DEVELOPMENT OF A ROBUST DRIED BLOOD SPOT METHOD FOR THE EVALUATION OF N-3 FATTY ACID STATUS OF INDIVIDUALS

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Abstract

Increased consumption of n-3 long chain polyunsaturated fatty acids (LCPUFA) is associated with higher n-3 LCPUFA status in the circulation, which has in turn been associated with a number of health benefits in humans (Calder *et al.* 2006; Makrides *et al.* 2009; Einvik *et al*. 2010).

The conventional approach to assay n-3 fatty acid status in humans involves invasive venous blood collection and expensive, time consuming multi-step processes as that limit its application in large-scare clinical trials and routine population screening (Risé *et al.* 2007). Recently, efforts have been made to adopt the dried blood spot (DBS) as a quick, inexpensive and minimally invasive alternative for the measurement of fatty acid status in humans (Marangoni *et al.* 2004). However, the existing DBS approaches have had only limited success in providing an accurate tool for the measurement of n-3 LCPUFA status in humans. This has been due to the presence of fatty acid contaminants in blood collection papers which are released during sample processing (Nishio *et al*. 1986; Ichihara *et al.* 2002), and the failure to prevent significant oxidative loss of the n-3 LCUFA in DBS sample during transportation and storage (Min *et al.* 2011; Bell *et al.* 2011).

This thesis aimed to develop a novel DBS technique which would overcome these limitations and enable the technology to be used for the accurate evaluation of n-3 LCPUFA status in human subjects. Firstly, a wide range of potential collection matrices and lab consumables were tested to determine which contained the lowest contaminant levels. A range of antioxidants and chelating agents were then tested with DBS in order to identity the optimal combination of these factors for protecting the

LCPUFA in DBS from oxidation. The protection system which provided optimal protection consisted of a combination of an antioxidant and a chelating agent applied to silica gel coated blood collection paper, and this resulted in more than 90% of the original n-3 LCPUFA content (expressed as a weight percentage in blood total lipids) in the DBS being retained following 2 months of storage at room temperature (20-25°C). This system (termed "PUFAcoat") represents a significant improvement in LCPUFA stability in DBS compared with previously reported standard DBS protection systems. For example, the standard Fluka system (Fluka blood collection kit) uses a single antioxidant (butylated hydroxytoluene, BHT) as protectant, and normal chromatography paper as a collection paper which retains only ~60% of the n-3 LCPUFA content in the applied DBS over the same time period (Min *et al.* 2011).

To explore the mechanisms underlying the protective effect of the "PUFAcoat" and to improve my understanding of the processes causing the rapid breakdown of LCPUFA in DBS, a novel *in vitro* system (comprising an oil blend on collection paper) was developed. Using this model I established that iron-induced oxidation was the principle driver of the rapid loss of the n-3 LCPUFA absorbed on the blood collection paper, and that iron chelating agent in the "PUFAcoat" system eliminated this process by binding the irons in the DBS samples.

The clinical validity of the "PUFAcoat" system was established in a human study that compared the fatty acid spectrum obtained from my DBS method (using capillary blood) with those obtained by traditional analytical techniques (using venous blood fractions). This study demonstrated strong correlations in fatty acid status between my DBS method and conventional measurements, which indicate the potential of use of

my DBS method as an appropriate alternative to conventional assessments. Morever, this clinical study showed that the n-3 LCPUFA status obtained using my DBS method reflected the habitual dietary n-3 fatty acid intakes of the study population.

This thesis is the first report of a protection system that is capable of stabilising the n-3 LCPUFAs in human DBS samples over 2 months storage at room temperature. Thus, my newly developed DBS method offers a significant improvement in the useability and reliability of the DBS technique for assessing n-3 LCPUFA status in humans. My DBS method has significant potential for use in large-scale clinical testing and population based screening diagnostics which focused on the role of n-3 fatty acid status in human health.

Declaration

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

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List of Abbreviations

AA	arachidonic acid
ALA	alpha-linolenic acid
ANOVA	analysis of variance
BHA	butylated hydraoxyanisol
BHT	butylated hydroxytoluene
CHD	coronary heart disease
DBS	dried blood spot
DHA	docosahexaenoic acid
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid
EDTA	ethylene diamine tetraacetic acid
EPA	eicosapentaenoic acid
FAMEs	fatty acid methyl esters
GC	gas chromatography
LA	linoleic acid
LCPUFA	long chain polyunsaturated fatty acids
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acids
RA	rheumatoid arthritis
RCT	randomized controlled trial
SD	standard deviation
SFA	saturated fatty acids
TBHQ	tert-Butylhydroquinone
TDM	Therapeutic drug monitoring
TG	triglycerides
TLC	thin-layer chromatography
TLE	total lipid extract

Chapter1

Literature review

1.1 Scope of the Review

The positive effect of dietary n-3 LCPUFA in maintaining good health has become an increasingly important area of research in recent years. Some epidemiological studies and clinical trials have demonstrated that increased intakes of n-3 LCPUFA, especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), are associated with certain improvements in neurodevelopmental outcomes in preterm infants (Makrides *et al.* 2009), primary and secondary protection against cardiovascular disease (Einvik *et al*. 2010) and strengthening immune defenses (Kasaga *et al*. 2007).

As a result of these health benefits, there has been a growing interest in assessing the n-3 LCPUFA status in human populations. Fatty acid profiling of human blood samples is a valuable biomarker of fatty acid status in humans, since blood levels are thought to reflect biological actions and blood is accessible for collection in human studies (Harris and von Schacky 2004; Baylin and Campos 2006). Through measurement of the levels of fatty acids in human blood, cardiovascular risk in human populations can be evaluated (Harris von Schacky *et al*. 2004) and those individuals who would benefit from n-3 LCPUFA supplementation can be identified (Metcalf *et al.* 2007). Conventional assays of fatty acid status in humans use a number of different tissues and blood fractions, including adipose tissue (Hirsch *et al.* 1960), cheek cells (Mcmurchie *et al.* 1984a), plasma, red blood cells, and whole blood (Risé *et al.* 2007). Of these approaches, whole blood fatty acid analysis represents the most

convenient way to assess fatty acid status, since separation of blood fractions is not required. Furthermore, some studies have indicated that the fatty acid analysis of DBS is a suitable method for fatty acid analysis (Marangoni *et al.* 2004; Marangoni *et al.* 2007; Bailey-Hall *et al.* 2008). The use of such DBS methods minimises the risks involved in the collection, handling and shipment of the whole blood samples, and considerably reduces the cost of sample processing and transportation (Parker and Cubitt 1999). However, the accuracy of the DBS assays for analysis of fatty acid composition has been questioned, since some studies have reported the successive degradation of LCPUFA in DBS during storage (Min *et al.* 2011; Bell *et al*. 2011; Metherel *et al.* 2013).

This literature review is focused on the current knowledge in the field of blood fatty acid status measurements, in particular the accuracy of the DBS methods for the determination of fatty acid status in human blood. The review aims to address the validity of the DBS technique as a quick and accurate tool for the determination of fatty acid composition in humans, and thereby provide the rationale for the experiments which form the basis of this thesis.

1.2 Fatty acids and their source

A fatty acid is a carboxylic acid with an aliphatic chain. Most natural sourced fatty acids have an unbranched chain containing an even number of carbon atoms, ranging from 4 to 28. Fatty acids mainly exist in esterified form in vegetable or animal fats, oils, or waxes. However, there are also small amounts of “free” fatty acids which are not attached to other molecules, but are often electrostatically bound to proteins

(Ashok and Ajit, 2009). Fatty acids can be saturated or unsaturated, depending on the number of double bonds. Saturated fatty acids (SFA) have no double bonds in the molecule, and the major food sources of saturated fats are animal-based products, such as red meat and whole milk products. Unsaturated fatty acids include monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). MUFA only contain one double bond in their aliphatic chain, whereas PUFA have two or more double bonds. Both types are predominantly found in plant products, such as vegetable oils and nuts, and some fish and seafoods (Drummond and Brefere 2009).

Polyunsaturated fatty acids include the essential fatty acids (EFAs). The two EFAs known for humans are linoleic acid (LA, 18:2 n-6) and alpha-linolenic acid (ALA, 18:3 n-3). The name "essential" were given because these fatty acids cannot be synthesised by animals and humans, and must therefore be derived from the diet. The main dietary sources of LA and ALA are vegetable oils and fats, followed by cereal products (Sioen *et al.* 2006). LA belongs to a group of fatty acids called the n-6 fatty acids while ALA belongs to a group of fatty acids called the n-3 fatty acids. The name n-3 or n-6 refers to the position of the first double-bond in the fatty acid molecule which is after the third or the sixth carbon from the methyl end, which is also known as the "n" end, in the n-3 and n-6 fatty acids, respectively . LA and ALA form the starting point for the synthesis of longer (≥20 carbon atoms) and more unsaturated (≥2 double bounds) fatty acids, which are referred to as LCPUFA. Arachidonic acid (AA, 20:4 n-6), eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are three of the most important LCPUFA. Humans can convert LA to AA through desaturation and chain elongation (Horrobin 1993), however, the the conversion of ALA to EPA and then DHA is very limited in healthy individuals

(Burdge and Calder 2005), thus the efficiency of obtain EPA and DHA from endogenous derivation in humans is much lower than the absorption of EPA and DHA from food. Major sources of AA in human diets are meat and eggs, while fish and marine products provide main sources of EPA and DHA (Sioen *et al.* 2006).

1.3 Health Benefits of N-3 LCPUFA

Many clinical studies have demonstrated that increased intakes of n-3 LCPUFA, especially EPA and DHA, are associated with beneficial health effects in humans, particularly for cardiovascular disorders (Saravanan *et al.* 2010), rheumatoid arthritis (Calder *et al.* 2006), pregnancy outcomes (Salvig *et al.* 2011) and neurodevelopment (Dunstan *et al.* 2008; Makrides *et al.* 2009). Therefore, health agencies worldwide have recommended that increasing n-3 fatty acids intake (either via supplementation or diretary consumption) may represent an effective strategy for reducing the risk of many of the diseases and improving the general health of the population (Table 1.1). For pregnant and lactating women the DHA dietary intake is recommended to be at least 200mg/day (Koletzko *et al.* 2007). The American Heart Association recommends consuming fatty fish at least twice a week to lower the risk of sudden death of heart (Kris-Etherton *et al.* 2003). Table 1.1 lists the recommendations for the consumption of EFAs and n-3 LCPUFA from various health agencies worldwide.

1.3.1 Dietary n-3 LCPUFA and the risk of cardiovascular disease

Certain evidence shows that dietary intakes of n-3 fatty acids EPA and DHA significantly reduce the risk of overall mortality, fatal myocardial infarction and sudden cardiac death in populations with and without established coronary heart

disease (CHD) (Bucher *et al.* 2002; Albert *et al.* 2002). Epidemiological studies and large randomised controlled clinical trials including the DART (diet and reinfarction trial), GISSI (Gruppo Italianoperlo Studio della Sopravvivenzanell Infartomiocardico)-Prevenzione trial and JELIS (Japan EPA Lipid Intervention Study), demonstrated that EPA and DHA supplementation is a promising treatment for both primary and in particular secondary prevention of cardiovascular diseases, including atherosclerosis, hypertension, cardiac arrhythmias, heart attack and stroke (Kromhout *et al.* 1985; Burr *et al.* 1989; GISSI-Prevenzione Investigators 1999; Yokoyama *et al.* 2007; Lavie *et al.* 2009). In the landmark DART study, the secondary prevention effect of long-term n-3 LCPUFA intervention was investigated in 2033 men who had recovered from myocardial infarction. A 30% reduction in overall mortality in the follow-up period of 2 year was reported in patients randomly advised to consumes fatty fish at least twice per week when compared with those did not receive this advised (Burr *et al.* 1989). In the GISSI-Prevenzione trial and its follow-up study, lower overall mortality was observed in post-myocardial infarction patients who took a low dose of n-3 fatty acids (1 g/d) for 3.5 years, and this was mainly attributed to fewer sudden cardiac deaths (GISSI-Prevenzione Investigators 1999; Marchioli *et al.* 2002). A further study in 992 individuals with stable coronary artery disease revealed that n-3 LCPUFA reduced the incidence of recurrent cardiovascular events by inhibiting systemic inflammation (Farzaneh *et al.* 2009).

However, more recently, the efficiency of n-3 LCPUFA in preventing cardiovascular disease in adult humans has been questioned by the results of some double-blind randomized controlled trials (RCTs), which have reported that daily intake low dose of EPA and DHA did not significantly reduce the rate of major cardiovascular events

in post-myocardial infarction patients (Kromhout *et al.* 2010) and in patients with dysglycemia (Bosch *et al.* 2012). Therefore, based on the overall estimate of effect across the entire body of evidence, the cardiac beneficial effects of n-3 LCPUFA seem not as great as previously believed, and further research in this area is necessary (Kotwal *et al.* 2012; Rizos *et al.* 2012).

1.3.2 Dietary n-3 LCPUFA and rheumatoid arthritis

Rheumatoid arthritis (RA) is defined as a chronic inflammatory disease characterised by joint inflammation which including clinical symptoms such as joint pain, swelling, and functional impairment, morning stiffness, osteoporosis, and muscle wasting (Feldman *et al.* 1993) A number of pathological studies have revealed the anti-inflammatory properties of n-3 fatty acids in patients with RA. For example, the n-3 LCPUFA supplementation was associated with reduced the concentration of many inflammatory biomarkers, including serum C-reactive protein, plasma interleukin-1 (Kremer *et al.* 1990), interleukin-1 beta (Kremer *et al.* 1985; Esperson *et al.* 1992), leukotriene B4 (Kremer *et al.* 1987; Cleland *et al.* 1988; van der Tempel *et al.* 1990), and also normalises the neutrophil chemotactic response (Sperling *et al.* 1987) in RA patients. Several RCTs have also reported the benefits of fish oil in treatment of RA. The dose of n-3 LCPUFA used in these trials was from 2.1 to 4.2g/d and averaged ≈3.0 g/d. The benefits of fish oil reported in these trials included reduced duration of morning stiffness, reduced number of tender or swollen joints, increased grip strength, reduced physician or patient assessment of joint pain or disease activity, reduced time to onset of fatigue, and decreased use of nonsteroidal anti-inflammatory drugs (Geusens *et al.* 1994; Volker *et al.* 2000; Adam *et al.* 2003; Berbert *et al.* 2005). Furthermore, there is no reported adverse side effects within the dose range used in

RA, and n-3 fatty acids are generally very well tolerated (Kremer *et al.* 2000). Systematic reviews of the clinical trials of fish oil in treatment of RA also concluded the positive role of fish oil (Kremer *et al.* 2000; Cleland *et al.* 2003; Miles and Calder 2012). Thus, there is relatively robust evidence in support of the clinical benefits of n-3 LCPUFA in treatment of RA.

1.3.3 Dietary n-3 LCPUFA and pregnancy outcomes

There is increasing evidence that maternal intake of n-3 LCPUFAs during pregnancy results in an increasing gestation length. In 1986, Olson first observed that pregnant women, who consume more n-3 LCPUFA-rich seafood, had a longer duration of gestation (Olsen *et al*. 1986). This result has been confirmed by two recent large RCTs in USA and Australia, in which pregnant women were provided with either DHA or placebo during the last half of pregnancy (Makrides *et al.* 2010; Carlson *et al.* 2013). The increasing in mean gestation length in women supplemented n-3 LCPUFA has also been associated to significant reductions of early preterm birth (< 34 weeks) in the majority (Olsen *et al.* 2000; Makrides *et al.* 2010; Carlson *et al.* 2013) but not all studies (Harper *et al.* 2010). Therefore, although the effect of n-3 LCPUFA supplementation in gestation length has been well demonstrated, further direct evidence is required for it to be possible for general recommendations for n-3 LCPUFA to be made to reduce the risk of preterm birth (Larque *et al.* 2012).

1.3.4 Dietary n-3 LCPUFA and neurodevelopment

The known importance of n-3 LCPUFA, in particularly DHA, for the early development of the brain and nervous system has led to suggestions that maternal n-3 exposure could improve neurodevelopmental outcomes in their children (Clandinin *et*

al. 1980; Hoffman *et al*. 2009). Epidemiological studies have linked higher intakes of fish (the major dietary source of DHA) in pregnant women to better performance on tests of cognitive function in their children (Hibbeln *et al.* 2007; Oken *et al.* 2008). The Danish National Birth Cohort studied 25,446 children born between 1997 and 2002 (Oken *et al.* 2008), and found that higher maternal higher fish intake (58.6g/day) was associated with improved cognitive performance in their children at 18 months of age. N-3 LCPUFA supplementation intervention studies also demonstrated positive association between maternal n-3 LCPUFA supplementation and neurodevelopmental outcomes (Helland *et al.* 2003; Helland *et al.* 2008; Dalton *et al.* 2009). In a double-blind RCT in Western Australia, children from 52 pregnant women who received fish oil supplementation (1.1g EPA and 2.2g DHA daily) from 20 weeks gestation until delivery achieved a higher score for hand-eye coordination than those children from placebo mothers (olive oil, n=46) (Dunstan *et al.* 2008). However, these positive results should be interpreted with caution, because it is always impossible to fully adjust all confounding variables in observational studies. Furthermore, results from the largest double-blind, multicenter RCT of maternal n-3 LCPUFA supplementation conducted to date showed that the use of fish oil supplements in pregnant mother did not result in improvement in cognitive and language development in their children during early childhood (18 months) when compared with control group (vegetable oil) (Makrides *et al.* 2010). Therefore, the effects of maternal n-3 supplementation during pregnancy on neurodevelopmental outcomes of offspring is still controversial.

There is plausible evidence that support the benefit of childhood supplementation of n-3 fatty acids, particularly DHA, in neurodevelopmental outcomes (Ryan *et al.* 2008; Dalton *et al.* 2009; McNamara *et al.* 2009). In a study that investigated healthy

preschool children who took 400 mg of DHA per day for 4 months, a positive association between the level of DHA in blood and the performance on a test of language acquisition was observed (Ryan *et al.* 2008). A dose of 60 mg/kg/day DHA in early life (0-18 months) improved the Mental Development Index scores of girls who were born very preterm(< 33 weeks) (Makrides *et al.* 2009). However, studies which administered low doses of DHA (≤100 mg/day) were failed in showing of significant cognitive improvement in DHA treatment group when compared with the placebo group (Osendarp *et al*. 2007; Muthayya *et al*. 2009). Since it is still unclear whether a certain level of DHA supplementation must be reached before a positive neurodevelopmental effect can be detected, well designed dose-ranging clinical trials are needed to help to establish optimal n-3 LCPUFA supplementation doses for optimizing neurodevelopment at different developmental ages (Ryan *et al.* 2010).

Table 1.1. The recommendations for EFAs and n-3 LCPUFA intake for adults (19+) from various organizations worldwide

Reference	Region	Organization	Rationale	Target population	Recommendations for daily intake		
					LA	ALA	n-3 LCPUFA
NHFA 2008	Australia	**NHFA**	↓ risk of CHD	General adults	-	-	0.5g /day EPA+DHA
			↓ risk of fatal CHD events	Patients with CHD	-	-	1.0g /day EPA+DHA
			Reach target TG level	Patients have hypertriglyceridemia	-	-	1.2g /day of EPA+DHA as first-line therapy, increase to 4.0g/day as needed
NHMRC 2006		**NHMRC**	General health	Australian adults	-	-	Upper level of intake: 3g/day
				Men(19+)	13g/day	1.3g/day	0.16g/day
				Women(19+)	8.0g/day	0.8g/day	0.09g/day
				Pregnancy(19-50yr)	10g/day	1.0g/day	0.115g/day
				Lactation(19-50yr)	12g/day	1.2g/day	0.145g/day
			Optimise chronic disease risk, notably CHD.	All adults	4-10% en	0.4-0.1%en	-
			↓risk of chronic disease	Men	-	-	0.61g/day
				Women	-	-	0.43g/day
Kris-Etherton ***et al.*** 2002	USA	**AHA**	General health	Adults without CHD	-	Eat a variety of (preferably oily) fish at least twice a week. Include oils and foods rich in ALA (flaxseed, canola, and soybean oils; flaxseed and walnuts)	
			Maintain cardiovascular health	Patients with CHD	-	-	Consume ≈1g /day of EPA+DHA; EPA+DHA supplements could be considered in consultation with the physician
			Lower triglycerides	Patients have hypertriglyceridemia	-	-	2-4g /day of EPA+DHA provided as capsules under a physician's care
IOM 2002		**IOM**	General health	Men (19-50 yr) Women (19-50 yr)	17g/day 12g/Day	1.6g/day ALA , 10% of which is consumed as EPA+DHA 1.1g/day ALA, 10% of which is consumed as EPA+DHA	
ADA 2007		**ADA**	General health	General Adults	-	-	0.5g/day n-3 LCPUFA

(Continued) EFSA 2010	Euro	**EFSA**	General health	General adults	**-**	**-**	0.25g /day EPA+DHA
				Pregnant & Lactating women	**-**	**-**	0.1-0.2g/day in addition to normal adult requirements
BNF 1999		**BNF**	General health	Adults	**-**	**-**	A weekly intake of 1.0-1.5 g/day
DGE&SGE 2008		**DGE&SGE**	Primary prevention of CVD	General adults	**-**	**-**	0.25g/day n-3 LCPUFA or 0.5% of total energy n-3 PUFA intake
			Secondary prevention of CVD	CHD patients	**-**	**-**	1g/day n-3 LCPUFA
			General health	Pregnant women	**-**	**-**	At least 0.2g/day DHA
AFFSA 2010		**AFFSA**	↓metabolic disease risk ↓CVD risk ↓AMD risk ↓breast and colon cancer risk	General adults	**-**	**-**	0.5-0.75g/day
			General health	Pregnant women	**-**	**-**	0.5g/day EPA +DHA
FAO/WHO 2008	**Global**	**FAO/WHO**	↓risk of fatal CHD events; Probably ↓ risk of total CHD events, stroke	General adults	2.5-9%en (AMDR)	≥0.5en (L-AMDR)	0.5g/day EPA +DHA
ISSFAL 2004		**ISSFAL**	General health	General adults	2%en	0.7%en	-
				Pregnant women	**-**	**-**	0.3g/day DHA
			Maintaining CVD health	General adults	**-**	**-**	0.5g/day EPA +DHA
Koletzko *et al.* 2007		**WAPM**	General health	Pregnant and Lactating Women	**-**	**-**	0.2g/day DHA

***Abbreviations used in this table:**

NHFA: National Heart Foundation of Australia
AHA: American Heart Association
ADA: American Dietetic Association and Dieticians of Canada
BNF: British Nutrition Foundation
AFFSA: French food safety agency
ISSFAL: International Society for the Study of Fatty Acids and Lipids
AMDR: Acceptable macronutrient distribution range
NHMRC: National Health and Medicines Research Council
IOM: Institute of Medicine
EFSA: European Food Safety Agency
DGE&SGE: German Society for Nutrition& Swiss Society for Nutrition Research
FAO/WHO: Food and Agriculture Organization of the United Nations/ World Health Organization Joint
WAPM: World Association of Perinatal Medicine
L-AMDR: Lower level of Acceptable macronutrient distribution range

1.4 Monitoring N-3 LCPUFA status

Since lower n-3 LCPUFA levels in humans has been associated with the increasing risk of many diseases, such as cardiovascular disease, inflammatory disease and mental health, there has been a growing interest in assessing the n-3 LCPUFA status in human populations (Prescott and Dunstan 2007; Ramakrishnan 2011; Miles 2012). The suggestion is that by determining the n-3 LCPUFA status, the risk of diseases in human populations can be evaluated and those individuals who would benefit from n-3 LCPUFA supplementation can be identified. This of course is based on the assumption that there is a relationship between n-3 LCPUFA status and disease risk.

1.4.1 N-3 LCPUFA status and the risk of Cardiovascular disease

Strong concentration-risk dependence has been reported between the n-3 LCPUFA status in blood and the risk of cardiovascular disease (Albert *et al.* 2002; Harris and von Schacky 2004). In a physicians' health study, physicians with an average n-3 fatty acids level of 6.87% in whole blood had a 90% lower risk of sudden cardiac death, when compared to physicians with an average level of 3.58% n-3 fatty acids (Albert *et al.* 2002). Similar results were also observed in a case-control study in Seattle showed that subjects with an average of 6.5% n-3 LCPUFA in red blood cell membranes had a 90% lower risk for sudden cardiac death compared to subjects with an average level of 3.3% n-3 LCPUFA (Siscovick *et al.* 1995). Meta-analysis of the studies which have determined the relationships between blood n-3 LCPUFA status and cardiovascular disease has resulted in the suggestion that the sum of EPA + DHA% in erythrocyte membranes (the "Omega-3 Index") provides a novel biomarker for the risk of CHD mortality (Harris and von Schacky 2004). Based on the collective results of these studies, the "Omega-3 Index" of >8% is associated with a 90% reduction in the risk of

sudden cardiac death, as compared to an “Omega-3 Index” of <4% (Harris and von Schacky 2004). Since the “Omega-3 Index” has been shown to reflect the n-3 fatty acid status in a given individual (Harris and von Schacky 2004), changes in the “Omega-3 Index” can be expected in a given individual after a change in diet, or during treatment with n-3 fatty acids supplements. Therefore, the dose and duration of n-3 fatty acid supplementation can be oriented by the baseline “Omega-3 Index”of an individual to achieve the 8% “Omega-3 Index” goal which is required for cardioprotection. However, there was research showed that in a population with a low intake of fish and fish oils (“Omega-3 index” ~3.6%, expressed in a weight percentage of total fatty acids), the “Omega-3 index” did not predict fatal events following hospitalization in patients with acute chest pain and suspected acute coronary syndrome (De La Fuente *et al.* 2013). Since the evidence reporting the usefulness of “Omega-3 Index” is limited, further clinical trials is needed before this index can be used as a universal biomarker for cadioprotection.

1.4.2 N-3 LCPUFA status and Inflammatory diseases

Several RCTs have reported beneficial effects of n-3 fatty acid supplementation in treatment of RA (Adam et al. 2003; Berbert et al. 2005). For example, a dietary intervention study involving the addition of 4 gm EPA and DHA in the diet to achieve plasma phospholipids EPA levels of equal to or greater than 3.2% was shown to produce a substantial inhibition of production of interleukin-1 beta and tumor necrosis factor alpha in patients with RA (James and Cleland, 1997).

There is also evidence that the n-3 LCPUFA status of pregnant women may influence allergy and athma risk in their children. Systematic reviews of RCTs of maternal n-3

PUFA supplementation during pregnancy concluded that this was associate with a lower risk for childhood asthma (Dunstan *et al.* 2003; Olsen *et al.* 2008), decreased response to an egg skin prick test (Dunstan *et al.* 2003; Furuhjelm *et al.* 2009), reduced inflamematory marker levels (Dunstan *et al.* 2003; Krauss-Etschmann *et al.* 2008), however, it was in the absence of an effect on the incidence of atopy (Klemens *et al.* 2011). A follow up of a large RCT of maternal n-3 LCPUFA supplementation from Australia also reported that while n-3 LCPUFA supplementation in pregnancy did not reduce the overall incidence of immunoglobulin E associated allergies in the first year of life, it did result in a reduction in the incidence of atopic eczema and egg sensitisation (Palmer *et al.* 2012). Although the majority of clinical studies in which n-3 LCPUFA supplementation were given to postnatal infants showed that while n-3 supplemntation improved infant n-3 status, it did not have any significant impact on the incidence of childhood allergic disease (Lauritzen *et al.* 2005; D'Vaz *et al.* 2012), a recent Australia multicnter RCT showed that postnatal n-3 supplementation in preterm baby (> 33 weeks of gestation) reduce bronchopulmonary dysplasia and reported hay fever in boys at either 12 or 18 months (Manley *et al.* 2011).

AA is a precursor of pro-inflammatory eicosanoids which biologically active in very small quantities however contribute to the formation of thrombosis, atheromatosis, allergic and inflammatory disorders if they are formed in large amounts (Benatti *et al.* 2004). The n-3 LCPUFA, especially EPA, suppresses and counteracts the activity of AA-derived pro-inflammatory eicosanoids (Kromann and Green 1980; Hirai *et al.* 1982) through competition with AA for incorporation into cell membrane (Wortman *et al.* 2009), and by generating their own eicosanoids which have a lower inflammatory activity than AA-derived eicosanoids (Prickett *et al.* 1981) or have anti-

inflammatory activity (Kasuga *et al.* 2008; Serhan *et al.* 2009). On this basis, Sears proposed that an AA/EPA ratio of more than 10 indicates excess levels of cellular inflammation, and indicates that individuals may be more at the risk of future development of inflammatory disease (Sears *et al.* 2008). A recent Italian study has reported that the AA/EPA ratio was always greater than 15 in patients with established inflammatory diseases,and their AA/EPA ratio can be lowered to ~5 with daily supplementation of 2-3 grams of EPA+DHA (Rizzo *et al.* 2010).

1.4.3 N-3 LCPUFA status and Mental health

In addition to cardiovascular and inflammatory diseases, n-3 LCPUFA status may also play a role in indicating mental health status, and there is some evidence suggesting that the level of n-3 LCPUFA in patients with mental health disorders is lower than the healthy individuals (Peet *et al.* 1998; Conquer *et al.* 2000). A study from Peet *et al.* showed that the n-3 LCPUFA status in subjects with symptom of depression were significantly lower than those in healthy control subjects (5.4% Vs. 9.0%) (Peet *et al.* 1998). A study investigating the attention-deficit/hyperactivity disorder (ADHD) also showed that the mean value of n-3 LCPUFA status was significantly lower in plasma phospholipids (3.34% Vs. 4.87%) and erythrocytes (6.57% Vs. 8.04%) in the ADHD group versus controls (Caryl *et al.* 2006). Brain autopsies of patients who had Alzheimer's disease (AD) showed significantly higher levels of saturated fat and lower levels of PUFA, particularly DHA, in the hippocampus and frontal lobes compared to age-matched controls (Söderberg *et al.* 1991). The role of n-3 LCPUFA status in AD is supported by a study from Conquer *et al.* which showed that the n-3 fatty acid status in plasma total phospholipids in AD patients were significantly lower than in an age-matched group of healthy control

subjects (5.6% Vs. 7.8%) (Conquer *et al.* 2000). Although the health benefits of n-3 LCPUFA supplementation on mental health is still controversial (Ramakrishnan *et al.* 2009; Milte *et al.* 2009; Karr *et al.* 2011), the abnormal n-3 LCPUFA status in patients with mental health disorders may indicate the therapeutic potential for n-3 LCPUFA supplementation in mental illness patients with very low n-3 LCPUFA status.

1.5 Conventional Measurement of Fatty Acid Status

Depending on the scientific question, the conventional assays of fatty acid status can be conducted in a range of tissues and blood fractions, including adipose tissue (Hirsch *et al.* 1960), cheek cells (Mcmurchie *et al.* 1984a), plasma, red blood cells and whole blood (Risé *et al.* 2007).

1.5.1 Adipose tissue

The fatty acid status of subcutaneous adipose tissue has been used as an index for the long-term habitual dietary intake of fatty acids in humans since the early 1960s (Hirsch *et al.* 1960). The adipose tissue biopsy used in this measurement is mostly collected from the upper buttock with a needle and a disposable syringe (Hirsch *et al.* 1960). Lipids from adipose tissue are then extracted from a mixture of hexane and isopropanol and transesterified in acidified methanol (Dayton *et al.* 1966) or boron trifluoride methanol (Metcalfe *et al.* 1966), and the resultant fatty acid methyl esters (FAME) are analysed by gas-liquid chromatography (Dayton *et al.* 1966). The adipose tissue fatty acids are considered the best choice for the study of long-term fatty acids intake (> 6 months) in weight stable individuals because of their slow turnover (2-3

years) and lack of response to acute disease (Dayton *et al.* 1966). However, the tissue-sampling site can have an impact on the fatty acids composition (Phinney *et al.* 1994; Malcom *et al.* 1989). A study comparing abdominal fat with fat from the inner and outer thigh showed higher levels of SFA (expressed as a weight percentage of total fatty acids) in the abdominal fat (Phinney *et al.* 1994). Differences in PUFA levels were also reported in this study, with the highest levels found in the outer thigh, the lowest levels were in the abdomen and intermediate levels were in the inner thigh. These differences were as great as 30% for SFA and 17% for the PUFA (Phinney *et al.* 1994). A comparison of fatty acid profiles from a deep-seated site (perirenal) and two subcutaneous sites (abdomen and buttocks) from autopsies of a group of adults of mixed ethnicity showed that the proportion of SFA were highest in the perirenal adipose tissue and lowest in the gluteal depot, whereas MUFA were highest in the gluteal depot, PUFA profiles however were not significantly different across these three sites. (Malcom *et al.* 1989). Katan et al. have reported that changes in fatty acid composition of abdominal subcutaneous adipose tissue are more evident than adipose tissue from the gluteal pool in reflecting the dietary n-3 LCPUFA intakes (Kanta et al. 1997). Therefore, the adipose tissue sampling must be carried out with caution and precision to ensure that the results between individuals are comparable. Although adipose tissue often considered a gold standard biomarker for long-term dietary changes (>6 months), due to the difficulties in obtaining the tissue samples, adipose tissue probably is not a good choice for use in large clinical studies or for routine population screening (Beynen and Katan, 1985).

1.5.2 Cheek cells

McMurchie *et al.* established an assay of FA status in cheek (buccal epithelial) cells

and showed that this provided a reliable measure of dietary lipids in human body. The analysis involves an initial collection of cheek cells by rinsing the mouth vigorously with 30-35ml of glass distilled water. The total lipids in cheek cells are then extracted in the presence of an antioxidant, and the phospholipids separated from the other lipid classes by thin-layer chromatography (TLC), and methylated in 1% (v/v) H_2SO_4 in methanol. The resultant FAME are then analysed by gas-liquid chromatography (Mcmurchie *et al.* 1984a). Their results showed that cheek cell fatty acid composition provided a sensitive marker of fatty acids intakes in humans (Mcmurchie *et al.* 1984a). In another study, the same approach was used to estimate the fatty acid status in infants, and also showed that oral mucosal cell LCPUFA composition in preterm infants is closely relate to dietary LCPUFA intake (Koletzko *et al.* 1999). In addition, the DHA content of cheek cell phospholipids of newborn infants was highly correlated with that of both red blood cell lipids and plasma phospholipids (Hoffman *et al.* 1999). However, in a 12 week diet intervention trial, the proportion of LA in the cheek cell phospholipids responded to a change from a low to a high PUFA/ SFA diet but not to the reverse manipulation (McMurchie *et al.* 1984b). This suggests that the response of cheek cell phospholipids to the changes in dietary intake of different fatty acids is not the same. In particular, cheek cells appear to be more sensitive changes in dietary LCPUFA, but les responsive to the changes of SFA content in diet (Skeaff *et al.* 2003). Therefore, the validity of using of check cells for investigating the fatty acid status remains uncertain in dietary studies.

1.5.3 Plasma

Another important biomarker for measuring the fatty acid status in humans is plasma levels of individual fatty acids, and this technique has been used extensively since the

1950s (Bronte-Stewart *et al.*1956; Ahrens *et al.* 1957). When analyzing the plasma fatty acid levels, 5-10ml of venous blood is usually collected in a tube which contains an anticoagulant, such as ethylenediaminetetraacetic acid (EDTA) or heparin. Plasma is obtained by centrifugation of the whole blood sample to spin down celluar components, and the plasma extracted with chloroform/methanol (2:1) to obtain plasma total lipids (Folch *et al.* 1957). The plasma total lipids can then be directly transmethylated with acidified methanol at 70°C, and the resultant FAME are analysed by gas chromatography (GC) to obtain a fatty acid composition of plasma total lipids (Agostoni *et al.* 1998). However, given the variability in the concentration of triglycerides, cholesterol ester, and phospholipid might cause difficulty in interpretation of fatty acid status in plasma total lipid, lipid classes are usually separated from plasma total lipids by one-dimensional TLC. The resultant plasma lipid fractions (triglycerides, cholesterol esters, and phospholipids) are collected separately and transesterified and analysed in the same way as for plasma total lipids (Ma *et al.* 1995). Plasma fatty acid levels have been shown to provide a sensitive indicator of changes in dietary intake of LCPUFA (Dougherty *et al.* 1987). However, the strength of the relationships between the estimated dietary intake of specific fatty acids and the proportions in plasma lipids vary for different fatty acids (Glatz *et al.* 1989; Vessby 2003). The relationships between the amount of PUFA in the diet and the proportions of corresponding fatty acids in plasma lipids are relatively strong. The relationships for SFA are weaker, and those between MUFA in the diet and the levels of corresponding fatty acids in plasma lipids are virtually non-existent (Ma *et al.* 1995). This may reflect the fact that while the content of PUFA in human tissue and blood lipids are ultimately derived from the diet (Riboli *et al.* 1987; James *et al.* 1993), there is considerable endogenous synthesis of MUFA and SFA in the human body (Ma

et al. 1995). A limitation for using plasma fatty acids as a biomarker of fatty acid status in human body is the short lifespan of circulating fats in plasma. The proportions of fatty acids in plasma only mirror the dietary fat composition over the last few days (serum cholesterol ester and plasma phospholipid fatty acids) or meals (plasma triglyceride) (Kohlmeier *et al.* 1995). Therefore, plasma fatty acid status lack the ability to provide information on long term dietary fat intake.

1.5.4 Red blood cells

Due to the turn over of fatty acid profile in red blood cells is slower than plasma, the measurement of red blood cell lipids has been regarded by many investigators as a more reliable indicators of relatively long term (few weeks to few months) dietary fatty acid consumption than plasma lipids (Glatz *et al.* 1989; Siscovik *et al.* 1995; Poppitt *et al.* 2005). For the measurement of fatty acid composition in red blood cell phospholipids, 5-10ml of venous blood are collected in a tube which contains an anticoagulant such as EDTA or heparin, and the sample is centrifuged to separate plasma from red blood cells. After removal of plasma and buffy coat, the packed red cells are washed in sodium chloride buffer, and extracted with chloroform/isopropanol (2:1) to obtain erythrocyte total lipids (Broekhuyse, 1974). The phospholipid fraction of red blood cells can be isolated from total lipids by TLC, and transesterified to FAME prior to GC profiling of fatty acids (Christie 1989). In metabolic studies, red blood cells fatty acid profile was less affected by changes in short-term (a few days) dietary intake of EPA and DHA, when compared to the fatty acid profiles of plasma free fatty acids or of plasma or platelet phospholipids (von Schacky *et al.* 1985, Harris *et al*. 2007). On this basis, the “Omega-3 Index” which obtained from the sum of EPA + DHA % (expressed as a weight percentage in total fatty acids) in red blood cells was

suggested as an indicator of the risk of death from coronary heart disease (Harris and von Schacky 2004). Harris *et al.* showed that this "Omega-3 Index" was able to identify persons at risk for sudden cardiovascular death in both the general population and among patients with established coronary artery disease (Harris *et al.* 2008). The "Omega-3 Index" was reported to be more easily measured and discriminative than most other cardiovascular risk indicators thus far proposed (von Schacky *et al.* 2011). However, the clinical evidences of the usefulness of the "Omega-3 Index" is limited, and the need of labour intensive and time consuming processing (centrifugue, lipid extraction and TLC seperation) immediately after phlebotomy limits the application of red blood cells in population-based clinical trials and diagnosis.

1.5.5 Whole blood

Whole blood as a fatty acid biomarker is an attractive option for epidemiologic studies when compared with plasma or red blood cells samples, because of its accessibility and simpler sample processing requirements. For whole blood fatty acid status analysis, 5-10ml of venous blood is collected in a tube which contains an anticoagulant such as EDTA or heparin. Total lipids from the whole blood sample are extracted with chloroform/isopropanol (2:1) and transesterified to FAME. The resultant FAME are analysed by GC for fatty acid status (Baylin *et al.* 2005). A study by Baylin *et al.* revealed that fasting whole blood reflects dietary ALA and LA intake, and provides results that were comparable to those obtained from fasting plasma and adipose tissue (Baylin *et al.* 2005). However, the interpretation of whole blood fatty acid status is more difficult, because the proportion of blood volume that is occupied by red blood cells varies with gender and age (Cirillo *et al.* 1992), and can be altered by factors such as hypertension (Cirillo *et al.* 1992), pulmonary and cardiac diseases,

and pregnancy (Belo *et al.* 2002). Thus, the potential for differences in the percentage of whole blood composed of plasma and red blood cells needs to be considered carefully to avoid the misinterpretation of whole blood fatty acid data (Risé *et al.* 2007).

1.5.6 Small blood volume

Conventional measurement of FA status in blood involves multi-step procedures, which are time-consuming and associated with using large volumes of organic solvents. For example, in lipid extraction, the disposal of organic solvents is always an expensive and time-consuming procedure, since they need to be removed by evaporation in the absence of oxygen (evaporation under an inert gas stream, usually nitrogen) to minimise the oxidative losses of fatty acids (Risé *et al.* 2005).

Ohta *et al.* described a method to obtain lipid status from a small volume of plasma (Ohta *et al.* 1990). The method involved directly applying a plasma sample to TLC to separate phospholipids from other lipid classes without any prior lipid extraction. The separated phospholipids were then transerterified with boron trifluoride (BF_3) reagent at 100°C for 30 mins and the resultant FAME were analysed by GC. Their results showed that the direct application method gave very similar fatty acids composition to the standard method of applying plasma total lipids extract to TLC. After comparison of different amounts of plasma (from 20µl to 120µl) applied on TLC, the authors claimed that there was no significant differences in the results of the fatty acid composition measurement between small and large volumes of samples if the amount of plasma applied on TLC was higher than 20µl, which meant that this simplified method was suitable for analyzing the fatty acids in plasma lipids from a 50µl of

fingertip blood sample (Ohta *et al.* 1990). However, an cautious examination of the data provided in the paper revealed that the fatty acid composition results from different volumes of sample varied. Although all SFA and most MUFA showed similar results when the volume of plasma varied between 20μl to 120μl, a number of LCPUFA showed significant differences between different blood volumes. For example, the difference in DHA content (expressed as a weight percentage of total fatty acids) measured in 20μl of plasma and 120μl of plasma was nearly 40%. Thus, this rapid measurement of plasma lipids needs to be further validated before it can be applied to the clinical practise.

Recently, an even simpler method which employed direct transmethylation in measurement of the FA status using 100μl of fasting whole blood was developed (Rizzo *et al.* 2010). Their studies demonstrated that the AA/ EPA ratio and the n-3/ n-6 ratio in the fasting whole blood samples were closely correlated with the corresponding ratios in the red blood cell phospholipids. Given the impracticality of assessing red blood cell phospholipids in some large cohort studies, they suggested the using of small volumes of whole blood as an attractive alternative to use of red blood cells since this eliminated a major sample processing step (centrifuge) (Rizzo *et al.* 2010).

1.6 Dried blood spot (DBS) technique

The DBS technique is a method of screening biomarkers for metabolic disorders. In this method, a drop of blood obtained via heel or fingerprick with an automatic lancet is applied to a sampling paper, and allowed to dry. DBS can then be posted to a

laboratory for further analysis. The DBS technique was first used to screen for phenylketonuria in neonates in the early 1960s (Guthrie and Susi, 1963), and more recently for the quantitation of human immunodeficiency virus (HIV) and the assay of thyroid status (Parker and Cubitt 1999). The limitation of sensitivity and specificity when screening such small volumes of blood (~20µl) has restricted use of the DBS technique for other application for many years. However, recent advances in analytical techniques, in particular the progress in mass spectrometry, the production of monoclonal antibodies, and the introduction of the polymerase chain reaction have overcome many of these problems, and allowing the potential of a vast bank of stored DBS to be realised by biochemists, geneticists, and microbiologists (Parker and Cubitt 1999). Over the past decade, the DBS technique has become a significant tool in newborn screening, epidemiological studies, and drug monitoring.

1.6.1 DBS technique in newborn screening

Newborn screening programmes aim to identify neonates that do not have symptoms but are at risk of developing serious health conditions. Identifying such neonates through screening can, depending on their condition, improve the accuracy of early diagnosis of diseases, enable early treatment to improve health or ameliorate illness (Oliver *et al.* 2004). The concept that DBS could be used to screen for metabolic disease in large population of neonates were first introduced in Scotland in 1963 when Dr. Robert Guthrie used DBS specimens to measure phenylalanine in newborns to detect phenylketonuria (Guthrie and Susi, 1963). Since then, DBS samples from 2-9 day old babies, has been collected rountinely in over 20 countries. It has becomes routine to test these newborns DBS samples in the Western World in order to detect metabolic disorders in inborn errors of metabolism for numerous of biological

markers including amino acids enzymes, organic acids, hormones, vitamins, trace elements, specific antibodies, and antigens (Nyambi *et al.* 1994; Eyles *et al.* 2010; Keevil 2011).

1.6.2 DBS technique in epidemiological studies

Large scale epidemiological surveys can be performed rapidly and enable strategies to be made targeting at risk populations. This may be particularly valuable for monitoring the immunity of general population and the extent of vaccination coverage at a time when the eradication of several viruses is in prospect (Parker and Cubitt 1999). The application of DBS technique in epidemiological studies has been used to provide information on the prevalence of an antibody to a pathogen in large population, thus giving a more representative indication of the impact of a disease outbreak on a community than screening blood donors, who are often unrepresentative. Following the historical use of DBS to study the prevalence of HBV (Farzadegan *et al.* 1978), rubella (Vejtorp and Leerhoy 1981) and measles (Wassilak*et al.* 1984), the feasibility of using similar technology for sentinel surveillance of HIV in the developing countries was evaluated (Arya 1988). Meanwhile, the need for an inexpensive and effective means for monitoring the extent of HIV in Europe (Varnier *et al.* 1988) and the USA (Hoff *et al.* 1988) was also being widely recognised. The potential for the using of DBS for the detection of nucleic acid was realised in 1987, when deoxyribonucleic acid (DNA) was released from the filter paper by micro-extraction for the purposes of newborn screening (McCabe *et al.* 1987). The molecular analysis of DNA obtained from neonatal DBS samples has enabled detection of genetic mutations responsible for cystic fibrosis (Hopfer *et al.* 1995),

oncogenesis (Fishbein and Kirsch 1993), athalassaemia (Harada *et al.* 1994), and markers of type-1 diabetes (Sjöroos *et al.* 1995).

1.6.3 DBS technique in Therapeutic drug monitoring

Therapeutic drug monitoring (TDM) is a branch of clinical chemistry and clinical pharmacology that specialises in the measurement of medication concentrations in blood. TDM is aimed at improving the efficacy of patient treatment by individually adjusting the dose of drugs to which that clinical experience or clinical trials have shown it to improve outcomes in the general or specific populations (Watson *et al.* 2001). It mainly focuses on drugs with a narrow therapeutic range, in particular drugs that can easily be underdosed or overdosed (Marshall and Bangert 2008). Given the significant advantage of DBS technique in blood collection and sample transportation, this provides an attractive alternative for use in TDM. The DBS technique has been put forward as a suitable tool for monitoring various classes of therapeutic drugs: antibiotics, antimalarials, antiepileptics and antiretrovirals (Lampe *et al.* 1987; Koal *et al.* 2005; ter Heine *et al.* 2008). Monitoring of many medications using DBS technique have already been published, including theophylline, an antiasthmatic drug (Watson *et al.* 2001), paracetamol, an analgesic (Oliveira *et al.* 2002), and metformin, an antidiabetic (Aburuz *et al.* 2006). The DBS technique thus seems to be a useful tool in TDM, and assays have been already developed for many medicines. However, a number of factors in current DBS tests have the potential to introduce error, such as the quality of the blood spot and the sampling paper, contamination in blood collection and sample handling, degradation loss of sample during transport and storage. Therefore, more quality assurance and standardization in DBS method is

needed if this method is going to be universally applied in TDM (Edelbroek *et al.* 2009).

1.6.4 Stability of biomarkers on DBS

For more than 30 years, investigators have studied the stability of the markers in DBS during storage (Levy *et al.* 1985; Chace *et al.* 1999; Strnadova *et al.* 2007). Newman *et al.* demonstrated that DBS, which were stored at room temperature, showed no significant loss of vitamin D detection on repeated analysis over more than 4 months (Newman *et al.* 2009), whilst tacrolimus and everolimus have been shown to be stable at room temperature for at least 1 month (van der Heijden *et al.* 2009). A low but steady decrease (~5% per year) of the amino acids has been found in DBS samples over long-term (5 years) storage at room temperatures (Strnadová *et al.* 2007). Temperature has been found to be one of major factors which caused natural degradation loss of markers in DBS, and various temperature conditions have been investigated in an attempt to define optimal storage condition for DBS. Amino acids in DBS were found to lose 20% of their initial concentrations after storage for one month at 37°C (Chace *et al.* 1999). Thyroid hormones and thyrotropin in DBS were also unstable after four weeks of storage at either 37°C or room temperature (Waite *et al.* 1987). Similarly, the activity of galactosylcerebrosidase and neutral α-glucosidase in DBS samples slowly decreased if the DBS samples were stored at room temperature. Humidity is another major factor which causes the loss of markers in DBS, in a study which investigated the stabilities of 35 markers in DBS stored at an elevated temperature (37 °C) and at low (below 30%) or high (above 50%) relative humidity showed that after a month of storage in the high humidity environment, only 6 markers in DBS lost less than 20% of initial concentrations, while 7 markers in DBS

lost more than 90% of their initial levels (Adam *et al.* 2011). Therefore, in general, the ideal storage conditions recommended for stabilise markers in DBS during prolonged period of time are -20°C in sealed bags with desiccant (Adam *et al.* 2011). However, for practical reasons, having a DBS method that samples can be stored at room temperature will offer significant advantages, since it elimates the costs and logistical problems of couriering the DBS samples on dry ice from the hospital to the diagnostic laboratory.

1.7 Application of the DBS technique to measure fatty acid status

Recently, the DBS technique has been adopted for quick, inexpensive and minimally invasive measurement of fatty acid status in humans. Nishio *et al.* first introduced the DBS technique for the measurement of fatty acid levels in whole blood. He described a fatty acid assay of using a DBS absorbed on filter paper to diagnose adrenoleukodystrophy, an x-linked metabolic disorder, characterised by progressive neurologic deterioration due to demyelination of the cerebral white matter. The method required ~100µl of blood and involved simultaneous fatty acid extraction and transesterification with MeOH/HCL at 100°C for 1 hour, the resultant FAME were analysed by gas chromatography-mass spectrometry (Nishio *et al.* 1986). Nishio's method demonstrated the potential for the DBS technique to be used for fatty acid analysis of human tissue samples. However, since their studies only focused on the level of biomarkers of adrenoleukodystrophy (C22:0, C24:0, C26:0), the usefulness of DBS technique in measurement of whole fatty acid composition in human blood samples was not established (Nishio *et al.* 1986).

Ichihara *et al.* developed a quick alkaline methanolysis procedure for analysis of LCPUFA status in dried blood spots or dried breast milk spots. This method involved an initial wash out of any contaminants from the collection paper by soaking the paper for a few minutes in an acetone solution prior to placing the blood or breast milk sample on the paper. After applying the sample onto the collection paper, a direct alkaline methanolysis procedure without any fatty acid extraction or TLC separation was employed, and the resultant FAME were analysed by GC (Ichihara *et al.* 2002). The authors claimed that this method had two major merits: firstly, it enabled analysis of the fatty acid composition of small volumes of sample; secondly, it allowed the researcher to follow up the successive changes in C_{20-22} PUFA composition of blood lipids in response to changes in the diet. However, the alkaline methanolysis procedure used in this method makes it only available for analysing the fatty acid composition of glycerolipids in body fluid, because the free fatty acids, steryl esters and sphingomyelins are not methylated under alkaline conditions.

Recently, Marangoni *et al.* described a rapid fatty acid profiling assay to analyse the fatty acids level in whole blood using DBS collected from the fingertip. After comparing the fatty acids composition measured from capillary DBS samples with that measured from venous blood samples by conventional procedures in six subjects, they claimed that the DBS method provided virtually identical information to conventional measurement (Marangoni *et al.* 2004). Further application of this DBS method in 108 healthy subjects showed that it was suitable for assessing associations between circulating fatty acids and various life style and dietary parameters in a large number of human subjects, and the authors suggested that it could therefore be

applicable to epidemiological studies and clinical intervention practice (Marangoni *et al.* 2007). Marangoni's method improved Ichihara's blood spot assay, however, the sample size they used for comparison between DBS method and conventional measurements was small (n=6), and the coefficient of variation (CV) in some LCPUFA among the subjects was large. For example, the proportion of DHA in whole blood total lipids obtained from conventional assay and DBS method was 4.59±3.01% and 4.02±2.10%, respectively. Given the CV of DHA level among the subjects was higher than 50% in both the conventional assay group and the DBS group, it is perfunctory to conclude that these two groups provided identical information through direct comparison of mean values. It should also be noted that, the mean value of DHA from conventional blood total lipids assay (4.59%) was about 15% higher than that from DBS method (4.0%), however this difference was ignored by author.

Armstrong *et al.* reported an even quicker estimation of n-3 fatty acid status in DBS using a one minute direct microwave transesterification to replace one hour conventional heat transesterification. The author claimed that this newly described method enabled the determination of a single individual's blood n-3 LCPUFA status within 30mins. It was claimed to be particularly useful when screening research participants for n-3 LCPUFA intervention studies, since when the data was expressed as a percentage of the n-3 LCPUFA in total blood fatty acids, the result determined from microwave transesterification showed no significant difference with the result from conventional oven incubation (Armstrong *et al.* 2008). However, the method significantly underestimated the absolute values of PUFA and MUFA concentrations in whole blood samples. Total MUFA concentrations determined by microwave transesterification was only 58% of the total MUFA as determined by conventional

heat treatment. In addition, among all PUFA in whole blood, the concentrations of LA and ALA obtained using the microwave method were only about 60% of those obtained from using the conventional heat treatment, while the concentrations of AA, EPA and DHA determined by microwave transesterification were around 70% of the levels obtained from the conventional assay. Therefore, additional modifications of microwave transesterification would seem to be needed before this can be applied to clinical practise.

When compared with the conventional measurement of fatty acid status, the DBS technique has many merits. The collection of capillary blood on filter paper has significant advantages over venepuncture, particularly in neonates and elderly. It is simple to perform, requires minimal training, and does not involve the risks associated with the use and disposal of needles and syringes. Handling of potentially infective material is also reduced since the need to centrifuge and separate serum/plasma from red blood cells is eliminated. Processing of DBS is also considerably cheaper and quicker than samples collected by venepuncture, because there is no need for lipid extraction and TLC separation. Biohazard risks associated with shipping are also minimised as DBS can be shipped in sealed envelopes to reference centres, whereas blood or tissue samples need to be packed in break-proof containers to prevent any leak or breakage in transit. Furthermore, with DBS technique, there is no requirement for transporting samples on dry ice, thus substantially reduce the transport cost (Parker and Cubitt 1999).

However, the stability of LCPUFA in DBS has been questioned since oxidation of LCPUFA in blood spots has been reported (Ichihara *et al.* 2002), and it has therefore

been recommended that samples are either processed immediately (Agostoni *et al.* 2008a) or treated by BHT and stored at -20°C to help stabilise the LCPUFA (Bell *et al.* 2011). Ichihara *et al.* proposed to impregnate the filter paper with 0.05% BHT to stabilise LCPUFA on blood spot, and claimed that this method could make AA and DHA stable at room temperature for nearly one week (Ichihara *et al.* 2002). Maragoni *et al.* suggested that without any antioxidant, a storage temperature of 4°C can stabilise the LCPUFA in DBS for 2 weeks and the incorporation of 50ug BHT per sample can further prolong the stability of LCPUFA in DBS (Maragoni *et al.* 2004). Based on Maragoni *et al.*'s research, a commercial blood collection card (Fluka) for fatty acid composition test has been developed. According to the manufacturer's instruction, the blood sample collected on the paper stick can be stored at room temperature with a humidity of less than 30% for up to 2 months and at 4°C for up to 2 years. However, a recent long term stability study conducted on DBS samples collected on Fluka collection card (pre-treated by 0.5mg/ml BHT in ethanol) showed that there was an approximately 40% loss of the n-3 LCPUFA levels (expressed as a weight percentage of total fatty acids) in DBS samples after either 1 month storage at room temperature or 3 month storage at 4°C (Min *et al.* 2011). Another study conducted on Whatman 903 blood collection cards showed that even when DBS samples were stored at 4°C in the presence of BHT (0.5mg/ml BHT in ethanol), progressive reductions of AA, EPA and DHA were seen from 48 hrs onwards with reductions of 15, 29 and 25% observed after 1 month. It has therefore been recommended that blood spot samples are either processed immediately or stored at -20°C to help stabilise the LCPUFA (Bell *et al*. 2011). A recent study from Metherel *et al.* showed that the pre-treatment of chromatography paper with 5mg/ml BHT for DBS sampling, and subsequent storage of DBS in vacuum sealed containers/bags

after nitrogen purging may prevent LCPUFA degradation in DBS for up to 8 weeks at room temperature (Metherel *et al.* 2013). However, the study was based on blood which contained relatively low level of n-3 LCPUFA (EPA 0.86%, DHA 1.64%, expressed as a weight percentage of total fatty acids). Therefore, the result is hard to apply to clinical practice where the n-3 LCPUFA content in DBS are likely to cover a wide range. Furthermore, the complicated sample processing required for this method including use of a vacuum sealer and nitrogen purging, also limits the potential for this method to be applied to large scale clinical trial and epidemiological studies.

Table 1.2 provides information about the characteristics of current published DBS assays which have been used to assess fatty acid status using the following items: country, controls, method of collection blood, assay range, procedure for fatty acid preprocessing, antioxidant, reported fatty acid stability, and reported contaminants. Using these information, the limitations and the quality of the respective assays can be assessed.

