 0.11	4.82 ± 0.08	4.79 ± 0.17
C 18:0 [3,5]	3.24 ± 0.08	3.41 ± 0.05	3.33 ± 0.06	3.42 ± 0.18	3.66 ± 0.22*	3.73 ± 0.18*	3.60 ± 0.10*
C 18:1n-9	16.9 ± 0.4	16.9 ± 0.3	17.3 ± 0.9	17.4 ± 0.6	17.0 ± 0.3	17.3 ± 0.8	17.4 ± 0.5
C 18:2n-6 [4]	15.6 ± 0.2	15.5 ± 0.1	15.6 ± 0.1	15.6 ± 0.2	15.5 ± 0.1	15.5 ± 0.3	15.4 ± 0.2
C 18:3n-3	57.2 ± 0.5	57.3 ± 0.2	56.9 ± 0.9	56.6 ± 0.5	56.5 ± 0.4	56.1 ± 0.7	56.3 ± 0.8
SFA [3,5]	8.45 ± 0.11	8.47 ± 0.04	8.40 ± 0.12	8.59 ± 0.14	8.94 ± 0.37	9.17 ± 0.14*	8.96 ± 0.17*
PUFA	73.0 ± 0.55	73.0 ± 0.2	72.7 ± 1.0	72.4 ± 0.6	72.2 ± 0.5	71.8 ± 0.8	72.0 ± 0.7
		40°C					
		60% Amplitude			**100% Amplitude**		
Name		**20min**	**40min**	**60min**	**20min**	**40min**	**60min**
C 16:0 [5]		4.75 ± 0.14	4.65 ± 0.11	4.80 ± 0.06	4.69 ± 0.11	4.61 ± 0.11	4.81 ± 0.10
C 18:0 [3,5]		3.69 ± 0.15*	3.36 ± 0.05	3.60 ± 0.05*	3.69 ± 0.15*	3.35 ± 0.14	3.54 ± 0.10
C 18:1n-9		16.8 ± 0.4	17.4 ± 0.4	17.7 ± 0.6	17.1 ± 0.8	17.2 ± 0.4	16.9 ± 0.4
C 18:2n-6 [4]		15.5 ± 0.1	15.7 ± 0.1	15.4 ± 0.1	15.4 ± 0.1	15.4 ± 0.2	15.6 ± 0.2
C 18:3n-3		56.8 ± 0.6	56.6 ± 0.4	56.1 ± 0.7	56.6 ± 1.0	56.2 ± 0.9	56.8 ± 0.3
SFA [3,5]		9.03 ± 0.30*	8.54 ± 0.08	8.99 ± 0.10*	8.96 ± 0.29*	8.47 ± 0.23	8.91 ± 0.21
PUFA		72.5 ± 0.6	72.5 ± 0.4	71.7 ± 0.7	72.2 ± 1.0	71.8 ± 0.9	72.6 ± 0.4

*Indicates fatty acid values significantly lower than 24 hr standard Folch extraction as determined by Tukey's HSD post hoc test following a significant F-value by one-way ANOVA. [3], indicates a significant temperature x time interaction; [4], indicates a significant amplitude x time interaction; [5], indicates a significant temperature x amplitude interaction as determined by three-way ANOVA. Data is presented as % weight of fatty acid in total fatty acids, mean ± SD. SFA, saturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid

CHAPTER 9

THE DEVELOPMENT OF FATTY ACID PREDICTIVE EQUATIONS FROM FINGERTIP PRICK WHOLE BLOOD FOR ESTIMATION IN PLASMA AND ERYTHROCYTE BLOOD FRACTIONS

INTRODUCTION

Fatty acid compositions, and as such, n-3 biomarkers are commonly reported in a variety of blood fractions, and most commonly in erythrocytes, plasma PL and TAG. The numerous blood fractions utilized for fatty acid analysis and the implications of each can make it difficult to compare between various scientific studies. Erythrocytes tend to reflect long-term dietary intake, plasma PL cell membrane content, plasma TAG adipose tissue stores and CE short-term dietary intake (Stark, 2008a). Fatty acid profiling in each of these blood fractions requires numerous analytical steps to 1) separate erythrocytes and plasma and 2) separate lipid fractions within plasma by thin layer chromatography. Whole blood, on the other hand, is a relatively equal distribution of both erythrocytes and plasma and, therefore, could theoretically be utilized for the estimation of both erythrocyte and plasma fatty acid or n-3 biomarker profiles. Whole blood is also a simpler method of analysis requiring no additional manipulation following collections, and is amenable to high-throughput FTP fatty acid analysis that does not require a lipid extraction step prior to transesterification to fatty acid methyl esters (Armstrong, Metherel, and Stark, 2008; Marangoni, Colombo, and Galli, 2004).

Previously, predictive equations have been developed for plasma PL and TAG HUFA from n-3 and n-6 fatty acid intake (Lands et al., 1992), total cholesterol and low-density lipoprotein cholesterol content in plasma (Yu et al., 1995) and serum (Muller, Kirkhus, and

Pedersen, 2001) from trans fatty acid and SFA intake and plasma TAG from n-3 fatty acid intake (Musa-Veloso et al., 2010). Although, correlational data and predictive values of various fatty acid measures have been assessed between multiple blood fractions (Bell et al., 2011; Kawabata et al., 2011; Rizzo et al., 2010; Stark, 2008b), to our knowledge no previous study has extended this to the development of predictive equations to accompany these assessments. In the present study, blood samples were collected from human volunteers and SFA, MUFA, PUFA and n-3 biomarkers EPA, DHA, % EPA+DHA and % n-3 HUFA in total HUFA (% n-3 HUFA) were measured. The predictive ability of FTP blood fatty acids and biomarkers were determined for estimation in erythrocytes and plasma TLE, PL, TAG, CE and NEFA, with accompanying predictive equations also developed.

METHODS AND MATERIALS

Study Design

Three individuals (2 men, 1 woman) between the ages of 25 and 30 and with different % n-3 HUFA status were recruited for blood collection. Blood was collected into a single 8mL EDTA-lined vacutainer. Aliquots of 25µL of whole blood was applied to chromatography strips for FTP fatty acid analysis. These FTP blood samples were used as the independent variable in the regression modeling and baseline values are presented (**Table 13**). An extra 500µL of whole blood was aliquoted. The remaining whole blood was centrifuged and the top plasma layer was aliquoted and stored at -75°C for later fatty acid analysis. The remaining erythrocytes were washed with saline and centrifuged twice before 500µL aliquots were removed and placed in storage at -75°C for future fatty acid analysis.

Data and Statistical Analyses

Differences in the subject pools average fatty acid composition were determined between blood fractions by one-way ANOVA by significant F-value, and post hoc analysis was performed using Tukey's Honestly Significant Difference test. A linear regression model was applied to the data set using FTP blood as the predictive independent variable and the remaining blood fractions as the predicted dependent variables. With the small subject pool (n = 3), statistically significant correlations were determined at a level of $p < 0.10$. All correlations reaching this significance level also obtained an R^2 of greater than 0.90. The relative contribution to whole blood of specific fatty acids and fatty acid subclasses in erythrocyte and plasma blood fractions (**Table 14**) were determined by participants measured hematocrit values and quantitative fatty acid values.

