

Nutrient Composition and Digestibility of Chloroplast Rich Fractions from Green Leaf Materials

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Abstract

Green leaf material is recognized as a good source of many valuable nutrients including vitamins, fatty acids and minerals. Chloroplast-rich-fractions (CRFs) were prepared from green plant species and their nutrient composition compared with the whole leaf materials (WLMs). Digestibility of CRFs from spinach was also compared with the WLM using simulated *in vitro* oral, stomach and small intestinal conditions. The impact of pancreatic lipase-related protein 2 from guinea pig (gPLRP2) on the hydrolysis of galactolipids compared to neutral lipid, Tributyrin was subsequently determined *in vitro*. Porcine pancreatic lipase was also used against the same substrates compared to gPLRP2. Furthermore, spinach CRFs and WLMs were fed to zebrafish and the impact of CRFs and WLMs on growth performance and transition of certain compounds into zebrafish body was evaluated.

Results indicated that compared with the WLM, the CRFs were enriched in more lipids and fatty acids, and more proteins and amino acids. Spinach CRFs possessed the highest α -tocopherol (62 mg 100 g⁻¹, dry weight (DW)), β -carotene (336 mg 100 g⁻¹ DW) and lutein (341 mg 100 g⁻¹ DW) contents, whilst grass CRFs had the highest alpha-linolenic acid (ALA) concentration (69.5 mg g⁻¹). Of the minerals, iron was most notably concentrated in CRF.

The digestive conditions caused changes in the structure and composition of the material providing some indication of its digestibility and bioaccessibility.

Whilst PLRP2 was more active on galactolipids, with moderate reaction against the neutral lipid, pancreatin indicated higher activity on Tributyrin with almost no activity against MGDG.

Diets with 10% zebrafish meal reduction improved growth rate with significant reduction in feed conversion ratio (FCR) compared to the control. CRFs diets showed greater ALA content and distinct pigmentation of zebrafish egg and flesh due to the CRF carotenoids.

Overall, the results indicated that CRF is a potential digestible source of vital nutrients.

Dedication

This work is dedicated to the mercy and merciful God (Allah) for all graces God gives me in my entire life. "Allâh has brought you out from the wombs of your mothers while you know nothing. And He gave you hearing, sight, and hearts that you might give thanks (to Allâh)" [Surat An-Nahl: 78].

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List of Abbreviations

1,2-di-O- α -linolenoyl-3- O- β -D-galactopyranosyl-sn-glycerol (DLGG)

Age related macular degradation (AMD)

Alpha linolenic acid (ALA)_v

Alpha-Tocopherol (transfer) protein (α -TPP).

Analysis of variance (ANOVA)

Arachidonic acid (ARA)

Bicinchoninic acid (BCA)

Bile-salt stimulated lipase (BSSL)

Butylated hydroxytoluene (BHT)

Carbohydrate (CHO)

Carboxyl ester hydrolase (CEH)

Chloroplasts rich fraction (CRF)

Collision-cell technology-kinetic energy discrimination (CCT-KED)

Dehydroascorbic acid (DAA)

Diacylglycerol (DAG)

Digalactosyldiacylglycerols (DGDG)

Docosahexaenoic acid (DHA)

Eicosapentaenoic acid (EPA)

Fatty acid methyl esters (FAMES)

Feed conversion ratio (FCR)

Fish in fish out (FIFO)

Fish meal ratio (FMR)

Food and agriculture organization (FAO)

Free fatty acid (FFA)

Freeze dried whole materials (F-WLM)

Galactolipids (GL)

Gas chromatography-mass spectrometry (GC-MS)

Gastrointestinal tract (GIT)

High performance liquid chromatography (HPLC)

High performance thin layer chromatography (HPTLC)

Hydrochloric acid (HCL)

Inductively coupled plasma mass spectrometry (ICP-MS)

L-Ascorbic acid (AA)

Linoleic acids (LA)

Lipoprotein lipase (LPL)

Methyl-terta-butyle ether (MTBE)

Mini bead beater (MBB)

Mono unsaturated fatty acids (MUFA)

Monogalctosyldiglyceride (MGDG)

Nutrient reference intake (RNI)

Pancreatic lipase related protein 2 (PLRP2)

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylglycerol (PG)

Phosphatidylserin phospholipase A1 (PLA1)

Photo Diode Array (PDA)

Poly unsaturated fatty acids (PUFAs)

Protein conversion ratio (PCR)

Protein efficiency ratio (PER)

Reactive oxygen species (ROS)

Retinol equivalents (RE)

Saturated fatty acids (SFA)

Simulated gastric fluid (SGF)

Simulated intestinal fluid (SIF)

Simulated salivary fluid (SSF)

Sodium dodecyl sulphate (SDS)

Sodium taurodeoxycholate (NaTDC)

Specific growth rate (SGR)

Sucrose extracted freeze dried-chloroplasts rich fractions (SEF-CRFs)

Sucrose extracted wet chloroplast rich fractions (SEW-CRFs)

Sulfoquinosyldiacylglycerol (SQDG)

Thin layer chromatography (TLC)

Transmission electron microscope (TEM)

Triacylglycerols (TAGs)

Very low-density lipoproteins (VLDL)

Whole leaf material (WLM)

World health organization (WHO)

β , β -carotene-15,15'-oxygenase (BCOX)

Chapter One

Introduction

1.1. Thesis Layout and General Introduction

1.1.1. Thesis Layout

The first chapter of this thesis gives an introduction to chloroplasts and green leaves; their chemical composition [lipid and fatty acid, protein and amino acids, chlorophylls and carotenoids, minerals, antioxidant vitamins (provitamin A, E and C), as well as a non-vitamin antioxidant carotenoid (lutein)]. A brief background information on *in vitro* digestion models, bioaccessibility and human digestive processes - from ingestion to excretion is also discussed in this chapter. Lipid hydrolysis and lipolytic enzymes are subsequently addressed. Finally, fish digestive systems and carotenoid transportation to organs within fish are discussed.

The second chapter presents the materials and methods used for each working chapter.

In Chapter 3, the first results chapter - the compositional analysis of chloroplasts rich fractions (CRFs) and the parent whole leaf materials (WLMs) from spinach, kale, nettles (stinging) and grass are presented. Compounds assayed in this chapter include lipid, protein, chlorophyll, amino acids, fatty

acids, tocopherols (α -tocopherol) carotenoids (total and individual), ascorbic acid and minerals.

In Chapter 4, the results of digestibility and carotenoid (β -carotene and lutein) bioaccessibility of CRFs and WLMs from spinach with or without oil treatment are presented. Spinach was included and selected from the rest of green materials for its availability throughout the year.

In the subsequent Chapter (5), lipolysis by pancreatic lipase related protein 2 (PLRP2) and pancreatin on pure and natural substrates is demonstrated; these substrates include monogalactosyldiglyceride (MGDG), tributyrin and naturally occurring lipids (mainly galactolipids) in spinach CRFs and WLMs.

Chapter 6 (the last working chapter) is all about fish (zebrafish) feeding trials.

The impact of WLM and CRF on zebrafish growth parameters, carotenoid uptake and egg coloration after fish meal was reduced with 10%, 20% and 50% of either WLM or CRF is presented. Zebrafish (*Danio rerio*) was used as they display several characteristics which make them an ideal model species.

The last chapter of this thesis, Chapter 7 provides some general conclusions to the studies conducted, as well as recommendations for future work.

1.1.2. General Introduction

Epidemiological studies suggest that high dietary intakes of fruits and vegetables is associated with reduced risks of developing a range of chronic

diseases, including cancer and cardiovascular diseases (Hung et al., 2004, Dauchet et al., 2006, Mirmiran et al., 2009, Liu et al., 2001). Antioxidant vitamins such as tocopherols (vitamin E), β -carotene (provitamin A) and ascorbic acid (vitamin C) are found in high concentrations in fruits and vegetables (Bergquist et al., 2006). It is suggested that these antioxidant-rich compounds are involved in the protection against these chronic diseases (Rice-Evans and Miller, 1995, Abuajah et al., 2015).

Green leafy vegetables represent a potentially valuable source of a range of macro (lipids, proteins and carbohydrates) and micro (vitamins and minerals) nutrients. However, such plant material also contains large amounts of indigestible carbohydrate (largely cellulose) and potential anti-nutritional factors (Gibson et al., 2006). According to some research (Padayachee, 2011, Phan et al., 2015, Ribas-Agustí et al., 2014) plant cell walls restrict the bioaccessibility of nutrients during the consumption of plant-based material. Furthermore, there is continual increase in global population and therefore increasing pressure on finite food resources. This exacerbates the limited access to healthy plant-based foods by those on low incomes. Sustainable nutrition can be defined as “protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable, nutritionally adequate, safe, and healthy, while optimizing natural and human resources” (Burlingame and Dernini, 2012); the liberation of intact, nutrient-rich chloroplasts from plant cells of abundant green biomass (green waste or green biomass currently not used in the food chain), offers, in theory, an increase in the nutrient delivery per unit mass of green plant material.

Furthermore, due to concentration of nutrients liberated and stabilised, chloroplasts could be used as a functional food ingredient/fortifying agent at relatively low levels of addition to a food/feed formulation.

Many of the most valuable plant nutrients are synthesized and stored in the chloroplasts, (Leister and Pesaresi, 2005). These include, fatty acids and lipids, amino acids, plant hormones, nucleotides, vitamins and secondary metabolites (Gross, 1991, Walker and Barber, 1976). The isolation of chloroplasts out of their cellular matrix may thus facilitate a breakthrough for utilizing these vital nutrients as functional food ingredients. Chloroplasts are a type of semi-autonomous organelle found in leaves which are responsible for photosynthesis, the process where sunlight energy is converted into chemical energy. By structure, chloroplasts are lens-shaped bodies with a diameter of approximately 5-10 μm (Block et al., 2007, Gross, 1991).

Recovering nutritionally-rich fractions of chloroplasts from unconventional plants that are comparable to that of well-known sources such as spinach and kale is expected to lay the basis for recovering sustainable new functional food ingredients from green plants, provided that the toxicity issues of these plants are initially resolved before use. This approach represents a sustainable way to ensure that poorer societies have access to food rich in valuable micronutrients. There are also a range of commercial opportunities to use isolated chloroplasts in food and feed formulations.

Although green vegetables are compositionally rich in nutrients before ingestion, *in vitro* and *in vivo* studies consistently reported challenges concerning low absorption efficiency of these nutrients (De Pee et al., 1998, Jeffery et al., 2012). Various models exist to estimate absorption efficiency including bioaccessibility (micellarization), uptake by intestinal cells and bioavailability. Bioaccessibility is defined as the degree to which a substance is released out of its matrix and transferred to the body (enterocytes) for absorption (Carbonell-Capella et al., 2014). The bioaccessible fraction transferred from the digested food to the micelle is generally considered as an indicator of bioavailability (Blanquet-Diot et al., 2009). Fat-soluble nutrients tend to be more bioaccessible in the presence of oil; this aspect of digestion will be explored in this research into the bioaccessibility of nutrients from liberated chloroplasts.

The thylakoid membranes of the chloroplasts are the specific site where photosynthesis in higher plants takes place. In thylakoid membranes, proteins and lipids account for 60–65% and 35–40% (w/w), respectively, depending on growing conditions (Amara et al., 2010). As 77% of the lipid composition in the thylakoid membranes are neutral galactosyldiacylglycerols, they are considered unique among eukaryotic cellular membranes (Sprague, 1987). Of these, 51% and 26% are represented by monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG), respectively, with lower sulfoquinosyldiacylglycerol (SQDG) contents found in the thylakoid membranes (Andersson et al., 1995). Some non-photosynthetic tissues such as potato tuber, apples and seed grains possess galactolipids, as well

(Galliard, 1968). In general, galactolipids are more dominant in plant kingdom compared to triacylglycerols, the major lipid compounds (99%) of vegetable oils from oil seeds (sunflower, maize, soybean, rapeseed, sesame and peanut). Galactolipids which are rich in essential fatty acids such as, α -linolenic (from plant leaves) and linoleic acid (from wheat and oat grains) are important food sources for both animals and humans. Recently, greater emphasis is being placed on understanding the hydrolysis of galactolipids (Amara et al., 2010, Amara et al., 2009, Andersson et al., 1995, Andersson et al., 1996, N'Goma et al., 2012, Sias et al., 2004, Carrière et al., 1994). Classical pancreatic lipase, the hydrolysing enzyme of triacylglycerols in combination with co-lipase are now known to have minimal impact on galactolipid lipolysis; instead, pancreatic lipase-related protein 2 (PLRP2) was found to have greater influence and possess high activity against galactolipids (Amara et al., 2009, Amara et al., 2013, Carrière et al., 1998).

1.1.2.1. Chloroplast Rich Fraction as a Feed Ingredient

Having gained an insight into the composition of chloroplasts, one can see that these organelles, isolated or a chloroplast-rich fraction from leaves, could deliver vital nutrients to a range of animals/insects in the food supply chain.

Ruminant animals represent one of the main food resources for man. These animals have the ability to digest plant foods along with their cell walls for they possess specific enzymes that can hydrolyse the cellulose rich plant foods.

It might be of interest to feed ruminants with CRF material to see if there is any improvement in nutrient uptake compared with eating grass.

The consumption of insects (directly or indirectly through incorporation animal feed) is gaining much interest as a sustainable route to feed humans. Liberated chloroplasts would provide a novel, easy to digest nutrient-rich feed ingredient for insects. Many species are able to assimilate the fatty acids they consume, so chloroplast consumption could alter the fatty acid profile of insects tailoring their nutrient profile for particular applications.

Fish and its products can be consumed in various forms and are a good source of vital nutrients including proteins, fats, vitamins and minerals to human populations at large (Gedi et al., 2015). The aquaculture industry has grown in recent years to provide approximately 51% of world fish and sea food in 2013 (FAO, 2015). The percentage of fish harvested for aquaculture feed increased to almost 70% in 2012 (FAO, 2014). Many feeds used in the fishing industry include fishmeal as a source of high quality protein, and fish oil, which are high in unsaturated fatty acids (FAO, 1986b) - to provide the necessary nutritional profiles for target species. Both materials are produced from reduction of captured fish and fish by-products (FAO, 1986a, FAO, 2014), therefore both commodities are highly affected by catch rates and raw material availability. As a result of increasing demand and stable production, the price of both commodities can increase and vary significantly. There is consequently a need to identify cheaper and more sustainable alternatives to both protein and oil materials, at least partially. Protein is the most expensive component of aquaculture feeds (Wilson, 2002); although the inclusion of

alternative materials has risen, the use of fish based materials has also declined. This trend is predicted to continue (Green, 2012).

A whole leaf material (WLM) and a more refined CRF materials were investigated as dietary ingredients to aid reduction of fishmeal in zebrafish diets. The major fatty acid (>60%) in chloroplast is the omega-3 fatty acid known as α -linolenic acid, a portion of which is suggested to be converted into long chain unsaturated fatty acids found in fish lipids (Barceló-Coblijn and Murphy, 2009). These lipids are an important dietary source for ω -3 long-chain polyunsaturated fatty acids (PUFAs) namely eicosapentaenoic acid (EPA, 20:5 ω -3), and docosahexaenoic acid (DHA, 22:6 ω -3), which have shown potential benefits for the proper growth of children and, prevention of cardiovascular diseases and cancer (Shahidi and Miraliakbari, 2004). Consumption of fish oils also contributes to energy supplies and assists in the proper absorption of fat soluble vitamins namely A, D, E, and K through digestion (Banda-Nyirenda et al., 2009).

Despite the apparent nutritional credentials of chloroplasts, no reports appear to have been published on their potential as functional food ingredients. In addition, while the nutritional composition and carotenoid bioaccessibility is well-documented, little is known about chloroplast digestibility and the physical state of the chloroplast carotenoids. Similarly, no-one to date has applied PLRP2 into complete *in vitro* digestion model (from oral, gastric to small intestines) using isolated chloroplasts rich fractions. Again, and to the

best of our knowledge no one has used CRFs as alternative feed for partial replacement of fish diet.

The main hypothesis of this study is that isolated CRFs represent a digestible source of vital nutrient for both human consumption and fish/animal feed

Aims and Objectives:

1. To concentrate a range of nutrients in the form of chloroplast-rich fractions (CRFs) compared to the parent whole leaf material (WLM) of selected green leafy plants. Spinach and kale were included as they are readily consumed and commercially available throughout the year, whilst grass and nettles were selected as they are generally not consumed as part of the typical human diet. The candidate nutrients/bioactive compounds are:

- a) Total lipid
- b) Fatty acid composition
- c) Total protein
- d) Amino acids composition
- e) Total chlorophylls and carotenoids
- f) Alpha-tocopherols (vitamin E)
- g) β -carotene (provitamin A)
- h) Lutein

- i) Ascorbic acid (vitamin C)
 - j) Minerals (major and trace).
2. To characterise the digestive fate and bioaccessibility (*in vitro*) of chloroplast rich fractions from spinach (as a model for green vegetables), compared with whole leaf material (WLM). Carotenoids namely, β -carotene and lutein were used as a marker of bioaccessibility of chloroplast nutrients in the presence or absence of oil
- a) CRF recovered by grinding leaves in a sucrose solution (0.3M) as an osmoticum to retain chloroplast integrity
 - b) CRF extracted using juicer (no added water)
 - c) CRFs from an Ultra-Turrax homogenizer (no added water)
 - d) CRFs from a microcavitator (no added water)
3. To examine the activity of pancreatic lipase related protein 2 (PLRP2) and pancreatin (cocktail of various porcine digestive enzymes including pancreatic lipase) on a preparation of pure MGDG, and on a chloroplast rich fraction where the MGDG is an integral part of the thylakoid membranes.
4. To assess the effectiveness of freeze-dried whole leaf spinach and its chloroplast rich fractions as alternative ingredients for fish feed. The impact of CRF incorporation into standard fish chow on general well-being factors (e.g. growth performance) and the resultant chemical

composition of Zebrafish were measured. Components assayed included, fatty acids, vitamin E, retinol, and carotenoids. In addition, flesh and egg colours were investigated and compared with the controls as qualitative markers of carotenoid transfer and digestibility in the zebrafish diet.

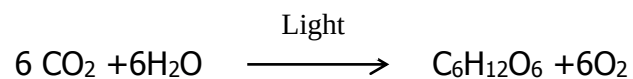
1.2. Background

Chloroplasts are among a group of organelles under the umbrella of plastids that are found in all living cells of plants. These organelles include chromoplasts, a carotenoid-rich plastid and leucoplasts, which are characterized by a lack of coloration. The leucoplasts can be further classified into amyloplasts (for starch storage), proteoplasts (for protein storage) and elaioplasts (for oil storage) (Møller, 2005). Of the plastid forms mentioned above, chloroplast is the only organelle that can produce energy as it is the site where all photosynthesis takes place (Møller, 2005, Walker and Barber, 1976). By structure, chloroplasts are lens-shaped bodies with a diameter of approximately 5-10 μm (Block et al., 2007, Gross, 1991) (See Fig.1.1). Surrounded by a double membrane envelope, permeability and transport of different metabolites into and out of the chloroplasts is controlled (Gross, 1991). Thylakoids, a complex membrane system that comprise grana and stroma lamellae, characterize mature chloroplasts (Møller, 2005). In general there are about 5-20 thylakoids per granum, but around 100 thylakoids in a granum of the chloroplasts from shaded plants (Meier and Lichtenthaler,

1981). Thylakoids are the site where photosynthetic apparatus of higher plants is located. Photosystem II complexes are concentrated more in the grana (Armond and Arntzen, 1977, Møller, 2005) whereas the stroma lamella is mainly found in photosystem I (Møller, 2005).

1.2.1. Function

The major function of chloroplasts is capturing light energy and using it for the conversion of carbon dioxide to carbohydrate and oxygen as shown below:



In addition, chloroplasts functions as the site where fatty acids and lipids, amino acids, plant hormones, nucleotides, vitamins and several other secondary metabolites are synthesised (Gross, 1991, Walker and Barber, 1976, Weaire and Kekwick, 1975).

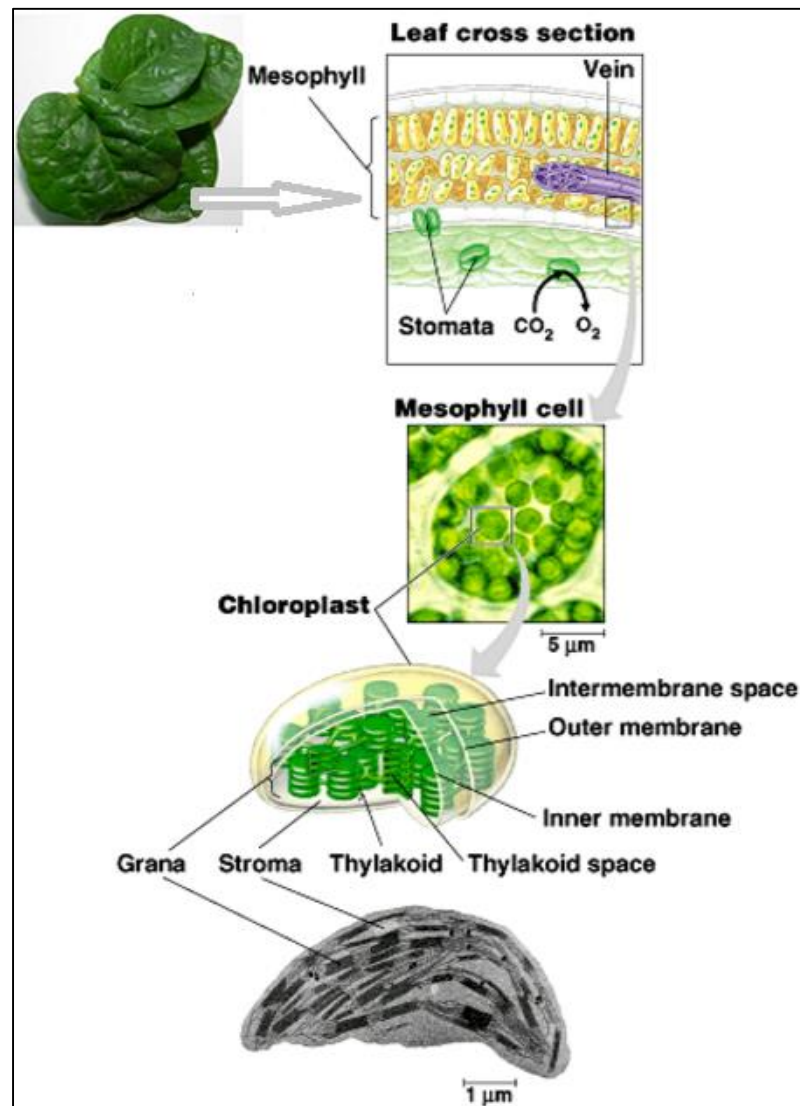


Figure 1.1. Cross section of leaf and its chloroplasts compartments adapted from: Biology-Article (2015)

1.2.2. Chemical Composition

About 70-80% of the chloroplast is water whereas about 60% of the chloroplasts dry weight is represented by proteins. These include structural proteins, which are bound in the lipoprotein complex of the lamellae (hydrophobic) and water-soluble or hydrophilic proteins (enzymes), found in

the complex and in the stroma (Gross, 1991). Lipids make up 30-40% of chloroplast dry weight and 50% of the photosynthetic thylakoid membranes; other pigments such as, chlorophylls, carotenoids and tocopherols being 5-10% of the total dry matter (Gross, 1991, Myers and Graham, 1956). Additional details of major chemical compositions of chloroplasts are discussed below.

1.2.2.1. Proteins and Amino Acids

Although proteins are among the major molecules in leafy vegetables, leafy tissues are not generally regarded as a good source of proteins. The protein value of vegetables is low due to their large moisture content (Bawa and Yadav, 1986). From a nutritional point of view, leafy vegetables can only contribute minor amounts of the daily recommended protein as compared to protein-rich sources from protein-rich plants (legumes) or animal sources. However, all essential amino acids, with varying concentrations depending on the individual vegetable, are found in these leafy vegetables. Chloroplasts containing 60% protein are an essential site for the synthesis of amino acids, vitamins, as well as other essential compounds (lipids, fatty acid and terpenoids). In most of these processes, proteins (such as those membrane proteins found in thylakoids and envelope membranes) play a key role (Salvi et al., 2000).

Whilst our body can produce some amino acids (alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine), for some others, we are dependent on external sources for the so-called "essential" amino acids (See Fig. 1.2); this is because humans lack the enzymes required for the biosynthesis of these amino acids, whereas plants can synthesise all their required amino acids. Some non-essential amino acids like tyrosine become essential if its precursor (phenylalanine) is deficient in the diet.

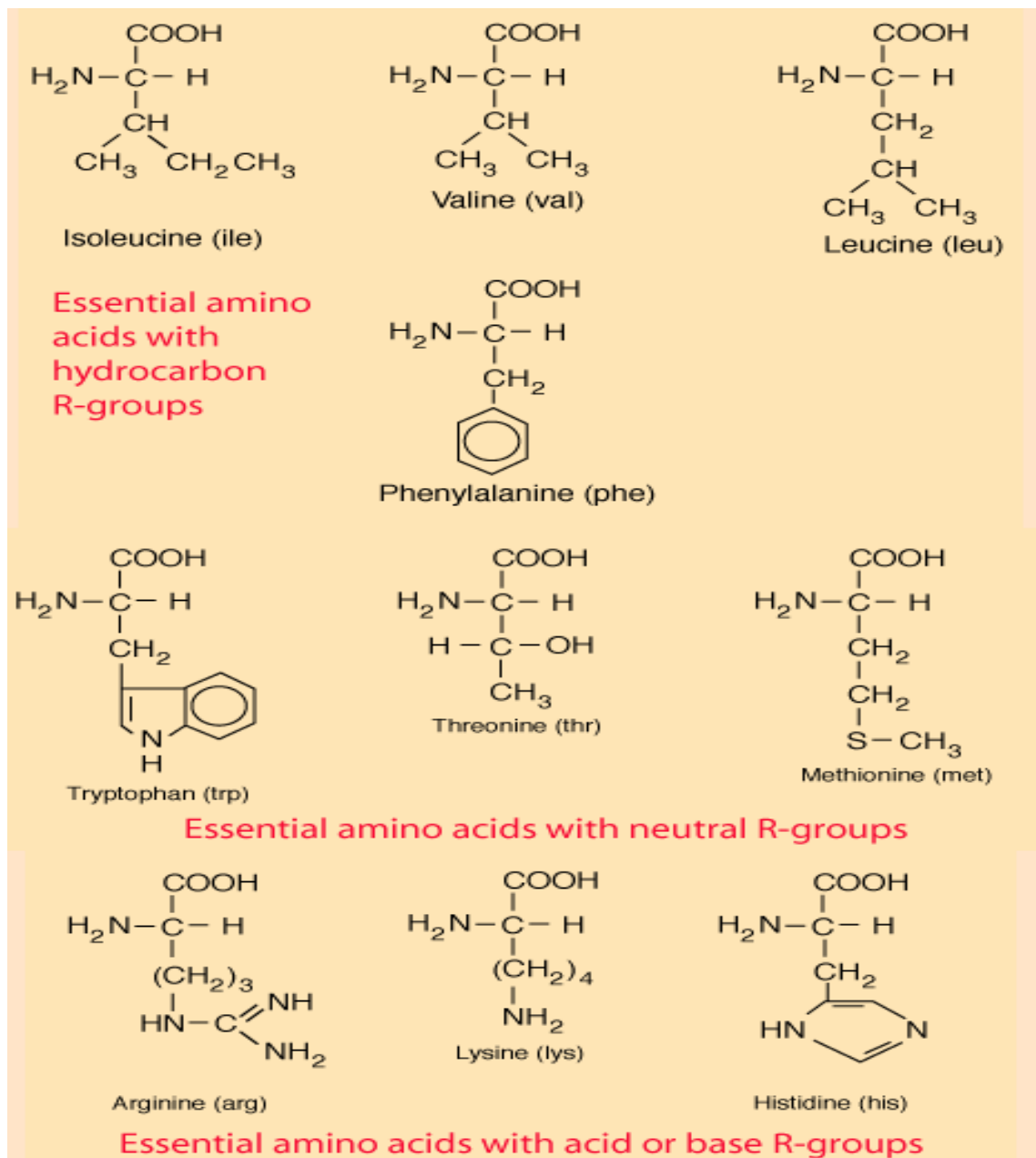


Figure 1.2. Essential amino acids (source: (Hyperphysics, 2012)).

Essential amino acids may depend on the age group, for instance, arginine is essential for young children but not for adults (Tucker and Dauffenbach, 2010). Failure to obtain an adequate amount of some of the essential amino acids can have serious health implications, and could potentially lead to the degradation of the body's proteins. Unlike carbohydrates and fats, excess amino acids are not stored by the body for later use, consequently amino acids must be consumed regularly as part of a diet.

1.2.2.2. Fatty acid Composition

The fatty acid composition of chloroplasts from a range of plant species is presented in Table (1.1). Apart from maize chloroplasts, where the saturated fatty acids (Palmitic and stearic) were dominant (Poincelot et al ,1976), unsaturated fatty acids, notably α -linolenic acid were the major fatty acids in all chloroplast leaves reported in these studies. The percentage of α -linolenic acid in spinach chloroplast reported by Poincelot et al (1976) was, however, lower than what others have found (Table 1.1). Poincelot et al (1976) suggested that this was probably due to the very young age of the leaves tested (two weeks old) , but the higher oven temperatures (60°C) used for drying the sample may have also degraded the highly thermo-sensitive unsaturated fatty acid e.g. α -linolenic acid.

Table 1.1.Fatty acid composition of chloroplasts from various plant leaf tissues

Plant	Membrane type	Fatty acid (%)							Reference
		16:0	16:1	16:3	18:0	18:1	18:2n6c	18:3n3	
Spinach	Intact chl	11.2	3.5	10.8	-	1.1	4.6	68.9	(Wolf et al., 1962)
Spinach	Envelope	41.4	3.4	2.4	10.5	4.6	8.4	28.9	(Poincelot, 1976)
Cauliflower*	Intact chl	4.8	0.9	28.6	1.4	6.9	3.6	47.5	(Schwertner and Biale, 1973)
Sunflower	Envelope	35.9	2.6	0.6	1.9	1.9	13.5	39.6	(Poincelot, 1976)
Maize	Envelope	77.3	Tr	5.4	11.7	1.0	1.5	0.4	(Poincelot, 1976)
Vicia faba	Envelope	13.3	1.8		4.31	5.8	11.5	63.3	(Mackender and Leech, 1974)
Vicia faba	Lamella	5.9	--	1.2	1.6	3.2	5.2	82.9	(Mackender and Leech, 1974)

C16:0 (Palmitic); **C16:1** (Palmitoleic); **C16:3** (Hexadecatrienoic); **C18:1** (Oleic); **C18:2** (Linoleic); **C18:3** (α -Linolenic); Tr: trace; -- = not detected; chl: chloroplasts; *: % mole.

1.2.2.3. Lipid Composition

The majority of lipids in green plants are glycosylglycerolipids such as, monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and sulfoquinosyldiacylglycerol (SQDG). These glycosylglycerolipids were not known until their presence in lipid extracts of wheat flour was reported by Carter et al. (1956). Later on, Benson et al. (1958) reported specific association between glycosylglycerolipids and photosynthetic tissues (thylakoid membranes of chloroplasts), before their currently accepted chemical structure was elucidated by Carter et al. (1961). These lipids are predominantly found in chloroplasts, and represent 90% of the total lipids in the chloroplast thylakoid membrane (Yunoki et al., 2009). Principally, glycosylglycerolipids function to enhance membrane stability. They are basically a glycerol moiety linked to a galactose group with a hydrophilic head group and a hydrocarbon chain with a hydrophobic head group (Chapman et al., 1983) as shown in Fig.1.3.

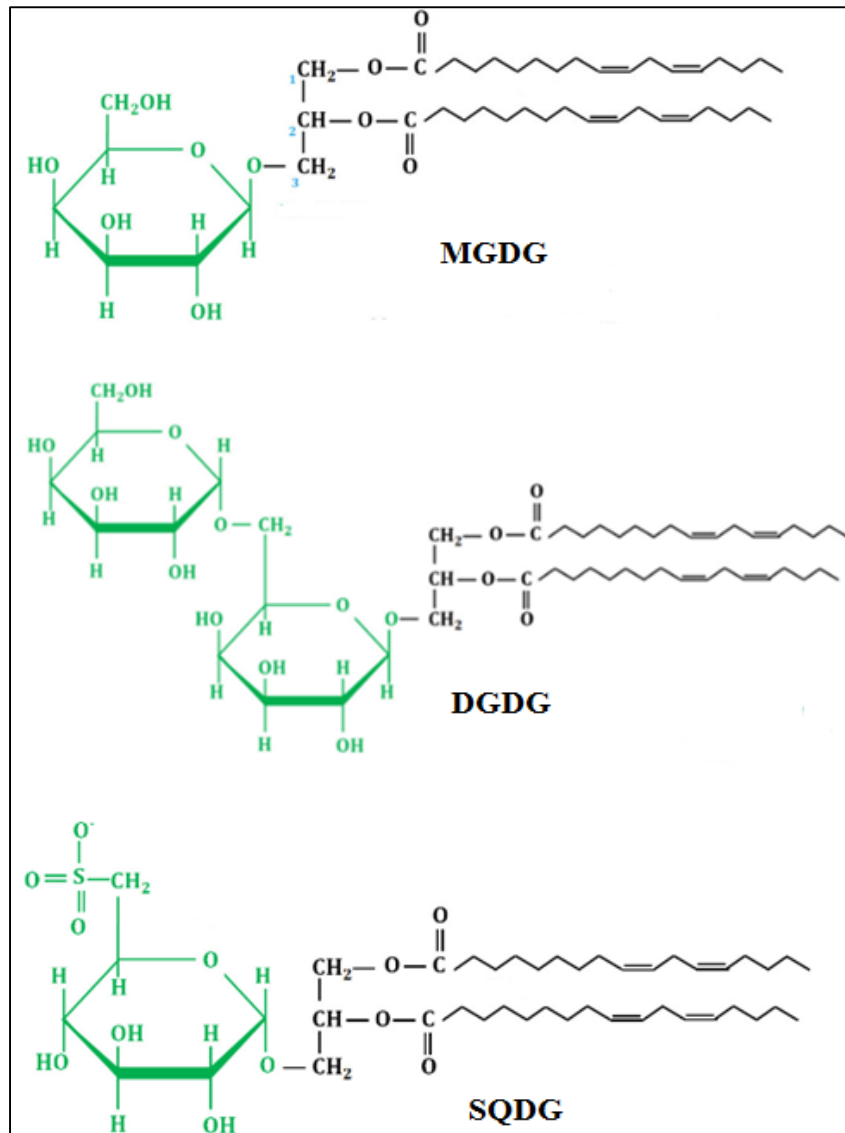


Figure 1.3. Galactolipid structure of MGDG, DGDG and SQDG. Adapted from Easybiologyclass (2016)

Previous published data on lipid composition of chloroplast-rich materials from cauliflower, avocado and spinach are also shown in Table (1.2).

Although phospholipids are not considered to be the principal lipid class in chloroplasts, non-negligible amounts of them was reported to be existing in chloroplasts as shown in Table (1.2). Mostly, phosphatidylcholine (PC) and phosphatidylglycerol (PG) are the predominant phospholipids in chloroplasts followed by phosphatidylethanolamine (PE) and phosphatidylinositol (Block et

al., 2007, Mackender and Leech, 1974, Poincelot, 1976, Schwertner and Biale, 1973).

Table 1.2. Fatty acid composition of lipid classes of cauliflower, avocado and spinach chloroplasts

Plant	chloroplast type		Fatty acid (%)											Reference
			14:0	14:3	16:0	16:1	16:2	16:3	17:0	18:0	18:1	18:2	18:3	
Cauliflower	Intact	Total lipids	--	--	11.3	4.0	1.5	13.9	--	1.7	9.6	7.2	40.2	(Schwertner and Biale, 1973)
		MGDG	--	--	2.8	0.9	2.8	28.6	0.8	1.4	6.9	3.6	47.5	
		DGDG	--	--	8.8	1.8	1.2	0.4	0.8	2.6	5.9	5.7	67.9	
		PG	--	--	24.1	31.6	1.6	--	2.0	1.9	2.1	5.2	2.8	
		PC	--	--	30.9	11.0	1.3	--	9.0	1.2	1.0	4.8	2.1	
		SL	--	--	27.8	16.1	--	--	--	3.2	18.3	11.7	18.8	
		Total phospho lipids	--	--	32.4	16.4	1.0	--	2.0	0.9	1.3	6.4	4.8	
		Total neutral lipids	--	--	26.9	7.0	0.6	4.4	3.7	0.6	8.7	3.2	2.5	
Avocado	Intact	Total lipids	--	--	11.2	1.4	0.1	0.2	--	0.5	57.4	13.2	13.1	(Schwertner and Biale, 1973)
		MGDG	--	--	4.4	2.1	0.9	0.9	0.9	2.8	43.2	7.8	31.7	
		DGDG	--	--	6.0	1.7	1.3	--	1.1	2.1	46.0	7.8	28.5	
Spinach	Envelope	Total Lipids (F4)	Tr	Tr	7.5	4.7	Tr	12.9	--	Tr	2.2	1.8	70.2	(Douce et al., 1973)
		MGDG (F4)	--	--	Tr	--	--	26.0	--	--	0.6	1.0	72.0	
		DGDG (F4)		0.9	4.6	--	--	4.0	--	Tr	2.5	0.8	87.0	
		SL(F4)	Tr	Tr	47.5	Tr	Tr	Tr	--	Tr	0.8	4.4	46.9	
		PG (F4)	1	Tr	18.3	39.6	--	Tr	--	Tr	0.5	1.6	38.5	
		PC(F4)	Tr		13.2	--	Tr	1.1	--		7.7	19.8	57.2	
		PI (F4)	2.1	Tr	42.1	0.5	4.1	2.0	--	1.0	7.0	14.3	26.3	

MGDG, monogalactosyldiglyceride; DGDG, digalactosyldiglyceride; SL, sulfoquinovosyldiglyceride; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; Tr= trace<0.5%; F= fraction; -- = not detected

1.2.2.4. Chlorophylls

Chlorophyll is the green photosynthetic (photoreceptor) pigment found in the chloroplasts of green plants and certain microorganisms (e.g. algae), and is what makes green plants, green. The basic structure of a chlorophyll molecule is a porphyrin ring, co-ordinated to a central atom. This is very similar in structure to the heme group found in hemoglobin, except that in heme the central atom is iron, whereas in chlorophyll it is magnesium (Fig.1.4).