Table 1.2. DBS assays and their limitations

Publications	Country	Sampling	Sample processing	antioxidant	Contaminants	Stability	Limitations
Nishio *et al.* 1986	Japan	Blood 100µl, Source not mentioned	Direct transmethylation in MeOH/HCL at 100°C for 1 hr	Nil	16:0 and 18:0 fatty acids were detected in collection paper	SFA stable at room temperature for 1 week	High blood volume; Only analysed SFA (no MUFA or PUFA data)
Ichihara *et al.* 2002	Japan	Blood and breast milk (40-60µl), source not mentioned	Direct alkaline methanolysis at 25 °C for 2 mins	BHT (0.05%) saturate the collection paper	Contaminants on collection paper were washed out prior to sampling	EPA + DHA were stable at room temperature for 1 week	Only suitable to test the fatty acid composition of glycerolipids
Marangoni *et al.* 2004	Italy	Fingertip blood (20-50 µl) **Vs.** Venous blood (500 µl)	Direct transmethylation in MeOH/HCL at 100°C for 1 hr **Vs.** Conventional procedure	50µg BHT for storage periods > 2weeks	No contaminant peak was obtained from blood collection paper	All fatty acid were stable at 4 °C for 3 weeks	Small sample size and large variation of EPA and DHA in subjects; Significant difference of DHA between conventional assay and DBS was ignored by author
Armstrong *et al.* 2008	Canada	Fingertip blood (20-50 µl) **Vs.** Venous blood (100 µl)	Direct 1 minute micro- wave transmethylation **Vs.** Conventional procedure	Nil	Not mentioned	All samples were processed immediately	40% underestimation of MUFA and 30% underestimation of LCPUFA when compared with conventional assay

(Continued) Min *et al.* 2011	UK	Fingertip blood (20-50 µl) **Vs.** Venous blood (500 µl)	Direct transmethylation in 15% Acetyl chloride in MeOH at 70°C for 3 hrs **Vs.** Conventional procedure	BHT (0.05%) saturate the collection paper	No contaminant peak was obtained from blood collection paper	~40% lose of the n-3 LCPUFA after 1 month storage at room temperature and 3 month storage at 4°C	Not reptoted
Bell *et al.* 2011	UK	Fingertip blood (20-50µl) **Vs.** Venous blood (~4 ml)	Direct transmethylation in MeOH/HCL at 70°C for 1 hr **Vs.** Conventional procedure	BHT (0.05%) saturate the collection paper	Not mentioned	Significant loss of LCPUFA was seen after 2 days storage at 4°C; LCPUFA in DBS were stable at -20°C for 1 month	Not reported
Metherel *et al.* 2013	Canada	Venous blood drop onto a chromatography paper saturated of an area of 1 cm^2	Direct transmethylation in 14% boron trifluoride in methanol with hexane at 95°C for 1 hr	BHT solution at three concentrations (0, 2.5, 5mg/ml) impregnate the collection paper	Not mentioned	LCPUFA in DBS with 5.0mg/ml BHT and stored in vacuum sealed containers/bags after N_2 purging were stable for 8 weeks at room temperature	Complicated procedure for sample storage. Conclusion is base on results from a single individual with low n-3 LCPUFA content in blood

1.8 Factors causing the inaccuracy in determining fatty acid status in DBS

If the DBS technique for evaluation LCPUFA status of individuals is going to be adopted universally, investigators need to be assured that the method is accurate and stable over time. However, since the contaminants released from blood collection paper (Nishio *et al.* 1986) and the oxidative loss of LCPUFA in DBS (Ichihara *et al.* 2002) have been reported, the accuracy of the LCPUFA status determined through the current DBS methods has been questioned (Min *et al.* 2011; Bell *et al.* 2011). The factors which may cause inaccuracy in determination of fatty acid status in DBS are also reviewed here.

1.8.1 Effect of drying

Insufficient drying is likely to negatively influence the accuracy of the DBS test, since moisture may harm the fatty acids in DBS specimens by inducing bacterial growth (McInerney *et al.* 1979). Therefore, it is suggested to dry blood spot specimens completely before storage or transportation. During storage and transportation it is also critical to protect against humidity and moisture by packing DBS in low gas-permeable zip-lock bags with desiccant packages (Mei *et al.* 2001). The drying time should be varied depending on the paper type and the applied blood volume. In general, it is suggested that blood spot specimens should be dried for at least 3 hours over an open nonabsorbent surface at 15-22°C (Hannon *et al.* 2003). During the drying process, it is also important to keep the blood spot away from direct sunlight and heat, the blood spots should not be stacked or allowed to touch other surfaces to avoid contaminantion (Mei *et al.* 2001).

1.8.2 Contamination during sampling and processing

The contamination problem in DBS measurement of fatty acid status in humans was first reported at 1986 (Nishio *et al.* 1986). They found that there were contaminating substances released from their blood collection papers (Toyoroshi, Japan), and the structures were elucidated as SFA, palmitic acid (Cl6:0) and stearic acid (C18:0). Since their studies only focused on the concentration of certain very long chain SFA (22:0, 24:0, 26:0) which had longer retention times than that of palmitic acid and stearic acid, these contaminants did not negatively influence the test result. Therefore, they did not further quantitate the contaminants, and a method to eliminate the contaminants was also not investigated. In another study using Whatman 3MM paper as a blood collection paper to test fatty acid composition of glycerolipids in blood and breast milk, Ichihara *et al.* suggested a method to eliminate the contaminants (Ichihara *et al.* 2002). In their method, the collection papers were soaked for a few minutes in acetone reagent prior to use, so as to wash out the contaminants (Ichihara *et al.* 2002). Interestingly, some studies on DBS tests claimed that no contaminant was observed from the blood collection papers they used (Marangoni *et al.* 2004; Min *et al.* 2011), this is probably because the sources of blood collection papers are different. Since in such a small blood sample volume (20~50μl per blood spot), even a small amount of contaminant could contribute to an alteration in the results of blood fatty acid composition tests, there is clearly a need to investigate the possibility that the amount of contaminants varies from different sources of collection papers and to carefully select the most appropriate blood collection papers for fatty acid test.

1.8.3 Oxidative loss of LCPUFA in DBS during sample storage

Loss of fatty acids from DBS samples due to oxidation is thought to make a

contribution to inaccuracy measurements of fatty acids in DBS (Min *et al.* 2011; Bell *et al.* 2011). Unsaturated fatty acids, in particular the LCPUFA, are very susceptible to oxidation, and the oxidative reactions can produce free radicals, which then catalyse further lipid oxidation (Jain *et al.* 2001). Given that drying blood on filter paper will naturally expose all the fatty acids in blood to the air, investigators have been concerned about the stability of DBS samples collected and stored in this manner (Ichihara *et al.* 2002). Therefore, a standard practise is using BHT to impregnate the collection paper prior to blood collection (Ichihara *et al.* 2002; Marangoni *et al.* 2004). BHT is an antioxidant which has been widely used as a food additive to prevent oxidative damage to fat (Babich 1982). It inhibits oxidation by removing free radical intermediates and being oxidised itself, thereby terminating further oxidative reactions. However, recent studies showed that if the current DBS methods in measurement of fatty acid profiles in blood is used, a time dependent oxidative loss of LCPUFA will occur regardless of whether the DBS was protected by BHT (Min *et al.* 2011). Therefore, long term storage of DBS samples at room temperature or even at 4°C, can result in significant changes in the profile of the PUFA in DBS (Bell *et al*. 2011). The oxidative degradation of LCPUFA in DBS samples may be retarded if the DBS are protected by high dose of BHT (5mg/ml) and stored in a vaccum sealed bag after nitrogen purging (Metherel *et al.* 2013) or stored at -20°C (Bell *et al*. 2011). However, this is generally inconvenient and can be cost prohibitive, and therefore limits the ability to apply the DBS technique to large-scale clinical trials and epidemiological studies.

The mechanism involved in such a fast loss of LCPUFA content in DBS during storage is unclear. However, breakdown of the LCPUFA in DBS could occur as a

result of the presence of iron ions in blood, such as ferrous ion and ferric ion, which have the potential to accelerate hydroperoxide decomposition and give rise to the formation of free radicals such as peroxyl radical (ROO.) and alkyl radical (R.). These free radicals are highly reactive and can cause rapid oxidative degradation of LCPUFA (Sies 1997). Previous studies showed that in long term storage of blood samples, the release of sequestered iron from red blood cells enhanced the degradation of LCPUFA in blood (Chiu *et al.* 1989). However, this enhancement could be prevented by prior addition of an iron chelating agent or an antioxidant, and store the blood sample at -50°C (Chiu *et al.* 1989; Otto *et al.* 1997).

1.9 Rationale for Thesis

Conventional approaches to assaying fatty acids in blood involve invasive venous blood collection and expensive, time consuming multi-step processes, including centrifugation, extraction and purification which limit its usefulness when analyzing large sample numbers from clinical trials or in population screening tests. In addition, the collection of venous blood from some patients, particularly young infants and the elderly, can prove difficult and can raise ethical concerns. DBS technique for the determination of blood fatty acids is less invasive, and much quicker and cheaper than the conventional method, thus making it ideal for large clinical trials and as a diagnostic test.

Commercial DBS collection cards (Fluka, Whatman 903) for fatty acid composition test is already being advertised to consumers inviting them to post in samples on prepared papers to obtain an assessment of their n-3 and n-6 status. Such a test

assumes that the fatty acids in the blood, most notably AA, EPA and DHA, are stable from the time of spotting until the sample is analysed by the distant laboratory. However, the reported progressive oxidative loss of LCPUFA in DBS (Bell *et al.* 2011; Min *et al.* 2011) makes all the existing DBS methods impractical for measurement of fatty acid levels in humans, since it would necessitate that all samples are analysed at a fixed time after collection if results are to be compared.

The objective of the present study was to develop an accurate and robust DBS method that would allow samples to be stored at room temperature for at least two months without significant change in the LCPUFA content.

Chapter 2

Materials and Methods

2.1 Ethical considerations

Ethical approval for the study was obtained from The University of Adelaide Human Research Ethics Committee. All volunteers were fully informed of the nature of the study and received written information sheets regarding the background and requirements of the study. Volunteers who met the selection criteria were informed about the study protocol and the possibility of risks associated with the procedure, and their eligibility determined. Each volunteer was given the opportunity to discuss their participation in the study with a family member or friend. Prior to the blood collection, consent form was signed in front of our research staff by each volunteer. All the blood from volunteers were only used for the fatty acid analysis, and any remaining blood samples were discarded after completion of the study. During the experiment, the samples were stored in a secure facility and only accessible to the project investigators. All individual information and results were kept confidential.

2.2 Subjects

All subjects were recruited from the University of Adelaide and the Royal Adelaide Hospital. Once potential participants make contact with the investigators, they were given an information sheet which explained the background and aims of the study. A further selection of volunteers was conducted on the basis of each participant's responses to questions in a questionnaire regarding their general health status, habitual dietary consumption, and the frequency of n-3 supplements intake. Volunteers who

had bleeding disorders, serious diseases transmissible through blood or were unwilling to donate blood for fatty acids test were excluded from participating in the study.

2.3 Blood collection

Following an overnight fast, the participants selected for the blood collection stage were invited to attend a separate appointment where an appropriately qualified and trained member of our research staff collected 5 ml of blood from a vein in the antecubital fossa to a 10ml collection tube containing heparin as anticoagulant. Blood spot samples were obtained by absorbing 25 to 50 µl of fresh blood (both from venous and fingertip) on blood collection papers and all blood spot samples were air dried at room temperature for 5 hrs prior to further processing.

2.4 Fatty acid analysis

2.4.1 Chemicals and reagents

Organic solvents including chloroform, methanol, isopropanol and *n*-heptane were all analytical grade and obtained from Chem-Supply (Gillman, Australia). Butylated hydraoxyanisol (BHA), BHT, tert-Butylhydroquinone (TBHQ) and ahydrous sodium sulphate (Na_2SO_4) were purchased from Sigma-Aldrich (Missouri, USA). Sulphuric acid (H_2SO_4) was purchased from BDH Pty Ltd (Sussex, UK), and isotonic saline (0.9% w/v of NaCl) was purchased from Baxer Healthcare (NSW, Australia). Gases for GC analysis and sample preparation including ultra-high purity helium and high

purity hydrogen, medical air and high purity nitrogen were supplied by Coregas (SA, Australia), FAME standards were purchased from Nu-chek Prep Inc. (Elysian, USA).

2.4.2 Fatty acid analysis of blood spot

Blood spot was mixed with 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex ,UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA) and allowed to transmethylate for 3 hrs in a 70°C oven. After cooling down the vial to room temperature, the resultant FAME were extracted with 250 µl of distilled water and 750 µl of *n*-heptane. The top heptane layer was then transferred into 2 ml Agilent GC auto-injector vials containing a few grains of ahydrous Na_2SO_4 as the dehydrating agent.

2.4.3 Conventional fatty acid measurements of blood lipid fractions

2.4.3.1 Fatty acid extraction of whole blood

Antioxidant BHA (0.005%, w/v) was added to chloroform and isopropanol prior to extraction. 500 µl of whole blood was transferred into Kimble glass tubes (Kimble Chase, Vineland, USA). 1.0 ml of cold 0.9% w/v of NaCl was added into the tube to make up the total volume to 1.5ml. The total lipids from whole blood were extracted by chloroform/isopropanol (2:1, v/v) (Broekhuyse 1974). After addition of the chloroform/isopropanol, the blood sample was shaken vigorously and allowed to stand at room temperature for 5 mins. The sample was then centrifuged at 3000 rpm for 10 mins to separate the aqueous and organic phase. The organic layer (bottom layer) was transferred into a scintillation vial (Wheaton, USA), and evaporated to dryness under a steady nitrogen stream.

2.4.3.2 Fatty acid extraction of plasma

Antioxidant BHA (0.005%, w/v) was added to chloroform and methanol prior to extraction. 500 µl of plasma was transferred into Kimble glass tubes (Kimble Chase, Vineland, USA). 1.0 ml of cold 0.9% w/v of NaCl was added into the tube to make up the total volume to 1.5ml. The total lipids from plasma were extracted by chloroform/methanol (2:1, v/v) (Folch *et al.* 1957). After addition of the chloroform/methanol, the plasma sample was shaken vigorously and allowed to stand at room temperature for 5 mins. The sample was then centrifuged at 3000 rpm for 10 mins to separate the aqueous and organic phase. The organic layer (bottom layer) was transferred into a scintillation vial (Wheaton, USA) and evaporated to dryness under a steady nitrogen stream.

2.4.3.3 Fatty acid extraction of red blood cell

Antioxidant BHA (0.005%, w/v) was added to chloroform and isopropanol prior to extraction. 500 µl of red blood cells were transferred into Kimble glass tubes (Kimble Chase, Vineland, USA). 1.0 ml of cold 0.9% w/v of NaCl was added into the tube to make up the total volume to 1.5ml. The total lipids from red blood cells were extracted by chloroform/isopropanol (2:1, v/v) (Broekhuyse 1974). After addition of the chloroform/isopropanol, the red blood cells sample was shaken vigorously and allowed to stand at room temperature for 5 mins. The sample was then centrifuged at 3000 rpm for 10 mins to separate the aqueous and organic phase. The organic layer (bottom layer) was transferred into a scintillation vial (Wheaton, USA) and evaporated to dryness under a steady nitrogen stream.

2.4.3.4 Phospholipids separation

The total lipid extract from plasma or red blood cells was resuspended in 150ul chloroform/methanol (9:1, v/v) for phospholipids separation. The phospholipids fraction in plasma or red blood cells total lipids was separated by TLC on silica gel plates (Silica gel 60H; Merck, Darmstadt, Germany). The mobile phase for TLC was petroleum spirit/acetone (3:1, v/v). The phospholipids band was visualised under ultraviolet light and scraped into scintillation vials for fatty acid transmethylation.

2.4.3.5 Fatty acid transmethylation

Lipid sample (total lipid extract or phospholipids fraction) was mixed with 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex ,UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA) and allow to transmethylate for 3 hrs in a 70°C oven. After cooling the vial to room temperature, the resultant FAME were extracted with 250 µl of distilled water and 750 µl of *n*-heptane. The top heptane layer (containing FAME) was then transferred into 2 ml Agilent GC auto-injector vials containing a few grains of anhydrous Na_2SO_4 as the dehydrating agent for GC analysis.

2.4.4 Gas chromatography analysis of fatty acid methyl esters

FAME were separated and quantified by using a GC (Hewlett-Packard 6890; Palo Alto, CA, USA) equipped with a BPX70 capillary column 50m x 0.32mm, film thickness 0.25µm (SGC Pty Ltd., Victoria, Australia), programmed temperature vaporization injector (PTV) and a flame ionisation detector (FID). The PTV temperature was set at 250 °C and the FID temperature at 300 °C, a programmed temperature ramp (140-240°C) was used. Helium gas was utilised as a carrier at a

flow rate of 35 cm per second in the column and the inlet split ratio was set at 20:1. The identification and quantification of FAME was achieved by comparing the retention times and peak area values of unknown samples to those of commercial lipid standards (Nu-Chek Prep Inc., Elysian, MN, USA) using the Hewlett-Packard Chemstation data system.

Chapter 3

Developing a Dried Blood Spot Technique For Determining the Fatty Acid Status of Individuals – Overcoming the Problem of Contaminants

3.1 Introduction

Fatty acid profiling of human blood samples which is used as a marker of fatty acid status in the human body, has become an important tool for understanding the relationship between dietary fatty acid intake and health (Kaaks *et al.* 1997). This is because blood fatty acid levels are thought to reflect dietary and biological actions, and blood is accessible for collection in human studies (Kaaks *et al.* 1997; Harris and von Schacky 2004; Marangoni *et al.* 2007). However, conventional approach to assaying fatty acids in blood involves invasive venous blood collection, time consuming multi-step processes, and expensive transport and storage as those limit its usefulness as a screening tool.

Recently, a simplified method for the determination of blood fatty acids was reported (Marangoni *et al.* 2004). In this method, a drop of blood from a finger prick is collected on a piece of chromatography paper to make a blood spot, and all lipid classes in the blood spot are transmethylated directly in acidic methnol, the resulting FAME are analysed by GC to evaluate fatty acid status (Marangoni *et al.* 2004). This blood spot method is quicker and cheaper than the conventional methods, thus making it an attractive alternative for use in large clinical trials and in population based screening diagnostics. However, recent attempts in our laboratory to validate this methodology found that contaminants from collection papers may reduce the accuracy

of the fatty acid results obtained from DBS tests (Liu *et al.* 2010).

The objective of this study was to systematically evaluate the levels of contaminants from different types of collection papers and identify a strategy to minimise the contaminant problem.

3.2 Design of the study

Fresh venous blood was analysed either directly or following application to a strip of blood collection paper (1.5x1.5cm). Six different types of collection papers were processed to identify any contaminants released from the blood collection papers during the methylation process. Further studies were also conducted to investigate whether other standard laboratory consumables (eg. gloves and storage bags) with the potential to come in contact with the DBS contained any contaminants which could affect the fatty acid analysis results.The potential strategies for minimise the negative influence of contaminants were also investigated in the current study.

3.3 Materials and Methods

3.3.1 Blood spot collection papers

Three sources of commercially developed blood spot collection products and three types of chromatography papers were tested. The commercial collection products included: Fluka blood collection kit (Sigma-Aldrich, Buchs, Switzerland), Hemaform-80 plate kit (Spot on science, Austin, USA), Whatman 903 specimen collection paper (3 batches, all from the same manufacture) (Whatman, Buckingham, UK); The chromatography papers included: Whatman 3MM chromatography paper (46x57cm,

Whatman, Buckingham, UK), Whatman ion exchange chromatography paper (46x57cm, Whatman, Buckingham, UK) and Whatman glass microfiber filter paper (GF/B 47mm, Whatman, Buckingham, UK). The Fluka blood collection kit and the Hemaspot-80 plate kit are commercially developed products, which are marketed for the measurement of blood n-3 LCPUFA status. The Whatman 903 specimen collection paper is a routine blood collection paper for the screening test of various metabolic disorders performed on every infant in the Western World. The Whatman 3MM chromatography paper was reported as a blood collection paper in clinical trials for the identification of blood disorder (Tangvarasittichai *et al.* 2008), and for the measurement of blood cholesterol and triglyceride levels (Lakshmy *et al.* 2010). Whatman ion exchange chromatography paper and Whatman glass microfiber filter paper were chosen to compare with other blood collection papers, since they are readily available in our lab, and have the potential to provide an alternative collection matrix.

3.3.2 Blood collection

Approximately 5 ml of blood was collected from the antecubital vein of one healthy volunteer aged 30 by an appropriately qualified and trained member of our research staff.

3.3.3 Fatty acid analysis

3.3.3.1 Fatty acid analysis of whole blood

In order to evaluate the levels of fatty acids in whole blood, 25µl of fresh whole blood was direcly added to 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex ,UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA) and

transmethylated by heating in a 70°C oven for 3 hrs as described in Chapter 2. The resultant FAME were extracted into heptane and injected into a GC for analysis as described in Chapter 2. All samples were processed in triplicate.

3.3.3.2 Fatty acid analysis of blood spots

At the time of blood collection, two further aliquots of venous blood sample (25 μl and 50 μl, respectively) were spotted onto the collection area (1.5x1.5cm) of the strips of blood collection papers. After the blood was completely absorbed (~5mins), the blood spots were excised from the collection papers and placed into the methylating agent and processed following the same procedure as described for the fresh blood. All samples were processed in triplicate.

3.3.4 Evaluation of contaminant sources

An equivalent area (1.5x1.5cm) of the six types of blank collection papers without blood was subjected to the same methylation process as blood spot samples to evaluate the natural level of contaminants that were released by each type of collection paper during the transmethylation process.

In a separate experiment trying to eliminate the contaminants from collection papers, all types of blank collection papers in same size (1.5x1.5cm) were either soaked in acetone at room temperature twice (2mins each time), or soaked in methylating agent at room temperature for 1 hr, 3 hrs and 10 hrs or at 70°C for 1 hr, 2 hrs and 3 hrs. After soaking, the collection paper strips were dried in air at room temperature, and processed in the fresh methylating agent using the same methylation method described for the blood spot samples to evaluate the residual amount of contaminants

in each type of paper. All the samples were processed in triplicate.

To evaluate the potential contaminants from sources other than paper during sample collection, processing and storage, blank Whatman glass microfiber filter paper strips (1.5x1.5cm) were wiped on a $10cm^2$ surface area of various materials routinely used during the fatty acid analysis procedure, including a latex glove (Ansell Limited, Red Bank, NJ, USA), a nitrile glove (Ansell Limited, Red Bank, NJ, USA), a polyethylene (PE) snap lock bag (Glad limited, Australia), an aluminium foil-barrier ziplock bag (Whatman, Buckingham, UK), and a cellophane bag (BSB packaging Limited, Melbourne, Australia). The wiped filter papers were then processed using the same methylation method described for the blood spot samples to evaluate the amount of contaminants released. The contaminant content of blank glass microfiber filter paper was subtracted from wiped glass microfiber filter papers to assess the contaminants derived from the gloves and bags. All samples were processed in triplicate.

3.3.5 Statistical Analysis

All statistical analysis were conducted using PASW Statistic 18 (SPSS, Chicago, IL, US). Values are expressed as mean ± standard deviation (SD). The comparisons of contaminants released from collection papers before and after soaking in acetone at room temperature were made by Student's *t*-test with significance inferred at $P<0.01$. One-way ANOVA with comparison of individual means by Tukey's post-hoc test was used to determine the significant difference bwtween groups. Due to the number of statistical comparisons made, $P< 0.01$ was chosen to report statistically significant findings.

3.4 Results and Discussion

3.4.1 Contaminants from blood collection papers

3.4.1.1 Comparison of fatty acids results obtained from blood spot samples and fresh blood

When compared with direct measurement of fresh blood, analysis of 25µl blood spotted onto either the Whatman ion exchange paper or Whatman glass microfiber filter paper dose not produced different fatty acid results (Table 3.1). In contrast, the analysis of 25µl blood spotted on the Fluka collection paper or the Whatman 903 collection paper batch No.1 produced significantly lower levels of n-3 LCPUFA and AA (expressed as a weight percentage of total fatty acids) when compared with the fresh blood measurement (Table 3.1). This apparent reduction in the proportion of n-3 LCPUFA and AA in the blood spot samples was offset by a significant increase in the percentages of the SFA, palmitic acid and stearic acid measured in these samples. When the volume of blood on the bloods spot was increased to 50µl, the fatty acid composition measured from the blood spots collected on Fluka paper was not significantly different with fresh blood, however, analysis of 50 µl blood spotted on the Whatman 903 collection paper batch No.1 still produced a significantly different fatty acid profile to that in fresh blood (Table 3.1).

The blood spot technique is regarded as a quick and inexpensive method for determining the total lipid fatty acid composition in whole blood, and its effectiveness has been validated in a small number of clinical studies (Marangoni *et al.* 2007; Risé *et al.* 2008). However, none of these validation studies have assessed the accuracy of the DBS method for measuring the fatty acids in individuals in comparison to direct transmethylation of fresh whole blood. The results presented here showed that the

fatty acid status test results from blood spot samples could vary significantly from those obtained from fresh whole blood. The Fluka blood collection paper and the Whatman 903 collection paper have been on the market for many years. However, low volumes of blood (25μl) collected on any of these two types of papers produced dubious fatty acid results when compared to direct assessment of fatty acid status in the whole blood. My data clearly demonstrated that the use of these collection papers may result in the inaccurate determination of the amount of the respective classes of fatty acids as a proportion of total lipids, especially when the volume of blood collected on paper is low. This may due to the contaminants released from collection papers during the transmethylation process (Nishio *et al.* 1986). Thus, caution needs to be exercised when selecting the type of paper used in the collection of blood spot samples for fatty acid analyses.

Table 3.1. Fatty acid composition determined in 25 µl and 50 µl of blood spotted on five types of collection paper compared with direct transmethylation of 25 µl fresh blood

Fatty acid[1] (%)	**Fresh blood**	**Blood spot**									
		Whatman								**Fluka collection paper**	
		Glass fibre filter paper		**Ion exchange paper**		**3MM paper**		**903 Paper #1**			
	25 µl	**25 µl**	**50 µl**	**25 µl**	**50 µl**	**25 µl**	**50 µl**	**25 µl**	**50 µl**	**25 µl**	**50 µl**
16:0	22.8±0.3[a]	22.9±0.2[a]	22.9±0.3[a]	23.0±0.2[a]	22.9±0.2[a]	23.7 ±0.1[b]	23.2 ±0.1[ab]	26.2±0.4[d]	25.2±0.2[c]	25.0±0.3[c]	23.6±0.2[ab]
18:0	10.5±0.1[a]	10.6±0.1[a]	10.5±0.1[a]	10.7±0.2[a]	10.6±0.1[a]	11.3±0.2[ab]	10.8±0.1[a]	11.6±0.2[c]	11.2±0.1[b]	11.2±0.1[b]	10.9±0.2[ab]
18:1n-9	22.2±0.2[a]	22.2±0.1[a]	22.2±0.2[a]	22.0±0.2[a]	22.2±0.2[a]	22.0 ±0.1[ab]	22.3 ±0.1[a]	21.2±0.2[b]	21.5±0.3[ab]	21.6±0.3[ab]	22.0±0.3[a]
18:2n-6	25.8±0.3[a]	25.7±0.3[a]	25.7±0.1[a]	25.8±0.2[a]	25.7±0.2[a]	25.2 ±0.2[ab]	25.6 ±0.1[a]	24.5±0.2[c]	25.0±0.3[bc]	25.0±0.2[bc]	25.4±0.2[ab]
18:3n-3	0.4±0.01	0.4±0.01	0.4±0.03	0.4±0.02	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.02	0.4±0.01
20:4n-6	9.8±0.1[a]	9.7±0.1[a]	9.8±0.2[a]	9.7±0.1[a]	9.8±0.1[a]	9.5±0.2[ab]	9.7±0.1[a]	8.9±0.1[c]	9.2±0.1[b]	9.3±0.1[b]	9.5±0.2[ab]
20:5n-3	1.3±0.03[a]	1.3±0.02[a]	1.3±0.03[a]	1.3±0.01[a]	1.3±0.03[a]	1.2±0.02[ab]	1.3±0.03[a]	1.1±0.01[b]	1.2±0.02[ab]	1.2±0.02[ab]	1.2±0.02[ab]
22:4n-6	0.9±0.02	0.8±0.02	0.9±0.02	0.8±0.03	0.9±0.03	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.01	0.8±0.02	0.8±0.02
22:5n-3	1.2±0.02	1.2±0.03	1.2±0.02	1.2±0.01	1.2±0.02	1.2±0.03	1.2±0.03	1.1±0.02	1.2±0.03	1.2±0.02	1.2±0.02
22:6n-3	3.9±0.02[a]	3.8±0.02[a]	3.9±0.02[a]	3.8±0.03[a]	3.9±0.03[a]	3.8±0.02[ab]	3.8±0.02[a]	3.4±0.03[c]	3.6±0.02[bc]	3.6±0.03[bc]	3.7±0.02[ab]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between groups, $P<0.01$. #**1**: batch No.1

3.4.1.2 Contaminants from different papers

The presence of contaminants in blood collection papers was assessed by transmethylating blank paper segments which no blood had been added. I found that the two main contaminants in all types of collection papers corresponded to the SFA, palmitic acid and stearic acid (Table 3.2). This explains the increase in the percentages of palmitic acid and stearic acid detected in blood spot samples when compared with those in fresh blood. Furthermore, the total amount of contaminants varied between different types of collection papers. For example, the mean value of contaminants in Whatman ion exchange paper was only 0.12μg/cm^2, whereas the Whatman 903 paper batch No.1 had about 20 times of this amount. It is also noteworthy that even the same type of paper from the same manufacturer has the potential to release different amounts of contaminants between different batches. For example, the contaminants released from 3 batches of Whatman 903 paper ranged from 0.18μg/cm^2 to 2.4μg/cm^2 (Table 3.2).

Earlier studies conducted on Fluka paper and Whatman 903 paper claimed that no contaminant peak was observed in GC after processing the collection paper alone (Marangoni *et al.* 2004; Min *et al.* 2011). In contrast, I found contaminant peaks in all types of collection papers tested. It may be the case that small quantities of contaminants in the collection paper do not significantly alter the fatty acid composition test result. For example, blood spot samples collected on Whatman ion exchange paper and Whatman glass microfiber filter paper showed an identical fatty acid composition with that of fresh blood. However, for those papers which contained high amounts of contaminants, such as the Fluka collection paper and Whatman 903 collection paper batch No.1, the fatty acid results were clearly different to fresh blood,

especially when the volume of blood collected was small (Table 3.1).

Contaminating substances released from a Japanese blood collection paper (Toyoroshi, Japan) was first reported by Nishio *et al.* at 1986, and the structures of contaminants were elucidated as palmitic acid and stearic acid (Nishio *et al.* 1986). Another study using Whatman 3MM paper as a blood collection paper to test the fatty acid composition of glycerolipids in blood and breast milk suggested a method to wash out the contaminants from collection paper by soaking the paper in acetone for a few minutes prior to use (Ichihara *et al.* 2002). However, the structures of the contaminants in Whatman 3MM paper were not identified in their study. Given that the GC signals of contaminants released from collection papers corresponded to palmitic acid and stearic acid, one possibility is that they may be resin acids or fatty acids, because both are present in wood (from which these papers are ultimately synthesised) either as free acids or various esters (Alen *et al.* 2000). Resins and fatty acids from processed water samples obtained from a paper mill, including palmitic acid and stearic acid have been reported recently (Valto *et al.* 2011). Thus, it appears that these acids or their esters can remain as impurities in the final paper product and lead to the introduction of contaminants when assessing fatty acid composition of blood spots collected on these papers.

Table 3.2. Amount of contaminants released from collection papers during methylation

Contaminants corresponding to fatty acid peaks[1]	Whatman glass fibre filter paper	Whatman ion exchange paper	Whatman 3MM paper	Fluka Collection paper	Hemaspot-80	Whatman 903 paper #1	Whatman 903 paper #2	Whatman 903 paper #3
	1.5x1.5cm	**1.5x1.5cm**	**1.5x1.5cm**	**1.5x1.5cm**	**1.5x1.5cm**	**1.5x1.5cm**	**1.5x1.5cm**	**1.5x1.5cm**
16:0	0.08±0.03[a]	0.14±0.03[a]	0.40±0.02[b]	1.0±0.1[c]	0.31±0.03[b]	1.5±0.1[d]	1.0±0.1[c]	0.22±0.03[ab]
18:0	0.02±0.01[a]	0.07±0.02[a]	0.10±0.01[a]	0.3±0.04[b]	0.10±0.02[a]	0.5±0.1[c]	0.30±0.02[b]	0.06±0.03[a]
Total	0.10±0.03[a]	0.21±0.03[ab]	0.50±0.02[c]	1.3±0.06[d]	0.41±0.05[bc]	2.0±0.1[e]	1.3±0.1[d]	0.28±0.03[ab]

[1]The unit for contaminants is μg/cm^2 on paper, values are presented as mean ± SD (n=3), different superscripts indicate significant differences between groups, P<0.01.

#1: batch No.1; **#2**: batch No.2; **#3**: batch No.3

3.4.1.3 Possible Ways to overcome the contaminant problem in collection paper

3.4.1.3.1 Washing out the contaminants by soaking collection papers in acetone or in methylating agent

Previous study claimed that soaking the blood collection paper in actone prior to blood collection could wash out the contaminants in the collection papers (Ichihara *et al.* 2002). My experimental data showed that acetone soaking could remove ~80% of contaminants from Fluka paper, however, it was only able to remove less than 50% of contaminants from other types of collection papers (Table 3.3). Thus, the effectiveness of washing the papers with acetone varied between different types of papers.

For all collection papers, ~60% of the contaminants that were present initially were retained in collection papers after 3 hrs of soaking in methylating agent at room temperature (Table 3.4). Furthermore, the results obtained from collection papers soaked for 10 hrs were very similar with those from the same collection paper soaked for 3 hrs, which suggested that it is unlikely to be possible to remove all the contaminants from the collection papers by further extending the soaking period at room temperature. Soaking the collection papers in methylating agent at a relatively high temperature (70°C) removed ~70% of contaminants after 3 hrs. However, this process destroyed the texture of collection papers and made them unusable for blood collection. Therefore, although pre-treatment of the collection papers with methylating agent at 70°C may remove a considerable amount of the contaminants from all types of collection papers, it is not a practical solution.

Thus, I could find no effective way to eliminate the influence of contaminants in collection paper on fatty acid analysis by pre-treating the collection papers prior to use.

Table 3.3. Amount of contaminants released from collection papers during methylation process before and after soaking in acetone at room temperature

Sources of papers[1]	**Before soaking**	**After soaking in acetone at room temperature**
	1.5x1.5cm	**1.5x1.5cm**
Whatman glass fibre filter paper	0.11±0.02	0.08±0.02
Whatman Ion exchange paper	0.23±0.04	0.12±0.03
Whatman 3MM	0.48±0.05	0.32±0.02
Fluka paper	1.4±0.1	0.26±0.03*
Hemaspot-80	0.38±0.03	0.33±0.02
Whatman 903 paper #1	2.0±0.1	1.0±0.1*
Whatman 903 paper #2	1.2±0.05	0.79±0.03*
Whatman 903 paper #3	0.31±0.03	0.28±0.05

[1]The unit for contaminants is μg/cm^2 on paper, values are presented as mean ± SD (n=3).

*Indicate significantly different than paper before soaking, Student's *t*-test, $P<0.01$.

#1: batch No.1; **#2**: batch No.2; **#3**: batch No.3

Table 3.4. Amount of contaminants released from collection papers during methylation process before and after soaking in 1% (v/v) H_2SO_4 in anhydrous methanol

Sources of papers[1]	Before Soaking	Soaking at room temperature			Soaking at 70°C		
		1 hour	3 hours	10 hours	1 hour	2 hours	3 hours
	1.5x1.5cm	1.5x1.5cm	1.5x1.5cm	1.5x1.5cm	1.5x1.5cm	1.5x1.5cm	1.5x1.5cm
Whatman glass fibre filter paper	0.11±0.02	0.1±0.05	0.06±0.03	0.07±0.01	0.06±0.03	0.03±0.01	0.03±0.01
Whatman Ion exchange paper	$0.23±0.04^{b}$	$0.2±0.03^{b}$	$0.17±0.03^{ab}$	$0.15±0.03^{ab}$	$0.16±0.03^{ab}$	$0.10±0.02^{ab}$	$0.07±0.02^{a}$
Whatman 3MM	$0.48±0.03^{b}$	$0.42±0.02^{b}$	$0.35±0.02^{ab}$	$0.32±0.02^{ab}$	$0.35±0.03^{ab}$	$0.24±0.02^{a}$	$0.21±0.03^{a}$
Fluka paper	$1.4±0.1^{e}$	$1.2±0.1^{de}$	$1.0±0.1^{cd}$	$0.9±0.1^{bc}$	$1.0±0.1^{cd}$	$0.76±0.03^{b}$	$0.32±0.03^{a}$
Hemaspot-80	$0.38±0.03^{b}$	$0.35±0.03^{b}$	$0.30±0.02^{ab}$	$0.30±0.03^{ab}$	$0.30±0.05^{ab}$	$0.21±0.02^{ab}$	$0.17±0.03^{a}$
Whatman 903 paper #1	$2.0±0.1^{d}$	$1.9±0.1^{d}$	$1.3±0.1^{c}$	$1.2±0.1^{c}$	$1.3±0.1^{c}$	$0.70±0.02^{b}$	$0.35±0.02^{a}$
Whatman 903 paper #2	$1.2±0.05^{c}$	$1.2±0.02^{c}$	$0.82±0.01^{b}$	$0.80±0.03^{b}$	$0.75±0.03^{b}$	$0.45±0.03^{a}$	$0.31±0.03^{a}$
Whatman 903 paper #3	$0.31±0.03^{b}$	$0.30±0.01^{b}$	$0.25 ±0.02^{ab}$	$0.25±0.01^{ab}$	$0.24±0.01^{ab}$	$0.17±0.01^{ab}$	$0.12±0.01^{a}$

[1]The unit for contaminants is μg/cm^2 on paper, values are presented as mean ± SD (n=3), different superscripts indicate significant differences between groups, $P<0.01$.

#1: batch No.1; **#2**: batch No.2; **#3**: batch No.3

3.4.1.3.2 Subtraction of the contaminants using a blank control

A simple method to diminish the impact of collection paper contaminants on the fatty acid profile of a blood spot sample is to use an equivalent area of blank paper (of the same type as used for the blood spot collection) as a control, and subtract the result of this control from those results obtained from blood spot samples collected on the same type of paper. This method was found to be effective in individual samples, since after subtracting the contaminant peaks from the blood spot results, the fatty acid composition obtained from the blood spot samples were no longer different from those obtained from fresh blood (Table 3.5). The results suggested that using blank collection paper as a control to correct for contaminants is feasible. However, given the fact that different types of blood collection papers release variable amount of contaminants, and the amount of contaminants present in same type of paper from a same manufacture varies across batches (Table 3.2), it is difficult to apply a correction factor which is based on the contaminants of blank paper with any degree of certainty or accuracy, especially in large clinical trials. Thus, this subtraction method is unlikely to be practical when performing fatty acid composition tests in a large group of subjects.

Table 3.5. Fatty acid compositions of blood spots determined after subtraction of paper contaminants compared with fresh blood

Fatty acid[1] (%)	Fresh blood	Blood spot				
		Whatman				Fluka paper
		Glass fibre filter paper	Ion exchange Paper	3MM paper	903 paper #1	
	25 µl	25 µl	25 µl	25 µl	25 µl	25 µl
16:0	22.8±0.3	22.8±0.3	22.8±0.4	22.7 ±0.1	23.1±0.5	22.8±0.4
18:0	10.5±0.1	10.6±0.1	10.5±0.1	10.6±0.2	10.6±0.2	10.5±0.1
18:1n-9	22.2±0.2	22.1±0.1	22.0±0.3	22.1 ±0.2	21.9±0.3	22.0±0.3
18:2n-6	25.8±0.3	25.8±0.3	25.8±0.2	25.7 ±0.1	25.5±0.3	25.7±0.2
18:3n-3	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02
20:4n-6	9.8±0.1	9.8±0.1	9.8±0.1	9.9±0.2	9.7±0.2	9.8±0.1
20:5n-3	1.3±0.03	1.3±0.02	1.3±0.02	1.3±0.02	1.2±0.03	1.3±0.02
22:4n-6	0.9±0.02	0.9±0.02	0.9±0.02	0.9±0.03	0.9±0.03	0.9±0.03
22:5n-3	1.2±0.02	1.2±0.03	1.2±0.02	1.2±0.03	1.2±0.02	1.2±0.02
22:6n-3	3.9±0.02	3.8±0.02	3.8±0.05	3.8±0.02	3.9±0.05	3.9±0.05

[1]Values are presented as mean ± SD (n=3), no significant differences were found in the fatty acid composition between groups.

3.4.2 Potential contaminants from other sources

Other potential sources of contaminants in the fatty acid analysis of DBS were tested in our laboratory by touching glass microfiber filter papers to several commonly used lab consumables including latex gloves, nitrile gloves, polyethylene (PE) ziplock bags, aluminum foil ziplock bags and cellophane bags (Table 3.6). Among all materials, latex gloves contained the highest amount of contaminants, whereas there was virtually no contaminants detected in the nitrile glove. The PE bag and the aluminium bag each contained certain amounts of contaminants which corresponded to oleic acid (18:1 n-9) and erucic acid (22:1 n-9), whereas the cellophane bags did not appear to produce any significant contaminants.

Latex gloves, PE bags and aluminium bags are widely used in many laboratories in the process of analysing fatty acid status in DBS. There have been no previous study which has evaluated latex gloves as a source of contamination in the experiments, with existing investigations reporting only that latex glove can release endotoxins which may cause an allergic reaction (Williams *et al.* 1997). However, my experiments have indicated that even these widely used laboratory items have the potential to release hydrophobic contaminants and could contribute to alterations in the results of blood fatty acid composition tests, especially when the blood sample volume is low. Thus, to minimise potential contamination problems from gloves and bags, it appears that it is important to use nitrile gloves and cellophane bags during collection, processing and storage of the blood spot samples.

Table 3.6. Amount of potential contaminants from lab consumables

Contaminants corresponding to fatty acid peaks[1]	Nitrile glove	Latex glove	PE ziplock bag	Aluminum foil ziplock bag	Cellophane bag
16:0	0.03±0.01[a]	0.60±0.02[b]	0.02±0.01[a]	-	-
18:0	-	0.60±0.01[b]	0.01±0.01[a]	-	0.01±0.002[a]
18:1 n-9	-	-	0.31±0.02	-	-
22:1 n-9	-	-	0.48±0.03	0.32±0.02	-
Total	0.03±0.01[a]	1.2±0.02[d]	0.80±0.05[c]	0.32±0.02[b]	0.01±0.002[a]

[1]The unit for contaminants is μg/cm^2 on glass microfiber filter paper after wiped on the surface of gloves or bags. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between groups, *P*<0.01. "–" referred to not detected.

3.5 Summary

All types of blood collection papers tested in the present studies release contaminants, and these contaminants may alter the analytical results of the blood spot samples, particularly when the volume of the blood spotted on paper is low. I showed that washing the paper prior to transmethylation or even subtracting a blank value are not fully effective strategies for overcoming the negtive influences of the contaminants from paper to the fatty acid results measured from blood spot samples. However, I was unable to detect any significant contaminants released from the Whatman ion exchange paper and Whatman glass microfiber filter paper, and the blood spots collected on either papers showed identical fatty acid composition results with that in fresh blood even when the amount of blood on the paper was low (25μl). Furthermore, my experimental results also revealed that using nitrile gloves and cellophane bags can prevent blood spot samples from potential contamination during sample collection, processing and storage. Therefore, the choice of collection paper, gloves, and storage bags which contain the minimum amount of contaminants is essential to reduce the impact of external contaminants on the analytical results of the blood spot fatty acid composition.

Since the Whatman ion exchange paper and the Whatman glass microfiber filter paper release much less contaminants than the Fluka collection paper and Whatman 903 papers, they may have the potential to replace Fluka and Whatman 903 papers in the blood spot fatty acid composition test and provide more accurate results.

Chapter 4

Developing a Dried Blood Spot Technique For Determining the Fatty Acid Status of Individuals – Stabilising Long Chain Polyunsaturated Fatty Acids in Dried Blood Spots at Room Temperature

4.1 Introduction

The n-3 LCPUFA status in blood is a useful marker for evaluating cardiovascular risk in human populations and identifying those individuals who would benefit from n-3 LCPUFA supplementation (Metcalf *et al.* 2007). The conventional assay of n-3 fatty acids in blood involves venous blood collection and expensive, time consuming, multi-step processes that limit its usefulness as a screening tool.

Recently, a DBS method for the determination of blood fatty acids was reported (Marangoni *et al.* 2004). This DBS method is much quicker and cheaper than conventional methods, thus making it ideal for large clinical trials and as a diagnostic test (Marangoni *et al.* 2004). However, the stability of the LCPUFA in DBS collected in this manner is questioneable, since the oxidative loss of LCPUFA in DBS has been reported (Ichihara *et al.* 2002), and it was therefore been recommended that samples are either processed immediately (Agostoni *et al.* 2008a; Agostoni *et al.* 2011) or treated with the antioxidant BHT and stored at 4°C to stabilise the LCPUFA (Marangoni *et al.* 2004). Based on the current DBS methodology for assessing fatty acids, a commercial product, "Fluka blood collection kit" has been developed by Sigma-Aldrich for the direct evaluation of the n-3 and n-6 LCPUFA status in blood lipids. However, the only long term stability study to date, which has assessed the

stability of fatty acids in DBS samples collected on Fluka test cards showed significant oxidative losses in all LCPUFA after 1 month storage at room temperature (Min *et al.* 2011). Thus, the apparently substantial loss of LCPUFA due to the oxidation makes the current DBS method impractical for use in large clinical and population screening studies, in which samples are collected over a long period of time and need to be transported among multiple centres.

The objective of the present study was to develop a robust method for stabilising the LCPUFA in DBS that would allow the DBS samples to be stored at room temperature for at least two months without significant change in the LCPUFA content.

4.2 Design of the study

The basic design of all the experiments was that the venous blood were obtained from a volunteer who consumed a high amount of fish oil regularly, and spotted on to varied types of blood collection papers. The collection papers were pre-treated with different protectant formulations prior to the blood collection. The DBS were stored at room temperature for up to 9 weeks and the stability of LCPUFA in the DBS over this period was assessed. The best protection system was then tested under different storage conditions, and applied to two other biological fluids, plasma and breast milk.

4.3 Materials and Methods

4.3.1 Blood collection papers

Four types of paper were used for blood spot collection: Fluka blood collection paper (Sigma-Aldrich, Buchs, Switzerland), Whatman 903 specimen collection paper (46x57cm, Whatman, Buckingham, UK), Whatman 3MM chromatography paper (46x57cm, Whatman, Buckingham, UK), and Whatman ion exchange paper (46x57cm, Whatman, Buckingham, UK). Two commercial phenolic antioxidants, BHT and TBHQ were purchased from Sigma-Aldrich (St Louis, MO). Iron chelation agents, L-ascorbic acid, citric acid and EDTA were purchased from Chem-supply Company (Gillman, Australia).

4.3.2 Protectant solutions

Twenty-one protectant formulations which consisted of phenolic antioxidants with or without chelating agents were prepared to stabilise the fatty acids in blood spots (Table 4.1). Two kinds of antioxidants (BHT and TBHQ) at three different concentrations (0.5mg/ml, 2mg/ml and 4mg/ml), and three iron chelators (L-ascorbic acid, citric acid and EDTA) at three levels (1mg/ml, 5mg/ml and 20mg/ml) were tested (Table 4.1) in order to determine the optimal protectant concentration to limit lipid oxidation in the blood spots. 50µl of each protectant formulation was spread evenly on the collection area (~1.5x1.5 cm) of blood collection paper strips (~1.5 x3cm) and air dried prior to collection of blood onto the paper.

4.3.3 Blood collection

Blood was collected through the antecubital vein from one healthy volunteer who had

consumed 15ml of MaxEPA fish oil daily (≈ 2.3g EPA + 1.6g DHA per day) for several years and had a very high blood content of EPA and DHA (each ~7% of the total fatty acids). It was reasoned that blood from such a donor would give me the greatest chance of detecting losses in n-3 LCPUFA if they occurred. It is also important to note that It is also important to note that previous studies which have reported reductions of EPA and DHA (Min *et al*. 2011, Metherel *et al.* 2013) over 2 months at room temperature were utilising subjects whose n-3 LCPUFA content in blood were much lower (EPA 0.5-1%, DHA1.5-3%, expressed in percentage of total fatty acids) than I used here.

4.3.4 Fatty acid analysis of DBS

A baseline measurement for fatty acid status in whole blood was achieved by direct transmethylation of the fresh blood (~50μl) added to 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex, UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA) and heating at 70°C for 3 hrs. The resultant FAME were extracted into heptanes, and injected into a GC for analysis as described in Chapter 2.

Blood spot samples were obtained by absorbing a drop of fresh blood (~50 μl) on blood collection paper strips in the absence or presence of protectant formulations (Table 4.1), and all blood spots were air dried at room temperature for 5 hrs. Once the blood spots were dried, a group of DBS were measured immediately following the same procedure as for the fresh blood to determing the stability of LCPUFA during air drying. The remaining DBS were stored under various conditions to enable testing of fatty acid stability over long term storage. The fatty acid composition in these samples were measured either 1 week, 2 weeks, 4 weeks or 9 weeks after the time of blood

collection. The contaminant peaks from collection papers were substrated from the GC peaks of DBS fatty acids to minimise the impact of contaminants from paper on the analytical results of the DBS fatty acid composition. All samples were processed in triplicate.

4.3.5 Statistical Analyses

All statistics analyses were conducted using PASW Statistic 18. Values are expressed as mean ± SD. One-way ANOVA and Tukey's post-hoc test were used to determine the significant differences in fatty acids percentage between groups in the case of storage-losses study. The effects of paper type and protectant formulation on the changes in fatty acids content (expressed as a weight percentage of total fatty acids) over time was deterrmined using two-way ANOVA. Where significant interactions were determined in the ANOVA, the significant differences in fatty acids percentage between groups was determined by one-way ANOVA with comparison of individual means by Tukey's post-hoc test. Due to the number of statistical comparisons made, $P<0.01$ was chosen as the level of statistical significance.

Table 4.1. Protectant formulations used for treatment of collection papers

Formulation	Protectant formulations	Formulation	Protectant formulations	Formulation	Protectant formulations
1	TBHQ 0.5mg/ml	**8**	L-ascorbic acid 5mg/ml	**15**	EDTA 20mg/ml
2	TBHQ 2mg/ml	**9**	L-ascorbic acid 20mg/ml	**16**	BHT 2mg/ml & L-ascorbic acid 5mg/ml
3	TBHQ 4mg/ml	**10**	Citric acid 1mg/ml	**17**	BHT 2mg/ml & Citric acid 5mg/ml
4	BHT 0.5mg/ml	**11**	Citric acid 5mg/ml	**18**	BHT 2mg/ml & EDTA 5mg/ml
5	BHT 2mg/ml	**12**	Citric acid 20mg/ml	**19**	TBHQ 2mg/ml & L-ascorbic acid 5mg/ml
6	BHT 4mg/ml	**13**	EDTA 1mg/ml	**20**	TBHQ 2mg/ml & Citric acid 5mg/ml
7	L-ascorbic acid 1mg/ml	**14**	EDTA 5mg/ml	**21**	TBHQ 2mg/ml & EDTA 5mg/ml

*Concentrations expressed in mg/ml in 70% ethanol/water solution.

4.4 Results and Discussion

4.4.1 Stability of DBS collected by Fluka test kit

The objective of this experiment was to evaluate the stability of fatty acids in DBS samples collected using the Fluka test kit. Blood spots were obtained by absorbing 50μl of fresh blood onto the collection area (~1.5x1.5cm) of Fluka blood collection papers in the presence of the antioxidant solution (0.5mg/ml BHT) provided with the Fluka test kit. After being air drying at room temperature for 5 hrs, DBS were divided into 7 treatment groups. The first group was immediately processed, and analysed for fatty acid composition by GC using the procedure described in Chapter 2. The results from DBS were compared with those from fresh blood direct transmethylation to determine the oxidative loss of LCPUFA in DBS samples that occurred during preliminary air drying. The DBS in the remaining groups were stored in sealed cellophane bags in the dark at either room temperature or in a refrigerator (4°C) in the presence of desiccants. The fatty acid composition in these samples was measured either 1 week, 2 weeks or 4 weeks after the time of blood collection. All samples were processed in triplicate.

The DBS samples collected by the Fluka test kit exhibited significant reductions in the levels of all n-3 LCPUFA (expressed as a percentage of weight in total blood fatty acids) after being dried in air for only 5 hours when compared with the corresponding n-3 fatty acid levels measured following the direct transmethylation of fresh blood samples (Table 4.2). Storage of DBS samples for longer periods of time revealed that, irrespective of the storage temperature, DBS collected by Fluka test kit exhibited significant losses in the percentage of EPA + DHA in total fatty acids measured in the DBS over the 4 weeks of storage period, even in the presence of BHT solution

(0.5mg/ml) (Figure 4.1). Although DBS samples stored at low temperatures (4°C) exhibited significantly higher retention of LCPUFA content when compared with samples stored at room temperature, there were still ~ 30% losses in the percentage of n-3 LCPUFA (from an initial weight percentage of 14.2% decreased to 9.8%) measured in the DBS after 4 weeks of storage at 4°C when compared with the corresponding fatty acid levels measured in fresh blood (Table 4.2). Conversely, the percentage of total SFA and oleic acid measured in the DBS samples increased steadily across the storage period (Table 4.2).

If the DBS technique is going to be adopted universally for evaluation of LCPUFA status of individuals, investigators need to be assured that the method is accurate and that the results are constant over time. The commercially available DBS product (Fluka blood collection kit) is being advertised to consumers as an accurate method for home monitoring of n-3 LCPUFA status, people are invited to post samples on prepared collection papers to the company to receive an assessment of their n-3 status. Such a test assumes that the fatty acids in the blood, most notably the n-3 LCPUFA, are stable from the time of spotting until the sample is analysed by the company's laboratory.

Given that spotting blood on filter paper will naturally expose the LCPUFA in blood to the air, investigators have been concerned about the stability of the LCPUFA in DBS samples collected in this manner (Ichihara *et al.* 2002). The stability of fatty acids in a DBS format should be considered in two stages: the stability during air drying process and the stability during storage (D'Arienzo *et al*. 2010; Liu *et al*. 2011). The present study used the fatty acid status which obtained from direct

tranesterification of 50μl of whole blood immediately after phlebotomy as a baseline control, this is because I have shown that there is significant loss of n-3 LCPUFA in DBS after only 5 hrs air drying even in the presence of BHT (Table 4.2). Interestingly, studies that reported low levels of oxidation of DBS samples during storage (Marangoni *et al.* 2004; Metherel *et al.* 2013) tend to compare these values with a control that blood had been spotted onto paper and dried in air, a process I have shown to result in significant losses in LCPUFA even without subsequent periods of storage. Therefore, this is an inappropriate control which may lead to underestimation of the extent of oxidative losses of LCPUFA in these DBS systems.

Several groups have reported the use of the antioxidant BHT to protect LCPUFA in DBS during storage (Marangoni *et al.* 2004; Bailey-Hall *et al.* 2008). However, the current experiment demonstrated that even after pre-treating the collection paper with BHT, a time-dependent loss of the n-3 fatty acids in DBS samples still occured during storage, regardless of whether the paper is stored at room temperature or at 4°C. This is in line with other studies that have reported significant reductions in LCPUFA content in DBS collected on both Fluka and Whatman 903 blood collection papers (Min *et al.* 2011; Bell *et al.* 2011; Metherel *et al.* 2013).

Thus, the instability of LCPUFA in DBS during storage makes all current DBS methods impractical for measurements of fatty acid profiles in a large numbers of samples, since it necessitates that all samples are analysed at a fixed time after collection if results were to be compared.