RESULTS

Blood Fraction Fatty Acid Characteristics

Presently, FTP whole blood was utilized in an attempt to develop equations that allow for the translation of FTP blood fatty acid values for the determination of the same fatty acid values in alternate blood fractions. The averages of the fatty acid compositions were determined to provide a comprehensive understanding of where these fatty acids are preferentially located in the blood (**Table 15**). In blood fractions that contain phospholipids (i.e. FTP, erythrocyte, plasma TLE and plasma PL) the predominant fatty acids subclasses are SFA and n-6 PUFA, followed by MUFA. Plasma TLE, however, has roughly equal distribution of these three fatty acid subclasses. Conversely, plasma TAG are predominantly MUFA and SFA, while plasma CE are predominantly MUFA and n-6 PUFA and plasma NEFA are SFA and MUFA. Both

erythrocytes and plasma PL contain more relative n-3 PUFA and HUFA compared to other blood fractions. EPA is higher in plasma TLE and PL, and DHA is higher in erythrocytes and plasma PL. Interestingly, % n-3 HUFA values are relatively consistent across all phospholipid containing blood fractions.

Translational Equation Development for Fatty Acid Subclasses

Linear regression models were applied to FTP blood as the independent predictive variable for the purpose of estimating the composition of the n-3 biomarkers % n-3 HUFA (**Figure 23**) and EPA+DHA (**Figure 24**), n-3 fatty acids EPA and DHA and fatty acid subclasses SFA, MUFA and PUFA in erythrocytes and plasma TLE and PL (**Table 16**) and plasma TAG, CE and NEFA (**Table 17**). Shaded out values in the tables represent regression models that did not reach significance of $P < 0.10$. The predictive ability of FTP % n-3 HUFA appears high as this biomarker in all six measured blood fractions could be accurately predicted with R^2 values of at least 0.98 for all (Figure 23). Interestingly, plasma NEFA was the only blood fraction to demonstrate an inverse relationship to FTP % n-3 HUFA. Visually, it can be seen that all 3 data points in the model land on or very near the regression line, while there is more separation seen in the EPA+DHA regression model. EPA+DHA in FTP also shows strong predictive strength for erythrocytes and plasma TLE, PL and CE, however, this is not seen for plasma TAG ($R^2 = 0.62$) or NEFA ($R^2 = 0.06$) (Figure 24).

Of the 4 n-3 biomarkers measured (EPA, DHA, EPA+DHA and % n-3 HUFA), all 4 FTP values could be used to accurately estimate their values in erythrocytes, plasma TLE and PL, the 3 most commonly used blood fractions for determining n-3 status in individuals. However, beyond % n-3 HUFA, EPA+DHA in plasma CE was the only other n-3 biomarker that could be

accurately predicted from FTP values in all of plasma TAG, CE and NEFA. For the fatty acid subclasses tested, a reliable linear equation could not be developed for any blood fraction for SFA. A reliable MUFA equation could be developed for plasma TLE and NEFA, and a PUFA equation for plasma CE and NEFA.

Blood Fraction Contribution to Whole Blood Fatty Acid Content

To assist in explaining why FTP blood for specific fatty acid measures can be predictive in some blood fractions but not others, the percent contribution of the blood fractions for each fatty acid measure in FTP whole blood was determined (Table 18). In our three participants, plasma TLE accounted for nearly two-thirds of SFA, approximately three-quarters of MUFA, n-6 PUFA and PUFA and more than half of n-3 PUFA. The largest contributions from plasma blood fractions are attributed to PL in all fractions except MUFA that were located in plasma TAG one-third of the time in whole blood. MUFA are also high in plasma CE (16%) and PL (17%) in whole blood. In addition to plasma PL, plasma CE also contain significant amounts of n-6 PUFA and together account for nearly half of all n-6 PUFA in whole blood. HUFAs, and similarly EPA+DHA are much higher in erythrocytes (~40%) compared to other fatty acid subclasses, and together with plasma PL make up approximately 70% of all HUFA and EPA+DHA found in the whole blood.

DISCUSSION

Results of the current study indicate that fatty acid biomarkers in the simpler FTP whole blood collection method can be used as an estimator of those same biomarkers in the more difficultly measured erythrocyte and plasma blood fractions. Each of EPA, DHA and

EPA+DHA in FTP blood are strong predictors of these n-3 biomarkers in erythrocytes, plasma TLE and plasma PL. Interestingly, these three biomarkers provide measurements of n-3 HUFA content in blood and are most abundantly located in the *sn*-2 position of phospholipids (Stark, 2008a), and phospholipids are present in each of the aforementioned blood fractions and no other. This is not surprising as blood fractions of similar lipid composition (i.e. high in phospholipids) should correlate well, and subsequently demonstrate strong predictive strength for n-3 HUFA in these high phospholipid blood fractions. This is shown for n-3 HUFA in erythrocyte, plasma phospholipid and plasma TLE blood fractions (Table 16). For instance, EPA is relatively evenly distributed across erythrocytes (26%), plasma PL (32%) and is also relatively high in plasma CE (17%). These three blood fractions in addition to plasma TLE (74% of EPA) are strongly predicted by FTP EPA content. Similarly, DHA is distributed mostly in erythrocytes (44%) and plasma PL (31%), and these fractions as well as plasma TLE (56%) are strongly predicted from FTP DHA, and the same can be said for EPA+DHA in erythrocytes (40%) and plasma PL (31%).

To our knowledge, only two previous studies have analyzed the correlations between n-3 biomarkers in whole blood and erythrocytes. No other correlational analyses could be found for whole blood and any other blood fraction. It has been determined that the biomarkers AA/EPA (R^2=0.8765) and the n-6/n-3 PUFA ratio (R^2=0.7114) were highly correlated between the two blood fractions (Rizzo et al., 2010). Their goal was to verify that the simpler whole blood n-3 biomarker measures could be used in place of the more cumbersome but also more frequently used RBC measure. Subsequently, whole blood measures were used to determine the health benefits of individuals consuming n-3 supplements, however, whole blood measures were not used as a predictive tool for the determination of these measures in erythrocytes. In addition,

regression analyses have been performed for EPA, AA, DHA, AA:EPA ratio and % n-3 HUFA between FTP blood and erythrocytes with generally good correlational agreement (Bell et al., 2011). Both studies support our current findings that FTP blood n-3 HUFA content is a strong predictor of erythrocyte n-3 HUFA content.