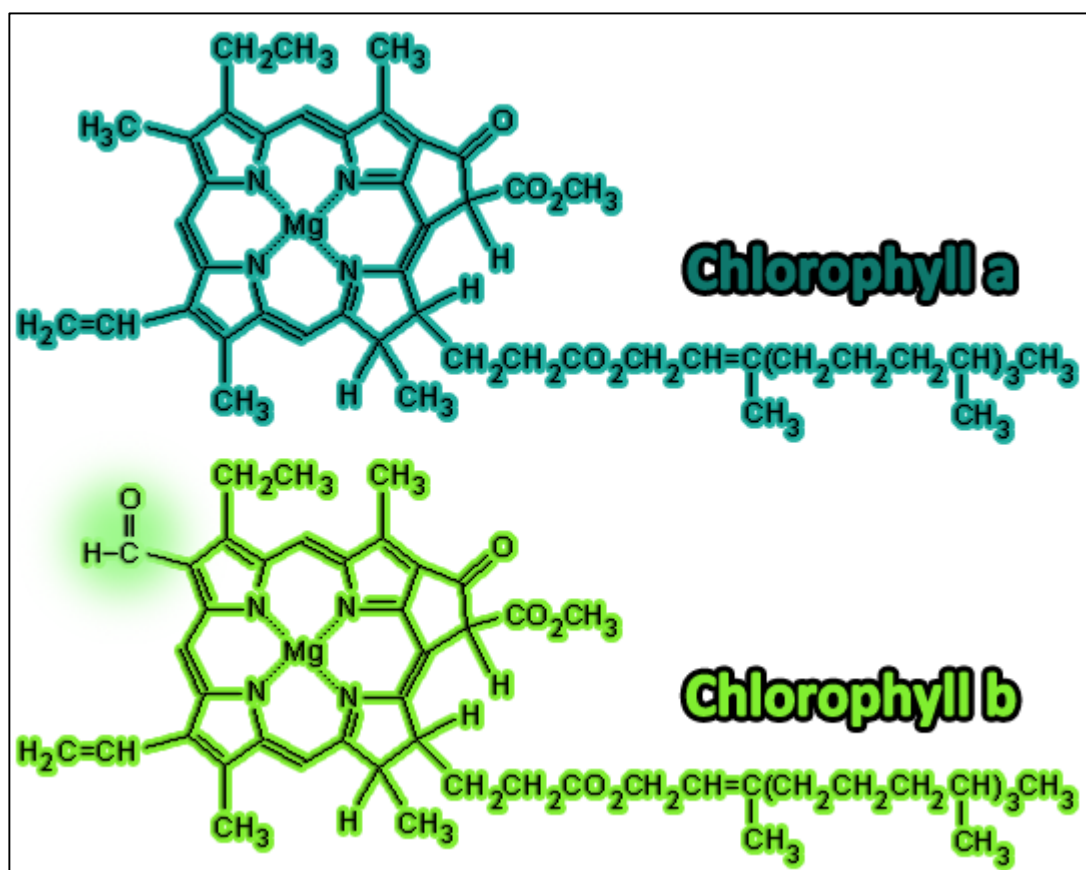


Figure 1.4. Chemical structure of chlorophylls *a* and *b*. Source: (Georgia-Virtual-Learning, 2015)

Chlorophylls are basically of two types, namely *a* and *b*; certain chemical or physical reactions such as acidification and heat can degrade and allomerize chlorophylls *a* and *b* into their derivatives (pheophytins *a* and *b*) (Ferruzzi et al., 2001). Chlorophylls *a* and *b* differ slightly only in the composition of a side chain (in *a* it is -CH₃, in *b* it is CHO). Both of these two chlorophylls are very effective photoreceptors (absorb violet, blue and red light). Chlorophyll *a* is directly involved in the light reactions of photosynthesis whilst chlorophyll *b* assists chlorophyll *a* in light energy, thus, it also known as an accessory pigment. Accessory pigments (including chlorophyll *b* and carotenoids) absorb colours that chlorophyll *a* cannot absorb, this allows plants to capture more energy from light. Chlorophyll *a* and *b* can be found in the ratio of ~3:1 (Coultate et al., 2002, Lichtenthaler et al., 1981, Seybold and Egle, 1970). A ratio of chlorophyll *a/b* ranging between 2.5 to 3.5 was also reported by Gross (1991).

1.2.2.5. Vitamin E

Tocopherols comprise methyl tocols or benzopynarols which are found in plant tissues and in oil seeds. In the tocopherols, the C16 side chain is saturated, and in the tocotrienols, three trans double bonds are found in the C16 side chain. Collectively these two groups are known as tocochromanols (Christie, 2014). These compounds are powerful lipid-soluble antioxidants, abundant in chloroplasts and are essential constituents of animal and human diets,

(DellaPenna and Pogson, 2006). Tocochromanols constitute a group of eight tocopherols and tocotrienols that together constitute vitamin E (Fig.1.5), and are essential nutrients in the diet of all mammals (DellaPenna and Pogson, 2006). Vitamin E is protective against about 80 diseases, including cancer, cardiovascular diseases and DNA damage by free radicals and oxidation of low-density lipoproteins (Gey and Puska, 1989, Weber et al., 1997). Alpha - tocopherol, the major common form of vitamin E existing in nature is the most biologically active (Bjorneboe et al., 1990), and preferentially retained in large quantities and transported to body components (Traber et al., 1990).

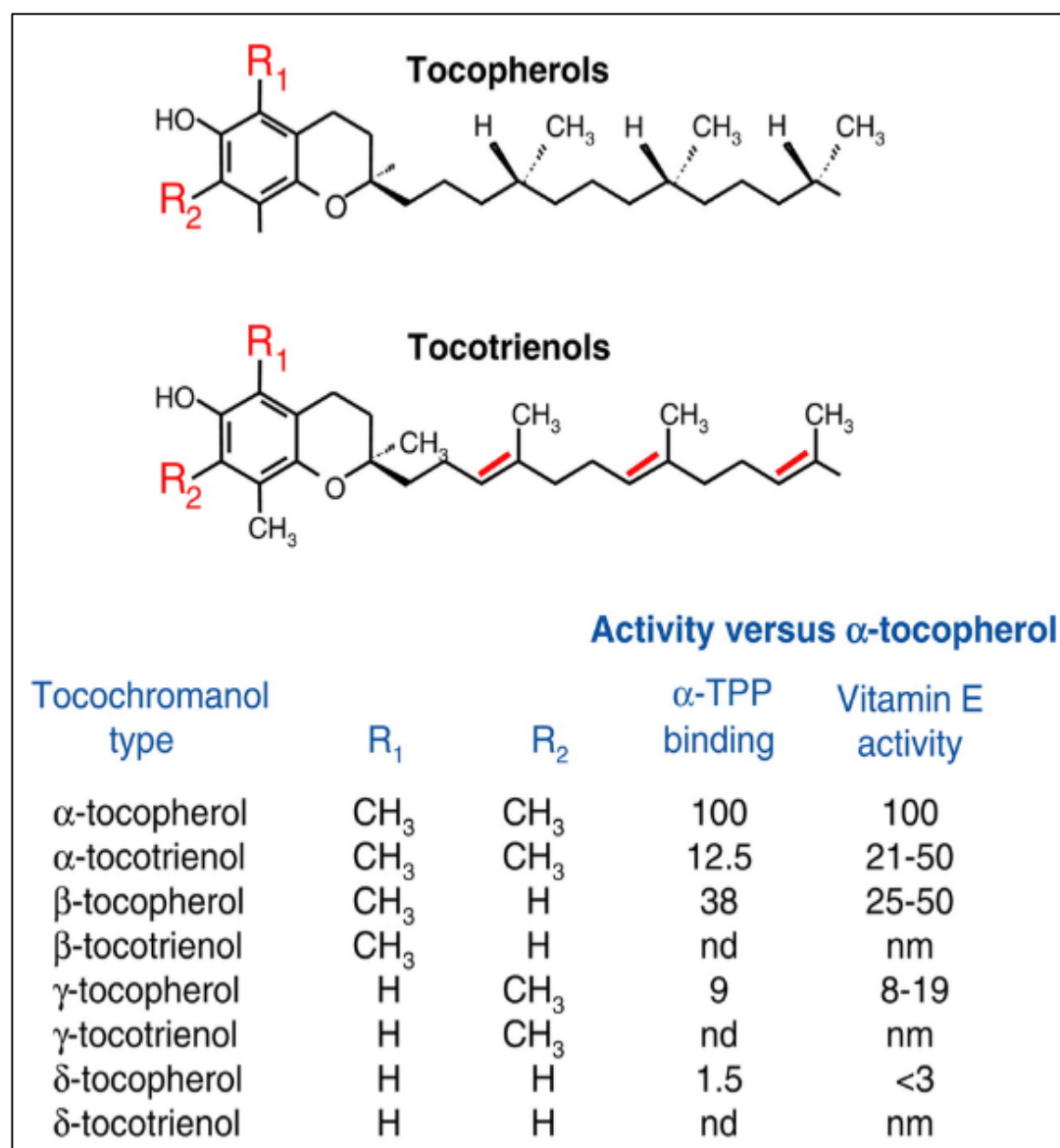


Figure 1.5. Chemical structures of tocochrominols and their activities adapted from DellaPenna and Pogson (2006). α -TPP: α -Tocopherol (transfer) protein.

In general, tocopherols are more concentrated in leaf tissues (α -tocopherol as the main isomer) and many seeds (γ -tocopherol as the primary tocopherol) whilst tocotrienols exist only in certain seeds and fruits, especially of monocots e.g. wheat, rice, oats and barley (Christie, 2014). The work of Chun et al. (2006) provides a long list of tocopherols and tocotrienol content in fruits and vegetables.

1.2.2.6. Carotenoids

Carotenoids are pigments synthesized exclusively by photosynthetic organisms including crop plants, algae, few fungi and certain bacteria (Britton et al., 2004). They constitute a group of over 700 structures responsible for the yellow, red and orange colours of many fruits, vegetables and flowers (Britton et al., 2004). Carotenoids belong to the C₄₀- based isoprenoid family termed tetraterpenes (Kaulmann and Bohn, 2014, Milani et al., 2016). Categorically, they are of two main groups: carotenes (e.g. α -carotene, β -carotene and lycopene) which are purely hydrocarbons and well distinguished for being devoid of oxygen, and xanthophylls (e.g. lutein, zeaxanthin, neoxanthin, violaxanthin and β -cryptoxanthin) with oxygen in their carbon chain. (See Fig. 1.6). Where green vegetables contain high amounts of both oxygenated and non-oxygenated carotenoids, lycopene an oxygenated carotenoid is present in ripe tomato fruit as a lipophilic red pigment and carrots are orange in colour as a result of β -carotene. Similarly, whilst the red colour of pepper is due to capsanthin the pink red coloration of crustaceans is accounted for by astaxanthin. Like tocochromanols, carotenoids are lipid-soluble antioxidants predominantly found in chloroplasts which are essential pigments in animal diets, humans included (DellaPenna and Pogson, 2006). These pigments also play an important role in photo system-I and photo system-II. Most notably, they act as light harvesters and protect chlorophylls from photo oxidation (Ahamad et al., 2007).

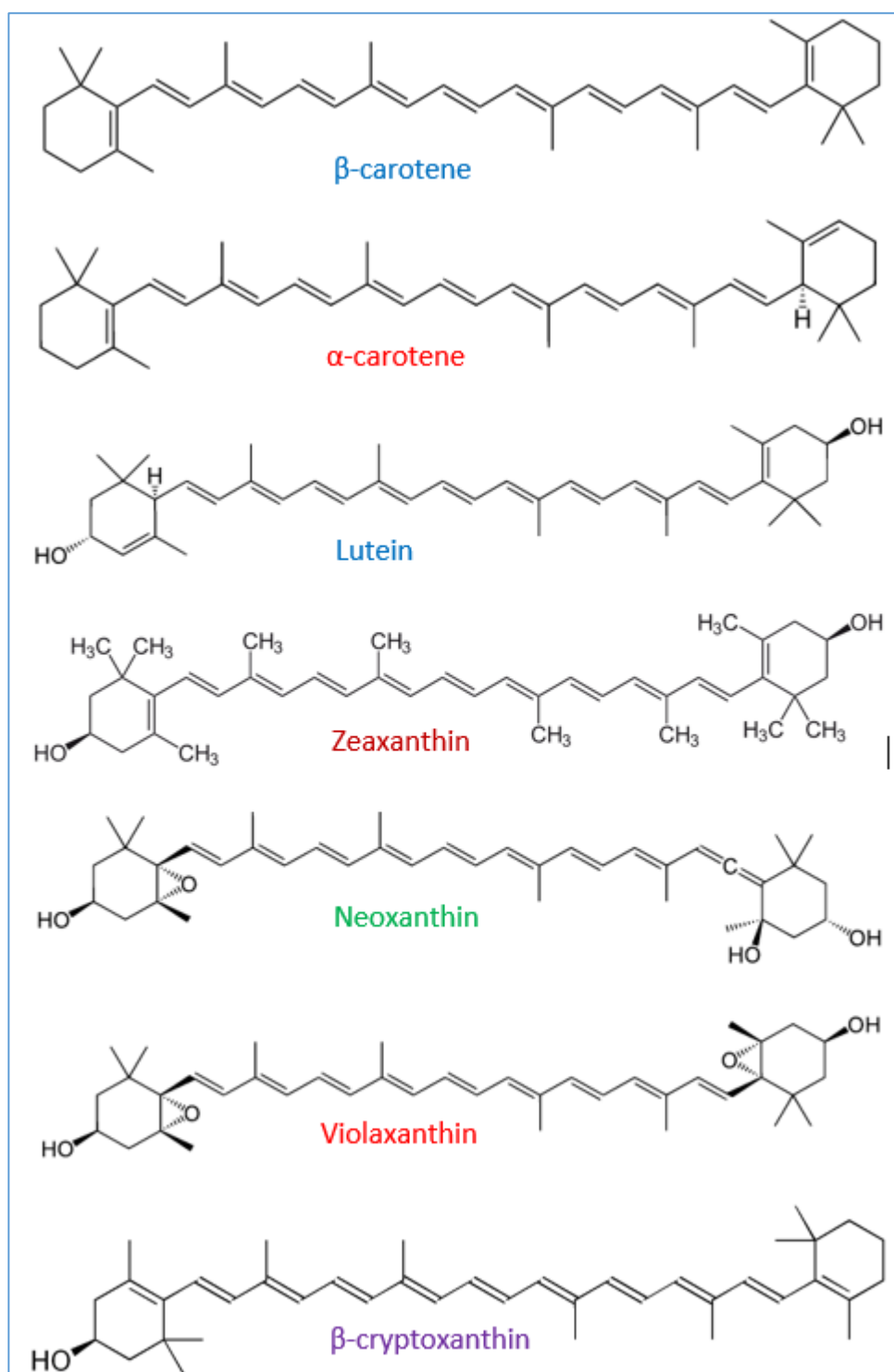


Figure 1.6. Major dietary carotenoids in vegetables and their chloroplasts

The two main groups of carotenoids (i.e. carotenes and xanthophylls) are found in spinach chloroplasts with β -carotene (29%) representing carotenes, as α -carotene is never detected in spinach. Xanthophylls in spinach

chloroplasts include lutein (31%), violaxanthin (20%) and neoxanthin (10%). These were identified as the four major carotenoids in chloroplasts of higher plants, spinach included (Konishi et al., 1968). Despite difficulties in comparing results of different laboratories who have treated samples and extraction procedures differently (different solvents, use or/no saponification step, difference in chromatographic conditions and type etc.), an attempt to draw β -carotene and lutein averages of spinach from the literature was made (Table 1.3).

Table 1.3. Spinach carotenoid composition (mg 100 g⁻¹ fresh weight) from literature as compared to the current study

Reference	Carotenes		Xanthophylls			
	β-carotene	α-carotene	Lutein	Zeaxanthin	violaxanthin	Neoxanthin
Hart and Scott (1995)	3.4	-	5.8	0.13	-	-
Bergquist et al. (2006)	5.2	-	9.1	-	7.3	3.1
Granado et al. (1992)	3.2	-	4.2	0.37	-	-
Tee Siong and Lam (1992)	2.6	-	3.9	-	-	-
Heinonen et al. (1989)	3.3	-	4.4	-	-	-
de Azevedo-Meleiro and Rodriguez-Amaya (2005)	5.0	-	7.21	-	3.9	2.2
Holden et al. (1999)	5.6	-	-	-	-	-
Average	4.0	-	5.7	0.25	5.6	2.7
This study	4.6	-	5.2	-	-	-

-: not detected or assayed

1.2.2.6.1. β -Carotene

Beta-carotene is an organic pigment (red-orange colour) widely found in plants mainly in fruits and vegetables, as well as, algae (e.g. *Dunaliella Salina*). In some cases, specific carotenoids are essential components of mammalian diets as precursors for vitamin A synthesis and approximately 10 % of the carotenoids are convertible to vitamin A (retinol) by enzymatic (β -carotene 15, 15'-monooxygenase) cleavage in the body (Byers and Perry, 1992), mainly from β -carotene but also from α -carotene, β -cryptoxanthin and γ -carotene. However, excessive β -carotene consumption (mainly in the form of supplement and not from common dietary sources) could increase the risk of cancer in smokers (Russell, 2002).

Intakes of vitamin A are normally expressed in terms of retinol equivalents (RE), the biological activity associated with a specific amount of all-trans retinol. Despite ongoing discussions in the literature for carotene conversion rates, 6 μg of all-trans β -carotene or 12 μg of other provitamin A carotenoids, have been retained as the conversion values equivalent to 1 RE (FAO and WHO, 2005).

Vitamin A is essential for the maintenance of normal epithelial cellular differentiation, eye health, and strong immune system. Vitamin A deficiency remains a significant global health problem, with specific impact on the poorest segments of populations in low and middle income countries as shown in Fig 1.7. The status of vitamin A deficiency in Somalia, for instance, is among the

worst in the world and lies at the bottom list of the table with highly severe vitamin A deficiency (Fig.1.7). According to WHO (WHO, 2009a), vitamin A deficiency is among the most common causes of preventable childhood blindness and greatly contributes to morbidity and mortality of infections mainly in children and pregnant women.

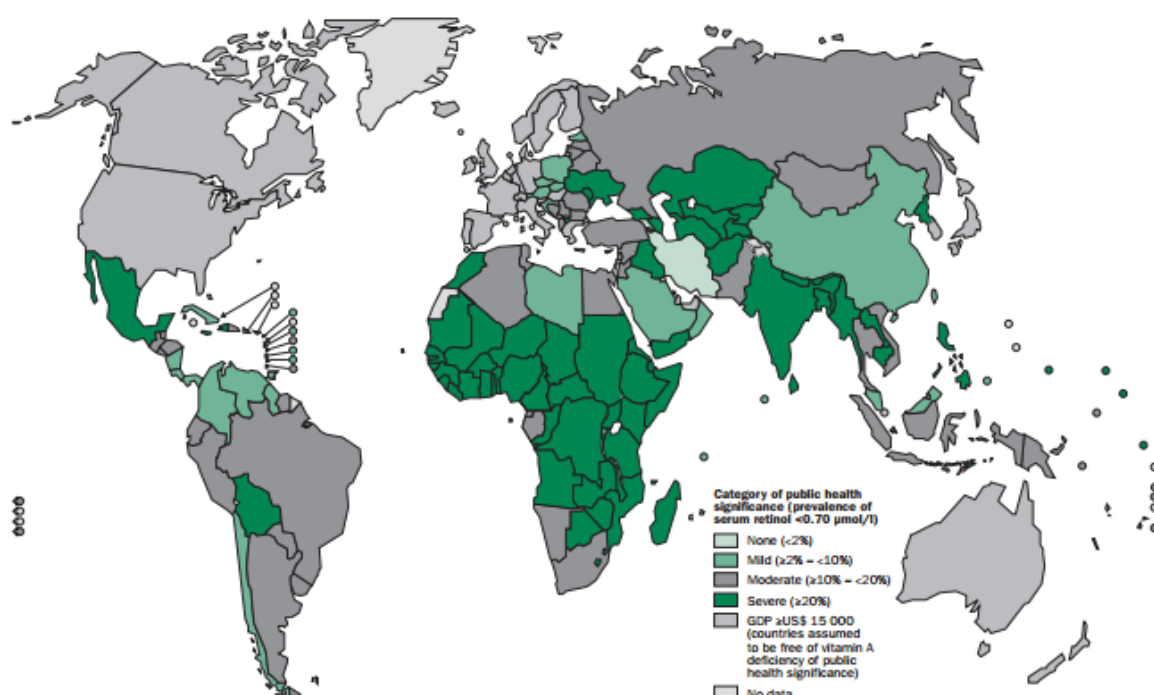


Figure 1.7. Night blindness as a public health problem by country 1995–2005: Preschool-age children (source: WHO, 2009b)

Vitamin A concentrated in its inactive form (provitamin A) from chloroplasts rich fractions (CRFs) of various unconventional green leafy plants as well as green waste for example, pea vine (which represents thousand/million tons waste each year worldwide); could theoretically contribute nearly 80% of vitamin A requirement based on rough estimates of nutrient reference intake (RNI) calculations using data provided in section 3.7.

1.2.2.6.2. Lutein

Lutein occurs widely in green leafy vegetables such as kale and spinach, with its orange colour masked by the major pigments of green plants - chlorophyll (Nwachukwu et al., 2016). Over 30 mg of lutein is present in 100 g of a dark green leafy vegetables e.g. raw kale, but less than 1 mg for a cooked portion of edible foods with a yellow/orange colour such as baby carrots, peaches, corn, papaya, and raw oranges (Holden et al., 1999). According to USDA (2015), the highest amount of lutein and zeaxanthin exists in egg yolk, chicken (broiler) and cheese and due to their lipid-dissolved physical state, xanthophylls in these animal sources are greatly bioavailable (Schweiggert and Carle, 2015). The amount of lutein and lutein esters in foods and food products made from wheat flour are low, nevertheless, these products represent an important source of carotenoids due to their regular consumption and being a staple food in many regions in the world.

Despite the fact that lutein does not possess provitamin A activity like β -carotene, its important role in the reduction of risk of macular degeneration and cataracts, and as antioxidant is well recognized (Abdel-Aal et al., 2013, Fullmer and Shao, 2001, Kris-Etherton et al., 2002, Roberts et al., 2009, Semba and Dagnelie, 2003). Along with its stereo-isomer zeaxanthin, it is believed that lutein in the macula lutea protects from blue light to reach the underlying structures in the retina. Lutein/zeaxanthin also appears to have significant antioxidant role in the body; and like many other antioxidant vitamins such as, vitamin C, provitamin A (β -carotene) and vitamin E, it

prevents against the formation of reactive oxygen species (ROS) and hence free radicals (Roberts et al., 2009). A previous study suggested that higher dietary intake of lutein/zeaxanthin resulted in decreased likelihood of having age related macular degradation (AMD) (Seddon et al., 1994). Recently, a prospective study (20 years) also confirmed that higher intake of lutein/zeaxanthin is correlated with long-term reduced risk of advanced AMD (Wu et al., 2015).

A more recent study by Giampietri et al. (2016) proposed neurodevelopmental effects of lutein in preterm infants. Arterial cord blood level of lutein was reported to be higher in preterm infants, especially at the early stage of the third trimester, than in full term infants. It is therefore speculated that a high carotenoid level in this period has a role in promoting central nervous system development. Giampietri and co-workers recommended further studies to be conducted before any conclusions are drawn.

Although there are no reference nutrient intake (RNI) for lutein, some experts suggest that one should consume at least 6 mg of lutein per day for health benefits (Gary, 2016).

1.2.2.7. Vitamin C (ascorbic acid)

L-Ascorbic acid (AA) and its oxidized derivative, dehydroascorbic (DAA) acid, are the two forms of ascorbates found in plants. However, the former is generally regarded to be the major contributing form of vitamin C perhaps due

to its higher concentrations in plant tissues (95% AA versus 5% DAA). Besides its participation in a number of vital processes, such as photosynthesis, photoprotection, resistance to environmental stresses, cell wall growth, cell expansion etc., L-Ascorbic acid functions as an antioxidant, an enzyme cofactor, and as a precursor for oxalate and tartrate synthesis (Smirnoff and Wheeler, 2000). Localization of ascorbate in various compartments of plant cell is shown in Fig. 1.8. These cellular locations include cytosol, chloroplasts (*C*), vacuole (*V*), appoplasts, mitochondria (*M*) and peroxisome (*P*).

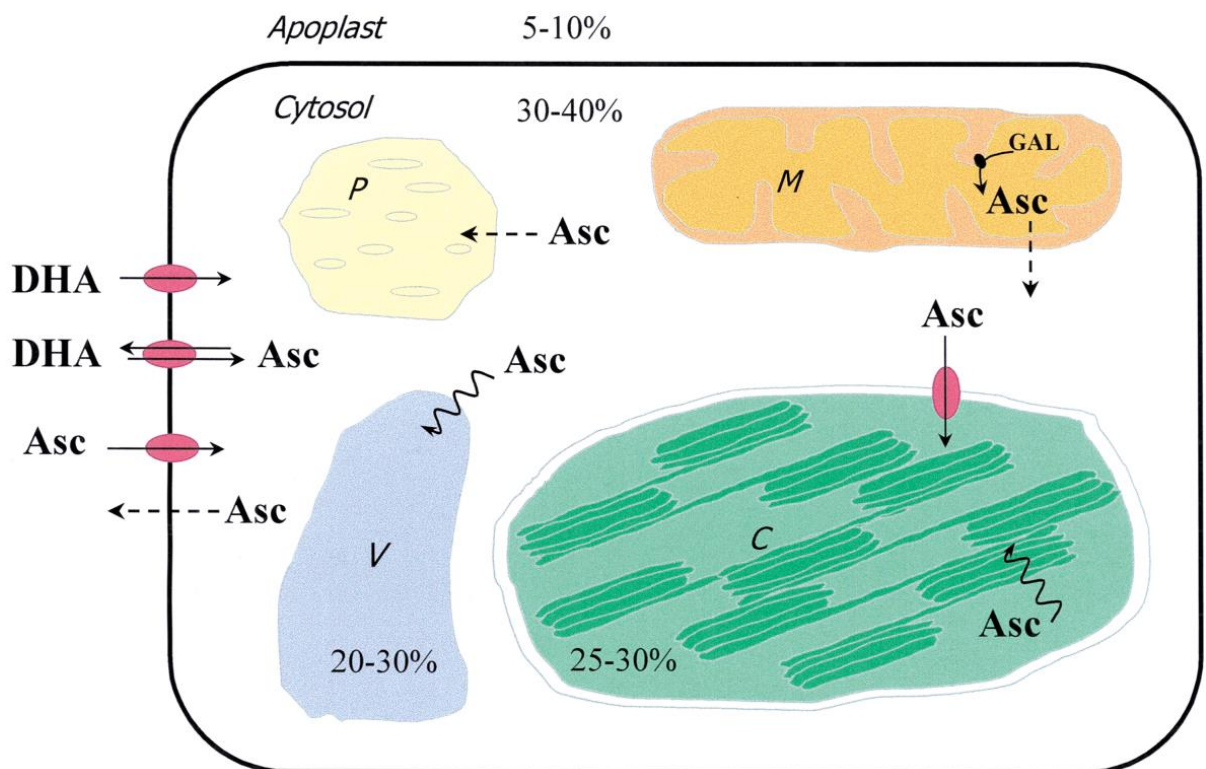


Figure 1.8. The relative occurrence of ascorbate (Asc) in these different compartments is given as a percentage. Since no separate data are available, the peroxisomal and mitochondrial Asc concentration is included in the percentage of the cytosol. The final step of Asc biosynthesis is indicated as the activity of L-galactono- γ -lactone (GAL) dehydrogenase, on the inner mitochondrial membrane, converting GAL to Asc. Asc translocation through the different membranes is presented as follows: identified Asc or dehydroascorbate (DHA) carrier systems, $\bullet \rightarrow$; transport that is not carrier mediated, $\sim$; and translocation that has not been studied so far, $-----\rightarrow$. For the plasma membrane, the possible presence of different Asc and DHA transporters as well as the possibility of carrier working as a DHA/Asc exchange carrier is indicated. Source: Horemans et al. (2000).

Among other components characterised, ascorbates highly contribute to the nutritive value of fruits and vegetables.

1.2.2.8. Minerals

Leafy vegetables play a vital role in the nourishment and in the balanced diets of a large number of the global population. In addition, vegetables occupy a crucial place in the vegetarian diets of certain populations. Chloroplasts rich fractions from green leafy vegetables could be few of the sustainable sources rich in numerous compounds with great nutritional value as a single collection. Such compounds include minerals and vitamins. Minerals and their principal functions in living organisms have been known for a long time and it is currently accepted that many of them are vital for the normal functioning of these organisms. In green leafy vegetables, minerals are classed in two groups i.e. major (e.g. K, Ca, P, Mg, Na) and trace (e.g. Fe, Cu, Zn Mn) minerals. As leafy vegetables contain more nutritionally important chemical compounds than other groups of vegetables, greater attention is being paid to leafy vegetables as a sources of sustainable nutrition. (Kalt, 2005, Lisiewska et al., 2009).

The element profile of the ash fraction of different plant species is shown in Table 1.5.

Table 1.5. Comparative composition of the ash of chloroplasts and whole leaf tissues

Chloroplast	Ash %	Fe	Ca	Mg	P	Mn	K	Na	Cu
Trifolium pratense	8.04	0.44	6.15	1.99	9.20		*	0.48	0.10
Onoclea sensibilis	5.23	1.08	6.33	3.04	4.90		*	0.74	0.33
Elodea canadensis	9.74	2.56	-	2.07	4.01	0.24	*	2.57	0.17
Artium minus	5.32	1.18	9.62	3.00	6.39		*	0.39	0.16
Whole leaf									
Trifolium pratense	9.91	0.27	20.87	3.03	3.84	-	20.01	3.21	0.04
Onoclea sensibilis	9.75	0.44	13.31	5.12	3.80	0.06	21.01	2.14	0.08
Elodea canadensis	12.89	1.14	-	3.41	3.56	0.52	25.28	5.16	0.07
Artium minus	11.75	0.59	14.02	4.86	2.64	-	21.68	3.35	0.03

Data in this table was reported by Neish (1939) in dry weight basis of the species. * Presence doubtful – not detected by the method used.

1.3. Human Digestive System and Nutrient Uptake

Digestion is a complex system in which a group of organs work together to convert complex molecules of the food into simpler compounds for energy and for basic nutrients to nourish the body. Generally, the process involves three main steps 1) ingestion 2) digestion and 3) excretion. Food passes via a long tube-like structure inside the body known as the gastrointestinal tract (GIT) or alimentary canal. The GIT includes the oral cavity (mouth), oesophagus,

stomach, small intestines, and large intestines. In addition to the GIT canal, several accessory organs ease the digestion process to take place, these include, teeth, tongue, salivary glands, pancreas, gall bladder and the liver. Human digestion system is illustrated in Fig. 1.9.

Digestion begins in the oral cavity (teeth, tongue and salivary glands) in which amylase is released to break starches into polysaccharides by means of salivary glands releasing saliva to moisten and lubricate the diet. After mastication, boluses are formed and the food is passed through the oesophagus to enter the stomach. In the stomach, the pH of the food is acidified between 2 and 4 by hydrochloric (HCL) acid. HCL acid also activates pepsinogen to pepsin, an enzyme that hydrolyses protein into smaller polypeptides. Initial lipolysis (around 10-20%) also occurs in the stomach through the action of gastric lipase. The partially digested food (chyme) is then passed through the pyloric valve to enter in the small intestines.

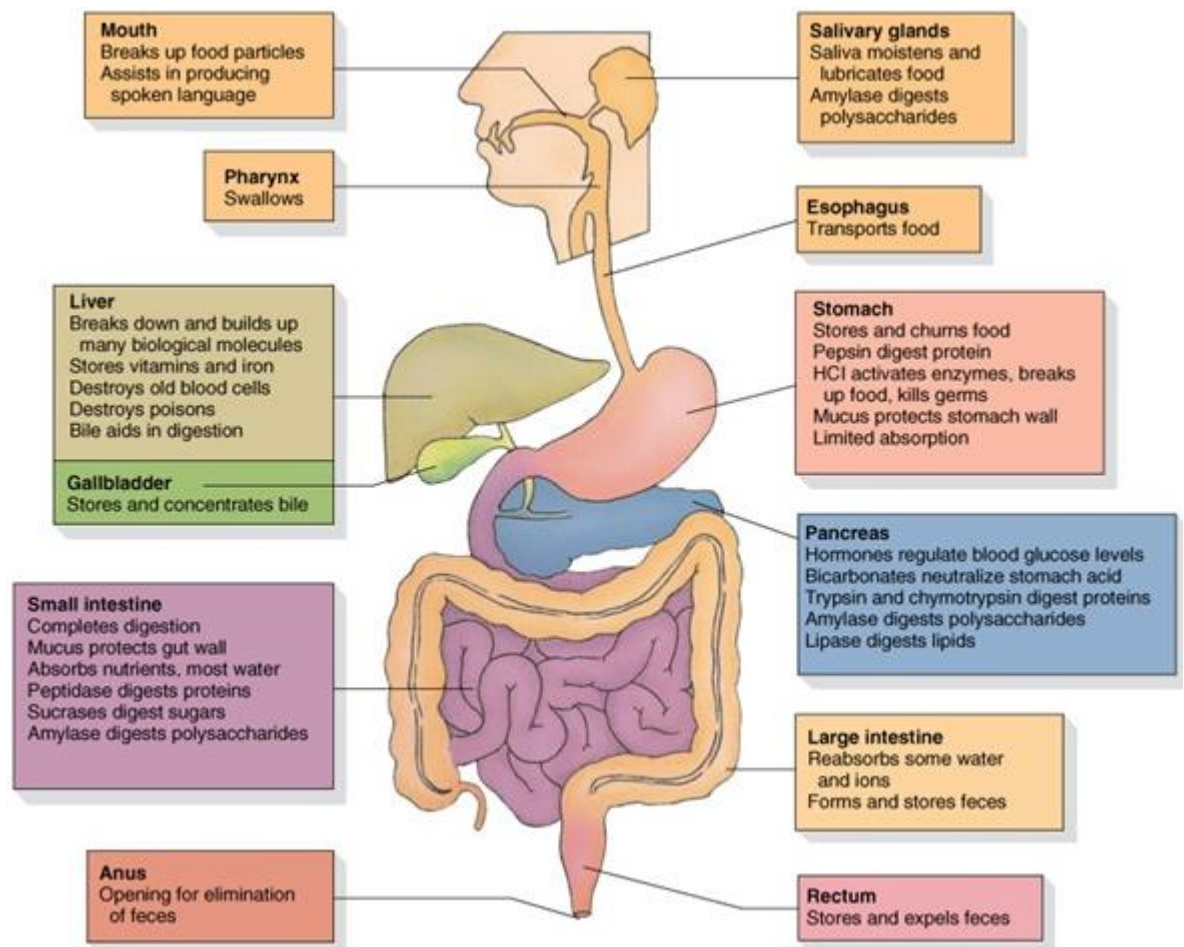


Figure 1.9. Human digestive system, digestion process by organ and functional biochemical reactions. (Source: (HEALTH, 2013))

The small intestines are tube-like stacks (6-7 m) containing three main sections; namely the duodenum (measuring around 25 cm), ileum (3.6 m) and jejunum (2.5 m). As soon as the chyme from stomach enters the first section of the small intestine (duodenum), digestion of the chyme is aided by pancreatic juice, bile salts and intestinal juice, and it is the duodenum where most of digestive enzymes are released. During this stage, juice containing sodium bicarbonate and pancreatic enzymes (lipase, amylase, trypsin chymotrypsin etc.) is secreted from the pancreas. Due to the mixtures of

pancreatic enzymes, bile salts and sodium bicarbonate neutralize the acid chyme into neutral condition.

Duodenum is the major site of digestion where the process of chemical digestion that began in the mouth is completed (Kidport, 2012, Taylor, 2016) and so small intestines are often referred to duodenum phase. For instance, in the duodenum, polysaccharides are converted into monosaccharides by the pancreatic amylase, sucrase, and lactase, whilst protein is digested to polypeptides and consequently to amino acids by trypsin. Fats are, in their turn, hydrolysed to monoglycerides and fatty acids by lipases with the help of bile salts, stored in the gall bladder for fat emulsification. Finally, digestion products can be absorbed by the villi in the ileum and the jejunum to be finally transported to their functional or storage sites in the body; for instance, polysaccharides are broken into monosaccharides like glucose which are transported into the blood stream and used for energy. Excess glucose (if any) is then converted into glycogen, by the action of insulin, and stored in the liver to be broken down to glucose later on when required, by the action of glucagon. The remaining undigested pellets are excreted through the large intestines to the rectum (HEALTH, 2013).