Table 4.2. Fatty acid composition (%) of DBS on Fluka paper determined over 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	After drying (5hrs)	Room temperature (20-25°C)			Fridge (4°C)		
			1 week	2 weeks	4 weeks	1 week	2 weeks	4 weeks
16:0	21.6±0.3^{a}	22.7±0.3^{ab}	23.7±0.3^{cd}	24.2±0.3^{d}	26.0±0.4^{e}	23.3±0.2^{bc}	23.7±0.3^{cd}	25.4±0.4^{e}
18:0	12.4±0.1^{a}	13.1±0.2^{ab}	13.6±0.1^{bc}	13.9±0.2^{cd}	14.9±0.2^{e}	13.4±0.1^{bc}	13.6±0.2^{bc}	14.3±0.1^{d}
18:1 n-9	18.9±0.2^{a}	19.4±0.2^{ab}	19.8±0.2^{b}	20.7±0.2^{c}	22.7±0.2^{e}	19.8±0.1^{b}	20.0±0.2^{bc}	21.6±0.3^{d}
18:2 n-6	20.2±0.2^{a}	20.1±0.1^{a}	19.7±0.2^{ab}	19.5±0.1^{b}	18.9±0.1^{c}	19.9±0.1^{ab}	19.7±0.2^{ab}	19.3±0.2^{bc}
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02	0.4±0.02	0.4±0.02	0.4±0.02	0.4±0.01
20:4 n-6	7.6±0.1^{a}	7.2±0.1^{ab}	6.9±0.1^{b}	6.6±0.1^{bc}	5.8±0.2^{d}	6.8±0.1^{b}	6.8±0.1^{b}	6.2±0.1^{cd}
20:5 n-3	6.9±0.1^{a}	6.2±0.1^{b}	5.7±0.1^{c}	5.2±0.1^{d}	4.1±0.1^{f}	5.9±0.1^{bc}	5.5±0.1^{cd}	4.6±0.1^{e}
22:4 n-6	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.01^{b}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.01^{b}
22:5 n-3	4.0±0.04^{a}	3.6±0.05^{b}	3.4±0.03^{bc}	3.0±0.03^{d}	2.2±0.04^{f}	3.4±0.05^{bc}	3.2±0.04^{cd}	2.6±0.02^{e}
22:6 n-3	7.3±0.1^{a}	6.6±0.1^{b}	6.2±0.1^{bc}	5.8±0.1^{c}	4.5±0.1^{e}	6.4±0.1^{b}	6.1±0.1^{bc}	5.2±0.1^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant difference between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

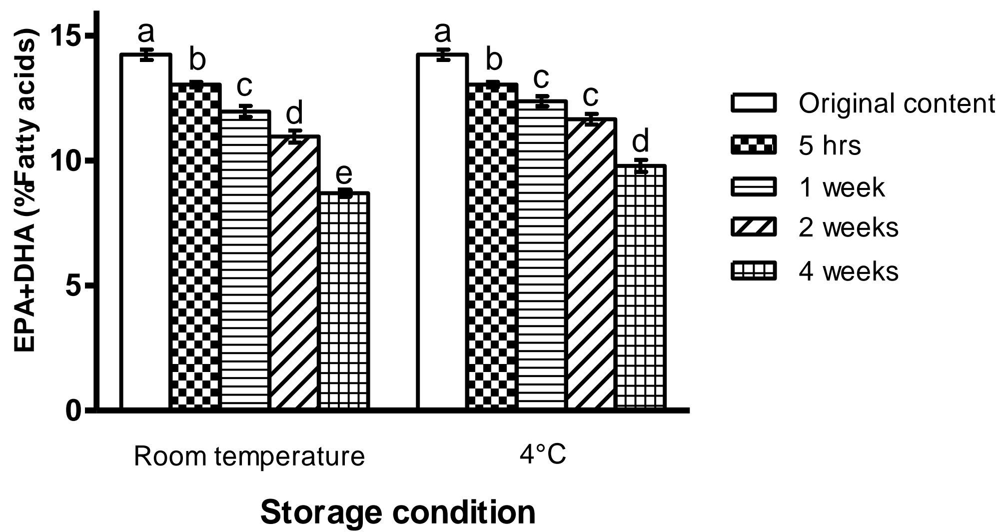


Figure 4.1. Stability of EPA+DHA in DBS on Fluka paper over 4 weeks of storage at room temperature (20~25°C) or at 4°C. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between the treatments in the same group, *P*<0.01. The "Original content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

4.4.2 Optimization of DBS method

4.4.2.1 Stability of DBS absorbed on different types of collection papers

The objective of this experiment was to compare the stability of fatty acid in the DBS collected on 4 types of collection papers. Blood spots were obtained by absorbing 50μl of fresh blood onto the collection area (~1.5x1.5cm) of 4 types of blood collection papers (Fluka blood collection paper, Whatman 903 blood collection paper, Whatman 3MM chromatography paper and Whatman ion exchange paper) in the presence of standard antioxidant solution (0.5mg/ml BHT). After being air dried at room temperature for 5 hrs, DBS samples were processed, and analysed by GC as describe in Chapter 2 to determine fatty acid composition either immediately after air drying, or after 1 week, 2 weeks or 4 weeks of storage at room temperature in cellophane bags in the dark and in the presence of desiccants.

A significant decrease in the levels of all n-3 LCPUFA measured as a weight percentage of total blood fatty acids in DBS samples was observed after 5 hrs of air drying for all collection papers. The levels of n-3 LCPUFA continued to decline over the 4 weeks of storage period even in the presence of BHT solution (0.5mg/ml) (Tables 4.3-4.6). However, there was a significant interaction between storage time and paper type in relation to fatty acid composition of the DBS samples, such that different types of paper showed different stabilising ability. There was no significant difference in the fatty acid composition of the DBS samples collected on Fluka blood collection paper, Whatman 3MM paper or Whatman 903 paper. However, the DBS samples collected on Whatman ion exchange paper exhibited a significant higher degree of residual n-3 LCPUFA levels when compared with those DBS collected on other types of papers following 4 weeks of storage at room temperature (Figure 4.2).

Table 4.3. Fatty acid composition of DBS on Fluka paper in the presence of antioxidant (0.5mg/ml BHT) determined over 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	After drying (5hrs)	Fluka		
			1 week	2 weeks	4 weeks
16:0	21.5±0.3[a]	22.6±0.3[b]	23.6±0.3[c]	24.2±0.2[c]	25.8±0.2[d]
18:0	12.6±0.2[a]	13.1±0.1[ab]	13.5±0.2[bc]	13.8±0.2[c]	14.8±0.2[d]
18:1 n-9	18.8±0.2[a]	19.3±0.2[ab]	19.8±0.2[b]	20.7±0.2[c]	22.6±0.2[d]
18:2 n-6	20.2±0.1[a]	20.0±0.1[ab]	19.8±0.2[ab]	19.5±0.1[bc]	18.9±0.1[c]
18:3 n-3	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.02	0.4±0.01
20:4 n-6	7.7±0.1[a]	7.3±0.1[ab]	6.9±0.1[bc]	6.7±0.1[c]	5.8±0.1[d]
20:5 n-3	6.9±0.1[a]	6.3±0.1[b]	5.7±0.1[c]	5.2±0.1[d]	4.1±0.1[e]
22:4 n-6	0.5±0.01[a]	0.5±0.01[a]	0.5±0.02[a]	0.4±0.01[b]	0.4±0.01[b]
22:5 n-3	3.9±0.04[a]	3.5±0.05[b]	3.4±0.05[b]	3.1±0.04[c]	2.3±0.05[d]
22:6 n-3	7.3±0.1[a]	6.6±0.1[b]	6.2±0.1[b]	5.7±0.1[c]	4.6±0.2[d]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.4. Fatty acid composition of DBS on Whatman 903 paper in the presence of antioxidant (0.5mg/ml BHT) determined over 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	After drying (5hrs)	Storage periods		
			1 week	2 weeks	4 weeks
16:0	21.5±0.3[a]	22.8±0.3[b]	23.8±0.2[c]	24.3±0.2[c]	26.1±0.4[d]
18:0	12.6±0.2[a]	13.0±0.2[ab]	13.5±0.1[bc]	13.9±0.2[c]	14.8±0.2[d]
18:1 n-9	18.8±0.2[a]	19.3±0.2[ab]	19.9±0.2[b]	20.8±0.2[c]	22.0±0.2[d]
18:2 n-6	20.2±0.1[a]	20.1±0.1[a]	19.7±0.1[ab]	19.4±0.1[bc]	18.9±0.2[c]
18:3 n-3	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02
20:4 n-6	7.7±0.1[a]	7.2±0.1[b]	6.9±0.1[bc]	6.6±0.1[c]	5.7±0.2[d]
20:5 n-3	6.9±0.1[a]	6.2±0.1[b]	5.7±0.1[c]	5.2±0.1[d]	4.0±0.2[e]
22:4 n-6	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.4±0.01[b]	0.4±0.01[b]
22:5 n-3	3.9±0.04[a]	3.6±0.05[b]	3.3±0.06[bc]	3.0±0.04[c]	2.3±0.06[d]
22:6 n-3	7.3±0.1[a]	6.5±0.1[b]	6.1±0.1[b]	5.6±0.1[c]	4.5±0.1[d]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.5. Fatty acid composition of DBS on Whatman 3MM paper in the presence of antioxidant (0.5mg/ml BHT) determined over 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	After drying (5hrs)	Storage periods		
			1 week	2 weeks	4 weeks
16:0	21.5±0.3^{a}	22.5±0.1^{b}	23.5±0.3^{c}	23.8±0.3^{c}	25.7±0.3^{d}
18:0	12.6±0.2^{a}	13.1±0.2^{ab}	13.4±0.2^{bc}	13.8±0.1^{c}	14.9±0.2^{d}
18:1 n-9	18.8±0.2^{a}	19.2±0.1^{ab}	19.8±0.2^{b}	20.8±0.2^{c}	22.6±0.3^{d}
18:2 n-6	20.2±0.1^{a}	20.1±0.1^{a}	19.9±0.1^{ab}	19.5±0.1^{b}	18.9±0.1^{c}
18:3 n-3	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.7±0.1^{a}	7.3±0.1^{ab}	7.0±0.1^{bc}	6.7±0.1^{c}	5.8±0.1^{d}
20:5 n-3	6.9±0.1^{a}	6.3±0.1^{b}	5.9±0.1^{b}	5.3±0.1^{c}	4.1±0.1^{d}
22:4 n-6	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.01^{b}
22:5 n-3	3.9±0.04^{a}	3.6±0.05^{b}	3.4±0.06^{b}	3.1±0.02^{c}	2.3±0.02^{d}
22:6 n-3	7.3±0.1^{a}	6.6±0.1^{b}	6.2±0.1^{bc}	5.8±0.1^{c}	4.6±0.2^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

Table 4.6. Fatty acid composition of DBS on Whatman ion exchange paper in the presence of antioxidant (0.5mg/ml BHT) determined over 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	After drying (5hrs)	Storage periods		
			1 week	2 weeks	4 weeks
16:0	21.5±0.3^{a}	22.4±0.3^{ab}	22.8±0.3^{b}	23.0±0.2^{bc}	23.7±0.2^{c}
18:0	12.6±0.2^{a}	13.1±0.2^{ab}	13.4±0.1^{b}	13.5±0.2^{b}	13.7±0.1^{b}
18:1 n-9	18.8±0.2^{a}	19.1±0.2^{ab}	19.3±0.2^{ab}	19.8±0.1^{b}	20.9±0.3^{c}
18:2 n-6	20.2±0.1^{a}	20.1±0.1^{a}	20.0±0.1^{ab}	19.9±0.1^{ab}	19.5±0.1^{b}
18:3 n-3	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.02	0.4±0.01
20:4 n-6	7.7±0.1^{a}	7.4±0.1^{ab}	7.2±0.1^{ab}	7.0±0.1^{bc}	6.6±0.1^{c}
20:5 n-3	6.9±0.1^{a}	6.3±0.1^{b}	6.2±0.1^{b}	5.9±0.1^{bc}	5.6±0.1^{c}
22:4 n-6	0.5±0.01^{a}	0.5±0.02	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.9±0.04^{a}	3.7±0.05^{ab}	3.6±0.03^{bc}	3.4±0.04^{cd}	3.2±0.04^{d}
22:6 n-3	7.3±0.1^{a}	6.7±0.1^{b}	6.5±0.1^{bc}	6.2±0.1^{cd}	5.7±0.1^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

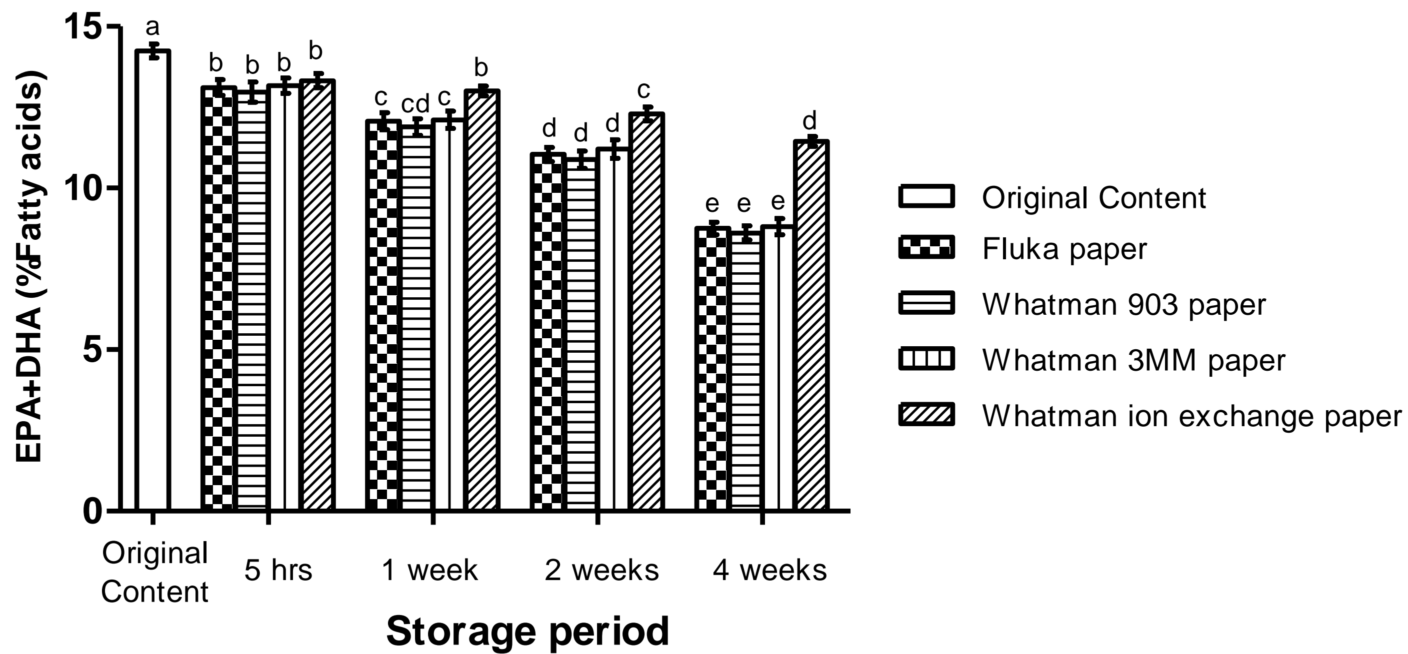


Figure 4.2. Stability of EPA+DHA in DBS on the 4 types of collection papers in the presence of antioxidant (0.5mg/ml BHT) over 4 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

4.4.2.2 Stability of DBS treated with different concentration of single antioxidant

The objective of this experiment was to optimise the concentration of single antioxidant for stabilising the LCPUFA in the DBS collected on collection papers. Blood spots were obtained by absorbing 50μl of fresh blood onto the collection area (~1.5x1.5cm) of two different types of collection papers (Fluka blood collection paper and Whatman ion exchange paper) in the absence of antioxidant or in the presence of a single phenolic antioxidant (BHT or TBHQ) at three concentrations (0.5mg/ml, 2mg/ml, 4mg/ml) (Table 4.7). After being air dried at room temperature for 5 hrs, DBS samples were processed, and analysed by GC as described in Chapter 2 to determine the fatty acid composition, samples were analysed either immediately after air drying, or after 2 weeks or 4 weeks of storage at room temperature in cellophane bags in the dark and in the presence of desiccants.

DBS collected onto either Fluka blood collection paper or Whatman ion exchange paper in the absence of antioxidant exhibited significant declines in the percentage of all the LCPUFA measured in total blood fatty acids after 4 week storage at room temperature (Table 4.9, 4.12). Interestingly, irrespective of the protectant formulations used, DBS collected on Whatman ion exchange papers consistently exhibited a higher residual LCPUFA percentage when compared with the DBS samples collected on Fluka blood collection papers (Figure 4.3).

The same concentration of antioxidant BHT and TBHQ showed similar abilities to retard the decline of LCPUFA levels in DBS collected on Fluka paper over the 4 week periods of storage at room temperature (Tables 4.7-4.9). However, the DBS collected on BHT pre-treated Whatman ion exchange papers retained a significant higher

percentage of LCPUFA than those collected on TBHQ pre-treated Whatman ion exchange papers at all three antioxidant concentrations following 4 weeks of storage at room temperature (Tables 4.10-4.12). The DBS samples which were collected on papers pre-treated with either BHT or TBHQ at a concentration of 2mg/ml exhibited a significantly higher residual n-3 LCPUFA percentage over the 4 week storage period than those DBS samples collected on papers pre-treated with 0.5mg/ml of antioxidant (Figure 4.3). Further increasing the BHT concentration to 4mg/ml did not result in any significant improvements in the level of n-3 LCPUFA retained in blood spots after storage. Increasing the concentration of TBHQ to 4mg/ml actually resulted in a significant reduction of n-3 LCPUFA percentage of total blood fatty acids measured in DBS after 4 weeks of storage when compared with 2mg/ml TBHQ (Figure 4.3).

As a result, antioxidants at a concentration of 2mg/ml were chosen as the optimal protectant concentrations for subsequent experiments.

Table 4.7. Fatty acid composition of DBS on Fluka paper pre-treated with single antioxidant after 5 hrs of air drying

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3±0.2[a]	24.6±0.3[b]	24.2±0.4[b]	24.0±0.4[ab]	24.2±0.3[b]	23.9±0.3[ab]	23.9±0.4[ab]	24.1±0.4[ab]
18:0	13.4±0.2[a]	14.5±0.2[b]	13.9±0.2[a]	13.8±0.2[a]	13.7±0.2[a]	13.9±0.2[a]	13.9±0.1[a]	14.0±0.1[ab]
18:1 n-9	18.4±0.1[a]	19.3±0.2[b]	18.9±0.2[ab]	18.8±0.1[ab]	18.7±0.3[ab]	18.9±0.1[ab]	18.7±0.2[ab]	19.0±0.2[ab]
18:2 n-6	19.7±0.2	19.3±0.1	19.5±0.2	19.5±0.2	19.6±0.1	19.5±0.1	19.7±0.1	19.6±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1	7.1±0.1	7.2±0.1	7.3±0.1	7.2±0.1	7.3±0.1	7.3±0.1	7.2±0.01
20:5 n-3	6.5±0.1[a]	5.5±0.1[b]	5.8±0.1[b]	5.8±0.1[b]	5.9±0.1[b]	5.9±0.1[b]	5.9±0.1[b]	5.8±0.1[b]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06[a]	3.2±0.04[c]	3.5±0.05[ab]	3.5±0.05[ab]	3.5±0.05[ab]	3.5±0.03[ab]	3.5±0.03[ab]	3.4±0.05[bc]
22:6 n-3	6.9±0.1[a]	5.9±0.1[b]	6.2±0.1[b]	6.3±0.1[b]	6.3±0.1[b]	6.2±0.1[b]	6.3±0.1[b]	6.2±0.2[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.8. Fatty acid composition of DBS on Fluka paper pre-treated with single antioxidant after 2 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3 ± 0.2^{a}	26.5 ± 0.3^{c}	25.3 ± 0.3^{b}	25.1 ± 0.3^{b}	24.9 ± 0.4^{b}	25.3 ± 0.4^{b}	25.1 ± 0.3^{b}	25.4 ± 0.3^{b}
18:0	13.4 ± 0.2^{a}	15.3 ± 0.1^{c}	14.5 ± 0.2^{b}	14.4 ± 0.2^{b}	14.2 ± 0.2^{b}	14.7 ± 0.1^{b}	14.2 ± 0.2^{b}	14.6 ± 0.2^{b}
18:1 n-9	18.4 ± 0.1^{a}	20.6 ± 0.2^{d}	20.2 ± 0.3^{cd}	19.5 ± 0.2^{b}	19.8 ± 0.2^{bc}	20.2 ± 0.1^{cd}	19.4 ± 0.1^{b}	20.1 ± 0.1^{cd}
18:2 n-6	19.7 ± 0.2^{a}	19.0 ± 0.2^{b}	19.2 ± 0.2^{ab}	19.3 ± 0.2^{ab}	19.2 ± 0.1^{ab}	19.1 ± 0.1^{ab}	19.3 ± 0.1^{ab}	19.1 ± 0.2^{ab}
18:3 n-3	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01
20:4 n-6	7.6 ± 0.1^{a}	6.3 ± 0.1^{c}	6.6 ± 0.1^{bc}	6.9 ± 0.1^{b}	6.9 ± 0.1^{b}	6.6 ± 0.1^{bc}	7.0 ± 0.1^{ab}	6.6 ± 0.1^{bc}
20:5 n-3	6.5 ± 0.1^{a}	4.4 ± 0.1^{c}	5.0 ± 0.1^{b}	5.3 ± 0.1^{b}	5.4 ± 0.1^{b}	5.0 ± 0.1^{b}	5.4 ± 0.1^{b}	5.1 ± 0.1^{b}
22:4 n-6	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01	0.5 ± 0.01
22:5 n-3	3.7 ± 0.06^{a}	2.7 ± 0.05^{c}	2.9 ± 0.05^{bc}	3.1 ± 0.04^{b}	3.1 ± 0.03^{b}	2.9 ± 0.04^{bc}	3.1 ± 0.04^{b}	2.9 ± 0.03^{bc}
22:6 n-3	6.9 ± 0.1^{a}	4.9 ± 0.1^{c}	5.4 ± 0.1^{b}	5.6 ± 0.1^{b}	5.7 ± 0.1^{b}	5.4 ± 0.2^{b}	5.7 ± 0.1^{b}	5.4 ± 0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.9. Fatty acid composition of DBS on Fluka paper pre-treated with single antioxidant after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3±0.2[a]	28.2±0.3[d]	27.1±0.3[c]	26.4±0.4[bc]	26.3±0.4[b]	27.0±0.2[bc]	26.2±0.3[b]	27.1±0.4[bc]
18:0	13.4±0.2[a]	16.4±0.2[d]	15.8±0.2[cd]	15.2±0.2[bc]	15.0±0.2[b]	15.8±0.2[cd]	15.1±0.2[bc]	15.6±0.2[c]
18:1 n-9	18.4±0.1[a]	22.4±0.2[d]	21.8±0.3[cd]	20.0±0.3[b]	20.3±0.2[b]	21.7±0.1[c]	20.1±0.1[b]	21.5±0.1[c]
18:2 n-6	19.7±0.2[a]	18.2±0.2[c]	18.4±0.3[bc]	18.8±0.2[b]	18.9±0.2[b]	18.5±0.1[bc]	18.9±0.2[b]	18.6±0.1[bc]
18:3 n-3	0.4±0.01[a]	0.3±0.01[b]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.01[a]
20:4 n-6	7.6±0.1[a]	5.4±0.1[d]	5.8±0.1[cd]	6.5±0.1[b]	6.4±0.1[b]	5.8±0.1[cd]	6.5±0.1[b]	5.9±0.1[c]
20:5 n-3	6.5±0.1[a]	3.3±0.1[d]	3.7±0.1[c]	4.7±0.1[b]	4.7±0.1[b]	3.7±0.1[c]	4.7±0.1[b]	3.8±0.1[c]
22:4 n-6	0.5±0.01[a]	0.4±0.01[b]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]
22:5 n-3	3.7±0.06[a]	2.0±0.06[d]	2.4±0.03[c]	2.8±0.05[b]	2.8±0.04[b]	2.4±0.05[c]	2.8±0.03[b]	2.4±0.04[c]
22:6 n-3	6.9±0.1[a]	3.7±0.1[d]	4.3±0.1[c]	5.0±0.1[b]	5.0±0.1[b]	4.3±0.1[c]	5.0±0.1[b]	4.4±0.1[c]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.10. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single antioxidant after 5 hrs of air drying

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3±0.2	24.1±0.3	23.9±0.3	24.0±0.3	23.8±0.4	23.9±0.4	24.0±0.3	23.9±0.3
18:0	13.4±0.2^{a}	14.3±0.2^{b}	14.0±0.2^{ab}	13.9±0.2^{ab}	13.9±0.1^{ab}	13.9±0.1^{ab}	13.8±0.2^{ab}	14.0±0.2^{ab}
18:1 n-9	18.4±0.1	19.0±0.2	18.8±0.1	18.7±0.1	18.6±0.1	18.9±0.1	18.7±0.1	18.8±0.1
18:2 n-6	19.7±0.2	19.5±0.1	19.5±0.1	19.6±0.1	19.6±0.1	19.7±0.1	19.7±0.1	19.7±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1	7.2±0.1	7.3±0.1	7.4±0.1	7.4±0.1	7.3±0.1	7.3±0.1	7.4±0.2
20:5 n-3	6.5±0.1^{a}	5.8±0.1^{b}	5.9±0.1^{b}	5.9±0.1^{b}	5.9±0.1^{b}	5.8±0.1^{b}	5.9±0.1^{b}	5.8±0.1^{b}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06^{a}	3.4±0.04^{b}	3.5±0.05^{ab}	3.5±0.04^{ab}	3.6±0.03^{ab}	3.5±0.04^{ab}	3.5±0.05^{ab}	3.4±0.05^{b}
22:6 n-3	6.9±0.1^{a}	6.2±0.1^{b}	6.2±0.1^{b}	6.3±0.1^{b}	6.3±0.1^{b}	6.2±0.1^{b}	6.3±0.1^{b}	6.2±0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

Table 4.11. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single antioxidant after 2 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3±0.2[a]	25.5±0.4[d]	24.5±0.2[bc]	24.3±0.4[bc]	24.0±0.3[b]	25.0±0.3[cd]	24.7±0.2[bc]	25.1±0.3[cd]
18:0	13.4±0.2[a]	15.0±0.2[c]	14.2±0.2[b]	14.1±0.2[b]	14.0±0.2[ab]	14.5±0.2[b]	14.4±0.2[b]	14.6±0.2[b]
18:1 n-9	18.4±0.1[a]	20.0±0.2[c]	19.1±0.1[b]	19.0±0.2[ab]	18.8±0.1[ab]	19.9±0.1[c]	19.3±0.1[b]	19.8±0.2[c]
18:2 n-6	19.7±0.2[a]	19.0±0.1[b]	19.3±0.1[ab]	19.5±0.1[ab]	19.6±0.1[a]	19.2±0.1[ab]	19.4±0.1[ab]	19.1±0.1[ab]
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1[a]	6.8±0.1[b]	7.1±0.1[ab]	7.2±0.1[ab]	7.3±0.1[ab]	6.8±0.1[b]	7.1±0.1[ab]	6.8±0.1[b]
20:5 n-3	6.5±0.1[a]	4.9±0.1[d]	5.6±0.1[bc]	5.6±0.1[b]	5.7±0.1[b]	5.2±0.1[cd]	5.5±0.1[bc]	5.2±0.1[cd]
22:4 n-6	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06[a]	3.0±0.05[c]	3.3±0.03[b]	3.4±0.05[b]	3.4±0.04[b]	3.0±0.03[c]	3.2±0.04[bc]	3.0±0.06[c]
22:6 n-3	6.9±0.1[a]	5.3±0.1[d]	6.0±0.1[bc]	6.1±0.1[b]	6.2±0.1[b]	5.5±0.1[cd]	5.7±0.1[cd]	5.6±0.1[cd]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood.

Table 4.12. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single antioxidant after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Antioxidant	0.5mg/ml BHT	2mg/ml BHT	4mg/ml BHT	0.5mg/ml TBHQ	2mg/ml TBHQ	4mg/ml TBHQ
16:0	23.3±0.2^{a}	26.8±0.3^{d}	25.3±0.3^{bc}	24.9±0.4^{bc}	24.5±0.3^{b}	26.5±0.3^{d}	25.7±0.3^{c}	26.5±0.3^{d}
18:0	13.4±0.2^{a}	15.7±0.1^{d}	14.6±0.2^{b}	14.4±0.2^{b}	14.3±0.2^{b}	15.4±0.2^{cd}	14.9±0.1^{bc}	15.3±0.1^{cd}
18:1 n-9	18.4±0.1^{a}	20.6±0.1^{d}	19.7±0.1^{bc}	19.2±0.1^{b}	19.2±0.1^{b}	20.4±0.2^{d}	20.0±0.2^{cd}	20.6±0.1^{d}
18:2 n-6	19.7±0.2^{a}	18.7±0.2^{b}	19.4±0.1^{ab}	19.4±0.1^{ab}	19.3±0.2^{ab}	18.8±0.3^{b}	18.9±0.2^{b}	18.7±0.1^{b}
18:3 n-3	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1^{a}	6.3±0.1^{c}	6.8±0.1^{bc}	7.0±0.1^{ab}	7.1±0.1^{ab}	6.4±0.1^{c}	6.7±0.1^{bc}	6.5±0.1^{c}
20:5 n-3	6.5±0.1^{a}	4.0±0.1^{d}	5.0±0.1^{bc}	5.3±0.1^{b}	5.5±0.1^{b}	4.3±0.2^{cd}	4.8±0.1^{c}	4.3±0.1^{d}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}
22:5 n-3	3.7±0.06^{a}	2.7±0.06^{de}	3.1±0.03^{bc}	3.3±0.03^{b}	3.3±0.03^{b}	2.6±0.04^{e}	2.9±0.05^{cd}	2.6±0.04^{e}
22:6 n-3	6.9±0.1^{a}	4.5±0.1^{d}	5.4±0.1^{c}	5.9±0.1^{b}	6.0±0.1^{b}	4.7±0.1^{d}	5.3±0.1^{c}	4.7±0.1^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

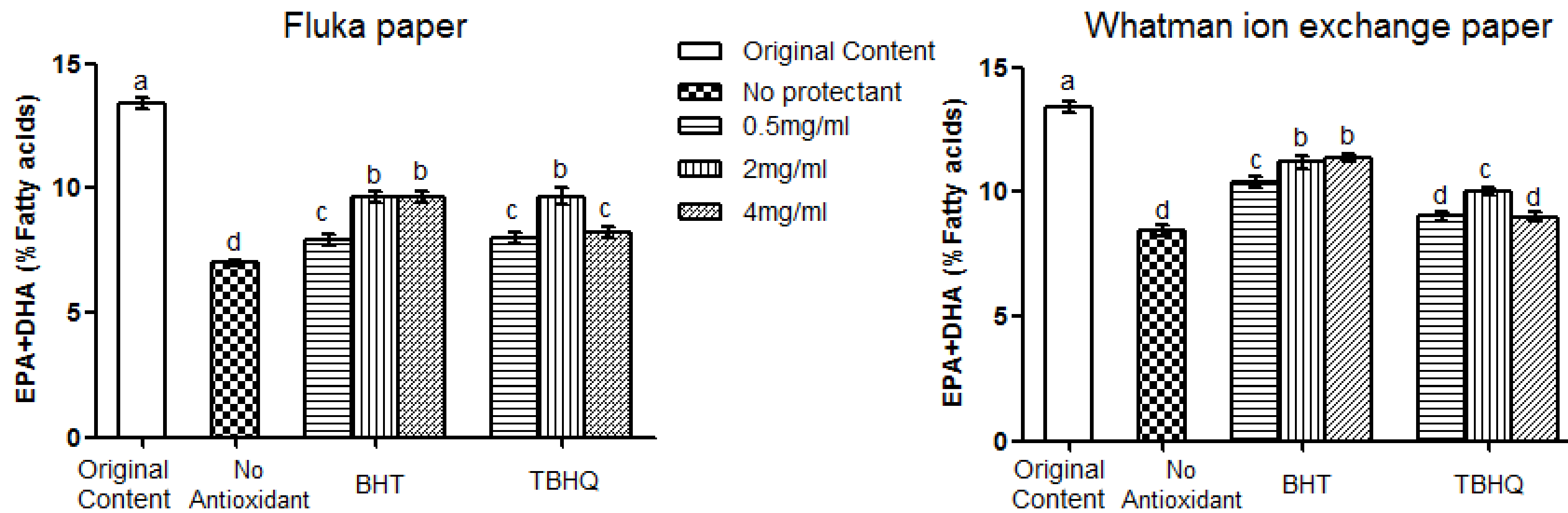


Figure 4.3. EPA+DHA% of DBS on Fluka paper or Whatman ion exchange paper in the presence or absence of single antioxidant determined after 4 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

4.4.2.3 Stability of DBS treated with single chelating agent

The objective of this experiment was to optimise the concentration of single antioxidant for stabilising the LCPUFA in the DBS collected on collection papers. Blood spots were obtained by absorbing 50µl of fresh blood onto the collection area (~1.5x1.5cm) of two different types of collection papers (Fluka blood collection paper and Whatman ion exchange paper) in the absence or presence of a single chelating agent (L-ascorbic acid, citric acid or EDTA) at three concentrations (1mg/ml, 5mg/ml, 20mg/ml) (Table 4.13). After being air dried at room temperature for 5 hrs, DBS samples were processed, and analysed by GC as described in Chapter 2 to determine fatty acid composition, samples were analysed either immediately after air drying, or after 2 weeks or 4 weeks of storage at room temperature in cellophane bags in the dark and in the presence of desiccants.

DBS collected onto either Fluka blood collection paper or Whatman ion exchange paper in the absence of chelating agent exhibited significant declines in the percentage of all the LCPUFA measured in total blood after 4 week storage at room temperature (Tables 4.15, 4.18). Interestingly, irrespective of the chelating agents used, DBS collected on Whatman ion exchange papers consistently exhibited a higher residual LCPUFA percentage when compared with the DBS samples collected on Fluka blood collection papers (Figure 4.4).

Regardless of the type of chelating agent used, the DBS samples collected on papers pre-treated with a chelating agent at a concentration of 5mg/ml showed a significantly higher residual n-3 LCPUFA content than the samples collected on papers pre-treated with 1mg/ml chelating agent over the 4 week storage period (Figure 4.4). Further

increasing the chelating agent concentration to 20mg/ml did not result in any further improvements in the residual levels of n-3 LCPUFA retained in DBS (Tables 4.13-4.18).

As a result, chelating agents at a concentration of 5mg/ml were chosen as the optimal protectant concentrations for subsequent experiments.

Table 4.13. Fatty acid composition of DBS on Fluka paper pre-treated with single chelating agent after 5 hrs of air drying

Fatty acids[1]	Original Content[2]	No Chelator	1mg/ml L-ascorbic acid	5mg/ml L-ascorbic acid	20mg/ml L-ascorbic acid	1mg/ml Citric acid	5mg/ml Citric acid	20mg/ml Citric acid	1mg/ml EDTA	5mg/ml EDTA	20mg/ml EDTA
16:0	23.3±0.2[a]	24.6±0.3[b]	24.5±0.3[b]	24.1±0.3[ab]	24.1±0.3[ab]	24.7±0.3[b]	24.1±0.2[ab]	24.1±0.3[ab]	24.5±0.3[b]	24.1±0.4[ab]	24.1±0.3[b]
18:0	13.4±0.2[a]	14.5±0.2[bc]	14.4±0.1[b]	13.7±0.1[a]	14.0±0.1[ab]	14.4±0.2[b]	13.8±0.2[a]	14.0±0.1[a]	14.2±0.2[b]	13.9±0.2[ab]	14.0±0.2[ab]
18:1 n-9	18.4±0.1[a]	19.3±0.2[b]	19.0±0.1[b]	19.0±0.1[b]	18.8±0.1[ab]	19.1±0.1[b]	19.1±0.1[b]	19.0±0.1[b]	19.2±0.2[b]	19.1±0.2[b]	19.1±0.1[b]
18:2 n-6	19.7±0.2	19.3±0.1	19.3±0.1	19.6±0.2	19.6±0.1	19.4±0.1	19.5±0.2	19.4±0.1	19.4±0.1	19.4±0.1	19.4±0.2
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1	7.1±0.1	7.1±0.1	7.3±0.1	7.2±0.1	7.1±0.1	7.2±0.1	7.2±0.1	7.1±0.1	7.2±0.1	7.3±0.1
20:5 n-3	6.5±0.1[a]	5.5±0.1[c]	5.7±0.1[bc]	6.0±0.1[ab]	6.0±0.1[ab]	5.6±0.1[b]	5.9±0.1[bc]	5.9±0.1[bc]	5.6±0.1[bc]	5.8±0.1[bc]	5.8±0.1[bc]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06[a]	3.2±0.04[b]	3.3±0.04[b]	3.4±0.03[b]	3.3±0.03[b]	3.2±0.03[b]	3.4±0.05[b]	3.3±0.03[b]	3.2±0.05[b]	3.4±0.04[b]	3.3±0.05[b]
22:6 n-3	6.9±0.1[a]	5.9±0.1[b]	6.0±0.1[b]	6.2±0.1[b]	6.3±0.1[b]	6.0±0.1[b]	6.2±0.1[b]	6.2±0.1[b]	6.0±0.1[b]	6.2±0.1[b]	6.1±0.1[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.14. Fatty acid composition of DBS on Fluka paper pre-treated with single chelating agent after 2 weeks of storage at room temperature

Fatty acids[1]	**Original Content[2]**	**No Chelator**	**1mg/ml L-ascorbic acid**	**5mg/ml L-ascorbic acid**	**20mg/ml L-ascorbic acid**	**1mg/ml Citric acid**	**5mg/ml Citric acid**	**20mg/ml Citric acid**	**1mg/ml EDTA**	**5mg/ml EDTA**	**20mg/ml EDTA**
16:0	23.3±0.2^{a}	26.5±0.2^{c}	26.0±0.3^{bc}	25.4±0.3^{b}	25.3±0.3^{b}	26.1±0.3^{bc}	25.6±0.3^{b}	25.6±0.3^{b}	26.3±0.2^{c}	25.8±0.3^{bc}	25.7±0.3^{b}
18:0	13.4±0.2^{a}	15.3±0.1^{c}	15.1±0.2^{bc}	14.6±0.2^{b}	14.5±0.1^{b}	15.2±0.1^{bc}	14.8±0.1^{b}	14.6±0.1^{b}	15.2±0.2^{c}	15.1±0.2^{bc}	15.1±0.2^{bc}
18:1 n-9	18.4±0.1^{a}	20.6±0.2^{bc}	20.5±0.1^{bc}	19.9±0.1^{b}	19.8±0.1^{b}	20.5±0.1^{c}	20.0±0.1^{bc}	19.9±0.2^{bc}	20.4±0.2^{bc}	20.1±0.2^{bc}	20.0±0.2^{bc}
18:2 n-6	19.7±0.2^{a}	19.0±0.2^{b}	18.9±0.1^{b}	19.3±0.1^{ab}	19.4±0.1^{ab}	18.7±0.1^{b}	19.2±0.1^{ab}	19.3±0.1^{ab}	18.9±0.2^{b}	19.2±0.2^{ab}	19.2±0.1^{ab}
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1^{a}	6.3±0.1^{b}	6.4±0.1^{b}	6.7±0.1^{b}	6.6±0.1^{b}	6.4±0.1^{b}	6.6±0.2^{b}	6.6±0.1^{b}	6.4±0.2^{b}	6.5±0.1^{b}	6.5±0.1^{b}
20:5 n-3	6.5±0.1^{a}	4.4±0.1^{d}	4.7±0.1^{cd}	5.1±0.1^{b}	5.2±0.1^{b}	4.6±0.1^{cd}	5.0±0.2^{bc}	5.0±0.2^{b}	4.5±0.1^{cd}	4.9±0.1^{bc}	4.9±0.1^{bc}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06^{a}	2.6±0.05^{c}	2.8±0.06^{bc}	3.0±0.06^{b}	3.0±0.05^{b}	2.8±0.07^{bc}	2.9±0.03^{b}	3.0±0.04^{b}	2.7±0.02^{c}	2.8±0.04^{bc}	2.8±0.03^{bc}
22:6 n-3	6.9±0.1^{a}	4.9±0.1^{d}	5.0±0.1^{cd}	5.5±0.1^{bc}	5.6±0.1^{b}	5.1±0.1^{c}	5.4±0.1^{bc}	5.4±0.1^{bc}	5.0±0.1^{cd}	5.2±0.1^{bc}	5.3±0.1^{bc}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.15. Fatty acid composition of DBS on Fluka paper pre-treated with single chelating agent after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Chelator	1mg/ml L-ascorbic acid	5mg/ml L-ascorbic acid	20mg/ml L-ascorbic acid	1mg/ml Citric acid	5mg/ml Citric acid	20mg/ml Citric acid	1mg/ml EDTA	5mg/ml EDTA	20mg/ml EDTA
16:0	23.3±0.2^{a}	28.2±0.3^{d}	28.0±0.3^{c}	27.0±0.3^{b}	27.6±0.2^{bc}	28.0±0.3^{c}	27.2±0.3^{bc}	27.2±0.2^{b}	28.1±0.3^{c}	27.6±0.3^{bc}	27.6±0.3^{bc}
18:0	13.4±0.2^{a}	16.4±0.2^{c}	16.4±0.1^{c}	15.6±0.1^{b}	16.1±0.1^{bc}	16.4±0.2^{c}	15.8±0.3^{bc}	15.8±0.2^{bc}	16.3±0.3^{c}	16.0±0.2^{bc}	16.0±0.2^{bc}
18:1 n-9	18.4±0.1^{a}	22.4±0.2^{cd}	22.4±0.1^{cd}	21.6±0.2^{b}	21.8±0.2^{bc}	22.4±0.2^{cd}	21.6±0.1^{b}	21.6±0.2^{b}	22.5±0.2^{d}	22.7±0.2^{d}	21.6±0.2^{b}
18:2 n-6	19.7±0.2^{a}	18.2±0.2^{b}	18.1±0.2^{b}	18.5±0.1^{b}	18.1±0.1^{b}	18.0±0.1^{b}	18.5±0.1^{b}	18.5±0.1^{b}	18.0±0.2^{b}	18.1±0.2^{b}	18.1±0.3^{b}
18:3 n-3	0.4±0.01^{a}	0.3±0.01^{b}	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.3±0.01^{b}	0.4±0.02^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.02^{a}	0.4±0.01^{a}
20:4 n-6	7.6±0.1^{a}	5.4±0.1^{b}	5.6±0.2^{b}	5.9±0.1^{b}	5.8±0.1^{b}	5.5±0.1^{b}	5.8±0.1^{b}	5.9±0.1^{b}	5.5±0.2^{b}	5.9±0.1^{b}	5.8±0.1^{b}
20:5 n-3	6.5±0.1^{a}	3.3±0.1^{c}	3.4±0.1^{c}	4.0±0.1^{b}	3.7±0.1^{b}	3.3±0.1^{c}	4.0±0.1^{b}	4.0±0.1^{b}	3.4±0.1^{c}	3.9±0.1^{b}	3.9±0.1^{b}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.02^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.02^{a}
22:5 n-3	3.7±0.06^{a}	2.0±0.06^{c}	2.0±0.06^{c}	2.2±0.03^{bc}	2.1±0.03^{b}	2.0±0.02^{c}	2.3±0.05^{b}	2.2±0.03^{bc}	2.1±0.04^{bc}	2.2±0.02^{bc}	2.2±0.08^{bc}
22:6 n-3	6.9±0.1^{a}	3.7±0.1^{d}	3.8±0.1^{cd}	4.3±0.1^{b}	4.1±0.1^{bc}	3.8±0.1^{cd}	4.3±0.1^{b}	4.3±0.1^{b}	3.7±0.1^{d}	4.2±0.1^{b}	4.2±0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.16. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single chelating agent after 5 hrs of air drying

Fatty acids[1]	Original Content[2]	No Chelator	1mg/ml L-ascorbic acid	5mg/ml L-ascorbic acid	20mg/ml L-ascorbic acid	1mg/ml Citric acid	5mg/ml Citric acid	20mg/ml Citric acid	1mg/ml EDTA	5mg/ml EDTA	20mg/ml EDTA
16:0	23.3±0.2[a]	24.1±0.3	24.0±0.3	23.9±0.2	24.1±0.4	24.1±0.2	23.9±0.2	23.9±0.3	24.0±0.4	23.9±0.3	23.9±0.3
18:0	13.4±0.2[a]	14.3±0.2[b]	13.9±0.2[ab]	14.0±0.1[ab]	14.0±0.1[ab]	14.0±0.2[ab]	13.9±0.1[ab]	13.9±0.1[ab]	14.1±0.1[b]	13.8±0.2[ab]	14.0±0.2[ab]
18:1 n-9	18.4±0.1	19.0±0.2	18.9±0.1	18.9±0.1	18.9±0.1	18.9±0.1	18.8±0.1	18.8±0.1	19.0±0.1	18.9±0.1	18.9±0.2
18:2 n-6	19.7±0.2	19.5±0.1	19.5±0.1	19.5±0.1	19.6±0.1	19.5±0.1	19.6±0.1	19.6±0.1	19.4±0.2	19.5±0.1	19.5±0.2
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1	7.2±0.1	7.3±0.1	7.3±0.1	7.2±0.1	7.2±0.1	7.3±0.1	7.3±0.1	7.3±0.1	7.4±0.1	7.2±0.1
20:5 n-3	6.5±0.1[a]	5.80±0.1[b]	5.8±0.1[b]	6.0±0.1[b]	6.1±0.1[ab]	5.8±0.1[b]	5.9±0.1[b]	6.0±0.1[b]	5.8±0.1[b]	5.9±0.1[b]	5.9±0.1[b]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06[a]	3.4±0.04[b]	3.4±0.04[b]	3.4±0.03[b]	3.5±0.04[ab]	3.4±0.03[b]	3.4±0.04[b]	3.5±0.06[ab]	3.4±0.03[b]	3.4±0.03[b]	3.4±0.09[ab]
22:6 n-3	6.9±0.1[a]	6.2±0.1[b]	6.2±0.1[b]	6.2±0.1[b]	6.4±0.1[b]	6.2±0.1[b]	6.2±0.1[b]	6.3±0.1[b]	6.2±0.1[b]	6.3±0.1[b]	6.2±0.1[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.17. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single chelating agent after 2 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Chelator	1mg/ml L-ascorbic acid	5mg/ml L-ascorbic acid	20mg/ml L-ascorbic acid	1mg/ml Citric acid	5mg/ml Citric acid	20mg/ml Citric acid	1mg/ml EDTA	5mg/ml EDTA	20mg/ml EDTA
16:0	23.3±0.2^{a}	25.5±0.3^{d}	24.7±0.2^{bc}	24.4±0.3^{b}	24.2±0.2^{b}	24.8±0.2^{bc}	24.5±0.3^{bc}	24.3±0. 2^{b}	25.2±0.3^{cd}	24.6±0.3^{bc}	24.5±0.3^{bc}
18:0	13.4±0.2^{a}	15.0±0.2^{c}	14.3±0.1^{b}	14.2±0.1^{b}	14.0±0.1^{b}	14.3±0.2^{b}	14.2±0.1^{b}	14.1±0.2^{b}	14.5±0.1^{b}	14.1±0.1^{b}	14.1±0.1^{b}
18:1 n-9	18.4±0.1^{a}	20.0±0.2^{c}	19.3±0.1^{b}	19.0±0.1^{b}	19.1±0.1^{b}	19.4±0.1^{b}	19.1±0.1^{b}	19.1±0.1^{b}	19.4±0.2^{b}	19.2±0.1^{b}	19.2±0.1^{b}
18:2 n-6	19.7±0.2^{a}	19.0±0.1^{b}	19.4±0.1^{ab}	19.4±0.1^{ab}	19.5±0.1^{ab}	19.4±0.1^{ab}	19.4±0.1^{ab}	19.5±0.1^{ab}	19.2±0.2^{ab}	19.4±0.2^{ab}	19.4±0.2^{ab}
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1^{a}	6.8±0.1^{b}	7.0±0.1^{b}	7.1±0.1^{ab}	7.1±0.1^{ab}	6.9±0.1^{b}	7.1±0.1^{ab}	7.1±0.1^{ab}	6.8±0.1^{b}	7.1±0.1^{ab}	7.1±0.1^{ab}
20:5 n-3	6.5±0.1^{a}	4.9±0.1^{d}	5.5±0.1^{c}	5.8±0.1^{bc}	5.9±0.1^{b}	5.5±0.1^{bc}	5.8±0.1^{bc}	5.8±0.1^{bc}	5.4±0.1^{c}	5.8±0.1^{bc}	5.8±0.1^{bc}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.06^{a}	3.0±0.05^{c}	3.2±0.08^{bc}	3.3±0.05^{b}	3.3±0.03^{bc}	3.2±0.04^{bc}	3.2±0.04^{bc}	3.3±0.06^{b}	3.2±0.09^{bc}	3.2±0.05^{bc}	3.2±0.08^{bc}
22:6 n-3	6.9±0.1^{a}	5.3±0.1^{d}	5.8±0.1^{bc}	6.1±0.1^{b}	6.1±0.1^{bc}	5.7±0.1^{bc}	6.0±0.1^{bc}	6.0±0.1^{bc}	5.6±0.1^{cd}	6.0±0.1^{bc}	6.0±0.2^{bc}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.18. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with single chelating agent after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Chelator	1mg/ml L-ascorbic acid	5mg/ml L-ascorbic acid	20mg/ml L-ascorbic acid	1mg/ml Citric acid	5mg/ml Citric acid	20mg/ml Citric acid	1mg/ml EDTA	5mg/ml EDTA	20mg/ml EDTA
16:0	23.3±0.2[a]	26.8±0.3[d]	25.8±0.3[bc]	25.1±0.4[b]	25.4±0.4[bc]	26.0±0.3[bc]	25.1±0.4[b]	25.2±0.3[b]	26.2±0.3[c]	25.4±0.3[bc]	25.5±0.3[bc]
18:0	13.4±0.2[a]	15.7±0.1[d]	15.0±0.1[bc]	14.6±0.1[b]	14.7±0.1[b]	15.0±0.1[bc]	14.7±0.2[bc]	14.6±0.2[b]	15.3±0.2[cd]	15.0±0.2[bc]	14.7±0.1[bc]
18:1 n-9	18.4±0.1[a]	20.6±0.1[b]	20.7±0.1[b]	20.1±0.2[b]	20.2±0.2[b]	20.8±0.2[b]	20.1±0.1[b]	20.2±0.2[b]	20.8±0.2[b]	20.2±0.2[b]	20.1±0.2[b]
18:2 n-6	19.7±0.2[a]	18.7±0.1[b]	18.9±0.2[b]	19.3±0.2[ab]	19.1±0.1[ab]	19.0±0.1[b]	19.2±0.2[ab]	19.2±0.1[ab]	18.8±0.2[b]	19.0±0.1[b]	19.0±0.2[b]
18:3 n-3	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.6±0.1[a]	6.3±0.1[b]	6.5±0.1[b]	6.8±0.1[b]	6.7±0.1[b]	6.4±0.1[b]	6.8±0.1[b]	6.7±0.1[b]	6.3±0.1[b]	6.6±0.2[b]	6.7±0.1[b]
20:5 n-3	6.5±0.1[a]	4.0±0.1[d]	4.5±0.1[c]	5.1±0.1[b]	5.0±0.1[b]	4.6±0.1[c]	5.0±0.1[b]	5.0±0.1[bc]	4.4±0.1[c]	4.9±0.2[b]	4.9±0.1[b]
22:4 n-6	0.5±0.01[a]	0.4±0.01[b]	0.4±0.01[b]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]
22:5 n-3	3.7±0.06[a]	2.7±0.06[d]	2.9±0.02[cd]	3.1±0.06[bc]	3.0±0.05[bc]	2.8±0.04[cd]	3.1±0.03[bc]	3.2±0.04[b]	2.8±0.04[cd]	3.0±0.05[bc]	3.0±0.03[bc]
22:6 n-3	6.9±0.1[a]	4.5±0.1[d]	5.1±0.1[c]	5.5±0.1[b]	5.4±0.1[bc]	4.9±0.1[c]	5.5±0.1[b]	5.4±0.1[b]	4.9±0.1[c]	5.3±0.1[bc]	5.3±0.1[bc]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

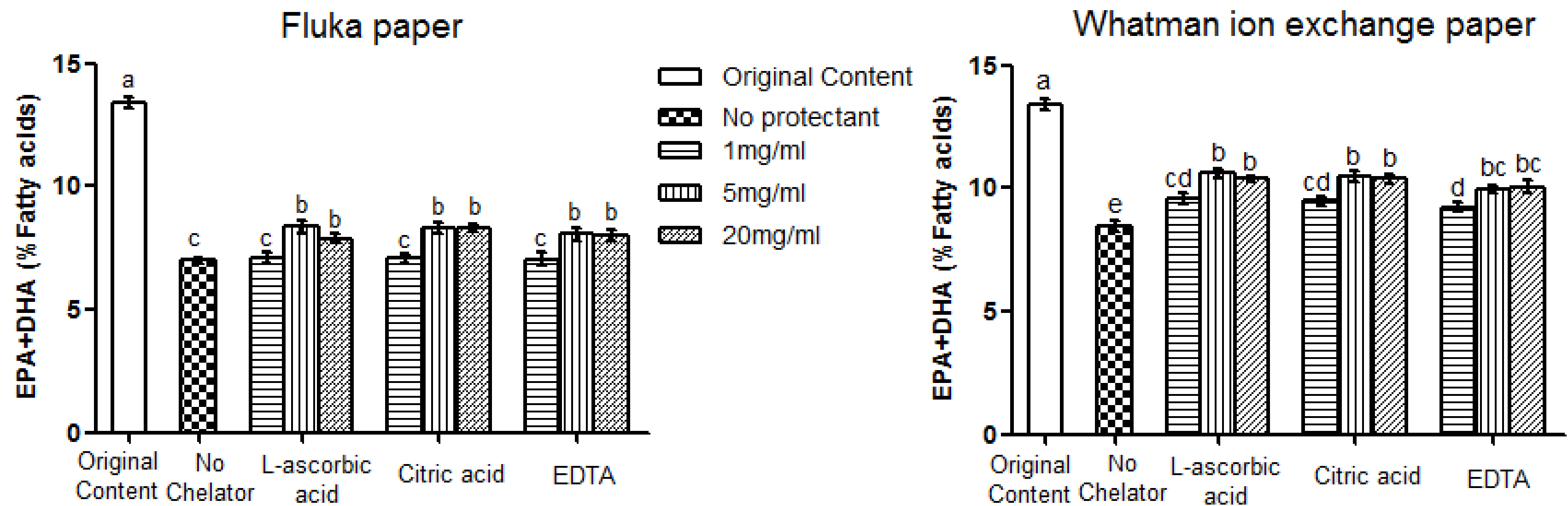


Figure 4.4. EPA+DHA% of DBS on Fluka paper or Whatman ion exchange paper in the presence or absence of single chelating agent determined after 4 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

4.4.2.4 Stability of DBS treated with a mixture of an antioxidant and a chelating agent

The objective of this experiment was to test the effectiveness of a mixture of an antixoidant and a chelating agent in stabilising the LCPUFA in DBS. Blood spots were obtained by absorbing 50μl of fresh blood onto the collection area (~1.5x1.5cm) of two types of blood collection paper (Fluka blood collection paper and Whatman ion exchange paper) in the absence or presence of a single phenolic antioxidant, or a mixture of a phenolic antioxidant and a chelating agent (Table 4.19). After being air dried at room temperature for 5 hrs, DBS samples were processed, and analysed by GC as describe in Chapter 2 to determine fatty acid composition, either immediately after air drying, or after 2 weeks, 4 weeks or 9 weeks of storage at room temperature in cellophane bags in the dark and in the presence of desiccants.

Consistent with the results of the previous experiments, DBS collected on both types of collection papers in the absence of protectant exhibited significantly lower levels of all LCPUFA as a proportion of total blood fatty acids measured in the blood spot after 9 weeks of storage at room temperature when compared with those DBS collected on papers pre-treated with protectant (Figures 4.5-4.6). Furthermore, DBS collected on Whatman ion exchange paper consistently exhibited a fatty acid composition that was more similar to that in fresh blood when compared with DBS collected on Fluka blood collection paper, irrespective of the storage periods and the protectant formulations used (Tables 4.19-4.26).

After 5 hrs of air drying at room temperature, there was a significant reduction in the levels of all n-3 LCPUFA measured as a weight percentage of total blood fatty acids

in the DBS collected on both types of collection papers which had not been pre-treated with any antioxidant or chelating agent (Tables 4.19). Significant declines in the levels of EPA and DHA was also detected in DBS samples which were collected on either of the collection papers treated with 2mg/ml of BHT or TBHQ alone when compared with those measured in fresh blood by direct transmethylation (Tables 4.19, 4.23). However, after air drying, the fatty acid levels measured in DBS collected on collection papers which were pre-treated with the mixture of an antioxidant (BHT or TBHQ) and either of the iron chelators (L-ascorbic acid, citric acid or EDTA) were not significantly different from the results obtained from direct transmethylation of fresh blood, irrespective of the type of collection paper used (Tables 4.19, 4.23).

The DBS samples collected on Fluka blood collection paper at all time points during the 9 weeks of storage showed significantly different fatty acid compositions compared with those obtained from fresh blood by direct transmethylation, irrespective of the protectant formulation used (Tables 4.20-4.22). The levels of n-6 (LA, AA) and n-3 (EPA, Docosapentaenoic acid (DPA, 22:5 n-3), DHA) LCPUFA measured in the DBS collected on Fluka blood collection paper were all significantly lower than the corresponding LCPUFA values obtained by direct transmethylation of fresh blood as early as 2 weeks after collection (Table 4.20), and continued to decline over the 9 week storage period (Figure 4.5a, Figure 4.6a, Tables 4.21-4.22). Conversely, the levels of SFA, palmitic acid and stearic acid, measured as a percentage of total blood fatty acids in the DBS increased steadily across the storage period (Tables 4.20-4.22).

In DBS collected on Whatman ion exchange paper, there was a significant interaction

between time and protectant formulation in relation to fatty acid composition of the samples, such that different protectant formulations showed different stabilising ability. The DBS samples collected on Whatman ion exchange papers which were treated with 2mg/ml BHT alone exhibited significant declines in the percentage of EPA and DHA in total fatty acids after only 2 weeks of storage when compared with the corresponding LCPUFA values measured from the fresh blood direct transmethylation (Figure 4.5b, Table 4.24). However, following the addition of 5mg/ml of either of the iron chelators (L-ascorbic acid, citric acid or EDTA) to the BHT treated Whatman ion exchange paper, the levels of EPA and DHA in the DBS samples did not differ significantly from those measured in fresh blood by direct transmethylation, even after 9 weeks of storage at room temperature (Figure 4.5b, Tables 4.24-4.26). However, the same effect was not seen in TBHQ treated Whatman ion exchange paper, which showed significant losses of all n-3 LCPUFA after 2 weeks of storage at room temperature even after the addition of an iron chelator (Figure 4.6b, Tables 4.24-4.26).