Interestingly, these aforementioned correlational analyses were strongest in the % n-3 HUFA measure, a finding supported by our study as we demonstrate that the % n-3 HUFA biomarker is a strong predictor of % n-3 HUFA in every blood fraction measured. We demonstrate R^2 value of no lower than 0.98, with erythrocytes, plasma TLE and plasma PL all with $R^2 = 1.0$. This advantage of % n-3 HUFA over EPA+DHA and n-6/n-3 ratio has previously been shown (Stark, 2008b) with the % n-3 HUFA in blood being more robust when predicting for % n-3 HUFA in liver, heart and brain compared to EPA+DHA. Expression of an individual's n-3 status as % n-3 HUFA reduces the differences between different blood fractions by focusing on the HUFA pool. HUFA tend to be trans-esterified into the *sn*-2 position of PL (Stark, 2008a), and this reflects their structural and cell-signaling importance in membranes (Stark, 2008b). This aids in explaining why n-3 biomarkers in FTP blood are highly predictive of erythrocyte, plasma PL and plasma TLE n-3 biomarkers as each of these fractions are high in phospholipids. The % n-3 HUFA measure is a relative measure of n-3 HUFA to n-6 HUFA and relative incorporation rates may be similar within a blood fraction. For this reason, predictive ability of this measure extends across each blood fraction measured, although generally speaking is representative of the competition between HUFA for the incorporation into the *sn*-2 position of PL (Stark, 2008a).

Similar to the predictive ability of FTP blood for n-3 HUFA biomarkers, SFA, MUFA and PUFA can also be predicted, however, the relationships are not as clear. SFA were not

statistically correlated between FTP blood and any other blood fraction and as such predictive equations developed are likely to be inaccurate. MUFA in FTP blood were only predictive of plasma TLE where 77% of all MUFA were located. High correlations, however, were determined for MUFA between FTP blood and plasma TAG (R^2=0.85), plasma CE (R^2=0.82) and plasma PL (R^2 = 0.69) and represent 33, 16 and 17% of total MUFA in whole blood, respectively. A larger subject pool in this case may provide significant correlations between the blood fractions and subsequently more accurate predictive equations may be produced. Plasma CE contains 20% of the PUFA in whole blood and represents the only blood fraction that is significantly correlated to FTP blood. This does not follow our general theory of FTP blood predicting the more similar blood fractions, which in the case of PUFA is plasma CE as well as erythrocytes and plasma PL that are each represented by 26% of all whole blood PUFA. Linoleic acid (LA, 18:2n-6) is the most abundant PUFA in blood and tends to drive PUFA composition. In our subject pool we can see that 31% of whole blood linoleic acid is found in plasma CE, and represents the most abundant LA-containing lipid pool, and likely explains the high correlation between plasma CE and FTP blood PUFA.

CONCLUSION

Based on a subject pool of 3 individuals with low, moderate and high n-3 HUFA status it can be concluded that FTP blood can be used to estimate EPA, DHA, EPA+DHA and % n-3 HUFA in erythrocytes, plasma PL and plasma TLE. These predicted fractions are the most commonly utilized blood fractions for determining the n-3 status of an individual. This is a significant finding as FTP blood is a much simpler method of analysis that can be performed in a single one-step procedure that does not require venous blood collection, blood separation or lipid

class separations. Stronger conclusions about the predictive strength of the developed equations could be made with a larger subject pool, and the developed equations may only apply to a healthy population, and further predictive equations may be required for specific diseased population that can affect lipid components of the blood such as diabetes or anemia. In addition, further regression analysis needs to be performed between all blood fractions to allow for easier interpretation of multiple data sets analyzed from various blood fractions.

Table 13 – Baseline Participant FTP Blood Fatty Acid Composition

	Participant (omega-3 status)		
Fatty Acid	**Low**	**Moderate**	**High**
C 16:0	19.4	22.5	18.9
C 18:0	14.2	9.4	12.2
C 24:0	2.06	1.53	2.01
SFA	**40.5**	**37.5**	**38.1**
C 18:1n-7	1.85	1.93	1.86
C 18:1n-9	13.4	16.8	19.1
C 24:1n-9	2.19	2.09	2.02
MUFA	**19.1**	**23.9**	**24.8**
C 18:2n-6	19.9	17.6	17.8
C 20:4n-6	10.4	8.7	7.8
N-6	**33.4**	**30.4**	**28.4**
C 18:3n-3	0.40	0.52	0.46
C 20:5n-3	0.46	0.66	1.27
C 22:5n-3	1.17	0.99	1.61
C 22:6n-3	2.43	3.51	2.81
N-3	**4.49**	**5.73**	**6.21**
PUFA	**37.9**	**36.1**	**34.6**
HUFA	**17.0**	**17.4**	**15.8**
EPA+DHA	**2.89**	**4.16**	**4.08**
N-6/N-3	**7.43**	**5.30**	**4.57**
% N-3 HUFA	**24.0**	**30.0**	**36.4**
Total (μg/100μL)	**393.2**	**395.2**	**335.0**

Values expressed as weight % of fatty acid in total fatty acids. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Table 14 – Average Relative Blood Fraction Contribution of Fatty Acids and Subclasses in Whole Blood

	Whole Blood		Plasma			
Name	**RBC**	**Plasma**	**PL**	**TAG**	**CE**	**NEFA**
C 16:0	28.9	71.1	35.8	16.0	7.86	4.95
C 18:0	41.5	58.5	35.6	6.47	3.94	10.5
C 24:0	72.4	27.6	15.6	0.70	0.54	1.20
SFAs	36.3	63.7	34.1	12.0	6.35	6.41
C 18:1n-7	21.8	78.2	29.0	25.5	10.6	3.22
C 18:1n-9	20.3	79.7	15.2	36.2	17.5	4.53
C 24:1n-9	58.8	41.2	25.9	0.69	0.55	0.63
MUFAs	23.2	76.8	17.3	32.5	16.3	4.47
C 18:2n-6	12.1	87.9	24.1	12.9	31.1	1.25
C 20:4n-6	39.5	60.5	29.3	2.52	11.2	0.22
N-6	22.8	77.2	26.2	9.39	23.1	1.00
C 18:3n-3	7.96	92.0	11.2	39.5	19.1	4.97
C 20:5n-3	26.4	73.6	31.6	4.73	16.5	0.88
C 22:5n-3	59.9	40.1	20.0	5.91	1.01	0.80
C 22:6n-3	43.8	56.2	30.8	3.47	3.35	0.60
N-3	41.4	58.56	27.1	7.29	6.25	1.09
PUFAs	25.7	74.3	26.3	9.07	20.5	1.01
HUFAs	42.1	57.9	29.4	3.43	8.09	0.48
EPA+DHA	40.0	60.0	31.0	3.74	6.25	0.66
Total	29.0	71.0	27.2	15.6	14.5	3.83

Values expressed as percentage of blood fraction fatty acid in whole blood. RBC, red blood cell; PL, phospholipid; TAG, triacylglycerol; CE, cholesteryl ester; NEFA, non-esterified fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid.