1.3.1. *In vitro* Digestion Model Systems

In vitro digestion systems are extensively applied for studying digestibility, bioaccessibility and structural changes of foods or pharmaceuticals under simulated oral and gastrointestinal conditions. However, one important

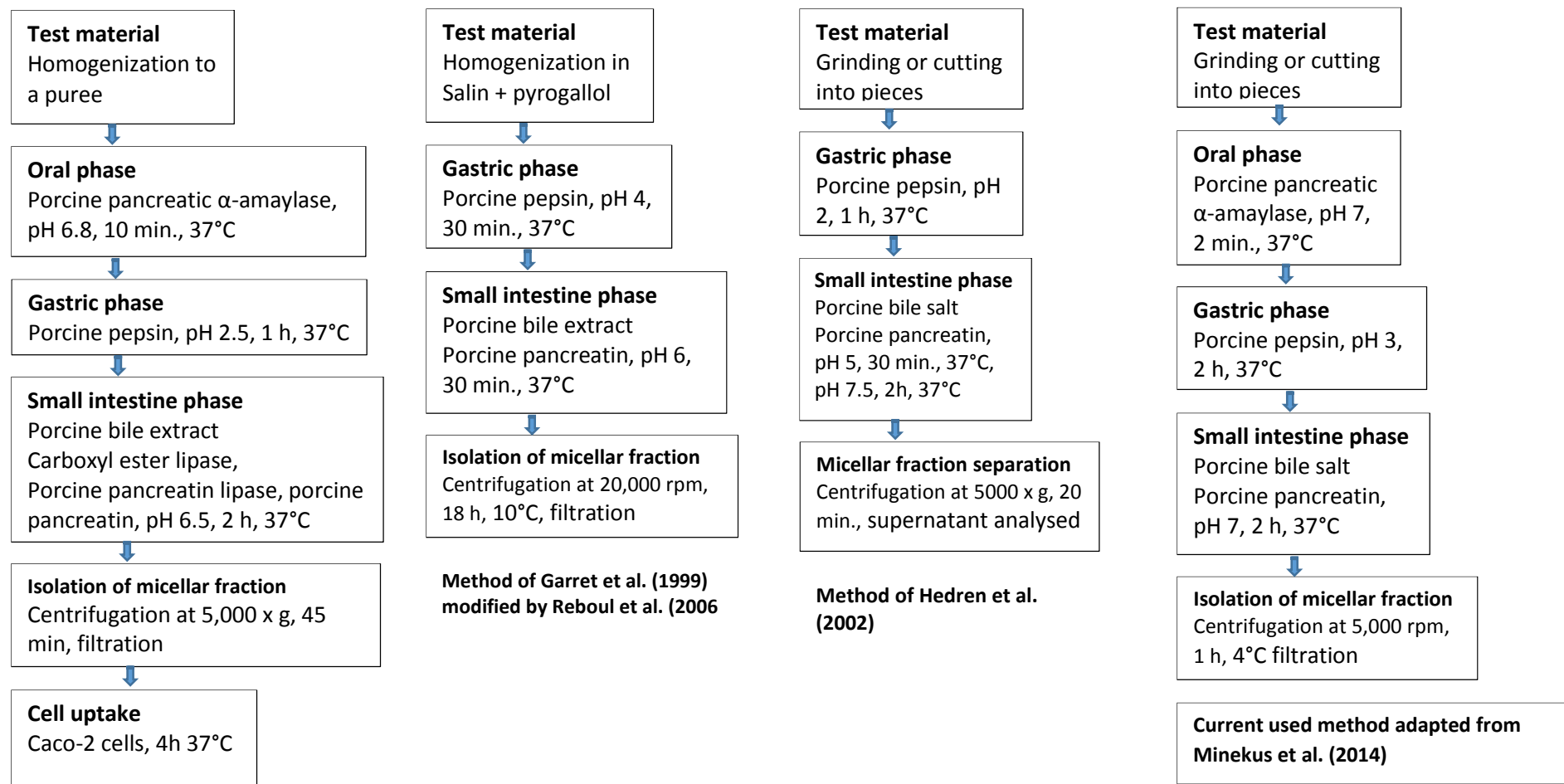
limitation in the *in vitro* digestion model is that results are often different from those found by *in vivo* models due to difficulties in accurately simulating the numerous complex physiochemical and physiological factors involving in the animal and human digestive tracts (Minekus et al., 2014). *In vivo* feeding trials using either animals or humans generally yield more accurate results compared to those of *in vitro*. However, they are time consuming, highly costly and ethically disputable, and with high inter-individual response variability, therefore, great effort has been put into refining *in vitro* based models (Boisen and Eggum, 1991, Hur et al., 2011, Minekus et al., 2014). *In vitro* digestion methods could be carried out using either static or dynamic assays. The *in vitro* digestion models discussed hereafter are mainly for carotenoids as they are the main components targeted and dedicated almost one chapter in this study for bioaccessibility purposes.

1.3.1.1. Static Assays

Static methods are generally rapid, reproducible, simple and inexpensive. Owing to these advantageous aspects, several researchers have developed *in vitro* methods and employed to estimate bioaccessibility of carotenoids (Cilla et al., 2012, Colle et al., 2010, Garrett et al., 1999, Granado-Lorencio et al., 2007, Hedrén et al., 2002). The physiological conditions and the processes that take place during gastrointestinal digestion in humans is simulated in these assays. Developers of the assays have tried their best to mimic as much

as possible the physiological conditions in the human gastrointestinal tract. However, static methods are different from *in vivo* models in several aspects, including food sample preparation, inclusion of oral phase or not, choice and concentration of enzymes, pH, duration of digestion (gastric and intestinal phases) and isolation of micellar fraction (Rodriguez-Amaya, 2010). These variations can have significant influence on the obtained results.

Several studies measuring carotenoid bioaccessibility widely follow the method proposed by Garrett et al. (1999) with or without slight modifications. The carotenoid concentrations of the digesta (whole digested mixtures at the end of the intestinal phase) and the micellar fraction are analysed by high performance liquid chromatography (HPLC).



Method of Garret et al. (1999)
Modified by Thakkar et al. (2007),
Ferruzzi et al. (2006), and
Chitchumroonchokchai and Failla (2006)

Figure 1.10. Widely used methods for the *in vitro* assessment of bioaccessibility, modified from Rodriguez-Amaya (2015). These widely used methods considered a gastric pH ranging 2 to 4, however, it is now recognised that gastric pH can vary between 2 to 6 depending on the gastric emptying conditions (Sams et al., 2016), as such reconsideration of the gastric pH provided by these methods seems timely.

In several cases, the method of Garrett et al. (1999) has been modified to better fit physiological conditions (Fig.1.10). As the test material was rich in corn oil, Ferruzzi et al. (2006), for instance, incorporated porcine pancreatic lipase into the small intestine phase (with the pH adjusted also to 7.0) along with pancreatin to ensure complete digestion of the oil. An oral phase with α -amylase was also included for a test material (cassava) containing high starch, with the gastric pH adjusted to 2.5 rather than 2.0 and that of small intestinal adjusted from 6.5 to 7.0 (Thakkar et al., 2007). Furthermore, centrifugation force was modified from 167, 000 x g for 35 min to 5000 x g for 45 min to obtain micelle. Carboxyl ester lipase, which was shown to catalyse the hydrolysis of different substrates of xanthophylls esters (lutein, capsanthine, β -cryptoxanthin and zeaxanthin), was also added to the intestinal phase (Breithaupt et al., 2002, Chitchumroonchokchai and Failla, 2006).

A recently published paper by 29 researchers (Minekus et al., 2014) proposed a more general standardized and practical static digestion assay which was adopted in this work. The method also shown in Fig. (1.10) is based on physiologically relevant parameters and amendable to specific requirements.

Unlike Garrett and co-authors, this method considered the oral phase for 2 min using α -amylase. In addition, the method proposed a pH of 3 for 2 h in the gastric phase and a pH of 7.0 for 2 h in the intestinal phase.

1.3.1.2. Dynamic *in vitro* Digestion Models

Certain mechanical and physiological processes that occur *in vivo* including peristalsis and pH change are not simulated by static models (Rodriguez-Amaya, 2015). Therefore, dynamic gastric assays have been suggested to better mimic these physico-biochemical processes (Blanquet-Diot et al., 2009, Déat et al., 2009, Kong and Singh, 2010). An example of one advantage out of several in employing such a method is simulating the *in vitro* physiological processes occurring within the stomach and small intestinal lumen using, for example, a computer controlled gastrointestinal tract model (TIM)(Blanquet-Diot et al., 2009). However, dynamic *in vitro* models have had limited applications due to their high complexity and the sophisticated equipment they require (Biehler et al., 2010, Rodriguez-Amaya, 2015).

1.3.2. Bioaccessibility

In digestion models (*in vitro* or *in vivo*), there is great intra and/or inter-relationship between terms, such as, bioaccessibility, bioavailability and bioactivity as the concept of bioavailability involves bioactivity as well as bioaccessibility. Bioaccessibility was hence defined as the fraction of compound that is released from its matrix in the gastrointestinal tract to become available for intestinal absorption via the blood stream (Benito and Miller, 1998, Fernández-García et al., 2009). On the other hand, the fraction

of ingested nutrient, readily available to the body to be used for physiological functions or for storage is termed bioavailability. Bioaccessibility is generally determined through *in vitro* digestion models; bioavailability is best evaluated via human (Rodriguez-Amaya, 2015) or animal studies.

1.3.3. Human Uptake of Dietary Carotenoids

Owing to their high hydrophobicity, carotenoids cannot be easily dispersed in aqueous media without amphipathic compounds, such as bile salts and bile-derived phospholipids. These compounds including others such as, dietary lipids and carotenoids released from food matrix need to be dispersed in the gastrointestinal tracts, before being subsequently solubilised in the form of mixed micelles. Bioaccessible (solubilised) carotenoids are then taken up by the intestinal epithelia. A simple diffusion across the plasma membrane of the epithelial cells is believed to mediate the cellular uptake (Scita et al., 1992). More lipophilic compounds penetrate readily into the lipid bilayer of the cells by diffusion and thus the uptake of carotenoids from the mixed micelles by the intestinal cells and the solubilised process in the digestive tracts determines the bioavailability of dietary carotenoids (Nagao, 2011) as shown in Fig. 1.11.

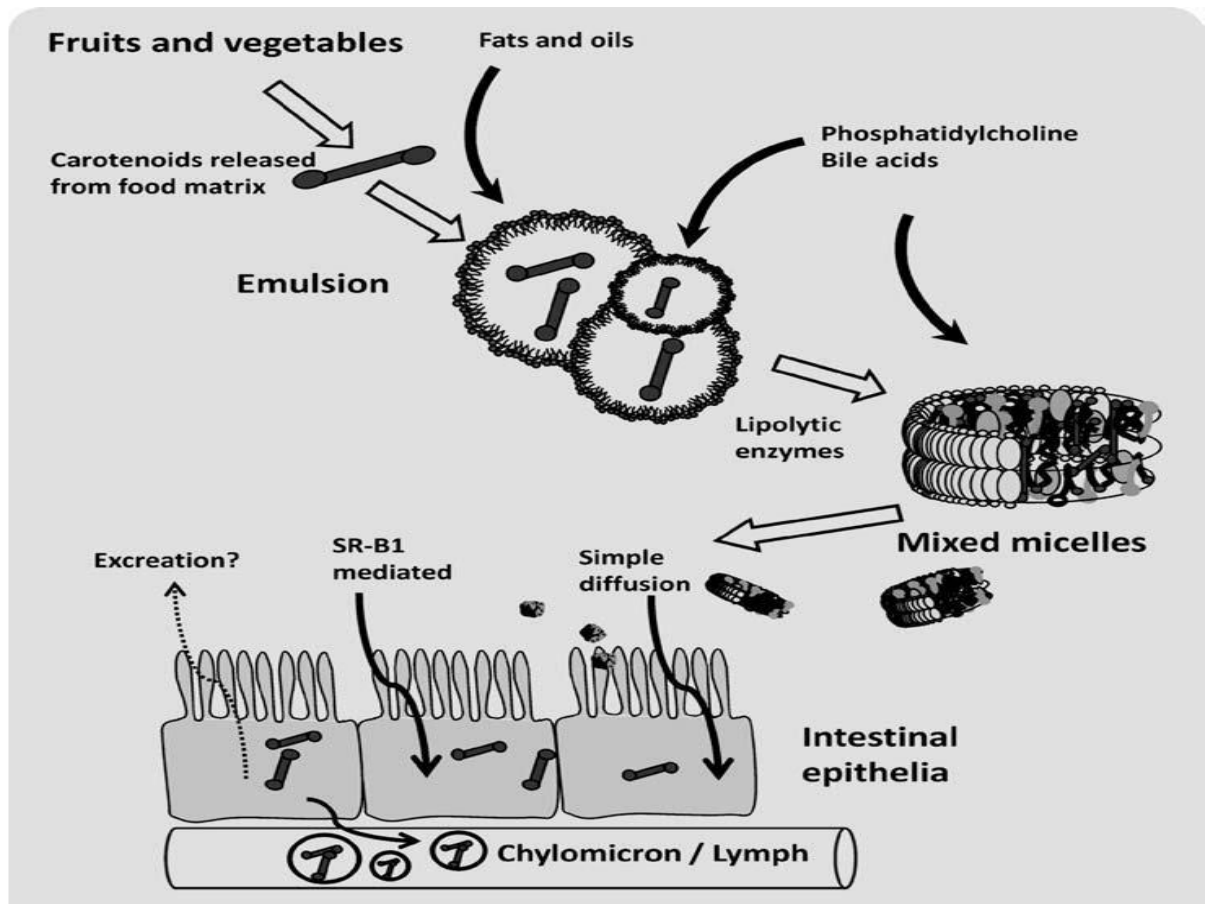


Figure 1.11. Solubilisation and intestinal absorption of dietary carotenoids source: Nagao (2011).

For instance, 2-50% of the ingested β -carotene (depending on the source and treatment) mixed with micelles, is absorbed by the mucosal cells (enterocytes) and the rest (50-98%) is not absorbed. Absorbed β -carotene is then converted to vitamin A and retinol esters and then released into the blood stream via lymphatic system in the form of chylomicrons (Fig.1.12) and stored in the stellate cells in the liver (Ball, 1998)

The amount of naturally occurring lipids in most fruits and vegetables is quite low and so it is realistic to add extra lipids to the system during food processing and/or digestion and hence, enhancement of the carotenoid bioaccessibility (Lemmens et al., 2014).

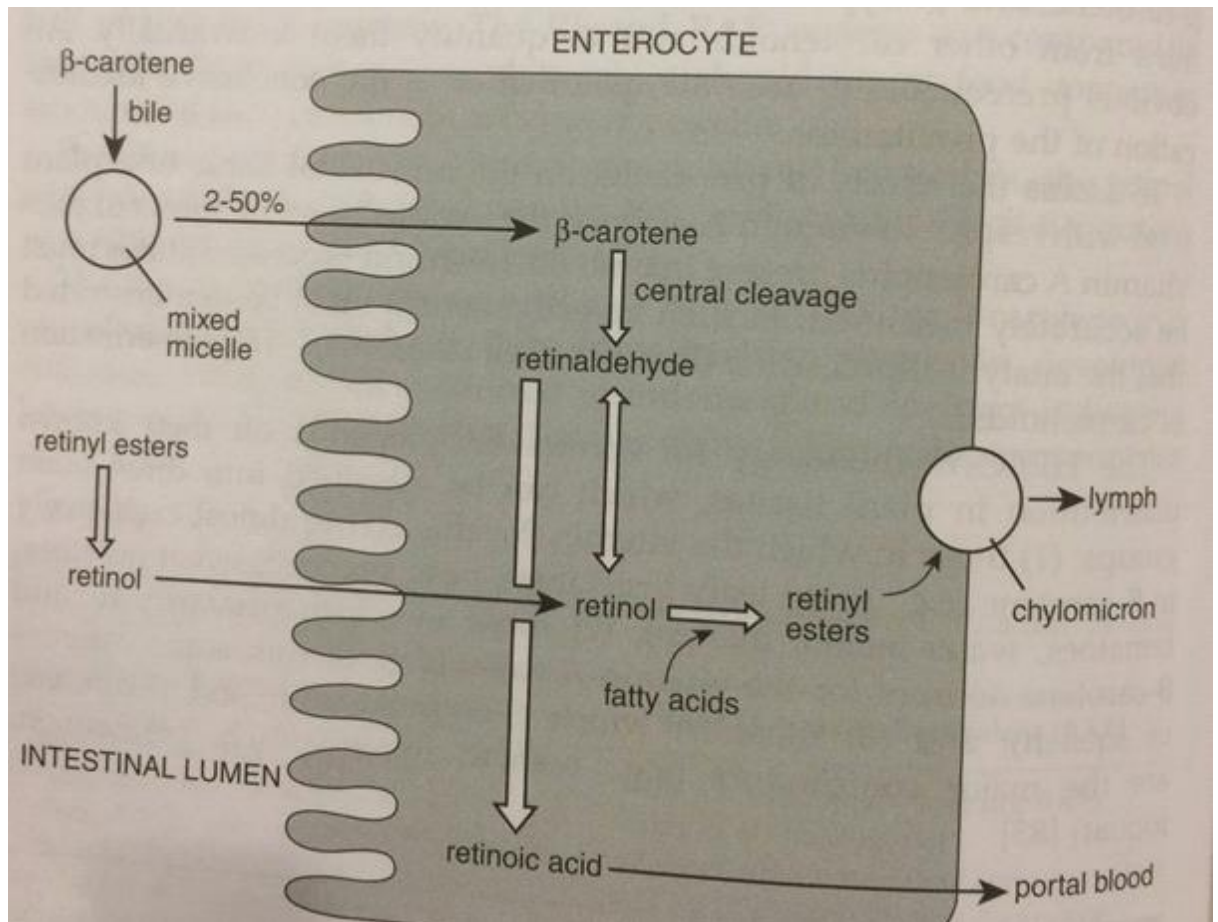


Figure 1.12. Intestinal absorption and metabolism of β -carotene and retinyl esters (adapted from Erdman Jr et al. (1988)).

Generally, carotenes (e.g. α -carotene, β -carotene and lycopene) are not bioaccessible as readily as xanthophylls (e.g. lutein), as illustrated in Fig.1.13 (Goñi et al., 2006, Huo et al., 2007), apparently due to their higher hydrophobicity.

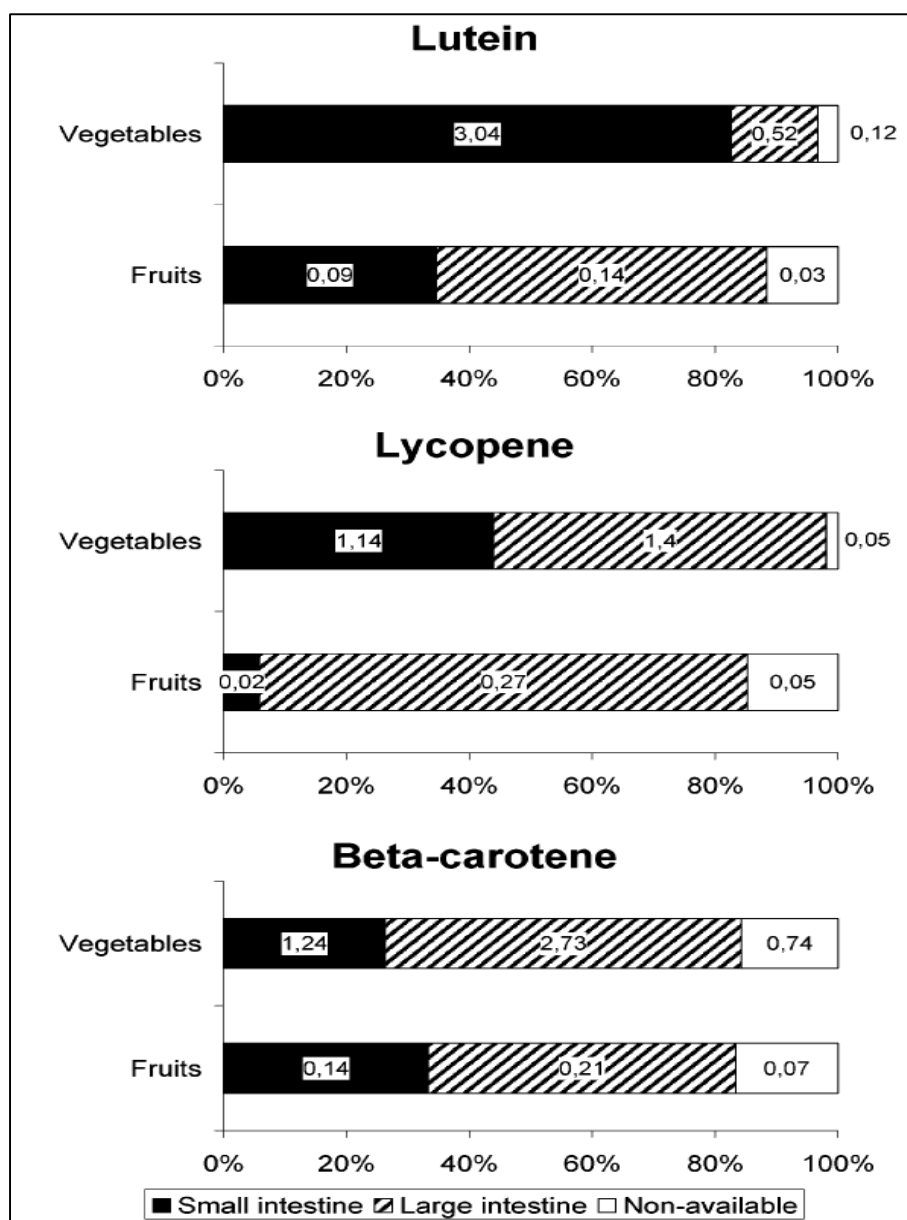


Figure 1.13. Small and large intestine bioaccessibility of lutein, lycopene and β -carotene in fruit and vegetable groups. Values within bars indicate the estimated intake of carotenoids (mg/person/day) from fruits and vegetables in the Spanish diet, this was adapted from Goñi et al (2006).

The physical nature and the form of delivery may also have an impact on β -carotene bioaccessibility. A previous study by Micozzi et al. (1992) investigated plasma response to supplementation with 12 or 30 mg/d of purified β -carotene in capsule form as compared to consumption of carrots or tomatoes containing

equivalent amounts of β -carotene for 6 wk, followed by a 4-wk washout period of self-selected diet without supplementation. The 12- and 30-mg/d supplements increased plasma β -carotene within the first week, which continued to increase up to 6 wk and then gradually declined during the 4-wk washout period. The study concluded that β -carotene in supplement form was more effective than an equal amount of β -carotene in foods for increasing plasma β -carotene concentration.

1.3.4. Enzyme Selection for *In Vitro* Digestion Models

A number of parameters, including sample type and characteristics, degree of matrix homogeneity and particle size, enzyme activity, ionic strength, and digestion times have significant impact on the findings of *in vitro* digestion models. A recent standardized static *in vitro* study (an international consensus) by Minekus et al. (2014) emphasized the role of enzyme activity in the *in vitro* digestion. In their *in vitro* protocol, the authors provided two ways that enzymes could be used: a) single enzyme protocol, which is useful for predicting the digestibility of one nutrient e.g. by the use of α -amylase, pepsin or lipase for the hydrolysis of carbohydrates, proteins and lipids, respectively, and b) complex biological mixture e.g. pancreatin, containing all these three enzymes in one cocktail.

Coles et al. (2005) reported that using single purified enzyme is often advantageous over a complex biological cocktail for it may lead to the

standardization of *in vitro* digestion systems. However, the digestion of one nutrient often influences the digestion of others; as a result, preferring complex mixture of enzymes over single purified one was suggested (Boisen and Eggum, 1991, Minekus et al., 2014). Enzyme characteristics are the most determinant factors in an *in vitro* digestion model but several other factors have an influence on enzyme activity, they include temperature, pH, stability, activators, inhibitors, concentration and incubation time (Hur et al., 2011, Boisen and Eggum, 1991).

1.3.5. Lipid Hydrolysing Enzymes

Gastric and pancreatic lipases are the hydrolysing enzymes of dietary lipids. In general, pancreatic lipase and its specific protein cofactor - colipase are the main lipases acting on the hydrolysis of dietary fats in the presence of bile salts, during small intestinal digestion. The functional significance of a number of lipases and their gene family was studied (Wong and Schotz, 2002, Lowe, 1997). Authors highlighted three mammalian baseline lipases namely pancreatic lipase (PL), lipoprotein lipase (LPL) and hepatic lipase (HL) and four additional lipases were subsequently added, based on amino acid homology: pancreatic lipase-related protein (PLRP) 1 and 2, phosphatidylserine phospholipase A1 (PLA1), and endothelial lipase.

PL and PLRPs are further discussed in the subsequent sections as they are part of the lipolytic enzymes used in this study. Other lipase members (LPL,

LPL, PLA1 and endothelial lipase) have different functions in the metabolism of circulating lipoproteins (for further details refer to these references (Lowe, 1997, Olivecrona and Bengtsson-Olivecrona, 1993, Wong and Schotz, 2002)).

1.3.5.1. Gastric Lipase

Although the majority of lipolysis (> 70%) takes place in the intestines (Fig1.14), some lipid hydrolysis (10-20%) occurs in the stomach (Carriere et al., 1993, Carrière et al., 2000). Gastric lipase can be active and stable at a wide pH range (2 -7), however, its optimum activity lies between pH 4 and 4.5 (Sams et al., 2016). Gastric lipase concentrations are also influenced by the rate of gastric emptying. The association between gastric emptying rate, the regulatory processes (governing enzyme secretions such as, hormones or neurotransmitters), and the physical properties of test meals have been studied (Fried et al., 1988, Fried et al., 1989, Hunt, 1980, Hunt et al., 1985, Hunt and Stubbs, 1975, Moberg and Carlberger, 1973, Moore et al., 1981). When food is ingested, the human gastric lipase concentrations in the stomach is low due to the high dilution of basal gastric juice.

1.3.5.2. Pancreatic Lipase and Colipase

Pancreatic lipase are the major lipid hydrolysing enzymes in the small intestines, and is maximally active and stable at a pH range of 7.0 and 7.5,

with significantly lower activity at pH below 5. PL is denatured at pH below 3 (N'Goma et al., 2012, Ranaldi et al., 2008).

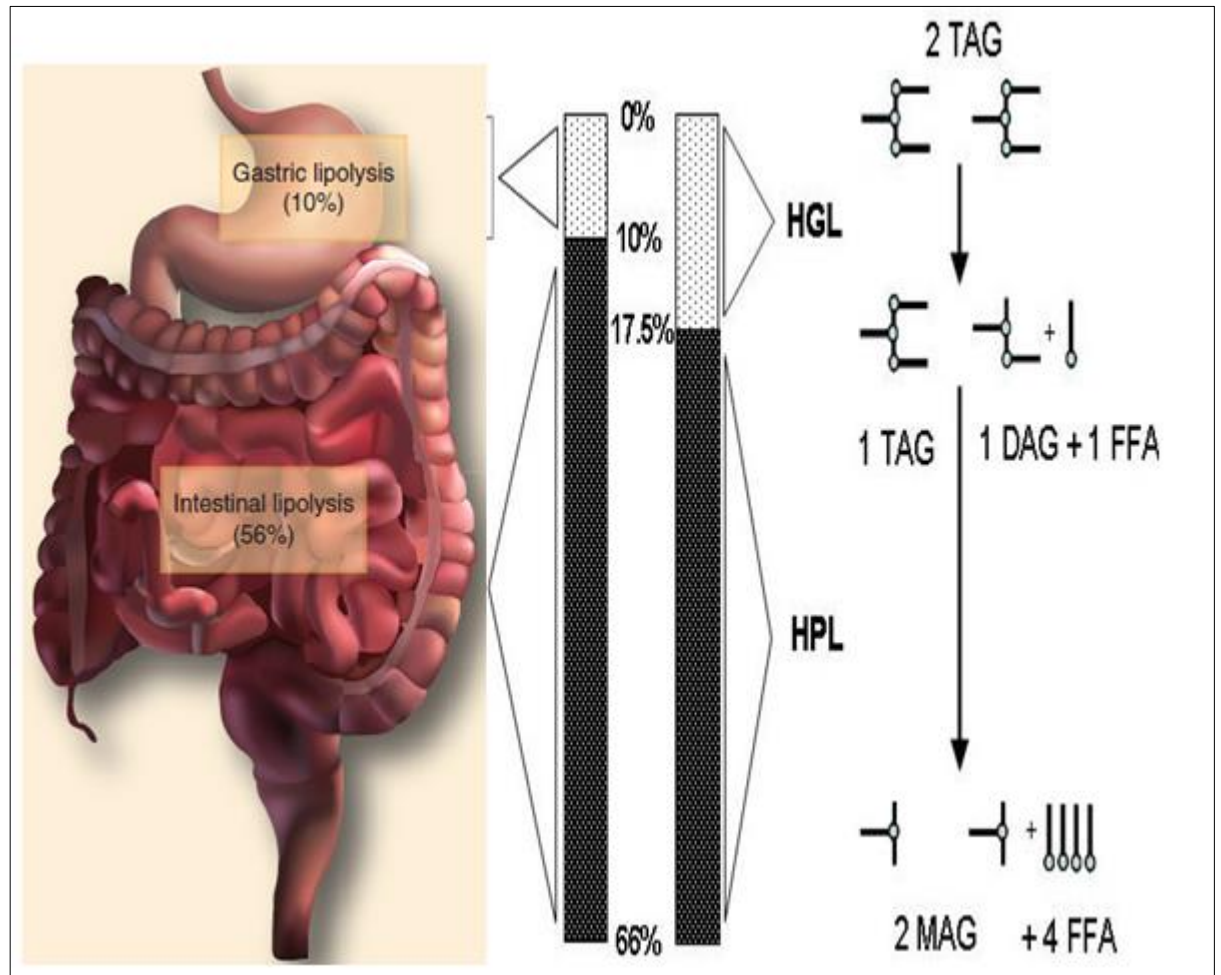


Figure 1.14. Typical contributions of human gastric and human pancreatic lipases to the gastrointestinal lipolysis. Depending on gastric emptying rate, meal type, and the pH, 10-17.5 % of lipolysis occur in the stomach, the rest, around 66% takes place in the small intestines. DAG: Diacylglycerol; FFA: Free fatty acids; HGL: Human gastric lipase; HPL: Human pancreatic lipase; MAG: Monoacylglycerol; TAG: Triacylglycerol; source: N'Goma et al., (2012).

PL is a glycosylated 50.5 kDa serine hydrolase produced by acinar cells in the pancreas (among many other enzymes in the zymogen granules) but secreted in its active form via the pancreatic duct into the intestinal lumen. The

secretion of this enzyme is stimulated by hormones in the intestine such as cholecystokinin-PZ and secretin; lipolysis products (free fatty acids) also promote further lipid digestion by stimulating the release of cholecystokinin (Lowe, 2002, N'Goma et al., 2012, Chu et al., 2009, Lowe, 1997). When the secretion site (acinar cells) of the enzyme is medically infected due to chronic pancreatic insufficiency or cystic fibrosis, its secretion rates are considerably reduced (N'Goma et al., 2012).

Previous studies revealed that human pancreatic lipase alone can act on triacylglycerols (TAGs) but their hydrolysis is inhibited in the presence of bile salts desorbing the lipase from the lipid-water interface (Brockman, 2000), and so the presence of colipase, a 10 kDa protein is required to counter-act the effects of bile salts. With the presence of lipids and amphiphiles, the affinity of colipase towards lipase is enhanced (N'Goma et al., 2012, Patton et al., 1978)

For its regioselectivity, pancreatic lipase targets only the ester bonds at the sn-1,3 positions (Fig. 1.15) of acylglycerols (Rogalska et al., 1990).

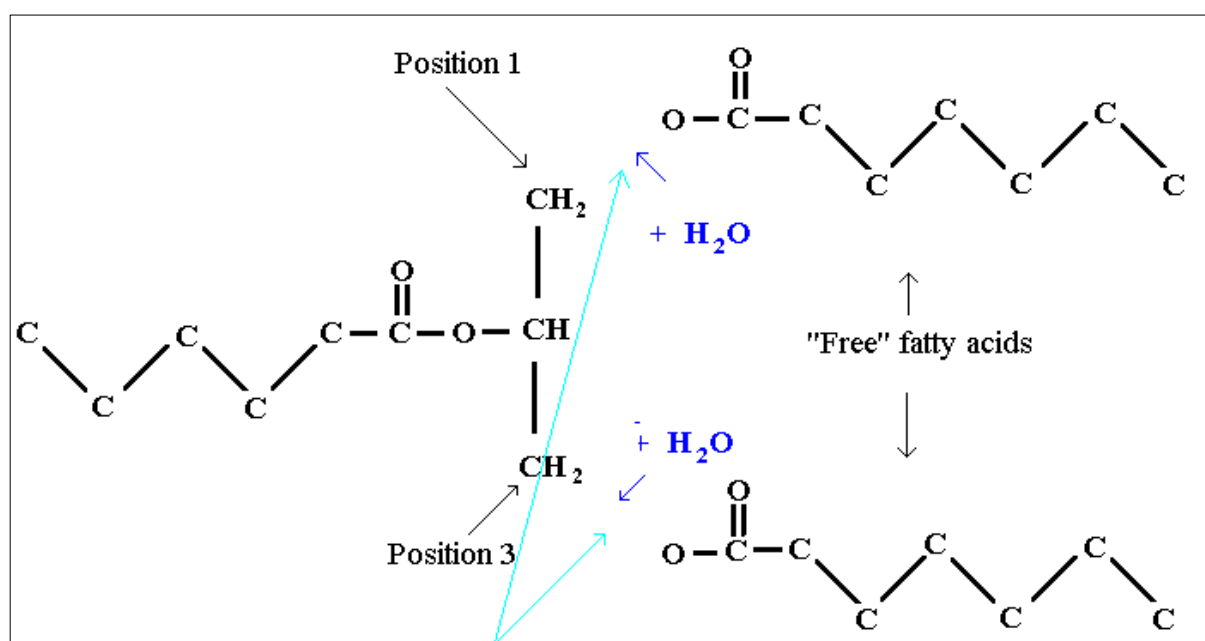


Figure 1.15. Pancreatic lipase breaking the bonds between glycerol and the fatty acids at positions 1 and 3 to liberate the 2 fatty acids (Chhabra, 2013)

1.3.5.3. Pancreatic Lipase Related Proteins

Pancreatic lipase-related protein (PLRP 1 and 2) are members of the lipase family and have been widely studied in recent years. These two proteins have similar structures of approximately 70% amino acid sequence homology with classical pancreatic lipases (Giller et al., 1992).

1.3.5.3.1. Pancreatic Lipase Related Protein 1

Great variations of levels for PLRP1 were found depending on the species investigated. Low levels of PLRP1 have been reported in humans, pigs and rats, whereas high levels have been recorded in dogs, with no enzymatic

activity observed for PLRP1 so far (Crenon et al., 1998, De Caro et al., 1998, De Caro et al., 2004, Roussel et al., 1998a).

1.3.5.3.2. Pancreatic Lipase Related Protein 2

PLRP2 is a 51 kDa serine enzyme that unlike PL, which preferentially and with great specificity acts on substrates interacting weakly with water (TAG and DAG forming oil-in water emulsions). It shows preference to substrates forming monomers. Although PLRP2 showed broader range of substrate specificities compared to PL, by hydrolysing triglycerides, phospholipids and galactolipids *in vitro*, it is inhibited by high bile salt concentrations and not reactivated by colipase, suggesting that TAGs are not significantly hydrolysed by PLRP2 *in vivo* (N'Goma et al., 2012, Sias et al., 2004).

PLRP2 is thought to be the major hydrolysing enzyme in the digestion of galactolipids in the GIT and it displays high galactolipase activity towards MGDG and DGDG. Higher levels of the enzyme are found in monogastric herbivores than in others species (Amara et al., 2010, Amara et al., 2009, Andersson et al., 1996, Carrière et al., 1998, Sias et al., 2004). Monogastric herbivores and omnivores consume plants and vegetables daily, their intake of galactolipids is quite variable and so galactolipase levels in their digestion systems could be varying considerably; for instance, guinea pigs can eat more than 500 mg galactolipids per day, whereas, humans only consume an average of about 200 mg a day (De Caro et al., 2008, Andersson et al., 1996). Lower

rate hydrolysis of MGDG but with no activity against DGDG or SQDG by purified human pancreatic lipase and colipase combined, was found; but when PLRP2 was used against these substrates, they were all hydrolysed (Andersson et al., 1996). Studies proposed that PLRP2 from guinea-pig and coypu might only hydrolyse phospholipids *in vivo*, as the pancreas of these two species produce a classical pancreatic lipase but no detectable phospholipase A₂ activity. In humans and rats, the phospholipase A₂ exists, therefore PLRP2 might have another physiological role to play (Andersson et al., 1996); this role might be the hydrolysis of galactolipids.

1.4. Fish as a Food for Human

Fish is a food of highly nutritional value and besides its high quality protein; it provides unique type of compounds, vital to normal brain development in unborn babies and infants. Indeed, fats from fatty fish species are an important dietary source for ω -3 long-chain polyunsaturated fatty acids (PUFAs) namely eicosapentaenoic acid (EPA, 20:5 ω -3), and docosahexaenoic acid (DHA, 22:6 ω -3), which have shown potential benefits on proper growth of children and, prevention of cardiovascular diseases and cancer (Shahidi and Miraliakbari, 2004). Fish also provides a range of vitamins and minerals notably, vitamins A and D. On the other hand, some fish species like zebrafish act as a good model for the studies of various biological, chemical and pharmaceutical studies.

1.4.1. Zebrafish as a Model for Animal Studies

Zebrafish are basically freshwater fish belonging to the cyprinid family. Along with minnows and carps they are classed under Actinoptergii, which are also known as ray –finned fishes. Besides insects, this omnivorous fish depends primarily on zoo- and phyto-plankton in the wild for its food (Hölttä-Vuori et al., 2010). For animal modelling, insects such as fly (*Drosophila melanogaster*) and the worm (*Caenorhabditis elegans*) possess powerful genetics but with different metabolic process from those of vertebrates in many aspects. Even though rodent animals (e.g. mouse) have some resemblance to humans at the whole-organism level, generation of their gene manipulation is slow and costly (Hölttä-Vuori et al., 2010). Zebrafish (*Danio rerio*), on the other hand, has received popularity as model organism for several aspects including developmental biology, genetics, neurobiology, pharmaceuticals and medicine (Bournele and Beis, 2016, Chakraborty et al., 2009, Hölttä-Vuori et al., 2010, Lampert et al., 2003b). As a vertebrate, zebrafish display several characteristics which make them an ideal model species for a pilot study into human digestion. Their small adult size requires less space, rapid development and maturation, they are also highly fecund and reproduce very frequently at a low cost (Tavares and Lopes, 2013, NC3R's, 2014).

Studies suggest that in zebrafish, $\Delta 6$ and $\Delta 5$ fatty acid desaturases are with similar functional fatty acyl desaturases to those in human are expressed. These enzymes are vital in the pathways for the biosynthesis of

polyunsaturates fatty acids (arachidonic, eicosapentaenoic and docosahexaenoic acids)(Hastings et al., 2001).

Another study employed zebrafish as model for obesity control by visualizing zebrafish adipocytes *in vivo* and labelling neutral lipids droplets with Nile Red; this study concluded that the cellular anatomy of zebrafish adipocytes is similar to mammalian white adipocytes (Flynn et al., 2009).

Carotenoid uptake (provitamin A conversion into retinoids) is among areas where zebrafish has been used as animal model. For this, Lampert and co-workers found that β -carotene in the egg yolk of zebrafish is cleaved by β , β -carotene-15,15'-oxygenase (bcox) into retinal. The authors revealed that retinoic acid generation, necessary for various developmental processes, depends on local *de novo* formation of retinal from provitamin A via bcox; concluding that carotenoids have an essential role in embryonic development. In a more recent study, zeaxanthin, which is naturally found in human retina along with lutein, was reported to have positive effect on the enhancement of visual acuity in zebrafish eyes, too (Saidi et al., 2015).

1.5. Fish Digestive System and Carotenoid Uptake

The digestive system in most fish is very similar. Depending on fish adaption with their diets there are major differences in the structure of the mouth and the teeth. There can be also differences in the gill rakers, the pharynx, and the stomach (if any) and in the length of the intestine (Fish-History, 2016), as shown in Fig. 1.16.