Table 4.19. Fatty acid composition of DBS on Fluka paper pre-treated with different protectants after 5 hrs of air drying at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3[a]	25.0±0.3[b]	24.2±0.3[ab]	23.8±0.2[a]	23.8±0.3[a]	23.8±0.2[a]	24.3±0.3[ab]	23.7±0.3[a]	23.7±0.3[a]	23.7±0.3[a]
18:0	13.4±0.1[a]	14.4±0.1[b]	13.8±0.2[ab]	13.5±0.1[a]	13.4±0.1[a]	13.5±0.1[a]	14.1±0.1[b]	13.5±0.1[a]	13.5±0.2[a]	13.5±0.1[a]
18:1 n-9	18.6±0.2[a]	19.4±0.2[b]	19.0±0.1[ab]	18.5±0.1[a]	18.5±0.1[a]	18.6±0.2[a]	19.0±0.2[ab]	18.7±0.2[a]	18.6±0.1[a]	18.7±0.2[a]
18:2 n-6	19.8±0.2	19.5±0.1	19.8±0.1	19.8±0.2	19.8±0.2	19.8±0.2	19.9±0.2	19.7±0.2	19.7±0.1	19.8±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.4±0.1[a]	6.8±0.1[b]	7.1±0.1[ab]	7.3±0.1[a]	7.4±0.2[a]	7.3±0.1[a]	7.1±0.1[ab]	7.3±0.1[a]	7.4±0.1[a]	7.3±0.1[a]
20:5 n-3	6.3±0.1[a]	5.3±0.1[c]	5.6±0.1[bc]	6.2±0.1[a]	6.2±0.1[a]	6.2±0.1[a]	5.7±0.1[bc]	6.3±0.1[a]	6.3±0.1[a]	6.3±0.01[a]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.6±0.06[a]	3.2±0.05[b]	3.4±0.03[ab]	3.6±0.03[a]	3.5±0.04[a]	3.5±0.11[ab]	3.4±0.03[ab]	3.6±0.03[a]	3.6±0.08[a]	3.6±0.06[a]
22:6 n-3	6.8±0.1[a]	5.9±0.2[b]	6.1±0.1[b]	6.8±0.1[a]	6.8±0.1[a]	6.7±0.1[a]	6.2±0.1[b]	6.7±0.1[a]	6.8±0.1[a]	6.7±0.1[a]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.20. Fatty acid composition of DBS on Fluka paper pre-treated with different protectants after 2 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3^{a}	26.9±0.3^{d}	25.7±0.3^{c}	24.6±0.3^{b}	24.7±0.3^{b}	24.8±0.3^{b}	25.6±0.3^{c}	24.8±0.3^{b}	24.7±0.2^{b}	24.8±0.2^{b}
18:0	13.4±0.1^{a}	15.2±0.1^{c}	14.4±0.2^{b}	14.4±0.2^{b}	14.4±0.3^{b}	14.5±0.2^{b}	14.5±0.1^{b}	14.6±0.2^{b}	14.6±0.2^{b}	14.6±0.2^{bc}
18:1 n-9	18.6±0.2^{a}	20.6±0.21^{c}	19.3±0.2^{b}	19.2±0.1^{b}	19.2±0.1^{b}	19.3±0.2^{b}	19.3±0.1^{b}	19.2±0.2^{b}	19.1±0.1^{b}	19.4±0.2^{b}
18:2 n-6	19.8±0.2^{a}	19.0±0.1^{b}	19.3±0.2^{ab}	19.5±0.1^{ab}	19.5±0.2^{ab}	19.3±0.2^{ab}	19.4±0.1^{ab}	19.4±0.2^{ab}	19.5±0.2^{ab}	19.4±0.2^{ab}
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.4±0.1^{a}	6.1±0.12^{c}	6.8±0.1^{b}	7.0±0.1^{ab}	7.0±0.1^{ab}	7.0±0.1^{ab}	6.8±0.1^{b}	6.9±0.1^{b}	7.0±0.1^{ab}	6.9±0.1^{b}
20:5 n-3	6.3±0.1^{a}	4.3±0.18^{c}	5.2±0.2^{b}	5.4±0.1^{b}	5.4±0.1^{b}	5.4±0.1^{b}	5.2±0.2^{b}	5.4±0.1^{b}	5.5±0.1^{b}	5.4±0.1^{b}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.6±0.06^{a}	2.6±0.05^{c}	3.0±0.03^{b}	3.1±0.08^{b}	3.2±0.08^{b}	3.1±0.06^{b}	3.0±0.04^{b}	3.1±0.04^{b}	3.1±0.04^{b}	3.1±0.04^{b}
22:6 n-3	6.8±0.1^{a}	4.8±0.18^{d}	5.6±0.1^{c}	6.2±0.1^{b}	6.1±0.1^{b}	6.1±0.1^{b}	5.7±0.1^{bc}	6.1±0.1^{bc}	6.1±0.1^{b}	6.0±0.1^{bc}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.21. Fatty acid composition of DBS on Fluka paper pre-treated with different protectants after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3^{a}	28.5±0.3^{d}	26.8±0.3^{c}	25.7±0.2^{b}	26.1±0.3^{bc}	25.8±0.3^{b}	26.8±0.3^{c}	25.6±0.3^{b}	25.5±0.2^{b}	25.6±0.3^{b}
18:0	13.4±0.1^{a}	16.3±0.2^{c}	15.1±0.1^{b}	14.9±0.2^{b}	15.1±0.2^{b}	14.9±0.1^{b}	15.2±0.2^{b}	15.0±0.2^{b}	15.1±0.2^{b}	15.0±0.1^{b}
18:1 n-9	18.6±0.2^{a}	22.3±0.2^{d}	20.1±0.2^{b}	20.5±0.1^{bc}	20.8±0.2^{c}	20.6±0.1^{bc}	20.1±0.2^{bc}	20.5±0.2^{bc}	20.5±0.1^{bc}	20.6±0.2^{bc}
18:2 n-6	19.8±0.2^{a}	18.3±0.1^{c}	18.8±0.2^{bc}	19.0±0.2^{b}	18.8±0.2^{bc}	19.0±0.1^{b}	19.0±0.2^{b}	19.1±0.2^{b}	19.0±0.1^{b}	19.0±0.1^{b}
18:3 n-3	0.4±0.01^{a}	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}
20:4 n-6	7.4±0.1^{a}	5.3±0.2^{c}	6.3±0.2^{b}	6.5±0.1^{b}	6.3±0.1^{b}	6.5±0.1^{b}	6.2±0.1^{b}	6.5±0.1^{b}	6.6±0.1^{b}	6.6±0.2^{c}
20:5 n-3	6.3±0.1^{a}	3.2±0.2^{c}	4.4±0.1^{b}	4.6±0.1^{b}	4.5±0.1^{b}	4.5±0.1^{b}	4.4±0.1^{b}	4.7±0.1^{b}	4.7±0.1^{b}	4.6±0.1^{b}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}
22:5 n-3	3.6±0.06^{a}	1.9±0.06^{c}	2.9±0.06^{b}	2.9±0.1^{b}	2.9±0.05^{b}	2.9±0.06^{b}	2.9±0.03^{b}	3.0±0.03^{b}	3.0±0.06^{b}	2.9±0.05^{b}
22:6 n-3	6.8±0.1^{a}	3.7±0.3^{c}	4.9±0.2^{b}	5.3±0.2^{b}	5.1±0.2^{b}	5.3±0.1^{b}	5.0±0.2^{b}	5.3±0.1^{b}	5.2±0.1^{b}	5.2±0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.22. Fatty acid composition of DBS on Fluka paper pre-treated with different protectants after 9 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3^{a}	29.7±0.3^{c}	27.7±0.3^{b}	27.1±0.3^{b}	27.3±0.3^{b}	27.3±0.2^{b}	27.7±0.2^{b}	27.7±0.3^{b}	27.6±0.3^{b}	27.6±0.3^{b}
18:0	13.4±0.1^{a}	16.9±0.2^{c}	15.8±0.1^{b}	15.6±0.1^{b}	15.7±0.1^{b}	15.4±0.2^{b}	15.8±0.2^{b}	15.7±0.2^{b}	15.6±0.2^{b}	15.7±0.2^{b}
18:1 n-9	18.6±0.2^{a}	23.1±0.2^{c}	21.1±0.2^{b}	21.4±0.2^{b}	21.7±0.2^{b}	21.3±0.2^{b}	21.3±0.2^{b}	21.4±0.2^{b}	21.3±0.2^{b}	21.3±0.2^{b}
18:2 n-6	19.8±0.2^{a}	17.5±0.2^{c}	18.6±0.2^{b}	18.8±0.2^{b}	18.7±0.1^{b}	18. 8±0.2^{b}	18.7±0.2^{b}	18.8±0.2^{b}	18.7±0.2^{b}	18.7±0.2^{b}
18:3 n-3	0.4±0.01^{a}	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.02^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}
20:4 n-6	7.4±0.1^{a}	4.8±0.2^{c}	5.4±0.1^{b}	5.5±0.1^{b}	5.4±0.1^{b}	5.6±0.2^{b}	5.3±0.1^{b}	5.4±0.1^{b}	5.4±0.1^{b}	5.4±0.1^{b}
20:5 n-3	6.3±0.1^{a}	2.7±0.2^{c}	4.0±0.2^{b}	4.2±0.1^{b}	4.1±0.1^{b}	4.2±0.1^{b}	4.1±0.1^{b}	4.1±0.1^{b}	4.1±0.1^{b}	4.2±0.1^{b}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.02^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}
22:5 n-3	3.6±0.06^{a}	1.7±0.12^{c}	2.4±0.04^{b}	2.4±0.06^{b}	2.4±0.06^{b}	2.5±0.04^{b}	2.4±0.05^{b}	2.4±0.06^{b}	2.4±0.06^{b}	2.5±0.05^{b}
22:6 n-3	6.8±0.1^{a}	3.1±0.1^{c}	4.3±0.2^{b}	4.4±0.1^{b}	4.3±0.1^{b}	4.5±0.2^{b}	4.3±0.1^{b}	4.3±0.1^{b}	4.4±0.2^{b}	4.4±0.1^{b}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.23. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with different protectants after 5 hrs of air drying at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3[a]	24.4±0.3[b]	24.3±0.2[ab]	23.7±0.2[a]	23.7±0.2[ab]	23.6±0.2[a]	24.0±0.3[ab]	23.7±0.3[ab]	23.7±0.3[ab]	23.7±0.3[ab]
18:0	13.4±0.1[a]	14.0±0.2[b]	13.9±0.2[ab]	13.4±0.1[a]	13.4±0.1[a]	13.4±0.2[a]	14.1±0.1[b]	13.5±0.1[ab]	13.5±0.1[ab]	13.5±0.2[ab]
18:1 n-9	18.6±0.2[a]	19.1±0.1[b]	19.0±0.2[ab]	18.5±0.1[a]	18.5±0.1[a]	18.5±0.1[a]	19.1±0.1[b]	18.6±0.2[ab]	18.6±0.2[ab]	18.6±0.2[ab]
18:2 n-6	19.8±0.2	19.7±0.2	19.8±0.1	19.9±0.2	19.7±0.2	19.9±0.1	19.8±0.2	19.8±0.1	19.7±0.1	19.8±0.2
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.4±0.1	7.1±0.2	7.2±0.1	7.3±0.1	7.4±0.1	7.3±0.1	7.1±0.1	7.4±0.2	7.4±0.1	7.3±0.1
20:5 n-3	6.3±0.1[a]	5.6±0.1[b]	5.7±0.1[b]	6.3±0.1[a]	6.3±0.1[a]	6.3±0.1[a]	5.7±0.1[b]	6.3±0.1[a]	6.3±0.1[a]	6.3±0.1[a]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.6±0.06[a]	3.3±0.05[b]	3.5±0.08[ab]	3.6±0.05[a]	3.6±0.05[a]	3.6±0.05[a]	3.4±0.05[ab]	3.6±0.06[a]	3.6±0.05[a]	3.6±0.06[a]
22:6 n-3	6.8±0.1[a]	6.1±0.1[b]	6.2±0.1[b]	6.9±0.2[a]	6.9±0.1[a]	6.8±0.1[a]	6.2±0.1[b]	6.7±0.1[a]	6.7±0.1[a]	6.8±0.1[a]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.24. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with different protectants after 2 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3[a]	26.6±0.2[d]	24.7±0.2[c]	23.9±0.3[ab]	23.9±0.3[ab]	23.9±0.2[ab]	25.0±0.2[c]	24.4±0.3[bc]	24.6±0.3[bc]	24.5±0.2[bc]
18:0	13.4±0.1[a]	14.7±0.2[c]	14.0±0.1[bc]	13.6±0.2[ab]	13.5±0.2[a]	13.5±0.1[a]	14.4±0.2[c]	14.2±0.1[bc]	14.2±0.2[bc]	14.1±0.2[bc]
18:1 n-9	18.6±0.2[a]	19.6±0.2[c]	19.1±0.1[bc]	18.6±0.1[ab]	18.7±0.1[ab]	18.7±0.1[ab]	19.3±0.2[c]	19.2±0.2[bc]	19.3±0.1[bc]	19.3±0.2[bc]
18:2 n-6	19.8±0.2	19.2±0.1	19.7±0.2	19.7±0.1	19.7±0.1	19.7±0.2	19.6±0.1	19.7±0.2	19.7±0.2	19.6±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.4±0.1[a]	6.6±0.1[b]	7.0±0.1[ab]	7.2±0.1[a]	7.3±0.1[a]	7.2±0.1[a]	7.0±0.1[ab]	7.1±0.1[a]	7.0±0.1[ab]	7.2±0.1[a]
20:5 n-3	6.3±0.1[a]	4.8±0.1[c]	5.5±0.1[b]	6.2±0.1[a]	6.2±0.1[a]	6.2±0.1[a]	5.3±0.1[b]	5.5±0.1[b]	5.5±0.1[b]	5.4±0.1[b]
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.6±0.06[a]	2.9±0.03[d]	3.4±0.05[ab]	3.5±0.04[a]	3.5±0.04[a]	3.5±0.03[a]	3.1±0.04[cd]	3.2±0.05[bc]	3.2±0.06[bc]	3.2±0.03[bc]
22:6 n-3	6.8±0.1[a]	5.2±0.2[d]	5.9±0.1[b]	6.6±0.1[a]	6.7±0.1[a]	6.6±0.1[a]	5.8±0.1[b]	6.2±0.1[b]	6.1±0.1[b]	6.2±0.1[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.25. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with different protectants after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3^{a}	27.3±0.4^{d}	25.3±0.3^{b}	24.1±0.3^{a}	24.1±0.2^{a}	24.2±0.2^{a}	26.4±0.4^{c}	25.5±0.4^{b}	25.6±0.2^{b}	25.4±0.3^{b}
18:0	13.4±0.1^{a}	15.8±0.2^{d}	14.4±0.2^{bc}	13.7±0.1^{a}	13.7±0.1^{a}	13.8±0.2^{ab}	15.0±0.2^{c}	14.8±0.1^{c}	14.9±0.1^{c}	14.8±0.1^{c}
18:1 n-9	18.6±0.2^{a}	20.6±0.2^{b}	19.2±0.1^{a}	18.9±0.1^{a}	18.9±0.1^{a}	19.0±0.2^{a}	20.1±0.1^{b}	20.2±0.2^{b}	20.3±0.2^{b}	20.0±0.2^{b}
18:2 n-6	19.8±0.2^{a}	18.7±0.2^{c}	19.3±0.1^{ab}	19.6±0.2^{ab}	19.6±0.2^{ab}	19.5±0.1^{ab}	19.1±0.1^{bc}	19.2±0.2^{ab}	19.1±0.1^{bc}	19.3±0.2^{ab}
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.4±0.1^{a}	6.2±0.1^{cd}	6.8±0.1^{bc}	7.1±0.1^{a}	7.1±0.1^{a}	7.1±0.1^{a}	6.4±0.1^{cd}	6.6±0.1^{c}	6.6±0.2^{b}	6.7±0.1^{bc}
20:5 n-3	6.3±0.1^{a}	3.9±0.1^{d}	5.3±0.1^{bc}	6.0±0.1^{a}	6.1±0.1^{a}	6.0±0.2^{a}	4.5±0.1^{d}	4.8±0.1^{c}	4.7±0.1^{c}	4.9±0.1^{c}
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.6±0.06^{a}	2.7±0.04^{d}	3.3±0.04^{b}	3.5±0.05^{ab}	3.5±0.05^{ab}	3.5±0.08^{ab}	2.9±0.06^{cd}	3.0±0.05^{c}	3.0±0.06^{c}	3.0±0.02^{c}
22:6 n-3	6.8±0.1^{a}	4.4±0.1^{d}	5.7±0.1^{b}	6.5±0.1^{a}	6.5±0.1^{a}	6.5±0.1^{a}	5.1±0.1^{c}	5.4±0.1^{bc}	5.3±0.1^{c}	5.5±0.1^{bc}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

Table 4.26. Fatty acid composition of DBS on Whatman ion exchange paper pre-treated with different protectants after 9 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	No Protectant	BHT	BHT & Cítric acid	BHT & L-ascorbic acid	BHT & EDTA	TBHQ	TBHQ & Citric acid	TBHQ & L-ascorbic acid	TBHQ & EDTA
16:0	23.6±0.3^{a}	28.0±0.3^{e}	26.0±0.3^{c}	24.4±0.3^{ab}	24.5±0.2^{b}	24.2±0.3^{ab}	27.1±0.2^{d}	26.9±0.3^{d}	27.3±0.3^{d}	27.1±0.3^{d}
18:0	13.4±0.1^{a}	16.1±0.1^{e}	14.7±0.2^{c}	14.0±0.1^{b}	14.1±0.1^{bc}	14.0±0.2^{b}	15.5±0.2^{de}	15.4±0.2^{d}	15.4±0.1^{d}	15.4±0.2^{d}
18:1 n-9	18.6±0.2^{a}	21.4±0.2^{c}	19.3±0.2^{a}	19.0±0.2^{ab}	19.0±0.1^{ab}	19.0±0.2^{b}	21.1±0.1^{c}	21.0±0.1^{c}	21.0±0.2^{c}	21.1±0.2^{c}
18:2 n-6	19.8±0.2^{a}	18.3±0.1^{b}	19.3±0.2^{a}	19.6±0.1^{a}	19.4±0.1^{a}	19.5±0.1^{a}	18.7±0.1^{b}	18.5±0.2^{b}	18.7±0.2^{b}	18.7±0.2^{b}
18:3 n-3	0.4±0.01^{a}	0.3±0.01^{b}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.01^{a}	0.4±0.02^{a}	0.4±0.01^{a}
20:4 n-6	7.4±0.1^{a}	6.0±0.2^{c}	6.6±0.1^{b}	7.0±0.1^{ab}	7.0±0.1^{a}	7.1±0.1^{a}	6.0±0.1^{c}	6.2±0.1^{bc}	6.2±0.1^{bc}	6.2±0.1^{bc}
20:5 n-3	6.3±0.1^{a}	3.4±0.1^{e}	4.9±0.1^{c}	5.8±0.2^{ab}	5.7±0.2^{b}	5.9±0.2^{ab}	4.2±0.1^{d}	4.3±0.1^{d}	4.1±0.03^{d}	4.2±0.1^{d}
22:4 n-6	0.5±0.01^{a}	0.4±0.01^{b}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}
22:5 n-3	3.6±0.06^{a}	2.4±0.04^{c}	3.2±0.04^{b}	3.4±0.05^{ab}	3.5±0.05^{a}	3.4±0.05^{ab}	2.5±0.06^{c}	2.5±0.06^{c}	2.5±0.06^{c}	2.5±0.05^{c}
22:6 n-3	6.8±0.1^{a}	4.0±0.1^{d}	5.5±0.1^{b}	6.3±0.2^{a}	6.3±0.2^{a}	6.4±0.2^{a}	4.4±0.1^{cd}	4.6±0.1^{c}	4.3±0.1^{cd}	4.4±0.1^{cd}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

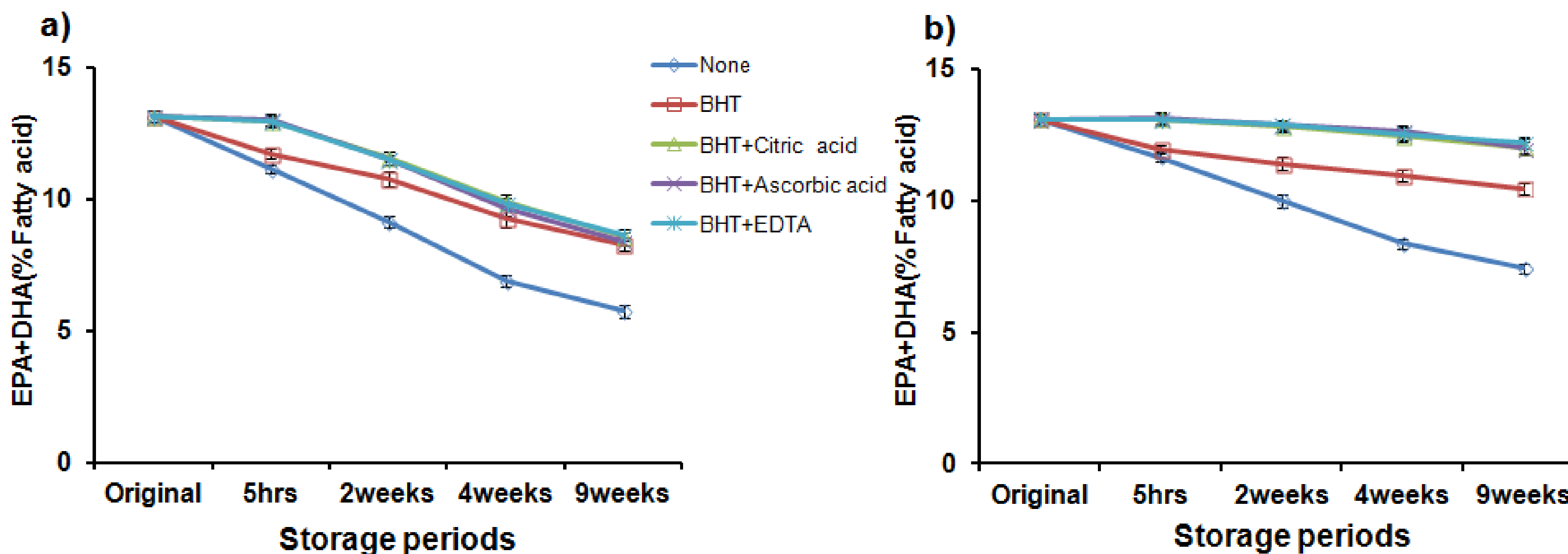


Figure 4.5. Stability of EPA+DHA% in DBS on Fluka paper (a) or Whatman ion exchange paper (b) determined over 9 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

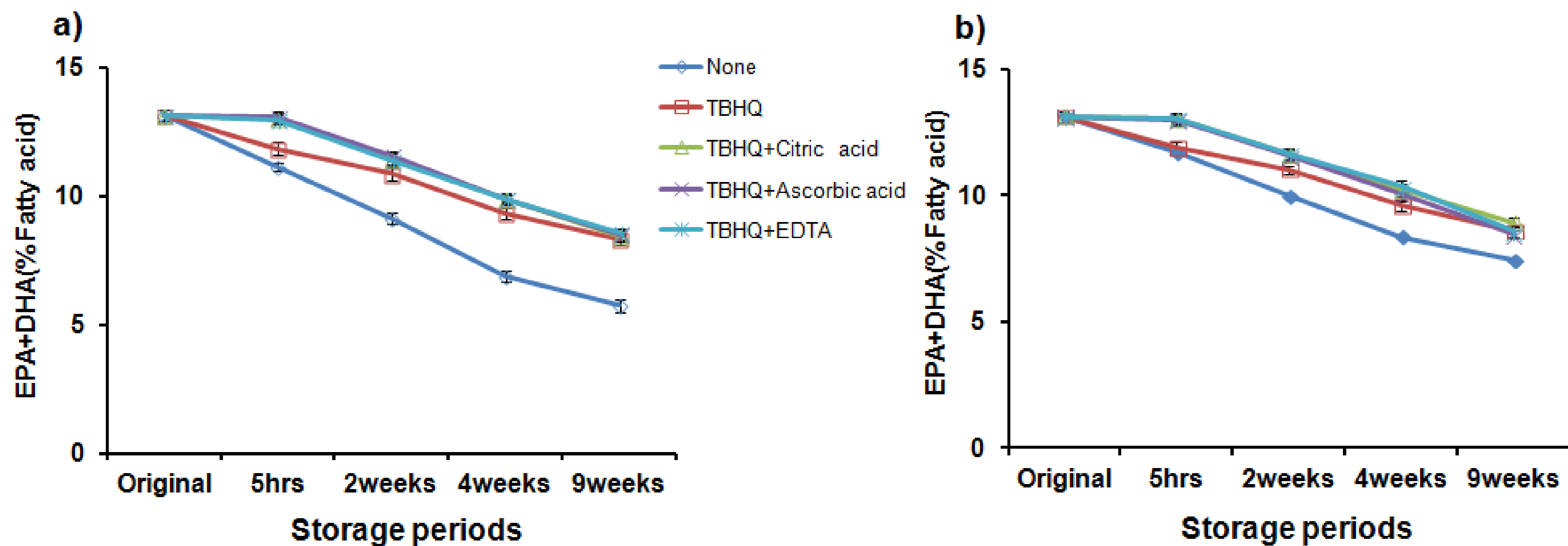


Figure 4.6. Stability of EPA+DHA% in DBS on Fluka paper (a) or Whatman ion exchange paper (b) determined over 9 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

The present study has demonstrated that appropriately selected protection systems can greatly retard the degradation of LCPUFA in DBS samples during air drying and the subsequent storage at room temperature. The greatest stability of fatty acid composition in the blood spots was achieved by combing the antioxidant BHT with an iron chelating agent (citric acid, L-ascorbic acid or EDTA) and using a silica gel coated paper (Whatman ion exchange paper) as the collection paper, as this allowed for the maintenance of original EPA and DHA levels in the DBS samples after even 2 months of storage at room temperature. This represents a significant improvement over the protection system used in previous studies, consisting of BHT treatment and Fluka collection paper, in which only ~60% of the n-3 LCPUFA contents in the samples were retained after 2 months storage at room temperature (Min *et al.* 2011).

Therefore, my study revealed the need for all three components, namely an antioxidant, an iron chelator, and a silica gel coated paper to ensure the stability of LCPUFA in DBS samples. The mechanisms by which all three components achieve optimal stability of LCPUFA can be explained by the respective actions of these three components.

LCPUFA are particularly susceptible to oxidation, and the oxidative reactions can produce free radicals, which then catalyse further lipid oxidation. Antioxidants are frequently used in inhibiting the oxidation of lipids because they compete with lipids for oxidation, and stop the propagation of free radicals by undergoing oxidation to produce stable and relatively unreactive antioxidant radicals (Frankel *et al.* 1994). Two kinds of antioxidants, BHT and TBHQ, were tested in the present study. Both of these antioxidants are widely used as an additive in food processing to prevent

oxidative damage to fat in food products (Babich 1982; Jain *et al.* 2011). In DBS samples absorbed on normal chromatography paper, the protective ability of both antioxidants was similar at a concentration of ≤ 2mg/ml. However, a significant drop in LCPUFA content in TBHQ treated DBS samples was observed when the concentration of TBHQ was increased from 2mg/ml to 4mg/ml, which may indicate the pro-oxidant property of TBHQ at a relatively higher concentration. TBHQ has been reported for its dual capacity to act both as an antioxidant and as a producer of reactive oxygen species (Pinkus *et al.* 1996). Some researchers even used TBHQ as pro-oxidant to induce oxidative stress in monocytes (Messer *et al.* 2005). The pro-oxidant property of TBHQ might be partially due to the fact that TBHQ has two hydroxyl groups that are available to donate protons to reactive free radicals (van Aardt *et al.* 2004).

Interestingly, DBS samples absorbed on Whatman ion exchange paper and treated with BHT showed significantly higher residual n-3 LCPUFA levels than those treated with TBHQ at all three concentrations tested in my experiments. This may because the antioxidative activity of TBHQ can be altered depending on the medium to which it is applied. For example, in previous studies, TBHQ showed higher antioxidative activity than BHA in vegetable oil systems (Sherwin and Thompson 1967), however, its antioxidative activity was 3 folds lower than BHA when applied in silica monolayer system (Porter *et al.* 1977).

The use of chelating agents in my study was not accidental, but based on the hypothesis that by binding the iron ions in blood with chelator, the LCPUFA losses from DBS could be greatly retarded. This hypothesis is based on the known role of

ferrous and ferric forms of iron as oxidisers, and that even trace amounts of these substances enables the catalyses the degradation of unsaturated lipids either by direct initiation and formation of lipid peroxyl radical and alkyl radical or by inducing the production of hydroxyl radicals through a Fenton reaction (Halliwell and Gutteridge 1997). These free radicals are highly reactive and can cause rapid oxidative degradation of LCPUFA (Sies 1997). A study of lipid peroxidation in iron-loaded heart cells showed significant reductions in the levels of LCPUFA and a relative increase in the levels of SFA (expressed as a weight percentage of total fatty acids) in liposomal vesicles in response to iron loading (Link *et al.* 1989). These changes were identical to those reported in previous studies of the fatty acid composition of red blood cells membrane lipids in beta-thalassemia, a disease in which iron overload is frequently observed in the blood (Rachnilewitz *et al.* 1976). Thus, the fact that similar changes in n-3 LCPUFA and SFA levels observed in DBS in the absence of a chelator in the present experiment supports the hypothesis that iron in the DBS samples makes an important contribution to the oxidative loss of LCPUFA.

In healthy human blood, iron is usually firmly bound to haemoglobin and enclosed in red blood cells (Gurzau *et al.* 2003). However, once the red bloodcells in blood spots are broken during air drying, haemoglobin molecules may be released and oxidised in air which will liberate the iron from haemoglobin (Nagy *et al.* 2010). Therefore, in the presence of air, the liberated iron has the potential to cause oxidative loss of LCPUFA. Cell culture studies have shown that addition of a chelating agent to the media protects cells in culture from lipid peroxidation induced by iron overload (Link *et al.* 1989; Sharma et al 1990). The key property shared by all chelating agents is that metal ions are bound very tightly to the chelator, thus rendering them chemically inert.

Studies investigating strategies for protection of fatty acid oxidation in substrates other than cell have also shown that a protection system consisting of both antioxidant and iron chelators performs much better than systems which used antioxidant alone. Examples include protecting LCPUFA in avocado (Grajales-Lagunes *et al.* 1999) and fish oil (Hamilton *et al.* 1998) and vitamin A palmitate in ultra rice (Li *et al.* 2009) from oxidation during storage. Consequently, an iron chelator was used in my protection system to prevent ferrous and ferric ions from forming reactive free radicals and hence improve the stability of LCPUFA in DBS samples.

In Chapter 3 I have shown that Whatman ion exchange paper (silica gel coated paper) is likely to be the optimal blood collection paper because it was unable to detect any significant contaminants released from Whatman ion exchange paper. According to the data presented in this Chapter, DBS samples absorbed on Whatman ion exchange paper also showed the highest residual LCPUFA after storage when compared with samples absorbed on other types of colletion papers. It is likely that the silica gel coated paper worked as a desiccant to control the moisture content as well as the water activity in dried blood spots. Studies have shown that lipid oxidation rates are reduced with decreasing water activity on the surface of lipids (Karel 1980; Stapelfeldt *et al.* 1997), therefore, silica gel coated paper may diminish the lipid oxidation rate by reducing the water activity close to the water monolayer on the surface of DBS (Velasco *et al.* 2003).

In conclution, I have developed a three components protection system (BHT, iron chelator and silica gel coated paper), I have called "PUFAcoat", which is capable of stabilising the LCPUFA in human DBS samples for extended periods when stored at

room temperature. The development of this system was based on a complete understanding of the processes causing the breakdown of fatty acids. Overcoming this breakdown by chemically impregnating the collection matrix with a mixture of an antioxidant and an iron chelator meant that the n-3 LCPUFA content was retained in the DBS even after 9 weeks of storage at room temperature.

4.4.3 Further investigations on "PUFAcoat" system

4.4.3.1 Effect of desiccant and oxygen absorber

This experiment was aimed to test the influence of a desiccant and an oxygen absorber on the stability of LCPUFA in DBS protected by "PUFAcoat" system. Blood spots were obtained by absorbing 50µl of fresh blood onto the collection area (~1.5x1.5cm) of Whatman ion exchange paper strips in the presence of 2mg/ml BHT and 5mg/ml EDTA. After being air dried at room temperature for 5 hrs, DBS samples were stored at room temperature in cellophane bags in the dark in the absence or presence of a desiccant and an oxygen absorber (Table 4.27), and analysed by GC as describe in Chapter 2 to determine fatty acid composition after either 1 week, 2 weeks or 4 weeks of storage.

There was no significant difference in fatty acid composition in DBS treated with "PUFAcoat" system over 4 weeks of storage at room temperature, irrespective of the presence or absence of desiccant or oxygen absorber (Figure 4.7, Table 4.27-4.29).

Table 4.27. Fatty acid composition of DBS in the absence or presence of a desiccant and an oxygen absorber determined after 1 week of storage at room temperature

Fatty acids[1]	**Original Content[2]**	**Nil**	**Nil**	**Desiccant**	**Desiccant**
		Nil	**oxygen absorber**	**Nil**	**oxygen absorber**
16:0	22.4±0.3	22.6±0.3	22.6±0.3	22.5±0.3	22.6±0.3
18:0	13.1±0.2	13.3±0.1	13.3±0.1	13.3±0.2	13.2±0.2
18:1 n-9	18.5±0.2	18.5±0.2	18.5±0.2	18.5±0.2	18.5±0.2
18:2 n-6	19.7±0.1	19.8±0.1	19.8±0.1	19.8±0.1	19.8±0.1
18:3 n-3	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.9±0.1	7.8±0.1	7.8±0.1	7.8±0.1	7.8±0.1
20:5 n-3	6.7±0.1	6.6±0.1	6.6±0.1	6.5±0.1	6.6±0.1
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.02
22:5 n-3	3.7±0.04	3.6±0.04	3.6±0.06	3.7±0.05	3.6±0.05
22:6 n-3	7.1±0.1	6.9±0.1	6.9±0.1	7.0±0.1	7.0±0.1

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0

Table 4.28. Fatty acid composition of DBS in the absence or presence of a desiccant and an oxygen absorber determined after 2 weeks of storage at room temperature

Fatty acids[1]	**Original Content[2]**	**Nil**	**Nil**	**Desiccant**	**Desiccant**
		Nil	**oxygen absorber**	**Nil**	**Oxygen absorber**
16:0	22.4±0.3	22.8±0.3	22.7±0.3	22.7±0.3	22.7±0.3
18:0	13.1±0.2	13.4±0.2	13.4±0.2	13.3±0.1	13.3±0.1
18:1 n-9	18.5±0.2	18.6±0.2	18.7±0.2	18.7±0.1	18.7±0.2
18:2 n-6	19.7±0.1	19.6±0.1	19.6±0.1	19.6±0.2	19.7±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02
20:4 n-6	7.9±0.1	7.7±0.1	7.7±0.1	7.7±0.1	7.7±0.1
20:5 n-3	6.7±0.1	6.5±0.1	6.5±0.1	6.5±0.1	6.5±0.1
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.04	3.6±0.05	3.6±0.06	3.6±0.05	3.6±0.03
22:6 n-3	7.1±0.1	6.9±0.1	6.9±0.1	6.9±0.1	6.9±0.1

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0

Table 4.29. Fatty acid composition of DBS in the absence or presence of a desiccant and an oxygen absorber determined after 4 weeks of storage at room temperature

Fatty acids[1]	Original Content[2]	Nil	Nil	Desiccant	Desiccant
		Nil	oxygen absorber	Nil	Oxygen absorber
16:0	22.4±0.3	22.8±0.3	22.9±0.3	22.9±0.3	22.8±0.3
18:0	13.1±0.2	13.5±0.1	13.4±0.2	13.4±0.1	13.4±0.1
18:1 n-9	18.5±0.2	18.8±0.2	18.8±0.2	18.8±0.1	18.8±0.2
18:2 n-6	19.7±0.1	19.6±0.1	19.6±0.1	19.6±0.1	19.6±0.1
18:3 n-3	0.4±0.01	0.4±0.01	0.4±0.02	0.4±0.02	0.4±0.01
20:4 n-6	7.9±0.1	7.7±0.1	7.6±0.1	7.6±0.1	7.6±0.1
20:5 n-3	6.7±0.1	6.4±0.1	6.4±0.1	6.4±0.1	6.4±0.1
22:4 n-6	0.5±0.01	0.5±0.01	0.5±0.02	0.5±0.01	0.5±0.01
22:5 n-3	3.7±0.04	3.6±0.05	3.7±0.03	3.7±0.05	3.6±0.02
22:6 n-3	7.1±0.1	6.8±0.1	6.8±0.1	6.8±0.1	6.8±0.1

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0

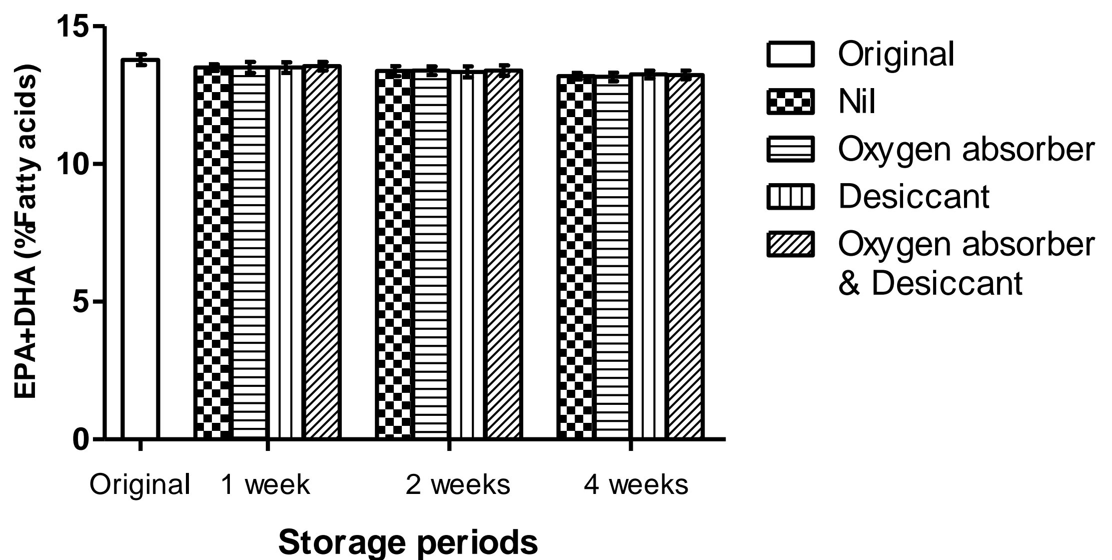


Figure 4.7. EPA+DHA% of DBS in the absence or presence of desiccant and oxygen absorber determined over 4 weeks of storage at room temperature. Values are presented as mean ± SD (n=3), no significant difference were found between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood.

4.4.3.2 Effect of storage temperature

The objective of this experiment was to test the influence of storage temperature on the stability of LCPUFA in DBS protected by "PUFAcoat" system. Blood spots were obtained by absorbing 50μl of fresh blood onto the collection area (~1.5x1.5cm) of Whatman ion exchange paper strips in the presence of 2mg/ml BHT and 5mg/ml EDTA. After being air dried at room temperature for 5 hrs, DBS samples were analysed by GC as described in Chapter 2 to determine fatty acid composition either immediately after air drying, or after 1 week, 2 weeks or 4 weeks of storage at four different temperatures (-20°C, 4°C, room temperature, or 40°C) in cellophane bags in the dark and in the presence of a desiccant (Table 4.30).

There was no significant differences in fatty acid composition among the DBS samples stored at -20°C, 4°C, and room temperature over 4 weeks of storage (Figure 4.8). Although the DBS samples stored at -20°C showed a relatively higher residual EPA percentage over 4 weeks of storage when compared with those DBS samples stored at 4°C or room temperature, the difference was not statistically significant (Table 4.30). However, for those DBS samples stored at 40°C, significant reductions in both EPA and DHA was seen as early as 2 weeks after collection and continued to decline over the 4 weeks storage period (Figure 4.8). There was a ~20% loss in the percentage of EPA and DHA measured in the DBS which were stored at 40°C after 4 weeks (Table 4.30).

Table 4.30. Fatty acid composition of DBS stored at different temperatures over 4 weeks

Fatty acids[1]	Original Content[2]	After drying (5 hrs)	–20°C		4°C		Room Temperature (20-25°C)		40°C	
			2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
16:0	22.5±0.2[a]	22.6±0.1[a]	22.6±0.2[a]	22.7±0.2[a]	22.7±0.2[a]	22.8±0.2[a]	22.8±0.3[a]	22.9±0.2[a]	23.7±0.2[b]	24.8±0.2[c]
18:0	13.1±0.2[a]	13.2±0.2[a]	13.3±0.2[a]	13.4±0.1[a]	13.3±0.1[a]	13.4±0.1[a]	13.3±0.1[a]	13.4±0.2[a]	13.7±0.1[ab]	14.1±0.1[b]
18:1 n-9	18.5±0.1[a]	18.5±0.1[a]	18.5±0.2[a]	18.8±0.1[a]	18.7±0.1[a]	18.9±0.1[ab]	18.6±0.1[a]	18.9±0.1[ab]	19.0±0.1[ab]	19.3±0.1[b]
18:2 n-6	19.7±0.1[a]	19.6±0.1[a]	19.6±0.1[a]	19.6±0.1[a]	19.6±0.1[a]	19.6±0.1[a]	19.7±0.2[a]	19.5±0.1[a]	19.1±0.1[ab]	18.7±0.1[b]
18:3 n-3	0.4±0.01[a]	0.4±0.01[a]	0.4±0.02[a]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.01[ab]	0.4±0.01[a]	0.4±0.01[a]	0.4±0.02[a]	0.3±0.01[b]
20:4 n-6	7.8±0.1[a]	7.8±0.1[a]	7.7±0.1[a]	7.6±0.1[a]	7.6±0.1[a]	7.5±0.1[ab]	7.7±0.1[a]	7.5±0.1[ab]	7.5±0.1[ab]	7.1±0.1[b]
20:5 n-3	6.7±0.1[a]	6.7±0.1[a]	6.7±0.1[a]	6.6±0.1[a]	6.6±0.1[a]	6.4±0.1[ab]	6.5±0.1[a]	6.4±0.1[ab]	6.0±0.1[bc]	5.6±0.1[c]
22:4 n-6	0.5±0.01[a]	0.5±0.02[a]	0.5±0.02[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.01[a]	0.5±0.02[a]	0.5±0.01[a]	0.5±0.01[a]	0.4±0.01[b]
22:5 n-3	3.7±0.03[a]	3.6±0.02[a]	3.7±0.06[a]	3.6±0.04[a]	3.7±0.02[a]	3.6±0.05[a]	3.6±0.05[a]	3.6±0.05[a]	3.4±0.06[a]	3.3±0.05[b]
22:6 n-3	7.1±0.1[a]	7.0±0.1[a]	7.0±0.1[a]	6.9±0.1[a]	6.9±0.1[a]	6.8±0.1[a]	6.9±0.1[a]	6.8±0.1[a]	6.4±0.1[b]	6.0±0.1[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

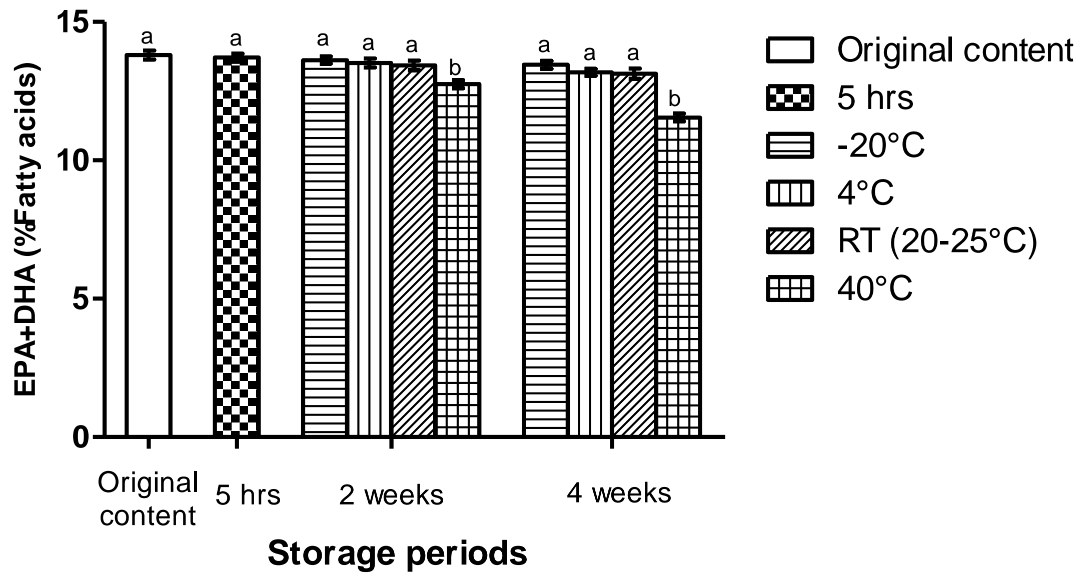


Figure 4.8. EPA+DHA% of DBS stored at different temperatures over 4 weeks. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

4.4.3.3 The effect of storage on "PUFAcoat" collection papers

The objective of this experiment was to evaluate the shelf-life of pre-made "PUFAcoat" collection papers. Two groups of Whatman ion exchange paper strips were treated with a mixture of 2mg/ml BHT and 5mg/ml EDTA, and stored for either 1 month or 2 months prior to use. 50μl of fresh blood was spotted onto the collection area (~1.5x1.5cm) of both stored and freshly made collection papers. After being air dried at room temperature for 5 hrs, DBS samples were analysed by GC as described in Chapter 2 to determine fatty acid composition either immediately after air drying, or after 2 weeks or 4 weeks of storage at room temperature in cellophane bags in the dark and in the presence of a desiccant.

The blood spotted on pre-made "PUFAcoat" papers prepared either 1 month or 2 months prior to use did not show significant difference fatty acid composition when compared with same blood sample spotted on freshly made "PUFAcoat" papers over 4 weeks of storage at room temperature (Figure 4.9, Table 4.31).

Table 4.31. Fatty acid composition of DBS on freshly made or pre-made "PUFAcoat" papers stored at room temperature over 4 weeks

Fatty acids[1]	Original Content[2]	Fresh made papers			1 month old papers			2 month old papers		
		5hrs	2 weeks	4 weeks	5hrs	2 weeks	4 weeks	5hrs	2 weeks	4 weeks
16:0	24.7±0.2	24.8±0.1	24.9±0.1	25.2±0.2	24.9±0.1	24.9±0.2	25.3±0.2	24.8±0.2	25.0±0.2	25.2±0.2
18:0	13.1±0.1	13.1±0.1	13.2±0.1	13.5±0.2	13.2±0.1	13.2±0.2	13.5±0.1	13.2±0.1	13.3±0.2	13.6±0.2
18:1 n-9	19.3±0.1	19.2±0.2	19.7±0.1	19.9±0.2	19.2±0.1	19.6±0.1	19.9±0.2	19.3±0.2	19.7±0.1	19.8±0.1
18:2 n-6	18.8±0.1	18.7±0.1	18.5±0.1	18.3±0.1	18.7±0.1	18.5±0.1	18.4±0.1	18.7±0.1	18.6±0.2	18.3±0.1
18:3 n-3	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.01	0.4±0.02	0.4±0.01	0.4±0.01	0.4±0.01
20:4 n-6	7.2±0.1	7.2±0.1	7.2±0.03	7.1±0.1	7.2±0.1	7.1±0.1	7.1±0.1	7.2±0.1	7.2±0.1	7.1±0.1
20:5 n-3	6.3±0.1	6.3±0.1	6.2±0.1	6.0±0.1	6.3±0.1	6.1±0.1	6.1±0.1	6.3±0.1	6.1±0.1	6.1±0.1
22:4 n-6	0.6±0.03	0.6±0.01	0.5±0.02	0.5±0.02	0.6±0.01	0.5±0.02	0.6±0.01	0.6±0.01	0.6±0.01	0.6±0.02
22:5 n-3	3.0±0.03	2.9±0.03	2.9±0.03	2.9±0.05	3.0±0.02	3.0±0.02	2.9±0.04	3.0±0.03	3.0±0.04	2.9±0.06
22:6 n-3	6.6±0.1	6.6±0.1	6.5±0.1	6.4±0.1	6.5±0.1	6.5±0.1	6.3±0.1	6.6±0.1	6.4±0.1	6.3±0.1

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $P<0.01$.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh blood at time 0.

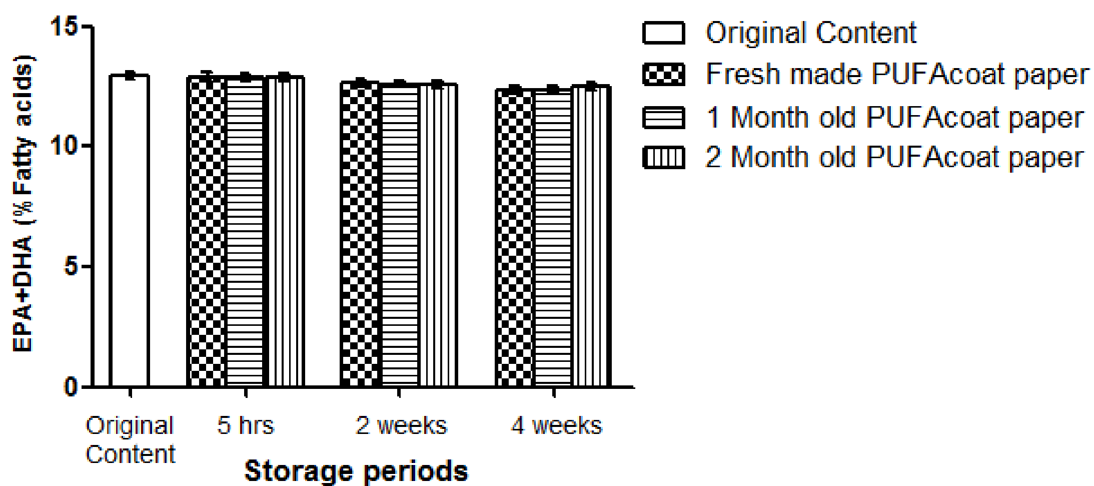


Figure 4.9. Fatty acid composition of DBS on freshly made or pre-made "PUFAcoat" collection papers stored at room temperature over 4 weeks. Values are presented as mean ± SD (n=3), no significant differences were found between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh blood at time 0.

4.4.3.4 Stability of other bio-fluids spots collected on "PUFAcoat" paper

This experiment was aimed to compare the stability of LCPUFA in plasma spots and breast milk spots collected using "PUFAcoat" collection paper with those collected using Fluka collection kit. Ethical approval for the collection of body fluids for this study was obtained from The University of Adelaide Human Research Ethics Committee. Plasma and breast milk were collected from volunteers, and spotted onto two blood collection systems: the Fluka collection kit (Fluka paper + 0.5mg/ml BHT) and the "PUFAcoat" paper (Whatman ion exchange paper + 2mg/ml BHT+ 5mg/ml EDTA). After being air dried at room temperature for 5 hrs, plasma spots and breast milk spots were processed in methylating agent, and analysed by GC following the same procedure as described for DBS in Chapter 2 to determine fatty acid composition either immediately after air drying, or after 1 week, 2 weeks or 4 weeks of storage at room temperature in cellophane bags in the dark and in the presence of a desiccant (Table 4.32, 4.33).

The plasma spots collected on BHT (0.5mg/ml) pre-treated Fluka paper did not show significant loss of any LCPUFA after 1 week storage at room temperature (Figure 4.10). However, significant losses of EPA and DHA were observed after 2 weeks of storage at room temperature (Figure 4.10), and after 4 weeks of storage there was a ~20% loss of all n-3 LCPUFA content (expressed as a weight percentage of total blood fatty acids) in the plasma spotted on BHT pre-treated Fluka paper (Table 4.32). However, there was no significant change in the fatty acid composition of plasma spots collected using the "PUFAcoat" paper over 4 weeks of storage at room temperature (Table 4.32).

In relation to the breast milk spots, irrespective of the protection system used, there was no significant difference in fatty acid composition in dried breast milk spots over 4 weeks of storage at room temperature (Figure 4.11, Table 4.33).

Table 4.32. Fatty acid composition of plasma spots on Fluka collection kit or "PUFAcoat" paper stored at room temperature over 4 weeks

Fatty acids[1]	Original Content[2]	"PUFAcoat" paper				Fluka collection kit			
		5 hrs	1 week	2 weeks	4 weeks	5 hrs	1 week	2 weeks	4 weeks
16:0	21.3±0.4[a]	21.3±0.3[a]	21.4±0.4[a]	21.2±0.4[a]	21.4±0.3[a]	21.3±0.2[a]	21.5±0.3[a]	22.1±0.2[ab]	22.4±0.2[b]
18:0	5.8±0.2[a]	5.8±0.1[a]	5.9±0.2[a]	5.9±0.1[a]	6.0±0.3[ab]	5.9±0.1[a]	6.0±0.1[ab]	6.3±0.1[ab]	6.5±0.1[b]
18:1 n-9	27.7±0.2[a]	27.8±0.1[a]	27.6±0.2[a]	27.9±0.4[a]	27.9±0.3[a]	27.6±0.2[a]	28.0±0.3[ab]	28.3±0.2[ab]	28.6±0.2[b]
18:2 n-6	32.4±0.3[a]	32.4±0.2[a]	32.2±0.2[a]	32.2±0.3[a]	31.8±0.3[ab]	32.4±0.2[a]	31.9±0.2[ab]	31.7±0.2[ab]	31.4±0.2[b]
18:3 n-3	0.3±0.02	0.3±0.01	0.3±0.02	0.3±0.02	0.3±0.02	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.01
20:4 n-6	6.9±0.1[a]	6.8±0.1[a]	6.7±0.1[a]	6.7±0.1[a]	6.6±0.1[a]	6.8±0.1[a]	6.7±0.1[a]	6.5±0.1[ab]	6.2±0.1[b]
20:5 n-3	1.5±0.02[a]	1.5±0.01[a]	1.5±0.02[a]	1.5±0.05[a]	1.4±0.04[a]	1.5±0.03[a]	1.4±0.04[a]	1.3±0.02[b]	1.2±0.03[b]
22:4 n-6	0.6±0.02	0.6±0.01	0.6±0.01	0.6±0.02	0.6±0.02	0.6±0.02	0.6±0.02	0.5±0.02	0.5±0.02
22:5 n-3	0.8±0.03	0.8±0.01	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.02	0.8±0.03	0.7±0.02
22:6 n-3	3.0±0.1[a]	3.0±0.1[a]	3.0±0.1[a]	3.0±0.1[a]	2.9±0.1[a]	3.0±0.1[a]	2.8±0.1[ab]	2.6±0.1[bc]	2.3±0.1[c]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01.

[2]The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh plasma at time 0.

Table 4.33. Fatty acid composition of breast milk spots on Fluka collection kit or “PUFAcoat” paper stored at room temperature over 4 weeks

Fatty acids[1]	**Original Content**[2]	**“PUFAcoat” paper**				**Fluka collection kit**			
		5 hrs	**1 week**	**2 weeks**	**4 weeks**	**5 hrs**	**1 week**	**2 weeks**	**4 weeks**
16:0	30.2±0.2	30.2±0.2	30.2±0.2	30.3±0.1	30.3±0.1	30.4±0.2	30.4±0.1	30.5±0.2	30.5±0.2
18:0	8.1±0.1	8.2±0.1	8.1±0.1	8.2±0.1	8.1±0.1	8.2±0.1	8.2±0.1	8.3±0.1	8.3±0.1
18:1 n-9	43.1±0.3	43.3±0.2	43.3±0.1	43.4±0.2	43.4±0.1	43.3±0.2	43.3±0.2	43.4±0.1	43.3±0.1
18:2 n-6	15.1±0.1	14.9±0.1	14.9±0.1	14.7±0.1	14.8±0.1	14.9±0.1	14.8±0.1	14.7±0.1	14.6±0.1
18:3 n-3	2.1±0.1	2.1±0.02	2.1±0.03	2.0±0.04	2.1±0.01	2.0±0.03	2.0±0.02	2.0±0.03	1.9±0.04
20:3 n-9	0.09±0.00	0.09±0.00	0.09±0.00	0.09±0.00	0.08±0.00	0.09±0.00	0.08±0.00	0.08±0.00	0.08±0.00
20:4 n-6	0.56±0.01	0.54±0.01	0.54±0.01	0.53±0.02	0.54±0.01	0.54±0.01	0.52±0.01	0.52±0.01	0.50±0.01
20:5 n-3	0.11±0.01	0.11±0.01	0.10±0.01	0.10±0.01	0.11±0.01	0.10±0.01	0.10±0.01	0.09±0.01	0.09±0.01
22:4 n-6	0.1±0.00	0.1±0.00	0.1±0.01	0.1±0.00	0.1±0.01	0.1±0.00	0.1±0.01	0.1±0.00	0.1±0.00
22:5 n-3	0.22±0.01	0.22±0.01	0.22±0.02	0.23±0.01	0.22±0.01	0.23±0.01	0.22±0.01	0.21±0.01	0.20±0.02
22:6 n-3	0.31±0.02	0.3±0.02	0.31±0.02	0.30±0.01	0.30±0.01	0.30±0.01	0.29±0.01	0.29±0.01	0.28±0.01

[1]Values are presented as mean ± SD (n=3), no significant differences were found between treatments for any of the fatty acids, $P<0.01$.

[2]The “Original Content” refers to the fatty acid composition obtained from direct transmethylation of 50 μl fresh breast milk at time 0.