Table 15 – Average Fatty Acid and Subclass Profiles in Weight Percent for Multiple Blood Fractions

			Plasma				
Fatty Acid	**Fingertip Prick**	**Erythrocytes**	**Total Lipid Extract**	**Phospholipids**	**Triacylglycerols**	**Cholesteryl Esters**	**Non-Esterified FA**
			% weight in total fatty acids				
C 16:0	20.3 ± 1.9^{a}	20.1 ± 1.7^{a}	20.2 ± 2.1	26.6 ± 2.3^{a}	20.7 ± 4.0^{a}	11.0 ± 1.8^{b}	26.1 ± 1.9^{a}
C 18:0	11.9 ± 2.4^{ab}	14.6 ± 0.8^{a}	8.40 ± 1.80^{bc}	13.3 ± 1.3^{ab}	4.23 ± 0.42^{c}	2.77 ± 0.29^{c}	27.9 ± 4.5^{d}
C 24:0	1.87 ± 0.29^{a}	3.35 ± 0.21^{b}	0.52 ± 0.18^{cd}	0.77 ± 0.31^{c}	0.06 ± 0.02^{d}	0.05 ± 0.01^{d}	0.42 ± 0.19^{cd}
SFAs	**38.7 ± 1.6^{ab}**	**44.5 ± 1.2^{a}**	**31.8 ± 2.3^{bc}**	**44.5 ± 1.9^{a}**	**27.3 ± 4.4^{c}**	**15.6 ± 2.0^{d}**	**59.3 ± 6.6^{e}**
C 18:1n-7	1.88 ± 0.05^{a}	1.29 ± 0.05^{a}	1.89 ± 0.16^{a}	1.83 ± 0.10^{a}	2.81 ± 0.47^{b}	1.26 ± 0.19^{a}	1.44 ± 0.47^{a}
C 18:1n-9	16.4 ± 2.8^{a}	11.5 ± 1.3^{a}	18.4 ± 4.9^{a}	9.14 ± 2.33^{a}	38.0 ± 7.7^{b}	19.8 ± 6.9^{a}	19.4 ± 3.3^{a}
C 24:1n-9	2.10 ± 0.09^{b}	3.22 ± 0.23^{a}	0.92 ± 0.25^{c}	1.51 ± 0.43^{bc}	0.07 ± 0.04^{d}	0.06 ± 0.01^{d}	0.26 ± 0.01^{d}
MUFAs	**22.6 ± 3.1^{a}**	**17.3 ± 1.3^{a}**	**23.4 ± 5.2^{a}**	**13.7 ± 2.0^{a}**	**45.2 ± 7.6^{b}**	**24.3 ± 6.3^{a}**	**25.2 ± 3.9^{a}**
C 18:2n-6	18.4 ± 1.3^{c}	8.66 ± 0.45^{ab}	25.7 ± 3.3^{c}	18.4 ± 1.3^{c}	17.2 ± 6.1^{bc}	44.6 ± 4.7^{d}	6.80 ± 2.54^{a}
C 20:4n-6	8.96 ± 1.31^{ab}	12.8 ± 1.0^{a}	8.02 ± 1.88^{ab}	10.2 ± 1.4^{ab}	1.52 ± 0.81^{c}	7.26 ± 2.45^{b}	0.55 ± 0.18^{c}
N-6	**30.7 ± 2.5^{ab}**	**26.6 ± 1.02^{ab}**	**36.7 ± 4.5^{a}**	**32.5 ± 1.0^{a}**	**20.3 ± 7.0^{bc}**	**53.8 ± 6.5^{d}**	**8.80 ± 2.61^{c}**
C 18:3n-3	0.46 ± 0.06^{ab}	0.14 ± 0.03^{a}	0.66 ± 0.04^{b}	0.21 ± 0.05^{a}	1.29 ± 0.35^{c}	0.67 ± 0.17^{b}	0.66 ± 0.18^{b}
C 20:5n-3	0.79 ± 0.42	0.87 ± 0.46	0.99 ± 0.59	1.11 ± 0.77	0.29 ± 0.18	1.09 ± 0.35	0.22 ± 0.15
C 22:5n-3	1.26 ± 0.32^{bc}	2.67 ± 0.71^{a}	0.73 ± 0.12^{bcd}	0.95 ± 0.10^{bcd}	0.49 ± 0.09^{cd}	0.09 ± 0.01^{d}	0.27 ± 0.10^{d}
C 22:6n-3	2.92 ± 0.55^{b}	5.10 ± 1.02^{a}	2.67 ± 0.44^{b}	3.82 ± 0.72^{ab}	0.75 ± 0.21^{c}	0.78 ± 0.20^{c}	0.53 ± 0.15^{c}
N-3	**5.48 ± 0.88^{bc}**	**8.81 ± 1.42^{a}**	**5.08 ± 0.81^{bcd}**	**6.14 ± 1.05^{b}**	**2.88 ± 0.39^{cde}**	**2.66 ± 0.46^{de}**	**1.75 ± 0.27^{e}**
PUFAs	**36.2 ± 1.7^{a}**	**35.4 ± 0.9^{a}**	**41.8 ± 3.8^{a}**	**38.7 ± 0.4^{a}**	**23.2 ± 7.1^{b}**	**56.4 ± 6.1^{c}**	**10.6 ± 2.9^{d}**
HUFAs	**16.7 ± 0.8^{bc}**	**26.2 ± 1.0^{a}**	**14.7 ± 1.5^{c}**	**19.5 ± 1.6^{b}**	**3.96 ± 0.52^{e}**	**10.1 ± 2.5^{d}**	**2.25 ± 0.36^{e}**
EPA+DHA	**3.71 ± 0.71^{bc}**	**5.98 ± 1.15^{a}**	**3.66 ± 0.69^{bc}**	**4.93 ± 0.99^{ab}**	**1.04 ± 0.32^{d}**	**1.87 ± 0.33^{cd}**	**0.75 ± 0.08^{d}**
N-6/N-3	**5.77 ± 1.48^{a}**	**3.08 ± 0.62^{a}**	**7.44 ± 2.19^{a}**	**5.43 ± 1.2^{a}**	**7.09 ± 2.40^{a}**	**20.9 ± 6.4^{b}**	**4.95 ± 0.87^{a}**
% N-3 HUFA	**30.1 ± 6.2^{ab}**	**33.1 ± 5.2^{ab}**	**30.6 ± 8.2^{ab}**	**30.6 ± 6.2^{ab}**	**41.4 ± 15.6^{ab}**	**21.1 ± 8.3^{a}**	**48.8 ± 1.1^{b}**

Values with different letters within a row are significantly different by Tukey's HSD post hoc procedure ($P < 0.05$) after a significant F-value by one-way ANOVA ($P < 0.05$). Values are Mean ± S.D, n = 3. FA, fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