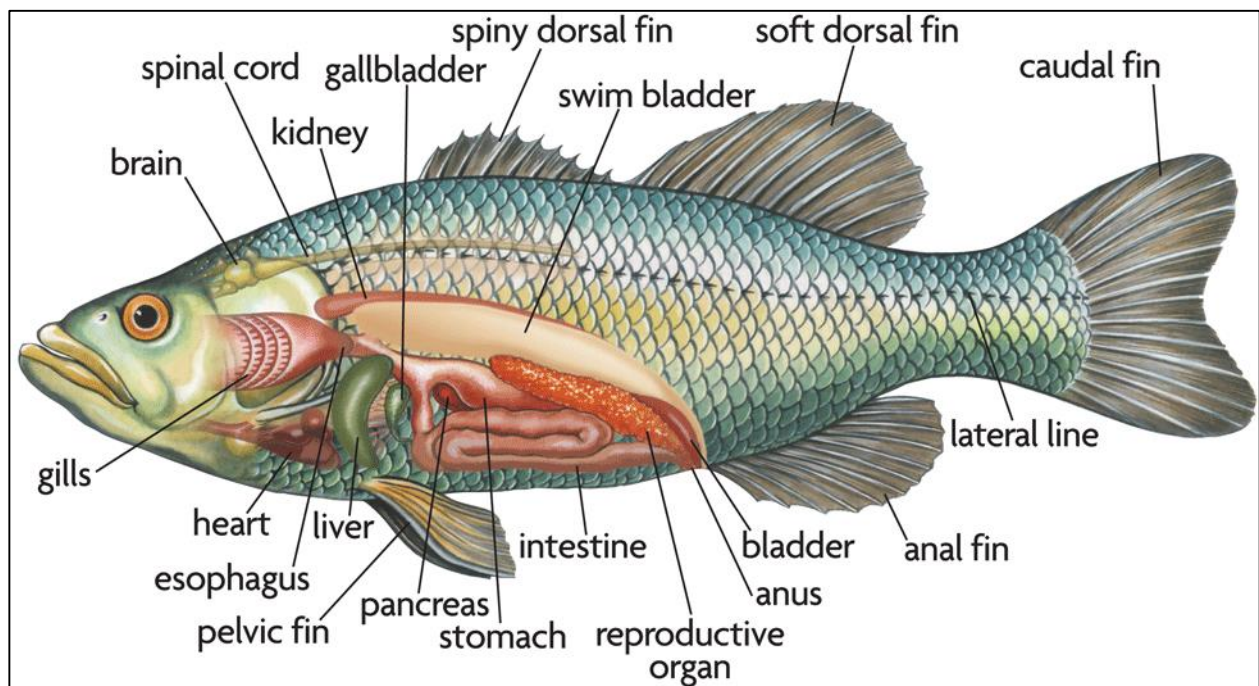


Figure 1.16. The digestive system of fish. Source: Laurent (2016)

1.5.1. The Stomach

The stomach of fish is less well-defined compared to higher vertebrates, and in some cases, it is absent; for instance, whilst carnivorous fish have stomach or foregut, herbivorous fish lacks one and rely on a much elongated small area. Even when an actual stomach exists, it is rather a muscular bag or tube with low pH for its internal environment; this low pH environment of the stomach fluctuates depending on its fullness or emptiness. As the food enters, the stomach walls stretch or expand stimulating secretion of hydrochloric acid (HCl), which, in turn increases stomach acidity. For example, it is less acidic when it is empty than when it is full. In most fish, the pH of the stomach varies between 2 and 4. Like many higher vertebrates, the main enzymes active in

the stomach are pepsins (Pchelarov, 2016). Food which is largely digested in the stomach, leaves in the form of liquid to the intestine.

In mammals preduodenal lipase activity exists in lingual, pharyngeal and gastric tissues, and have high stability and activity under acidic conditions, contrary to pancreatic lipases (PLs) (Carriere et al., 1994). However, information on the enzymology of digestive lypolysis in fish is scarce, and when available provides information on mammalian-type PLs (Norman and Benjamin, 2000).

1.5.2. The Intestines

The intestine is quite variable in length from fish to fish and depends upon diet (Parenti and Weitzman, 2015). It is the site where food is finally digested and absorbed. Intestines have a large surface area to allow the absorption of nutrients across its walls. In sharks and rays, and a few other ancient fish such as lampreys, lungfish, paddlefish and sturgeons, the internal surface area of the intestines is greatly increased by a structure called the spiral valve. Spiral valve is a corkscrew-like structure that runs down the centre of part of the intestine; being twisted helps it to pack more surface area into a given length. The spiral valve is simple in the ancient fish, but often highly evolved in sharks and rays (Parenti and Weitzman, 2015).

1.5.3. The Pancreas

The pancreas is a diffuse gland found on the pyloric caeca (Pchelarov, 2016). The pancreas of some fish species such as, lungfish, sharks and rays, as well as, most juvenile fishes is well developed. It is either distinct from the liver (sharks and rays) or embedded (teleosts, where it is found). Pancreatic juice consists of enzymes such as trypsin, amylases and lipases for the hydrolysis of protein, carbohydrates and fats, respectively. Enzymes are discharged into the intestines either through sharing one of the hepatic ducts (those belonging to the liver), or through its own pancreatic duct (Pchelarov, 2016).

1.5.4. The Liver

Liver is one of the main vital organs in fish with numerous functional roles such as glycogen storage, production of digestive enzymes and secretion of hormones plus a number of other important molecules. Fish liver lacks any specific shape and tends to mould itself onto the space around the stomach and the heart. Often, fish liver shape reflects the fish's body shape. For instance, it is long and thin in eels and wide in rays and skates. The gall bladder is attached to the liver to secrete lipolysis substances. With its ducts, liver secretes various substances into the first part of the intestines (Pchelarov, 2016).

1.5.5. Fish Carotenoid Uptake and Transport into Blood

The manner in which carotenoids are metabolized is very dependent on the fish species. Most studies have been conducted on the absorption of astaxanthin by salmon, which could give an idea of carotenoids absorption in fish. Being fat-soluble, dietary astaxanthin is assumed to be in micellar form in the intestine, together with bile salts, fatty acids, monoglycerides and other fat-soluble vitamins (Choubert et al., 1994). It is believed to passively diffuse into the intestinal lumen, together with fatty acids , and the uptake seems to be a slow process taking between 18 and 30 hours (Yeum and Russell, 2002b). Once absorbed the fatty acids are converted into triacylglycerols (TAG), and the astaxanthin, like other xanthophylls, is incorporated together with TAGs in lipoprotein spheres called chylomicrons, shown in Fig.1.17. These are then transported directly into the blood and due to its polarity, astaxanthin is assumed to be attached to the surface of the chylomicron spheres (Sheridan et al., 1985).

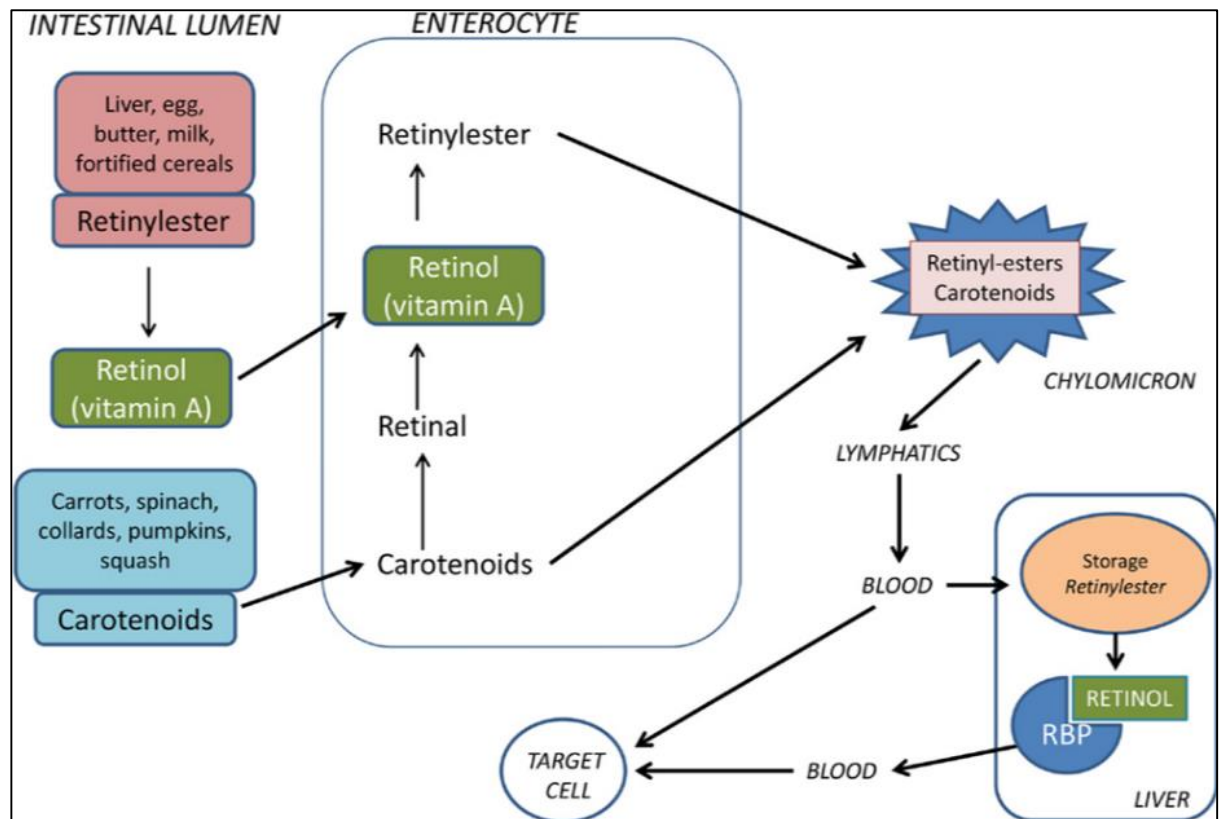


Figure 1.17 Vitamin A is provided from the food either as preformed vitamin A (retinyl esters) or as provitamin A carotenoids. Retinyl esters are hydrolysed by pancreatic and intestinal enzymes and free retinol is taken up by the enterocytes. Half of the carotenoids is oxidized to retinal and then reduced to retinol. Retinol is esterified with long-chain fatty acids and incorporated into chylomicrons together with intact carotenoids and then carried by the lymphatics. The chylomicrons are taken up by hepatocytes in the liver where vitamin A is stored as retinyl esters. Before being released from the liver to the circulation, retinyl esters are hydrolysed to retinol which binds to retinol-binding protein (RBP). Source : Henning et al. (2015).

In mammals, transport of chylomicrons from the intestinal lumen into the blood is carried out through lymphatic vessels. While being transported along the blood stream, chylomicrons undergo hydrolysis by lipoprotein lipase (LPL), a triacylglycerol lipase found on the surface of endothelial cells of the tissue capillaries, to yield free fatty acids. The fatty acids and monoglycerides that are derived from chylomicrons in this way are subsequently taken up by the tissues or serum albumin (Schneider, 2002). Changes in the lipid composition

of a chylomicron modify the affinity of the associated lipoproteins for its surface, causing the chylomicron to change its apolipoprotein signature (Vance, 2002). When the chylomicrons have lost about 80% of their initial TAG content, they become small enough to pass through the endothelium in the liver, and in addition their apolipoprotein signature can then be recognized by specific receptors in the liver.

Studies in salmon have provided evidence that astaxanthin is strongly associated with a protein likely to be serum albumin (Aas et al., 1999). Serum albumin associates with the surface of lipoprotein particles (Fielding and Fielding, 2002), and it is the major transporter of free fatty acids released during lipolysis by LPL to tissues (including liver and muscle). This suggests that albumin may acquire astaxanthin from chylomicrons during lipolysis as well as directly from chylomicrons in the bloodstream.

1.5.6. Liver Metabolism and Excretion

Liver is the main metabolic and excretory organ for carotenoids and is considered to have the major responsibility for the metabolic loss of astaxanthin. The liver secretes bile into the intestine to aid in lipid digestion as well as in the excretion of metabolic breakdown products, and radioactive labelling studies with canthaxanthin found bile radioactivity levels of canthaxanthin to be 8 times higher than the level in blood (Torrissen and Ingebrigtsen, 1992). The astaxanthin metabolites in the bile are secreted into

the intestine and re-absorbed (Hardy et al., 1990). The liver is therefore either catabolizing astaxanthin to other pigments or to metabolites that no longer have a chromophore (Page and Davies, 2003).

The exact process of astaxanthin metabolism in the liver is unknown, as is the case with β -carotene metabolism (Parker, 1996). We do know, however, that the chylomicron-delivered astaxanthin that is not metabolized is repackaged into very low-density lipoproteins (VLDL) before being sent out into the blood once more.

1.5.7. Carotenoid Conversion to Vitamin "A" In Fish

Like many other aquatic animals, carotenoids represent a major part in fish diet. Beta-carotene is not a prevalent carotenoid in the marine environment. Information on provitamin A activity of β -carotene in salmonids is scarce. The provitamin A character of β -carotene has been established for other fish species such as Atlantic halibut.

In salmonids, the red colour of the flesh is due to astaxanthin. As fish is among animals that cannot form carotenoids *de novo*, it depends entirely on dietary supplements to achieve their natural pigmentation. Generally, astaxanthin and canthaxanthin are usually used in the diets of farmed salmonids to produce coloration similar to wild fish. Conversion of astaxanthin into vitamin A is well established in salmonids (Barbosa et al., 1999).

The egg yolk of vertebrates contains carotenoids, which account for its characteristic yellow colour in some species. As egg yolk also contains stored vitamin A, carotenoids have been solely discussed as pigments for the coloration of the offspring. Lampert et al. (2003a) provided strong evidence that, for several developmental processes, retinoic acid generation depends on local *de novo* formation of retinal from provitamin A via a β,β -carotene-15,15'-oxygenase, revealing an unexpected, essential role for carotenoids in embryonic development.

Another study by Moren et al. (2002), investigated the ability of Atlantic halibut juveniles to convert β -carotene, astaxanthin and canthaxanthin to vitamin A. Three levels of each of the carotenoids and retinyl acetate were fed to Atlantic halibut juveniles for 60 days. A vitamin A and carotenoid deficient diet was fed in triplicate as control. Results showed that the increasing levels of carotenoids in the diets were reflected in increasing levels of vitamin A in both whole fish and liver samples. All carotenoids were converted to vitamin A, but to different degrees. Retinyl acetate and β -carotene resulted in whole fish vitamin A levels significantly higher than canthaxanthin and astaxanthin.

Materials and methods employed for the whole thesis are displayed in the subsequent chapter (2).

Chapter Two

Materials and Methods

2.1. Materials and Methods for CRFs And WLMs Compositional Analysis

2.1.1. Sample (Green Materials) Preparation

Spinach (*Spinacia oleracea*) and kale (*Brassica oleracea* var *acephala*) were obtained from local supermarkets (Sainsbury's and/or TESCO). Whereas, nettles (*Urtica dioica*) and grass (*Paspalum notatum*) were freshly harvested from the grounds of the School of Biosciences Campus (The University of Nottingham, Sutton Bonington, UK). When samples were brought to the laboratory, they were washed with tap water, cut into smaller pieces and split into two portions; one designated to make chloroplast-rich fractions (CRFs) and the other for whole leaf materials (WLMs).

2.1.1.1. Whole Leaf Material (WLM)

WLMs (100 g) were frozen at -80 °C for couple of hours prior to freeze drying (Edwards Freeze Dryer, Super Modulyo) for 48 h at -60 °C. During the freeze drying process samples were protected from light exposure. Samples were then ground to homogeneous powder (1 mm) using mortar and pestle under dim light.

2.1.1.2. Chloroplast-Rich Fraction (CRF)

Green leaf tissues (100 g) were homogenised in a kitchen blender for 30 s with 0.3 M sucrose solution in the ratio of 1:6 (w/v). The homogenate was then filtered by pressing through a double-layered cheese cloth. The filtrate was centrifuged (Thermo Jouan CR3i multifunction centrifuge) at 1300 RCF for 10 minutes at 4 °C. The supernatant was decanted off from the chloroplast pellet and centrifuged again under the same conditions. The resulting supernatant was discarded and the chloroplast pellets were weighed. Chloroplast pellets were frozen at -80 °C prior to freeze drying (Edwards Freeze Dryer, Super Modulyo) for 48 hours at -60 °C. See Fig.2.1 for CRF recovery process.

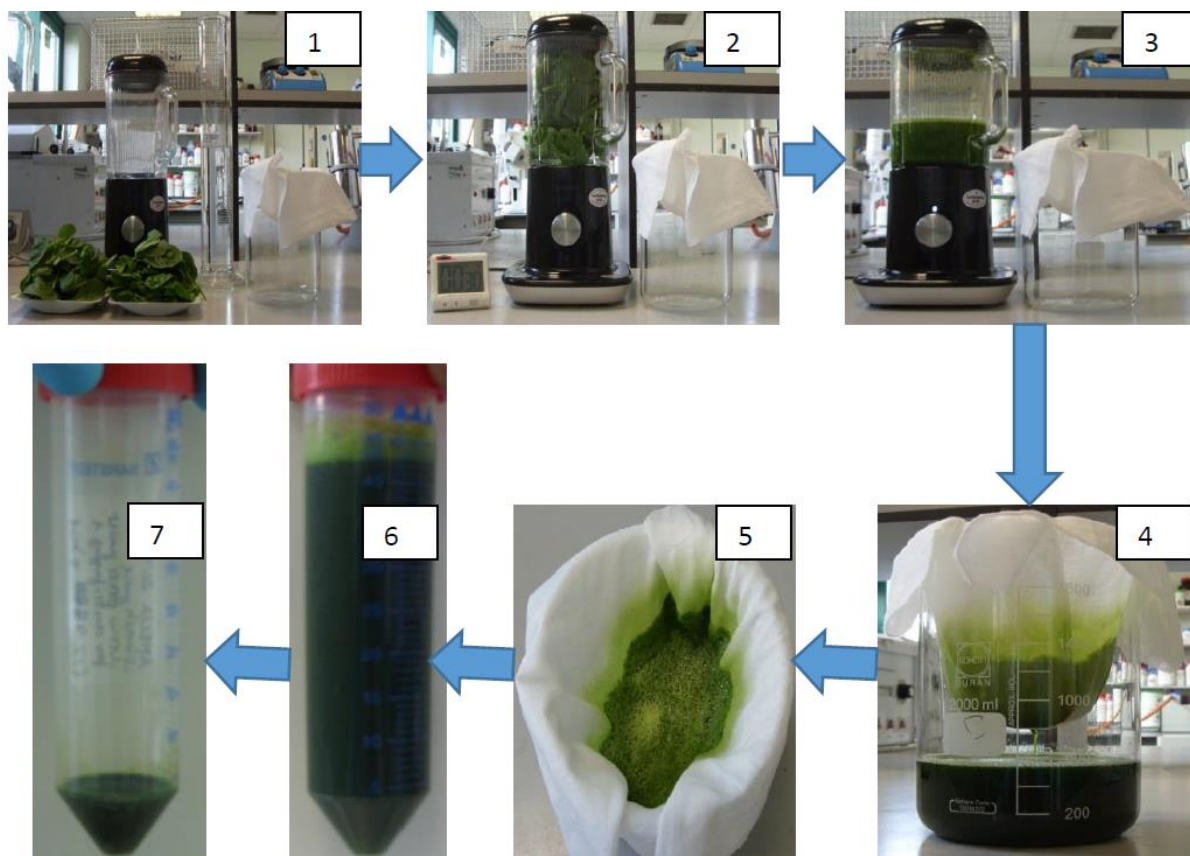


Figure 2.1. Process of chloroplasts rich fractions isolation from spinach: 1.prewieghed spinach; 2: spinach with 0.3 M sucrose soulution in blender; 3. blended spinach with sucrose solution (30 sec); 4. mixture filtered onto double layer of cheese cloth; 5. Remaining residue after filtration; 6: filtered solution for centrifugation (3000 rpm, 10 min at 4C); 7. Pellet rich in chloroplasts at the bottom of the tube after supenatant was removed.

2.1.2. Determination of Moisture Content

Three samples of each of the materials were weighed in trays and then placed in an oven at 105 °C and reweighed after 24 hours. The moisture content was then calculated gravimetrically using the following equation (Eq.1).

Moisture content (%)

$$= \frac{[\text{wet sample(g)} - \text{Dry sample (g)}]}{\text{wet sample (g)}} \times 100 \quad (\text{Eq. 1})$$

2.1.3. Determination of Sucrose (for CRFs) Content

Residual sucrose content was determined using high performance liquid chromatography (HPLC). A 0.1 g sample (CRFs) was diluted with 10 mL double distilled water and then centrifuged (Thermo Jouan CR3i multifunction centrifuge) at 2400 RCF for 5 min at 4 °C. The samples were then filtered through 0.45 µm syringe filters into amber HPLC tubes. A set of standards was prepared (concentrations of sucrose ranging from 7.5-0.5 mg mL⁻¹). The sucrose was analysed using HPLC (Jasco AS2055 sample + Pu-980 pump, Japan) coupled with a refractive index detector (Jasco RI-2031 Plus Intelligent RI Detector, Japan), and resolved using a Phenomenex Luna 5µm NH2 (100A 2 mm id x 25 cm) column with 80 % acetonitrile in water (HPLC grade) as the mobile phase. The injection volume was 10 µL and the mobile phase flow rate was set at 0.7 mL min⁻¹. When sucrose was quantified, residual sucrose in CRFs was adjusted i.e. the values were normalized by reducing the amount of sucrose from each analysed compound and all values presented in subsequent results of CRFs are non-sucrose.

2.1.4. Determination of Ash Content

Ash content was determined according to ISO (2002). Samples (0.5 g) from CRFs and WLMs were placed into pre-weighed silica crucibles in duplicate. The crucibles were then placed in a muffle furnace (Carbolite, AAF1100) and ignited for 8 h at 550 °C. The following day, samples were removed from the muffle furnace and crucibles reweighed.

The ash content was calculated as:

$$W_{FDS} = W_{(C+FDS)} - W_C$$

$$W_{IS} = W_{(C+IS)} - W_C$$

$$\% \text{ Ash} = W_{IS} * 100 / W_{FDS}$$

Where symbols refer to the weight of freeze dried samples (W_{FDS}), the crucible with freeze dried sample ($W_{(C+FDS)}$), weight of the crucibles (W_C) and the ignited sample (W_{IS}).

2.1.5. Analysis of Protein

Protein content of freeze dried CRFs and WLMs was determined using a bicinchoninic acid (BCA) Pierce protein assay. Sodium dodecyl sulphate (SDS) (1 mL of a 2 % solution) was added to 0.1 g of CRFs (2 mL SDS for 0.1 g WLMs). This was heated in a water bath at 60 °C for 30 min and then vortexed for 1 min. The sample was centrifuged (Thermo Jouan CR3i multifunction centrifuge) at 2400 RCF for 10 min and further diluted by a factor of 1 :100 using the SDS solution before 100 μ L was added to 2 mL of the BCA standard

working reagent (prepared using 50 parts reagent A: 1 part reagent B) and incubated for 30 min at 37 °C. The absorbance was read at 562 nm using a UV-vis spectrophotometer blanked with RO water and the concentration determined using the polynomial equation (produced from a range of bovine serum albumin (BSA) standards (25-2000 $\mu\text{g mL}^{-1}$)).

After the results of the BCA assay were analysed, unexpected values were found for certain samples such as CRF of nettles, where the protein content was lower than the nettles leaf itself. Therefore, a different protein assaying method that determines sample N₂ content was considered. In brief, samples were placed in a tared tin capsule and precisely weighed using a 4 digit balance. Samples ranged in weight from 2 to 3 mg. Two standards (Sulphanilamide STD) were also weighed. All samples, along with the standards, were run on an Organic Elemental Analysis Eager Experience (Flash 200, Fisher Scientific). The Nitrogen values was automatically calculated and then converted to protein using 6.25 as a conversion ratio.

2.1.6. Amino Acid Analysis

Amino acid concentrations in CRF and WLM were determined by oxidising samples with a hydrogen peroxide/formic acid/phenol mixture. Excess oxidation reagent was decomposed with sodium metabisulphite. The oxidised samples were then hydrolysed with 6M HCl acid for 18 h at 110 °C under nitrogen. The pH of the hydrolysate was then adjusted to 2.2 with 7.5 N NaOH. Aliquots of these samples were transferred to 20 mL centrifuge tubes and centrifuged

(Biofuge stratos) at 3000 rpm for 2 min; this was then syringe filtered through 0.22 μm filters in glass vials. The amino acids were separated by ion exchange chromatography (Pharmacia Biochrom, Cambridge) using sodium citrate buffers and determined by reaction with ninhydrin using photometric detection at 570 nm (440 nm for proline). For representative peak chromatographs, see Fig.2.2.

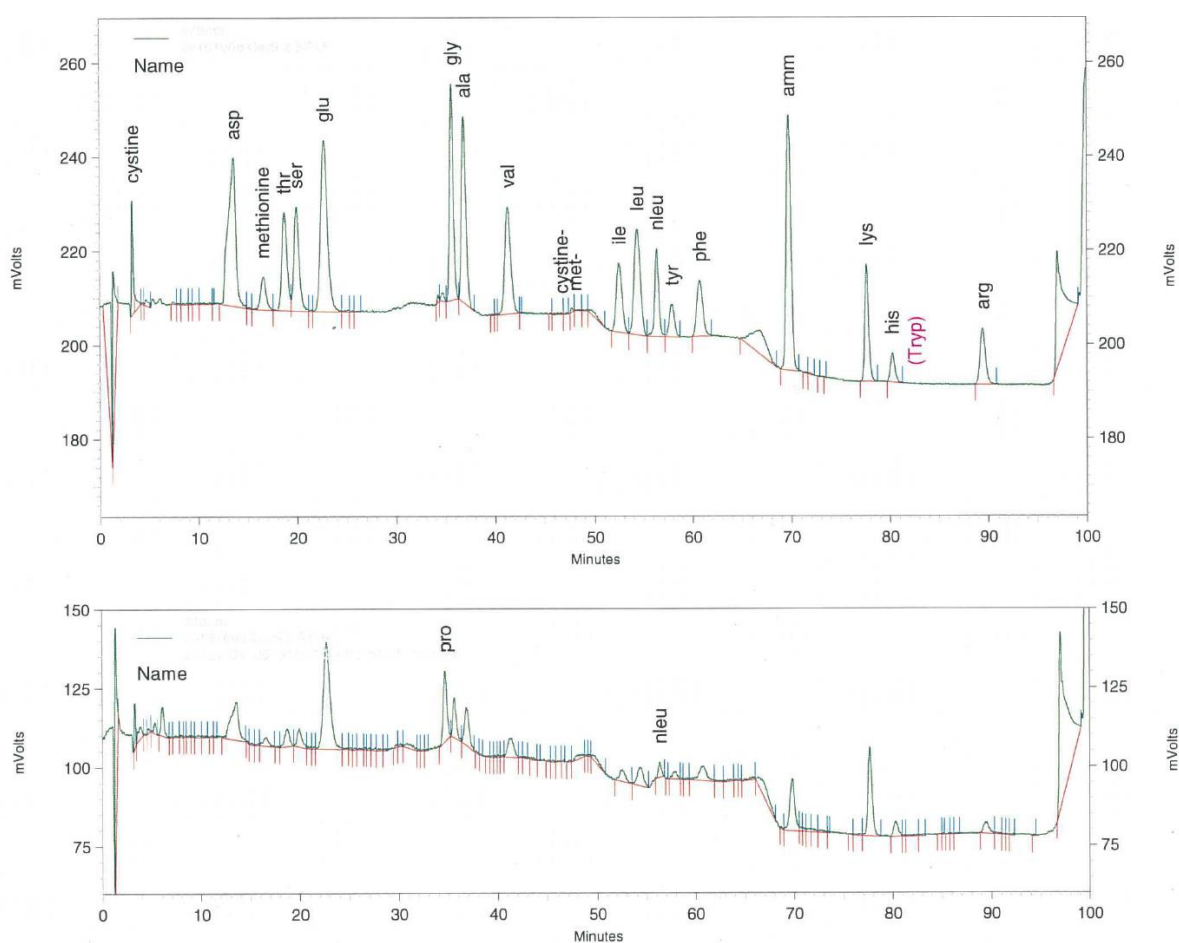


Figure 2.2. Representative chromatogram (spinach leaf) of 18 amino acids detected in freeze dried CRM and their total leaves from a range of green leafy tissues. Cys: Cysteine; Asp: aspartic acid (aspartate); Met: Methionine; Thr: Threonine; Ser: Serine; Glu: Glutamic acid (glutamate); Gly: Glycine; Ala: Alanine; Val: Valine; Ile: Isoleucine; Leu: Leucine; Tyr: Tyrosine; Phe: Phenylalanine; Lys: lysine; His: Histidine; Try: Tryptophan; Arg: Arginine; Pro: Proline.

2.1.7. Estimation of Carbohydrate Content

The percentage carbohydrate content of the freeze dried CRFs and their parent WLMs was estimated by difference (100- the percentage of the other macro-molecules; proteins, lipids and ash).

2.1.8. Lipid Extraction

Lipids were extracted from the freeze-dried materials (CRFs or WLMs) using a modified Folch et al. (1957) technique. A ratio of Chloroform: methanol (2:1) was firstly prepared, and 1.2 mL was added to 0.1 g of freeze dried sample and vortexed. To this 0.5 mL of a 0.9 % sodium chloride solution was added, and the mixture was vortexed again before being centrifuged, using a Thermo Jouan CR3i multifunction centrifuge (1300 RCF for 10 minutes at 4 °C) to separate into 3 layers. The lower phase containing the lipids and chloroform was transferred to a clean vessel. A further 1.2 mL of chloroform: methanol (2:1) was added to the remaining two phases which were then vortexed and centrifuged again. The lipid phase was removed and pooled with the original lipid layer before a third and final sequence was completed. The pooled lipid phases were centrifuged again to separate the lipids from any residual compounds content, and then dried under a flow of nitrogen. The combined lipid extracts were then weighed and quantified gravimetrically as follows:

$$\text{Total lipid (\%)} = \frac{\text{weight of dry lipid extract}}{\text{weight of "non - sucrose matter" (g)}} \times 100$$

2.1.9. Chlorophyll and Total Carotenoid Analysis

The pigment content (Chlorophylls *a* and *b*, as well as total carotenoids) of the lipid extracts was analysed using a spectrophotometer (CARY 50 Probe UV-visible). Total lipids (obtained from section 2.1.8) were dried and dissolved in 1 mL acetone (HPLC grade) and further diluted by a factor of 1:1000. An aliquot of this solution was added to a glass cuvette. Samples were exposed to selected wavelengths (661.6 nm for chlorophyll (*a*), 644.8 nm for chlorophyll (*b*) and 470 nm for total carotenoids) and the absorbance recorded (acetone was used as the blank). Equations (Eq. 2-4) by Lichtenthaler and Buschmann (2001) were used to calculate the concentration ($\mu\text{g mL}^{-1}$) of each pigment. The total chlorophyll content was obtained by adding the two concentrations of chlorophylls *a* and *b* together.

$$\text{Chlorophyll } a = (11.24 * A_{661.6}) - (2.04 * A_{644.8}) \quad (\text{Eq. 2})$$

$$\text{Chlorophyll } b = (20.13 * A_{644.8} - 4.19 * A_{661.6}) \quad (\text{Eq. 3})$$

$$\text{Carotenoid} = (1000 * A_{470}) - (1.90 * C_a) - (63.14 * C_b) / 214 \quad (\text{Eq. 4})$$

Where: A stands for absorbance, C_a for chlorophyll *a* and C_b for chlorophyll *b* concentrations.

2.1.10. Analysis of Fatty Acid Composition

The fatty acid content of the CRFs and WLMs was determined by esterification of total lipid extracts, obtained from section 2.1.8, to fatty acid methyl esters (FAMES) and analysed using gas chromatography-mass spectrometry (GC-MS). The dried lipid extracts were dissolved in 2 mL of chloroform; 100 μL (10 mg mL^{-1}) of methyl pentadecanoate (internal standard) was then added to 1 mL of the lipid extract in chloroform. Esterification was achieved through the addition of 200 μL of trimethylsulfonium hydroxide. The solution was then left, to ensure complete conversion, for 10 minutes before injection onto the GC-MS (Thermo Scientific, DSQII). The sample was injected at a volume of 10 μL onto a Phenomenex Zebron ZB-FFAP (30 m $\times$ 0.22 mm) column using a vaporising injector with a split flow of 50 mL min^{-1} of the carrier gas (helium). The oven temperature was maintained at 120 $^{\circ}\text{C}$ for 1 min, and then increased to 250 $^{\circ}\text{C}$ at a ramp of 5 $^{\circ}$ min^{-1} . The final temperature of 250 $^{\circ}\text{C}$ was held for 2 min. Detection was conducted using a mass spectrometer and identification of individual fatty acids was achieved using a mass spectrum library and its comparison of retention times to FAME standards. The percentage content of each fatty acid was calculated and the concentrations (mg mL^{-1}) were determined using the response factor of the internal standard. For representative chromatography refer to Fig.2.3.

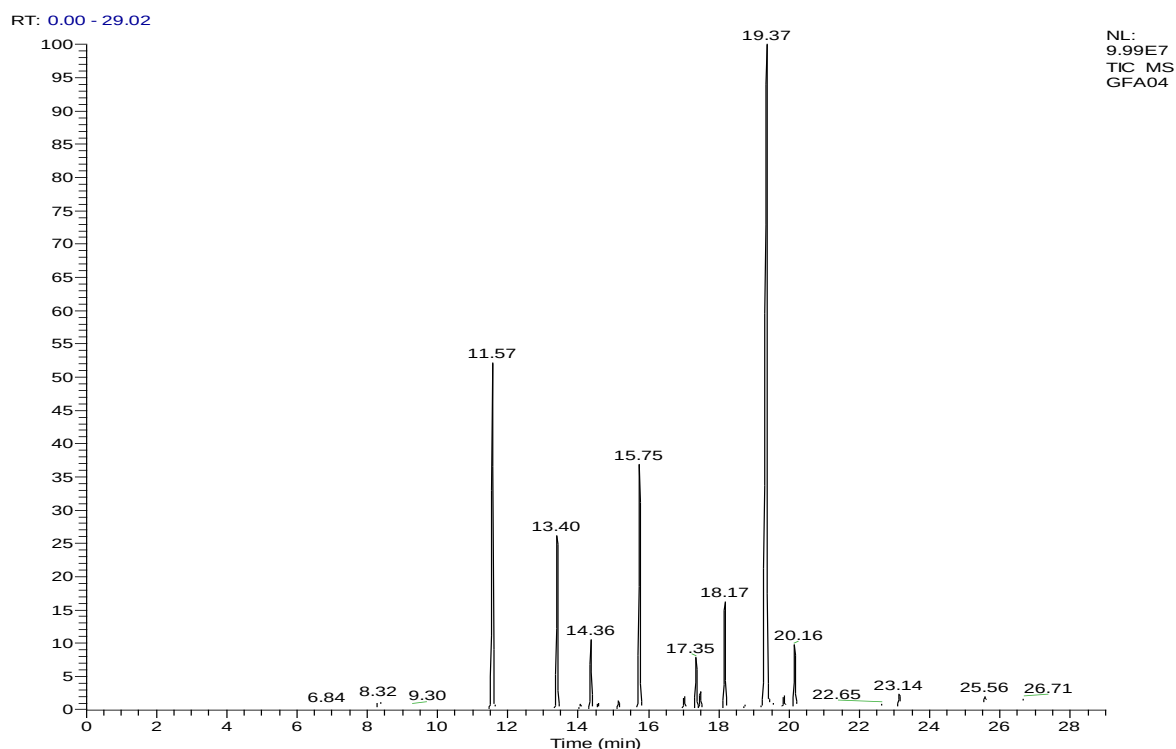


Figure 2.3. Representative gas chromatography of spinach chloroplast fatty acid composition. RT=retention time; RT 11.57=C15:0 (Pentadecanoic acid); RT13.40=C16:0 (Palmitic acid); RT14.36= C16:1 (Palmitoleic acid); RT15.75= C16:3 (Hexadecatrienoic acid); RT17.35= C18:1 (Oleic); RT18.17= C18:2 (Linoleic); RT19.37= C18:3 (α-Linolenic).

2.1.11. Vitamin E (Tocopherols) Analysis

Vitamin E content of freeze dried CRFs and WLMs was determined by measuring the concentration of α, β, γ and δ-tocopherols (see Fig. 2.4) by HPLC (using an Agilent 1100) with Fluorescence detector using a modification of the method of Rogers et al. (1993). Samples were prepared through the addition of 800 μL of methanol (containing 1 % butylated hydroxytoluene (BHT)) to 0.1 g freeze dried CRFs or WLMs. The material, weighed in an Eppendorf tube (2 mL), was physically broken up to assist in the solvent extraction using a mini bead beater (MBB-607EUR) at maximum speed (3450

RPM) for 1 min. The tube was then transferred to a centrifuge (Thermo Heraeus Fresco 21) and spun at 16200 RCF for 5 min at 4 °C; 400 µL of the supernatant was transferred into a clean vessel. A further 800 µL of methanol was added to the MBB tube, and the process repeated to ensure quantitative recovery of tocopherols. The pooled supernatants (2 mL total) were dried under a flow of nitrogen gas, and methanol (containing 1 % BHT) (2 mL) was added and vortexed before being syringe filtered (0.45µm) into an Eppendorf tube. This was centrifuged at 16200 RCF for 5 min at 4 °C, and the supernatant was transferred to an amber HPLC vial.

Samples (10 µL) were injected through a security guard-column (C18, 4 µm, 3.9×20 mm) and separated on a Zorbax RX-C8 5 µm (250 × 4.6 mm) column with the oven set at 20 °C. A gradient system of two mobile phases was employed; the first solvent (A) contained 45:45:5:5 Acetonitrile: Methanol: Isopropanol: 1 % Acetic Acid solution and the second solvent (B) contained 25:70:5 Acetonitrile: Methanol: Isopropanol. The flow rate of the mobile phase was set at 0.8 mL min⁻¹ starting with 100 % solvent A. After 6 min of solvent A running isocratically, the mobile phase changed linearly to 100 % solvent B over 10 min. This was held for 12 min before being returned to the initial conditions, with a total run time of 36 min. Detection of tocopherols, was achieved at excitation and emission wavelengths of 298 and 328 nm and quantitation achieved using the linear formula produced from a calibration curve of external (tocopherols) standards (4-100 µg mL⁻¹) in methanol containing 1 % BHT.