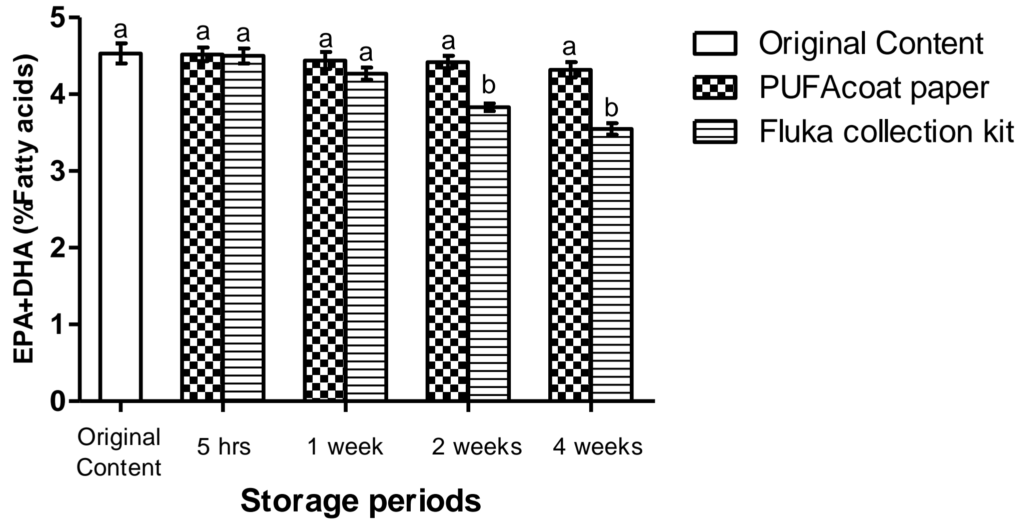


Figure 4.10. Fatty acid composition of plasma spots on Fluka collection kit or "PUFAcoat" paper stored at room temperature over 4 weeks. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh plasma at time 0.

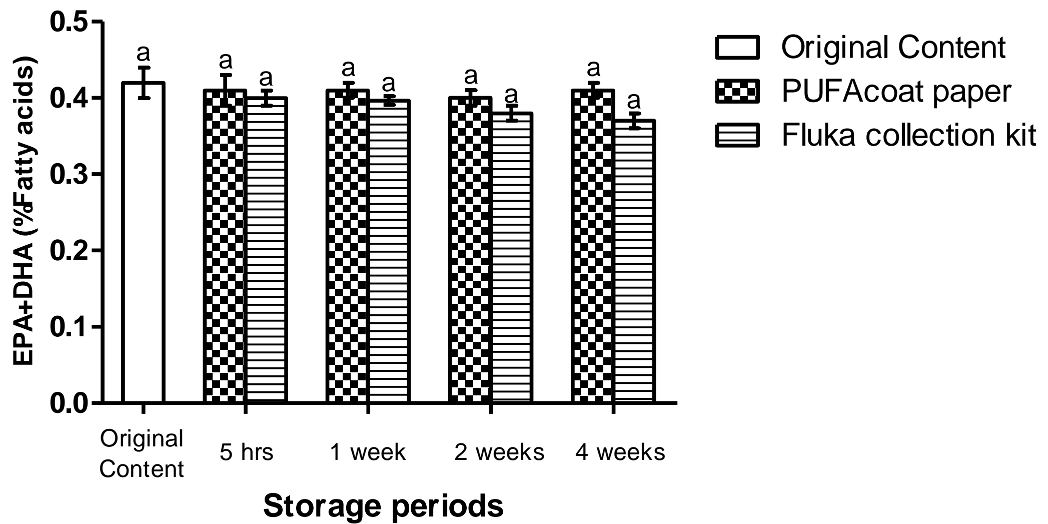


Figure 4.11. Fatty acid composition of breast milk spots on Fluka collection kit or "PUFAcoat" paper stored at room temperature over 4 weeks. Values are presented as mean ± SD (n=3), no significant differences were found between treatments, $P<0.01$. The "Original Content" refers to the fatty acid composition obtained from direct transmethylation of 50 µl fresh breast milk at time 0.

In general, physical conditions like moisture, oxygen and temperature can act independently or synergistically to cause oxidative losses of lipids (Kanner and Rosenthal 1992; Min and Ahn 2005). Moisture content increases may harm the fatty acids by either favouring the growth of micro-organisms (McInerney *et al.* 1979) or promoting lipid oxidation by increasing water activity (Labuza *et al.* 1971). This suggests that it may be advisable to use desiccant with DBS samples to minimise the moisture level (Marangoni *et al.* 2004; Metherel *et al.* 2013). Spontaneous reaction of atmospheric oxygen with lipids is also a common process leading to oxidative degradation of lipids during storage (Gordon *et al.* 2001). The use of an oxygen absorber has previously been reported to protect crackers and tofu against lipid oxidation (Berenzon and Saguy 1998; Yoko *et al.* 1999). However, data from the present study did not show any significant difference in fatty acid composition among DBS samples collected using the "PUFAcoat" paper during 4 weeks of storage at room temperature, irrespective of the presence of a desiccant or an oxygen absorber (Figure 6). This indicates that the use of either desiccant or oxygen absorber may not be necessary for DBS samples collected on the "PUFAcoat" paper. The "PUFAcoat" paper is highly efficient as an antioxidant system, because it combines an antioxidant, an ion chelator and silica gel coated paper. In this system, the antioxidant inhibits the lipid oxidation by blocking the propagation of free radicals (Frankel *et al.* 1994). The ion chelator inhibits the initiation of free radical production through binding of iron. In addition, the silica gel coated paper itself might work as a desiccant to control the moisture content as well as water activity in the DBS (Velasco *et al.* 2003). However, it is important to note that the current experiment was conducted in an environment where the relative humidity was low (~40%), and all the DBS samples were tested after only 1 month of storage. Therefore, further studies need to be conducted in

higher relative humidity environments, and with more prolonged storage periods to determine if the “PUFAcoat” system is effective in the absence of desiccant and oxygen absorber under these conditions.

The stability of LCPUFA in DBS was poor when the samples were stored at a high temperature (40°C) even they were proteced by the “PUFAcoat” system. This is likely to be because high temperature reduces the activation energy required for oxidation, and breakdown of preformed hydroperoxide into free radicals, which promotes lipid oxidation processes (Kanner 1994).

Commercial blood collection products, for example, the Fluka blood collection kit, generally require people to put few drops of 0.5mg/ml BHT solution onto the collection paper and leave it to dry prior to blood collection. However, this procedure is time consuming, cumbersome and subject to user error, especially when a large number of blood spot samples are going to be collected. This is because BHT solution is colourless, and there is no way to visually distinguish between papers that have been pre-treated and those which haven’t. A possible solution could be pre-treat the blood collection paper with protectant during the manufacture of the collection card, since BHT and EDTA are stable at room temperature for at least 1 year (Babich 1982; Dawson *et al*. 1986). From my experimental data, the “PUFAcoat” papers that had been made 2 months prior to being used for DBS collection had the same efficiency at stabilising LCPUFA as the freshly made ones. Although the present study only investigated the shelf-life of “PUFAcoat” paper for 2 months, it supports the potential for manufacturing pre-made “PUFAcoat” collection papers for subsequent distribution.

The plasma levels of individual fatty acids is considered as an important biomarker for measuring the fatty acid status in the diet, and has been used extensively since the 1950s (Bronte-Stewart *et al.* 1956; Ahrens *et al.* 1957). The importance of the fatty acid composition of human breast milk for an infant's growth and development has prompted extensive research into the determinants of breast milk n-3 LCPUFA, and how the n-3 LCPUFA concentration in breast milk varies across the populations (Koletzko *et al.* 1988). The present study also compared the stability of LCPUFA in plasma and in breast milk collected on "PUFAcoat" paper with those collected using Fluka blood collection kit. The plasma spots collected on Fluka collection paper were stable over the first week of storage at room temperature, however, showed a ~20% loss of n-3 LCPUFA after 4 weeks of storage, whereas the breast milk spots collected on Fluka collection paper were stable over 4 weeks of storage at room temperature. This is likely to be because neither plasma (< 2mg/L) (Huebers *et al.* 1987) nor breast milk (~0.35mg/L) (Suitor & Meyers, 2006) contains significant levels of iron, and thus the iron accelerated oxidative loss of LCPUFA observed in the DBS did not make a significant contribution to LCPUFA oxidation in plasma or breast milk. However, it is important to note that the LCPUFA in both plasma spots and breast milk spots collected by "PUFAcoat" paper remained stable over 4 weeks of storage at room temperature (Figures 10, 11). This indicates that the "PUFAcoat" paper might be suitable for protecting the LCPUFA in bio-fluids other than blood, such as plasma, breast milk and saliva.

In addition, the "PUFAcoat" system may also has the potential to protect other components in blood and tissue samples such as deoxyribonucleic acid (DNA) from degradation, because free radicals induce the strand cleavage of DNA (Breen *et al.*

1995) and previous studies have shown that chelating agents reduce DNA fragmentation by inhibiting the production of iron derived free radicals (Campo *et al.* 2004).

4.5 Summary

In conclusion, the results of this study indicate that a protection system that combines BHT, an iron chelator, and silica gel coated paper can prevent LCPUFA in DBS from significant oxidative loss for at least 2 months when stored at room temperature. This method allowed accurate evaluation of fatty acid status in DBS samples after long term storage at room temperature, and thus has the potential to enable comparisons between samples which have been stored for different periods of time. This is the first report of a system capable of stabilising LCPUFA in human blood samples when stored at room temperature, and the method has significant future potential in clinical research and diagnostic applications.

Chapter 5

Development of an *in vitro* Model of Blood Lipids for Studying the Oxidative Role of Iron in DBS

5.1 Introduction

Iron is a pro-oxidant which has potential to induce considerable oxidative degradation of lipids. This is because the ionic form of iron has the capability to catalyse lipid autoxidation either by directly initiating the formation of lipid radicals, or by inducing the production of highly reactive hydroxyl radicals through the Fenton reaction (Halliwell and Gutteridge 1997). In Chapter 4, I have shown that a combination of an antioxidant and an iron chelator provided better protection against LCPUFA oxidation in DBS than an antioxidant alone. I hypothesised that this was due to the iron chelator binding iron which was liberated from red blood cells, thereby preventing it from accelerating the autoxidation of LCPUFA degradation in DBS during storage. This hypothesis is supported by earlier studies which showed that in the long term storage of blood samples, red blood cell damage promoted the release of sequestered iron, and therefore enhanced the degradation of LCPUFA in blood (Chiu *et al.* 1989). This enhancement could be prevented by prior addition of an iron chelating compound (Chiu *et al.* 1989; Otto *et al.* 1997). However, these earlier studies were all based on liquid blood samples rather than DBS, therefore, there is a need to seek evidence that the same mechanism also operates in DBS.

In the previous Chapter, I used a blood sample with a relatively high content of EPA and DHA (both ~7%, expressed as a weight percentage of total fatty acids), in order to indentify the optimal method for preventing n-3 LCPUFA oxidation. However, the use

of blood sample for elucidating the mechanism of action is inappropriate, since it has limitations. Firstly, blood is not always available for collection from a donor with such a high content of n-3 LCPUFA in the blood. Secondly, even blood from the same person may show variations in LCPUFA content if it is collected at different time points. Such inconsistencies in LCPUFA content will make it difficult to compare the fatty acid status between samples. In addition, the components in blood other than iron, including specific proteins, enzymes and inorganic metals have the potential to catalyse the autoxidation of LCPUFA (Carlsen *et al.* 2004). Therefore, the use of whole blood in a mechanistic study makes it difficult to isolate the effect of iron as distinct from other components in DBS.

Thus, the main purpose of this study was to establish an *in vitro* model to directly test the hypothesis that the presence of iron in DBS can accelerate the degradation of LCPUFA, and to determine the effect of chelating agents in this process. The *in vitro* model also provides a system for rapidly screening the iron chelating efficiency of a range of chemicals that could potentially inhibit that acceleration by chelation of iron.

5.2 Design of the study

A lipid solution that contained a constant level of fatty acids was developed to mimic the fatty acid composition (especially the content of EPA and DHA) of the human blood used in the previous experiments in Chapter 4. A drop of the lipid solution was absorbed onto a piece of blood collection paper to make a lipid spot. Accelerated autoxidation of the LCPUFA in the lipid spot was achieved by exposing the spots to air in the presence of fixed levels of iron. This *in vitro* model was utilised to

investigate the role of iron and iron chelators in LCPUFA autoxidation and to assess the chelating properties of three different iron chelators.

5.3 Materials and Methods

5.3.1 Lipid solution preparation

The lipid solution comprised of a blend of fatty acid sources that mimicked the fatty acid composition in blood lipids in Chapter 4, and could be easily spotted on to papers to evaluate the effects of added iron compounds on autoxidation of LCPUFA and their interactions with chelating agents.

5.3.1.1 Lipid sources

Five kinds of oils were used as lipid sources. They were Omega brain fish oil (BlackMores, Sydney, Australia), Omega joint fish oil (BlackMores, Sydney, Australia), Eco krill oil (BlackMores, Sydney, Australia), Rice bran oil (King, Thailand) and Peanut oil (International collection, Hull, UK). These five oils were selected because the two kinds of fish oils provided triglyceride sources of EPA and DHA, while krill oil provided a phospholipid source of EPA and DHA. Rice bran oil and peanut oil provided SFA, oleic acid and linoleic acid.

5.3.1.2 Preparation of lipid solution

20μl of rice bran oil, 20μl of peanut oil, 5μl of Omega brain fish oil, 5μl of Omega joint fish oil and 5μl of Eco Krill oil was mixed together in a 20ml vial (Wheaton, Millville, USA), and the resultant mixture was dissolved in 20ml 100% ethanol (Chem-supply, Gillman, Australia) to make a lipid solution. 50 μl of this solution was

directly transesterified in 2ml of methanol containing 1% H_2SO_4 (v/v), and the resultant FAME were analysed by GC as described in Chapter 2. The fatty acid composition of the resulting lipid solution roughly matched the whole blood that I tested in Chapter 4 (Figure 5.1). The levels of SFA in the current lipid solution were lower than the whole blood that I tested in Chapter 4, while Oleic acid and Linoleic acid were relatively higher. However, a high level of the major fatty acids of interest, including the EPA and DHA was observed in the lipid solution, and these levels were similar to the whole blood which was used in Chapter 4 (Table 5.1). The lipid solution was stored at -80°C before use.

5.3.2 Collection matrix

Fluka blood collection paper (Sigma-Aldrich, Buchs, Switzerland) was cut into 1.5cm x 4.5cm paper strips and used as collection matrix for the lipid solution. I chose Fluka paper instead of silica gel coated paper (Whatman ion exchange paper) is because silica gel has certain ability in binding heavy metal (Tzvetkova and Nickolov 2012) which may make it difficult to isolate the effect of added iron chelators as distinct from silica gel.

5.3.3 Iron and iron chelator solutions

5.3.3.1 Total Iron solutions

A mixture of ferrous sulphate (British Drug House, London, UK) and ferric chloride (anhydrous, Chem-supply, Gillman, Australia) at a molar ratio of 1:1 was used as iron source for studies. The total iron (ferrous + ferric) concentrations used ranged from 0.625mM to 10mM.

5.3.3.2 Iron chelator solutions

EDTA (>99%, Sigma-Aldrich, St Louis, USA), deferoxamine mesylate salt (≥92.5%, Sigma-Aldrich, St Louis, USA) and baicalein (Sigma-Aldrich, St Louis, USA) were used as iron chelators for these studies. EDTA is a universal iron chelator which is widely used in industry, laboratory and medical practice (Knudtson *et al.* 2002). Deferoxamine is an iron chelator which has been widely used in the medical treatment of iron overload for decades (Olivieri and Brittenham 1997). Baicalein is a natural flavonoid, which has been reported to exhibit strong iron chelating properties in some previous studies (Perez *et al.* 2009; Melidou *et al.* 2005). The molar concentration of all iron chelators used in this study was 12.5mM which is as same as I used to protect the LCPUFA in DBS in Chapter 4.

5.3.4 Evaluation of the rate of autoxidation of LCPUFA

50μl of the lipid solution was spotted onto the collection area (1.5x1.5cm) of the Fluka paper strip. To evaluate the rate of autoxidation of LCPUFA in the spot, all spots were divided into four groups. The first group was immediately transesterified and tested by GC (as described in Chapter 2) to provide a baseline measurement for the fatty acid composition in the lipid spots. The remaining three groups of spots were exposed to air at room temperature and tested after either 6 hrs, 12 hrs or 24 hrs. The contaminant peaks from Fluka paper were substrated from the GC peaks of lipid spot to minimise the impact of contaminants from paper on the analytical results of the lipid spots. All samples were processed in triplicate.

5.3.5 Investigation of the role of iron and iron chelator

Based on an earlier report (Mladěnka *et al.* 2011), a molar ratio of chelator to total

iron (ferric + ferrous) no less than 10:1 gave an ideal chelation effect. Since the concentration of iron chelators I used in this study was 12.5mM, a total iron concentration of 0.625mM (20:1, chelator to total iron), 1.25mM (10:1, chelator to total iron) and 2.5mM (5:1, chelator to total iron) were tested in this experiment.

50μl of 12.5mM EDTA solution was spread evenly over the collection area of Fluka paper and air dried prior to loading the lipid solution. 50μl of the lipid solution and the same volume of total iron solution at four different concentrations (0mM, 0.625mM, 1.25mM and 2.5mM) were spotted onto the collection area (1.5x1.5cm) of the collection paper in the absence or presence of EDTA (Table 5.2). All samples were exposed to air at room temperature and test for fatty acid composition as described in Chapter 2 after either 6hrs, 12hrs or 24hrs. The contaminant peaks from Fluka paper were substrated from the GC peaks of lipid spot to minimise the impact of contaminants from paper on the analytical results of the lipid spots. All samples were processed in triplicate.

5.3.6 Comparison of different iron chelators

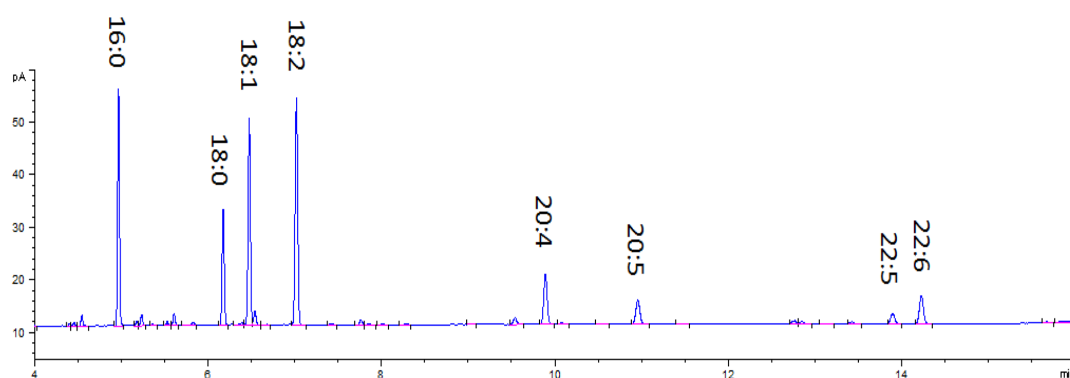
Three iron chelators were compared in this study (EDTA, deferoxamine and baicalein). 50μl of each iron chelator solution at a concentration of 12.5mM was spread evenly over the collection area (1.5x1.5cm) of the collection paper respectively, and air dried prior to loading the lipid solution. 50μl of the lipid solution and the same volume of total iron solution at five different concentrations (0mM, 1.25mM, 2.5mM, 5mM and 10mM) were spread evenly over the collection area of the collection paper in the absence or presence of 50μl of 12.5mM iron chelator solution (Table 5.3). All samples were exposed to air at room temperature and test for fatty acid composition as

described in Chapter 2 after either 1 day, 3 days or 7 days. The contaminant peaks from Fluka paper were substrated from the GC peaks of lipid spot to minimise the impact of contaminants from paper on the analytical results of the lipid spots. All samples were processed in triplicate.

5.3.7 Statistical analyses

All statistics analyses were conducted using PASW Statistic 18. Values are expressed as mean ± SD. One-way ANOVA and Tukey's post-hoc test were used to determine the significant differences in fatty acids percentage between groups, $P<0.01$ was chosen as the level of statistical significance in all analysis.

A. Whole blood



B. The lipid solution

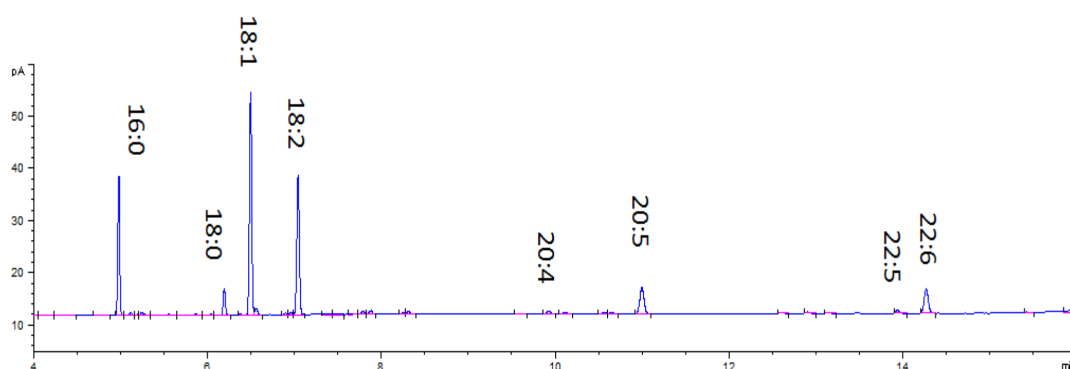


Figure 5.1. GC chromatographs of the whole blood (A) and the lipid solution (B) with peaks indentified as fatty acids.

Table 5.1. Comparison of the fatty acid composition of the whole blood and the lipid solution

Fatty acids[1]	Whole Blood	Lipid Solution
16:0	24.3±0.2	19.5±0.1
18:0	20.9±0.2	3.9±0.1
18:1 n-9	18.9±0.1	32.5±0.3
18:2 n-6	19.6±0.2	26.6±0.1
20:4 n-6	7.5±0.1	0.6±0.01
20:5 n-3(EPA)	5.9±0.1	7.7±0.1
22:5 n-3	3.3±0.1	1.4±0.1
22:6 n-3(DHA)	6.5±0.2	6.8±0.1

[1]Values are presented as mean ± SD (n=3)

Table 5.2. Investigation of the role of iron and iron chelator in the lipid spots over 24 hrs of air exposure at room temperature

Treatment No.	Iron (Fe^{2+} + Fe^{3+})	Iron chelator
1	0mM	Nil
2	0.625mM	
3	1.25mM	
4	2.5mM	
5	0mM	12.5mM EDTA
6	0.625mM	
7	1.25mM	
8	2.5mM	

Table 5.3. Comparison of the chelating properties of iron chelators in the lipid spots over 7 days of air exposure at room temperature

Treatment No.	Iron (Fe^{2+} + Fe^{3+})	Iron chelator
1	0mM	Nil
2	1.25mM	
3	2.5mM	
4	5mM	
5	10mM	
6	0mM	12.5mM EDTA
7	1.25mM	
8	2.5mM	
9	5mM	
10	10mM	
11	0mM	12.5mM Deferoxamine
12	1.25mM	
13	2.5mM	
14	5mM	
15	10mM	
16	0mM	12.5mM Baicalein
17	1.25mM	
18	2.5mM	
19	5mM	
20	10mM	

5.4 Results

5.4.1 The autoxidation of LCPUFA in the lipid spots

The objective of this experiment was to evaluate the rate of autoxidation of LCPUFA in the lipid spots after exposure to air at room temperature. The lipid spots were tested for fatty acid composition either immediately, or after 6, 12, or 24 hrs of air exposure at room temperature.

When the lipid spots were exposed to air, a slight reduction of the content of EPA and DHA (expressed as a weight percentage of total fatty acids) was observed after 6 hrs and 12 hrs at room temperature, however, the losses were not statistically significant. When the exposure period was further extended to 24hrs, both EPA and DHA showed significant reduction when compared with the baseline fatty acid composition of the lipid spot (Table 5.4 and Figure 5.2).

The significant autoxidation of EPA and DHA after 24 hrs of air exposure established that 24 hrs of air exposure is appropriate for the purpose of studying the autoxidative loss of n-3 LCPUFA in lipid spots. The loss of LCPUFA during this period was moderate, and it meant that if additional loss occurred as a result of adding a catalyst such as iron, it could earsily be detected.

Table 5.4. Fatty acid composition in the lipid spots over 24 hrs of air exposure

Fatty acids[1]	Air exposure periods			
	0hr	6hrs	12hrs	24hrs
16:0	19.6 ±0.2[a]	20.0±0.2[ab]	20.4±0.2[b]	20.8±0.1[b]
18:0	3.9±0.1	3.9±0.1	3.9±0.1	4.2±0.1
18:1 n-9	32.8±0.2	32.6±0.1	32.7±0.2	32.5±0.3
18:2 n-6	26.7±0.2	26.8±0.2	26.4±0.3	26.0±0.2
20:4 n-6	0.6±0.03	0.6±0.02	0.6±0.06	0.6±0.05
20:5 n-3(EPA)	7.7±0.1[a]	7.5±0.2[ab]	7.2±0.2[ab]	7.0±0.1[b]
22:5 n-3	1.4±0.1	1.3±0.1	1.3±0.1	1.3±0.1
22:6 n-3(DHA)	6.9±0.1[a]	6.6±0.2[ab]	6.5±0.2[ab]	6.3±0.1[b]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between time points, $P<0.01$.

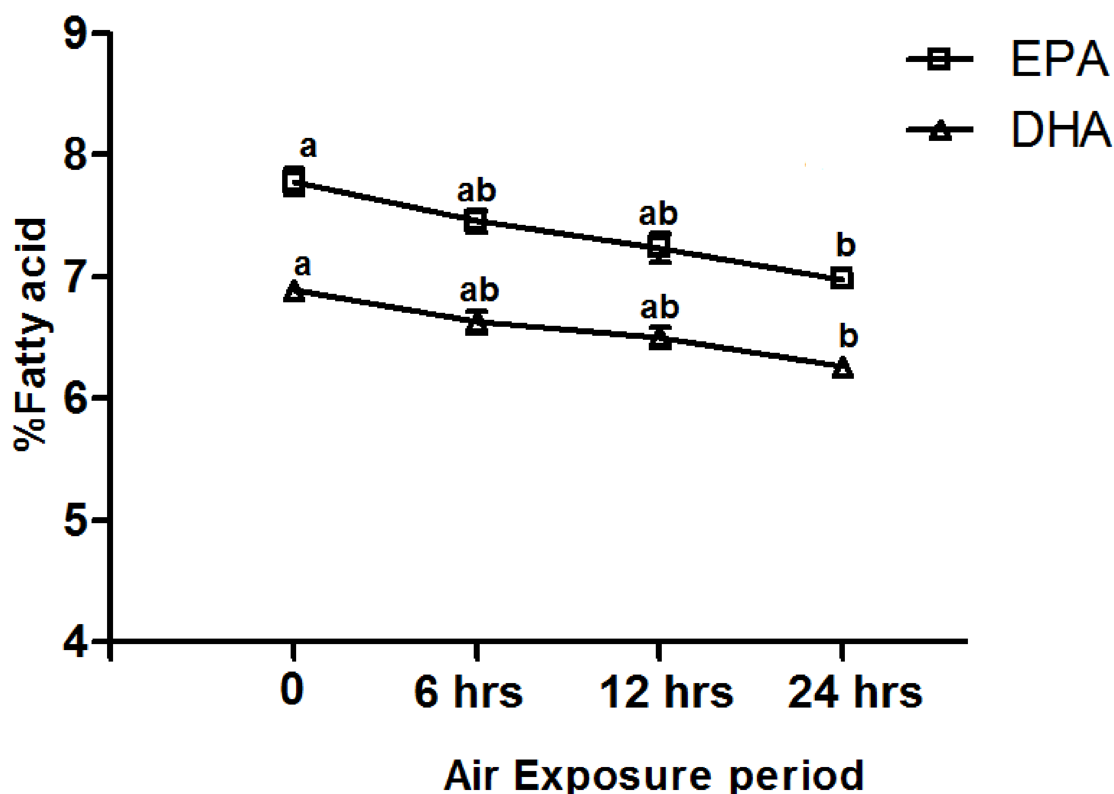


Figure 5.2. EPA and DHA contents in the lipid spots over 24 hrs of air exposure at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between time points, $P<0.01$.

5.4.2 The role of iron and iron chelator

The objective of this experiment was to validate the hypothesis that the presence of iron could accelerate the oxidative losses of LCPUFA in the lipid spots, and that the addition of an iron chelator would eliminate this effect. The lipid spots were tested at various iron concentrations in the presence or absence of EDTA (Table 5.2 in Method).

In the presence of iron but without EDTA, the lipid spots treated with the highest concentration of iron (2.5mM) showed significantly lower EPA and DHA levels after only 6 hrs of air exposure at room temperature when compared with the fatty acid composition of the spots in the control group after the same period of air exposure (0 EDTA, 0 iron) (Table 5.5, Figure 5.3a). However, after 12 hrs of exposure at room temperature, all iron intervention groups (0.625mM, 1.25mM, and 2.5mM) showed significant reductions of EPA and DHA contents when compared with the control group (Table 5.6, Figure 5.3b). Following 24 hrs of air exposure at room temperature, the levels of all LCPUFA in all iron intervention groups were significantly lower than the control group (Table 5.7, Figure 5.3c), and the reductions of EPA and DHA content in iron intervention groups increased with each increment of iron concentration (Figure 5.3). The lipid spots treated with 0.625mM iron showed an ~ 10% decline of EPA and DHA content when compared with the fatty acid composition of the control group, whereas the spots treated with 2.5mM iron showed an ~18% decline of EPA and DHA content (Table 5.7). However, the LCPUFA content of the lipid spots protected by 12.5mM EDTA did not have significantly different fatty acid profile to the control group regardless of both the iron concentrations with which they were treated and the exposure period (Tables 5.5-5.7, Figure 5.3).

Table 5.5. Fatty acid composition of the lipid spots following iron treatment in the presence or absence of chelating agent after 6 hrs of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions						
		0.625mM Iron	1.25mM Iron	2.5mM Iron	12.5mM EDTA	0.625 mM Iron & 12.5mM EDTA	1.25mM Iron & 12.5mM EDTA	2.5mM Iron & 12.5mM EDTA
16:0	20.1±0.2	20.5±0.3	20.6±0.3	20.7±0.3	19.7±0.2	20.1±0.3	20.3±0.1	20.3±0.2
18:0	4.0±0.1	4.0±0.3	4.0±0.3	4.2±0.2	4.0±0.1	4.1±0.1	4.0±0.2	4.1±0.2
18:1n-9	32.6±0.1	33.0±0.4	32.8±0.1	33.0±0.1	32.6±0.3	32.8±0.3	32.7±0.2	32.8±0.2
18:2n-6	26.9±0.2	26.7±0.2	26.7±0.2	26.1±0.2	27.0±0.3	26.6±0.6	26.8±0.2	26.9±0.2
20:4n-6	0.6±0.01	0.6±0.02	0.6±0.03	0.6±0.02	0.6±0.02	0.6±0.02	0.6±0.01	0.6±0.01
20:5n-3(EPA)	7.5±0.1^{a}	7.2±0.1^{ab}	7.0±0.1^{ab}	6.8±0.1^{b}	7.7±0.1^{a}	7.5±0.1^{a}	7.4±0.1^{a}	7.4±0.1^{a}
22:5n-3	1.4±0.01^{a}	1.4±0.04^{a}	1.3±0.01^{ab}	1.2±0.02^{b}	1.4±0.03^{a}	1.4±0.05^{a}	1.4±0.05^{a}	1.3±0.04^{ab}
22:6n-3(DHA)	6.6±0.1^{a}	6.3±0.1^{ab}	6.3±0.1^{ab}	6.0±0.1^{b}	6.7±0.1^{a}	6.7±0.1^{a}	6.6±0.1^{a}	6.7±0.1^{a}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, P<0.01.

[2]Control group, 0 EDTA and 0 iron.

Table 5.6. Fatty acid composition of the lipid spots following iron treatment in the presence or absence of chelating agent after 12 hrs of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions						
		0.625mM Iron	1.25mM Iron	2.5mM Iron	12.5mM EDTA	0.625 mM Iron & 12.5mM EDTA	1.25mM Iron & 12.5mM EDTA	2.5mM Iron & 12.5mM EDTA
16:0	20.4±0.1[a]	20.9±0.1[ab]	21.1±0.3[b]	21.1±0.2[b]	20.1±0.2[a]	20.3±0.2[a]	20.3±0.3[a]	20.4±0.2[ab]
18:0	3.9±0.1	4.0±0.1	4.2±0.2	4.1±0.2	3.9±0.2	4.1±0.2	4.0±0.2	3.9±0.1
18:1n-9	32.6±0.4[a]	33.1±0.4[ab]	33.5±0.2[b]	33.3±0.3[ab]	32.5±0.2[a]	32.7±0.2[a]	32.8±0.2[ab]	32.6±0.1[a]
18:2n-6	26.4±0.2	26.5±0.10	26.1±0.2	26.3±0.3	26.7±0.2	26.6±0.2	26.4±0.5	26.6±0.2
20:4n-6	0.6±0.01	0.6±0.01	0.6±0.02	0.6±0.02	0.6±0.01	0.6±0.01	0.6±0.02	0.6±0.01
20:5n-3(EPA)	7.2±0.1[a]	6.6±0.1[b]	6.4±0.1[b]	6.2±0.1[b]	7.5±0.1[a]	7.4±0.1[a]	7.4±0.1[a]	7.4±0.1[a]
22:5n-3	1.3±0.02	1.3±0.01	1.3±0.03	1.3±0.01	1.3±0.02	1.3±0.04	1.3±0.05	1.3±0.06
22:6n-3(DHA)	6.5±0.1[a]	5.9±0.1[bc]	5.7±0.1[c]	5.5±0.1[c]	6.7±0.1[a]	6.7±0.1[a]	6.4±0.2[ab]	6.5±0.1[a]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01.

[2]Control group, 0 EDTA and 0 iron.

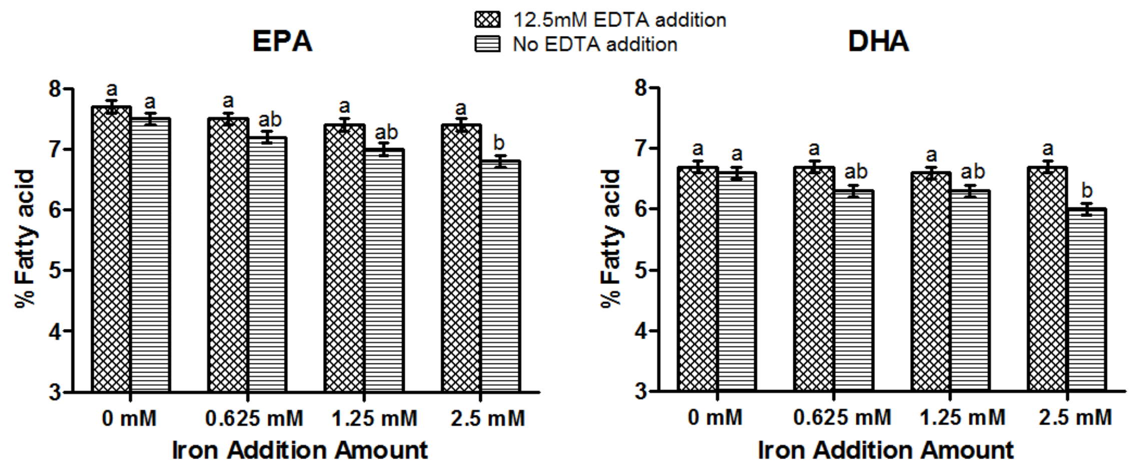
Table 5.7. Fatty acid composition of the lipid spots following iron treatment in the presence or absence of chelating agent after 24 hrs of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions						
		0.625mM Iron	1.25mM Iron	2.5mM Iron	12.5mM EDTA	0.625 mM Iron & 12.5mM EDTA	1.25mM Iron & 12.5mM EDTA	2.5mM Iron & 12.5mM EDTA
16:0	20.8±0.1^{ab}	21.2±0.1^{bc}	21.3±0.2^{bc}	21.4±0.1^{c}	20.4±0.3^{a}	20.2±0.2^{a}	20.7±0.3^{ab}	20.7±0.2^{ab}
18:0	4.2±0.3	4.1±0.1	4.2±0.2	4.3±0.1	3.8±0.2	3.9±0.2	4.1±0.2	4.2±0.1
18:1n-9	32.4±0.3^{a}	33.2±0.2^{bc}	33.7±0.1^{cd}	34.2±0.3^{d}	32.2±0.3^{a}	32.3±0.1^{a}	32.7±0.2^{ab}	32.5±0.4^{a}
18:2n-6	26.1±0.2	26.2±0.1	26.0±0.2	26.4±0.1	26.2±0.4	26.3±0.2	26.1±0.2	26.3±0.2
20:4n-6	0.6±0.02^{a}	0.5±0.03^{ab}	0.4±0.01^{b}	0.4±0.01^{b}	0.6±0.02^{b}	0.6±0.01^{a}	0.6±0.03^{a}	0.6±0.02^{a}
20:5n-3(EPA)	7.0±0.1^{a}	6.3±0.1^{b}	6.0±0.1^{bc}	5.7±0.1^{c}	7.3±0.1^{a}	7.2±0.1^{a}	7.2±0.1^{a}	7.2±0.1^{a}
22:5n-3	1.3±0.02	1.2±0.04	1.3±0.02	1.3±0.03	1.3±0.02	1.3±0.03	1.3±0.06	1.3±0.06
22:6n-3(DHA)	6.2±0.1^{a}	5.7±0.1^{b}	5.4±0.1^{bc}	5.1±0.1^{c}	6.5±0.1^{a}	6.5±0.1^{a}	6.3±0.1^{a}	6.3±0.1^{a}

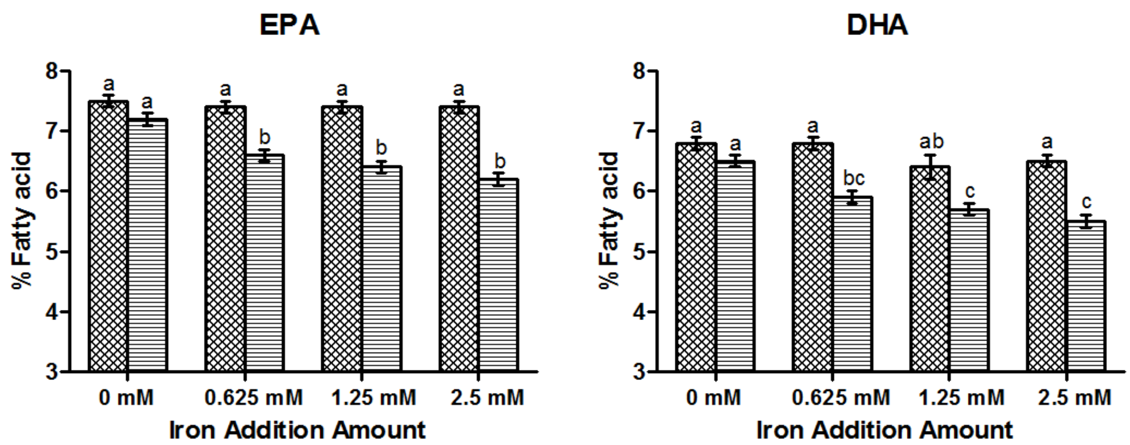
[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, $P<0.01$.

[2]Control group, 0 EDTA and 0 iron.

a. After 6 hrs of air exposure



b. After 12 hrs of air exposure



c. After 24 hrs of air exposure

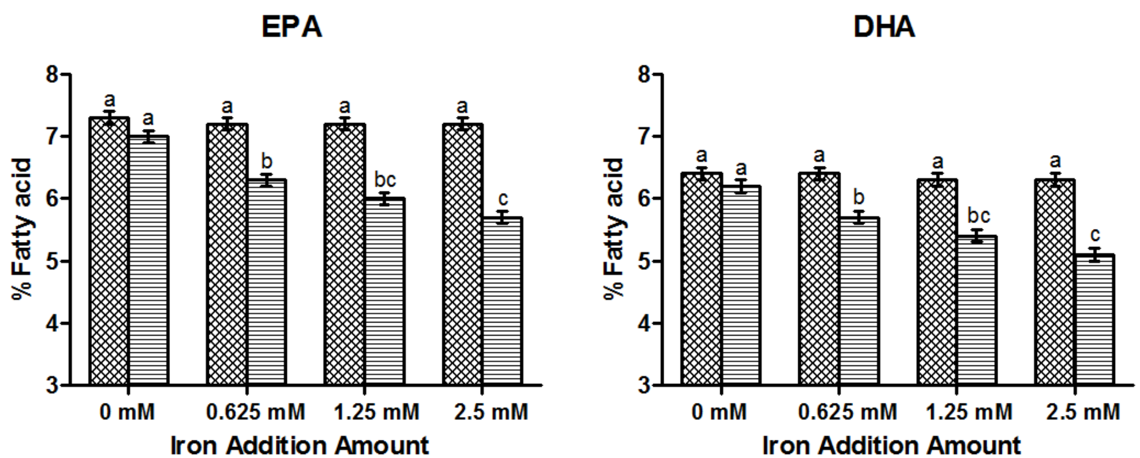


Figure 5.3. EPA and DHA content in the lipid spots under different conditions over 24 hrs of air exposure at room temperature. Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, P<0.01.

5.4.3 Comparison of three iron chelators in the in vitro model

The objective of this study was to compare the efficiency of three iron chelators in preventing n-3 LCPUFA autoxidation using the *in vitro* model. The lipid spots were tested at 4 iron concentrations in the presence or absence of iron chelator (Table 5.3 in Method).

In the presence of irons, but without chelator, the lipid spots showed significant declines of EPA and DHA content after only 1 day air exposure at all iron concentrations (1.25mM, 2.5mM, 5mM and 10mM) when compared with the control group (0 iron, 0 chelator) (Tables 5.8-5.10). Furthermore, the reductions of EPA and DHA were increased both by increasing the iron concentration (Figure 5.6, 5.7) and the extending the period of air exposure (Figures 5.4, 5.5).

However, the iron induced decline in the n-3 LCPUFA content in the lipid spots was significantly inhibited by the addition of chelating agents.

After 1 day of air exposure at room temperature, the fatty acid composition of both 12.5mM EDTA and 12.5mM deferoxamine treated lipid spots did not differ significantly from that of the control group (0 iron, 0 chelator) at the three lower iron concentrations (1.25mM, 2.5mM and 5mM) (Table 5.8, 5.9). A similar result was observed for the fatty acid composition of the 12.5mM baicalein treated spots when the iron concentration was 1.25mM and 2.5mM. However, when the iron concentration of the baicalein treated spots was further increased to 5mM or higher, significant decreases in EPA and DHA contents were observed (Tables 5.10). In the absence of iron, although EDTA and deferoxamine treated spots showed slightly

higher residual EPA and DHA levels than the control group, the difference was not statistically significant. However, in the absence of iron, the baicalein treated spots showed a significantly higher residual EPA and DHA levels when compared with the control group. (Tables 5.8-5.10).

After 3 days of air exposure at room temperature, both 12.5mM EDTA and 12.5mM deferoxamine treated lipid spots showed significantly higher residual EPA and DHA levels than the control group at the three lower iron concentrations (1.25mM, 2.5mM and 5mM) (Table 5.11, 5.12). The 12.5mM baicalein treated spots showed similar results when the added iron concentration was 1.25mM (Table 5.13). Hower, further increasing of the iron concentration in baicalein treated spots to 2.5mM (Figure 5.4, 5.5) or higher resulted in significant decreases in the content of all n-3 LCPUFA when compared with the control group (Table 5.13). In the absence of iron, all chelator treated spots showed significantly higher residual EPA and DHA levels when compared with the control group, and baicalein treated spots retained the highest level of EPA and DHA (Table 5.11-5.13).

After 1 week of air exposure at room temperature, the fatty acid composition of the 12.5mM EDTA and the 12.5mM deferoxamine treated lipid spots did not differ significantly from each other at the two lowest iron concentrations (1.25mM and 2.5mM), however, the deferoxamine treated lipid spots showed significantly higher residual EPA and DHA levels than the EDTA treated spots when 5mM of iron was added. Both the EDTA and the deferoxamine treated lipid spots showed significantly higher residual EPA and DHA levels than the control group at the three lower iron concentrations (1.25mM, 2.5mM and 5mM). EDTA and the deferoxamine treated

spots retained more than 70% original content of EPA and DHA content in the lipid solution at two iron concentrations (1.25mM and 2.5mM), whereas the control group retained only around 50% (Table 5.14, 5.15, Figure 5.6, 5.7). Baicalein treated spots had significantly higher residual EPA level than the control group when the added iron concentration was 1.25mM. However, further increasing in the concentration of iron in the baicalein treated lipid spots to 2.5mM (Figure 5.4, 5.5) or higher resulted in significant decreases in the content of all n-3 LCPUFA when compared with the control group (Table 5.16, Figure 5.6, 5.7). In the absence of iron, the baicalein treated spots retained more than 95% of the original content of EPA and DHA in the lipid solution, whereas the EDTA and the deferoxamine treated spots only retained ~80% and 70% respectively (Figure 5.6, 5.7).

When the added iron concentration to the lipid spots was increased to 10mM, all lipid spots showed significant decreases of LCPUFA after only 1 day air exposure at room temperature when compared with the control group, irrespective of the presence of the chelating agents (Table 5.8-5.10). However, baicalein treated lipid spots had significantly higher residual levels of EPA and DHA after 1 week air exposure at room temperature compared to lipid spots which were treated with EDTA and deferoxamine (Figure 5.6, 5.7).

Table 5.8. Fatty acid composition of the lipid spots at four iron concentrations in the presence or absence of 12.5mM EDTA after 1 day of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	EDTA	1.25mM iron & EDTA	2.5mM iron & EDTA	5mM iron & EDTA	10mM iron & EDTA
16:0	20.3±0.2^{a}	21.4±0.4^{b}	22.0±0.2^{b}	22.9±0.3^{c}	24.4±0.2^{d}	20.3±0.3^{a}	20.4±0.3^{a}	20.2±0.3^{a}	20.5±0.3^{a}	23.1±0.8^{cd}
18:0	4.2±0.1^{ab}	4.2±0.1^{ab}	4.3±0.1^{b}	4.4±0.1^{b}	4.8±0.1^{c}	3.8±0.1^{a}	4.0±0.2^{ab}	4.1±0.1^{ab}	3.9±0.2^{a}	4.5±0.2^{b}
18:1n-9	32.7±0.6^{a}	33.9±0.5^{a}	34.3±0.6^{b}	35.3±0.4^{bc}	37.5±0.5^{d}	33.2±0.5^{a}	32.7±0.5^{a}	32.6±0.6^{a}	32.9±0.5^{a}	36.5±0.7^{cd}
18:2n-6	26.1±0.3^{a}	25.8±0.1^{a}	26.3±0.4^{a}	26.0±0.2^{a}	26.6±0.4^{a}	26.1±0.3^{a}	26.2±0.3^{a}	26.3±0.1^{a}	26.2±0.3^{a}	26.1±0.7^{a}
20:4n-6	0.6±0.01^{a}	0.5±0.01^{b}	0.4±0.01^{b}	0.4±0.02^{b}	0.3±0.01^{c}	0.6±0.01^{a}	0.6±0.01^{a}	0.6±0.01^{a}	0.6±0.01^{a}	0.4±0.03^{b}
20:5n-3(EPA)	7.0±0.1^{a}	6.1±0.2^{b}	5.7±0.2^{b}	5.1±0.2^{c}	3.4±0.1^{e}	7.5±0.1^{a}	7.2±0.1^{a}	7.2±0.1^{a}	7.1±0.1^{a}	4.3±0.2^{d}
22:5n-3	1.3±0.03^{a}	1.2±0.06^{a}	1.2±0.05^{a}	1.1±0.03^{b}	0.57±0.03^{c}	1.3±0.02^{a}	1.3±0.01^{a}	1.3±0.02^{a}	1.3±0.02^{a}	1.0±0.05^{b}
22:6n-3(DHA)	6.2±0.1^{a}	5.6±0.1^{b}	5.1±0.2^{bc}	4.7±0.13^{c}	2.6±0.1^{e}	6.5±0.1^{a}	6.5±0.2^{a}	6.5±0.1^{a}	6.4±0.2^{a}	3.5±0.4^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, $P<0.01$.

[2]Control group, 0 EDTA and 0 iron.

Table 5.9. Fatty acid composition of the lipid spots at four iron concentrations in the presence or absence of 12.5mM deferoxamine after 1 day of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	Deferoxamine	1.25mM iron & Deferoxamine	2.5mM iron & Deferoxamine	5mM iron & Deferoxamine	10mM iron & Deferoxamine
16:0	20.3±0.2^{a}	21.4±0.4^{b}	22.0±0.2^{b}	22.9±0.3^{c}	24.4±0.2^{d}	20.2±0.2^{a}	20.5±0.4^{a}	20.8±0.4^{ab}	21.5±0.4^{b}	21.9±0.6^{b}
18:0	4.2±0.1^{ab}	4.2±0.1^{ab}	4.3±0.1^{b}	4.4±0.1^{b}	4.8±0.1^{c}	4.0±0.2^{a}	4.2±0.1^{a}	3.9±0.3^{a}	3.9±0.1^{a}	4.0±0.2^{ab}
18:1n-9	32.7±0.6^{a}	33.9±0.5^{a}	34.3±0.6^{b}	35.3±0.4^{bc}	37.5±0.5^{d}	33.0±0.6^{a}	32.6±0.3^{a}	33.0±0.6^{a}	32.2±0.5^{a}	35.7±0.6^{b}
18:2n-6	26.1±0.3^{a}	25.8±0.1^{a}	26.3±0.4^{a}	26.0±0.2^{a}	26.6±0.4^{a}	26.0±0.4^{a}	26.1±0.3^{a}	26.0±0.2^{a}	26.1±0.4^{a}	26.4±0.5^{a}
20:4n-6	0.6±0.01^{a}	0.5±0.01^{b}	0.5±0.01^{b}	0.4±0.02^{b}	0.3±0.01^{c}	0.6±0.03^{a}	0.6±0.03^{a}	0.6±0.03^{a}	0.6±0.04^{a}	0.5±0.01^{b}
20:5n-3(EPA)	7.0±0.1^{a}	6.1±0.2^{b}	5.7±0.2^{b}	5.1±0.2^{c}	3.4±0.1^{e}	7.4±0.1^{a}	7.3±0.1^{a}	7.2±0.1^{a}	7.2±0.1^{a}	5.3±0.1^{bc}
22:5n-3	1.3±0.03^{a}	1.2±0.06^{a}	1.2±0.05^{a}	1.1±0.03^{b}	0.58±0.03^{c}	1.3±0.05^{a}	1.3±0.06^{a}	1.2±0.02^{a}	1.3±0.04^{a}	1.0±0.02^{b}
22:6n-3(DHA)	6.2±0.1^{a}	5.6±0.1^{b}	5.1±0.2^{bc}	4.7±0.1^{c}	2.6±0.1^{e}	6.4±0.1^{a}	6.4±0.1^{a}	6.4±0.1^{a}	6.3±0.1^{a}	4.8±0.2^{c}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01.

[2]Control group, 0 EDTA and 0 iron.

Table 5.10. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM baicalein after 1 day of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	Baicalein	1.25mM iron & Baicalein	2.5mM iron & Baicalein	5mM iron & Baicalein	10mM iron & Baicalein
16:0	20.3±0.2^{a}	21.4±0.4^{b}	22.0±0.2^{b}	22.9±0.3^{c}	24.4±0.2^{d}	20.0±0.4^{a}	20.1±0.3^{a}	20.9±0.2^{ab}	21.6±0.4^{b}	22.7±0.3^{c}
18:0	4.2±0.1^{ab}	4.2±0.1^{ab}	4.3±0.1^{ab}	4.4±0.1^{b}	4.8±0.1^{c}	4.0±0.1^{a}	4.1±0.2^{ab}	4.3±0.3^{ab}	4.3±0.2^{ab}	4.3±0.2^{ab}
18:1n-9	32.7±0.6^{a}	33.9±0.5^{a}	34.3±0.6^{b}	35.3±0.4^{b}	37.5±0.5^{c}	33.0±0.9^{a}	33.3±0.4^{a}	34.3±0.4^{b}	34.7±0.2^{b}	35.1±0.6^{b}
18:2n-6	26.1±0.3^{a}	25.8±0.1^{a}	26.3±0.4^{a}	26.0±0.2^{a}	26.6±0.4^{a}	26.0±0.3^{a}	26.0±0.3^{a}	25.9±0.4^{a}	26.2±0.3^{a}	26.2±0.4^{a}
20:4n-6	0.6±0.01^{a}	0.5±0.01^{b}	0.5±0.01^{b}	0.4±0.02^{b}	0.3±0.01^{c}	0.6±0.01^{a}	0.6±0.03^{a}	0.5±0.01^{b}	0.5±0.05^{ab}	0.4±0.02^{b}
20:5n-3(EPA)	7.0±0.1^{b}	6.1±0.2^{cd}	5.7±0.2^{d}	5.1±0.2^{e}	3.4±0.1^{f}	7.7±0.1^{a}	7.2±0.1^{b}	6.7±0.2^{bc}	6.3±0.2^{cd}	5.0±0.3^{e}
22:5n-3	1.3±0.03^{a}	1.2±0.06^{a}	1.2±0.05^{ab}	1.1±0.03^{b}	0.57±0.03^{a}	1.3±0.02^{a}	1.3±0.02^{a}	1.3±0.01^{a}	1.2±0.01^{a}	1.2±0.05^{a}
22:6n-3(DHA)	6.2±0.1^{ab}	5.6±0.1^{bc}	5.1±0.2^{cd}	4.7±0.13^{d}	2.6±0.1^{e}	6.7±0.1^{a}	6.3±0.1^{ab}	5.8±0.1^{b}	5.3±0.3^{bc}	4.5±0.1^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01.

[2]Control group, 0 EDTA and 0 iron.

Table 5.11. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM EDTA after 3 days of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions									
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	EDTA	1.25mM iron & EDTA	2.5mM iron & EDTA	5mM iron & EDTA	10mM iron & EDTA
16:0	20.6±0.3[a]	22.6±0.3[b]	24.3±0.4[c]	27.4±0.5[d]	31.3±0.5[e]	19.4±0.2[a]	20.0±0.4[a]	20.0±0.4[a]	20.1±0.4[a]	25.4±1.2[c]
18:0	4.0±0.1[a]	4.2±0.2[a]	4.9±0.2[b]	4.8±0.2[b]	6.1±0.3[c]	3.7±0.1[a]	3.8±0.2[a]	3.7±0.2[a]	4.0±0.2[a]	4.6±0.3[b]
18:1n-9	35.0±0.5[ab]	35.6±0.4[b]	37.5±0.2[c]	39.9±0.5[d]	44.5±0.7[e]	33.7±0.5[a]	34.2±0.4[a]	34.1±0.7[ab]	34.5±0.7[ab]	38.2±1.0[c]
18:2n-6	26.8±0.3[a]	26.2±0.3[a]	25.8±0.4[a]	23.4±0.4[b]	16.6±0.2[c]	26.7±0.4[a]	26.8±0.2[a]	26.8±0.3[a]	26.8±0.5[a]	25.5±1.2[ab]
20:4n-6	0.5±0.01[b]	0.5±0.03[ab]	0.4±0.04[bc]	0.2±0.03[d]	0.2±0.04[d]	0.6±0.01[a]	0.6±0.01[a]	0.6±0.01[a]	0.6±0.02[a]	0.3±0.01[cd]
20:5n-3(EPA)	5.9±0.2[c]	4.4±0.4[d]	3.3±0.1[e]	1.6±0.1[f]	0.5±0.1[g]	7.4±0.1[a]	6.8±0.1[ab]	6.8±0.1[b]	6.5±0.1[b]	2.6±0.8[ef]
22:5n-3	1.1±0.04[b]	0.9±0.06[c]	0.6±0.01[d]	0.4±0.08[e]	0.2±0.02[f]	1.3±0.03[a]	1.3±0.05[a]	1.3±0.02[a]	1.2±0.02[b]	0.4±0.01[e]
22:6n-3(DHA)	5.3±0.1[c]	4.2±0.2[d]	2.5±0.3[e]	1.4±0.1[f]	0.7±0.1[g]	6.4±0.1[a]	5.8±0.1[b]	6.0±0.1[ab]	5.7±0.1[bc]	2.0±0.5[ef]

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, $P<0.01$.

[2]Control group, 0 EDTA and 0 iron.