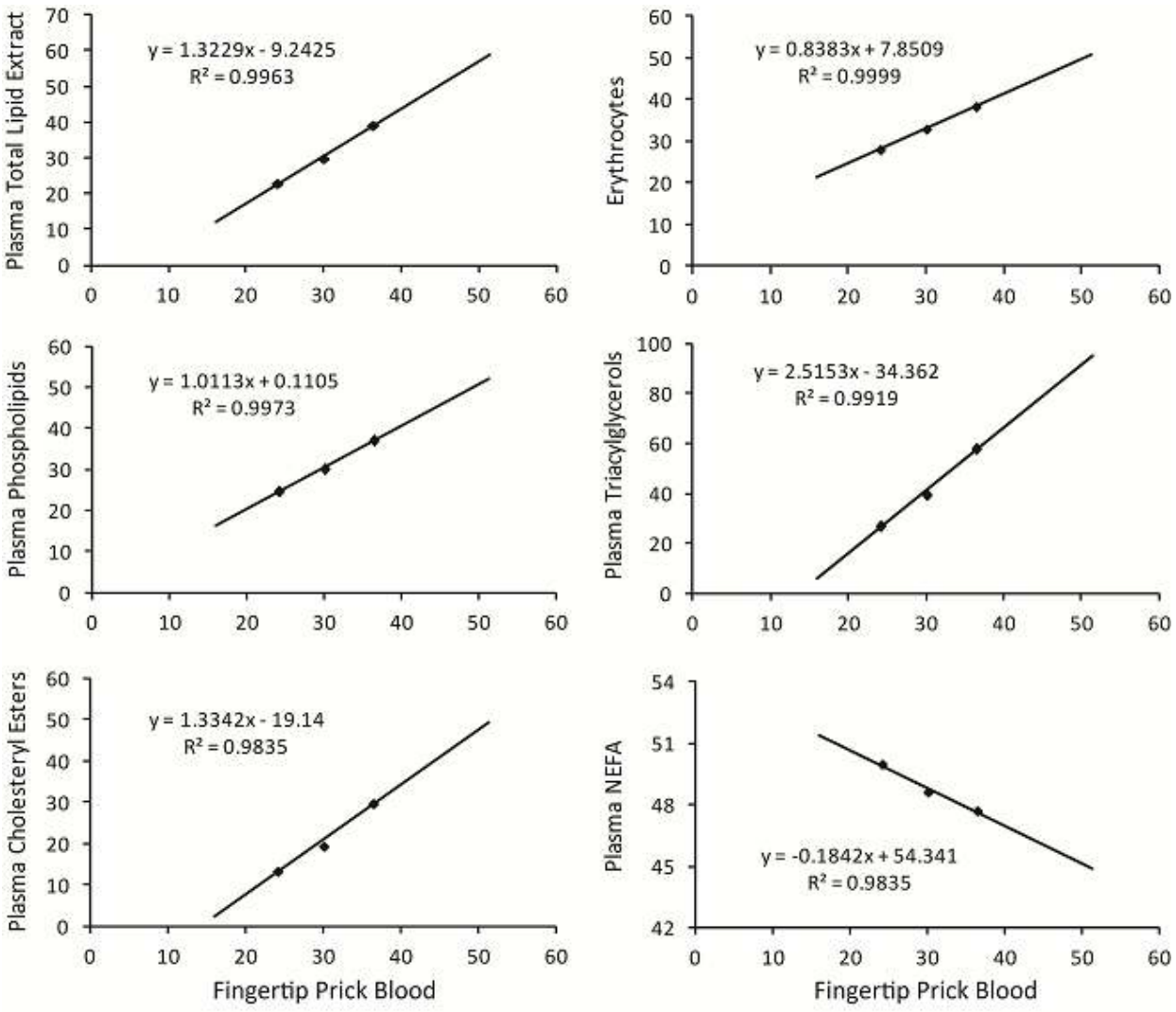

Figure 23 - Linear regression model for the estimation of % n-3 HUFA in blood fractions from FTP blood. NEFA, non-esterified fatty acids

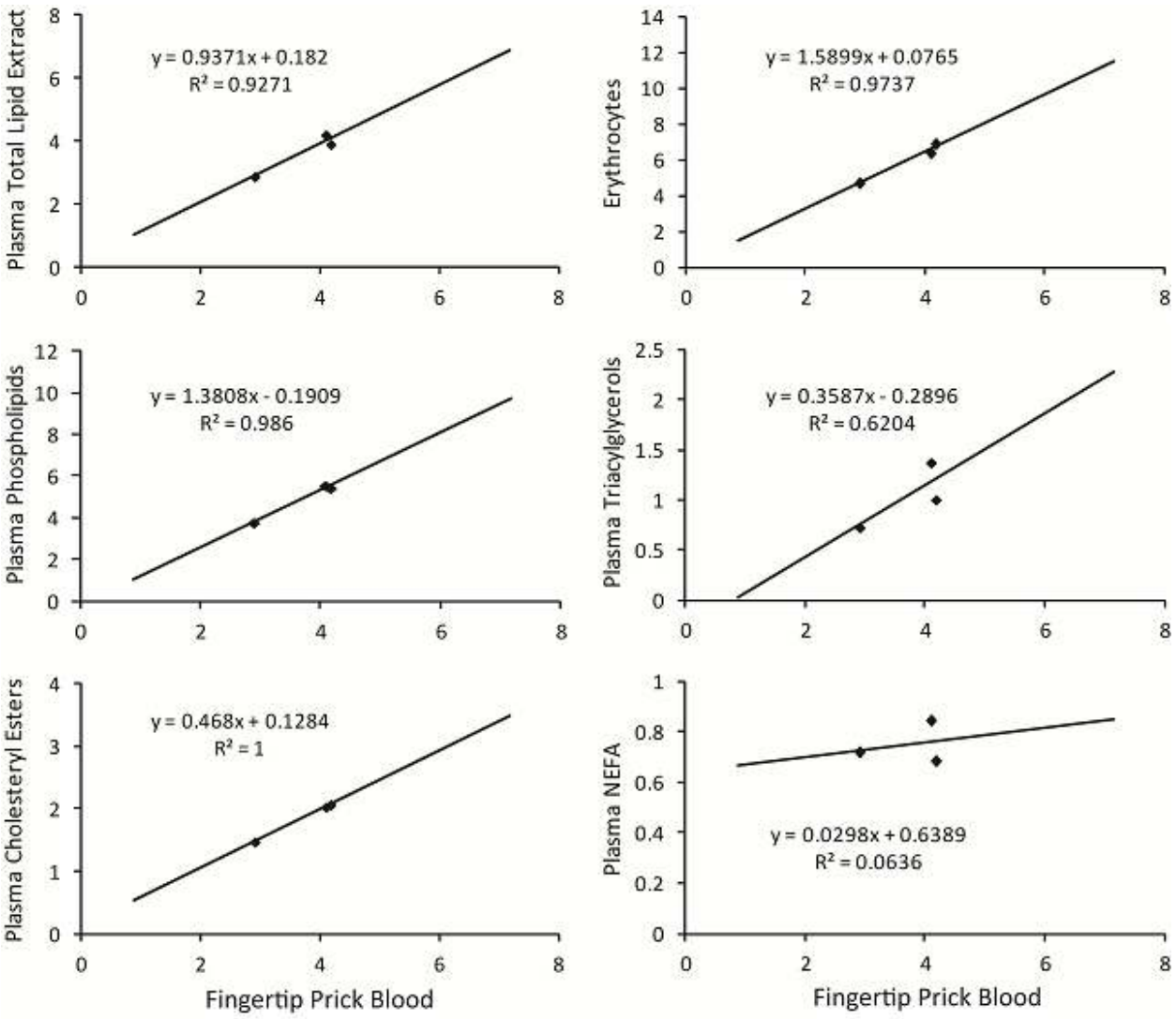

Figure 24 - Linear regression model for the estimation of % EPA+DHA in blood fractions from FTP blood. NEFA, non-esterified fatty acids.