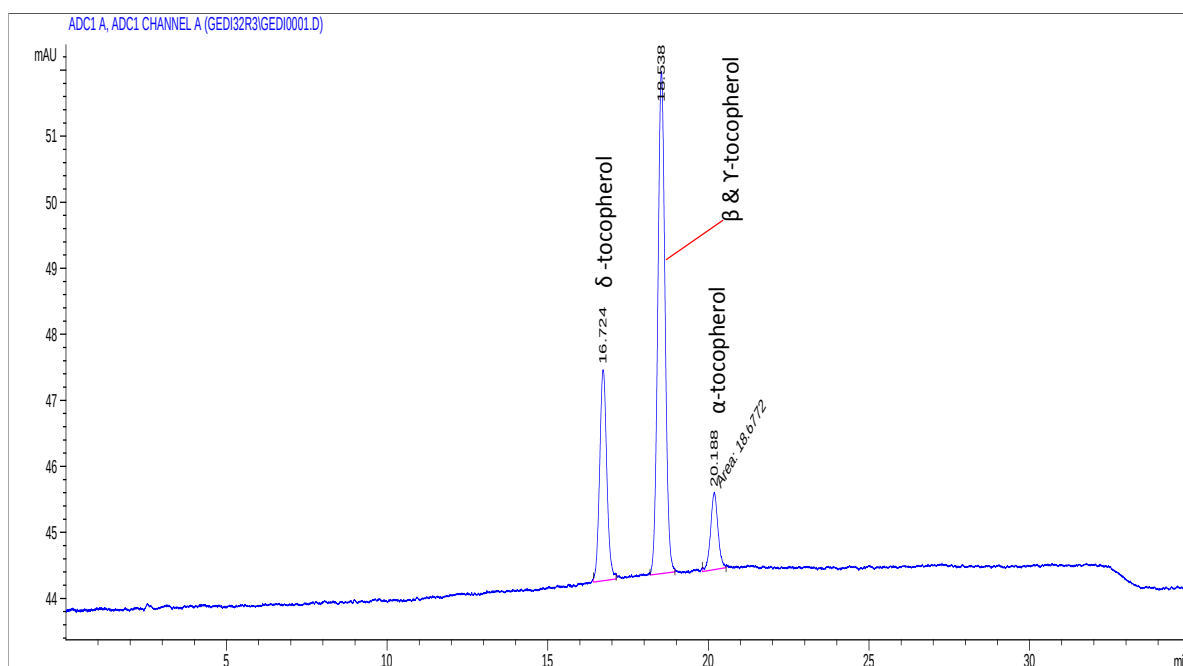


Figure 2.4. Tocopherol standards (α , β , γ and δ), it should be noted here that β and γ tocopherols coeluted at the same retention time with the FP detector on the Zorbax RX-C8, 5 μ m (250 $\times$ 4.6 mm) column, however, they were either very trace or could not be detected in the analysed green materials, so further effort to achieve separation (using other methods) was not considered.

2.1.12. Vitamin A (β -carotene) and Lutein Analysis

The β -carotene content of the freeze dried CRFs and WLMs was analysed using HPLC (Agilent 1100) with Photo Diode Array (PDA) detection using a method slightly modified from Kimura and Rodriguez-Amaya (2002). Material (0.1 g) was mixed with cold acetone containing 0.1 % butylated hydroxytoluene (BHT) (2 mL) to which 0.4 g of anhydrous sodium sulphate was added. The mixture was gently shaken for 30 s, and then centrifuged, (Thermo Jouan CR3i multifunction centrifuge) at 4 °C for 5 min at 1350 RCF. The supernatant was then syringe filtered (0.45 μ m) into a clean vessel. A further 2 mL of the

acetone solution was added to the remaining salt pellet and centrifuged again to liberate more lipids (into the supernatant) which were pooled together with the original fraction. This step was repeated until the supernatant became colourless or pale green. The collected lipid extract was dried under a flow of nitrogen, and the weight of the lipid fraction noted. The dried lipid extract was then dissolved in 10 mL of the acetone solution (containing 0.1 % BHT), and syringe filtered (0.45 μm) into an amber HPLC vial.

The flow rate of the mobile phase (Acetonitrile: Methanol: Ethyl Acetate) was set at 0.5 mL min⁻¹. Two gradient mobile phases were used from 95:5:0 to 60:20:20 in 20 min, maintaining this proportion until the end of the run. Re-equilibration took 15 min. Samples were injected at a volume of 10 μL through a Sentry guard-column (Waters, Nova-Pak C18, 4 μm , 3.9 $\times$ 20 mm), and separated using Waters Spherisorb S3ODS (3 μm , 4.6 $\times$ 15 cm) column, with the temperature set at 22 °C; β -Carotene was detected at 454 nm. The concentration of β -carotene and that of lutein was determined using a linear equation created using a calibration curve produced from a range of external (β -carotene) standards (10-100 $\mu\text{g mL}^{-1}$) dissolved in cold acetone containing 0.1 % BHT. For representative peaks see chromatographs (A-D) in Fig. (2.5).

2.1.13. Vitamin C (ascorbic acid) Analysis

The vitamin C in the freeze dried CRFs and WLMs was extracted by adding 5 mL of 0.3 M meta-phosphoric acid and 1.4 M acetic acid to 0.1 g of the sample. The samples were then vortexed for 1 min, sonicated (10 min at 10 °C, power 9 (HF45 kHz, VWR International, USC1700D) and vortexed again for 1 min. All samples were then centrifuged at 16200 RCF for 8 min at 4 °C, and filtered (0.45 µm). Each sample (200 µL) was placed into brown amber HPLC vials and diluted with 800 µL acetonitrile. HPLC conditions were according to Singh et al. (2007). Briefly, samples were injected (10 µL) onto a reverse phase C-18 (150×4.6mm 5µm) column and quantified using an external (ascorbic acid) standard (100 to 1 µg mL⁻¹). The mobile phase consisted of acetonitrile and 0.05 M KH₂PO₄ in the ratio of 75:25 at a flow rate of 1.5 mL min⁻¹. Ascorbic acid was detected at 261 nm using HPLC (Agilent 1100) with PDA detection.

2.1.14. Mineral Analysis

In preparation for mineral analysis, an acid digestion was conducted by adding 20 mL concentrated HNO₃ to 0.2 g of CRFs or ground WLMs. This was heated gently whilst stirring occasionally. After approximately 30-60 min when the sample had been reduced to approximately 5 mL, the flask was removed and left to cool (in the fume cupboard) for several minutes. Small quantities of deionised water were then added to each flask and the solution was filtered

through a Whatman No 42 (equivalent) filter paper and diluted up to 50 mL using deionised water.

Multi-element analysis of the diluted solutions was undertaken by inductively coupled plasma mass spectrometry (ICP-MS) (Thermo-Fisher Scientific iCAP-Q; Thermo Fisher Scientific). The instrument was run employing collision-cell technology with kinetic energy discrimination (CCT-KED) to remove polyatomic interferences; the collision cell gas used was helium (He). Samples were introduced using an auto sampler (Cetac ASX-520) incorporating an ASXpress™ rapid uptake module through a PEEK nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream on a separate line via the ASX press unit and comprised of Sc ($20 \mu\text{g L}^{-1}$), Rh ($10 \mu\text{g L}^{-1}$), Ge ($10 \mu\text{g L}^{-1}$) and Ir ($5 \mu\text{g L}^{-1}$) in 2% trace analysis grade (Fisher Scientific, UK) HNO_3 . External multi-element calibration standards (Claritas-PPT grade CLMS-2 from SPEX Certiprep Inc., Metuchen, NJ, USA) included As, Cd, Cu, Fe, Mn, Pb, Se and Zn, in the range $0 - 100 \mu\text{g L}^{-1}$ (0, 20, 40, $100 \mu\text{g L}^{-1}$). A bespoke external multi-element calibration solution (PlasmaCAL, SCP Science) was used to create Ca, Mg, Na and K standards in the range of $0-30 \mu\text{g L}^{-1}$. Phosphorus calibration utilized an in-house KH_2PO_4 solution standard ($10 \mu\text{g L}^{-1}$ P). Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific), utilizing external cross-calibration between pulse-counting and analogue detector modes as required.

2.2. Materials and Methods for *In Vitro* Digestion

Fresh raw spinach as vegetable source was purchased from Tesco the morning of the sample preparation. Spinach was selected as a model for green leaf biomass due to its availability throughout the year. In addition to spinach WLMs and CRFs isolated using sucrose, CRFs concentrated with a juicer, processed by means of Ultra-Turrax homogenization and microcavitation were included in this section to study carotenoid bioaccessibility of materials obtained with different matrix processes. The preparation of WLMs and sucrose extracted CRFs is as in sections 2.1.1.1 and 2.1.1.2.

2.2.1. Juicer Extraction of CRFs with Ultra-Turrax and Microcavitation

The juicer (Oscar Neo, DA-1000, Natures wonderland, Queensland, Australia) was put on setting 5 to gain maximum yield of juice in litres per kilogram of spinach material. Spinach (6 kg) was processed through the juicer, yielding 4 L of concentrated spinach juice. The material was only put through this process on one occasion. One litre of the material was then saved and termed juicer chloroplast rich fractions (J-CRFs) and the remaining 3 L were saved for further processing.

One litre of the juice saved from the Oscar Neo juicer was then further processed using the Ultra-Turrax (T18 basic, IKA works Inc, Germany). This

was done on setting 1 (6.000 rpm) for 30 s. Resultant juice was then called Ultra-Turrax chloroplast rich fractions (UT-CRFs).

The remaining 2 L of the juice from the Oscar Neo juicer was then further processed by microcavitation (Efficiency Technologies, Milton Keynes). This was done using a 1.4 cm cavitation pipe for 1 min. A higher quantity of juice needed to be processed through the microcavitation device, due to this piece of machinery requiring a minimum of this volume in order to process it adequately and efficiently via the pressure gradient necessary. Finally microcavitated samples were referred to as microcavitated chloroplast rich fractions (MC-CRFs). After all processes were done, J-CRFs, UT-CRFs and MC-CRFs were all stored and freeze dried similar conditions as those in section 2.1.1.

2.2.2. *In Vitro* Digestion Model

The digestibility of nutrients *in vitro* was determined by the method of Minekus et al. (2014) with slight modifications. Briefly, 0.5 g sample, made to 5 g with water and/or oil was subjected to 4.0 mL of simulated salivary fluid (SSF) electrolyte stock solution and mixed together. A 0.5 mL salivary α -amylase solution of 1500 U mL⁻¹ made up in SSF electrolyte stock solution (α -amylase from human saliva Type IX-A, 1000–3000 U mg⁻¹ protein, Sigma) was added followed by 25 mL of 0.3 M CaCl₂ and 0.475 mL of water, thoroughly mixed, and shaken (100 rpm, 2 min at 37°C). Next 8.0 mL of simulated gastric fluid

(SGF) electrolyte stock solution was added to the oral bolus; 1.0 mL of porcine pepsin (EC 3.4.23.1) and 5.0 μL of CaCl_2 were then added to achieve 2000 U mL^{-1} and 0.075 mM, respectively in the final digestion mixture. The pH was then reduced to 3 using 2M HCl and samples were again put in the shaking incubator (100 rpm for 2 h at 37°C). The digestate from gastric chyme (20 mL) was mixed with 11 mL of simulated intestinal fluid (SIF), electrolyte stock solution, 5.0 mL of a pancreatin solution of 800 U mL^{-1} (based on trypsin) made up in SIF electrolyte stock solution (pancreatin from porcine pancreas, Sigma), 2.5 mL fresh bile (160 mM), 40 μL of 0.3 M CaCl_2 , 0.15 mL of 1 M NaOH (volumes of HCl/ NaOH occasionally varied depending on sample nature) to reach pH 7.0. Samples were once more incubated in the shaker (100 rpm for 2 h at 37°C). Before each incubation step and at the end of the *in vitro* digestion, samples were flushed under a blanket of Argon gas to protect samples from oxidation and stored at -80°C until further analysis (maximum of one week). For detailed preparation of solutions refer to Minekus et al. (2014).

2.2.3. Ultra-structural Analysis

The structural changes of the samples with the simulated oral and gastro intestinal tract (GIT) were observed under a light microscope (Nikon Microscope Eclipse E400, Nikon Corporation) and transmission electron microscope (TEM) [Tecnai t12 Biotwin].

2.2.3.1. Ultra-structural Analysis by Light Microscope

To increase the integrity of the sample structure, optical microscopy with differential interference contrast microscopy (DIC) was used (Leitz Diaplan Microscope). A drop of each *in vitro* digestion step sample (oral, gastric and duodenal) was placed on a glass slide, topped with a cover slip and allowed to dry before being placed under the microscope. All images were taken using a 100X objective lens. A digital camera was attached to the microscope to capture images of the samples. The scales of the images were calibrated against a glass mounted graticule (1 mm, 0.01 mm division from Graticules Ltd, Tonbridge).

2.2.3.2. Ultra-structural Analysis by TEM

An aliquot (1 mL) of pre-digested, gastric and duodenal phases of the CRF from spinach was derived and fixed with 3% glutaraldehyde in 0.1M cacodylate buffer. Samples were then transferred into an Eppendorf tube and centrifuged at 1000 rpm. Supernatant was removed, resuspended in a 1% aqueous osmium tetroxide and left for one hour. Samples were twice (each 5 min) washed with distilled water followed by centrifugation and removal of the supernatant each time. They were then dehydrated with a series of different ethanol concentrations (50%, 70% and 90%; 2 times each for 15 min); and

further washed with pure ethanol (100%, 3 times each for 20 min) before propylene oxide (100%, 2 times each 15 min) was added to further wash. A resin (in the ratio of 1:3 and 1:1, resin: propylene oxide) was prepared. Initially 1 mL of resin (1:3) was added into the samples and left for 3 h. Samples were then centrifuged at 13,000 rpm, supernatant resins removed and a new resin (1:1) was added. Tubes were then left overnight under the fume hood with the lids off. Pure resin was this time suspended into the samples (3 times each 2.5 h) and left in the oven (60°C) for 48 h. Thin sections of the samples were cut with ultra-microtome (Leica EM) using diamond knife. Thin section of each samples in the Nano scale was then visualized under the TEM.

2.2.4. Bioaccessibility Determination

For carotenoid and other nutrient bioaccessibility, the micelle fraction, containing the bioaccessible carotenoid plus other nutrients was separated by centrifugation (5000 rpm at 4°C for 1 h). Centrifuged samples were separated into two phases (unless oil was added to the samples, in which case, three phases could be formed and the middle layer carries the mixed micelles): green sediment phase at the bottom and relatively light green at the top. The top phase was assumed to contain mixed micelles that entrapped the carotenoids and other nutrients, and hence, the bioaccessible fraction. The micelle fraction was passed through 0.45 µm Nylon syringe filters (It should be noted that samples could hardly pass through the 0.45 µm filter and occasionally required pre-filtration step. In such a case, syringe filters packed

with pre-filter of glass fibre with a pore size of 0.8 μm were used before being passed to the 0.45 μm filter). Filtered (micellar fraction) and non-filtered (total digestate) samples (5 mL each) were collected into pre-weighed falcon tubes. All samples were flushed with argon gas and stored in -80°C until further analysis (maximum of one week). Samples were then analysed for their content in specific carotenoids (β -carotene and lutein). See Fig.2.6.

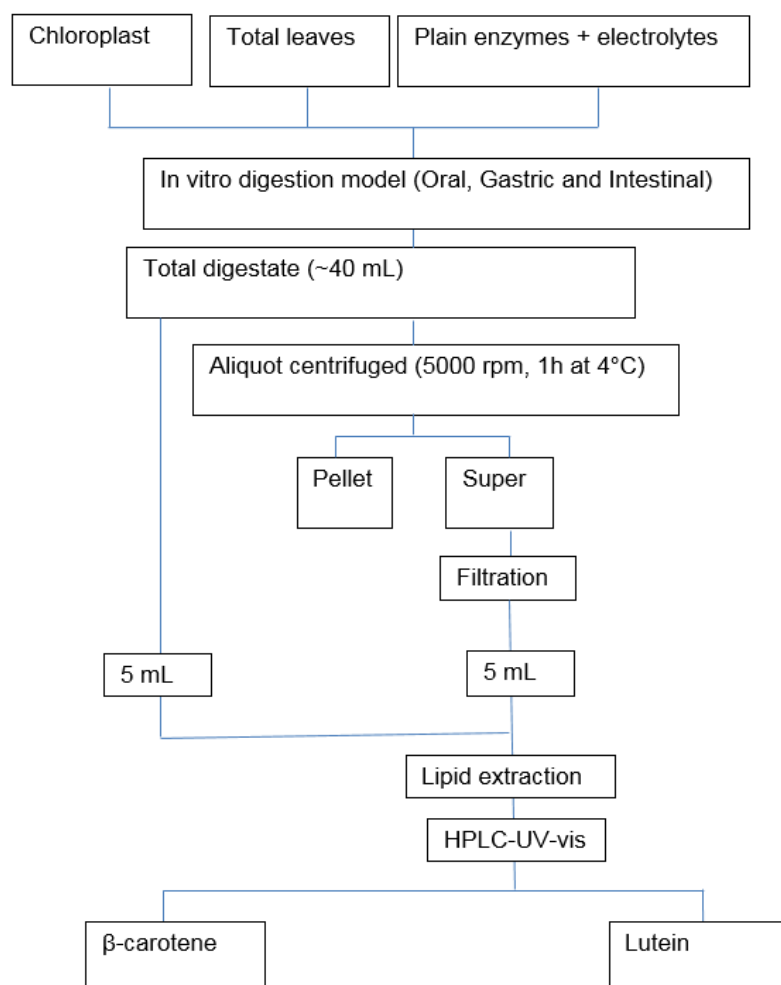


Figure 2.6. Diagram for sample bioaccessibility determination

Bioaccessibility was calculated using the following formula by (Garrett et al., 1999)

$$\text{Bioaccessibility (\%)} = \frac{[\text{C micelle}]}{[\text{C digesta}]} \times 100$$

Where C micelle stands for concentration of the target compound (nutrient) in the micelle fraction and C digesta is the concentration of the target compound in the overall sample (total digesta)

2.2.5. Carotenoid Extraction of Digested Materials

Lipids were extracted from the *in vitro* digested materials, CRF (freeze dried or wet) and WLM (freeze dried) using slightly modified Folch et al. (1957) method. Briefly, chloroform: methanol (2:1) was prepared and 5 mL was mixed with an equal volume (5 mL) of the digesta (either micellar or total phase) and vortexed for 1 min. To this, 1 mL of a 0.9% sodium chloride solution was added and the mixture was vortexed again (1 min) before being centrifuged, using a Thermo Jouan CR3i multifunction centrifuge (1300 RCF for 10 minutes at 4 °C) to separate the phases. The lowest phase containing the lipids and the chloroform was transferred to a clean vessel. A further 5 mL of the chloroform: methanol mixture was added to the remaining extract which was then vortexed and centrifuged again with the same conditions. The lipid phase was removed and pooled with the original lipid layer before a third and final sequence was completed. The pooled lipid phases were centrifuged again to separate the lipids from any residual compounds; this lipid layer was removed and dried under a flow of nitrogen. Lipid extracts were then weighed to calculate the total lipid content (when required) and to quantify the intended carotenoids (β -carotene and lutein) by HPLC analysis.

2.2.5.1. Carotenoid (β -Carotene and Lutein) Analysis

The Carotenoid content of materials was analysed by high performance liquid chromatography (HPLC) with PDA detection using an Agilent 1100 system. Dried lipid extracts obtained previously through Folch et al. (1957) method (section 2.2.5) were dissolved in a known volume of acetone solution (containing 0.1% BHT) and syringe filtered (0.45 μ m) into brown amber HPLC vials. HPLC conditions were similar to those in section 2.1.12.

2.3. Materials and Methods for Lipolysis Using pH-Stat

Due to limited amounts of pancreatic lipase related protein 2 (PLRP2), generously provided by Prof.Dr.Frédéric Carrière (Laboratory of Enzymology at Interface and Physiology of Lipolysis, Marseille, France), only CRFs isolated using sucrose and WLMs were included in the lipolysis section. Before material lipolysis, galactolipids which are the major form of lipids existing in the materials, were identified and quantified.

2.3.1. Separation of Polar Lipids from CRFs and WLM by 2-D-TLC

Solvent was removed from a portion of original sample (spinach CRFs and WLM) in chloroform and the weight of lipid recorded. To this, a known weight of C17:0 phosphatidylethanolamine (PE) was added, as internal standard. The

sample (14 µl for chloroplast and 20 µl for leaves) and standard mixture was spotted near the corner of the thin layer chromatograph (TLC) plate using 2-dimensional glass high performance thin layer chromatography (HPTLC) Silica gel 60 F₂₅₄ plates (Merck) with size dimension of 10 cm x 10 cm and separated into two directions using solvent mixtures of chloroform/methanol/water (65:25:2.8) for the first direction and chloroform/methanol/acetic acid/water (80:12:15:4) for the second direction.

2.3.1.1. Analysis of Fatty Acids by Gas Chromatography (GC)

Initially, lipid fractions (MGDG, DGDG, SQDG, TGDG and PE) were removed from the 2-D TLC plate before re-extracting from the silica. A second internal standard (C21:0) was added to the fractions and esterification was carried out. The esterification procedure was conducted by mixing the lipid fraction with 1 mL toluene and 2 mL of 1% sulfuric acid in a glass test tube. The mixture was then heated at 50°C for 14 – 16 h. After cooling, the mixture was shaken with 2 mL isohexane and 5 mL of 5% NaCl solution. The solvent was then transferred into a new glass tube. The original tube was shaken with another 2 mL of isohexane and the two solvents were combined in the new glass tube. The combined solvents were shaken with 3 mL of 2% KHCO₃ solution. The solvents mixture was then transferred into a new tube and 1 mL of toluene was added before blowing off the solvents into dryness with N₂ gas. Then isohexane and BHT was added to give a lipid concentration of 5 mg mL⁻¹. The

GC technique was carried out using an Agilent 6890. Fatty acid methyl esters (FAMES) were separated using a capillary column (Cp-wax 52CB, 30 mm x 0.25 mm internal diameter x 0.15 μm , Agilent, UK). Hydrogen was used as the carrier gas with the flow rate of 40 mL min^{-1} . The column temperature was initially held at 170°C for 3 min. The temperature was then increased from 170°C to 220 °C at 4°C min^{-1} and maintained at 220°C for 10 min. One μL of sample was injected in a 230°C inlet with a 50:1 split ratio. The detector used was flame ionization detector at 300°C. Results were an integration of the area under curve and reported as normalised area (%) as well as mg fraction g^{-1} lipid by reference to the internal standard.

2.3.2. *In Vitro* Digestion Analysis with pH-Stat

The oral, gastric and duodenal phases of the *in vitro* digestion model were carried out following procedure in section 2.2.2 except that pancreatin, PLRP2 and the bile salt solutions were (on this occasion) added into the duodenal system after the pH-stat conditions was made ready. The activity of pancreatin and PLRP2 was then determined potentiometrically at 37°C and at a constant pH value (7.0 for pancreatin and 8-9 for PLRP2) by continuously measuring the release of free fatty acids from mechanically stirred dispersion of the digested solutions using 0.1 M NaOH as titrant and a pH-stat device (702 SM Titrino, Metrohm).

2.3.3. Determination of gPLRP2 Activity on MGDG

The galactolipase activity measurement was slightly modified from Amara et al. (2009). Briefly, galactolipase activity was determined potentiometrically at 37°C and at a constant pH value by continuously measuring the release of free fatty acids from mechanically stirred dispersion of galactolipids, using 0.1 M NaOH as titrant and a pH-stat device (702 SM Titrino, Metrohm). To prepare the galactolipid dispersion, 25 mg of MGDG was mixed with 5 mL of 0.33 mM Tris–HCl buffer, containing 0.1M NaCl and the optimum point of galactolipid to bile salt (NaTDC) ratio (1.3) reported by Amara et al. (2009). Mixtures were then subjected to ultrasonic treatment for 6–10 min in a water bath (HF45 kHz, VWR International, USC1700D). The effects of pH on galactolipase activities were tested by adjusting the pH of the assay solution before adding the enzyme. When required, due to low levels of FFA ionization, back titration was performed. After incubating the enzyme with MGDG for 3 min at a given pH, the pH end-point value was then increased to pH 9.0 in order to ensure full ionization and titration of free fatty acids. Control experiments without any enzyme were performed in order to determine the amounts of NaOH required increasing the pH value to pH 9.0 regardless of the FFA titration. One international galactolipase unit corresponds to the release of one μmol of fatty acid per minute. The specific activity was expressed in international units (U) per mg of enzyme.

2.3.4. Determination of Pancreatin Activity on Tributyrin

A similar procedure as above (section 2.3.3) was used for lipase activity determination in pancreatin cocktail (containing mixture of enzymes such as lipases, trypsin, chymotrypsin etc.) but with different assay solutions. A 14.5 mL of an assay solution containing Tris (1 mM), NaCl (150 mM), CaCl₂ (1 mM) and sodium taurodeoxycholate (NaTDC) (0.5 mM) was firstly prepared and added to the pH-stat vessel thermo-stated at 37°C, then 0.5 mL tributyrin was added into the assay solution present in the pH stat vessel. The pH stat vessel was then put on the pH-stat device and the mechanical stirrer switched-on to form the emulsion. Tributyrin makes readily a fine emulsion upon stirring. The pH end point value has been set at 7.0. One international lipase unit corresponds to the release of one μ mol of fatty acid per minute. The activity was expressed in international units (U) per milligram of enzyme.

2.4. Materials and Methods for Zebrafish Feeding Trials

2.4.1. Preparation

Once a diet was formulated, the desired amounts of all ingredients required were mixed using a Hobart food mixer until thoroughly mixed. Water was added until the mixture achieved dough-like consistency. The dough mixture was then spread out on trays and dried for 24 h at 50°C using a nine shelf Parallexx Excalibur food dehydrator. Once dry, the diet was crushed and processed through a series of sieves with apertures of 425 µm and 850 µm. The desired pellet size fell between the two. All diets were stored at +4°C.

2.4.2. Subjects and Husbandry

All fish were bred in-house at The Institute of Integrative Biology aquarium facility, the University of Liverpool. The first trial involved 525 juvenile *D. rerio* (AB wild type strain). The fish were approximately 2 months old at trial start. Fish were housed in groups of 15 individuals in 35 identical 1.5 L zebrafish tanks, made by Aquatic Habitats, each measuring 25cm x 7cm x 15.5 cm. The tanks were connected to a central system which is maintained by a sump filtration system and 20% weekly water changes. Due to the small size of the fish, tanks were fitted with a 400 μ m fry mesh baffle; cleaning was conducted weekly during the weighing of the fish to prevent further disturbances. Water quality was subsequently kept stable with the following parameters, Ammonia (NH_4); 0 mg L⁻¹, Nitrite (NO_2); 0 mg L⁻¹, Nitrate (NO_3); <50 mg L⁻¹ and pH; 7.0. Fish were maintained at 28 $\pm$ 1°C and exposed to a 12/12 hour light cycle.

For the second trial, 84 juvenile *D. rerio* (Casper strain) were used. The fish were approximately 7 months old at trial start. Fish were housed in groups of 6 individuals, either all male or all female, in 14 identical tanks, the same 1.5 L tanks as above using the same baffles. The tanks were maintained on the same system as above with the same water quality parameters and environmental conditions. Cleaning of tanks was done while fish were housed in breeding tanks.

2.4.3. Experimental Procedure

2.4.3.1. Weighing Fish

Fish were weighed weekly to record growth throughout. Fish were weighed by tank; 15 fish as a whole due to time constraints. A separate 1.5 L tank was used, filled with one cm of system water, placed on a Fisher scientific SG-602 balance and tared. All 15 fish were caught in a small net, lifted from the housing tank, excess water was removed gently by dabbing the net on blue roll paper towel, the net preventing any physical harm to the fish, and then the fish were placed in the tank on the balance. The weight was recorded for each tank. While the fish were situated in the weighing tank, the housing tank and mesh baffle were cleaned before returning the fish.

2.4.3.2. Feeding and Sampling

After each tank of fish had been weighed, one week's worth of daily feeds was premeasured using a Kern 770 five-point balance at 4% body weight per day. This was repeated weekly to maintain a 4% body weight feed regime throughout the 8-week trial, this encouraged maximum growth and health. On the days when fish were weighed, feed was given afterwards in order to gain accurate fish weights, on all other days feed was given in the morning. Five replicate tanks were given the same experimental diet, 75 fish in total per diet. At the end of the trial period, the fish were humanely euthanized by fully

trained personnel via a Home Office approved Schedule 1 method; concussion followed by pithing of the brain to confirm death. Once dead, fish were individually weighed and measured. Fish from all five tanks fed the same feed were collated together, initially frozen at -20°C at the University of Liverpool, transported on dry ice to the Division of Food Science, School of Biosciences, University of Nottingham and stored at -80°C, this prevented samples thawing.

From the measurements collected, along with percentage growth during the trial period, the following feed efficiency and performance indicators were assessed: Feed Conversion Ratio (FCR), Protein Conversion Ratio (PCR), Protein Efficiency Ratio (PER), Fish Meal Ratio (FMR), (Boyd et al., 2007) and Specific Growth Rate (SGR) (Korkmaz and Cakirogullari, 2011). Further indicators can be used to analyze overall fish condition and health alongside growth performance. The Fulton type condition factor (K) is commonly used in fisheries science to describe the relationship of fish weight and length to overall condition (Nash et al., 2006, Kerambrun et al., 2011, Barnham and Baxter, 2003, Snyder et al., 2004)

2.4.4. Zebrafish Breeding for Spawning

Each of the seven diets (10, 20 and 50% of CRF and WLM plus the control) was fed to two tanks, one male and one female tank, six fish per tank. Feed was given daily *ad libitum* until satiation. After 3 weeks of feeding the fish were bred by mixing males and females together (1:1 male : female ratio) one

evening in medium (3 L) aquatic habitat zebrafish tanks, measuring 11.5cm x 25cm x 15cm. The tanks contained a mesh net, with a 2mm aperture, used to create a false raised base with a gradient to prevent cannibalism of the eggs by adults. The next morning, as soon as the lights came on, the flow was switched off and the net was raised slightly to stimulate breeding. After a two-hour period the fish were removed, sexed, and returned to all male and all female housing. Eggs were collected from the spawning tanks by hand, transferred to sample jars then snap frozen with liquid nitrogen; samples were stored at -80 until further analyses. Spawning was repeated every 3 days until sufficient embryos had been collected for the required sample size. Fish were then humanely euthanized by fully trained personnel via a Home Office approved Schedule 1 method; concussion followed by pithing of the brain to confirm death. Fish were also stored at -80°C until further analyses.

2.4.5. Ethical Issues

It is anticipated that the welfare standards of the subject fish will not be affected; they were housed using standard methods and fed on balanced diets formulated to meet their nutritional requirements. The subject fish were also bred at the University of Liverpool zebrafish facility and so endured minimal transport or housing stress prior to this experiment starting. The welfare was monitored independently, so had the welfare condition of any individual fish reached an unacceptable level, it would have been removed from the trial.

2.4.6. Post-trial Preparation and Chemical Analysis of Zebrafish

Zebrafish and its eggs were freeze dried for one week (Edwards Freeze Dryer, Super Modulyo). During the freeze drying process, samples were protected from light exposure. Samples were then ground to homogeneous powder using mortar and pestle under dim light and with liquid nitrogen.

2.4.6.1. Protein Analysis

Each freeze dried sample was placed in a tared tin capsule and precisely weighed using a 5-decimal place balance. Samples ranged in weight from 2 - 3 mg. Two standards (Sulphanilamide STD) were also weighed. All samples along with the standards were run on an Organic Elemental Analysis Eager Experience (Flash 200, Fisher Scientific). The Nitrogen values were automatically calculated and then converted to protein by using 6.25 as a conversion ratio.

2.4.6.2. Lipid Extraction

Lipids were extracted from the freeze dried materials (CRFs or WLMs) using a modified Folch et al. (1957) technique. A ratio of chloroform: methanol (2:1) was firstly prepared, and 6 mL was added to 0.5 g of freeze dried sample and vortexed. To this 2.5 mL of a 0.9 % sodium chloride solution was added, and

the mixture was vortexed again before being centrifuged, using a Thermo Jouan CR3i multifunction centrifuge (1300 RCF for 10 minutes at 4 °C) to separate into 3 layers. The lower phase containing the lipids and chloroform was transferred to a clean vessel. A further 6 mL of chloroform: methanol (2:1) was added to the remaining two phases which were then vortexed and centrifuged again. The lipid phase was removed and pooled with the original lipid layer. The pooled lipid phases were centrifuged again to separate the lipids from any residual compounds content, they were then filtered (0.45µm PTFE syringe filter) before drying under a flow of nitrogen. The combined lipid extracts were then weighed and quantified gravimetrically as follows:

$$\text{Total lipid (\%)} = \frac{\text{weight of dry lipid extract}}{\text{weight of "non - sucrose matter" (g)}} \times 100$$

2.4.6.3. Fatty acid Composition

The fatty acid content of the zebrafish was determined by esterification of total lipid extracts, obtained from section 2.4.5.2 to fatty acid methyl esters (FAMES) and analysed using GC-MS and according to section 2.1.10. For representative peaks, see Fig.2.7.

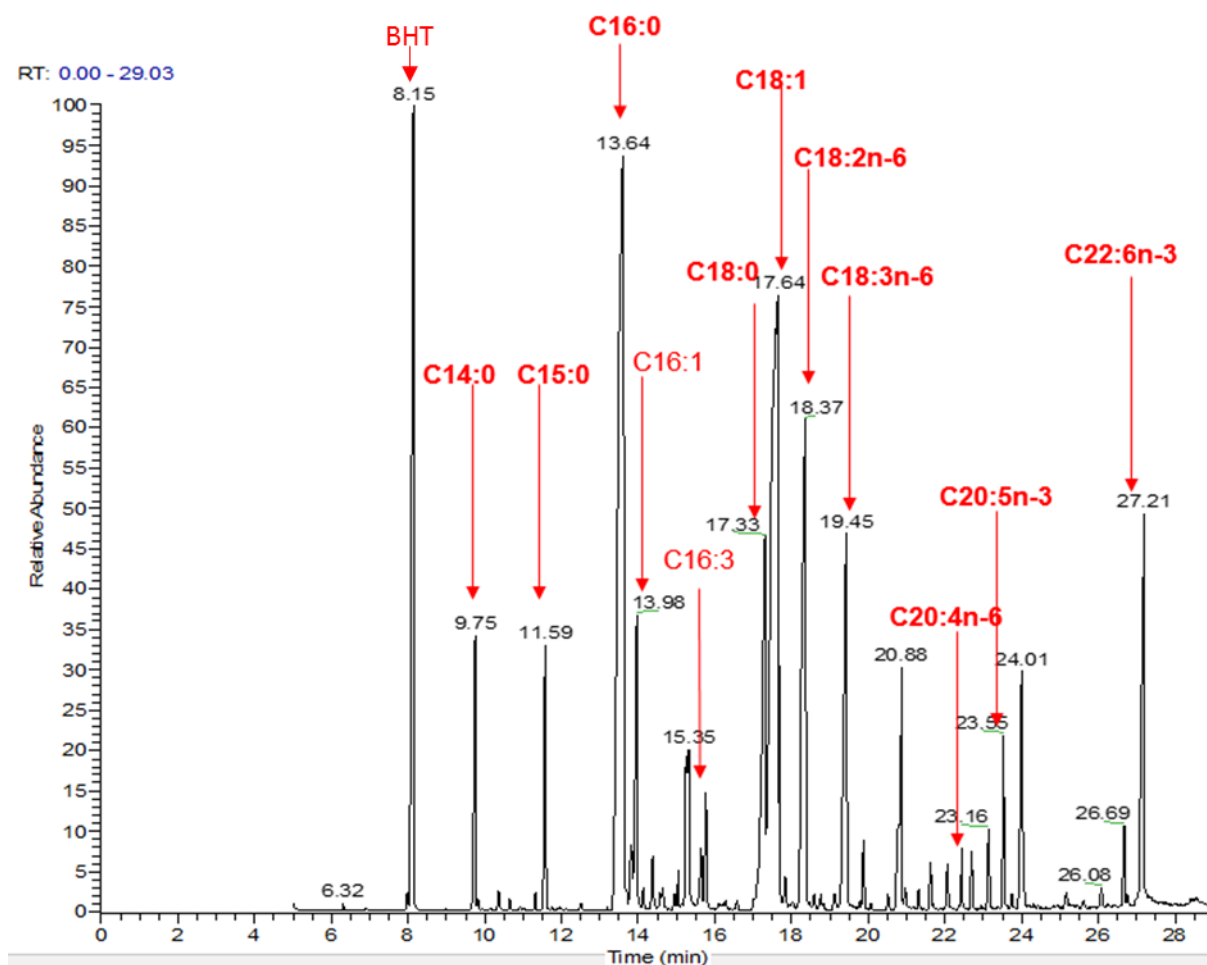


Figure 2.7. Representative chromatograph of zebrafish fatty acid profile (this trial was fed with 20% CRFs).

2.4.6.4. Zebrafish Vitamin E Analysis

Vitamin E content of freeze dried zebrafish feed in CRFs and WLMS was determined by measuring the concentration of α , β , γ and δ -tocopherols by HPLC (using an Agilent 1100) with Fluorescence detector using a modification of the method of Rogers et al. (1993). Samples were prepared through the addition of 2 mL chloroform containing 0.1% BHT to the total lipids obtained

from section 3.4.6.2. Lipids were vortexed before being syringe filtered (0.45µm) and injected to the HPLC. The HPLC conditions were same to those in section 3.1.13.

2.4.6.5. Zebrafish Retinol Analysis

The analysis and detection of all trans retinol in the whole zebrafish for test trials and controls was based on Li et al. (2005) with slight modifications. Briefly, nitrogen-dried lipids obtained from section 3.4.6.2 were redissolved in 1 mL acetone and vortexed. Extracts were then vigorously mixed with an equivalent volume of methyl-tetra-butyl ether (MtBE) and 0.5 mL distilled water. The aqueous phase was extracted further three times using the MtBE. The combined MtBE phases were evaporated to dryness under a gentle stream of nitrogen, suspended with 0.5 mL MtBE, vortexed and passed through a PTFE membrane filter (0.45µm). The extracts were sealed under nitrogen in brown glass vials and stored in the dark at -80°C until further analysis. Retinol analysis was carried out using an Agilent 1100 HPLC equipped with a diode array UV detector. Separation was achieved with a Gemini-NX C18 column (250 mm X 4.6 mm, 5 µm) coupled with a C18 guard column, both purchased from Phenomenex (UK). Absorption spectra were recorded at 325 nm for retinol. The mobile phase consisted of two components: (A) methanol and (B) MtBE. The solvents program was initially 90% A from 0-12 min, followed by linear gradients of 90-60%A from 12-13 min, maintained until 22 min followed by linear gradient back to 90% A initial conditions until end of run at 30 min.