Table 5.12. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM deferoxamine after 3 days of air exposure at room temperature

Fatty acids[1]	Control[2]	Intervention								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	Deferoxamine	1.25mM iron & Deferoxamine	2.5mM iron & Deferoxamine	5mM iron & Deferoxamine	10mM iron & Deferoxamine
16:0	20.6±0.3^{a}	22.6±0.3^{b}	24.3±0.4^{c}	27.4±0.5^{e}	31.3±0.5^{f}	20.0±0.4^{a}	20.4±0.5^{a}	20.1±0.4^{a}	20.0±0.5^{a}	25.9±0.5^{d}
18:0	4.0±0.1^{a}	4.2±0.2^{a}	4.9±0.2^{b}	4.8±0.2^{b}	6.1±0.3^{c}	3.8±0.2^{a}	3.8±0.2^{a}	3.9±0.2^{a}	3.9±0.3^{a}	4.9±0.3^{b}
18:1n-9	35.0±0.5^{ab}	35.6±0.4^{b}	37.5±0.2^{c}	39.9±0.5^{d}	44.5±0.7^{e}	34.5±0.4^{a}	34.1±0.5^{a}	34.5±0.4^{a}	34.7±0.3^{a}	38.2±0.9^{cd}
18:2n-6	26.8±0.3^{a}	26.2±0.3^{a}	25.8±0.4^{a}	23.4±0.4^{b}	16.6±0.2^{c}	26.9±0.2^{a}	27.0±0.4^{a}	26.9±0.3^{a}	27.0±0.21^{a}	25.3±0.6^{a}
20:4n-6	0.5±0.01^{b}	0.5±0.03^{ab}	0.4±0.04^{bc}	0.2±0.03^{d}	0.2±0.04^{d}	0.6±0.01^{a}	0.6±0.02^{a}	0.6±0.01^{a}	0.6±0.01^{a}	0.3±0.02^{cd}
20:5n-3(EPA)	5.9±0.2^{b}	4.4±0.4^{c}	3.3±0.1^{de}	1.6±0.1^{f}	0.5±0.1^{g}	6.6±0.1^{a}	6.5±0.2^{a}	6.5±0.1^{a}	6.5±0.11^{a}	2.6±0.4^{e}
22:5n-3	1.1±0.04^{a}	0.9±0.06^{b}	0.6±0.01^{c}	0.4±0.08^{d}	0.2±0.02^{e}	1.2±0.01^{a}	1.1±0.04^{a}	1.2±0.01^{a}	1.2±0.03^{a}	0.4±0.03^{d}
22:6n-3(DHA)	5.3±0.1^{b}	4.2±0.2^{c}	2.5±0.3^{d}	1.4±0.1^{e}	0.7±0.1^{f}	5.8±0.1^{a}	5.6±0.2^{ab}	5.7±0.1^{a}	5.7±0.15^{ab}	1.9±0.3^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01.

[2]Control group, 0 EDTA and 0 iron.

Table 5.13. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM baicalein after 3 days of air exposure at room temperature

Fatty acids[1]	Control[2]	Intervention								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	Baicalein	1.25mM iron & Baicalein	2.5mM iron & Baicalein	5mM iron & Baicalein	10mM iron & Baicalein
16:0	20.6±0.3^{b}	22.6±0.3^{c}	24.3±0.4^{d}	27.4±0.5^{e}	31.3±0.5^{f}	19.2±0.3^{a}	20.2±0.7^{ab}	22.0±0.6^{c}	22.6±0.9^{cd}	24.3±0.9^{d}
18:0	4.0±0.1^{ab}	4.2±0.2^{b}	4.9±0.2^{c}	4.8±0.2^{c}	6.1±0.3^{c}	3.7±0.1^{a}	4.12±0.3^{ab}	4.9±0.2^{bc}	4.9±0.2^{c}	4.7±0.5^{bc}
18:1n-9	35.0±0.5^{b}	35.6±0.4^{b}	37.5±0.2^{c}	39.9±0.5^{d}	44.5±0.7^{e}	33.3±3^{a}	33.4±0.7^{a}	34.1±1.1^{ab}	34.2±1.2^{ab}	37.5±1.1^{cd}
18:2n-6	26.8±0.3^{a}	26.2±0.3^{a}	25.8±0.4^{a}	23.4±0.4^{b}	16.6±0.2^{c}	26.6±0.2^{a}	26.2±0.6^{a}	25.8±0.6^{a}	26.0±0.7^{a}	25.3±1.0^{a}
20:4n-6	0.5±0.01^{b}	0.5±0.03^{ab}	0.4±0.04^{b}	0.2±0.03^{c}	0.2±0.04^{c}	0.6±0.01^{a}	0.6±0.01^{a}	0.4±0.02^{b}	0.4±0.03^{b}	0.4±0.07^{b}
20:5n-3(EPA)	5.9±0.2^{b}	4.4±0.4^{c}	3.3±0.1^{e}	1.6±0.1^{f}	0.5±0.1^{g}	7.7±0.1^{a}	6.4±0.2^{b}	4.7±0.2^{c}	4.0±0.1^{d}	3.7±0.1^{de}
22:5n-3	1.1±0.04^{b}	0.9±0.06^{bc}	0.6±0.01^{d}	0.4±0.08^{e}	0.2±0.02^{f}	1.3±0.02^{a}	1.3±0.03^{ab}	1.0±0.02^{c}	0.9±0.02^{c}	0.7±0.04^{d}
22:6n-3(DHA)	5.3±0.1^{b}	4.2±0.2^{c}	2.5±0.3^{e}	1.4±0.1^{f}	0.7±0.1^{g}	6.7±0.1^{a}	5.7±0.2^{b}	4.1±0.1^{c}	3.6±0.1^{d}	3.2±0.2^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, $P<0.01$.

[2]Control group, 0 EDTA and 0 iron.

Table 5.14. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM EDTA after 7 days of air exposure at room temperature

Fatty acids[1]	Control[2]	Interventions								
		1.25mM iron	2.5mM iron	5mM iron	10mM iron	EDTA	1.25mM iron & EDTA	2.5mM iron & EDTA	5mM iron & EDTA	10mM iron & EDTA
16:0	23.6±0.2^{c}	25.8±1.1^{c}	31.8±0.1^{d}	31.5±0.3^{d}	32.3±0.6^{d}	20.3±0.4^{a}	20.5±0.4^{a}	20.5±0.4^{a}	21.8±0.3^{b}	31.1±0.6^{d}
18:0	4.4±0.1^{a}	5.0±0.2^{b}	6.0±0.2^{c}	6.1±0.3^{c}	6.1±0.2^{c}	3.9±0.1^{a}	4.1±0.1^{a}	4.0±0.2^{a}	4.4±0.4^{a}	5.7±0.3^{bc}
18:1n-9	37.1±0.4^{b}	38.4±0.7^{b}	40.2±0.3^{c}	44.4±0.5^{d}	46.3±0.5^{e}	34.7±0.4^{a}	35.1±0.5^{a}	35.3±0.3^{a}	35.8±0.4^{a}	40.3±0.7^{c}
18:2n-6	26.4±0.5^{a}	24.7±0.7^{b}	19.5±0.3^{c}	16.6±0.2^{d}	15.2±0.2^{e}	26.9±0.3^{a}	26.7±0.3^{a}	26.8±0.2^{a}	26.5±0.3^{a}	20.3±0.6^{c}
20:4n-6	0.4±0.01^{b}	0.3±0.05^{b}	0.1±0.01^{c}	-	-	0.5±0.01^{a}	0.5±0.01^{a}	0.5±0.01^{a}	0.4±0.03^{ab}	-
20:5n-3(EPA)	4.1±0.1^{c}	2.9±0.5^{d}	1.0±0.1^{e}	0.5±0.1^{f}	-	6.3±0.1^{a}	5.8±0.1^{a}	5.8±0.3^{a}	5.0±0.1^{b}	1.1±0.1^{e}
22:5n-3	0.7±0.02^{b}	0.4±0.03^{c}	0.2±0.02^{d}	0.2±0.11^{d}	-	1.1±0.02^{a}	1.1±0.03^{a}	1.1±0.04^{a}	0.9±0.1^{ab}	-
22:6n-3(DHA)	3.5±0.3^{c}	1.9±0.3^{d}	0.9±0.1^{e}	0.7±0.1^{e}	-	5.5±0.1^{a}	5.3±0.1^{a}	5.4±0.2^{a}	4.6±0.1^{b}	1.0±0.1^{e}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, $P<0.01$. "-" not detected.

[2]Control group, 0 EDTA and 0 iron.

Table 5.15. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM deferoxamine after 7 days of air exposure at room temperature

Fatty acids[1]	**Control**[2]	**Intervention**								
		1.25mM iron	**2.5mM iron**	**5mM iron**	**10mM iron**	**Deferoxamine**	**1.25mM iron & Deferoxamine**	**2.5mM iron & Deferoxamine**	**5mM iron & Deferoxamine**	**10mM iron & Deferoxamine**
16:0	23.6±0.2^{b}	25.8±1.1^{c}	31.8±0.1^{d}	31.5±0.3^{d}	32.3±0.6^{d}	21.2±0.3^{a}	21.0±0.4^{a}	20.8±0.3^{a}	21.8±0.5^{a}	31.7±0.7^{d}
18:0	4.4±0.1^{b}	5.0±0.2^{c}	6.0±0.2^{d}	6.1±0.3^{d}	6.1±0.2^{c}	4.1±0.1^{ab}	4.1±0.1^{ab}	3.9±0.1^{a}	4.6±0.2^{b}	5.6±0.3^{cd}
18:1n-9	37.1±0.4^{b}	38.4±0.7^{b}	40.2±0.3^{c}	44.4±0.5^{d}	46.3±0.5^{e}	35.2±0.4^{a}	35.4±0.4^{a}	35.0±0.4^{a}	35.1±0.6^{a}	40.7±0.8^{c}
18:2n-6	26.4±0.5^{a}	24.7±0.7^{b}	19.5±0.3^{c}	16.6±0.2^{d}	15.2±0.2^{e}	26.0±0.3^{a}	26.5±0.2^{a}	26.1±0.2^{a}	25.8±0.9^{ab}	19.4±1.0^{c}
20:4n-6	0.4±0.01^{b}	0.3±0.05^{b}	0.1±0.01^{c}	-	-	0.5±0.01^{a}	0.4±0.04^{ab}	0.4±0.01^{b}	0.4±0.06^{ab}	-
20:5n-3(EPA)	4.1±0.1^{c}	2.9±0.5^{d}	1.0±0.1^{e}	0.5±0.1^{f}	-	5.6±0.2^{a}	5.7±0.1^{a}	5.6±0.1^{a}	5.5±0.1^{a}	1.2±0.2^{d}
22:5n-3	0.7±0.02^{b}	0.4±0.03^{c}	0.2±0.02^{d}	0.2±0.11^{d}	-	1.1±0.03^{a}	1.1±0.07^{a}	1.1±0.01^{a}	1.1±0.07^{a}	-
22:6n-3(DHA)	3.5±0.3^{c}	1.9±0.3^{d}	0.9±0.1^{e}	0.7±0.1^{e}	-	5.3±0.1^{a}	5.0±0.1^{a}	5.1±0.1^{a}	5.0±0.1^{a}	1.0±0.1^{d}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01. "-" not detected.

[2]Control group, 0 EDTA and 0 iron.

Table 5.16. Fatty acid composition at four iron concentrations in the presence or absence of 12.5mM baicalein after 7 days of air exposure at room temperature

Fatty acids[1]	**Control**[2]	**Intervention**								
		1.25mM iron	**2.5mM iron**	**5mM iron**	**10mM iron**	**Baicalein**	**1.25mM iron & Baicalein**	**2.5mM iron & Baicalein**	**5mM iron & Baicalein**	**10mM iron & Baicalein**
16:0	23.6±0.2^{c}	25.8±1.1^{c}	31.8±0.1^{e}	31.5±0.3^{e}	32.3±0.6^{e}	19.3±0.3^{a}	22.6±0.2^{b}	23.8±0.5^{c}	24.7±0.5^{c}	29.1±0.6^{d}
18:0	4.4±0.1^{b}	5.0±0.2^{c}	6.0±0.2^{d}	6.1±0.3^{d}	6.1±0.2^{d}	3.7±0.1^{a}	5.2±0.2^{b}	5.2±0.2^{b}	5.2±0.3^{b}	5.8±0.7^{bc}
18:1n-9	37.1±0.4^{bc}	38.4±0.7^{c}	40.2±0.3^{d}	44.4±0.5^{e}	46.3±0.5^{f}	33.5±0.4^{a}	36.1±0.8^{b}	37.0±0.4^{b}	37.8±0.3^{c}	40.6±0.6^{d}
18:2n-6	26.4±0.5^{a}	24.7±0.7^{b}	19.5±0.3^{c}	16.6±0.2^{d}	15.2±0.2^{e}	26.8±0.2^{a}	26.4±0.3^{a}	26.0±0.6^{ab}	25.3±0.5^{b}	20.3±0.4^{c}
20:4n-6	0.4±0.01^{b}	0.3±0.05^{bc}	0.1±0.01^{d}	-	-	0.6±0.01^{a}	0.4±0.01^{b}	0.3±0.02^{bc}	0.3±0.02^{bc}	0.2±0.02^{c}
20:5n-3 (EPA)	4.1±0.1^{c}	2.9±0.5^{de}	1.0±0.1^{g}	0.5±0.1^{h}	-	7.6±0.1^{a}	4.8±0.1^{b}	3.4±0.1^{d}	2.7±0.1^{e}	1.8±0.2^{f}
22:5n-3	0.7±0.02^{c}	0.4±0.03^{d}	0.2±0.02^{e}	0.2±0.11^{e}	-	1.3±0.01^{a}	1.0±0.06^{b}	0.6±0.04^{c}	0.4±0.01^{d}	0.3±0.13^{de}
22:6n-3 (DHA)	3.5±0.3^{b}	1.9±0.3^{d}	0.9±0.1^{f}	0.7±0.1^{f}	-	6.6±0.1^{a}	4.1±0.1^{b}	2.7±0.1^{c}	2.0±0.1^{d}	1.5±0.1^{e}

[1]Values are presented as mean ± SD (n=3), different superscripts indicate significant differences between interventions, *P*<0.01. "-" not detected.

[2]Control group, 0 EDTA and 0 iron.

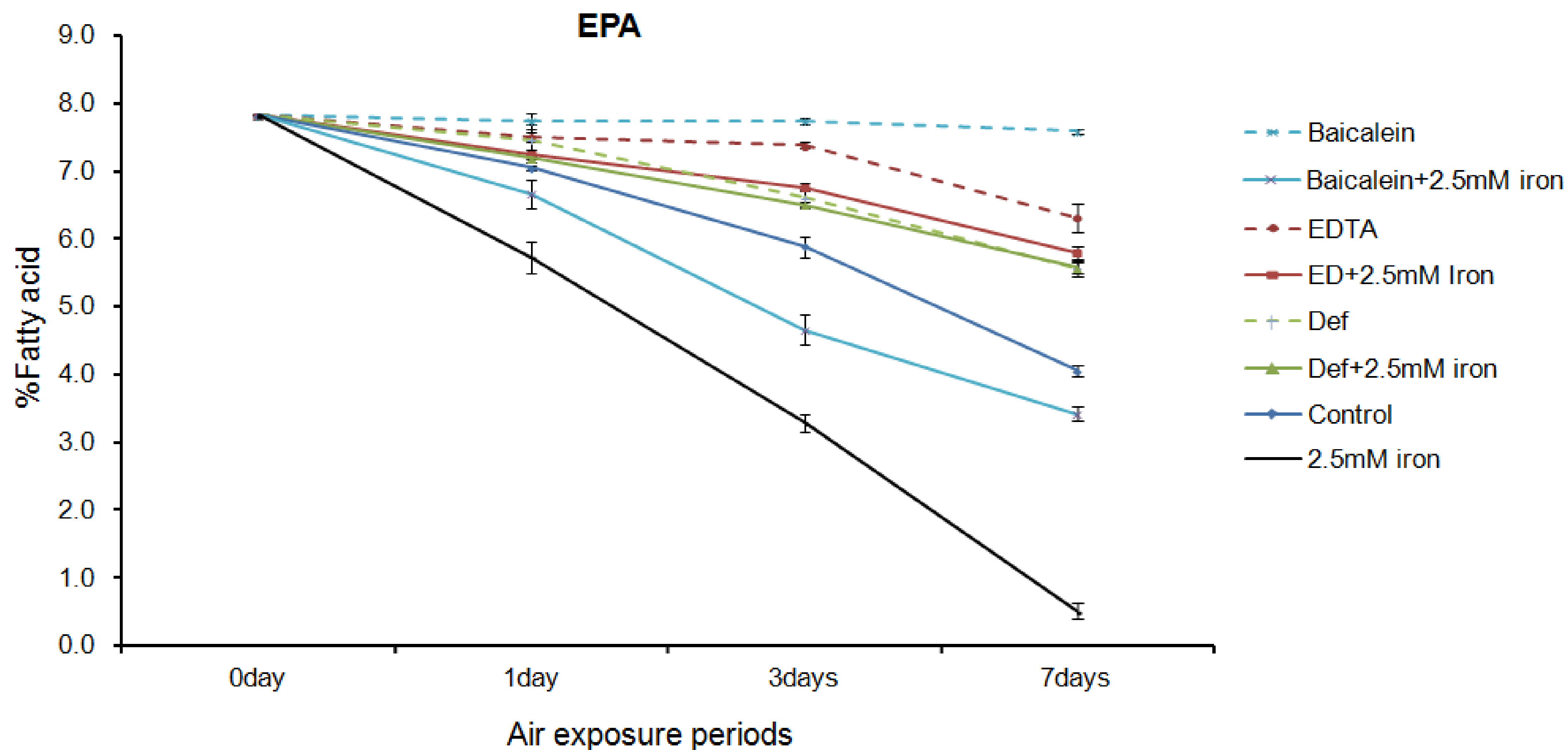


Figure 5.4. EPA content of the lipid spots in the presence or absence of iron chelator and in the presence or absence of 2.5mM of iron over 1 week of air exposure at room temperature. Values are presented as means ± SD (n=3). “Control”, 0 chelator and 0 iron. “0 day” refers to the fatty acid composition obtained from direct transmethylation of 50µl lipid solution at time 0.

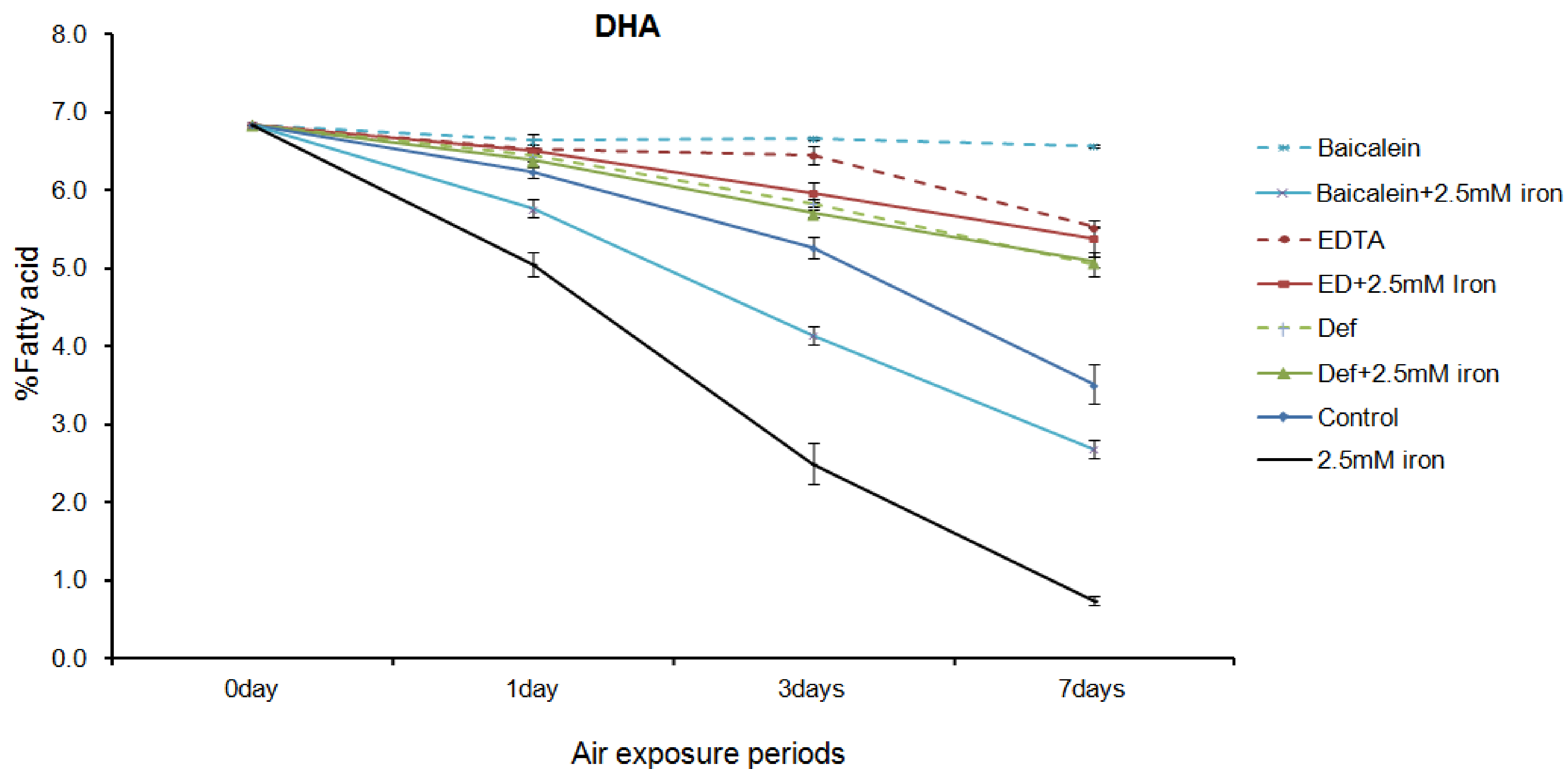


Figure 5.5. DHA content of the lipid spots in the presence or absence of iron chelator and in the presence or absence of 2.5mM of iron over 1 week of air exposure at room temperature. Values are presented as means ± SD (n=3). "Control", 0 chelator and 0 iron. "0 day" refers to the fatty acid composition obtained from direct transmethylation of 50µl lipid solution at time 0.

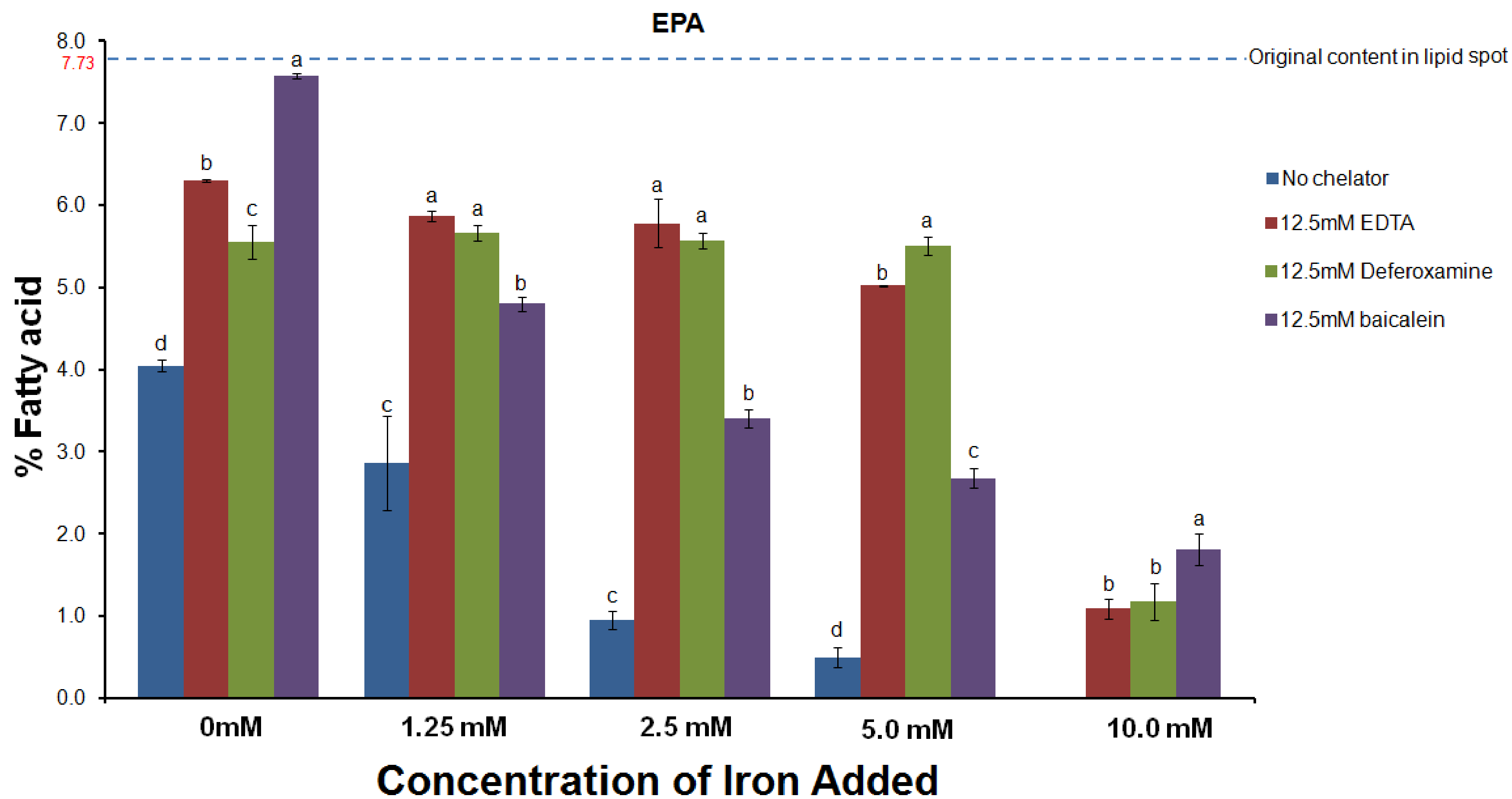


Figure 5.6. EPA content of the lipid spots protected by iron chelator at different iron concentrations after 1 week of air exposure at room temperature. Values are presented as means ± SD (n=3), different superscripts indicate significant differences between treatments, *P*<0.01. The "Original content" refers to the fatty acid composition obtained from direct transmethylation of 50μl lipid solution at time 0.

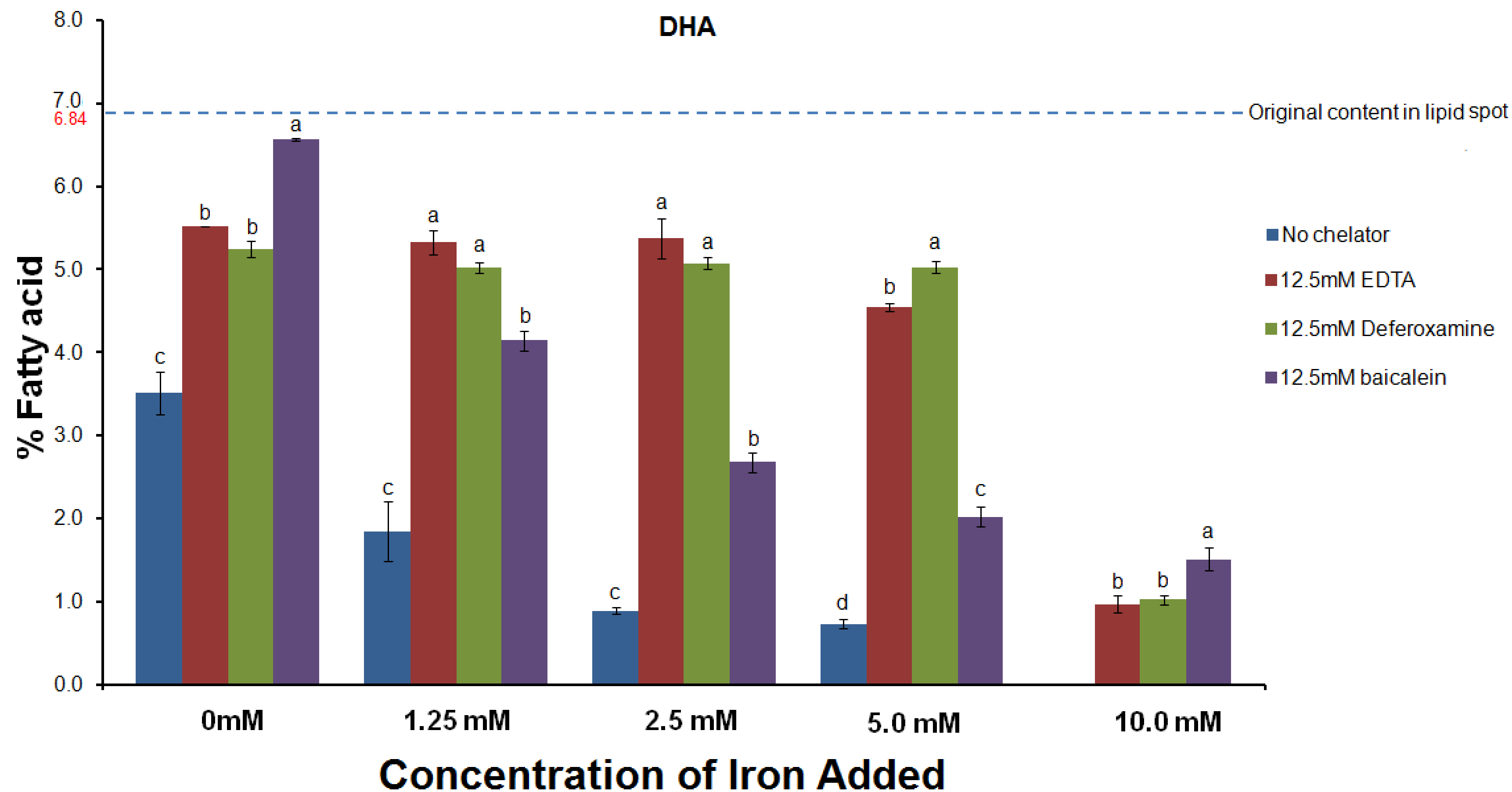


Figure 5.7. DHA content of the lipid spots protected by iron chelator at different iron concentrations after 1 week of air exposure at room temperature. Values are presented as means ± SD (n=3), different superscripts indicate significant differences between treatments, $P<0.01$. The "Original content" refers to the fatty acid composition obtained from direct transmethylation of 50μl lipid solution at time 0.

5.4 Discussion

The oxidative property of ionic iron in food and in the human body has been extensively reviewed (Eric *et al.* 1992, Carlsen *et al.* 2004). Although there is limited information regarding the amount of ionic iron in DBS, about 2.5 g of total iron is sequestered in human red blood cells, which corresponds to a molar concentration of ~ 10mM total iron in human blood (Fraga and Oteiza 2002). Under normal conditions, there is no appreciable concentration of ionic iron in the blood of healthy individuals, since iron is usually firmly bound to haemoglobin and enclosed in red blood cells (Gurzau *et al.* 2003). However, studies have shown that damaging red blood cells causes the release of sequestered iron which enhances the degradation of LCPUFA in blood (Chiu *et al.* 1989). It is almost certain that the red blood cells in the DBS are broken during air drying, resulting in haemoglobin molecules being released and oxidised in the air which will liberate the iron from haemoglobin (Nagy *et al.* 2010). Therefore, I hypothesised that the iron liberated from red blood cells has the potential to accelerate the degradation of LCPUFA in the DBS during storage, and that the iron chelator may prevent it from catalysing the production of undesirable oxidative radicals by binding these iron molecules (Sies 1987).

In order to validate the hypothesis that iron from red blood cells has the capability to accelerate the oxidation of LCPUFA in DBS, an *in vitro* model was developed for directly evaluating the effect of iron on the degradation of LCPUFA, and determining the effect of chelating agent in this process. The first step was to develop a lipid mixture containing n-3 LCPUFA sources that would approximate the n-3 LCPUFA status in the human blood samples I used in Chapter 4, and which could easily spotted on to papers to evaluate the effects of added iron (ferrous + ferric) on the oxidation of

n-3 LCPUFA and their interactions with chelating agent. In order to isolate the oxidative catalysis effects of iron, it was also critically important that this model was free from other components in blood such as proteins, enzymes and inorganic metals, which could contribute to the degradation of lipids (Carlsen *et al.* 2004). Therefore, a pure chemical system that comprised of fixed levels of iron ions (ferrous + ferric) and a mixture of marine and plant oils was developed.

The standard storage protocol is to store the DBS samples in a zip-lock bag with desiccants (Mei *et al.* 2001), however, this approach would reduce the rate of degradation of the LCPUFA in lipid spots, and makes it time consuming to detect the differences among the treatments in their ability to accelerate or impede the degradation process. Earlier studies reported that air exposure caused a rapid acceleration in lipid degradation (Scopesi *et al.* 2004; Metherel *et al.* 2013). On this basis, the lipid spots were exposed to air to achieve a quick degradation of LCPUFA. In the present study, significant degradation of LCPUFA in lipid spots without any added catalyst appeared within 24 hrs of air exposure, and the losses of LCPUFA during this period were moderate, which meant that if additional losses occurred as a result of adding a catalyst such as iron, they could clearly be detected.

My results from the *in vitro* model have confirmed that in the absence of any external interventions, oxidative reduction of LCPUFA occured relatively slow. However, even the presence of small amounts of added iron in the lipid spots greatly accelerated the oxidative reduction of LCPUFA. Furthermore, the degradation of LCPUFA appeared to be dependent on iron concentrations, and increased in proportion to the amount of iron that was added. Importantly, further results from the *in vitro* model showed that

the negative influence of iron can be eliminated by the addition of an iron chelator. Therefore, this study has reported for the first time that the addition of iron can induce significant autoxidative loss of LCPUFA in samples absorbed on blood collection paper. However, this negative influence of iron can be eliminated by pre-treatment of the collection paper with chelating agents.

In vitro models have been widely used for studying the iron chelating properties of compounds (Kontoghiorghes *et al.* 1986; Das *et al.* 2012). In the present study, I evaluated the iron chelating capacity of three iron chelators (EDTA, deferoxamine and baicalein) using my *in vitro* model. Consistent with earlier studies (Kontoghiorghes *et al.* 1986; Mladěnka *et al.* 2011), I demonstrated that all chelators inhibited iron-induced degradation of LCPUFA in the lipid spots at a high chelator to iron molar concentration ratio (10:1). Both EDTA and deferoxamine chelated total iron (ferric + ferrous) effectively when the molar ratio of chelator to iron was at least 2.5:1. At a 2.5:1 (chelator: iron) molar ratio, the fatty acid composition of deferoxamine treated lipid spots did not differ significantly from those of EDTA treated spots after 3 days of air exposure. However, the deferoxamine treated samples had significantly higher residual EPA and DHA levels than EDTA treated spots after 1 week of air exposure. This may suggests that the iron chelating capacity of deferoxamine is superior to that of EDTA. Deferoxamine is widely regarded as a suitable reference for the comparison of iron chelating activity because of its high affinity for ferric iron (Kalinowski and Richardson 2005). However, the results from my study revealed that it chelates both ferric and ferrous iron with high affinity under aerobic conditions. This is in line with other studies which also reported the ability of deferoxamine to chelate ferrous iron (Mladěnka *et al.* 2011). Baicalein has shown comparable iron chelation activity to

deferoxamine at a chelator to iron molar ratio of 10:1 in previous *in vitro* studies (Mladěnka *et al.* 2011). Consistent results were also observed in my study where baicalein showed similar iron chelation property is to EDTA and deferoxamine when its molar ratio to iron is 10:1.

Theoretically, in the absence of added iron, the stability of LCPUFA in lipid spots with or without pre-treatment of chelators should be similar to each other. However, significantly higher residual EPA and DHA levels were observed in chelator pre-treated spots when compared with control spots (0 chelator, 0 iron). This may be due to the presence of trace amount of iron contaminants in the collection paper that can accelerate the autoxidation of EPA and DHA in those lipid spots in the absence of iron chelators (Meakin 1973). It is also interesting to note that in the absence of added iron, baicalein treated lipid spots showed the highest residual LCPUFA content among all three iron chelator treated spots. This may be because baicalein inhibits the production of free radicals in the lipid spots through both iron chelation and free radical scavenging, as previously reported by others (Hamada *et al.* 1993; Shieh *et al*. 2000; Perez *et al.* 2011).

5.5 Summary

In the present study, an *in vitro* model of blood lipids has been established for studying the interaction among iron, iron chelators and the autoxidation of LCPUFA in DBS. The data from *in vitro* studies clearly demonstrated that the presence of iron plays a significant role in the autoxidation of LCPUFA in the lipid spots and this activity can be inhibited by the pre-treatment of the collection papers with chelating

agent. Furthermore, I demonstrated that my *in vitro* model has capacity to show clear differences in the protective properties between different types of iron chelators, which indicates that the model provides a suitable tool for quickly distinguishing the metal-chelating efficiency of different chelators.

Chapter 6

Clinical Validation of Newly Developed Dried Blood Spot Method for Measuring Fatty Acids

6.1 Introduction

Fatty acids carry out many functions that are necessary for maintaining optimal health (Spector 1999; De La Cruz 2000). The fatty acid status in humans has the potential to provide valuable information that relate to the susceptibility of individuals to a range of diseases, and assist clinicians in identifying individuals who would be likely to benefit from dietary n-3 supplementation (Vessby, 2003; Metcalf *et al.* 2007). The evaluation of n-3 status in human is particularly important in view of the fact that n-3 LCPUFA supplementation is now adopted in the management and treatment of several diseases (Cleland *et al.* 2003; Einvik *et al.* 2010).

However, the potential for using fatty acid profiles as biomarkers in large-scare RCTs and population screening programs has been limited by the logistical difficulties associated with the collection of blood from the antecubital vein, blood storage and transport, and the expensive and time consuming analytical procedure which traditionally used to assess blood fatty acid composition involves plasma and red blood cells preparation, lipid extraction, and TLC separation (Marangoni *et al.* 2007). Thus, there is a need for a rapid, inexpensive and reliable fatty acid test method to enable analysis of a large number of samples in small quantities of starting material.

The DBS technique enables analysis of the fatty acids profiles in whole blood from a drop of blood from fingertip. This technique overcomes many of the problems

associated with standard procedures for blood sampling, transportation and storage (Parker and Cubitt, 1999). However, the reliability of the DBS method for measuring fatty acid status has been called into question by data showing that LCPUFA levels measured in DBS progressively declined during storage either at room temperature (Min *et al.* 2011) or at 4°C (Bell *et al.* 2011). This makes it impractical for measurement of fatty acid profiles in large clinical studies, since it would necessitate that all samples are stored and transported at low temperatures and analysed at a fixed time after collection if results are to be comparable.

In Chpater 4, I described a novel DBS assay which stabilised the n-3 LCPUFA in DBS for up to 9 weeks when stored at room temperature, and I proposed a mechanism through which this was achieved in Chapter 5. However, the fatty acid results obtained from DBS samples using my newly developed DBS method have not been compared with those obtained using conventional measurements. Such a comparison is important for establishing the clinical applicability of my DBS method, because the fatty acid status from conventional measurements are currently widely used as indicators of dietary fatty acid intake (Kaaks *et al.* 1997), or biomarkers of disease risks (Einvik *et al*. 2010). For example, plasma fatty acid composition has been shown to reflect habitual fatty acids intake (Ma *et al.* 1995), and red blood cells n-3 status was suggested as a biomarker for coronary heart disease risk (Harris and von schacky *et al.* 2004). In addition, whole blood fatty acid status has been related to the risk of sudden heart death (Albert *et al.* 2002). Thus, the next step in validating the my newly developed DBS method is to determine how it compares to conventional measurements, in which fatty acid status is assessed in whole blood, plasma or red blood cells through total lipid extraction, lipid class separation, transmethylation, and

finally GC analysis.

Therefore, the purpose of this study was to compare the fatty acid status obtained from my DBS method with those obtained from conventional measurements. The correlation between the n-3 fatty acid status obtained from my DBS method and individual's habitual dietary n-3 fatty acid intakes was also investigated.

6.2 Design of the study

In a total of 50 subjects, the fatty acid composition of capillary blood was compared with that of venous blood samples to validate the equivalence of capillary blood and venous blood in the measurement of fatty acid status. The levels of 11 predominant fatty acids obtained from capillary blood using my DBS method were compared with those obtained using conventional assays, involving whole blood total lipid extraction, plasma total lipid extraction, plasma phospholipids and red blood cells phospholipids (Figure 6.1). A questionnaire about health and dietary habits was used to obtain information on fish and fish oil intake from all the subjects, and people's self-reported dietary parameters was correlated with the fatty acid composition obtained from capillary DBS samples.

6.3 Materials and Methods

6.3.1 Subjects

A total of 50 subjects (26 females and 24 males) aged between 22 and 71 years were

recruited from The University of Adelaide and Royal Adelaide Hospital. All participants gave informed consent prior to the appointment and were asked to complete a questionnaire about their dietary habits and fish oil supplement intake within the last six months. The study was approved by The University of Adelaide Human Research Ethics Committee. The subjects in my clinical study included 23 people who consumed relatively high amounts of fish oil supplements (≥3gram per day), 14 people who seldom consumed any fish oil supplements (<1 gram per week) but have a regular consumption of fish (≥1 serving per week), and 13 people who seldom consumed any fish (<1 serving per week) or fish oil supplements (<1 gram per week) (Table 6.1). I deliberately recruited individual with a wide range of blood n-3 LCPUFA intakes in order to ensure that I had a broad range of n-3 LCPUFA concentrations across the participant group, so as to verify that my DBS method is reliable across a wide range of blood n-3 status.

6.3.2 Blood collection papers

Whatman ion exchange paper (Grade SG81, 46x57cm, Whatman, Buckingham, UK) was cut into 1.5cm x 4.5cm paper strips and used as the blood collection paper. 50 µl of protectant formulation (2mg/ml BHT+5mg/ml EDTA) was spread evenly over the collection area (1.5cm x 1.5cm) of all the paper strips and air-dried prior to collection of the blood onto the paper.

6.3.3 Blood collection and preparation

All participants were instructed to fast overnight (10pm-8am) before attending a clinic appointment on the following morning at which blood samples were collected. At the clinic appointment, both venous and capillary blood samples were collected from each

subject by appropriately qualified and trained members of our research staff. All clinic appointments were conducted between 8 am and 9 am.

6.3.3.1 Capillary blood

A drop of blood (~30μl) from each subject was obtained from the thumb using an automatic lancing device (Unistick 2, Owen Mumford, UK). The blood was spotted on the collection area of the "PUFAcoat" blood collection paper, and air dried for 5 hrs at room temperature.

6.3.3.2 Venous blood

Venous blood samples (~6 ml) were drawn from an antecubital vein of the forearm into Vacutainer tubes containing heparin as an anticoagulant (Vacutte, greiner bio-one, Austria). At the time of blood collection, a drop of venous blood (~30μl) was spotted on the collection area of the "PUFAcoat" blood collection paper, and air dried for 5 hours at room temperature for the comparison of fatty acid composition between capillary and venous blood. For the venous whole blood sample, 500μl of whole blood were left for total lipid extraction, and the remainder of the sample was centrifuged at 3200 rpm for 15 mins at 4°C to separate plasma and red blood cells. The red blood cells were then washed three times with 0.9% sodium chloride, and the buffy coat was removed.

6.3.4 Fatty acid analysis

6.3.4.1 Fatty acid analysis of DBS samples

All DBS samples were mixed with 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex ,UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA),

and allowed to transmethylate for 3 hrs in a 70°C oven as described in Chapter 2. The resultant FAME were extracted into heptane, and injected into a GC for analysis as described in Chapter 2.

6.3.4.2 Conventional fatty acid analysis of blood fractions

Total lipids from whole blood, plasma, and red blood cells were extracted, and the phospholipid fraction of plasma and red blood cells were separated from total lipids using TLC plates as described in Chapter 2. The lipid fractions were mixed with 2ml of 1% (v/v) H_2SO_4 (18M AR grade, BDH, Sussex ,UK) in anhydrous methanol in a 5 ml sealed vial (Wheaton, Millville, USA), and allowed to transmethylate for 3 hrs in a 70°C oven as described in Chapter 2. The resultant FAME were extracted into heptanes, and injected into a GC for analysis as described in Chapter 2.

6.3.5 Statistical analyses

All statistics analyses were conducted using PASW Statistic 18. Values are expressed as mean ± SD. The Bland-Altman agreement test was used to determine the agreement level in fatty acid test results between capillary blood and venous blood, and between direct transmethylation of whole blood and whole blood total lipids extraction. Spearman's rank correlation coefficient was calculated to determine the strength of the association between capillary DBS fatty acids and the corresponding fatty acids in various lipid fractions from venous blood. One way ANOVA with comparison of individual means by Tukey's post-hoc test was used to determine differences in fatty acid composition between groups, $P<0.01$ was chosen as the level of statistical significance in all analysis.

Table 6.1 N-3 consumption characteristics of the 50 participants

	Number	Age (mean and range)	Gender (M/F)
High n-3 intake (≥3 grams of fish oil/week)	23	56 (24-75)	14/9
Moderate n-3 intake (Seldom fish oil consumption, but ≥1 serving of fish/week)	14	42 (23-59)	5/9
Low n-3 intake (Seldom fish oil consumption, and<1 serving of fish/week)	13	35 (23-54)	5/8

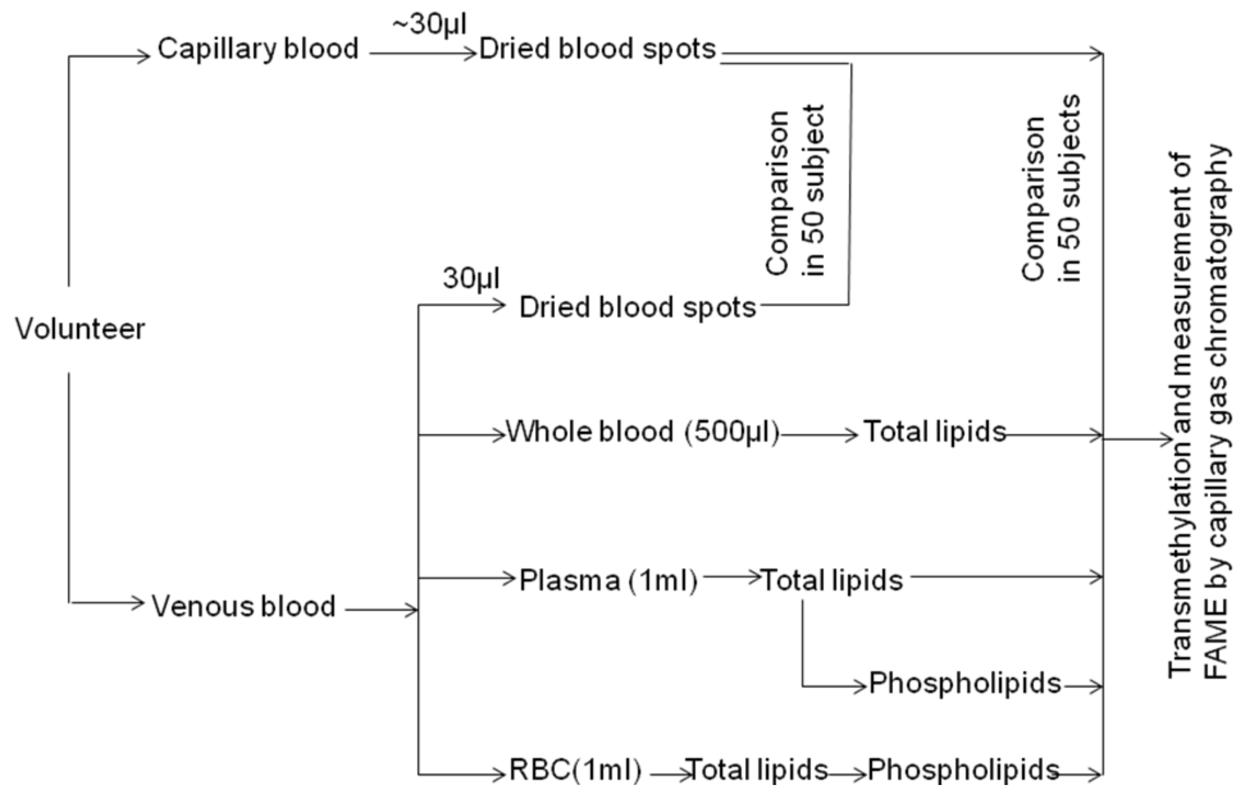


Figure 6.1. Study design for evaluating of my DBS method on human subjects

6.4 Results

6.4.1 Comparison of capillary DBS and venous DBS

Comparison of the fatty acid composition between capillary DBS and venous DBS on 50 healthy subjects showed very good agreement between the fatty acid composition of two whole blood sources (Figure 6.2). The geometric mean ratio of EPA+DHA% vaules (expressed as a weight percentage of total fatty acids) measured by capillary blood and venous blood was 1.00 with 95% limits of agreement 0.98 to 1.02. This indicated that capillary blood sample provide equivalent results for n-3 fatty acid status in humans as venous blood sample.

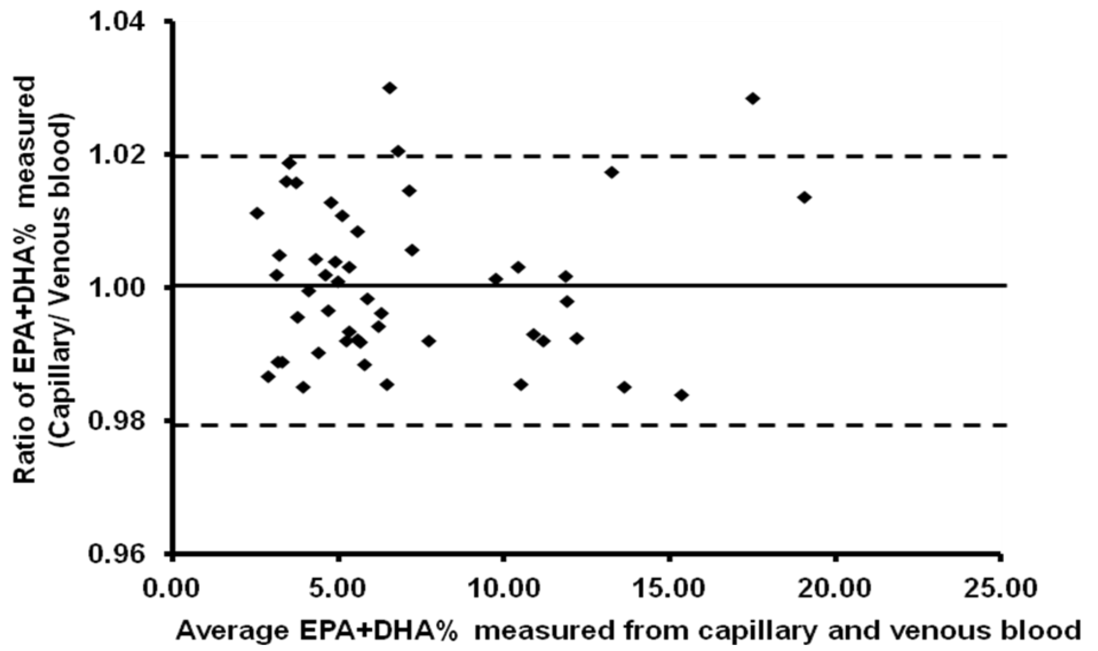


Figure 6.2. Ratio of EPA+DHA% measured from capillary blood and venous blood plotted against their average.

6.4.2 Comparison fatty acid composition obtained from different blood fractions

The levels of all major fatty acids of interest in capillary DBS did not differ significantly from that of whole blood total lipid extract (TLE) (Table 6.2). The geometric mean ratio of EPA+DHA% vaules measured by capillary DBS and venous whole blood TLE was 0.96 with 95% limits of agreement 0.92 to 1.00 (Figure 6.3).

The mean content for the majority of fatty acids in whole blood sample (both capillary and venous) differed significantly from those measured in plasma total lipid extracts, plasma phospholipids (PL) and red blood cell PL. The only exception was EPA, which did not exhibit any significant differences between different sample types. Plasma TLE showed the highest level of total n-6 PUFA, and the lowest level of total n-3 PUFA and SFA among all blood lipid fractions. The LA and the ALA levels in plasma TLE were significantly higher than those in other blood fractions, whereas the stearic acid, AA, adrenic acid (22:4 n-6), DPA and DHA levels were significantly lower.

Plasma PL showed a significantly higher percentage of SFA and lower percentage of MUFA when compared with other blood lipid fractions. The palmitic acid and stearic acid in plasma phospholipids were significantly higher than those in other blood lipid fractions, whereas the oleic acid, ALA and adrenic acid levels were significantly lower (Table 6.2). Red blood cells PL had the lowest levels of n-6 PUFA and the highest levels of n-3 PUFA. The AA, DPA, and DHA in red blood cells PL were higher than in other blood lipid fractions analysed, whereas the LA and ALA levels in red blood cells PL were significantly lower (Table 6.2). The n-6 PUFA/n-3 PUFA

ratio was highest in plasma TLE and lowest in red blood cell PL, whereas a moderate ratio was observed in capillary DBS.

Table 6.2. Fatty acid profiles of lipids in 50 subjects measured from capillary DBS, venous whole blood, plasma and red blood cells

Fatty acids	Capillary DBS	Whole blood TLE	Plasma TLE	Plasma PL	Red Blood Cell PL
16:0	[*]23.4±1.8[a]	23.6±2.0[a]	23.3±2.5[a]	29.4±1.9[c]	25.4±1.3[b]
18:0	11.7±1.2[b]	10.9±1.1[b]	7.4±0.9[a]	14.3±1.9[d]	13.7±1.4[d]
SFA	35.1± 1.9[b]	34.5±1.9[b]	30.7±2.5[a]	43.7±2.4[d]	39.0±1.4[c]
16:1 n-7	0.6±0.04[a]	0.6±0.05[a]	0.7±0.11[a]	0.4±0.02[b]	0.3±0.03[c]
18:1 n-9	19.0±2.8[a]	19.0±3.0[a]	20.8±3.9[a]	10.5±2.5[c]	15.7±1.7[b]
MUFA	19.6±2.7[a]	19.6±2.6[a]	21.5±3.7[a]	11.0±2.4[c]	16.0±1.5[b]
18:2 n-6(LA)	26.7±4.2[b]	27.0±4.6[b]	34.3±5.8[c]	24.4±4.7[b]	14.3±2.8[a]
20:4 n-6(AA)	9.1±1.6[b]	9.1±1.7[b]	6.5±1.2[a]	9.5±2.0[b]	14.1±2.4[c]
22:4 n-6	0.9±0.3[b]	1.0±0.3[b]	0.2±0.04[a]	0.3±0.12[a]	2.9±1.0[c]
n-6 PUFA	36.7± 4.9[b]	38.0±5.3[b]	40.9±6.0[c]	35.2±5.2[b]	31.3±5.2[a]
18:3 n-3(ALA)	0.5±0.1[b]	0.5±0.1[b]	0.7±0.2[c]	0.2±0.1[a]	0.2±0.1[a]
20:5 n-3(EPA)	2.5± 2.4	2.8±2.5	2.8±2.4	3.2±2.5	2.7±2.3
22:5 n-3(DPA)	1.8±0.5[b]	1.8±0.5[b]	0.8±0.3[a]	1.4±0.4[b]	3.9±1.1[c]
22:6 n-3(DHA)	4.4±1.3[b]	4.5±1.5[b]	3.3±1.4[a]	5.7±1.9[c]	7.3±1.9[d]
EPA+DHA	6.9±3.3[a]	7.3±3.5[a]	6.1±3.7[a]	8.9±4.0[ab]	9.9±3.3[b]
n-3 PUFA	9.2±3.3[b]	9.5±3.6[b]	7.6±3.7[a]	10.6±4.0[b]	14.0±3.8[c]
Total PUFA	45.9±3.5[a]	46.5±3.8[ab]	48.5±5.2[b]	45.8±2.8[a]	45.3±2.1[a]
AA/EPA	8.4 ± 4.9[ab]	7.3±5.1[ab]	5.3±3.9[a]	6.4±4.9[a]	10.7±6.9[b]
n-6 /n-3	4.9±2.0[b]	4.9±2.1[b]	7.2±3.4[c]	4.2±2.0[b]	2.6±1.0[a]

[*]Values in the same row with different superscripts indicate values which are significantly different between groups, $P<0.01$.

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.; TLE, total lipid extract; PL, phospholipids

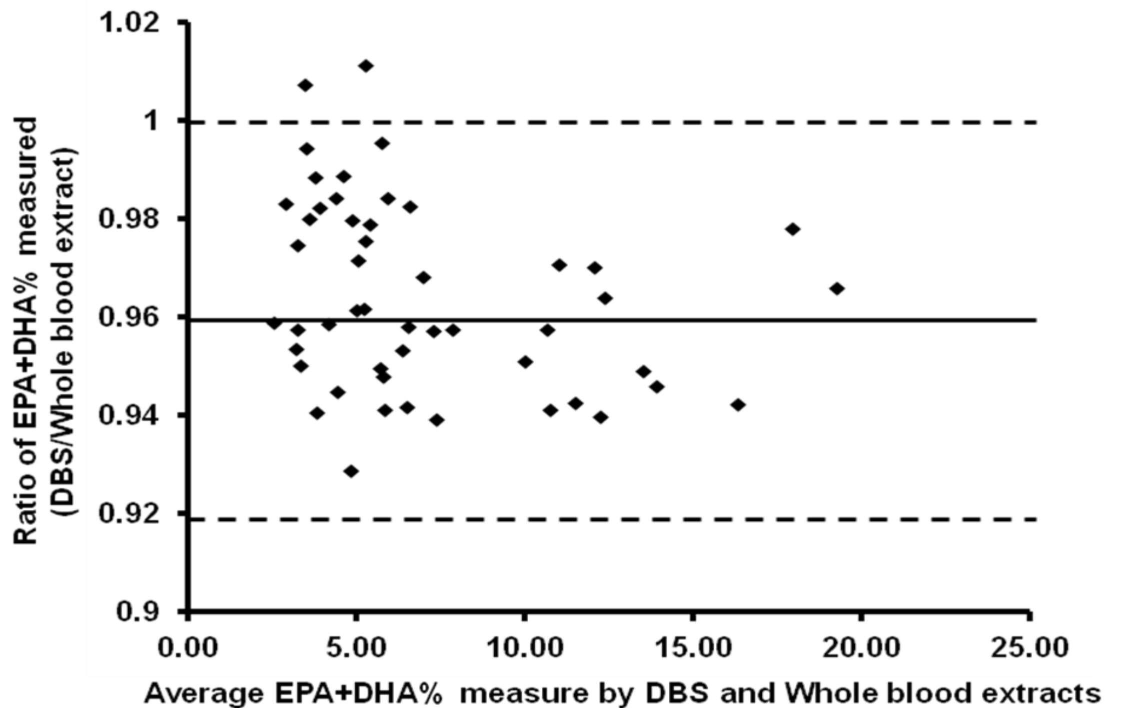


Figure 6.3. Ratio of EPA+DHA% measured from capillary blood and venous blood plotted against their average.