Table 16 – Predictive Equations for the Estimation of Fatty Acid Values in Erythrocytes, and Plasma PL and TLE from FTP Blood

	Erythrocytes		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{RBC} = 0.34(SFA_{FTP}) + 31.37$	0.21	0.349
MUFA	$MUFA_{RBC} = 0.13(MUFA_{FTP}) + 14.38$	0.10	0.397
PUFA	$PUFA_{RBC} = -0.30(PUFA_{FTP}) + 46.08$	0.32	0.309
EPA	$EPA_{RBC} = 1.10(EPA_{FTP}) + 0.00$	1.00	0.020**
DHA	$DHA_{RBC} = 1.87(DHA_{FTP}) - 0.35$	1.00	0.017**
EPA+DHA	$EPA+DHA_{RBC} = 1.56(EPA+DHA_{FTP}) + 0.08$	0.97	0.052*
% n-3 HUFA	$\%n3HUFA_{RBC} = 0.84(\%n3HUFA_{FTP}) + 7.85$	1.00	0.002**
	Plasma PL		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{PL} = 0.91(SFA_{FTP}) + 9.18$	0.91	0.223
MUFA	$MUFA_{PL} = 0.55(MUFA_{FTP}) + 1.42$	0.69	0.188
PUFA	$PUFA_{PL} = 0.04(PUFA_{FTP}) + 37.20$	0.04	0.440
EPA	$EPA_{PL} = 1.83(EPA_{FTP}) - 0.34$	1.00	0.018**
DHA	$DHA_{PL} = 1.31(DHA_{FTP}) - 0.00$	0.98	0.043**
EPA+DHA	$EPA+DHA_{PL} = 1.38(EPA+DHA_{FTP}) + 0.19$	0.99	0.038**
% n-3 HUFA	$\%n3HUFA_{PL} = 1.01(\%n3HUFA_{FTP}) +0.11$	1.00	0.017**
	Plasma TLE		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{TLE} = 0.96(SFA_{FTP}) - 5.42$	0.45	0.267
MUFA	$MUFA_{TLE} = 1.68(MUFA_{FTP}) - 14.63$	1.00	0.014**
PUFA	$PUFA_{TLE} = 1.86(PUFA_{FTP}) - 25.48$	0.66	0.199
EPA	$EPA_{TLE} = 1.40(EPA_{FTP}) - 0.12$	0.99	0.024**
DHA	$DHA_{TLE} = 0.79(DHA_{FTP}) + 0.36$	0.99	0.033**
EPA+DHA	$EPA+DHA_{TLE} = 0.94(EPA+DHA_{FTP}) + 0.18$	0.93	0.087*
% n-3 HUFA	$\%n3HUFA_{TLE} = 1.32(\%n3HUFA_{FTP}) -9.24$	1.00	0.019**

The coefficient of linear regression is indicated as R^2 and falls between 0 and 1, and statistical significance is determined by * indicating a $P < 0.10$ or ** indicating $P < 0.05$. PL, phospholipid; TLE, total lipid extract; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; RBC, red blood cell; FTP, fingertip prick.

Table 17 – Predictive Equations for the Estimation Fatty Acid Values in Plasma TAG, CE and NEFA from FTP Blood

	Plasma TAG		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{TAG} = -0.72(SFA_{FTP}) + 54.97$	0.06	0.419
MUFA	$MUFA_{TAG} = 2.27(MUFA_{FTP}) - 6.09$	0.85	0.128
PUFA	$PUFA_{TAG} = 3.35(PUFA_{FTP}) -97.69$	0.60	0.218
EPA	$EPA_{TAG} = 0.39(EPA_{FTP}) - 0.01$	0.84	0.131
DHA	$DHA_{TAG} = 0.27(DHA_{FTP}) - 0.04$	0.51	0.247
EPA+DHA	$EPA+DHA_{TAG} = 0.36(EPA+DHA_{FTP}) - 0.29$	0.62	0.211
% n-3 HUFA	$\%n3HUFA_{TAG} = 2.52(\%n3HUFA_{FTP}) - 34.36$	0.99	0.029**
	Plasma CE		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{CE} = -0.54(SFA_{FTP}) + 36.80$	0.19	0.355
MUFA	$MUFA_{CE} = 1.86(MUFA_{FTP}) - 17.64$	0.82	0.140
PUFA	$PUFA_{CE} = 3.66(PUFA_{FTP}) - 76.06$	0.97	0.06*
EPA	$EPA_{CE} = 0.79(EPA_{FTP}) + 0.46$	0.92	0.09*
DHA	$DHA_{CE} = 0.27(DHA_{FTP}) - 0.02$	0.57	0.228
EPA+DHA	$EPA+DHA_{CE} = 0.47(EPA+DHA_{FTP}) + 0.13$	1.00	0.000**
% n-3 HUFA	$\%n3HUFA_{CE} = 1.33(\%n3HUFA_{FTP}) - 19.14$	0.98	0.041**
	Plasma NEFA		
Fatty Acids	**Equation**	**R^2**	**P value**
SFA	$SFA_{NEFA} = 3.50(SFA_{FTP}) - 76.00$	0.68	0.193
MUFA	$MUFA_{NEFA} = 1.26(MUFA_{FTP}) - 3.27$	1.00	0.001**
PUFA	$PUFA_{NEFA} = -1.74(PUFA_{FTP}) + 73.54$	1.00	0.013**
EPA	$EPA_{NEFA} = -0.04(EPA_{FTP}) + 0.25$	0.01	0.457
DHA	$DHA_{NEFA} = 0.23(DHA_{FTP}) - 0.13$	0.69	0.188
EPA+DHA	$EPA+DHA_{NEFA} = 0.03(EPA+DHA_{FTP}) + 0.64$	0.06	0.419
% n-3 HUFA	$\%n3HUFA_{NEFA} = -0.18(\%n3HUFA_{FTP}) + 54.34$	0.98	0.041**

The coefficient of linear regression is indicated as R^2 and falls between 0 and 1, and statistical significance is determined by * indicating a $P < 0.10$ or ** indicating $P < 0.05$. TAG, triacylglycerol; CE, cholesteryl ester; NEFA, non-esterified fatty acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; RBC, red blood cell; FTP, fingertip prick.