The flow rate was 0.8 mL min⁻¹. Column conditions were maintained at ambient temperature. The injection volume was 10 µL and retinol quantified using external standards.

2.4.6.6. Analysis of Carotenoids of Whole Zebrafish and Its Eggs

Carotenoids were extracted as section 3.4.6.6, however the detection program of the HPLC was different and was based on Garner et al. (2010). Briefly, two mobile phases: (A) acetonitrile, methanol, 0.1M Tris-HCl pH=8 (7:1:1) and (B) methanol, hexane (4:1) were prepared. Separation began by injection of 10 µL of sample, followed by 4 min of 100% solvent A, 2.5 min linear gradient and 16 min at 100 solvent A to re-equilibrate the column before loading the next sample. The flow rate was 1 mL min⁻¹ with all solvents used being HPLC grade and the wavelength at 480 and 450 nm.

2.5. Statistical Analysis

Experiments were performed in triplicate. i.e. three separate samples for each experiment and the statistical analysis was carried out using the Minitab V. 17 statistical package (Minitab Inc., PA, USA) using post-hoc analysis of variance (ANOVA) and according to Fishers' test with statistical significance at $p \leq 0.05$. It should be noted here that in zebrafish feeding trials, i.e. experiments done during aquaculture of the zebrafish, 5 biological replicates were used for each

of the experimental and control diets. However, during post-trial (gravimetical and chromatographic) analysis three separate analytical replicates were performed instead of the biological replicates as biological zebrafish/egg replicates were combined to achieve an extractable amount of fish/egg sample for the subsequent gravimetical and chromatographic measurements.

Chapter 3

The Nutrient Composition of Chloroplasts from Range of Green Leaf Tissues

3.1. Introduction

Green leaf material is recognized as a good source of many valuable nutrients including vitamin E, β -carotene (pro-vitamin A), lutein and vitamin C. In addition, green leaf materials contains chlorophylls, lipids and fatty acids (α -linoleic acid), as well as proteins and their amino acids. Chloroplasts are the site where most of these essential nutrients are synthesized and stored. Chloroplast-rich-fractions (CRFs) were prepared from spinach (*Spinacia oleracea*), kale (*Brassica oleracea* var *acephala*), stinging nettles (*Paspalum notatum*) and grass (*Urtica dioica*), and the macro- and micro-nutrient composition compared with the parent whole leaf materials (WLMs) on a dry weight basis.

3.2. Sucrose and Moisture Content

The percentage of residual moisture and sucrose in the freeze dried CRFs and WLMs samples were determined. The moisture contents of the raw materials (i.e. WLMs) before the CRFs were prepared were $94\pm0.2\%$, $82\pm0.5\%$, $77\pm0.8\%$, and $80\pm1.2\%$, whereas, that for the CRFs were $79\pm0.3\%$,

82±0.4%, 70±0.7% and 72±0.5%, respectively for spinach, kale, stinging nettles and grass. The concentration of sucrose used for the improvement of CRFs recovery was also calculated from the HPLC data. The sucrose content of the CRFs ranged between 30 % (kale) and 37 % (stinging nettles). Moisture and sucrose values were taken into account when expressing nutrient concentration values relative to total leaf dry solids (non-sucrose CRF DW.).

3.3. Proximate Protein, Lipid, Carbohydrate and Ash Content

Protein, lipid, carbohydrate (CHO) and ash contents, as well as, chlorophyll per 100 parts of protein (will be explained later) from freeze dried CRFs and their parent WLMs from a range of green tissues were characterised and are presented in Table (3.1). The results show that CRFs contained more lipid and protein than the WLMs with lower CHO content except for CRF of stinging nettles, which did not follow the trend of the other three plant species and, in turn, showed lower protein and high CHO content. Lower ash content in CRFs compared to WLMs was also observed.

Among the selected green materials i.e. both the CRFs and WLMs, spinach and kale showed similar values in terms of lipid and protein content (Table 3.1). Although protein content for CRFs of nettles (18.3 %) was lower than grass CRFs, nettles and grass results were roughly similar for the rest of the parameters shown in Table (3.1). There is a scarcity of published data in the literature for the proximate composition of total leaves and chloroplasts from these selected green vegetables, except for spinach, which, as a result, was

used as reference for analytical purposes. Care must be taken on making these comparisons since our material (CRF) is not pure chloroplasts. A previous study (Singh et al., 2001) reported protein content of spinach (26.5 %) on dry weight (DW) that is lower than our current value, However, Kahlon et al. (2007) reported a spinach protein content of 38.2% D.W, showing closer agreement with this study (Table.3.1). Similarly Kuti and Kuti (1999) analysed spinach leaves, and reported protein content of 3.2 % on a fresh weight (FW) basis, which would correspond to 53.3 % (DW); if the moisture content is assumed to be similar (94 %) to the spinach in this study (Table 3.1). In addition, protein content in the WLMs of grass (23 %) is similar to that of Jancik et al. (2008) (21.1 %).

Table 3.1. Proximate composition of CRFs and WLMs from selected green leaves

	Non-sucrose CRF (% DW)				WLM (% DW)			
	Spinach	Kale	Nettles	Grass	Spinach	Kale	Nettles	Grass
Protein	42.6±0.1 ^{AB}	44.1±2.7 ^A	18.3±1.1 ^G	30.2±0.2 ^{DE}	35.3±4.5 ^{CD}	36.6±4.8 ^{BC}	28.0±0.3 ^{EF}	23.1±1.2 ^{FG}
Lipid	36.9±1.3 ^A	36.1±1.7 ^A	29.8±1.5 ^B	30.4±2.7 ^B	19.3±1.6 ^C	17.0±0.6 ^C	13.4±0.4 ^D	12.5±0.3 ^D
Ash	4.1±0.0 ^D	2.4±0.0 ^{DE}	2.1±0.0 ^E	1.8±0.0 ^E	18.8±0.2 ^A	14.5±0.1 ^B	16.4±0.0 ^B	8.8±2.5 ^C
Carbohydrate	16.4	17.4	49.8	37.6	26.6	31.9	42.2	55.6
Chlorophyll	7.4±2.7 ^A	5.4±0.7 ^{AB}	5.7±1.3 ^{AB}	4.8±1.2 ^B	0.8±0.2 ^C	0.8±0.1 ^C	1.0±0.1 ^C	1.1±0.3 ^C
ChI PHP	17.3	12.2	19.1	15.9	2.3	2.2	3.6	4.8

Abbreviations: ChI PHP: chlorophyll per 100 parts of protein; CRF: Chloroplast rich fractions; WLM: whole leaf material
 DW: dry weight. Chlorophyll is considered part of the lipid thus it is not included in the total sum (100) used to calculate carbohydrates by difference (100- [protein + lipid + ash]). Values with the same superscripts for each row are not significantly different at P>0.05.

Proteins in photo-synthetic tissues are closely associated with chlorophylls (Smith, 1941). The chlorophyll-protein ratio for spinach CRFs (Table 3.1) is 17.3 parts of chlorophyll per 100 parts of protein, which is in agreement with that (16.3 parts of chlorophyll per 100 parts of protein in spinach chloroplasts) found by Smith (1941). The chlorophyll-protein relationship for the rest of green materials is as displayed in Table (3.1)

Lipid (including lipid soluble pigments such chlorophylls, carotenoids, tocopherols etc.) content in the green materials of this study (DW), ranged between 29.8-36.9 %, and 12.5-19.3 % respectively for CRFs and WLMs (Table 3.1).

Around 10 % of the dry weight of green leaves of higher plants is represented by lipids, where they are found concentrated in the membranous organelles, particularly in the plastids (Walker and Barber, 1976). Lipids also make up 35 % of chloroplast dry weight, and 50 % of the photosynthetic thylakoid membranes (Myers and Graham, 1956). These findings are consistent with those reported in Table (3.1) for lipid content.

Although quite high CHO content was found for certain CRFs and WLMs (Table 3.1), in most cases CHO contents decreased after the chloroplast recovery process. As were hypothetically expected, CRFs contained lower CHO than WLMs as their cell wall was minimised during CRFs isolation. However, CRFs were crude chloroplasts instead of pure and hence the term 'CRFs'. In light of this, some of these fractions (like nettles and grass) were found to contain a white layer that looks like starch under polarised light (appendix A). The

estimated CHO value (42 %) for WLFs of nettles (for example) in Table (3.1) is slightly higher than that reported by Adhikari et al. (2015) (37.4 %), with very similar ash content (16.2 %) as found in this study (16.4 %). Further, our spinach WLM CHO content (26.6 %) is in agreement with that of Kahlon et al. (2007) who found a value of 29 % on a DW basis, suggesting that these estimations are roughly within the normal range of CHO content in nettles and spinach.

3.4. Chlorophyll and Total Carotenoid Content

Total chlorophyll and carotenoid concentrations of freeze dried CRFs and WLMs are shown in Table (3.2). Spinach CRFs chlorophyll content (73.86 mg g⁻¹) corresponding to 7.4 % is in line with the value (7.9 %) found by Smith (1941). The total carotenoid content (xanthophylls and carotenes) for CRFs presented in Table (3.2) is about 15 mg g⁻¹. Of this, β -carotene, a provitamin A carotenoid, accounted for 22 %; this agrees with the findings of Konishi et al. (1968) that β -carotene was the second most concentrated carotenoid (29 %) after lutein (31 %) in spinach chloroplasts.

Table 3.2. Chlorophyll and carotenoid levels and pigment ratios from CRFs and WLMs

Plant	Non-sucrose CRF (mg g ⁻¹ DW)				WLM (mg g ⁻¹ DW)			
	<i>a+b</i>	<i>x+c</i>	<i>a/b</i>	<i>a+b/x+c</i>	<i>a+b</i>	<i>x+c</i>	<i>a/b</i>	<i>a+b/x+c</i>
Spinach	73.8±27.0	14.7±4.6	2.2	5.02	7.8±1.6	2.2±0.2	3.2	3.5
Kale	54.0±7.1	7.3±1.2	3.0	7.39	8.2±1.0	1.7±0.3	3.0	4.8
Nettles	57.2±13.0	9.3±0.3	2.2	4.22	10.0±1.4	2.0±0.2	3.2	5.0
Grass	48.1±12.0	7.4±1.4	2.1	6.5	10.9±3.1	1.1±0.1	1.1	9.9

Abbreviations: *a + b*; total chlorophylls *a* and *b*; *x + c*, total carotenoids (xanthophylls and carotenes), CRF: Chloroplast rich fractions; WLM: whole leaf material; DW: dry weight.

The weight ratio of chlorophylls to total carotenoids ($a+b/x+c$) is an indicator of the greenness of plants. This ratio ranges between 4.2 and 5 in sun leaves and sun-exposed plants, and between 5.5 and 7.0 in shade leaves and shade-exposed plants. Lower values for the ratio ($a+b/x+c$) indicates senescence, stress, and damage to the plant and the photosynthetic apparatus (Lichtenthaler and Buschmann, 2001). Smith (1941) suggested that properties of green leaf pigments are best described in terms of a true stoichiometric arrangement of chlorophyll with protein. For this, chloroplasts from higher plants were found to contain 400 chlorophyll molecules, and roughly 83 molecules of carotenoids (that is a ratio of about 5:1 [chlorophylls : carotenoids]) (Gross, 1991). Therefore, chlorophyll per 100 parts of protein (discussed in protein section) of Table (3.1), as well as mg chlorophyll per mg compound was calculated (Table 3.4). A measure of chlorophyll concentration provides an indirect indication of the concentration of chloroplasts in the sample of interest; measuring nutrient concentration as a function of chlorophyll content also allows one to gauge the concentration of candidate

nutrients relative to the actual chloroplast material instead of the total dry matter.

3.5. Amino Acid Composition

The amino acid profiles after hydrolysis of freeze dried CRFs and WLMs are shown in Fig. (3.1). In general, higher glycine and leucine levels were observed in CRFs, whilst glutamate and glycine were the dominant amino acids in WLMs (Fig. 3.1) and tryptophan showed the lowest level (traces) in both CRFs and WLMs. Freeze-dried spinach leaves could be used as a representative example of amino acid distribution in these materials for analytical purpose. Amino acid profile in freeze dried spinach WLMs was in good agreement with that of Eppendorfer and Bille (1996). For instance, the values of glutamate and glycine (238 and $199\ \mu\text{mol g}^{-1}$) in this study and those values reported by Eppendorfer and Bille (1996) for glutamate and glycine (292 and $195\ \mu\text{mol g}^{-1}$) were similar and both of the amino acids were the dominant ones in both findings.

Whilst our body can produce some amino acids (alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine and tyrosine), for some others, we are dependent on external sources for the so-called "essential" amino acids (methionine, threonine, glutamate, valine, Leucine, isoleucine, phenylalanine, lysine, tryptophan, histidine and arginine); this is because humans lack the enzymes required for the biosynthesis of these

amino acids. Some non-essential amino acids like tyrosine becomes essential if its precursor (phenylalanine) is deficient in the diet. The essentiality itself is dependent on the age group, for instance, arginine is essential for young but not for adults (Tucker and Dauffenbach, 2010). The failure to obtain an adequate amount of even a single amino acid out of the 10 essential amino acids has serious health implications and could lead to the degradation of the body's proteins. Unlike carbohydrates and fats, excess amino acids are not stored by the body for later use, consequently the amino acids must be in the food every day.

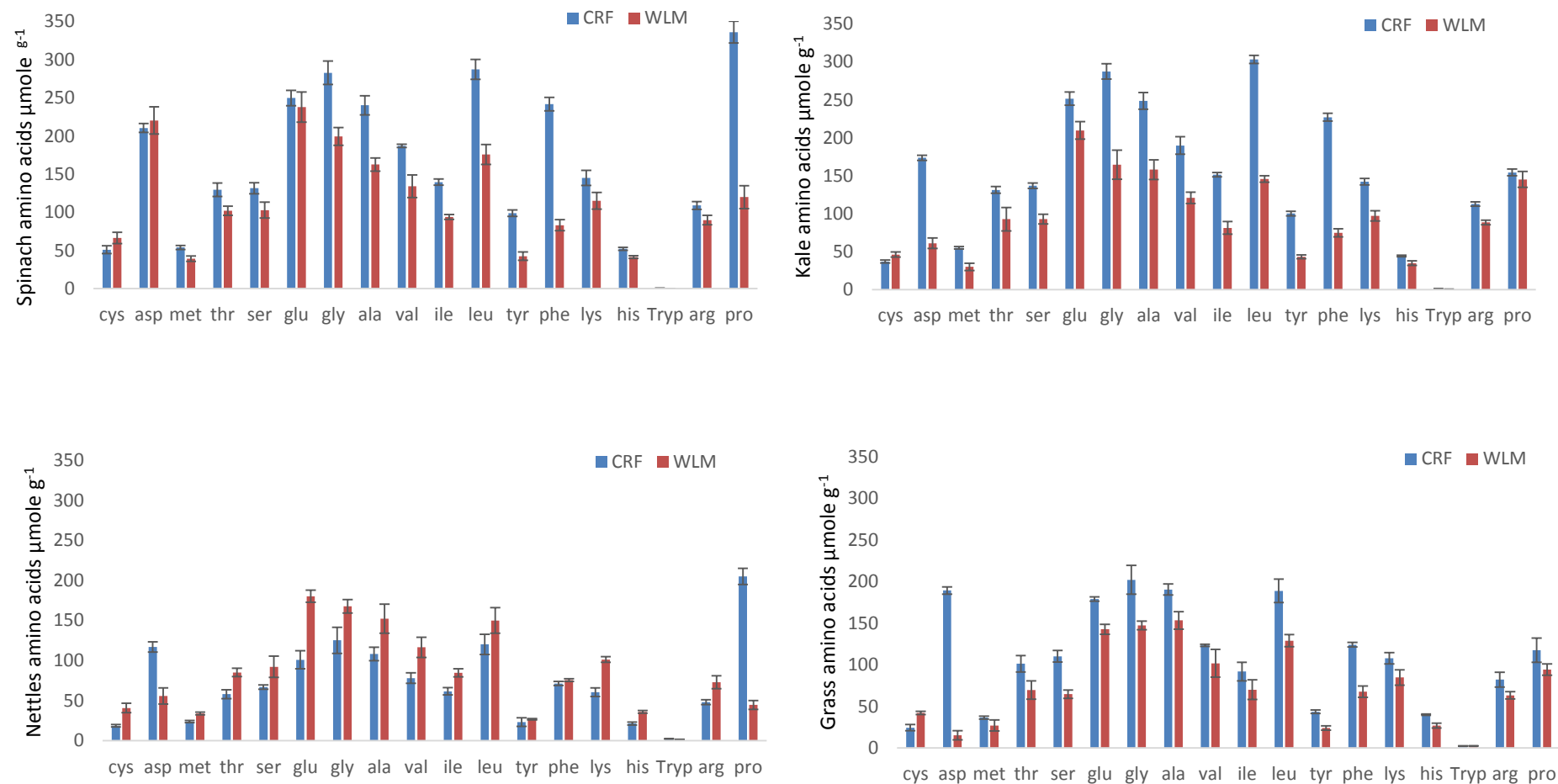


Figure 3.1 Amino acid profile of non-sucrose freeze dried CRFs and WLM from spinach, kale, stinging nettles and grass; Cys: Cysteine; Asp: aspartic acid (aspartate); **Met: Methionine**; **Thr: Threonine**; Ser: Serine; Glu: Glutamic acid (glutamate); Gly: Glycine; Ala: Alanine; **Val: Valine**; **Ile: Isoleucine**; **Leu: Leucine**; Tyr: Tyrosine; **Phe: Phenylalanine**; **Lys: lysine**; **His: Histidine**; **Try: Tryptophan**; **Arg: Arginine**; Pro: Proline. With bold are essential amino acids.

Total amino acid content of spinach and kale adapted from Eppendorfer and Bille (1996) is presented in Table 3.3. Amino acids in both studies were in close agreement e.g. spinach glycine (195.7 versus 199.3) and valine (134.6 versus 134.1). Further, amino acid content in spinach and kale in the current study in comparison with reference amino acid sources such as soybean and casein was also provided. It should be noted here that soybean seeds reported were cooked, therefore, dry weight basis of its amino acid composition would be higher than the values shown in this Table.

Table 3.3. Total amino acid profile of spinach and kale as representative of green leaf tissues (literature versus experimental data). Data is expressed in $\mu\text{mole g}^{-1}$.

Amino acid	Eppendorfer		Silva Soybean grain*	Hall Casein (powder)	This study	
	Spinach	Kale			Spinach	Kale
Lysine	110.7	88.4	150	567.7	115.1	97.1
Histidine	37.0	32.6	66	206.2	41.2	34.8
Arginine	87.8	80.6	128	218.1	89.7	88.5
Aspartic acid	179.6	150.6	277	548.5	220.4	61.0
Threonine	95.9	86.6	117	386.2	102.0	92.7
Serine	110.7	99.9	180	599.5	102.8	92.9
Glutamic acid	292.0	333.4	353	1515.7	238.0	209.8
Proline	106.6	167.3	153	912.0	119.8	145.2
Glycine	195.7	150.7	200	253.1	199.3	164.6
Alanine	173.5	143.5	176	347.9	162.5	158.1
Valine	134.6	102.9	103	631.6	134.1	120.9
Isoleucine	87.7	66.6	89	442.2	93.8	81.3
Leucine	159.7	124.6	176	769.9	175.8	145.8
Tyrosine	56.2	45.5	67	320.2	42.4	43.1
Phenylalanine	78.4	66.3	103	326.9	83.1	74.8

Adapted from Eppendorfer and Bille (1996).

Silva et al. (2009)

Hall et al. (2003)

* cooked

3.6. Fatty Acid Composition

Fatty acid composition of the CRFs and WLMs are presented in Fig. (3.2). Of the selected green materials, grass showed the highest α -linolenic (ALA) acid content (69.5% and 44.4 mg g⁻¹ DW, respectively for CRFs and WLMs), followed by spinach (57.8% and 32.5 mg g⁻¹ DW), kale (50.2% and 23.6 mg g⁻¹ DW) and nettles (33.7 and 31.8 mg g⁻¹ DW).

As a result of the lower amounts of ALA in nettles and kale, they displayed higher proportions of linoleic (C18:2) (12.6 and 19.2 mg g⁻¹ DW) and palmitic acids (C16:0) (13.6 and 21.9 mg g⁻¹ DW) compared to linoleic acid of spinach CRF (5.8 mg g⁻¹ DW) and grass CRF (13.7 mg g⁻¹ DW) both showing lower linoleic than nettles and kale. By weight percentage, the spinach fatty acid profile in this study is in agreement with that reported by Wolf et al. (1962).

Fatty acid biosynthesis in higher plants proceeds from *de novo* production of palmitate followed by addition of C2 units in conjunction with monenoic and polyenoic desaturation, particularly when the plant is very young (Poincelot, 1976).

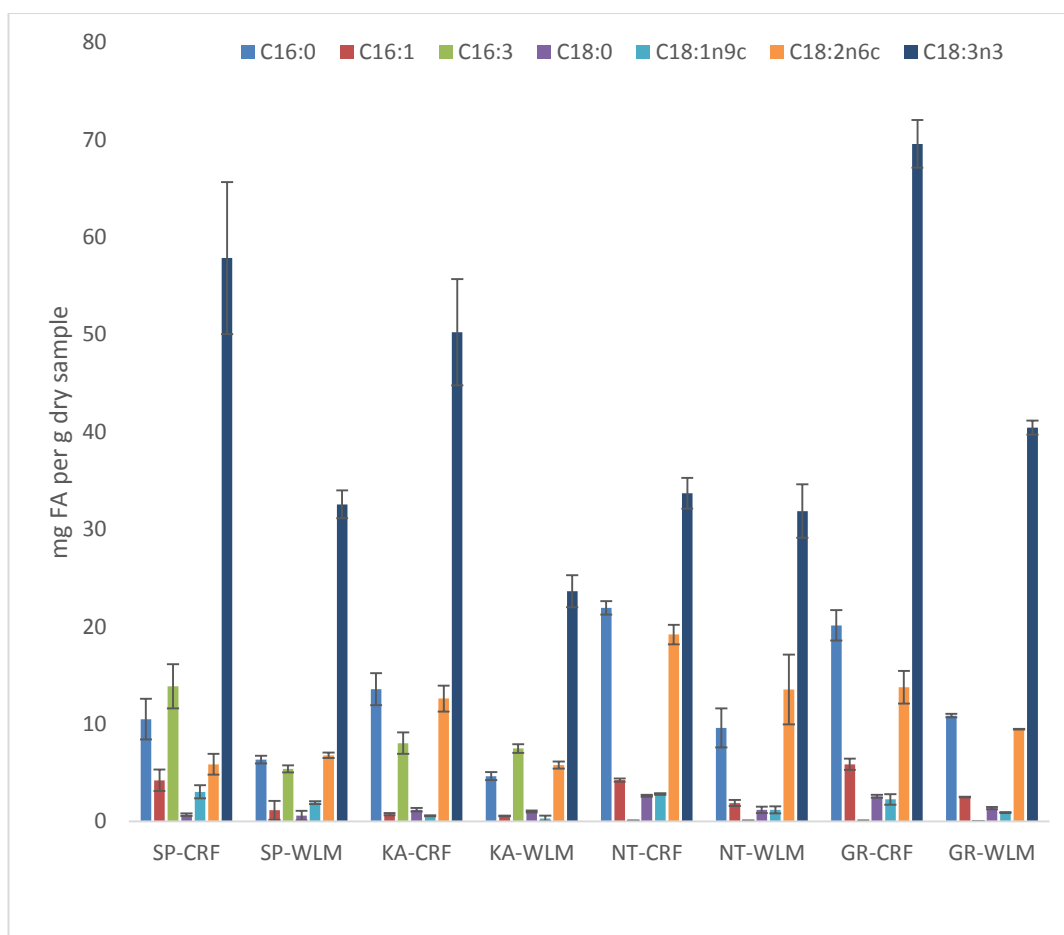


Figure 3.2. Fatty acid composition of CRFs and WLMs from four green plants; CRFs: chloroplasts rich fractions; WLMs: whole leaf materials refer; SP: spinach; Ka: Kale; NT: nettles (stinging); GR: grass.

On a dry mass basis, CRFs were significantly higher in fatty acids (mainly ALA) compared with WLMs except for nettles (see Fig.3.2).

Interestingly, CRFs from grass exhibited high and comparable values of ALA to those of conventional green leaves (spinach and kale). In this sense, ingredients with potential nutritional value could be obtained in the form of chloroplasts from unconventional sources such as grass and nettles, for human feed. ALA is an essential fatty acid (omega 3) that mammals, humans included, cannot produce *de novo* and is precursor of EPA and DHA. Studies suggested that an increase of ALA consumption elevates tissue EPA (eicosapentaenoic

acid) and, in some cases, DHA (docosahexaenoic acid) content (Barceló-Coblijn and Murphy, 2009).

3.7. Vitamin E (α -tocopherol)

HPLC quantification of vitamin E, expressed as α -tocopherol that was extracted from freeze dried CRFs and WLFs is presented in Table (3.4). Out of four different standards analysed for vitamin E (α , β , γ and δ - tocopherols), α -tocopherol was the only component detected in quantifiable amounts; others were either trace or not detected at all. Since α –tocopherol is the major form of vitamin E existing in nature, and the most biologically active (Bjorneboe et al., 1990), only α -tocopherol data was considered in this study. Among the analysed material, spinach CRFs (Table 3.4) exhibited the highest α -tocopherol (62 mg 100 g⁻¹ dry weight (DW)), followed by nettles; with kale and grass showing the lowest α -tocopherol content. In contrast, for the WLM, nettles exhibited higher α -tocopherol values than spinach (Table 3.4). It should be noted, though, that α -tocopherol content in spinach CRFs and nettles was not significantly different ($p \geq 0.05$). Previous studies on vitamin E content of various vegetables and fruits reported slightly higher values of α -tocopherol in nettles than in spinach (Piironen et al., 1986). Overall all CRFs contained significantly higher α -tocopherol concentrations than the parent WLMs (Table 3-4).

3.8. Pro-vitamin A (β -carotene) and Lutein

The content of β -carotene and lutein in different CRFs and their WLMs is presented in Table (3.4). The data showed that both β -carotene and lutein were highly concentrated in the CRFs compared with the WLMs, and β -carotene ranged from 247-336 and 67-87 mg 100g⁻¹ DW, for the CRF and WLM, respectively, whilst lutein concentration ranged from 214-341 and 75-100 mg 100g⁻¹ DW in the CRF and WLM material, respectively. The highest β -carotene and lutein contents was recorded in spinach CRFs (336 and 341 mg 100 g⁻¹ DW), whilst nettles had the highest β -carotene and lutein in the WLMs (87 and 100 mg 100 g⁻¹ DW). As with α -tocopherol data, no significant difference ($p>0.05$) between spinach and nettles CRFs for β -carotene content was apparent (Table 3.4). CRFs of kale and grass, with significantly lower ($p<0.05$) β -carotene content than spinach and nettles, exhibited comparable ($p>0.05$) β -carotene values as shown in Table (3.4).

Table 3.4. Alpha-Tocopherol, β -carotene lutein and ascorbic acid (AA) content of freeze dried CRFs and WLMs from selected leaf tissues

Species	mg 100 g ⁻¹ DW non-sucrose				mg Chlorophyll per mg nutrient			
	α -Toc	β -car	Lutein	AA	α -Toc	β -car	Lutein	AA
CRFs								
Spinach	62 $\pm$ 2 ^A	336 $\pm$ 6.1 ^A	341 $\pm$ 12.7 ^A	81 $\pm$ 20 ^C	119.2	22.0	21.6	91.2
Kale	22 $\pm$ 5 ^B	247 $\pm$ 6.3 ^B	214 $\pm$ 2.8 ^C	118 $\pm$ 22 ^C	245.5	21.9	25.2	25.8
Nettles	51 $\pm$ 8 ^A	327 $\pm$ 1.9 ^A	278 $\pm$ 8.0 ^B	112 $\pm$ 33 ^C	112.2	17.5	20.5	51.1
Grass	12 $\pm$ 1 ^{BC}	255 $\pm$ 6.3 ^B	267 $\pm$ 3.8 ^B	121 $\pm$ 23 ^C	400.8	18.9	18.0	39.8
WLFs								
Spinach	10 $\pm$ 1.2 ^{BC}	76 $\pm$ 8.5 ^C	87 $\pm$ 8.5 ^{DE}	472 $\pm$ 127 ^B	78.0	10.2	9.1	1.7
Kale	9 $\pm$ 0.3 ^C	77 $\pm$ 5.1 ^C	85 $\pm$ 5.0 ^{DE}	439 $\pm$ 42 ^B	91.1	10.6	9.6	1.9
Nettles	14 $\pm$ 0.1 ^{BC}	87 $\pm$ 14 ^C	100 $\pm$ 0.7 ^D	214 $\pm$ 37 ^C	71.4	11.5	10.0	4.7
Grass	06 $\pm$ 0.1 ^C	67 $\pm$ 6.2 ^C	75.8 $\pm$ 8 ^E	684 $\pm$ 36 ^A	181.7	16.3	14.4	1.6

Data were the mean $\pm$ SD of 3 separate experiments. Data was analysed by one-way ANOVA using Fisher method to assess differences of the samples. Values with the same superscripts for each column are not significantly different at $P>0.05$; DW: dry weight; α -Toc: α -tocopherol, β -car: β -carotene, AA: ascorbic acid; for CRFs and WLM: refer to Table 3.1.

The β -carotene values in spinach (WLMs) when calculated in terms of FW was 4.59 mg 100 g⁻¹; this is in agreement with the values of 4.65, 5.6 and 3.4 mg 100 g⁻¹ (FW) reported by Huck et al. (2000), Holden et al. (1999) and Hart and Scott (1995) respectively for β -carotene. Furthermore, the value 4.59 mg 100 g⁻¹ FW (8707 IU), was in line with that of 5.6 mg 100 g⁻¹ FW (9377 IU) by the USDA National Nutrient Database for Standard Reference Release 27, for raw spinach. In addition, β -carotene content in the leaves of kale (77 mg 100 g⁻¹ DW) was in close agreement to the β -carotene content in kale (Lefsrud et al., 2007).

The lutein content of spinach WLM in this study (87 mg 100 g⁻¹ (DW) corresponds to 5.2 mg 100 g⁻¹ FW; this compares with 9.1, 5.8 and 3.9 mg

100 g⁻¹ of fresh spinach leaf reported by the following authors respectively: Bergquist et al. (2006); Hart and Scott (1995); and Tee Siong and Lam (1992). It should be noted, though that Bergquist et al. (2006) found this value from spinach obtained from the farm right before the analysis and, hence, fresh compared to the rest of the literature or this study, which used spinach from the super market.

During the entire procedure, namely isolation of CRFs and preparation of WLMs, β -carotene extraction, and chromatographic analysis, precautions were taken to minimise the exposure of samples to light and air to preserve the pigments (the carotenoids) which are highly degradable compounds if exposed to light and oxygen.

Besides β -carotene and lutein, other unidentified carotenoids supposed (according to the literature) to be mainly violaxanthin and neoxanthin were detected (see appendix B4-B5). Bergquist et al. (2006) reported that baby spinach leaves contained four carotenoids namely, lutein, violaxanthin, β -carotene and neoxanthin; lutein being the major (39 %) among the four (See appendices A1-A4 for carotenoid identification). Previous studies on carotenoids also revealed these four as the major carotenoid constituents in the chloroplasts of higher plants, and also reported two other minor xanthophyll components designated as xanthophyll-443 and 439 (Konishi et al., 1968). Since carotenoids and tocopherols are both lipid-soluble molecules synthesised in chloroplasts (DellaPenna and Pogson, 2006), it is reasonable that CRFs showed higher α -tocopherol and β -carotene values than WLMs (Table 3.4).

Remarkable reference nutrient intake (RNI) contribution of vitamin A by CRFs that is three times greater than WLMs was found in this study. The RNI contributions per g freeze dried CRFs and WLMs of β -carotene expressed as retinol equivalents (RE) ranged between 63-86 % and 19-25 %, respectively. Intakes of vitamin A are normally expressed in terms of retinol equivalents (RE), the biological activity associated with one μg of all-trans retinol. Despite ongoing discussions in the literature for carotene conversion rates, 6 μg of all-trans β -carotene or 12 μg of other provitamin A carotenoids, have been retained as the conversion values equivalent to 1 RE (FAO and WHO, 2005).

Lutein does not possess provitamin A activity, but lutein and its coexisting isomer, zeaxanthin are reported to be very important for eye health. Studies suggest that lutein and zeaxanthin play a vital role in the reduction of risk of macular degeneration and cataracts; their concentration in the macula lutea is believed to protect from blue light to reach the underlying structures in the retina and their antioxidant potential is well recognized (Abdel-Aal et al., 2013, Fullmer and Shao, 2001, Kris-Etherton et al., 2002, Roberts et al., 2009, Semba and Dagnelie, 2003).

3.9. Vitamin C (Ascorbic Acid)

Data presented in Table (3.4) shows that ascorbic acid content in the CRFs of the green leaf tissues ranged between 81 and 121 mg 100 g⁻¹ DW, whereas that of WLMs was between 214 and 680 mg 100 g⁻¹ DW (Table 3.4). Unlike α -

tocopherol and β -carotene (which were higher in CRFs), ascorbic acid followed a different trend and was higher in the WLMs. As overall chlorophyll (mg) per ascorbic acid (mg) in CRF samples is increased, the ascorbic acid content is decreased; in contrast when overall chlorophyll per mg of ascorbic acid for WLMs is low, high ascorbic acid content is shown (Table 3.4). Horemans et al. (2000) reported that ascorbate accumulation in chloroplasts ranged between 12 to 30 % of the total ascorbate composition of the leaves, and the rest was distributed to other organelles, such as vacuole (20-30 %) and cytosol (30-40 %).

Spinach WLMs ascorbic acid composition (472 mg 100 g⁻¹ DW) reported in Table (3.4), corresponds to 283.2 mg kg⁻¹ (FW) and is in the range that Bergquist et al. (2006) reported (140-460 mg kg⁻¹ (FW), although spinach was freshly harvested from the farm in Bergquist's study, whereas, it was only fresh from supermarket in this study. In general, stability is an important concern in ascorbic acid analysis as it is hardly stable in aqueous solutions due to a number of factors that negatively affect ascorbic acid stability such as light, increased pH, temperatures and the occurrence of oxygen or metal ions (Nováková et al., 2008). As such, minimising negative consequences of these factors and analysing samples without delay may prevent from ascorbic acid losses.

3.10. Mineral Composition

The mineral content of CRFs and WLMs (Table 3.5) of four green leafy tissues was examined. The mineral matrix of these materials is predominantly comprised of K, Ca, P, Na and Mg. The materials also consisted of substantial amounts of iron (Fe), associated with trace occurrence of Mn and Zn. Mg is generally significant in all green vegetables as a result of its association with chlorophylls, however, the abundance of K, Ca, P and Na indicate the mineral-rich nature of these materials. Similar findings of the abundance of these minerals in spinach leaves and stems were previously reported by Bhattacharjee et al (1998). The data of spinach total leaf minerals presented in Table (3.5) is consistent with that of Lisiewska et al. (2009). For instance, spinach WLMs values ($\text{mg } 100 \text{ g}^{-1} \text{ DW}$) of K (1195.6), Ca (676.8), P (298.2), Fe (31.4) and Cu (2.0) in Table (3.5) corresponding to 802.8, 146.5, 54.1, 1.0 and $0.1 \text{ mg } 100 \text{ g}^{-1} \text{ (FW)}$, respectively, was comparable to the raw spinach values (596.0, 163.3, 66.8, 1.58 and $0.1 \text{ mg } 100 \text{ g}^{-1} \text{ (FW)}$, respectively for K, Ca, P, Fe and Cu) reported by Lisiewska et al. (2009).

Major minerals such as K, Ca, P and Mg mostly showed decreased values in CRFs compared to their corresponding WLMs (Table 3.5). This could be further supported by the higher ash content in WLMs compared to CRFs (Table 3.1) as major minerals account for the highest proportion in the studied green tissues, which in turn, contributed to their masses gravimetrically after ashing. Interestingly, Na concentration in WLM of spinach $644 \text{ mg } 100 \text{ mg}^{-1}$ was reduced to around $21 \text{ mg } 100 \text{ mg}^{-1}$ (30 times reduction) after CRF recovery

from spinach. In contrast to this, values of trace minerals such as Fe and Mn increased in almost all samples of CRFs (e.g. 31.4 and 7.7 mg 100 g⁻¹ DW, respectively for Fe and Mn of spinach CRF) as compared to those of WLMs (e.g. 16.0 and 5.1 mg 100 g⁻¹ DW, respectively for Fe and Mn of spinach WLM); see Table (3.5). Hence, this data may suggest that chloroplasts are enriched in these trace minerals, and thus CRFs may be a better source of Fe and Mn than WLM

Table 3.5. Mineral content (mg 100 g⁻¹ of freeze dried WLMs and CRFs from selected leaf tissues

	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
CRFs									
Spinach	21±5 ^C	337±24 ^{CD}	298±42 ^D	1196±68 ^D	677±15 ^{DE}	7.7±0.6 ^{BC}	31.4±3.1 ^{AB}	2.0±0.1 ^A	3.6±0.5 ^B
Kale	39±11 ^{BC}	322±17 ^{DE}	628±111 ^{BC}	518±95 ^E	920±30 ^D	6.5±0.7 ^C	25.7±9.1 ^{AB}	0.5±0.1 ^C	3.6±0.2 ^B
Nettles	63±2 ^{BC}	396±19 ^{BC}	370±54 ^D	872±64 ^{DE}	1736±11 ^C	7.9±0.6 ^{BC}	42.1±12 ^A	1.0 ^B ±0.1 ^{BC}	3.6±0.2 ^B
Grass	44±2 ^{BC}	232±28 ^F	436±94 ^{CD}	1195±234 ^D	354±4 ^F	12.0±3.4 ^{BC}	37.0±0.6 ^{AB}	1.1±0.4 ^C	4.0±0.7 ^B
WLFs									
Spinach	644 ±11 ^A	398±3 ^B	874±49 ^A	12949±403 ^A	2364±86 ^B	5.1±0.1 ^C	16.0±0.4 ^B	1.7±0.0 ^{AB}	14.9±0.6 ^A
Kale	9 ±37 ^B	372±11 ^{BCD}	756±63 ^{AB}	4169±95 ^B	2601±63 ^B	2.5±0.0 ^C	12.2±0.1 ^B	0.5±0.0 ^C	2.4±0.0 ^B
Nettles	21±11 ^C	737±30 ^A	416±47 ^{AB}	2947±73 ^C	6249±188 ^A	14.1±0.5 ^B	21.1±0.5 ^{AB}	1.0±0.0 ^{BC}	3.1 ±0.2 ^B
Grass	32±25 ^{BC}	244±31 ^{EF}	615±22 ^{BC}	4383±417 ^B	523±90 ^{EF}	32.8±2.9 ^A	12.1±3.1 ^B	1.2±0.1 ^{BC}	3.8±0.5 ^B

Data were the mean ± SD of 3 separate experiments. Element data displayed is in mg per 100 g DW. Data was analysed by one-way ANOVA using Fisher method to assess mineral difference of the samples. Values with the same superscripts for each column are not significantly different at P>0.05; DW: dry weight; for CRFs and WLM: refer to Table 3.1.