6.4.3 Correlations between fatty acids values obtained from DBS and conventional measurements

The correlation coefficients for the relationships of fatty acid composition between capillary DBS assay and various conventional measurements are summarised in Table 6.3. In general, strong correlations were observed between the fatty acid results from capillary DBS and venous whole blood TLE for the same fatty acid (r >0.9) (Table 6.3). Similarly, the majority of the fatty acids from capillary DBS correlated well with the corresponding fatty acids measured from plasma TLE, plasma PL and red blood cells PL (Table 6.3). Of all fatty acids evaluated, n-3 LCPUFA (EPA, DPA and DHA) in capillary DBS samples exhibited the strongest correlation (r >0.8) with the corresponding fatty acids from all of the respective blood fractions measured by conventional assays (Figures 6.4-6.7). There was a particularly tight relationship between EPA (r = 0.998) and DHA (r = 0.996) levels measured using capillary DBS assay and conventional assay of venous whole blood TLE (Figure 6.4).

Correlations of n-3 biomarkers, including "Omega-3 Index", AA/EPA ratio, and n-6/n-3 ratio were also determined and compared between capillary DBS and whole blood TLE, plasma TLE, plasma PL and red blood cells PL. In general, values for n-3 biomarkers obtained from capillary DBS were strongly correlated with those from conventional measurements (r >0.9) (Table 6.3). The AA/EPA ratio in capillary DBS was significantly corrlated with the AA/EPA ratio measured in various blood lipid fractions by conventional assays, and the coorelation coefficients tended to be higher than those for the n-6/n-3 ratio. Furthermore, the EPA+DHA content in the capillary DBS was significantly correlated (r = 0.972) with the "Omega-3 Index" (sum of

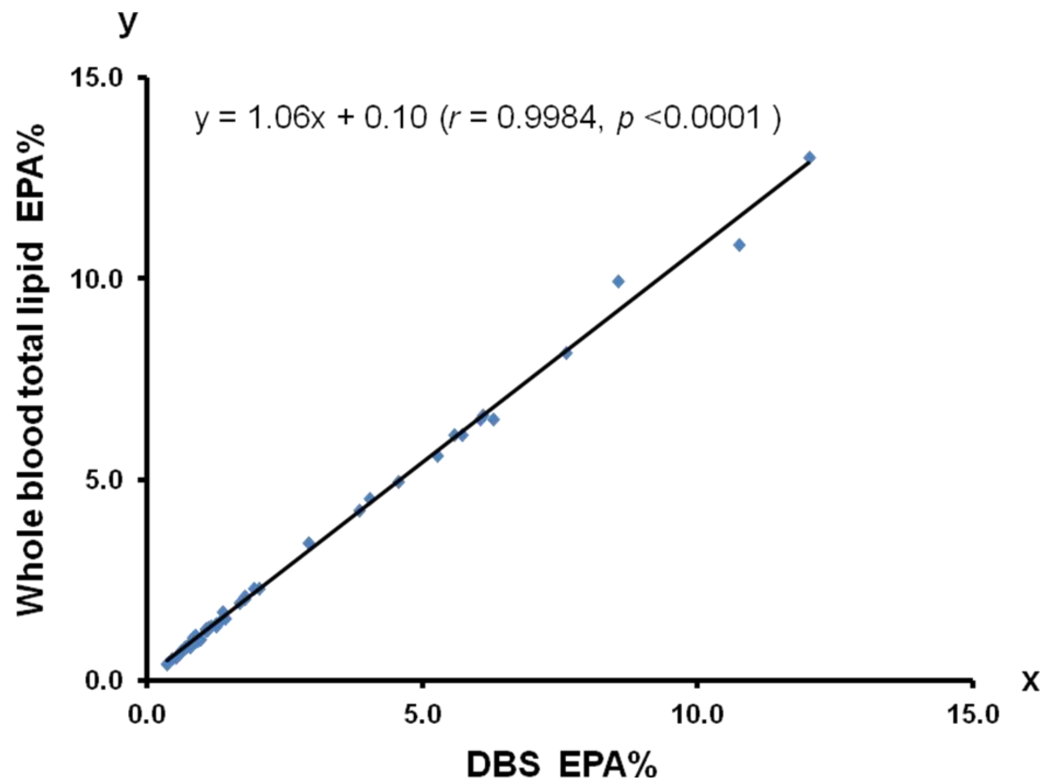
EPA+DHA% in red blood cell PL) which is used as a biomarker for the risk factor of coronary heart disease (Harris and von schacky *et al.* 2004) (Figure 6.8).

Table 6.3. Correlation for fatty acid composition between capillary DBS and conventional assays

Capillary DBS fatty acids	Corresponding fatty acids in venous blood fractions[a]			
	Whole blood TLE	Plasma TLE	Plasma PL	Red Blood Cell PL
16:0	0.961**	0.962**	0.771**	0.626**
18:0	0.938**	0.843**	0.551**	0.7**
16:1 n-7	0.946**	0.878**	0.623**	0.511*
18:1 n-9	0.982**	0.971**	0.463*	0.674**
18:2 n-6 (LA)	0.991**	0.854**	0.896**	0.824**
18:3 n-3 (ALA)	0.951**	0.927**	0.578**	0.441*
20:4 n-6 (AA)	0.993**	0.85**	0.693**	0.754**
20:5 n-3(EPA)	0.998**	0.995**	0.988**	0.973**
22:4 n-6	0.980**	0.587**	0.436*	0.889**
22:5 n-3 (DPA)	0.915**	0.878**	0.838**	0.847**
22:6 n-3(DHA)	0.996**	0.976**	0.964**	0.971**
EPA+DHA	0.996**	0.982**	0.962**	0.972**
AA/EPA	0.993**	0.970**	0.974**	0.955**
n-6 FA/n-3 FA	0.993**	0.936**	0.927**	0.925**

TLE, total lipid extract; PL, phospholipids
[a] Spearman's rank correlation coefficient
* P <0.001, ** P <0.0001

A.



B.

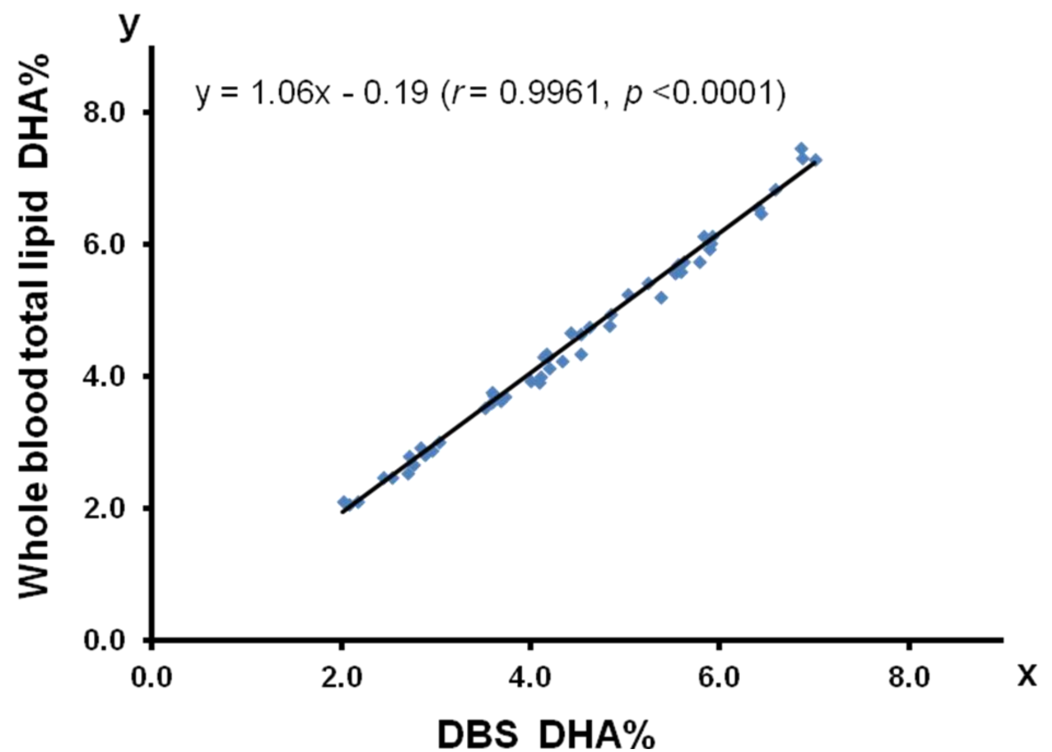
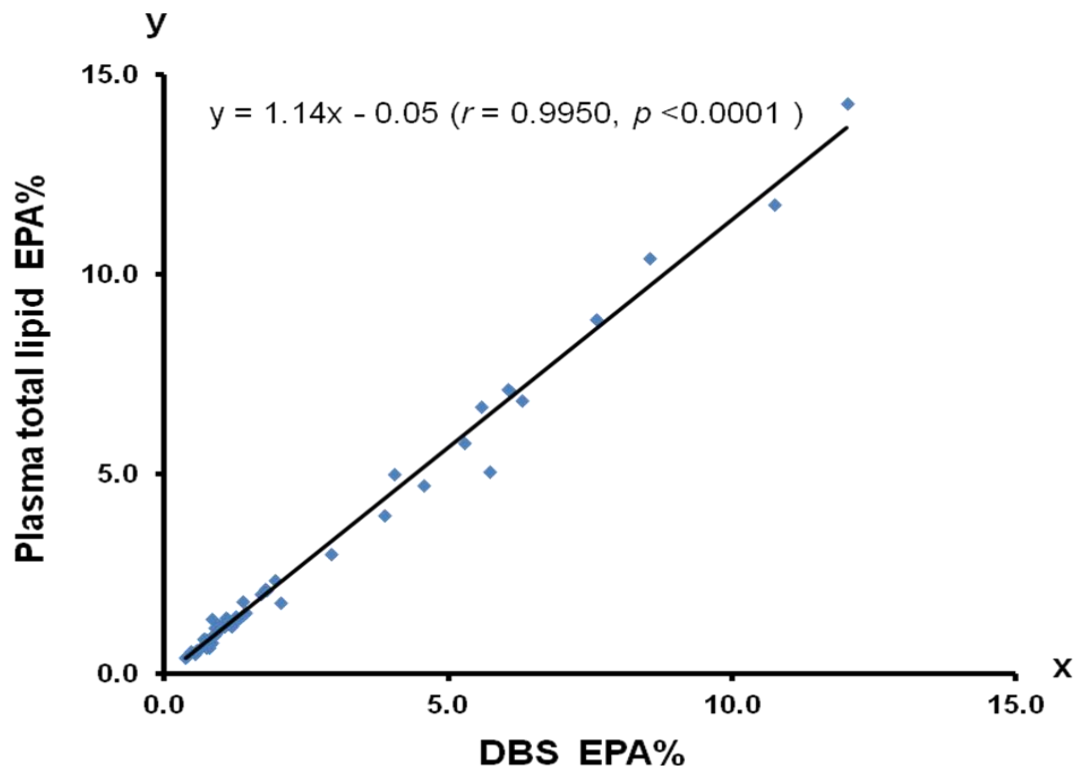


Figure 6.4. Correlation for EPA and DHA content between capillary DBS and whole blood extracted total lipids. A). EPA content in DBS(x axis) vs. in whole blood extracted total lipids (y axis); B). DHA content in DBS(x axis) vs. in whole blood extracted total lipids (y axis).

A.



B.

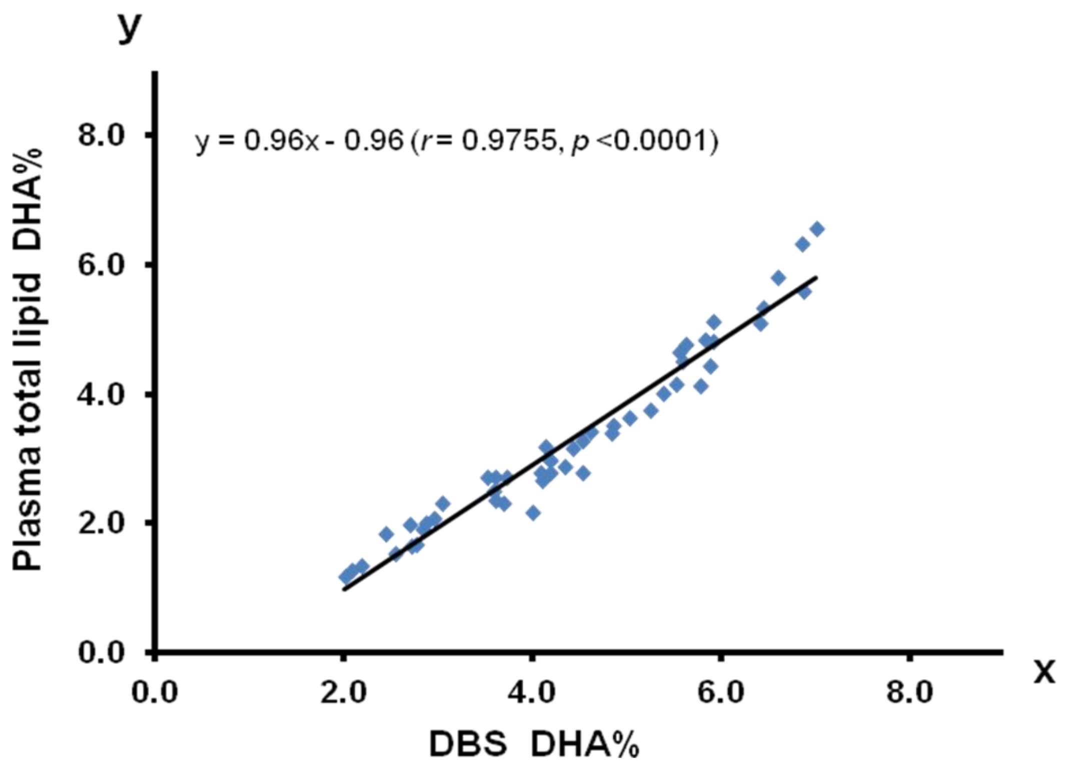
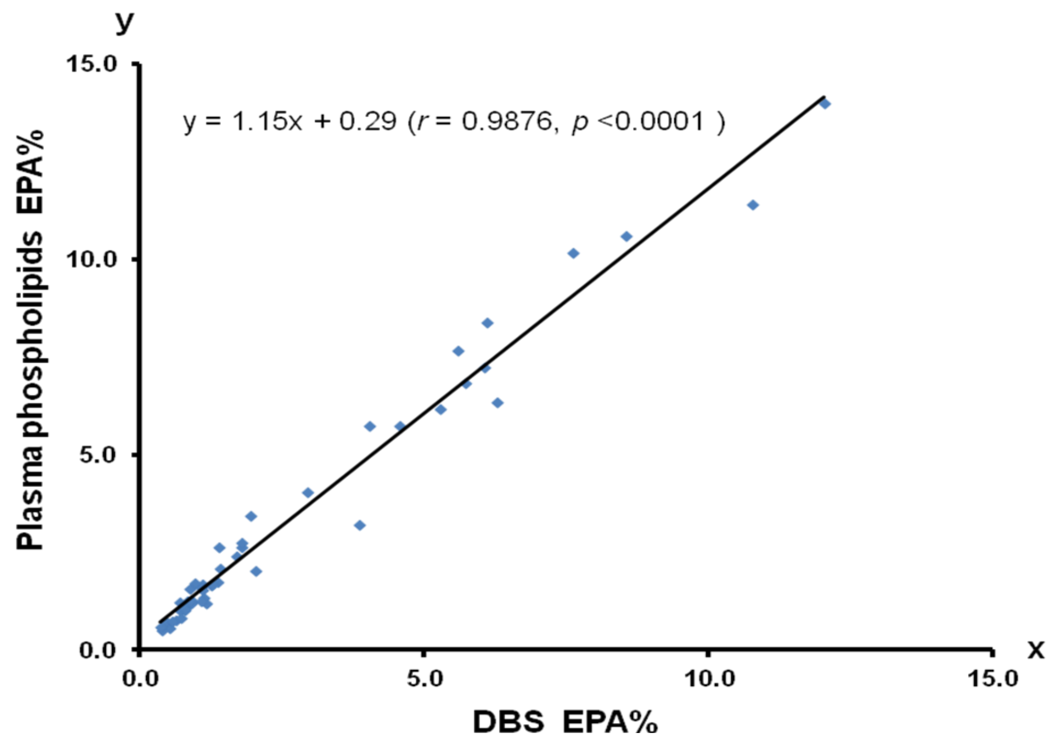


Figure 6.5. Correlations for EPA and DHA content between capillary DBS and plasma total lipids. A). EPA content in DBS(*x* axis) vs. in plasma total lipids (*y* axis); B). DHA content in DBS(*x* axis) vs. in plasma total lipids (*y* axis).

A.



B.

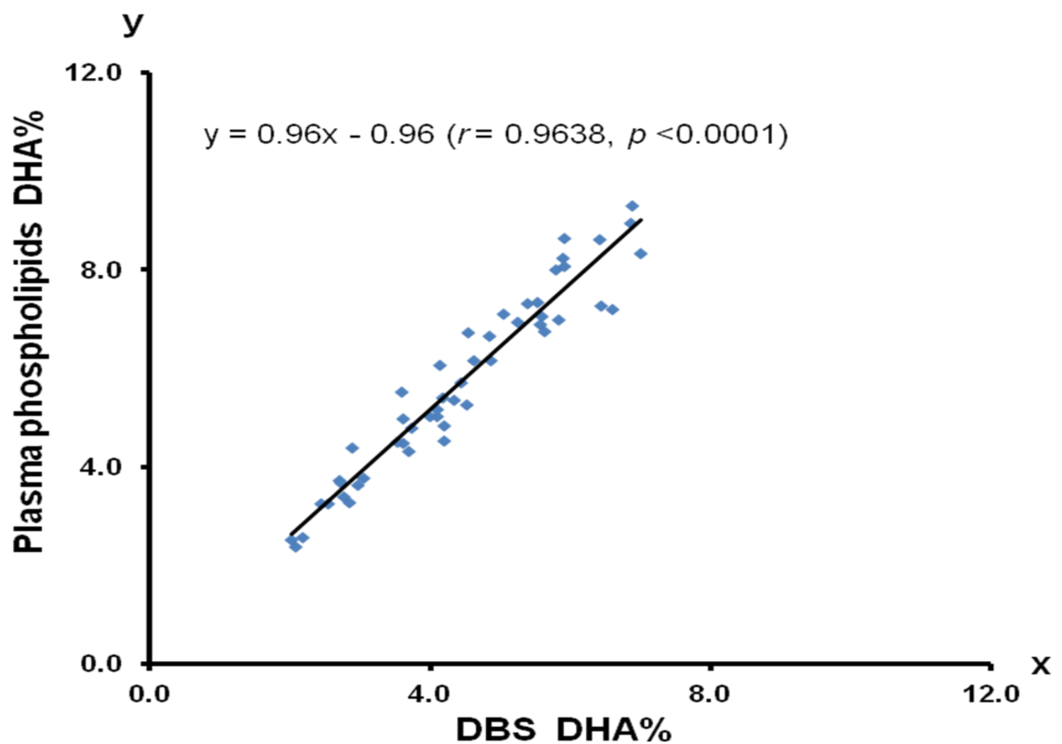
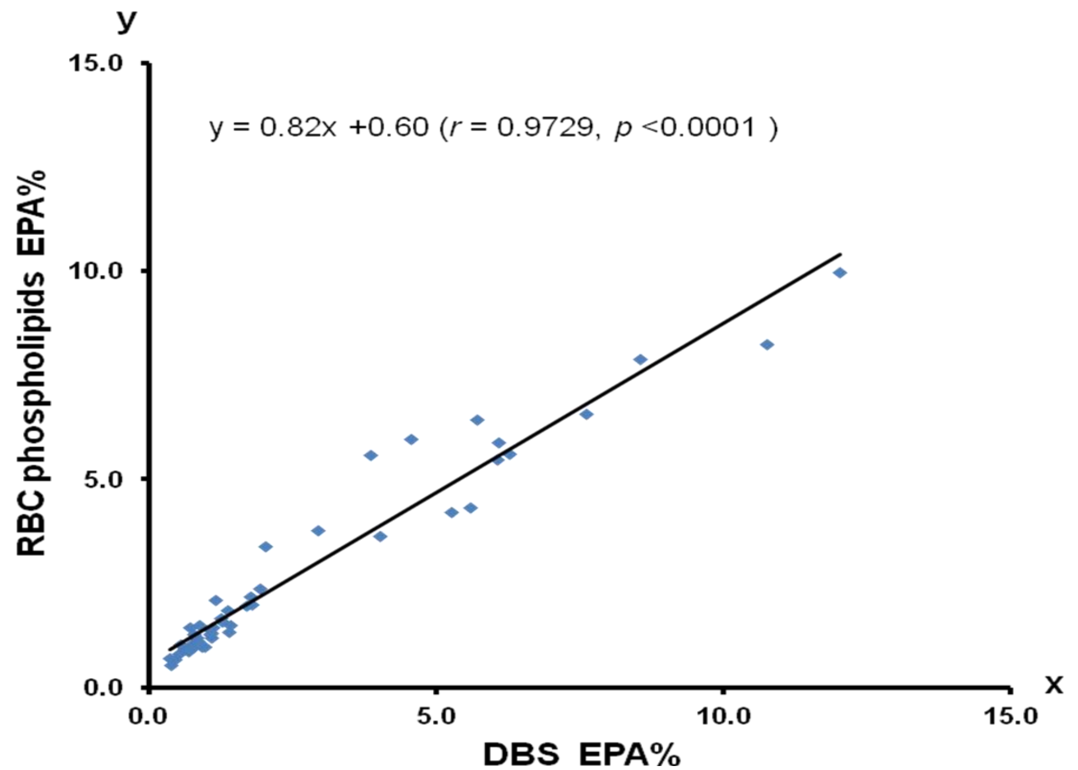


Figure 6.6. Correlations for EPA and DHA content between capillary DBS and plasma phospholipids. A). EPA content in DBS (*x* axis) vs. in plasma phospholipids (*y* axis); B). DHA content in DBS(*x* axis) vs. in plasma phospholipids (*y* axis).

A.



B.

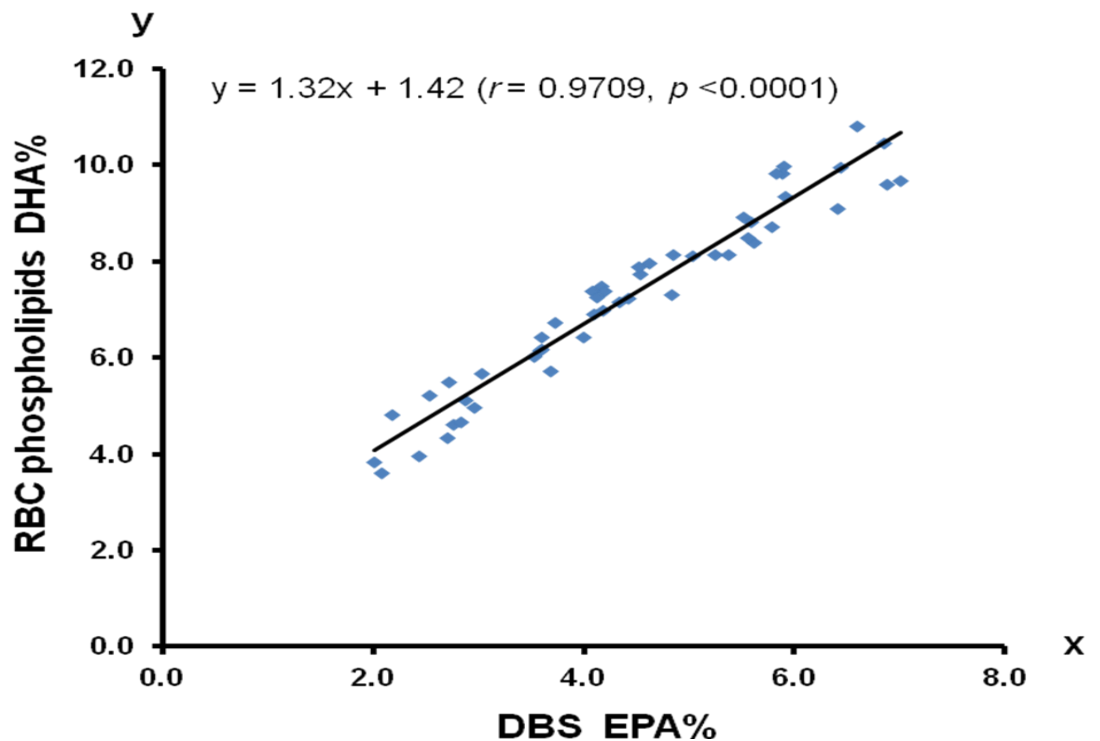


Figure 6.7. Correlations for EPA and DHA content between capillary DBS and red blood cells phospholipids. A). EPA content in DBS(*x* axis) Vs. in red blood cells (RBC) phospholipids (*y* axis); B). DHA content in DBS(*x* axis) Vs. in red blood cells (RBC) phospholipids (*y* axis).

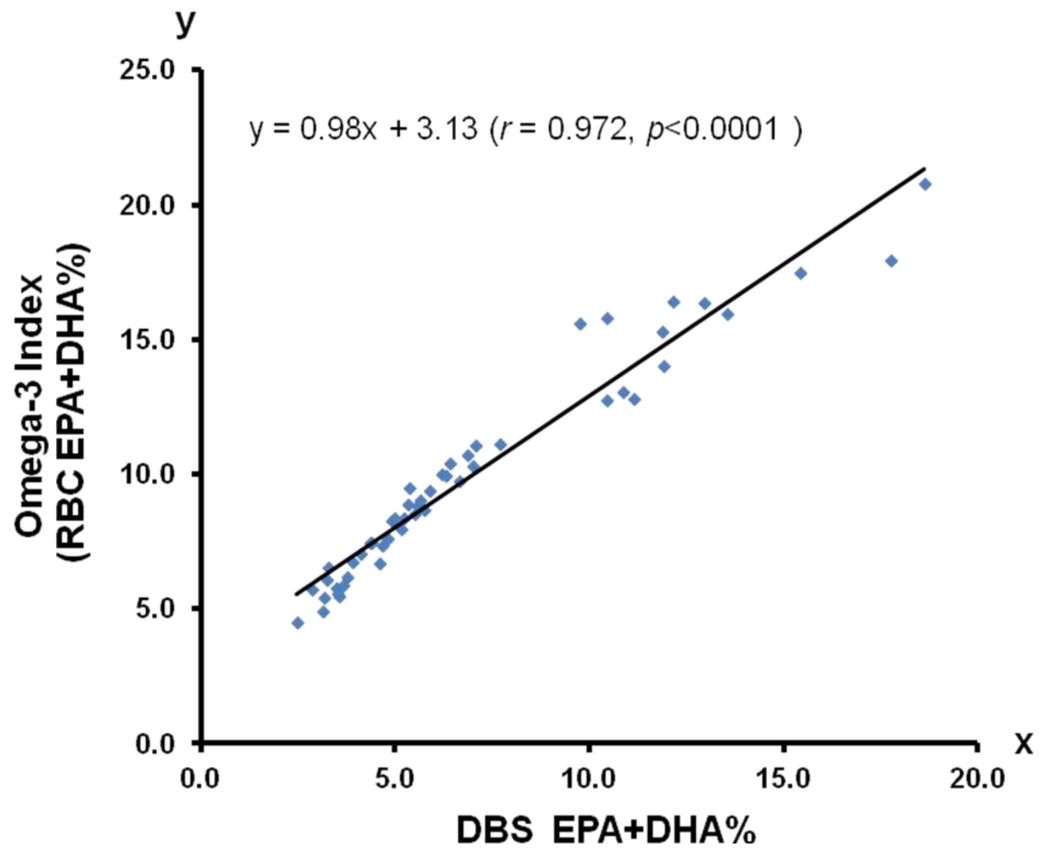


Figure 6.8. Correlation for level of EPA + DHA between capillary DBS (*x* axis) and Omega-3 Index (red blood cells EPA+DHA) (*y* axis).

6.4.4 Fatty acid profiles in DBS reflect dietary parameters

An important secondary aim of this experiment was to verify whether my DBS method would be an appropriate indicator of dietary fatty acid intakes, and I therefore assessed the relationships between whole blood n-3 status and habitual n-3 fatty acid intake as reported by dietary questionnaire.

On the basis of the data provided by the dietary questionnaire, the participants were divided into 3 subgroups corresponding low, moderate and high reported consumption of fish or fish oil supplements in the preceding 6 months. Based on this dietary information, 23 of the 50 subjects regularly consumed fish oil supplements (≥3 grams per week) in the preceding 6 months (high n-3 intake), whereas 27 participants seldom or never took fish oil supplements (<1 gram per week) during the same periods. Among the 27 participants who seldom or never took fish oil supplements, 14 of them regularly consumed higher amounts of fish (≥1 serving per week) (moderate n-3 intake) in the preceding 6 months, while the remaining 13 participants had a relatively low fish consumption (<1 serving per week) (low n-3 intake). The average fatty acid composition of the capillary DBS from all subjects and the levels of EPA and DHA in the 3 subgroups (corresponding to low, moderate and high n-3 LCPUFA intakes) are presented in Table 6.4. The subjects who consumed fish oil supplements of ≥3 grams per week showed significantly higher levels of both EPA and DHA in their capillary whole blood than the subjects who consumed <1 gram per week (Figure 6.9). Among those participants who had seldom or never took fish oil during the preceding 6 months, those subjects who reported having a higher dietary intake of fish (≥1 serving per week) were had significantly higher levels of DHA in their capillary whole blood when compared with those whose reported intake of fish was

<1 serving per week (Figure 6.9B). However, there was no difference in EPA levels between the subjects who had seldom took fish oil irrespective of their reported level of dietary fish intakes (Figure 6.9A).

Table 6.4. Fatty acid composition of capillary DBS from 50 participants and levels of EPA and DHA in subgroups

Fatty acids	Capillary DBS (n=50)	Regular Fish oil intake (n=23) (≥3 grams/week)	Seldom Fish oil intake (n=27) (<1 gram/week)	
			High fish consumption (n=14) (≥1 serving/week)	low fish consumption (n=13) (<1 serving/week)
20:4 n-6(AA)	9.1 ± 1.6	8.3 ± 1.5	9.9 ± 1.1	9.9 ± 1.6
20:5 n-3(EPA)	2.5 ± 2.4[b]	5.0 ± 3.1[a]	1.1 ± 0.3[b]	0.7 ± 0.2[b]
22:6 n-3(DHA)	4.4 ± 1.3[b]	5.6 ± 0.9[a]	4.2 ± 0.7[b]	2.9 ± 0.7[c]
AA/EPA	8.4 ± 4.9[b]	4.0 ± 2.1[a]	9.5± 2.0[b]	15.0 ± 3.9[c]

[*]Values with different superscripts are statistically significant different between groups, *P*<0.01.

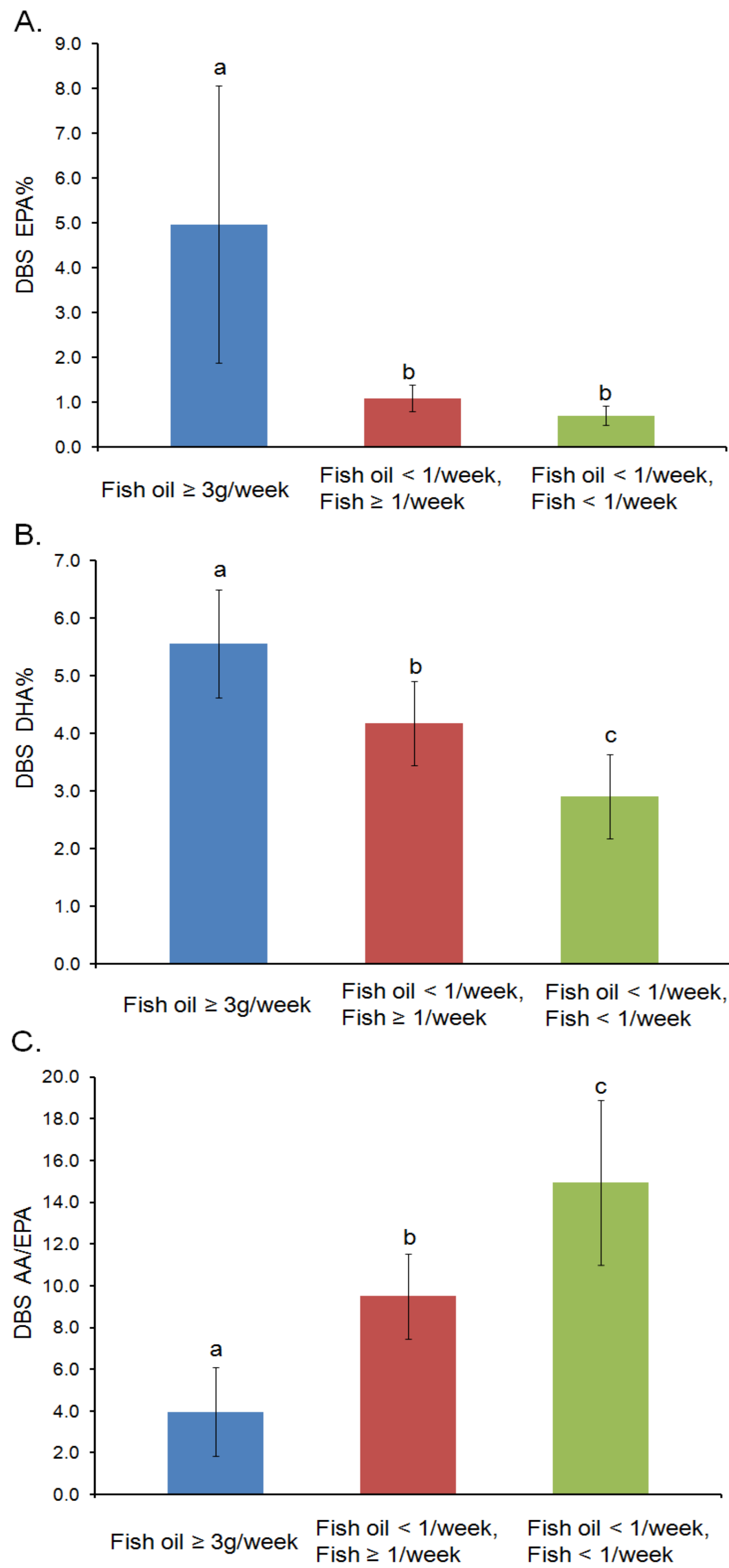


Figure 6.9. EPA (A), DHA (B), and AA/EPA (C) levels in capillary DBS of self-declared low and high fish or fish oil consumers. Values with different superscripts are statistically significant different between groups, P<0.01.

6.5 Discussion

In the present study, I have compared the human blood fatty acid test results obtained from my rapid and robust DBS method with those obtained using conventional measurements. In line with previous studies (Min *et al.* 2011; Arm strong *et al.* 2008), very good agreeemnt were observed between the n-3 fatty acid status of capillary whole blood and venous whole blood (Figure 6.2), confirming the suitability of capillary whole blood for evaluating of n-3 fatty acid status in humans. A similar comparison was also made between the fatty acid results obtained from venous whole blood total lipid extraction and direct transmethylation of capillary DBS (Table 6.2, Figure 6.3). This showed no significant differences in the fatty acid results obtained from whole blood analysis with or without lipid extraction (whole blood TLE vs. direct transmethylation of capillary DBS), indicating that the two methods provide comparable fatty acid results. This is consistent with previous studies (Marangoni *et al.* 2004; Armstrong *et al.* 2008).

As expected, I showed that the levels of majority fatty acids of interest differed between blood fractions (whole blood, plasma, red blood cells) (Table 6.2). These differences reflect the differing amount of free fatty acids, steryl esters, triglycerides (TG) and PL in each blood fraction (Rice *et al.* 2007). For example, the percentage of essential fatty acids that are derived exclusively from the diet, such as LA and ALA, are higher in plasma than in red blood cells (Table 6.2). This is due to the fact that the TG and cholesterol esters both of which are influenced by the type and amount of fat consumed during recent meals, represent the major lipid classes in plasma, but are almost absent in red blood cells (Hodson *et al*. 2008). The levels of AA and DHA were much higher in red blood cell PL than in other blood fractions (Table 6.2). This

is consistent with previous reports and relates to the fact that the predominant lipid class in red blood cells membranes is PL, which contain higher proportion of AA and DHA than TG (Dodge and Phillips 1967). The results presented in this Chapter demonstrated that both capillary DBS and venous whole blood TLE showed relatively balanced levels of the majority fatty acids of interest when compared with the corresponding fatty acid levels in plasma and red blood cells lipid fractions. The capillary DBS method is less invasive than venous blood collection, and considerably reduces the analytical time and cost when compared with conventional assays. This makes it the ideal choice for screening the blood n-3 status in large numbers of subjects.

In line with previous studies (Bailey-Hall, *et al.* 2008; Min *et al.* 2011), strong correlations (r >0.8) were observed between fatty acid results obtained from the capillary DBS assay and conventional measurements in my study for the majority of LCPUFA, and particularly for EPA and DHA (r >0.95). Similar trends were also observed for some of the most commonly used n-3 biomarkers. For example, the n-6/n-3 ratio in plasma and red blood cells PL is used for evaluating the balance of n-6 and n-3 fatty acids in humans (Lands *et al.* 1992; Orchard *et al.* 2012). In the present study, I found a correlation of 0.927 and 0.925 between the n-6/n-3 ratio in the capillary DBS vs. plasma PL and capillary DBS vs. red blood cells PL respectively. Based on these correlations, I developed equations to enable me to convert the fatty acid status obtained using my DBS method to the equivalent conventional measurements (Figure 6.4-6.8), which allow for a direct comparison of the fatty acid values obtained from DBS analysis with those reported from analyses carried out using conventional assays.

The fatty acid status measured from DBS has been correlated with physiological parameters, lifestyle factors, and disease risk in previous studies (Agostoni *et al.* 2008b; Marangoni *et al.* 2007; Risé *et al.* 2010). In a study by Agostoni *et al.* it was demonstrated that preterm infants had lower DBS DHA levels when compared with term infants (Agostoni *et al.* 2008b). Marangoni *et al.* also reported a 30% lower level of capillary DBS DHA in smokers (≥5 cigarettes per day) when compared with non-smokers (Marangoni *et al.* 2007). A similar trend was also found in infants that were born to mother who smoked in a case-control study (Agostoni *et al.* 2008a). Furthermore, the levels of DBS LA and DHA in patients with cystic fibrosis were significantly lower than in healthy control subjects (Risé *et al.* 2010), and this is in line with previous observations in plasma PL and red blood cells membrane lipids (Strandvik *et al.* 2001; Olveira *et al.* 2006).

I demonstrated in this Chapter that the fatty acid results obtained using my DBS method reflected habitual dietary fatty acid intake as assessed by a dietary questionnaire of fish or fish oil intake of the preceding 6 months period. The highest levels of EPA and DHA were present in DBS in subjects who consumed fish oil regularly (≥3 grams per week), confirming that the differences of EPA and DHA levels in capillary DBS reflect fish oil consumption. DHA, abundant in fish, is considered the fatty acid most influenced by dietary fish intake (Marangoni *et al.* 2004). In my study, the subjects who ate fish frequently (≥1 servings per week) showed significantly higher levels of DHA in the capillary DBS than those who did not (<1 serving per week). A lower AA/EPA ratio (from 1.5:1 to 6:1) is suggested to be good for health (Sears *et al.* 2008), because EPA suppresses and counteracts the

activity of AA-derived pro-inflammatory eicosanoids by generating its own less inflammatory (Prickett *et al.* 1981) or even anti-inflammatory eicosanoids (Kasuga *et al.* 2008; Serhan *et al.* 2009). However, the typical Western diet contains a high ratio of n-6 to n-3 fatty acids (15:1 to 25:1), due to the increased intake of meat/oil/spreads rich in n-6 and decreased dietary consumption of fish (Simopoulos 2002; Guebre-Egziabher 2008). Consistent with reports in other Western populations (Rizzo et al 2010; Holub *et al.* 2009), healthy subjects who seldom take fish or fish oil supplements had a relatively high AA/EPA ratio (~15:1) in whole blood in the present study. Conversely, a much lower AA/EPA ratio were observed in subjects who consumed fish oil supplements (~4:1) or reported regular fish consumption (~10:1) (Table 6.4). The differences in fatty acid status in participants who reported different patterns of fish or fish oil intake demonstrates the suitability of my capillary DBS method for assessing habitual consumption of n-3 fatty acids in humans. However, since self-reported fish and fish oil consumption cannot be considered as an exact quantitative measurement, further assessment is required.

The use of subjects with a very wide range of fish oil consumption (0-15ml fish oil per day) in my study is a possible limitation. Since it was associated with substantial variations in the fatty acid levels between subjects, which could have obscured differences in some specific n-3 fatty acid levels between the various blood fractions. For example, EPA levels in my study was not significantly different between blood lipid fractions, and this is probably due to the huge variation of EPA levels (almost 100%) between individual subjects in all blood lipid fractions (Table 6.2). However, the wide range of n-3 LCPUFA status in the study population can also be viewed as a strength, since the high correlations in fatty acid status between capillary DBS and

various blood fractions confirmed that my DBS method is suitable for fatty acid testing in subjects with a broad range of n-3 fatty acid status.

6.6 Summary

This Chapter presents the validation of a rapid, inexpensive and robust DBS method for determining of the fatty acid status in humans against conventional methodology. The equations developed from correlations between my DBS method and conventional measurements enable the direct comparison of DBS fatty acid status with results obtained using conventional measurements, which is important for comparison between studies. The analysis of capillary DBS samples may offer profound advantages when large numbers of study participants are involved, since its application does not necessitate the involvement of professional operators in blood sampling, and remarkably simplifies the analytical procedure when compared with conventional assays. Moreover, my DBS method may provide useful information on the habitual consumption of n-3 fatty acid in population groups, and therefore has significant potential for application in large-scale clinical or epidemiological studies focused on the role of n-3 fatty acids consumption in human health.

Chapter 7

General Discussion

The "holy grail" of fatty acid research has been the development of a DBS technique as a quick, inexpensive and minimally invasive tool for the measurement of fatty acid status in humans. There have been many published attempts and several systems have been commercialised (Ichihara *et al*. 2002; Marangoni *et al.* 2004; Bell *et al*. 2011). However, my investigations and the work of others have shown that most systems are not sufficiently robust to be accepted by the lipid community for general use. The shortcomings of existing systems are partly due to the fatty acid contaminants released from blood collection papers during sample processing (Nishio *et al*. 1986; Liu *et al*. 2010), and particularly due to the oxidative loss of LCPUFA in DBS samples during transportation and storage (Min *et al.* 2011; Bell *et al.* 2011).

This thesis is the first report of a three component protection system that is capable of stabilising the LCPUFA in human DBS samples for extended periods when stored at room temperature. This system, termed "PUFAcoat", comprises a combination of an antioxidant and an iron chelator absorbed onto a silica gel coated paper as the collection matix. The "PUFAcoat" system was found to prevent any significant loss of LCPUFA content in DBS samples following 9 weeks of storage at room temperature. This represents a significant improvement in stability when compared with all other reported DBS protection systems which tend to use BHT as the antioxidant and standard filter paper as a collection medium, where losses of up to 40% of the n-3 LCPUFA have been reported over the same period of storage at room temperature (Min *et al.* 2011; Bell *et al.* 2011).

The potential of our “PUFAcoat” system was further illustrated by the experimental data in Chapter 4, which demonstrated that the “PUFAcoat” paper could be produced at least 2 months prior to blood collection. The long shelf-life of the “PUFAcoat” paper means that collection papers could be impregnated during manufacture rather than adding the protectant solution immediately before blood collection which elimates a time consuming, cumbersome and error-prone procedure. Since this thesis only studied the shelf-life of pre-made “PUFAcoat” paper for 2 months, it wouldl be important in future studies to determine whether the shelf-life can be extended further without affecting the quality of the results.

The fact that the “PUFAcoat” system was so effective in preventing the oxidative loss of n-3 LCPUFA also provided me an opportunity to gain further understanding of the processes responsible for the breakdown of LCPUFA in DBS. By developing a novel *in vitro* system as described in Chapter 5, it was demonstrated that the rapid degradation of LCPUFA in DBS was mainly a result of iron-induced peroxidation, and the efficiency of the “PUFAcoat” system was due to the ability of the chelating agent to sequester the iron in the DBS. Although the use of *in vitro* model for studying the iron chelating properties of compounds has been documented in previous studies (Kontoghiorghes *et al.* 1986; Mladěnka *et al.* 2011; Das *et al.* 2012), my *in vitro* model showed for the first time that exogenous iron treatment significantly accelerated the oxidative loss of LCPUFA absorbed on blood collection papers, and that this degradation could be eliminated by pre-treating the collection paper with chelating agent. Furthermore, I demonstrated using my *in vitro* model that there were obvious differences in the protective capacity between different types of iron chelators, which suggests that the *in vitro* model provides a suitable tool for rapid comparison of

the metal-chelating efficiency of different chelators.

The clinical applicability of the “PUFAcoat” blood collection paper was validated in a clinical study reported in Chapter 6. The close correlations between the fatty acid status determined using capillary DBS (collected on “PUFAcoat” paper) with conventional blood fractions confirmed that the “PUFAcoat” paper is suitable for fatty acid testing in subjects with a broad range of n-3 fatty acid levels. The mathematical equations generated from the clinical study allow the quick conversion of a fatty acid values from capillary DBS to a range of values for standard lipid fractions (whole blood total fatty acids, plasma total fatty acids, plasma PL and red blood cell PL). This enables researchers to directly compare the fatty acid status obtained from capillary DBS with those of standard lipid fractions to answer research questions, and relate the DBS results to fatty acid levels reported in previous studies. Furthermore, n-3 status of subjects was shown to reflect their habitual dietary fish and fish oil consumption as assessed using the dietary recall questionnaire. This indicated the ability of using the “PUFAcoat” paper as a tool for assessing dietary n-3 fatty acids intake in humans. However, since self-reported fish and fish oil consumption cannot be considered as an exact quantitative measurement, further assessment is required to confirm. In addition, the time it takes for changes in n-3 fatty acid intake to be reflected in changes in capillary DBS n-3 status is still unclear.

Several commercial companies have already marketed DBS test produts for measuring the n-3 fatty acid status of individuals who are concerned about their health status. However, the oxidative losses of the n-3 fatty acids during sample transportation and storage severely limit the clinical relevance of these commercial

DBS methodologies. My DBS method has a much better ability to stabilise the n-3 fatty acids than any existing DBS system, and thus has potential to replace all current test kits in the market and provide great benefits to the clinical diagnostics industry. These benefits will flow on to individuals who have a low n-3 status as they can choose to change their diet to increase the consumption of the beneficial n-3 fatty acids (via seafood or fish oil) and routinely monitor the improvement in their n-3 status.

The DBS technique also represents a much more cost effective, easy to use, and portable platform for blood sample analysis compared to conventional approaches. A comparison of the cost of fatty acid analysis between capillary DBS sample and conventional venous blood sample is presented in table 7.1. When compared with conventional venous blood samples, DBS samples have the advantage that they can be collected by non-professionals, require minimal volumes of blood and do not require special storage and transportation conditions. Based on an evaluation in our lab, conventional analysis of venous blood fatty acid status cost ~45 Australian dollars (AUD) per sample, whereas my DBS method only cost ~10 AUD per sample (Table 7.1).

Although the results of the studies forming the basis of this thesis are encouraging, it is important, as in any studies, to consider the limitations of my research. Temperature sensitivity is a major limitation of the "PUFAcoat" system, and significant thermal deterioration of LCPUFA in DBS was observed in my study even in the presence of "PUFAcoat" system if the samples were stored at a high temperature (Chapter 4). This is likely to be because high temperatures reduce the activation energy required for

Table 7.1 Comparison the cost of blood fatty acid analysis between capillary DBS and conventional venous blood sample

	Conventional Methods	**DBS technique**
Hospital Cost	Nurse time (15mins, $11.25)	Nurse time (0min, $0.00)
	Alcohol wipe ($0.05)	"PUFAcoat" card ($3.00)
	Butterfly needle ($1.00)	Lancet ($0.20)
	Band-Aid ($0.05)	Desiccant ($0.1)
	Vacuum tube ($0.10)	Foil bag ($0.5)
Transportation Cost	$8.00 per sample ($24 for 3 samples, sent by courier)	$0.60 per sample (Sent by normal post)
Laboratory Cost	Storage ($1.00)	Storage ($0.00)
	Consumables in extraction and TLC separation ($4.00)	Consumables in extraction and TLC separation ($0.00)
	Technician time (30mins, $17.00)	Technician time (5mins, $3.00)
	Transesterification ($1.00)	Transesterification ($1.00)
	GC analysis ($2.00)	GC analysis ($2.00)
Total Cost	$45.45 per sample	$10.4 per sample

oxidation and breakdown of preformed hydroperoxide into free radicals, which promotes lipid oxidation (Kanner 1994). Some natural ingredients including tocopherols, rosemary extract, lecithin and oregano extract have shown an ability to prevent PUFA deterioration in foods and oils (Hamilton *et al.* 1998; Jiménez Alvarez *et al.* 2008; Azizkhani and Zandi 2009). A mixture of tocopherols, lecithin, and ascorbyl palmitate is commercially available and shows synergistic protection against thermal deterioration of n-3 LCPUFA during oil processing (Jacobsen 2010). Therefore, a systematic evaluation of all compounds with demonstrated ability as chelating agent and all known antioxidant compounds need to be conducted in future studies in order to enhance the understanding of the basic chemical processes involved in the stabilisation of LCPUFA in DBS, and identify ways of optimizing the potency of my current protection system.

Another limitation to be considered when applying the "PUFAcoat" system in clinical trial use is that the "PUFAcoat" paper only absorbs the blood/breast milk droplets in a relatively slow manner, taking up to 30mins for blood to dry to the point that drops do not smear, whereas this process only takes a few minutes for Whatman 903 paper. Therefore, it would be important in future studies to attempt to locate a more suitable matrix on which to base my DBS test. This objective will involve a systematic evaluation of commercially available cellulose and non-cellulose based matrices for: background contaminants, compatibility with my existing protectant solution and the ability of blood and other biological fluids (breast milk, sputum, semen) to quickly soak into the matrix. These activities will result in a full prototype of an improved system for collection, storage and transport of biological fluid for fatty acid testing.

It is important to note that in addition to its role in protecting n-3 LCPUFA in DBS from degradation, the "PUFAcoat" paper was also able to robustly stabilise LCPUFA in breast milk. Breast milk is the primary source of nutrition for breast fed infants, and it provides all the necessary vitamins, minerals, digestive enzymes, and hormones required for optimal development (Dewey *et al.* 2001; Chantry *et al.* 2011). If my DBS method can be applied to breast milk, this will offer huge potential for elucidating the full complement of components in human breast milk, in particular those susceptible to oxidation (unsaturated fatty acids, fat soluble vitamins, oxidation sensitive enzymes and hormones), and determine their roles in protecting infant against diseases. In addition, the "PUFAcoat" paper also has potential applications for testing the concentrations of a range of compounds in other bio-fluids, including sputum and semen.

Another potential area for the "PUFAcoat" paper is TDM, where circulating drug concentrations in blood need to be monitored, typically for drugs with a narrow therapeutic window and/or large inter-subject variability. It is worthwhile to note that there has recently been growing interest in the application of DBS technique in this area (Watson *et al.* 2001; Oliveira *et al.* 2002; Aburuz *et al.* 2006), chiefly because of the significant advantage that DBS offers in relation to ease of blood sampling, sample storage and transportation. However, the popularization of DBS technique in TDM is currently limited by the rapid degradation of unstable drug components during transport and storage (Edelbroek *et al.* 2009). If it is able to show that the "PUFAcoat" paper can stabilise such unstable analytes, it would allow the DBS technique to be universally applied in TDM, and thus provide an attractive alternative for current drug monitoring strategies.

The larger goal of our "PUFAcoat" system is to apply the system to every Guthrie card used for routine collection of blood samples for screening programs world-wide. Blood samples from a finger-prick or heel-prick from every newborn infant in Western world are rountinely spotted onto Guthrie cards for the screening of various metabolic compounds for diagnosis of inborn errors of metabolism. A wide range of metabolites indicative of many inborn errors of metabolism are routinely screened in laboratories using Guthrie DBS samples. However, many metabolites on DBS have been shown to be unstable at room temperature, and the current Guthrie cards lack the ability to protect them against oxidative degradation. For example, thyroid hormones and thyrotropin in DBS collected on Guthrie cards are unstable over 4 weeks of storage at room temperature (Waite *et al.* 1987). Similarly, the activity of galactosylcerebrosidase and neutral α-glucosidase in DBS samples collected on Guthrie cards slowly decreases during storage at room temperature. Therefore, storage of the Guthrie DBS at -20°C in an airtight bag during transportation and storage is recommended (Adam *et al.* 2011). However, the "PUFAcoat" system may have the potential to stabilise those biomarkers in DBS samples over long term storage at room temperature, which would enables the DBS to be stored at room temperature for long periods and to be sent through the regular mail service where permitted. This would thus elimate the cost and logistical problems of couriering the DBS samples on dry ice between clinical sites and the diagnostic laboratories. Furthermore, the incorporation of the "PUFAcoat" system into the Guthrie cards also has the potential to preserve as yet unidentified compounds on the Guthrie cards, and thus enable application of Guthrie cards to a much wider range of diagnoses.

In conclusion, my thesis described a new DBS method which has the capacity to

stabilise the n-3 LCPUFA for over 2 months when stored at room temperature. Thus, my new DBS method has significant potential for application to large-scale clinical research. Furthermore, this method could readily be applied to population based screening programs and home monitoring of key risk factors of diseases related to blood n-3 status.

References

Aburuz, S., Millership, J. and McElnay, J. (2006). Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin. *J Chromatogr B Analyt Technol Biomed Life Sci*. **832**: 202-207.

Adam, B. W., Hall, E. M., Sternberg, M., Lim, T. H., Flores, S.R., O'Brien, S., Simms, D., Li, L. X., De Jesus, V. R. and Hannon, W. H. (2011). The stability of markers in dried-blood spots for recommended newborn screening disorders in the United States. *Clin Biochem*. **44**: 1445-1450.

Adam, O., Beringer, C., Kless, T., Lemmen, C., Adam, A., Wiseman, M., Adam, P., Klimmek, R. and Forth, W. (2003). Anti-inflammatory effects of a low arachidonic acid diet and fish oil in patients with rheumatoid arthritis. *Rheumatol Int*. **23**: 27-36.

Agostoni, C., Zuccotti, G. V., Riva, E., Decarlis, S., Bernardo, L., Bruzzese, M.G. and Giovannini, M. (1998). Low levels of linoleic acid in plasma total lipids of HIV-1 seropositive children. *J Am Coll Nutr*. **17**: 25-29.

Agostoni, C., 2008a. Role of long-chain polyunsaturated fatty acids in the first year of life. *J Pediatr Gastroenterol Nutr*. **47**(suppl): S41-S44.

Agostoni, C., Marangoni, F., Stival, G., Gatelli, I., Pinto, F., Risé, P., Giovannini, M., Galli, C. and Riva, E. (2008b). Whole blood fatty acid composition differs in term versus mildly preterm infants: small versus matched appropriate for gestational age. *Pediatr Res*. **64**: 298-302.

Agostoni, C., Galli, C., Riva, E., Risé, P., Colombo, C., Giovannini, M. and Marangoni, F. (2011). Whole blood fatty acid composition at birth: from the maternal compartment to the infant. *Clin Nutr*. **30**: 503-505.

Ahrens, E. H. Jr., Insull, W. Jr., Blomstrand, R., Hirsch, J, Tsaltas, T. T. and Psterson M. L. (1957). The influence of dietary fats on serum-lipid levels in man. *Lanet*. **272**: 943-953.

Albert, C. M., Hennekens, C. H., O'Donnell, C. J., Ajani, U. A., Carey, V. J., Willett, W. C., Ruskin, J. N. and Manson, J. E. (1998). Fish consumption and risk of sudden cardiac death. Fish consumption and risk of sudden cardiac death. *JAMA*. **279**: 23-28.

Albert, C.M., Campos, H., Stampfer, M.J., Ridker, P.M., Manson, J.E., Willett, W.C., and Ma, J. (2002). Blood levels of long-chain n-3 fatty acids and the risk of sudden death. *N Engl J Med*. **346**: 1113-1118.

Alen, Y., Nakajima, S., Nitoda, T., Baba, N., Kanzaki, H. and Kawazu, K. (2000). Two antinematodal phenolics from Knema hookeriana, a Sumatran rainforest plant. *Z Naturforsch C*. **55**: 300-303.

Arab, L. 2003. Biomarkers of Fat and Fatty Acid Intake. *J Nutr.* **133**(suppl.): S925-932.

Armstrong, J. M., Metherel, A. H. and Stark, K. D. (2008). Direct microwave transesterification of fingertip prick blood samples for fatty acid determinations. *Lipids*. **43**: 187-196.

Arya, S. C. (1988). Testing for AIDS on samples of dried blood prepared on filter papers. *Vaccine*. **6**: 210.

Ashok, K. C. and Ajit, V. (2009). A Textbook of Molecular Biotechnology.

Azizkhani, M. and P. Zandi. (2009). Effects of some natural antioxidants mixtures on margarine stability. *World Academy of Science, Engineering and Technology*. **49**: 93-96.

Babich, H. (1982). Butylated hydroxytoluene (BHT): a review. *Environ Res*. **29**: 1-29.