CHAPTER 10

GENERAL DISCUSSION

Fatty acid analysis is a multi-step process comprising of numerous well documented and accepted options for collection, storage, extraction, separation, transesterification, detection and data handling, each of which may depend on the overall goal of analysis. However, novel method development techniques have the potential to simplify fatty acid analysis through the minimization of sample and solvent requirements, time and monetary commitments and through the use of less invasive collection techniques. Regarding the latter, FTP blood sampling does not require a trained phlebotomist to perform venipuncture sampling and has been implemented on a variety of populations including infant (Agostoni et al., 2005; Agostoni et al., 2011), young adult (Metherel et al., 2009a; Rise et al., 2008) and elderly populations (Fratesi et al., 2009). Due to the high-throughput nature of FTP sampling and the potential need for extended storage periods between collection and analysis, storage stability is important to understand, however, this has not been studied extensively in the literature (Marangoni, Colombo, and Galli, 2004; Min et al., 2011).

Presently, FTP blood stability was determined under various temperature, time, antioxidant and omega-3 status conditions. As expected, HUFA stability without antioxidant is longest when stored at -75°C, and unexpectedly is shortest when stored at -20°C with intermediate stability at room temperature and 4°C. Further research aimed at extending storage stability at room temperature and 4°C is required as storage at these temperatures are more frequently available under numerous research conditions. Instability during -20°C storage conditions (Chapter 7) is determined to be the result of hemolysis that releases free iron into the plasma and provides a catalyst for HUFA peroxidation that appears to remain active at -20°C.

This conclusion would be strengthened by the inclusion of an additional measure of fatty acid peroxidation, particularly the FOX assay that is specific to iron-mediated lipid peroxidation pathways (Gray, 1978). Interestingly, increasing the omega-3 content of whole blood (as well as erythrocytes) appears to modestly delay this rapid decline in HUFA degradation and suggests that hemolysis rates during -20°C may be slower in high omega-3 blood storage. Chromatography paper pretreatment methods for the prevention of HUFA degradation at -20°C were explored and a summary of FTP storage stability under all storage conditions (Chapters 6 and 7) tested is presented (**Figure 25**).

An increase in unsaturation of whole blood or FTP fatty acids due to omega-3 supplementation should result in an increased susceptibility to lipid peroxidation, however, current findings do not support this. Osmotic fragility, a marker of hemolytic susceptibility, has been positively associated with erythrocyte n-6 PUFA content (Ney et al., 2009), and is frequently used as a measure of the erythrocyte integrity (Kolanjiappan, Manoharan, and Kayalvizhi, 2002; Rai et al., 2009). Humans (Hagve, Lie, and Gronn, 1993) and rats (Hagve, Johansen, and Christophersen, 1991) receiving diets high in n-3 PUFA content demonstrate a decrease in osmotic fragility, a marker of cell membrane susceptibility to lysing. These changes suggest a more stable erythrocyte membrane that is more resistant to hemolysis as a result of fish oil supplementation, and provides further support for hemolysis and iron release as the primary trigger of HUFA degradation during -20°C storage. Other factors that can affect osmotic fragility include erythrocyte vitamin E (Ambali et al., 2010) and vitamin C (Ambali et al., 2011) among others that may also be responsible for hemolysis protection.

As the present study utilized only a single participant for all blood storage conditions, the effect of such hemolysis-related variables was not assessed and indicates that variability in blood

HUFA stability during storage may exist. Although, it is important to understand how these factors may affect blood HUFA stability, it is not feasible or necessary to screen for an individual's hemolytic susceptibility during storage, and selection of the most appropriate storage conditions should be made in an attempt to preserve samples of variable compositions. Methods to prevent fatty acid degradation during -20°C storage were developed specifically for this temperature, and require further examination at room temperature and 4°C in an attempt to further stabilize fatty acids during storage while minimizing subzero storage requirements.

Further support for hemolysis-induced fatty acid peroxidation is provided by the results of salmon storage at -20°C and -75°C for six months. Although reductions in salmon HUFA content did occur, there were no differences in HUFA content between the two subzero storage temperatures. This may be due to the absence of an iron-mediated trigger during cell lysis or significantly less lysis due to increased resistance of these cells to lysing compared to erythrocytes during storage. Canned pacific red sockeye salmon contains only 1.1mg of iron per 100g of tissue (Health Canada, 2012), which is approximately 50 times less than the previously reported 51.5mg of iron in 100mL (approximately 100g) of whole blood (Helmer O.M. and Emerson Jr., 1934). Relative stability of HUFA during storage of high omega-3 foods such as salmon provides certainty that consumption following long-term freezing continues to provide significant amounts of healthy omega-3 fatty acids from the diet.

HUFA peroxidation during sample analysis is not a storage-only phenomenon and can occur at multiple stages of processing. BHT, a general purpose antioxidant, is frequently added to extraction solvents (Metherel et al., 2009a) for the prevention of fatty acid losses. It has been determined here that under microwave-assisted direct transesterification conditions with more concentrated H_2SO_4 in methanol and longer reaction times, that HUFA degradation can occur in

FTP blood samples. Under these conditions BHT was not included in the sample and microwave-assisted FTP direct transesterification conditions of 5% and 10% H_2SO_4 in methanol for as little as 3min and 2min, respectively, can result in significant decrease in HUFA and PUFA yield. Although FTP sampling, in particular during -20°C storage and microwave-assisted direct transesterification appear susceptible to HUFA degradation, lipid peroxidation can occur in a wide range of tissues and samples. The microwave system employed here is limited as a single-sample apparatus and is not ideal for high-throughput analyses; however, multi-sample systems do exist and require further investigation.

Ultrasonic probes applied directly to oil have been shown to cause appreciable lipid oxidation in under 20 minutes (Canizares-Macias, Garcia-Mesa, and Luque de Castro, 2004), and the present study demonstrates some non-significant decreases in fatty acids when extending extraction time from 40min to 60min. However, no significant levels of fatty acid peroxidation have been demonstrated during 20min of direct probe sonication of flaxseed submerged in extraction solvents as measured by malondialdehyde formation (Metherel et al., 2009b). The potential for use of high-throughput fatty acid profiling outweighs the limitations as fatty acid peroxidation can be adequately prevented by altering processing and storage conditions such as FTP blood drying and antioxidant paper pretreatment. As advances in techniques increase throughput in certain stages of analysis, limitations in throughput shift to other stages. The development of fast gas chromatography shifted the limits towards sample handling and chemical processing. The advances presented herein in chemical processing and sample handling have shifted the limits of throughput to identification and integration of chromatographic outputs and the conversion of this raw data to fatty acid databases. Improvements in data processing software are required, specifically automatic peak translation

and data checking, in order to make further meaningful gains in analytical throughput of fatty acids. Fatty acid data generation is particularly challenging as 20-30 fatty acids are routinely identified in a simple (even chained, all cis isomers) biological fatty acid analysis.