3.11. Conclusion

This chapter accomplished characterisation of certain macro and micro-nutrients in isolated chloroplast rich fractions and their parent whole leaf materials. There is very limited data on nutritional composition, notably, on vitamins and minerals from chloroplasts. The results obtained for the WLMs and CRFs (when available) generally fall within the normal ranges of data reported in the literature for whole leaves. Total proteins and lipids were significantly ($p \leq 0.05$) greater (in most cases) in CRFs compared to WLMs. Compounds such as, α -tocopherol, β -carotene, lutein and iron, as well as total carotenoids were all present in greater concentrations in CRFs than they were in the WLMs. CRFs with high nutritional value were recovered from unconventional green plants (stinging nettles and grass). Excessive amounts of other green materials rich in those nutrients are also routinely produced as a waste globally. The high values of fatty acids (mainly the ω -3 α -linolenic acid), provitamin A, lutein and minerals in these green tissues have the potential to be used as a functional food ingredients that could benefit those with a sub-optimal intake of these nutrients e.g. the poor in developing countries, provided that safety concerns of the unconventional ingredients are resolved. They could also be used as alternative feed source for fish. The bioaccessibility and *in vitro* / *in vivo* digestibility of these materials are further studied in the subsequent chapters.

Chapter 4

Material Digestibility and *In Vitro* Bioaccessibility of Provitamin A (β -Carotene) and Lutein

4.1. Introduction

Green leaf materials are potentially beneficial to human health for their recognized nutrients; however, the bioaccessibility of nutrients seems to be hindered by the presence of cell walls. Therefore the digestibility of CRFs from spinach (prepared using different recovery techniques; either with sucrose or by juicing) was compared with the spinach WLM. A model that simulated *in vitro* oral conditions (simulated salivary fluid (SSF) and salivary amylase pH=7), stomach conditions (simulated gastric fluid (SGF) and pepsin pH=3) and small intestine conditions (simulated intestinal fluid (SIF) and pancreatin with bile pH=7) was used to compare digestibility. Micellar fractions of each digestate were collected at intervals, via centrifugation. Consequently β -carotene and lutein contents were quantified chromatographically.

4.2. Material Ultra-Structures

Before bioaccessibility tests are carried out, the digestibility and integrity of the chloroplasts in CRF and WLM materials were examined. Integrity was determined by visualising any morphological changes using light and transmission electron microscopes (TEM).

4.2.1. Light Microscope

Qualitative data from the microscope revealed that the size of chloroplast particles gradually decreased over time during four hours of *in vitro* digestion. Samples were treated with both digestive fluids and enzymes [Fig. 4.1(A1-E1 and A3-E3)] and others were treated merely with digestive fluids but not digestive enzymes [Fig. 4.1 (A2-E2 and A4-E4)].

It is difficult to compare morphological changes that occur in CRF with those that occur in WLM. From the images of CRF (A1-E1 with digestive enzymes, and A2-E2, without digestive enzymes) it is clear that disaggregation occurs as the samples move through the digestive model (probably due to pH changes and simple hydration of the freeze dried material). The presence of enzymes appears to promote further disaggregation, and, on close inspection, a reduction in chloroplast size (formation of residual particles). It appears that many intact chloroplasts remain in the absence of enzymes, but many are also transformed into organelle ghosts (loss of pigment); in the presence of enzymes, chloroplast size is reduced (residual particles) and the ghosts are significantly smaller. These changes are most dramatic during the later stages of the gastric phase, after that, the size of the residual particles reduces slightly in the presence of enzymes, but no clear changes occur to the chloroplasts or their ghosts in the control incubation during the duodenal phase.

Where it exists inside particles or organelles, a strong green colour appears to be retained in both incubations. One must be aware that chloroplasts will burst in the presence of water due to osmotic pressure differences; within this digestive model in miniature it is possible that the relatively high concentration of solids render the continuous phase rich in sugars (osmoticum carry over in the case of CRF and cellular in the case of WLM).

WLM images indicate tissue break up, cell wall folding/collapse, and a noticeable loss of chloroplasts in the presence of digestive enzymes; the leaf tissue in the control appears to change little during simulated digestion. The suggestion from this series of pictures is the digestive enzymes actually help to loosen plant cells in freeze-dried and powdered spinach leaves, and that this enzymic activity leads to the promotion of chloroplast release from cells. It seems unlikely that the digestive enzymes digest cell wall, so it may be that the tissue preparation (freeze-drying and powdering) has promoted a degree of cell wall breakage.

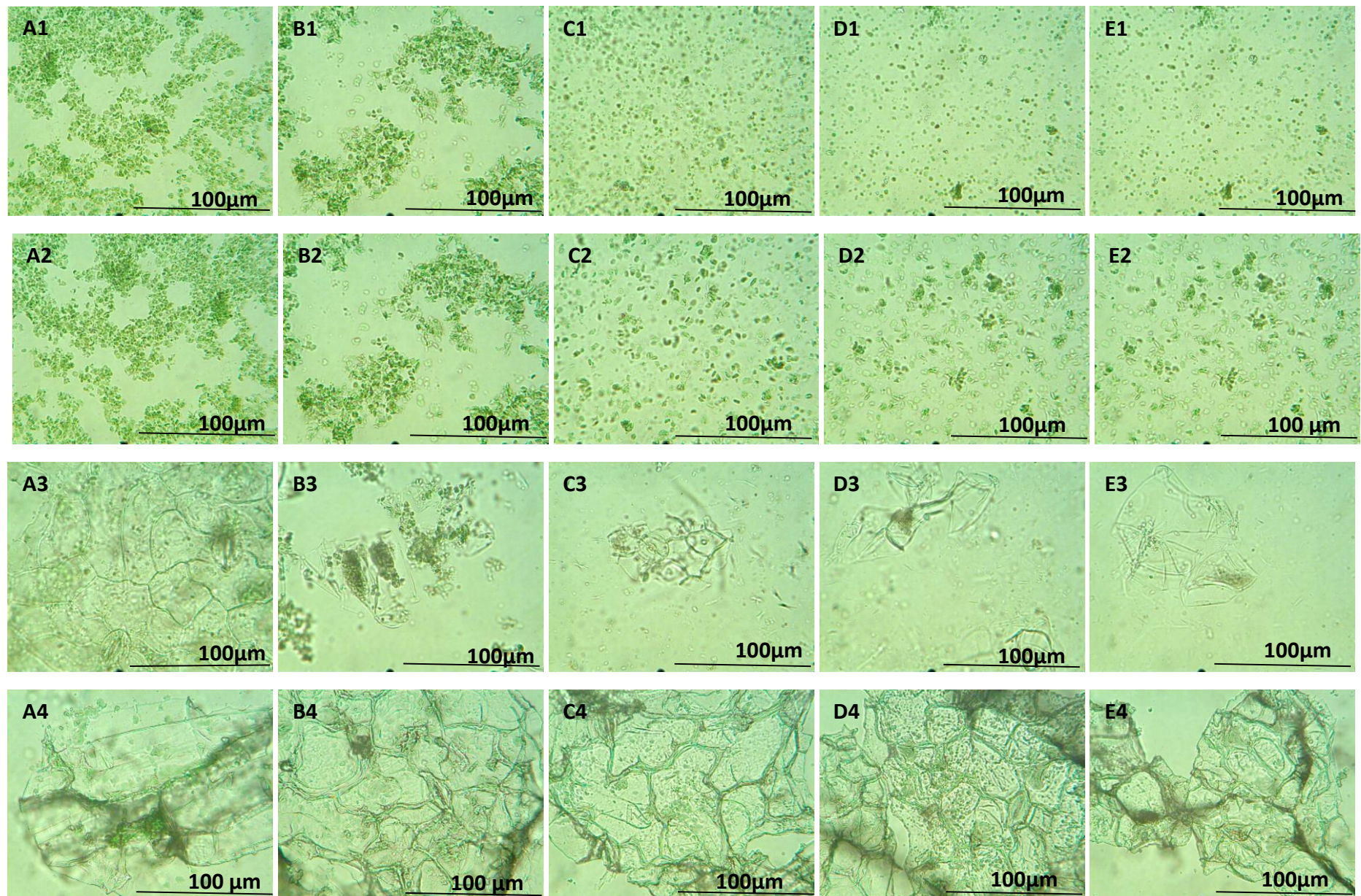


Figure 4.1. Representative light microscopic images of freeze dried spinach chloroplast rich fractions (A) Oral (2 min), (B) Gastric (1 h), (C) Gastric (2 h), (D) duodenal (1 h) and (E) duodenal (2h) during 4 hours of *in vitro* digestion model; A1-E1: CRFs with digestive fluids and enzymes A2-E2: CRFs with digestive fluids but no enzymes, A3-E3: WLM with digestive fluids and enzymes; A4-E4: WLM with only digestive fluids and no enzymes.

4.2.2. Transmissions Electron Microscope (TEM)

TEM was used to gain a more detailed picture of the morphological changes taking place in the CRF material during simulated digestion. A similar trend of structure reduction in spinach CRFs (as observed by light microscopy) from initial (pre-digestion Fig.4.2a), gastric (Fig.4.2b) and intestinal (Fig.4.2c) phases of *in vitro* digestion was observed using TEM.

Thylakoid architecture and packing is clearly visible in panel A, however, as the pH is lowered in the gastric phase (panel B), thylakoid membranes are disrupted. In the small intestines, thylakoid packing appears to have been broken down further and dismantled due to the action of intestinal enzymes. The TEM results suggest that chloroplast membranes with proven nutritional value by composition (chapter 3) are digestible *in vitro*.

Magnification and resolution of TEM is much higher than light microscopy owing to the smaller de Broglie (De Broglie, 1930) (matter behaving like a wave) wavelength of electrons and so single column of atoms, which is thousand times smaller than the smallest resolvable object in a light microscope could be examined.

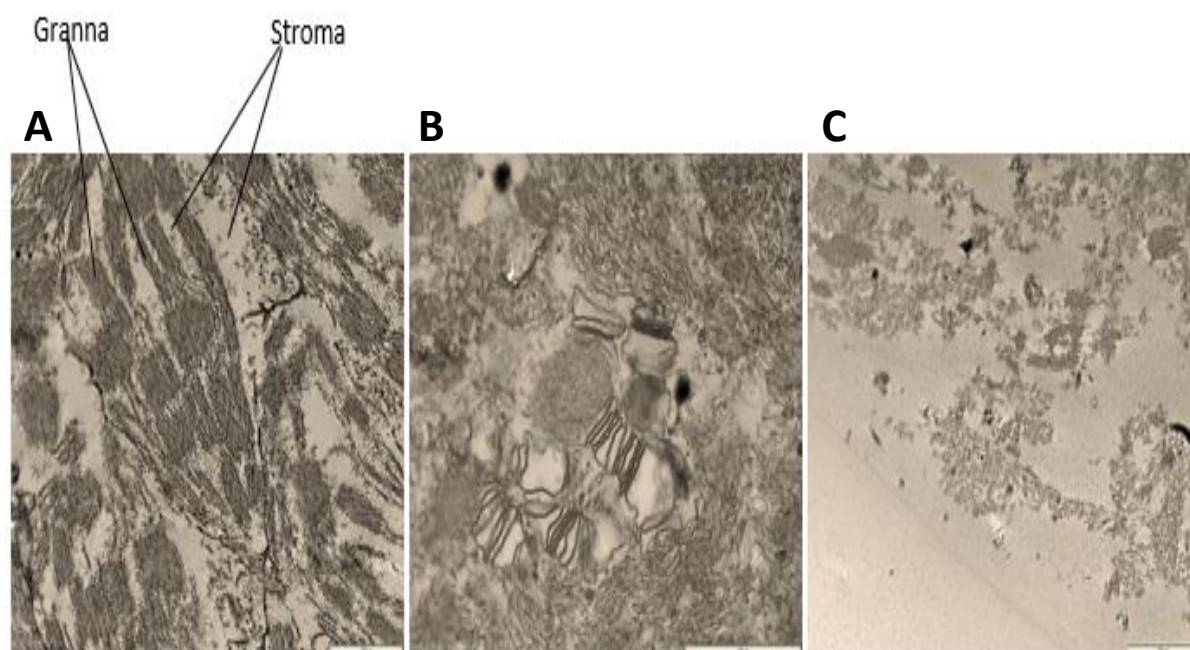


Figure 4.2. Representative Transmission Electron Microscopy (TEM) images of spinach chloroplast rich fractions in (a) Initial (Pre-digested), (b) Gastric and (c) Intestinal phases during in vitro digestion model.

These ultra-structural changes, from highly ordered to more amorphous structures, indicate that the chloroplasts organelles are appreciably digested. The features in these micrographs are distinct, suggesting particular changes at each stage of the digestion model.

4.3. Digestibility and Bioaccessibility of Organic Solids

The amount of organic matter precipitating into the bottom of the tube (pellet) or transferred to the top (supernatant) after centrifugation from freeze dried CRFs and WLMs is shown in Fig. (4.3 A-B). Lipid and protein content of the

digested (pellet and supernatant) and undigested WLM and CRF is also presented in Fig. (4.3.C-D). Results indicated that a considerable amount of CRF organic matter, greater than that of WLM (Fig.4.3 A-B) is sent into the supernatant after 4 h of *in vitro* digestion. Whilst slightly more CRF lipid than WLM is driven in to the micellar fraction, less CRF protein compared to WLM is lifted up into the supernatant fraction. The respective percent bioaccessible lipid and protein in these samples is 23.3 and 66.7 for WLM, and 33.6 and 28.3 for CRF. These values are based on percentages from digestate supernatant per unit mass of the initial (raw) undigested WLM or CRF shown in Fig. 4.3. C-D. The values could also be based on supernatant per unit mass of the total digestate and it results in similar figures. The values for undigested materials (WLM and CRF) for lipid and protein (Fig. 4.3. C-D) are consistent (independent experiment was carried out in each case) with those found in compositional analysis (Chapter 3).

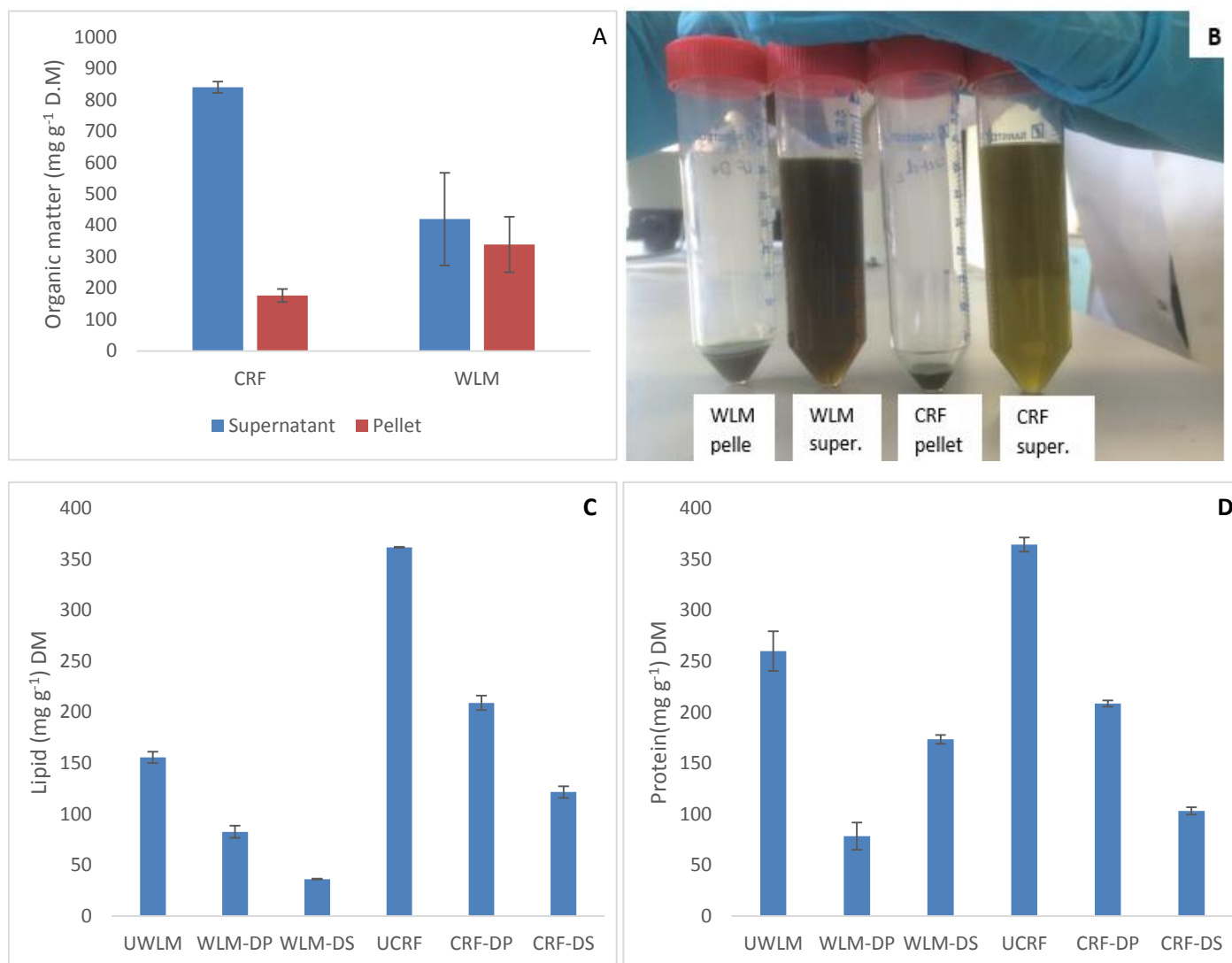


Figure 4.3. Organic matter (A & B), lipid (C) and protein (D) of freeze dried CRF and WLM either digested (*in vitro*) or analyzed before digestion, supper: supernatant; DP: digestate pellet; DS: digestate supernatant; U: undigested; WLM: whole leaf material; CRF: chloroplast rich fraction. Organic matter was determined by freeze drying samples in the Fig. (B), with a known mass (0.5 g) before digestion and the values normalized as digestive salts and enzymes add to the final masses. This was done by carrying out parallel tubes containing everything except test samples.

4.4. Digestibility and Carotenoid Release from the Chloroplast Matrix During Simulated *In vitro* Digestion

Carotenoid bioaccessibility, specifically β -carotene and lutein from spinach chloroplast-rich fractions and its whole leaves, in the presence or absence of

oil, was determined by an *in vitro* digestion model. The impact of the method used to prepare a chloroplast-rich fraction was also investigated. When carotenoids (lipophilic) are released from the chloroplast matrix they are taken up into nano-particles called micelles (hence micellar phase). Carotenoids retained in the matrix will be present in the pellet after centrifugation and so be destined, by implication, for the large intestine.

From the raw data in Table 4.1 it is apparent that the major carotenoids are released to a greater or lesser extent into the micellar phase during simulated digestion.

The concentration of freeze dried spinach carotenoids (β -carotene and lutein) in WLM (0.76 and 0.87 mg g⁻¹ D.W) and that of CRF (3.3 and 3.4 mg g⁻¹ D.W) was given in section 3.7 (chapter 3). Whereas that of juicer samples contained 2.5 and 1.8 mg g⁻¹ D.W, respectively for β -carotene and lutein. Given that we can account for most of the carotenoids being added to the system at time=0, we can see that sucrose-extracted chloroplasts have a higher concentration of carotenoids than those prepared via juicing. These alternative methods seem to generate a fraction that is contaminated with more cellular material. Not surprisingly the WLM has the lowest concentration of carotenoids as this material still contains cell wall material that makes up approximately 22-25% (Herranz et al., 1983, USDA, 2016) of the cell solids on dry weight basis.

Table 4.1. Partitioning of Beta-carotene and lutein from freeze dried and wet CRFs, and WLMs of spinach into micellar phase (small intestine) during simulated digestion

Treatment	β -carotene ($\mu\text{g g}^{-1}$ of digestate)			Lutein ($\mu\text{g g}^{-1}$ of digestate)		
	small intestine	large intestine	Total digestate	small intestine	large intestine	Total digestate
WLM	2.2 $\pm$ 0.5	6.7	8.9 $\pm$ 0.0	5.4 $\pm$ 0.2	0.5	5.9 $\pm$ 0.9
WLM+O (1:1)	3.4 $\pm$ 0.1	5.0	8.5 $\pm$ 1.6	3.7 $\pm$ 0.5	0.3	4.0 $\pm$ 0.3
SEF-CRF	3.8 $\pm$ 0.9	57.5	61.3 $\pm$ 7.9	11.5 $\pm$ 1.5	18.7	30.2 $\pm$ 0.3
SEF-CRF+O (1:1)	7.6 $\pm$ 1.3	49.6	57 $\pm$ 2 $\pm$ 5	20 $\pm$ 0.3	7.2	27.5 $\pm$ 0.7
SEW-CRF	6.8 $\pm$ 1.0	43.6	50.3 $\pm$ 6.2	11.1 $\pm$ 0.8	22.3	33.3 $\pm$ 0.8
SEW-CRF+O (1:1)	9.8 $\pm$ 0.6	19.9	29.7 $\pm$ 3.0	17.0 $\pm$ 1.6	12.6	29.6 $\pm$ 1.9
J-CRF	4.9 $\pm$ 1.9	8.9	13.8 $\pm$ 0.9	7.5 $\pm$ 1.3	1.7	9.2 $\pm$ 0.4
UT-CRF	6.2 $\pm$ 0.7	9.9	16.1 $\pm$ 1.1	7.6 $\pm$ 0.2	2.6	10.2 $\pm$ 0.9
MC-CRF	5.4 $\pm$ 0.4	10.3	15.7 $\pm$ 1.6	7.2 $\pm$ 0.3	2.9	10.1 $\pm$ 1.0

SEF: sucrose extracted and freeze dried; **CRF:** chloroplast rich fractions; **WLM:** whole leaf materials; **O:** oil; **SEW:** sucrose extracted wet; **J:** Juicer; **UT:** ultra-Turrax; **MC:** micro-cavitated.

The bioaccessibility data (%) was calculated from the concentration of the carotenoids in the micellar phase (small intestine) compared with that in the total digestate (see Table 4.1).

Goñi et al. (2006) studied freeze dried fruits and vegetables for their carotenoid bioaccessibility through *in vitro* digestion of small intestine or by colonic fermentation. Their results (of the vegetables) indicated greater lutein bioaccessibility (79%) than β -carotene (27%) in the small intestine, whilst bioaccessibility of 17 % (lutein) and 57% (β -carotene) was recorded in the large intestine. In this study, carotenoid concentration in the supernatant (bioaccessible) as well as those carotenoids found in the total digestates were quantitated; then the difference between the two is considered as carotenoids partitioning into the large intestines (Table 4.1). Despite the lower lutein transit into the colon, studies suggested lutein as the only carotenoid associated with reduced incidence of colon cancer among other carotenoids (α and β -carotene, lycopene, zeaxanthin and β -cryptoxanthin) studied by Slattery et al. (2000).

Bioaccessibility of nutrients in the large intestines will be affected by the metabolic/enzymatic activity by micro-flora bacteria in the colon. One study suggests that microflora cause the release of bioactive compounds such as carotenoids into the colon lumen (Goñi et al., 2006).

Percentage bioaccessible β -carotene and lutein out of sucrose-extracted, freeze-dried chloroplasts rich fractions (SEF-CRFs), freeze-dried whole leaf

materials (F-WLM) and sucrose-extracted, wet chloroplast rich fractions (SEW-CRFs) are displayed in Fig. 4.4.

The β -carotene bioaccessibility of the digested material ranged between 6.1% and 41.0%, whereas that of lutein was between 33% and 93.2% (Fig.4.4). SEW-CRFs displays greater β -carotene bioaccessibility compared to SEF-CRFs whilst SEF-CRFs shows higher lutein micellarization than SEW-CRFs whether oil is added or not.

Of the two carotenoids, lutein exhibited greater bioaccessibility than β -carotene in both spinach and spinach chloroplasts rich fractions; and in oil and non-oil treated samples (Fig.4.4). With the addition of oil, carotenoid bioaccessibility of SEF-CRFs, F-WLM and SEW-CRFs was nearly doubled except for lutein of the F-WLM. Although SEW-CRF materials were significantly ($p \leq 0.05$) lower than those of F-WLM for lutein bioaccessibility, no significant ($p \geq 0.05$) difference was apparent for both in β -carotene bioaccessibility.

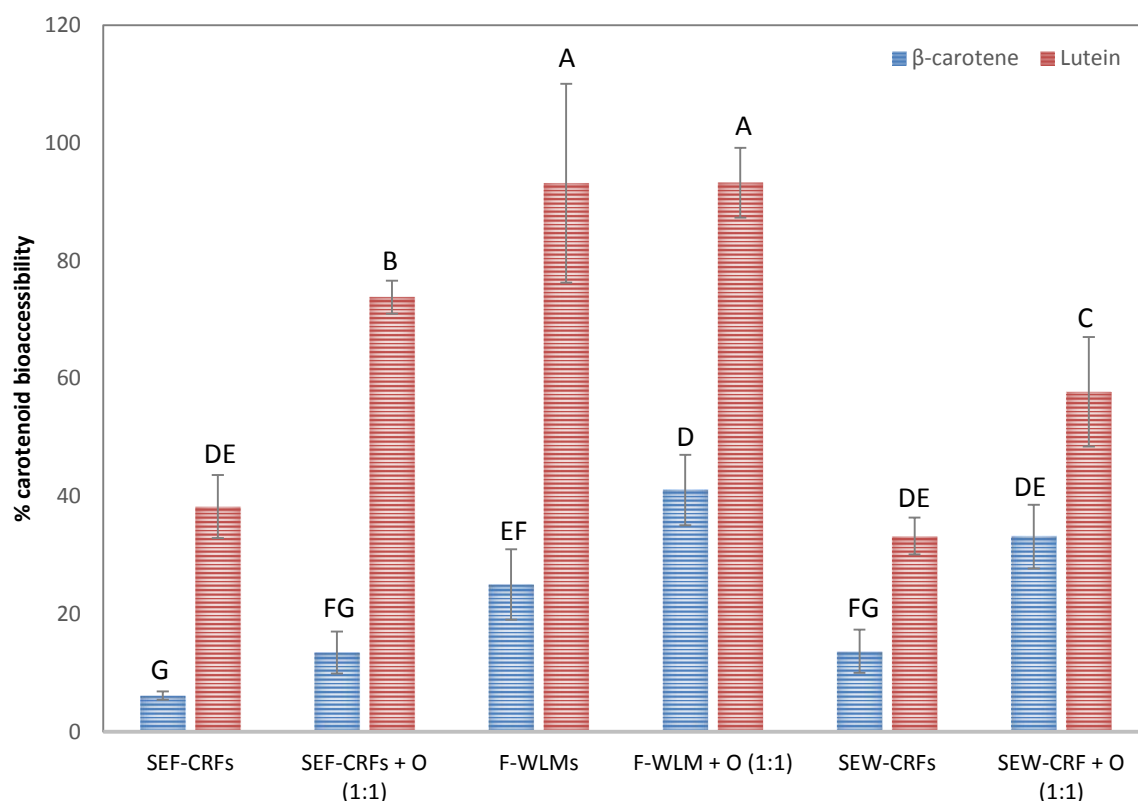


Figure 4.4. Bioaccessibility of β -carotene and lutein from digestates of SEF-CRF: sucrose extracted freeze dried chloroplast rich fractions; SEF-WLM: Sucrose extracted freeze dried whole leaf material; SEW-CRF: Sucrose extracted wet chloroplast rich fractions; with or without O: oil. Bars that do not share a letter are significantly ($p < 0.05$) different.

It is possible that the method of CRF preparation affects the bioaccessibility of carotenoids. Chloroplast rich fractions were obtained from spinach using a juicer (J) that removes most extracellular materials via its filter mesh; then a portion of the juice was processed using either Ultra-turrax (UT) homogenizer or microcavitation (MC). All samples were then freeze dried. The bioaccessibility of the target carotenoids after *in vitro* digestion is shown in Fig. 4.5.

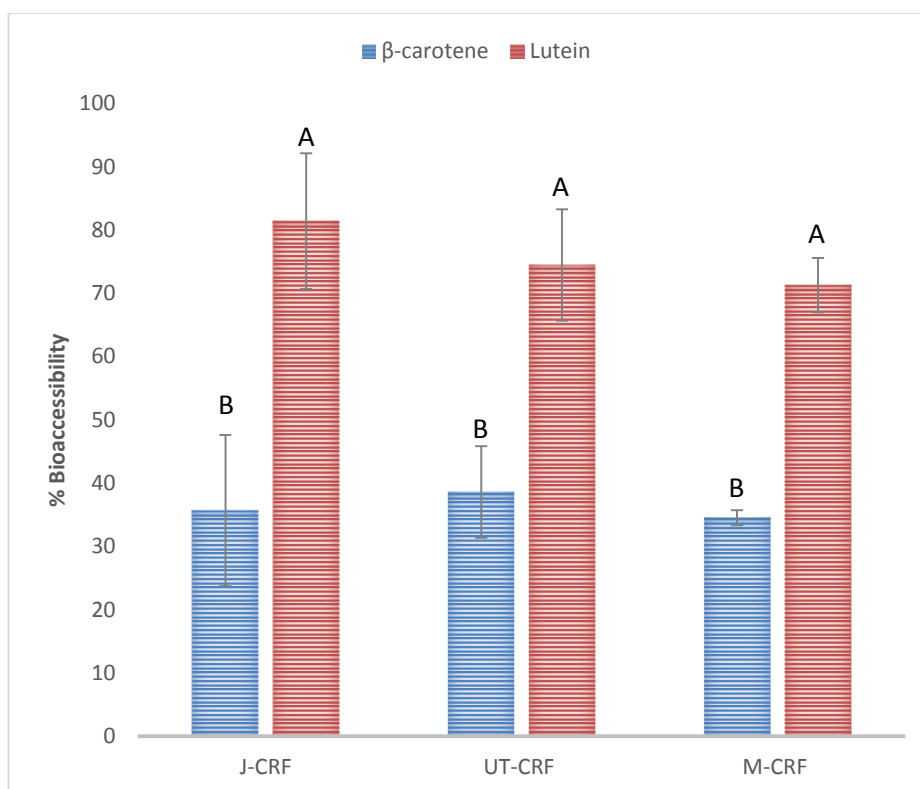


Figure 4.5. Beta-carotene and lutein bioaccessibility from freeze dried spinach chloroplasts rich fractions obtained using juicer and then processed either with Ultra-Turrax homogenizer or microcavitation. Columns that do not share a letter are significantly ($p < 0.05$) different. J-CRF: juicer extracted (freeze dried) chloroplast rich fractions; UT-CRF: juicer ultra-turraxed-chloroplast rich fraction; M-CRF: microcavitated-chloroplast rich fractions.

Findings indicated that CRFs obtained by juicing followed by processing noticeably improved micellarization of β -carotene and lutein without oil addition. For instance, the micellarization of SEF-CRFs (6%) shown in Fig.4.4 was increased to about 35%, 38% and 34%, respectively for JEF-CRF, JEFUT-CRF and JEFMC-CRFs (Fig.4.5). Similarly, lutein bioaccessibility of SEF-CRFs was enhanced from 38% (Fig.4.4) to around 81%, 74% and 72% respectively for J-CRF, UT-CRF and MC-CRFs. These data suggests greater carotenoid bioaccessibility of chloroplast rich fractions obtained via juicing compared to those obtained via grinding leaves with a blender in the presence of sucrose to act as an osmoticum. Qualitative assessment of all the CRF materials

indicated that the sucrose-extracted CRF was harder and less easily dispersed in water compared with the other CRF samples. This property is probably due to the presence of excess sucrose used as an osmoticum during leaf grinding to prevent chloroplasts bursting (see appendices B1-B2).

Dietary fat has an impact on carotenoid bioaccessibility (Furr and Clark, 1997, Yeum and Russell, 2002a). These include extraction of carotenoids from their food matrix and carotenoid solubilisation during the transfer of gastric chyme to the duodenum. In addition, fats act as stimulants for the secretion of pancreatic enzymes and bile salts, facilitating micelle formation (Rodriguez-Amaya, 2015). Olive oil is rich in unsaturated fatty acids and micellarization of carotenoids (β -carotene and lutein) is significantly enhanced with increasing acyl chain length of triacylglycerol used (Huo et al., 2007). Failla et al. (2014) found that *in vitro* bioaccessibility of β -carotene from mixed salad vegetables was increased by lipids rich in unsaturated fatty acids and olive oil ranked second after soybean out of four lipids included in the study (soybean oil > olive > canola > butter). In another study, the type of lipid was also found to have influence on β -carotene bioaccessibility after *in vitro* digestion. For instance, when red palm oil instead of sunflower oil was used with sun-dried Tanzanian green leaves (amaranth, cow pea, sweat potato, pumpkin and cassava), twice as much bioaccessible β -carotene was achieved (Hedrén et al., 2002).

Solubilisation of carotene and lutein into the oil phase is a relevant explanation, but since oil is digested by pancreatin, the release of fatty acids

may have some effect on carotenoid solubilisation. Studies suggest that increased acyl chain length increases the hydrophobicity of products of lipid digestion, which may facilitate carotene transfer from the food matrix (Huo et al., 2007). In addition, digestion products (fatty acids, included) with different chain length produced during gastric and duodenal phases influence the physiochemical characteristics of mixed micelles; longer acyl compounds increase the uptake of carotenes into micelles (Constantinides and Steim, 1985).

In our study, oil promoted a 2-fold increase in the bioaccessibility of carotenoids from CRF material.

Several studies measuring carotenoid bioaccessibility commonly follow a method proposed by Garrett et al. (1999) with some modifications. After small intestinal *in vitro* digestion is complete, the aqueous fraction is generally isolated from the total digestion mixtures using this method or its modified version. This is to determine bioaccessibility (the percent unit mass of carotenoid in the micellar fraction per that in the total digested mixtures). Hence, even when the carotenoid concentration (per unit mass) is different between fresh and dried food materials (e.g. fresh spinach versus freeze dried spinach), the bioaccessibility may not be affected. This allows one to compare bioaccessibility data from different food sources regardless of whether it is fresh or not. Using this approach, spinach β -carotene bioaccessibility was found to vary depending on treatment or processing. For example, Reboul et al. (2006) found that β -carotene bioaccessibility of boiled spinach (17.45%) was higher than that of minced spinach (5.2%) which was, its turn, higher

than the raw spinach leaves (2.43%). Following the method of Garrett and co-authors with slight modifications, O'Connell et al. (2007) found β -carotene bioaccessibility of about 29% for fresh spinach, which is in agreement with that of our freeze dried WLM data;. However, the lower spinach lutein (18.9%) bioaccessibility (lower than β -carotene) also reported by O'Connell et al. (2007), is surprising and contradicting to the trend found in the literature. In almost every study lutein was found to be less affected by material matrix with bioaccessibility greater than that of β -carotene (Erdman Jr et al., 1988, Failla et al., 2014, Garrett et al., 1999, Goñi et al., 2006, Reboul et al., 2006). It is recognized that xanthophyll carotenoids (e.g. lutein) from green tissues are more efficiently released by digestion and hence more bioaccessible than are carotenoids from carotenes such as α -carotene, β -carotene and lycopene (Granado-Lorencio et al., 2007), probably due to the lower hydrophobicity of xanthophyll carotenoids. During solubilisation of individual carotenoid into lipid globules, hydrocarbon (non-polar) carotenoids (carotenes) migrate to the lipid rich, hydrophobic core of the particles, whereas more polar xanthophylls are located at the surface along with proteins, phospholipids and partially ionized fatty acids (Borel et al., 1996, Tyssandier et al., 2002).

In general, carotenoids from green tissues are less bioaccessible compared to those from other sources e.g. fruits owing to subcellular localisation mechanisms and the form of carotenoids such as bound carotenoids in the photosystem I and II of the inner chloroplasts (green leafy tissues), globular in the chromoplasts (fruits e.g. mango, papaya and pumpkin) or crystalline (carrot) (De Pee et al., 1998, Rich et al., 2003). Therefore plastoglobuli,

crystals and membranes ease carotenoid release in descending order (Jeffery et al., 2012).

Bioaccessibility of carotenoids and their uptake after food ingestion is affected by many factors including food matrix, particle size, carotenoid hydrophobicity, gastric phase pH, indigestible fractions (soluble or insoluble), concentration of bile salts, amount and type of dietary fats, phospholipids, fibre content and interactions with other food constituents (Faulks and Southon, 2005, Granado-Lorencio et al., 2007). Gastric phase pH for instance, decreases the micellarization of carotenes. In an *in vitro* study identifying the main factors governing the bioaccessibility of beta-carotene incorporated into dispersions, it was found that the bioaccessibility was considerably low in acid pH such 2 and 3, and as the pH was slightly increased to 4 and 6 the bioaccessibility was enhanced (Wang et al., 2012).

As discussed above and according to Cilla et al. (2012), bioaccessibility is greatly dependent on the chemical and physical properties of the diet matrix and the way these change during digestion. Despite the cell wall being the main barrier for carotenoid uptake, bioaccessibility is not only determined by the cell wall integrity but also by interactions between structural components of the complex food matrix (Rodriguez-Amaya, 2015). For example, the cell matrix of different tomatoes was disrupted by high pressure homogenization (HPH) which decreased the particle size and increased product consistency in the form of fibre network (Panozzo et al., 2013). This resulted in decreased carotenoid bioaccessibility (lycopene, α -carotene and lutein), which was

attributed to the fibre network created by the HPH. Further, Panozzo and co-authors, observed that in one matrix that is red (homogenised) tomato, both carotenoids with the greatest (lutein) and the least (lycopene) bioaccessibility are present, suggesting that carotenoid bioaccessibility not only depends on the matrix but also carotenoid species.

Beta-carotene can act as a precursor to vitamin A (retinol); this conversion can happen in the epithelial cells of the small intestine, it is therefore difficult to fully account for β -carotene uptake into the body (bioavailability). In bioavailability studies 2-50% of the β -carotene found in mixed-micelles was reported to be absorbed and the remaining 50-98% goes unabsorbed (Ball, 1998). As such, the nature of β -carotene and the form it is localized within the food matrix (i.e. plastoglobuli, crystalin and membrane-bounded) plays a great role in its release from its matrix and its consequent bioavailability.