Bailey-Hall, E., Nelson, E. B. and Ryan, A. S. (2008) Validation of a rapid measure of blood PUFA levels in humans. *Lipids*. **43**: 181-186.

Baylin, A., Kim, M. K., Donovan-Palmer, A., Siles, X., Dougherty, L., Tocco, P., Campos, H. (2005). Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: comparison with adipose tissue and plasma. *Am J Epidemiol*. **162**: 373-381.

Baylin, A. and Campos, H. (2006). The use of fatty acid biomarkers to reflect dietary intake. *Curr Opin Lipidol*. **17**: 22-27.

Bell, J. G., Mackinlay, E. E., Dick, J. R., Younger, I., Lands, B. and Gilhooly, T. (2011). Using a fingertip whole blood sample for rapid fatty acid measurement: method validation and correlation with erythrocyte polar lipid compositions in UK subjects. *Br J Nutr*. **106**: 1408-1415.

Belo, L., Rebelo, I., Castro, E. M., Catarino, C., Pereira-Leite, L., Quintanilha, A., and Santos-Silva, A. (2002). Band 3 as a marker of erythrocyte changes in pregnancy. *Eur J Haematol*. **69**: 145-151.

Benatti, P., Peluso, G., Nicolai, R. and Calvani, M. (2004). Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties. *J Am Coll Nutr*. **23**:281-302.

Berbert, A. A., Kondo, C. R., Almendra, C. L., Matsuo, T. and Dichi, I. (2005). Supplementation of fish oil and olive oil in patients with rheumatoid arthritis. *Nutrition*. **21**:131-6.

Berenzon, S. and Saguy, I. S. (1998). Oxygen absorbers for extension of crackers shelf-life. *LWT - Food Science and Technology*. **31**: 1-5.

Beynen, A. C. and Katan, M. B. (1985). Rapid sampling and long-term storage of subcutaneous adipose-tissue biopsies for determination of fatty acid composition. *Am J Clin Nutr*. **42**: 317-322.

Bosch, J., Gerstein, H. C., Dagenais, G. R., Díaz, R., Dyal, L., Jung, H., Maggiono, A. P., Probstfield, J., Ramachandran, A., Riddle, M. C., Rydén, L. E. and Yusuf, S. (2012).

Omega-3 fatty acids and cardiovascular outcomes in patients with dysglycemia. *N Engl J Med*. **367**: 309-318.

Breen, P. A. and Murphy, A. J. (1995). Reactions of oxyl radicals with DNA. *Free Radical Biology & Medicine*, **18**: 1033-77.

Broekhuyse, R. M. (1974). Improved lipid extraction of erythrocytes. *Clin Chim Acta*. **51**: 341-343.

Bronte-stewart, B., Antonis, A., Eales, L., Brock, J. F. (1956). Effects of feeding different fats on serum-cholesterol level. *Lancet*. **270**: 521-526.

Bucher, H. C., Hengstler, P., Schindler, C. and Meier, G. (2002). N-3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. *Am J Med*. **112**: 298-304.

Burdge, G. C. and Calder, P. C. (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev*. **45**: 581-597.

Burr, M. L., Fehily, A. M., Gilbert, J. F., Rogers, S., Holliday, R. M., Sweetnam, P. M., Elwood, P. C . and Deadman, N. M. (1989). Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet*. **334**: 757-761

Calder, P. C. (2006). N-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr*. **83**(suppl.): S1505-1519.

Campo, G.M. (2004). Reduction of DNA Fragmentation and Hydroxyl Radical Production by Hyaluronic Acid and Chondroitin-4-sulphate in Iron Plus Ascorbate-induced Oxidative Stress in Fibroblast Cultures. *Free Radical Research*. **38**: 601-611.

Cantey, J. B., Bascik, S. L., Heyne, N. G., Gonzalez, J. R., Jackson, G. L., Rogers, V. L., Sheffield, J. S., Treviño, S., Sendelbach, D., Wendel, G. D. and Sánchez, P. J.

(2013). Prevention of mother-to-infant transmission of influenza during the postpartum period. *Am J Perinatol*. **30**: 233-40.

Carlsen, C. U. and Skibsted, L. H. (2004). Myoglobin species with enhanced prooxidative activity is formed during mild proteolysis by pepsin. *J Agric Food Chem*. **52**: 1675-1681.

Carlsen, C. U., Møller, J. K. S. and Skibsted L. H. (2005). Heme-iron in lipid oxidation. *Coordination Chemistry Reviews*. **249**: 485-498.

Carlson, S. E., Colombo, J., Gajewski. B. J., Gustafson, K. M., Mundy, D., Yeast, J., Georgieff, M. K., Markley, L. A., Kerling, E. H. and Shaddy, D. J. (2013). DHA supplementation and pregnancy outcomes. *Am J Clin Nutr*. **97**: 808-815.

Chace, D. H., Adam, B. W., Smith, S. J., Alexander, J. R., Hillman, S. L., Hannon, W. H. (1999). Validation of accuracy-based amino acid reference materials in dried-blood spots by tandem mass spectrometry for newborn screening assays. *Clin Chem*. **45**: 1269-1277.

Chantry, C. J., Wiedeman, J., Buehring, G., Peerson, J. M., Hayfron, K., k'Aluoch, O., Lonnerdal, B. and Israel-Ballard, K. (2011). Effect of Flash-Heat Treatment on Antimicrobial Activity of Breastmilk. *Breastfeed Med*. **6**: 111-116.

Chiu, D., Kuypers, F. and Lubin, B. (1989). Lipid peroxidation in human red cells. *Semin Hematol*. **26**: 257-276.

Christie, W. W. (1989) Gas Chromatography and Lipids: a Practical Guide, The Oily Press, Ayr, Scotland, pp. 39-40.

Cirillo, M., Laurenzi, M., Trevisan, M. and Stamler, J. (1992). Hematocrit, blood pressure, and hypertension: The Gubbio Population Study. *Hypertension*. **20**: 319-326.

Cleland, L. G., French, J. K., Betts, W. H., Murphy, G. A. and Elliott, M. J. (1988).

Clinical and biochemical effects of dietary fish oil supplements in rheumatoid arthritis. *J Rheumatol*. **15**: 1471-1475.

Cleland, L. G., James, M. J. and Proudman, S. M. (2003). The role of fish oils in the treatment of rheumatoid arthritis. *Drugs*. **63**: 845-853.

Conquer, J. A., Tierney, M. C., Zecevic, J., Bettger, W. J. and Fisher, R. H. (2000). Fatty acid analysis of blood plasma of patients with Alzheimer's disease, other types of dementia, and cognitive impairment. *Lipids*. **35**: 1305-1312.

Dalton, A., Wolmarans, P., Witthuhn, R. C., van Stuijvenberg, M. E., Swanevelder, S. A. and Smuts, C. M. (2009). A randomised control trial in schoolchildren showed improvement in cognitive function after consuming a bread spread, containing fish flour from a marine source. *Prostaglandins Leukot Essent Fatty Acids*. **80**: 143-149.

D'Arienzo, C. J., Ji, Q. C., Discenza, L., Cornelius, G. and Hynes, J. (2010). DBS sampling can be used to stabilize prodrugs in drug discovery rodent studies without the addition of esterase inhibitors. *Bioanalysis*. **2**: 1415-1422.

Das, P. , Mukhopadhyay, A., Mandal, S., Pal, B. C., Mishra, R., Mukherjee, D., Mukhopadhyay, S., Basak, J. and Kar, M. (2012). In vitro studies of iron chelation activity of purified active ingredients extracted from Triticum aestivum Linn (wheat grass). *European Journal of Medicinal Plants*. **2**: 113-124.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986). Data for Biochemical Research, 3rd Edition, Oxford University Press, New York, pp. 404.

Dayton, S., Hashimoto, S., Dixon, W. and Pearce, M. L. (1966). Composition of lipids in human serum and adipose tissue during prolonged feeding of a diet high in unsaturated fat. *J Lipid Res*. **7**: 103-111.

De La Cruz, J. P., Villalobos, M. A., Carmona, J. A., Martín-Romero, M., Smith-Agreda, J. M., de la Cuesta, F. S. (2000). Antithrombotic potential of olive oil

administration in rabbits with elevated cholesterol. *Thromb Res*. **100**: 305-315.

De La Fuente, R. L., Naesgaard, P. A., Nilsen, S. T., Woie, L., Aarsland, T., Gundersen, T. and Nilsen, D. W. (2013). Omega-3 index and prognosis in acute coronary chest pain patients with a low dietary intake of omega-3. *Scand Cardiovasc J*. **47**: 69-79.

Dewey, K.G. (2001). Nutrition, growth, and complementary feeding of the breastfed infant. *Pediatr Clin North Am*. **48**: 87-104.

Dodge, J.T. and Phillips, G. B. (1967). Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. *J Lipid Res*. **8**: 667-675.

Dougherty, R. M, Galli, C., Ferro-Luzzi A. and Iacono, J. M. 1987. Lipid and phospholipid fatty acid composition of plasma, red blood cells, and platelets and how they are affected by dietary lipids: a study of normal subjects from Italy, Finland, and the USA. *Am J Clin Nutr*. **45**: 443-455.

Drummond, K. E. and Brefere, L. M. (2009). Nutrition for Foodservice and Culinary Professionals, 7th Edition, John Wiley & Sons Inc., New York, pp. 151.

Dunstan, J. A., Mori, T. A., Barden, A., Beilin, L. J., Taylor, A. L., Holt, P. G. and Prescott, S. L. (2003). Fish oil supplementation in pregnancy modifies neonatal allergen-specific immune responses and clinical outcomes in infants at high risk of atopy: a randomized, controlled trial. *J Allergy Clin Immunol*. **112**: 1178-1184.

D'Vaz, N., Meldrum, S. J., Dunstan, J. A., Martino, D., McCarthy, S., Metcalfe, J., Tulic, M. K., Mori, T. A. and Prescott, S. L. (2012). Postnatal fish oil supplementation in high-risk infants to prevent allergy: randomized controlled trial. *Pediatrics*. **130**: 674-682.

Edelbroek, P. M., van der Heijden, J. and Stolk L. M. (2009). Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls. *Ther Drug Monit*. **31**: 327-336.

Einvik, G., Klemsdal, T. O., Sandvik, L. and Hjerkinn E. M. (2010). A randomized clinical trial on n-3 polyunsaturated fatty acids supplementation and all-cause mortality in elderly men at high cardiovascular risk. *Eur J Cardiovasc Prev Rehabil*. **17**: 588-592.

Espersen, G. T., Grunnet, N., Lervang, H. H., Nielsen, G. L., Thomsen, B. S., Faarvang, K. L., Dyerberg, J. and Ernst, E. (1992). Decreased interleukin-1 beta levels in plasma from rheumatoid arthritis patients after dietary supplementation with n-3 polyunsaturated fatty acids. *Clin Rheumatol*. **11**: 393-395.

Eyles, D. W., Morley, R., Anderson, C., Ko, P., Burne, T., Permezel, M., Mortensen, P. B., Nørgaard-Pedersen, B., Hougaard, D. M., McGrath, J. J. (2010). The utility of neonatal dried blood spots for the assessment of neonatal vitamin D status. *Paediatr Perinat Epidemiol*. **24**: 303-308.

Farzadegan, H., Noori, K. H. and Ala, F. (1978). Detection of hepatitis-B surface antigen in blood and blood products dried on filter paper. *Lancet*. **311**: 362-363.

Farzaneh-Far, R., Harris, W. S., Garg, S., Na, B. and Whooley, M. A. (2009). Inverse association of erythrocyte n-3 fatty acid levels with inflammatory biomarkers in patients with stable coronary artery disease: The Heart and Soul Study.Atherosclerosis. *Atherosclerosis*. **205**: 538-543.

Feldmann, M. and Maini, R. N. (1999). The role of cytokines in the pathogenesis of rheumatoid arthritis. *Rheumatology*. **38** (suppl.): 3-7.

Finigan, V. (2012). Breastfeeding and diabetes: Part 2. *Pract Midwife*. **15**: 33-4, 36.

Fishbein, W. N. and Kirsch, I. R. (1993). Detection chromosome 7 inversions peripheral blood specimens dried filter paper population screen potential oncogenesis exposure environmental genotoxins (abstract). *Clin Re*. **41**: A271.

Folch, J., Lees, M. and Sloane-Stanely, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. **226**: 497-507.

Fraga, C. G. and Oteiza, P. I. (2002). Iron toxicity and antioxidant nutrients. *Toxicology*. **180**: 23-32.

Frankel, E. N., Huang, S.W., Kanner, J., German, J. B. (1994). Interfacial Phenomena in the Evaluation of Antioxidants: Bulk Oils vs Emulsions. *J. Agric. Food Chem*. **42**: 1054-1059.

Furuhjelm, C., Warstedt, K., Larsson, J., Fredriksson, M., Böttcher, M. F., Fälth-Magnusson, K. and Duchén, K. (2009). Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy. *Acta Paediatr*. **98**: 1461-1467.

Geusens, P., Wouters, C., Nijs, J., Jiang, Y. and Dequeker, J. (1994). Long-term effect of omega-3 fatty acid supplementation in active rheumatoid arthritis. *Arthritis Rheum*. **37**: 824-9

GISSI-Prevenzione Investigators. (1999). Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. *Lancet*. **354**: 447-455.

Glatz, J. F., Soffers, A. E. and Katan, M. B. (1989). Fatty acid composition of serum cholesteryl esters and erythrocyte membranes as indicators of linoleic acid intake in man. *Am J Clin Nutr*. **49**: 269-276.

Gordon, M. H, Paiva-Martins, F. and Almeida, M. (2001). Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *J Agric Food Chem*. **49**: 2480-2485.

Grajales-Lagunes A., Garcia-Galindo, H. S., Angulo-Guerrero, O., Monroy-Rivera, J. A. (1999). Stability and sensory quality of spray dried avocado paste. *Drying technology*. **17**: 317-326.

Guebre-Egziabher, F., Rabasa-Lhoret, R., Bonnet, F., Bastard, J. P., Desage, M., Skilton, M. R., Vidal, H. and Laville, M. (2008). Nutritional intervention to reduce the n-6/n-3 fatty acid ratio increases adiponectin concentration and fatty acid oxidation in healthy subjects. *Eur J Clin Nutr*. **62**: 1287-1293.

Gurzau, E. S., Neagu, C. and Gurzau, A. E. (2003). Essential metals-case study on iron. *Ecotoxicol Environ Saf*. **56**: 190-200.

Guthrie, R. and Susi, A. (1963). A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics*. **32**: 338-343.

Halliwell, B. and Gutteridge, J. M. (1997). Lipid peroxidation in brain homogenates: the role of iron and hydroxyl radicals. *J Neurochem*. **69**: 1330-1331.

Hamada, H., Hiramatsu, M., Edamatsu, R. and Mori A. (1993). Free radical scavenging action of baicalein. *Arch Biochem Biophys*. **306**: 261-266.

Hamilton, R. J. Kalu C, McNeill GP, Padley FB, Pierce JH. (1998). Effects of tocopherols, ascorbyl palmitate, and lecithin on autoxidation of fish oil, *Journal of the American Oil Chemists' Society*. **75**: 813-822

Hannon, W. H. (2003). Performance evaluation for screening laboratories of the Asia-Pacific region. *Southeast Asian J Trop Med Public Health*. **34** (suppl.): 39-42.

Harada, F., Ireland, J. H., Hsia, Y. E. and Chui, D. H. (1994). Anti-zeta antibody screening for alpha-thalassemia using dried filter paper blood. *Biochem Med Metab Biol*. **51**: 80-84.

Harris, W. S. and von Schacky, C. (2004). The Omega-3 Index: a new risk factor for death from coronary heart disease? *Prev Med*. **39**: 212-220.

Harris W. S. (2008). The omega-3 index as a risk factor for coronary heart disease. *Am J Clin Nutr*. **87**: 1997-2002.

Harper, M., Thom, E. and Klebanoff, M. A. (2010) Omega-3 fatty acid supplementation to prevent recurrent preterm birth: a randomized controlled trial. *Obstet Gynecol*. **115**: 234-242.

Hirai, A., Hamazaki, T., Terano, T., Nishikawa, T., Tamura, Y., Kamugai, A. and Jajiki, J. (1980). Eicosapentaenoic acid and platelet function in Japanese. *Lancet*. **316**: 1132-1133.

Helland, I. B., Smith, L., Saarem, K., Saugstad, O. D. and Drevon, C. A. (2003) Maternal supplementation with very-long-chain n-3 fatty acids during pregnancy and lactation augments children's IQ at 4 years of age. *Pediatrics*. **111**: e39-e44.

Helland, I. B., Smith, L., Blomen, B., Saarem, K., Saugstad, O. D. and Drevon, C. A. (2008). Effect of supplementing pregnant and lactating mothers with n-3 very-long-chain fatty acids on children's IQ and body mass index at 7 years of age. *Pediatrics* **122**: e472–e479.

Hibbeln, J. R., Davis, J. M., Steer, C., Emmett, P., Rogers, I., Williams, C. and Golding, J. (2007). Maternal seafood consumption in pregnancy and neurodevelopmental outcomes in childhood (ALSPAC study): an observational cohort study. *Lancet*. **369**: 578-585.

Hirsch, J., Farquhar, J. W., Ahrenc, E. J., Peterson, M. L and Stoffel, W. (1960). Studies of adipose tissue in man. A microtechnic for sampling and analysis. *Am J Clin Nutr*. **8**: 499-511.

Hodson, L., Skeaff, C. M. and Fielding, B. A. (2008). Fatty acid composition of

adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res*. **47**: 348-80.

Hoffman, D. R., Birch, E. E., Birch, D. G. and Uauy, R. (1999). Fatty acid profile of buccal cheek cell phospholipids as an index for dietary intake of docosahexaenoic acid in preterm infants. *Lipids*. **34**: 337-342.

Hoff, R., Weiblin, B., Schwerzler, M., Deutch, G. and Shea, B. (1988). Testing for antibody to HIV in newborn blood samples collected on paper. *Infect Control Hosp Epidemiol*. **9**: 360.

Holub, B. J., Wlodek, M., Rowe, W. and Piekarski, J. (2009). Correlation of omega-3 levels in serum phospholipid from 2053 human blood samples with key fatty acid ratios. *Nutr J*. **8**: 58.

Hopfer, S. M., Makowski, G. S., Davis, E. L. and Aslanzadeh, J. (1995). Detection of cystic fibrosis delta F508 mutation by anti-double-stranded DNA antibody. *Ann Clin Lab Sci.* **25**: 475-484.

Horta B. L., Victora, C. G., Gigante, D. P., Santos, J. and Barros, F. C. (2007). Breastfeeding duration in two generations. *Rev Saude Publica*. **41**: 13-18.

Horrobin, D.F. (1993). Fatty acid metabolism in health and disease. *Am J Clin Nutr*. **57**(suppl.): S732-S737.

Hu, F. B., Cho, E., Rexrode, K. M., Albert, C. M. and Manson, J. E. (2003). Fish and long-chain omega-3 fatty acid intake and risk of coronary heart disease and total mortality in diabetic women. *Circulation*. **107**: 1852-1857.

Huebers, H. A., Eng, M. J., Josephson, B. M., Ekpoom, N., Rettmer, R. L., Labbé, R. F., Pootrakul, P. and Finch, C. A. (1987). Plasma iron and transferrin iron-binding capacity evaluated by colorimetric and immunoprecipitation methods. *Clin Chem*. **33**: 273-277.

Ichihara, K., Waku, K., Yamaguchi, C., Saito, K., Shibahara, A., Miyatani, S. and Yamamoto, K. (2002). A convenient method for determination of the C20–22 PUFA composition of glycerolipids in blood and breast milk. *Lipids*. **37**: 523-526.

Jacobsen, C. (2010). Enrichment of foods with omega-3 fatty acids: a multidisciplinary challenge. *Ann N Y Acad Sci*. **1190**: 141-50.

Jain, N., Shaily, G. and Ramawat, K. G. (2011). Evalution of antioxidant properties and total phenolic content of medical plants used in diet therapy during postpartum healthcare in Rajasthan. *International Journal of Pharmacy and Pharmaceutical Sciences*. **3**: 248-253.

James, M. J., Gibson, R. A., D'Angelo, M., Neumann, M. A. and Cleland, L. G. (1993). Simple relationships exist between dietary linoleate and the n-6 fatty acids of human neutrophils and plasma. *Am J Clin Nutr*. **58**: 497-500.

James, M. J. and Cleland, L. G. (1997). Dietary n-3 fatty acids and therapy for rheumatoid arthritis. *Semin Arthritis Rheum*. **27**: 85-97.

Jimenez-Alvarez, D., Giuffrida, F., Golay, P. A., Cotting, C., Lardeau, A. and Keely, B. J. (2008). Antioxidant activity of oregano, parsley, and olive mill wastewaters in bulk oils and oil-in-water emulsions enriched in fish oil. *J Agric Food Chem*. **56**: 7151-7159.

Kaaks, R. J. (1997). Biochemical markers as additional measurements in studies of the accuracy of dietary questionnaire measurements: conceptual issues. *Am J Clin Nutr*. **65**(suppl.): 1232S -1239S.

Kalinowski D. S. and Richardson, D. R. (2005). The evolution of iron chelators for the treatment of iron overload disease and cancer. *Pharmacol Rev*. **57**: 547-583.

Kanner J. and Rosenthal I. (1992). An assessment of lipid oxidation in foods. *Pure &Appl. Chern*., **64**: 1959-1964

Kanner, J. (1994). Oxidative process in meat and meat products: quality implications. *Meat Sci*. **36**: 169-189.

Karel, M. (1980). Lipid oxidation, secondary reactions and water activity of foods, in ‘‘Autoxidation in foods and biological systems’’, Plenum Press, New York, pp. 191-206.

Karr, J. E., Alexander, J. E. and Winningham, R. G. (2011). Omega-3 polyunsaturated fatty acids and cognition throughout the lifespan: a review. *Nutr Neurosci*. **14**: 216-225.

Ramakrishnan, U., Imhoff-Kunsch, B. and DiGirolamo, A. M. (2009). Role of docosahexaenoic acid in maternal and child mental health. *Am J Clin Nutr*. **89**(suppl.): S958- S962.

Kasuga, K., Yang, R., Porter, T. F., Agrawal, N., Petasis, N. A., Irimia, D., Toner, M. and Serhan, C. N. (2008). Rapid appearance of resolvin precursors in inflammatory exudates: novel mechanisms in resolution. *J Immunol*. **181**: 8677-8687.

Katan, M. B., Deslypere, J. P., van Birgelen, A. P., Penders, M. and Zegwaard, M. (1997). Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: an 18-month controlled study. *J Lipid Res*. **38**: 2012-22.

Keevil, B. G. (2011). The analysis of dried blood spot samples using liquid chromatography tandem mass spectrometry. *Clin Biochem*. **44**: 110-118.

Klemens, C. M., Berman, D. R. and Mozurkewich, E. L. (2011). The effect of perinatal omega-3 fatty acid supplementation on inflammatory markers and allergic diseases: a systematic review. *BJOG*. **118**: 916-925.

Knudtson, M. L., Wyse, D. G., Galbraith, P. D., Brant, R., Hildebrand, K., Paterson, D., Richardson, D., Burkart, C. and Burgess, E. (2002). Chelation therapy for

ischemic heart disease: a randomized controlled trial. *JAMA*. **287**: 481-486.

Koal, T., Burhenne, H., Römling, R., Svoboda, M., Resch, K. and Kaever, V. (2005). Quantification of antiretroviral drugs in dried blood spot samples by means of liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom*. **19**: 2995-3001.

Kohlmeier, L. and Kohlmeier, M. (1995). Adipose tissue as a medium for epidemiologic exposure assessment. *Environ Health Perspect*. **103**(suppl): 99-106.

Koletzko, B., Mrotzek, M. and Bremer, H. J. (1988). Fatty acid composition of mature human milk in Germany. *Am J Clin Nutr*. **47**: 954-959.

Koletzko, B., Knoppke, B., von Schenck, U., Demmelmair, H. and Damli, A. (1999). Noninvasive assessment of essential fatty acid status in preterm infants by buccal mucosal cell phospholipid analysis. *J Pediatr Gastroenterol Nutr*. **29**: 467-474.

Koletzko, B., Cetin, I. and Brenna, J. T. (2007). Dietary fat intakes for pregnant and lactating women. *Br J Nutr*. **98**: 873-877.

Kontoghiorghes, G. J., Jackson, M. J. and Lunec, J. (1986). *In vitro* screening of iron chelators using models of free radical damage. *Free Radic Res Commun*. **2**: 115-124.

Kotwal, S., Jun, M., Sullivan, D., Perkovic, V. and Neal, B. (2012). Omega 3 Fatty acids and cardiovascular outcomes: systematic review and meta-analysis. *Circ Cardiovasc Qual Outcomes*. **5**: 808-818.

Krauss-Etschmann, S., Hartl, D., Rzehak, P., Heinrich, J., Shadid, R., Ramírez-Tortosa, M., Campoy, C., Pardillo, S., Schendel, D. J., Decsi, T., Demmelmair, H., Koletzko, B. V.; Nutraceuticals for Healthier Life Study Group. (2008). Decreased cord blood IL-4, IL-13, and CCR4 and increased TGF-beta levels after fish oil supplementation of pregnant women. *J Allergy Clin Immunol*. **121**: 464-470.

Kremer. J. M., Bigauoette, J., Michalek, A. V., Timchalk, M. A., Lininger, L., Rynes, R. I., Huyck, C., Zieminski, J., Bartholomew, L. E. (1985). Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. *Lancet*. **325**: 184-187.

Kremer, J. M., Jubiz, W., Michalek, A., Rynes, R. I., Bartholomew L. E., Bigaouette, J., Timchalk, M., Beeler, D. and Lininger, L. (1987). Fish-oil fatty acid supplementation in active rheumatoid arthritis. A double-blinded, controlled, crossover study. *Ann Intern Med*. **106**: 497-503.

Kremer, J. M., Lawrence, D. A., Jubiz, W., DiGiacomo, R., Rynes, R., Bartholomew, L. E. and Sherman, M. (1990). Dietary fish oil and olive oil supplementation in patients with rheumatoid arthritis: Clinical and immunologic effects. *Arthritis Rheum*. **33**: 810-820.

Kremer, J. M. (2000). N−3 fatty acid supplements in rheumatoid arthritis. *Am J Clin Nutr.* **71**(suppl): 349S-351S.

Kris-Etherton, P. M., Harris, W. S. and Appel, L. J. (2002). Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation*. **106**: 2747-2757.

Kromann, N. and Green, A. (1980). Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med Scand*. **208**: 401-406.

Kromhout, D., Giltay, E.J. and Geleijnse, J. M. Alpha Omega Trial Group. (2010). N-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med*. **363**: 2015-26

Labuza, T. B., Silver, M., Cohn, M., Heidelbaugh, N. D and Karel, M. (1971). Metal-catalyzed oxidation in the presence of water in foods. *J Am Oil Chem Soc*. **48**: 527-531.

Lakshmy, R., Gupta, R., Prabhakaran, D., Snehi, U. and Reddy, K. S. (2010). Utility of dried blood spots for measurement of cholesterol and triglycerides in a surveillance study. *J Diabetes Sci Technol*. **4**: 258-262.

Lampe, D., Scholz, D., Prümke, H. J., Blank, W. and Hüller, H. (1987). Capillary blood, dried on filter paper, as sample for monitoring cyclosporin A concentrations. *Clin Chem*. **33**:1643-1644.

Lands, W. E., Libelt, B., Morris, A., Kramer, N. C., Prewitt, T. E., Bowen, P., Schmeisser. D., Davidson, M. H. and Burns, J. H. (1992). Maintenance of lower proportions of (n - 6) eicosanoid precursors in phospholipids of human plasma in response to added dietary (n - 3) fatty acids. *Biochim Biophys Acta*. **1180**: 147-162.
Larqué, E., Gil-Sánchez, A., Prieto-Sánchez, M. T. and Koletzko, B. (2012). Omega 3 fatty acids, gestation and pregnancy outcomes. *Br J Nutr*. **107**(suppl): S77-S84.

Lavie, C. J., Milani, R. V., Mehra, M. R., Ventura, H. O. (2009). Omega-3 polyunsaturated fatty acids and cardiovascular diseases. *J Am Coll Cardiol*. **54**: 585-594.

Lauritzen, L., Kjaer, T. M., Fruekilde, M. B., Michaelsen, K. F. and Frøkiaer, H. (2005). Fish oil supplementation of lactating mothers affects cytokine production in 2 1/2-year-old children. *Lipids*. **40**: 669-676.

Levy, H. L., Simmons, J. R. and MacCready, R. A. (1985). Stability of amino acids and galactose in the newborn screening filter paper blood specimen. *J Pediatr*. **107**: 757-760.

Li, Y. O., Lam, J., Diosady, L. L. and Jankowski, S. (2009). Antioxidant system for the preservation of vitamin A in Ultra Rice, *Food Nutr. Bull*., **30**: 82-89.

Link, G., Pinson, A., Kahane, I. and Hershko, C. (1989). Iron loading modifies the fatty acid composition of cultured rat myocardial cells and liposomal vesicles: effect of ascorbate and alpha-tocopherol on myocardial lipid peroxidation. *J Lab Clin Med*.

114: 243-249.

Liu, G., Apps, D. and Gibson R. A. (2010). Contaminants from Paper Can Cause Erroneous Results in Rapid Testing for Blood Fatty Acids. *ISSFAL Meeting, Lipids in Metabolic Health and Disease, Maastricht*. P255

Liu, G., Ji, Q. C., Jemal, M., Tymiak, A. A. and Arnold, M. E. (2011). Approach to evaluating dried bloodspot sample stability during drying process and discovery of a treated card to maintain analyte stability by rapid on-card pH modification. *Anal Chem*. **83**: 9033-9008.

Ma, J., Folsom, A. R., Shahar, E. and Eckfeldt, J. H. (1995). Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk in Communities (ARIC) Study Investigators. *Am J Clin Nutr*. **62**: 564-571.

Malcom, G. T., Bhattacharyya, A. K., Velez-Duran, M., Guzman, M. A., Oalmann, M. C. and Strong, J. P. (1989). Fatty acid composition of adipose tissue in humans: differences between subcutaneous sites. *Am J Clin Nutr*. **50**: 288-291.

Manley, B. J., Makrides, M., Collins, C. T., McPhee, A. J., Gibson, R. A., Ryan, P., Sullivan, T. R. and Davis, P.G., DINO Steering Committee. (2011). High-dose docosahexaenoic acid supplementation of preterm infants: respiratory and allergy outcomes. *Pediatrics*. **128**: e71-e77.

Makrides, M., Gibson, R. A., McPhee, A. J., Collins, C. T., Davis, P. G., Doyle, L. W., Simmer, K., Colditz, P. B., Morris, S., Smithers, L. G., Willson, K. and Ryan, P. (2009). Neurodevelopmental Outcomes of Preterm Infants Fed High-Dose Docosahexaenoic Acid: A Randomized Controlled Trial. *JAMA*. **301**: 175-182.

Makrides, M., Gibson, R. A., McPhee, A. J., Yelland, L., Quinlivan, J., Ryan, P., DOMInO Investigative Team. (2010). Effect of DHA supplementation during pregnancy on maternal depression and neurodevelopment of young children: a

randomized controlled trial. *JAMA*. **304**: 1675-1683.

Marangoni, F., Colombo, C. and Galli, C. (2004). A method for the direct evaluation of the fatty acid status in a drop of blood from a fingertip in humans: applicability to nutritional and epidemiological studies. *Anal Biochem*. **326**: 267-272.

Marangoni, F., Colombo, C., Martiello, A., Negri, E. and Galli, C. (2007). The fatty acid profiles in a drop of blood from a fingertip correlate with physiological, dietary and lifestyle parameters in volunteers. *Prostaglandins Leukot Essent Fatty Acids*. **76**: 87-92.

Marchioli, R., Barzi, F., Bomba, E., Chieffo, C., Di Gregorio, D., Di Mascio, R., Franzosi, M. G., Geraci, E., Levantesi, G., Maggioni, A. P., Mantini, L., Marfisi, R. M., Mastrogiuseppe, G., Mininni, N., Nicolosi, G. L., Santini, M., Schweiger, C., Tavazzi, L., Tognoni, G., Tucci, C. and Valagussa, F., GISSI-Prevenzione Investigators. (2002). Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation*. **105**: 1897-1903.

Marshall, W. J. and Bangert, S. K. (2008). Clinical Chemistry, 6th Edition., London: Mosby Elsevier, Edinburgh, pp. 355.

McCabe, E. R., Huang, S. Z., Seltzer, W. K and Law, M. L. (1987). DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening. *Hum Genet*. **75**: 213-216.

McInerney, M. J., Bryant, M. P. and Pfenning, N. (1979). Anaerobic Bacterium that Degrades Fatty Acids in Syntrophic Association with Methanogens. *Archives of Microbiology*. **122**: 129-135.

McMurchie, E. J., Potter, J. D., Rohan, T. E. and Hetzel, B. S. (1984a). Human cheek cells: A Non-invasive method for determining tissue lipid profiles in dietary and

nutritional studies. *Nutrition reports international*. **29**: 519-526.

McMurchie, E. J., Margetts, B. M., Beilin, L. J., Croft, K. D., Vandongen, R. and Armstrong, B. K (1984b). Dietary-induced changes in the fatty acid composition of human cheek cell phospholipids: correlation with changes in the dietary polyunsaturated/saturated fat ratio. *Am J Clin Nutr*. **39**: 975-980.

McNamara, R. K., Able, J., Jandacek, R., Rider, T., Tso, P., Eliassen, J. C., Alfieri, D., Weber, W., Jarvis, K., DelBello, M. P., Strakowski, S. M. and Adler, C. M. (2010). Docosahexaenoic acid supplementation increases prefrontal cortex activation during sustained attention in healthy boys: a placebo-controlled, dose-ranging, functional magnetic resonance imaging study. *Am J Clin Nutr*. **91**: 1060-1067.

Meakin, J. C. (1973). Trace-metal contaminants in filter paper. *Clin Chem*. **19**: 141.

Mei, J. V., Alexander, J. R., Adam, B. W. and Hannon, W. H. (2001). Use of filter paper for the collection and analysis of human whole blood specimens. *J Nutr*. **13**: 1631S-1636S.

Melidou, M., Riganakos, K. and Galaris, D. (2005). Protection against nuclear DNA damage offered by flavonoids in cells exposed to hydrogen peroxide: the role of iron chelation. *Free Radic Biol Med*. **39**: 1591-1600.

Messer, R. L., Lockwood, P. E., Tseng, W. Y., Edwards, K., Shaw, M., Caughman, G. B., Lewis, J. B. and Wataha, J. C. (2005). Mercury (II) alters mitochondrial activity of monocytes at sublethal doses via oxidative stress mechanisms. *J Biomed Mater Res B Appl Biomater*. **75**: 257-263.

Metcalfe, L. D., Schmitz A.A and Pelka, J. R. (1966). Rapid Preparation of Fatty Acid Esters from Lipids for Gas Chromatographic Analysis. *Anal. Chem*. **38**: 514-515.

Metcalf, R. G., James, M. J., Gibson, R. A., Edwards, J. R., Stubberfield, J., Stuklis, R., Roberts-Thomson, K., Young, G. D. and Cleland, L. G. (2007). Effects of fish-oil supplementation on myocardial fatty acids in humans. *Am J Clin Nutr*. **85**: 1222-1228.

Metherel, A. H., Hogg, .R. C., Buzikievich, L. M., Stark, K. D. (2013). Butylated hydroxytoluene can protect polyunsaturated fatty acids in dried blood spots from degradation for up to 8 weeks at room temperature. *Lipids Health Dis*. **12**: 22.

Miles E. A. and Calder, P. C. (2012). Influence of marine n-3 polyunsaturated fatty acids on immune function and a systematic review of their effects on clinical outcomes in rheumatoid arthritis. *Br J Nutr.* **107**(suppl): S171-S184.

Milte, C. M., Sinn, N. and Howe, P. R. (2009). Polyunsaturated fatty acid status in attention deficit hyperactivity disorder, depression, and Alzheimer's disease: towards an omega-3 index for mental health? *Nutr Rev*. **67**: 573-590.

Min, B., and Ahn, D. U. (2005). Mechanism of lipid peroxidation in meat and meat products -A review. *Food Science and Biotechnology*, **14**: 152–163.

Min, Y., Ghebremeskel, K., Geppert, J. and Khalil, F. (2011). Effect of storage temperature and length on fatty acid composition of fingertip blood collected on filter paper. *Prostaglandins Leukot Essent Fatty Acids*. **84**: 13-18.

Mladěnka, P., Macáková, K., Filipský, T., Zatloukalová, L., Jahodář, L., Bovicelli, P., Silvestri, I. P., Hrdina, R. and Saso, L. (2011). *In vitro* analysis of iron chelating activity of flavonoids. *J Inorg Biochem.***105**: 693-701.

Muthayya, S. (2009). Maternal nutrition & low birth weight - what is really important? *Indian J Med Res*. **130**: 600-608.

Nagy, E., Eaton, J. W., Jeney, V., Soares, M. P., Varga, Z., Galajda, Z., Szentmiklósi, J., Méhes, G., Csonka, T., Smith, A., Vercellotti, G. M., Balla, G. and Balla, J. (2010). Red cells, hemoglobin, heme, iron, and atherogenesis. *Arterioscler Thromb Vasc Biol*.

30: 1347-1353

Newman, M. S., Brandon, T. R., Groves, M. N., Gregory, W. L., Kapur, S. and Zava, D. T. (2009). A liquid chromatography/tandem mass spectrometry method for determination of 25-hydroxy vitamin D2 and 25-hydroxy vitamin D3 in dried blood spots: a potential adjunct to diabetes and cardiometabolic risk screening. *J Diabetes Sci Technol*. **3**: 156-162.

Nishio, H., Kodama, S., Yokoyama, S., Matsuo, T., Mio, T. and Sumino, K. (1986). A simple method to diagnose adrenoleukodystrophy using a dried blood spot on filter paper. *Clin Chim Acta*. **159**: 77-82.

Nyambi, P. N., Fransen, K., De Beenhouwer, H., Chomba, E. N., Temmerman, M., Ndinya-Achola, J. O., Piot, P. and van der Groen, G. (1994). Detection of human immunodeficiency virus type 1 (HIV-1) in heel prick blood on filter paper from children born to HIV-1-seropositive mothers. *J Clin Microbiol*. **32**: 2858-2860.

Ohta, A., Mayo, M. C., Kramer, N. and Lands, W. E. (1990). Rapid analysis of fatty acids in plasma lipids. *Lipids*. **25**: 742-747.

Oken, E., Osterdal, M. L., Gillman, M. W., Knudsen, V. K., Halldorsson, T. I., Strom, M., Bellinger, D. C., Hadders-Algra, M., Michaelsen, K. F. and Olsen, S. F. (2008) Associations of maternal fish intake during pregnancy and breastfeeding duration with attainment of developmental milestones in early childhood: a study from the Danish National Birth Cohort. *Am J Clin Nutr*. **88**: 789–796.

Oliver, S., Dezateux, C., Kavanagh, J., Lempert, T. and Stewart, R. (2004). Disclosing to parents newborn carrier status identified by routine blood spot screening. *Cochrane Database Syst Rev*. **4**: CD003859.

Oliveira, E. J., Watson, D. G., Morton, N. S. (2002). A simple microanalytical technique for determination of paracetamol in blood spots. *J Pharm Biomed Anal*. **29**: 803-809.

Olivieri, N. F., and Brittenham, G. M. (1997). Iron-Chelating Therapy and the Treatment of Thalassemia. *Blood*. **89**: 739-761.

Olsen, S. F., Hansen, H. S., Sørensen, T. I., Jensen, B., Secher, N. J., Sommer, S. and Knudsen, L. B. (1986). Intake of marine fat, rich in (n-3)-polyunsaturated fatty acids, may increase birthweight by prolonging gestation. *Lancet*. **328**: 367-369.

Olsen, S. F., Secher, N. J., Tabor, A., Weber, T., Walker, J. J. and Gluud, C. (2000). Randomised clinical trials of fish oil supplementation in high risk pregnancies. Fish Oil Trials In Pregnancy (FOTIP) Team. *BJOG*. **107**: 382-95.

Olsen, S. F., Østerdal, M. L., Salvig, J. D., Mortensen, L. M., Rytter, D., Secher, N. J. and Henriksen, T. B. (2008). Fish oil intake compared with olive oil intake in late pregnancy and asthma in the offspring: 16 y of registry-based follow-up from a randomized controlled trial. *Am J Clin Nutr*. **88**: 167-175.

Olveira, G., Dorado, A., Olveira, C., Padilla, A., Rojo-Martínez, G., García-Escobar, E., Gaspar, I., Gonzalo, M. and Soriguer, F. (2006). Serum phospholipid fatty acid profile and dietary intake in an adult Mediterranean population with cystic fibrosis. *Br J Nutr*. **96**: 343-349.

Orchard, T. S., Ing, S. W., Lu, B., Belury, M. A., Johnson, K., Wactawski-Wende, J. and Jackson, R. D. (2013). The association of red blood cell n-3 and n-6 fatty acids with bone mineral density and hip fracture risk in the women's health initiative. *J Bone Miner Res*. **28**: 505-515.

Otto, S. J., Foreman-von Drongelen, M. M., von Houwelingen, A. C. and Hornstra, G. (1997). Effects of storage on venous and capillary blood samples: the influence of deferoxamine and butylated hydroxytoluene on the fatty acid alterations in red blood cell phospholipids. *Eur J Clin Chem Clin Biochem*. **35**: 907-913.

Palmer, D. J., Sullivan, T., Gold, M. S., Prescott, S. L., Heddle, R., Gibson, R. A. and

Makrides, M. (2012). Effect of n-3 long chain polyunsaturated fatty acid supplementation in pregnancy on infants' allergies in first year of life: randomised controlled trial. *BMJ*. **344**: e184.

Parker, S. P. and Cubitt, W. D. (1999). The use of the dried blood spot sample in epidemiological studies. *J Clin Pathol*. **52**: 633-639.

Peet, M., Murphy, B., Shay, J. and Horrobin, D. (1998). Depletion of omega-3 fatty acid levels in red blood cell membranes of depressive patients. *Biol Psychiatry*. **43**: 315-319.

Perez, C. A., Wei, Y. and Guo, M. (2009). Iron-binding and anti-Fenton properties of baicalein and baicalin. *J Inorg Biochem*. **103**: 326-332.

Phinney, S. D., Stern, J. S., Burke, K. E., Tang, A. B., Miller, G. and Holman, R. T. (1994). Human subcutaneous adipose tissue shows site-specific differences in fatty acid composition. *Am J Clin Nutr*. **60**: 725-729.

Pinkus, R., Weiner, L. M. and Daniel, V. (1996). Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. *J Biol Chem*. **271**: 13422-13429.

Ponka, P., Beaumont, C., Richardson, D. R. (1998). Function and regulation of transferrin and ferritin. *Semin Hematol*. **35**: 35-54.

Poppitt, S. D., Kilmartin, P., Butler, P. and Keogh, G. F. (2005). Assessment of erythrocyte phospholipid fatty acid composition as a biomarker for dietary MUFA, PUFA or saturated fatty acid intake in a controlled cross-over intervention trial. *Lipids Health Dis*. **4**: 30.

Porter, W. L., Levasseur, L. A. and Henick, A. S. (1977). Evaluation of some natural and synthetic phenolic antioxidants in linoleic acid monolayers on silica. *J Food Sci*. **42**: 1533-1535.

Prescott S. L. and Dustan A. J. (2007). Prenatal Fatty Acid Status and Immune Development: The Pathways and the Evidence. *Lipids*. **42**: 801-810.

Prickett, J. D., Robinson, D. R. and Steinberg, A. D. (1981). Dietary enrichment with the polyunsaturated fatty acid eicosapentaenoic acid prevents proteinuria and prolongs survival in NZB x NZW F1 mice. *J Clin Invest*. **68**: 556-559.

Rachmilewitz, E. A. (1976). The role of intracellular hemoglobin precipitation, low MCHC, and iron overload on red blood cell membrane peroxidation in thalassemia. *Birth Defects Orig Artic Ser*. **12**: 123-133.

Ramakrishnan, U. (2011). Fatty acid status and maternal mental health. *Matern Child Nutr*. **7**(suppl.): 99-111.

Riboli, E., Rönnholm, H. and Saracci, R. (1987). Biological markers of diet. *Cancer Surv*. **6**: 685-718.

Risé, P., Salvetti, F. and Galli, C. (2005). Application of a direct transmethylation method to the analysis of fatty acid profile in circulating and cultured cells. Anal *Biochem*. **346**: 182-184.

Risé, P., Eligini, S., Ghezzi, S., Colli, S. and Galli, C. (2007). Fatty acid composition of plasma, blood cells and whole blood: relevance for the assessment of the fatty acid status in humans. *Prostaglandins Leukot Essent Fatty Acids*. **76**: 363-369.

Risé P., Marangoni, F., Martiello, A., Colombo, C., Manzoni, C., Marconi, C., Cattabeni, F. and Galli, C. (2008). Fatty acid profiles of blood lipids in a population group in Tibet: correlations with diet and environmental conditions. *Asia Pac J Clin Nutr*. **17**: 80-85.

Risé, P., Volpi, S., Colombo, C., Padoan, R. F., D'Orazio, C., Ghezzi, S., Melotti, P., Bennato, V., Agostoni, C., Assael, B. M. and Galli, C. (2010). Whole blood fatty acid

analysis with micromethod in cystic fibrosis and pulmonary disease. *J Cyst Fibros*. **9**: 228-333.

Rizos, E. C., Ntzani, E. E., Bika, E., Kostapanos, M. S. and Elisaf, M. S. (2012). Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis. *JAMA*. **308**: 1024-1033.

Rizzo, A. M., Montorfano, G., Negroni, M., Adorni, L., Berselli, P., Corsetto, P., Wahle, K. and Berra, B. (2010). A rapid method for determining arachidonic:eicosapentaenoic acid ratios in whole blood lipids: correlation with erythrocyte membrane ratios and validation in a large Italian population of various ages and pathologies. *Lipids Health Dis*. **9**: 7.

Ryan, A. S. and Nelson, E. B. (2008). Assessing the effect of docosahexaenoic acid on cognitive functions in healthy, preschool children: a randomized, placebo-controlled, double-blind study. *Clin Pediatr.* **47**: 355-362.

Ryan, A. S., Astwood, J. D., Gautier, S., Kuratko, C. N., Nelson, E. B. and Salem, N. Jr. (2010). Effects of long-chain polyunsaturated fatty acid supplementation on neurodevelopment in childhood: a review of human studies. *Prostaglandins Leukot Essent Fatty Acids*. **82**: 305-314.

Salone, L. R., Vann, W. F. Jr. and Dee, D. L. (2013). Breastfeeding: an overview of oral and general health benefits. *J Am Dent Assoc*. **144**: 143-51.

Saravanan, P., Davidson, N. C., Schmidt, E. B. and Calder, P. C. (2010). Cardiovascular effects of marine omega-3 fatty acids. *Lancet*. **376**: 540-550.

Scopesi, F., Zunin, P., Bellini, C., Sacchi, R., Boggia, R., Evangelisti, F. and Serra, G. (2004). Cholesterol oxidation in intravenous lipid emulsions: safety of preparations before and after experimental hyperoxia. *J Parenter Enteral Nutr.* **28**: 342-347.

Sears, B. (2008). Toxic Fat: When Good Fat Turns Bad, TN: Thomas Nelson, Nashville, pp. 78-82

Serhan, C. N., Yang, R., Martinod, K., Kasuga, K., Pillai, P. S., Porter, T. F., Oh, S. F., and Spite, M. (2009). Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *J Exp Med*. **206**: 15-23.

Sharma, B. K., Bacon, B. R., Britton, R. S., Park, C. H., Magiera, C. J., O'Neill, R., Dalton, N., Smanik, P. and Speroff, T. (1990). Prevention of hepatocyte injury and lipid peroxidation by iron chelators and alpha-tocopherol in isolated iron-loaded rat hepatocytes. *Hepatology*. **12**: 31-39.

Sherwin, E. R. and Thompson, J. W. (1967). Tertiarybutylhydroquinone-An antioxidant for fats and oils and fat containing foods. *Food Technology*. **21**: 912-916.

Shieh. D. E., Liu, L. T. and Lin, C. C. (2000). Antioxidant and free radical scavenging effects of baicalein, baicalin and wogonin. *Anticancer Res*. **20**: 2861-2865.

Sies, H. (1997). Oxidative stress: oxidants and antioxidants. *Exp Physiol*. **82**: 291-295.

Simopoulos, A. P. (2002). The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed Pharmacother*. **56**: 365-79.

Sioen, I. A., Pynaert, I., Matthys, C., De Backer, G., Van Camp, J. and De Henauw, S. (2006). Dietary intakes and food sources of fatty acids for Belgian women, focused on n-6 and n-3 polyunsaturated fatty acids. *Lipids*. **41**: 415-422.

Siscovick, D. S., Raghunathan, T. E., King, I., Weinmann, S., Wicklund, K. G., Albright, J., Bovbjerg, V., Arbogast, P., Smith, H. and Kushi, L. H. (1995). Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *JAMA*. **274**: 1363-1367.

Sjöroos, M., Iitiä, A., Ilonen, J., Reijonen, H. and Lövgren, T. (1995). Triple-label

hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques*. **18**: 870-877.

Skeaff, C. M., McLachlan, K., Eyles, H. and Green, T. (2003). Buccal cells as biomarkers of fat intake. *Asia Pac J Clin Nutr*. **12**(suppl.): S55.

Söderberg, M., Edlund, C., Kristensson, K. and Dallner, G. (1991). Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. *Lipids*. **26**: 421-425.

Spector, A. A. (19990. Essentiality of fatty acids. *Lipids*. **34**(suppl.): S1-S3.

Sperling, R. I., Weinblatt, M., Robin, J. L., Ravalese, J., Hoover, R. L., House, F., Coblyn, J. S., Fraser, P. A., Spur, B. W. and Robinson, D. R. (1987). Effects of dietary supplementation with marine fish oil on leukocyte lipid mediator generation and function in rheumatoid arthritis. *Arthritis Rheum*. **30**: 988-997.

Stapelfeldt, H., Nielsen, B. R. and Skibsted, L. H. (1997). Effect of heat treatment, water activity and storage temperature on the oxidative stability of whole milk powder. *Int. Dairy J*., **7**: 331-339.

Strandvik, B., Gronowitz, E., Enlund, F., Martinsson, T. and Wahlström, J. (2001). Essential fatty acid deficiency in relation to genotype in patients with cystic fibrosis. *J Pediatr*. **139**: 650-655.

Strnadová, K. A., Holub, M., Mühl, A., Heinze, G., Ratschmann, R., Mascher, H., Stöckler-Ipsiroglu, S., Waldhauser, F., Votava, F., Lebl, J. and Bodamer, O. A. (2007). Long-term stability of amino acids and acylcarnitines in dried blood spots. *Clin Chem*. **53**: 717-722.

Suitor, C. W. and Meyers, L. D. (2006). Dietary Reference Intakes Research Synthesis: Workshop Summary, THE NATIONAL ACADEMIES PRESS, Washington, D.C., pp. 96.

Tangvarasittichai, O., Jongjitwimol, J. and Tangvarasittichai, S. (2008). Whatman 3 MM dried blood spots for identifying alpha-thalassemia-1. *Clin Lab*. **54**: 281-283.

ter Heine, R., Rosing, H., van Gorp, E. C., Mulder, J. W., van der Steeg, W. A., Beijnen, J. H. and Huitema, A. D. (2008). Quantification of protease inhibitors and non-nucleoside reverse transcriptase inhibitors in dried blood spots by liquid chromatography-triple quadrupole mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. **867**: 205-212.

Tzvetkova, P. and Nickolov, R. (2012). Modified and unmodified silica gel used for heavy metal ions removal from aqueous solutions. *Journal of the University of Chemical Technology & Metallurgy*. **47**: 498-504.

Valto, P., Knuutinen, J., Alén, R. (2011). Direct injection analysis of fatty and resin acids in papermaking process waters by HPLC/MS. *J Sep Sci*. **34**: 925-30.

van Aardt, M., Duncan, S. E., Long, T. E., O'Keefe, S. F., Marcy, J. E. and Sims, S. R. (2004). Effect of antioxidants on oxidative stability of edible fats and oils: thermogravimetric analysis. *J Agric Food Chem*. **52**: 587-91.

van der Heijden, J., de Beer, Y., Hoogtanders, K., Christiaans, M., de Jong, G. J., Neef, C. and Stolk, L. (2009). Therapeutic drug monitoring of everolimus using the dried blood spot method in combination with liquid chromatography-mass spectrometry. *J Pharm Biomed Anal*. **50**: 664-670.

van der Tempel, H., Tulleken, J. E., Limburg, P. C., Muskiet, F. A. and van Rijswijk, M. H. (1990). Effects of fish oil supplementation in rheumatoid arthritis. *Ann Rheum Dis*. **49**: 76-80.

Varnier, O. E., Lillo, F. B., Reina, S., De Maria, A., Terragna, A. and Schito, G. (1988). Whole blood collection on filter paper is an effective means of obtaining samples for human immunodeficiency virus antibody assay. *AIDS Res Hum Retroviruses*. **4**: 131-136.

Vejtorp, M. and Leerhoy, J. (1981). Rubella IgG antibody detection by ELISA using capillary blood samples collected on filter paper and in microtainer tubes. *Acta Pathol Microbiol Scand B*. **89**: 369-370.

Velasco, P. J., Dobarganes, C., Márquez-Ruiz, G. (2003). Variables affecting lipid oxidation in dried microencapsulated oils. *International journal of fats and oils*. **54**: 304-314.

Vessby, B. (2003). Dietary fat, fatty acid composition in plasma and the metabolic syndrome. *Curr Opin Lipidol*. **14**: 15-19.

Volker, D., Fitzgerald, P., Major, G. and Garg, M. (2000). Efficacy of fish oil concentrate in the treatment of rheumatoid arthritis. *J Rheumatol*. **27**: 2343-2346.

von Schacky, C., Fischer, S. and Weber, P. C. (1985). Long-term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. *J Clin Invest*. **76**: 1626-1631.

Velasco, P. J., Dobarganes, C. and Márquez-Ruiz, G. (2003). Variables affecting lipid oxidation in dried microencapsulated oils. *International journal of fats and oils*. **54**: 304-314

Waite, K. V., Maberly, G. F. and Eastman, C. J. (1987). Storage conditions and stability of thyrotropin and thyroid hormones on filter paper. *Clin Chem*. **33**: 853-855.

Wassilak, S. G., Bernier, R. H., Herrmann, K. L., Orenstein, W. A., Bart, K. J. and Amler, R. (1984). Measles seroconfirmation using dried capillary blood specimens in filter paper. *Pediatr Infect Dis*. **3**: 117-121.

Walker, A. (2010). Breast milk as the gold standard for protective nutrients. *J Pediatr*. **156**(suppl.): S3-S7.

Watson, D. G., Oliveira, E. J., Boyter, A. C., Dagg, K. D. (2001). A rapid and sensitive method for the determination of the amount of theophylline in blood spots. *J Pharm Pharmacol*. **53**: 413-416.

Williams, P. B. and Halsey, J. F. (1997). Endotoxin as a factor in adverse reactions to latex gloves. *Ann Allergy Asthma Immunol*. **79**: 303-310.

Wortman, P., Miyazaki, Y., Kalupahana, N. S., Kim, S., Hansen-Petrik, M., Saxton, A. M., Claycombe, K. J., Voy, B. H., Whelan, J. and Moustaid-Moussa, N. (2009). n3 and n6 polyunsaturated fatty acids differentially modulate prostaglandin E secretion but not markers of lipogenesis in adipocytes. *Nutr Metab (Lond)*. **6**: 5.

Yoko, I., Mio, M., Momoko, Y. and Yukio, F. (1999). Prevention of lipid oxidation in dried tofu with oxygen absorber during storage. *Naganoken Tanki Daigaku Kiyo*. **54**: 15-20.

Yokoyama, M., Origasa, H., Matsuzaki, M., Matsuzawa, Y., Saito, Y., Ishikawa, Y., Oikawa, S., Sasaki, J., Hishida, H., Itakura, H., Kita, T., Kitabatake, A., Nakaya, N., Sakata, T., Shimada, K., Shirato, K., Japan EPA lipid intervention study (JELIS) Investigators. (2007). Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. *Lancet*. **369**: 1090-1098.

Clandinin MT, Chappell JE, Leong S, Heim T, Swyer PR, Chance GW. (1980). Extrauterine fatty acid accretion in infant brain: implications for fatty acid requirements. *Early Hum Dev*. **4**: 131-138.

Hoffman DR, Boettcher JA, Diersen-Schade DA. (2009). Toward optimizing vision and cognition in term infants by dietary docosahexaenoic and arachidonic acid supplementation: a review of randomized controlled trials. *Prostaglandins Leukot Essent Fatty Acids*. **81**: 151-158.

Osendarp SJ, Baghurst KI, Bryan J, Calvaresi E, Hughes D, Hussaini M, Karyadi SJ, van Klinken BJ, van der Knaap HC, Lukito W, Mikarsa W, Transler C, Wilson C;

NEMO Study Group. 2007. Effect of a 12-mo micronutrient intervention on learning and memory in well-nourished and marginally nourished school-aged children: 2 parallel, randomized, placebo-controlled studies in Australia and Indonesia. *Am J Clin Nutr*. **86**: 1082-1093.

Muthayya S, Eilander A, Transler C, Thomas T, van der Knaap HC, Srinivasan K, van Klinken BJ, Osendarp SJ, Kurpad AV. 2009. Effect of fortification with multiple micronutrients and n-3 fatty acids on growth and cognitive performance in Indian schoolchildren: the CHAMPION (Children's Health and Mental Performance Influenced by Optimal Nutrition) Study. *Am J Clin Nutr*. **89**: 1766-75.