Based on the preliminary predictive equation development (Chapter 9), FTP blood sampling also has the potential to provide estimates of the omega-3 blood biomarkers in erythrocytes, plasma total lipid extract and plasma phospholipids, however, only three data sets were used to develop these equations, limiting statistical power. Nonetheless, these equations also have potential use in predicting the level of fatty acid peroxidation that has occurred in a stored blood sample. Further to the benefits of FTP predictive equations, if a research study requires erythrocyte omega-3 profiling, FTP blood sampling may still be utilized as a quick screening tool to estimate omega-3 composition in erythrocytes for study entrance. This study is further limited by only assessing the predictive strength of FTP fatty acids for other blood fractions, and more extensive predictive equation development will allow for comparison of fatty acid profiles between research studies utilizing different blood fractions. These equations can improve knowledge transfer when comparisons of data would otherwise be difficult to interpret.

Understanding acceptable storage conditions for FTP blood should further promote its use in a variety of research situations including field study researchers that may have been previously weary of utilizing this simple and cost effective method due to the unknowns associated with storage in the absence of a deep freezer. Depending on storage conditions, stability of FTP samples can be achieved for as long as 120 days at room temperature, 90 days at 4°C, 30 days at -20°C and 180 days at -75°C. Using FTP sampling methods in conjunction with microwave-assisted direct tranesterification will increase sample throughput and allow for more frequent analysis and a greater understanding of changes in fatty acid profiles in response to

research study parameters. Compared to erythrocytes, FTP sampling and microwave-assisted techniques significantly increases sample transesterification throughput to as high as 180 samples per hour if a 10 sample microwave system was employed. Similar improvements in throughput could also be seen in ultrasound-assisted lipid extraction techniques compared to tedious standard methods, however, the current method was designed specifically for lipid extraction from flaxseed and applicability to additional tissue and food matrices requires investigation. Better understanding of storage stability of high-throughput sampling allows for enhanced prevention of sample degradation and longer storage periods as sample volumes subsequently increase. Energy-assisted analysis techniques help to ameliorate some of these issues by reducing required storage times.

Taken together, high-throughput fatty acid analysis and omega-3 determinations can enable the necessary research that may facilitate the implementation of standard omega-3 screening protocols by health professionals. The application of fatty acid profiling to clinical populations may assist in compliance and adherence to dietary advice and recommendations to increase omega-3 fatty acid consumption. It is anticipated that fatty acid profiles have the potential to yield additional valuable information regarding dietary habits and lipid metabolism beyond omega-3 fatty acids as well. The development of increased throughput methodologies can also reduce the cost and improve reporting and monitoring of omega-3fatty acids in the food supply. The combination of improved fatty acid labeling of food and the ability to ascertain one's personal fatty acid profile will allow consumers to make healthier choices, and enable health professionals to target advice and assess intervention strategies.

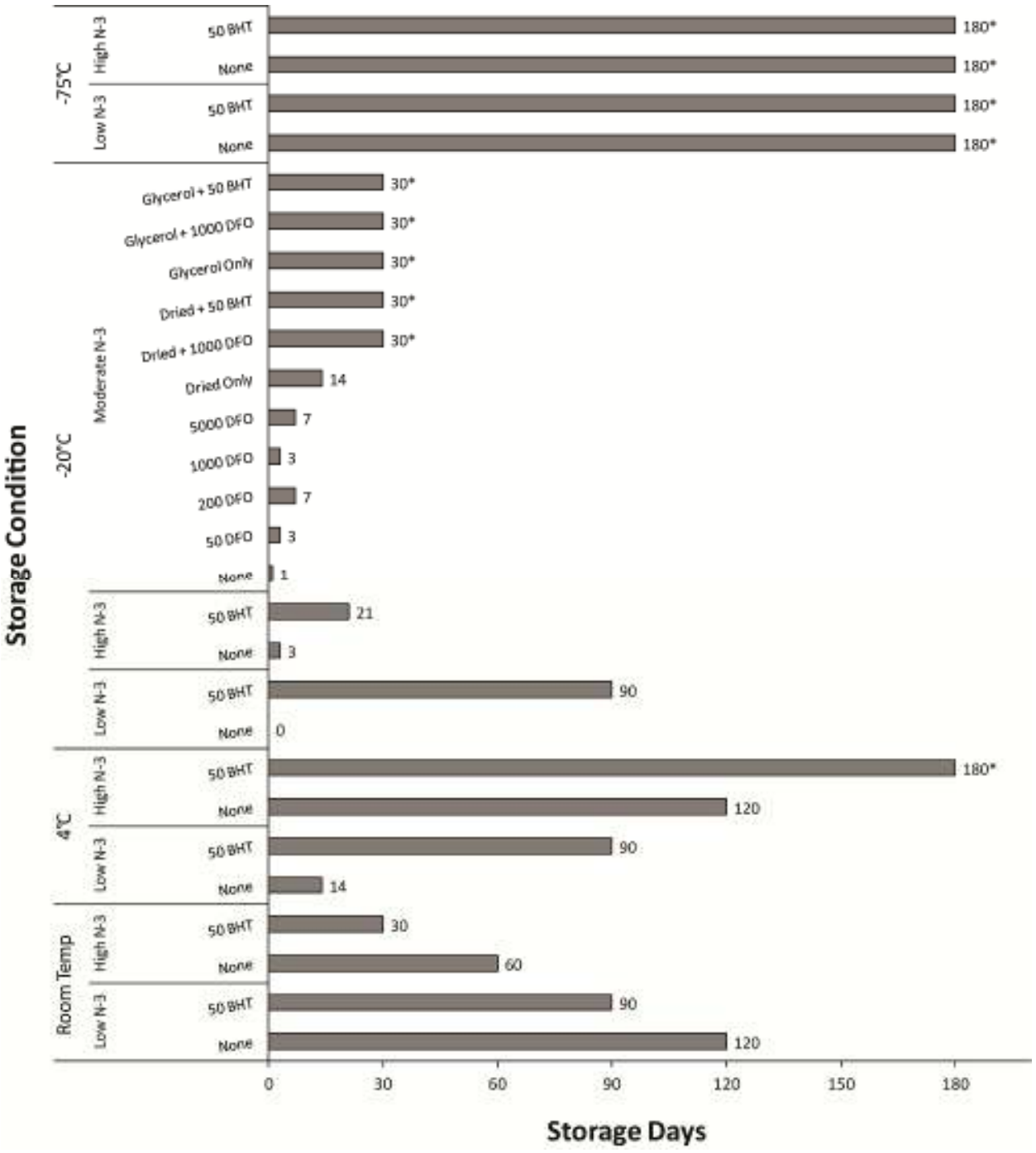

Figure 25 – Summary of qualitative highly unsaturated fatty acid stability (HUFA) in FTP blood during various storage conditions. HUFA stability determined to be changes in HUFA values of no more than 10% of baseline values. *Indicates HUFA stability that has occurred for the entire storage period measured. HUFA, highly unsaturated fatty acids; BHT, butylated hydroxytoluene (μg); FTP, fingertip prick; DFO, deferoxamine (μg); N-3, omega-3.

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