In an *in vivo* study, the effect of variously processed spinach products and of dietary fibre on bioavailability was assessed. Apart from the controls, subjects received over a 3 week period, one spinach product out of four: whole leaf spinach (with an almost intact food matrix), minced spinach (partially disrupted matrix), enzymatically liquefied spinach (with further disrupted matrix) and enzymatically liquefied spinach with fibre (10 g kg⁻¹ wet weight). Total β -carotene in the serum varied significantly between whole leaf and liquefied spinach groups and between the minced and liquefied spinach

groups. No significant difference for lutein response was observed among spinach groups. Therefore, the nature of spinach processing and the nature of the food matrix had a greater and significant influence on β -carotene bioavailability compared with lutein; that is, lutein was relatively more bioavailable (45 to 55% than β -carotene (~6 to 10%) and less affected by food matrix (Castenmiller et al., 1999). In vegetables, bioavailability of lutein (67%) was 5 times greater than that of β -carotene (14%) (van het Hof et al., 1999); and green leafy vegetables generally show the least β -carotene bioavailability as such greater attention has recently shifted to other carotenoids for their health benefits (e.g. Lutein).

Although β -carotene is minimally bioaccessible from the chloroplast rich fractions, an appreciable amount of lutein with recently recognized health benefits mainly for the eye is co-micellarized. In a previous study by Slattery et al. (2000), lutein was also reported to have positive effect on colon cancer; and it is lutein that was solely associated with reduced incidence of colon cancer among different carotenoids (α and β -carotene, lycopene, zeaxanthin and β -cryptoxanthin) studied.

From a sustainable nutrition point of view, chloroplast rich fractions enriched in these lipophilic isoprenoid carotenoids could be obtained from various unconventional green plant species. Therefore, despite the limited bioaccessibility, and probably bioavailability, of the pro-vitamin A carotenoid

(β -carotene), their wide availability throughout nature in the form of chloroplasts (green leafy plants) makes them a good source for provitamin A carotenoids as well as other clinically proven carotenoids for human health (lutein and zeaxanthin) (Abdel-Aal et al., 2013, Fullmer and Shao, 2001, Kris-Etherton et al., 2002, Roberts et al., 2009, Semba and Dagnelie, 2003).

Further, Lutein was found to be more concentrated in a zebrafish (whole and egg) fed with spinach CRFs compared to its WLM for 8 weeks (subsequent chapters). Although β -carotene was not measured (in the eggs) there was an increase in retinol concentration in the eggs of fish that consumed CRF as part of their diet.

4.5. Conclusion

Both structural and analytical data indicated digestibility of chloroplast rich fractions (CRFs) during simulated digestion. Carotenoid release was accompanied by dramatic ultra-structural changes to the chloroplasts. In general, lutein displayed more bioaccessibility than β -carotene in both CRFs and WLMs. Clumping due to hardness of the material probably explains in part the lower bioaccessibility of β -carotene from freeze dried SEF-CRFs as compared with SEW-CRFs or J-CRFs, UT-CRFs and MC-CRFs. However, bioaccessibility of both β -carotene and lutein in SEW-CRFs, as well as, SEF-CRFs was enhanced in the presence of oil, but with varying degrees. Employment of the juicer and its subsequent homogenization processes (Ultra Turrax and microcavitation) to break down the CRFs matrix, improved

carotenoid micellarization without oil treatment. The chemical nature of carotenoids (mainly carotenes compared to xanthophylls) their hydrophobicity characteristics and their strong attachment to pigment proteins in green leafy vegetables makes it difficult for efficient carotenoid micellarization. Further purification and removal of more extra-cellular green leafy constituents and/or adding other lipidic foods may facilitate release of additional amounts of these carotenoids.

Chapter Five

Hydrolysis of Galactolipids from Spinach Chloroplast Rich Fractions by Pancreatic Lipase Related Protein 2

5.1. Introduction

The impact of pancreatic lipase-related protein 2 from pig (porcine) and guinea pig (gPLRP2) on the hydrolysis of galactolipid substrates from commercial (pure Tributyrin and monogalactosyldiacylglycerol [MGDG]) or from natural sources was determined *in vitro*. These natural galactolipids (polar) were extracted from spinach chloroplast rich fractions (CRF) and its whole leaf material (WLM) and then analysed by preparative 2D thin layer chromatography (TLC).

5.2. Polar Lipid Profile in Freeze Dried Spinach and Spinach Chloroplast Rich Fractions

Polar lipids were extracted from spinach whole leaf materials (WLM) and its chloroplast rich fractions (CRFs). The lipid analysis, by preparative 2-directional thin layer chromatography (TLC), is presented in Fig. 5.1. Spinach WLM and its CRFs contain significant amounts of glycerol glycolipids i.e. monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinosyldiacylglycerol (SQDG) and certain phospholipids (e.g. PE) per unit mass of lipid. Although, different lipid classes were identified by the

preparative two direction TLC using commercial standards (Fig.5.1), only major polar lipids in spinach (MGDG, DGDG, SQDG) were quantified and results summarised in Table (5.1).

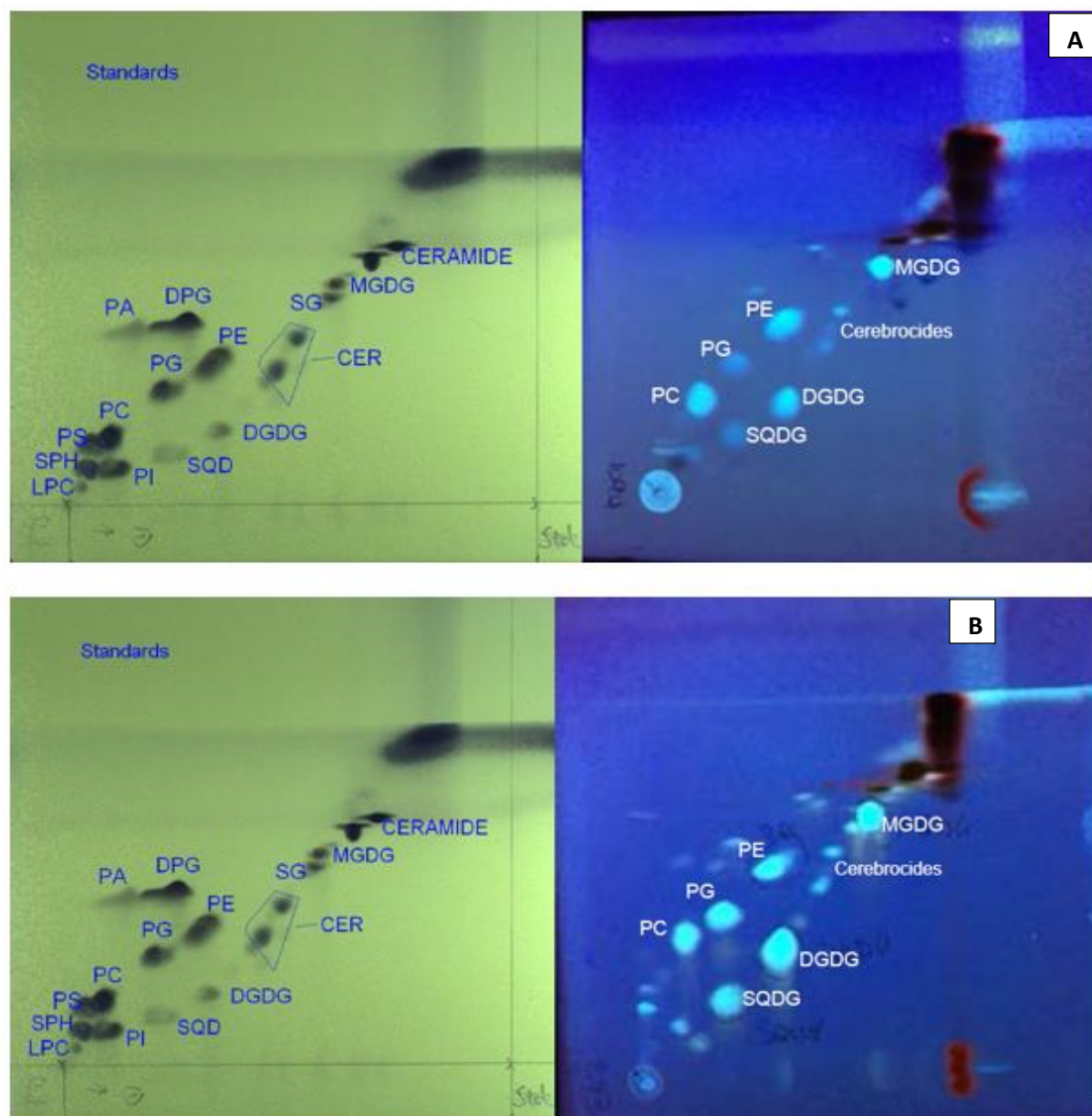


Figure. 5.1. TLC of polar lipids prepared from spinach WLMs (A) and spinach CRF (B). LPC – lyso phosphatidylcholine; SPH – sphingomyelin; PS – phosphatidylserine; PC – phosphatidylcholine; PG – phosphatidylglycerol; PA – phosphatides acid; DPG - diphosphatidylglycerol; PE – phosphatidylethanolamine; SG – steryl glycoside; Ceram – ceramides; MGDG – monogalactosyl diacylglycerol; Cereb – cerebrocides; DGDG - digalactosyl diacylglycerol; SQDG - sulphoquinovosyl diacylglycerol; PI – phosphatidylinositol.

The ratio of MGDG:DGDG:SQDG in spinach WLM and spinach CRFs in this study was 5:3:1 (W/W/W), which is in agreement with data reported by Yunoki et al. (2009) and Amara et al. (2010). Markers for differentiation of green leaf galactolipids could be the fatty acids C16:3 (hexadecatrienoic acid) and C18:3 (alpha-linolenic acid). These two fatty acids are concentrated in the chloroplasts of green leafy vegetables, thus the characterization of some green plants as being C16:3 or C18:3 plants. An example of C18:3 plants include Asteraceae (e.g. lettuce and sunflower) and Fabaceae (legumes/beans) families, whereas, C16:3 plants are represented by members of Apiaceae (e.g. cow parsley) and Brassicaceae (e.g. Arabidopsis and rape seed); spinach a member of the Amaranthaceae family, previously known as Chenopodiaceae, is also a C16:3 plant (Heinz and Roughan, 1983). The C16:3 in the MGDG of spinach (WLMs) calculated from Table (5.1) is around 20%, which is in accordance with that of spinach leaves (30%), reported by Allen et al. (1964). In addition, data provided in Table (5.1), shows C16:3 of MGDG is 9 times higher than that of DGDG. The C16:3 concentration varies in plants due to the origin of galactolipid synthesis i.e. prokaryotic versus eukaryotic-type lipid (see Fig. 5.2). For instance, MGDG, which is predominantly formed from prokaryotic diacylglycerol is characterized by its high content of C16:3; whereas, DGDG is mainly from eukaryotic origin and thus contains minimal concentrations of C16:3 (Dörmann and Benning, 2002).

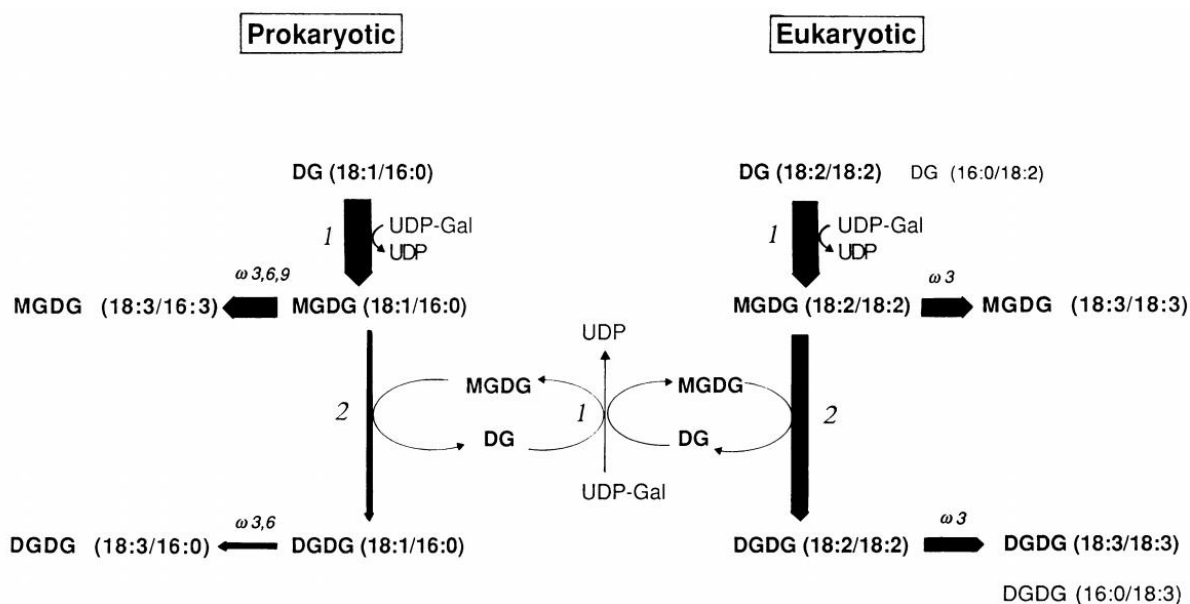


Figure 5.2. Simplified scheme for the synthesis of pro- and eukaryotic galactolipids in spinach and other 16:3 plants. De novo-made diacylglycerol, DG(18:1/16:0), is formed in chloroplasts by the prokaryotic pathway, is galactosylated to MGDG(18:1/16:0) and, at a slow rate, to DGDG(18:1/16:0). Desaturation of MGDG(18:1/16:0) competes with galactosylation and results, finally, in MGDG(18:3/16:3). Desaturation of DGDG(18:1/16:0) results in DGDG(18:3/16:0). The galactosylation of polyunsaturated MGDG species is thought to be a minor pathway and is not indicated. Eukaryotic diacylglycerol, DG(18:2/18:2), transported or released to the chloroplasts by an unknown mechanism, is galactosylated relatively fast to MGDG and to DGDG. Both galactolipids are subsequently desaturated to the C183/C183 molecular species. A similar reaction sequence, starting from DG(16:0/18:2), may lead to the formation of eukaryotic DGDG(16:0/18:3). The corresponding MGDG(16:0/18:3) is not well documented in the literature, and is therefore omitted. During the formation of DGDG by galactolipid:galactolipid galactosyltransferase diacylglycerol is produced, which is reconverted into MGDG. In 18:3 plants the prokaryotic pathway is absent. Note that this scheme includes only one enzyme transferring galactose residues from a water-soluble nucleotide of cytosolic origin. Abbreviations for enzymes: 1, UDPGal:diacylglycerol galactosyltransferase (EC 2.4.1.46); 2, galactolipid:galactolipid galactosyltransferase (EC 2.4.1.184); w3,6,9, 3, 6, and w9 desaturase (EC 1.3.?.?) (Heemskerk et al., 1991).

Table 5.1. Fatty acid profile of the Galactolipids of CRFs and WLMs from spinach expressed as mg fraction per g lipid

Fatty Acid	Polar lipids in mg g ⁻¹ phospholipid (PL*)							
	SQDG		PE		MGDG		DGDG	
	CRF	WLM	CRF	WLM	CRF	WLM	CRF	WLM
14:0	0.2	0.2	0.2	0.2	0.0	0.1	0.2	0.2
15:0	0.0	0.1	0.0	0.2	0.0	0.0	0.0	0.0
16:0	16.3	10.4	3.4	10.7	1.4	0.7	5.4	4.6
16:1	1.3	0.1	0.0	0.7	0.6	0.1	1.4	0.1
16:2	0.3	0.2	0.4	0.1	0.0	0.1	0.3	0.2
17:0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
16:3 n-3	0.2	0.2	0.0	0.2	37.7	23.4	4.1	2.6
18:0	1	0.5	0.6	0.7	0.6	0.2	1.0	0.9
18:1(n-9)	1.1	0.1	0.8	2.2	0.0	0.1	0.5	0.7
18:1(n-7)	0.2	0.1	0.7	1.3	0.9	0.4	1.9	1.1
18:2	2.1	0.7	4.6	14.5	1.9	1.1	1.3	1.6
18:3(n-3)	15.8	9.6	2.9	11.9	159.3	86.8	106.9	66.2
20:0	0.2	0.1	0.2	0.2	0.0	0.1	0.2	0.2
20:1	0.0	0.0	0.2	0.9	0.0	0.0	0.0	0.0
20:3(n-3)	0.0	0.0	0.0	0.1	0.0	0.1	0.4	0.3
22:0	0.2	0.1	0.3	0.4	0.0	0.1	0.2	0.2
22:1	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0
24:0	0.2	0.2	1.1	1.7	0.0	0.2	0.3	0.6
Total	39.1	22.6	15.4	46.2	202.3	113.7	124.2	79.6
Total								
CRFs mg g ⁻¹	39.1		15.4		202.3		124.2	381.1
WLMs mg g ⁻¹	22.6		46.1		113.7		79.6	262.0

SQDG—sulphoquinovosyl diacylglycerol; **PE**—phosphatidylethanolamine; **MGDG**—monogalactosyl diacylglycerol; **DGDG**—digalactosyl diacylglycerol; **CRF**—chloroplast rich fraction; **WLM**—whole leaf material. *— data is expressed in mg g⁻¹ PL, e.g. there are 600 mg PL g⁻¹ lipid of CRF (data not given) i.e. $600 - 381.1 = 218.9$; $381.1 / (218.9 + 381.1) \times 100$ is equal to 63.5% galactolipid. PE is used as internal standard for TLC analysis.

Quantitation of major lipids in WLMs and CRFs facilitated estimation of the ratio of bile salt to galactolipid (1.3 mole mole⁻¹) to be used for the subsequent galactolipid (GL) digestion analysis by pH-stat technique mimicking digestion in the small intestine; at this ratio, the PLRP2 reaches its optimum activity against GLs (Amara et al., 2009).

5.3. Impact of gPLRP2 on Galactolipid Digestion in an In-Vitro Model

In-vitro assays were carried out on pure GLs to establish the ability of pancreatin and PLRP2 to hydrolyse GLs by determining their activity against GL substrate. From these results the impact of the addition of PLRP2 during the small intestinal phase of an *in-vitro* model on fatty acids release was tested.

Physiologically relevant conditions were used for pancreatin, however, no proven physiologically relevant parameters are known for PLRP2 *in vivo* and so conditions where it showed to be maximally active *in vitro* was employed. For instance, to determine the activity of gPLRP2, the pH was set at 8.0 to 9.0 for those substrates rich in GLs such as MGDG (with the GLs/bile salt ratio of 1.3). In 100 g fresh spinach (6 g dry weight), there is approximately 1 g CRF on dry weight basis. In 1.0 g CRF there is 350 mg lipid, of which 10% is other pigments (chlorophylls, carotenoids, tocopherols e.tc.). About 80% of this lipid is Glyceroglycolipids mainly MGDG and DGDG (Table 5.1). Humans consume an average of 200 mg GLs per day which is slightly lower than 100 g fresh spinach whereas guinea pigs can ingest treble (>600 mg GLs per day) of what humans eat (De Caro et al., 2008, Andersson et al., 1996, Carriere et al., 1993).

5.3.1. Hydrolysis of Pure Galactolipids (Commercial) by gPLRP2 and Pancreatin in an Optimised Assay

The activity of gPLRP2 and that of pancreatin cocktail was investigated either on MGDG or Tributyrin, a triacylglycerol (TAG) rich lipid as commercial lipid standards using the pH-stat technique. Apart from the individual application, combination of gPLRP2 with pancreatin cocktail on each of these pure lipids was also tested to assess synergistic effects and whether other substances in the pancreatin cocktail would hinder or inhibit gPLRP2 activity. Similarly, the activity of both of these enzyme preparations was examined for their ability to hydrolyse CRF and WLMs obtained from simulated *in vitro* gastric chyme.

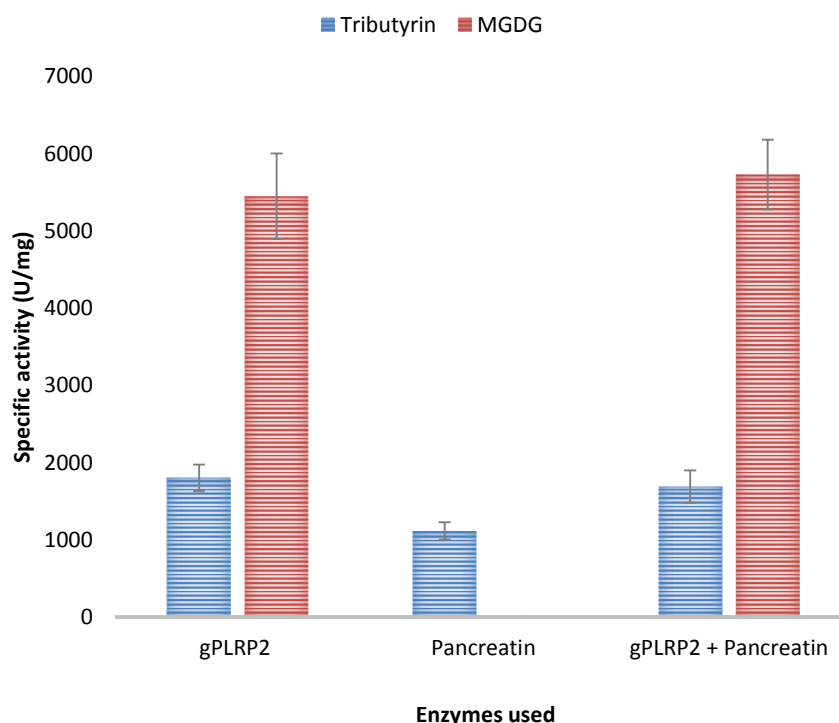


Figure. 5.3. Pancreatin and pancreatic lipase related protein 2 (gPLRP2) activities against two substrates: Triacylglycerol (TAG) rich lipid (Tributyrin) and Monoglactocyldiglyceride (MGDG) as glycerglycolipid. Galactolipase (PLRP2) activity was determined at 37 and pH 8.0 with a bile salt/MGDG ratio of 1.33 using NaTDC under conditions described in the respective materials and methods. Pancreatin activity was determined at 37 and pH 8.0 under conditions described in the respective materials methods. Knowing the volume (μL) of the enzyme added in the pH stat vessel, the activity (A) expressed in U mL^{-1} can be determined as: $A = (x.1000) \text{ v}^{-1}$. The activity was measured in the first 10 min of the reaction using mean linear values shown in appendix D1

Results of the individual enzyme activity showed that pancreatin was greatly active on Tributyrin with almost no activity against MGDG. Unlike pancreatin, gPLRP2 was active against both MGDG and tributyrin but to varying degrees (Fig. 5.3-5.4). However, no synergistic effect between gPLRP2 and pancreatin was apparent, and the activity of the combined enzyme preparations was similar to that of gPLRP2 against MGDG. The entire activity was thus attributed to gPLRP2 since pancreatin showed nearly zero activity against MGDG in Fig. (5.3). Unlike classical pancreatic lipases, PLRP2 displays broad substrate specificity, which could be further explained by the free fatty release shown in Fig.5.3. On one hand, when substantial amounts of free fatty acids (corresponding to 5450 U mg⁻¹ Fig.5.3) were released by gPLRP2 using MGDG as a substrate (Fig 5.4. A), very low amounts of free fatty acids (corresponding to 6 U mg⁻¹ Fig.5.3) were produced by pancreatin with the same substrate (Fig.5.4 C). On the other hand, gPLRP2 can release free fatty acids from both substrates (MGDG and Tributyrin) but at a lower rate in the case of the triacylglycerol substrate (Tributyrin). These results are consistent with those in the literature (Amara et al., 2009, Amara et al., 2013). Similar gPLRP2 activity on pure MGDG (5420 U mg⁻¹) to that shown in Fig.5.3 at the ratio of 1.3 NaTDC/MGDG (mole/mole) was established by Amara et al. (2009), who optimized the effect of different concentrations of bile salt on galactolipid

hydrolysis. In a previous study, Andersson et al. (1995) found lower rate hydrolysis of pure MGDG but with almost no activity against DGDG or SQDG by purified human pancreatic lipase (HPL) and colipase combined. However, Anderson and co-workers also found activity towards all three galactolipids by two proteins, when human pancreatic juice was separated on a Sephadex G100 gel filtration chromatography. One protein (100 kD) migrated with carboxyl ester lipase and the other (50 kD) with HPL. Therefore, Anderson and co-workers proposed that there is an enzyme with galactolipase activity in the pancreatic juice, with similar molar mass as HPL. Subsequently, Andersson et al. (1996) emphasized that it was PLRP2, expressed previously from guinea pig (Carrière et al., 1994) and other species that was responsible for those galactolipase activities.

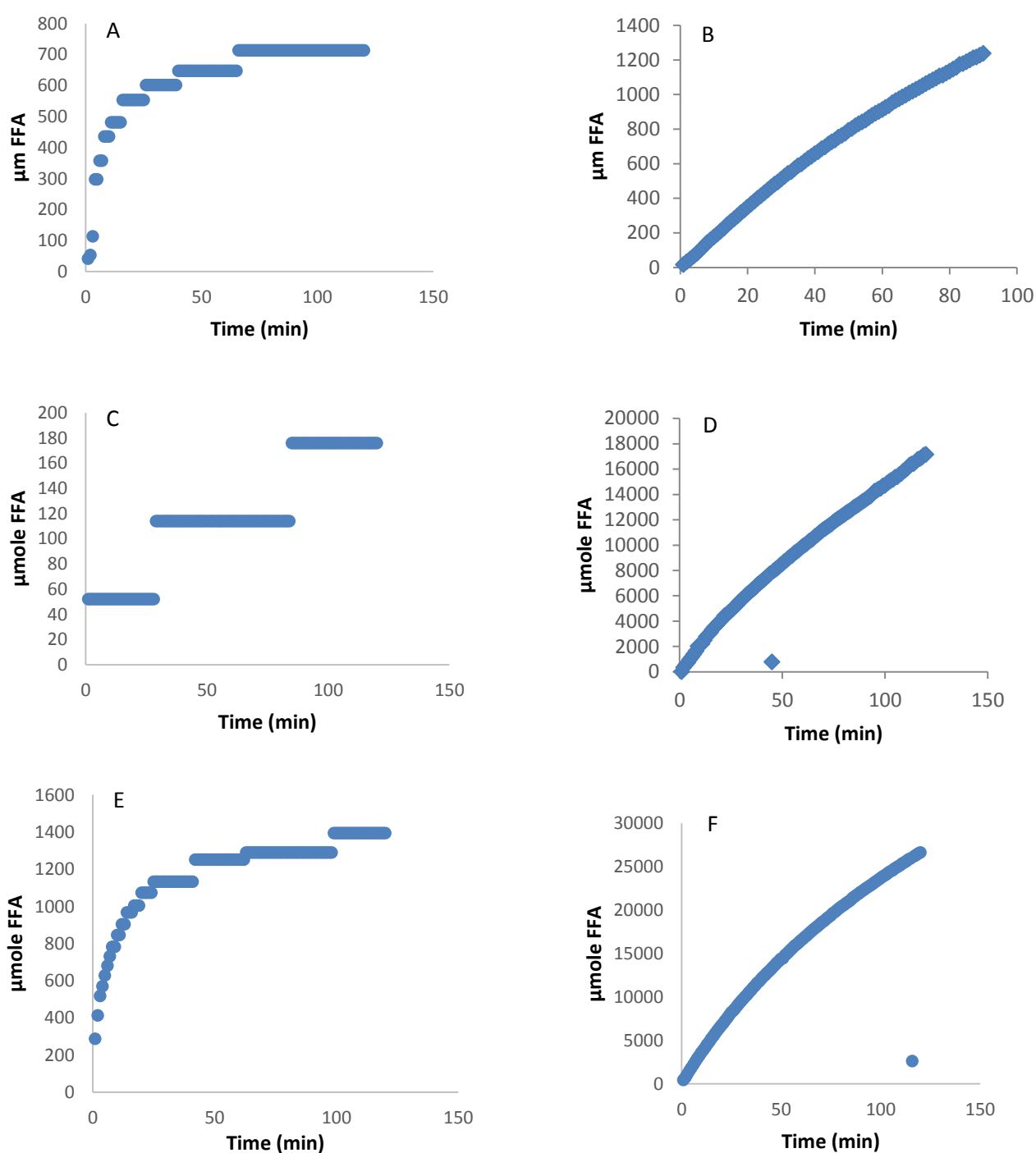


Figure 5.4. Rate of free fatty acid release (μ mole per min) from MGDG or Tributyrin in the presence of guinea pig PLRP2 or porcine pancreatin. The ratio of NaTDC to MGDG was 1.3 mole mole⁻¹; concentration of PLRP2 was 1.0 mg mL⁻¹ and 10 μ L was used; and that of pancreatin was 1.0 mg mL⁻¹ but 200 μ L was used. The pH was set at the maximum activity of gPLRP2 according to Amara et al. (2013). A: gPLRP2 against MGDG; B: gPLRP2 against Tributyrin; C: Pancreatin against MGDG; D: Pancreatin against Tributyrin; E: gPLRP2 and pancreatin combined against MGDG; gPLRP2 and pancreatin combined against Tributyrin.

De Caro et al. (2008), who investigated the existence of PLRP2 and other pancreatic lipases in various mammalian species found no evidence for the presence of PLRP2 in the pancreas of pigs; this explains the zero activity of porcine pancreatin against MGDG (Fig 5.3). However, it should be noted here that although PLRP2 protein could not be detected, and presumably not present in pancreatin, its mRNA is present in the porcine pancreas (Xiao et al., 2013). In the presence of colipase (to restore activity of pancreatic lipase inhibited by bile salts, phospholipids and proteins), conventional pancreatic lipase is preferentially active and hydrolyses triacylglycerol substrates, (Bezzine et al., 1999), whereas, PLRP2 is selectively active on substrates forming monomers or small aggregates (micelles) dispersed in solutions, such as monoacyl glycerols, phospholipids and galactolipids (Sias et al., 2004, N'Goma et al., 2012). Galactolipids are lipids of class II that normally form liquid crystals in water (vesicles and multilamellar liposomes), nevertheless, they can still form micelles similar to glycerophospholipids when they are mixed with bile salts (Amara et al., 2009, Small, 1968). However, PLRP2 (for triacylglycerol hydrolysis) from certain species including horse, coypu and guinea pig is inhibited by micellar bile salts concentrations and not reactivated by colipase (Lowe, 2002).

5.3.2. Activity of gPLRP2 in a Gastrointestinal Model System Against Galactolipid Rich Materials from Spinach CRF and Spinach WLM

For the first time, the activity of gPLRP2 against releasable galactolipids (via *in vitro* digestion), naturally occurring in spinach CRFs (wet or freeze dried) and WLMs was measured (see Fig. 5.5). For all the materials tested, fatty acids were released indicating that pPLRP2 is active against GLs in their natural state. The rate of fatty acid hydrolysis was higher in CRF than WLM samples, and the wet/fresh sample was a better substrate than the freeze-dried CRF sample, this might have something to do with the material dispersion during the simulated *in vitro* digestion. During the simulated digestion fresh CRF dispersed easily, but freeze dried CRF did not (aggregates of material persisted, see appendix C1-C2). The solidification of remnant sugar (grinding medium) may cause a hardening of the CRF material during freeze-drying. Given the similar concentration of GLs to bile salt (1.3) across the samples used in these assays (in the WLM and CRF incubation mixtures), the greater activity (~ 2.0 - 4.0 x, Fig 5.5) of gPLRP2 in the presence of CRF material compared with WLM suggests greater access to the GL substrate by the PLRP2 enzyme. This is probably due to the absence of cell walls impeding the access of the enzyme to the substrate.

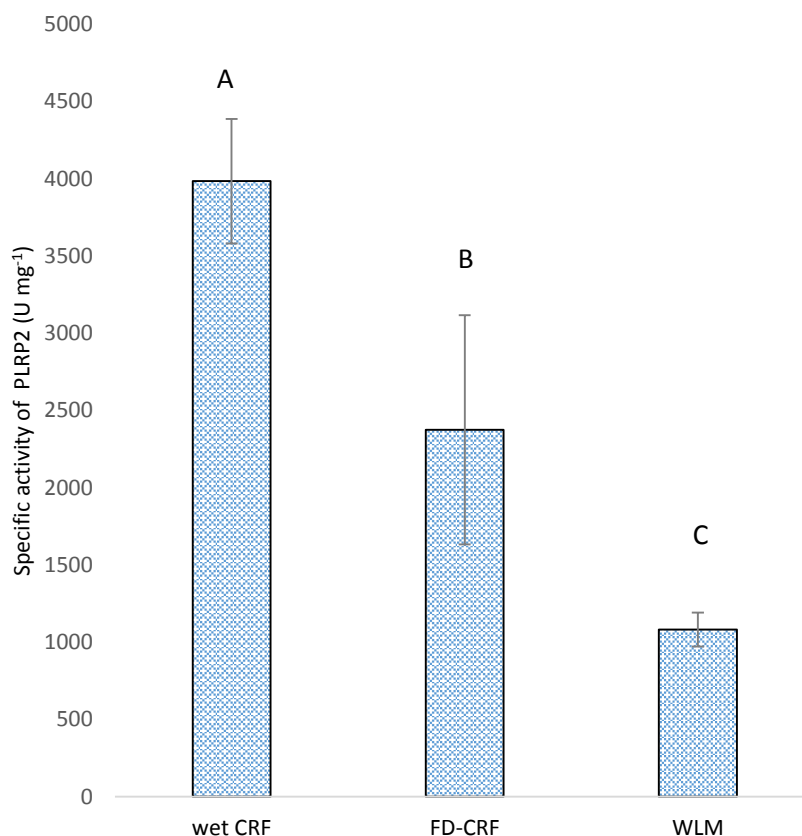


Figure 5.5. Specific activity of wet and freeze dried CRF, as well as WLM due to lipid hydrolysis by gPLRP2.

Since pancreatin showed almost no activity against galactolipids in the optimized system Fig.5.3 and 5.4C, we can assume that the free fatty acid release in this digestion model is caused by gPLRP2 activity. The initial rate of fatty acid released in these incubation mixtures by means of gPLRP2 activity is shown in Fig.5.5, and the kinetics over the incubation period is shown in Figure 5.6 (the trend over 2 hours remained the same after 20 minutes). It appears that across the samples, free fatty acids were released over the first 10-15 minutes and then reached a plateau. The reason for this was not tested but it is not unusual to see this sort of kinetic in *in vitro* models measuring lipase activity. The kinetics of gPLRP2-induced free fatty acid released from

freeze-dried material suggests a punctuated access to substrate, perhaps due to irregular material dispersion behaviour over the duration of the incubation.

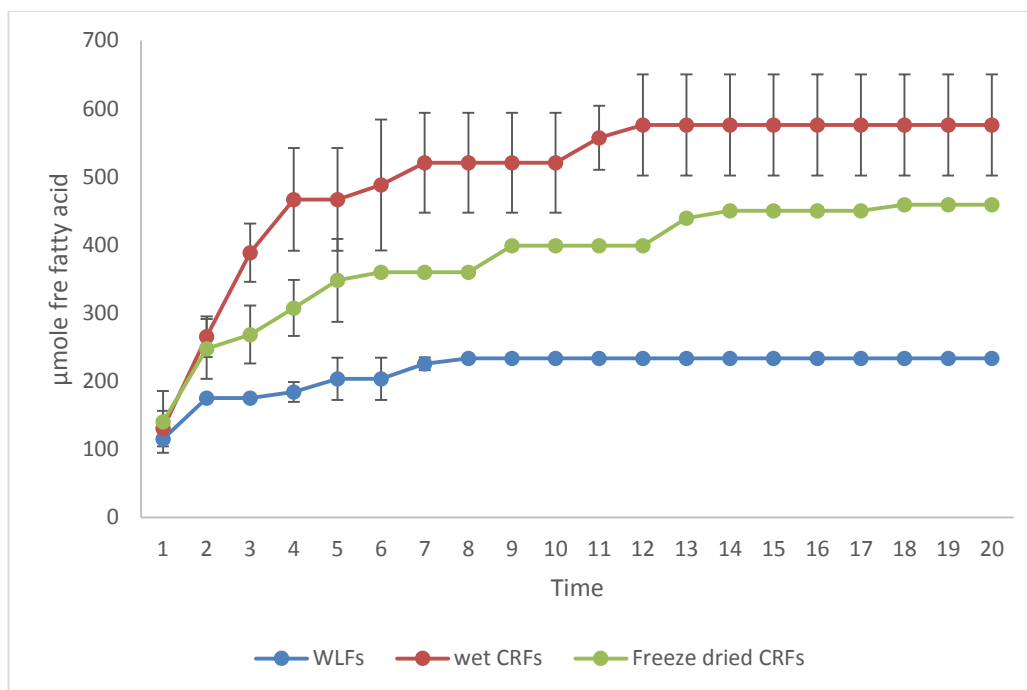


Figure 5.6. Free fatty acid release from leaf material and CRF material derived in the presence of PLRP2, during four hours of *in vitro* digestion.

Despite the smattering amount of gPLRP2 (10 μ L) with the low concentration (1 mg mL⁻¹) used, gPLRP2 indicated release of FFAs from the CRF and WLM *in vitro*, with CRFs (wet or freeze dried) showing higher FFA release compared to the WLMs (Fig.5.6). This volume and concentration of gPLRP2 was roughly based on the *in vitro* model used, as discussions on physiological conditions i.e. actual *in vivo* function of the enzyme towards GLs is still ongoing. The time required to digest most of the galactolipids was between 5-15 min depending on the sample type and homogeneity. After 15 min, and in certain rare cases 20 min, no further release FFA was apparent.

Kinetic reactions of the FA release becomes faster in the first few minutes, with low FA generation after 10 min. Since no lag time was observed for the gPLRP2 against commercial substrates (MGDG and Tributyrin) during the first few minutes, retardation of lipolysis due to the chloroplast thylakoids might explain the lower FFA released by the CRFs and WLMs. Studies suggest that there is strong binding between pancreatic lipase (with co-lipase) and the surface of thylakoid membranes, which in turn, induces to the prolonging of lipid digestion by inhibiting lipase-colipase activity temporarily. This may also be the case for PLRP2 and similar kinetic reactions i.e. binding between gPLRP2 and the surface of thylakoid membranes in the CRF/WLM might take place, because the two lipases (classical pancreatic lipase and PLRP2) display about 70% amino acid sequence homology (Carrière et al., 1998). However, since thylakoids comprise of proteins and lipids, they will eventually be hydrolysed in the gastrointestinal tract (Albertsson et al., 2007, Emek et al., 2013).

In a study designed to test thylakoid digestibility, Emek et al. (2011) reported that light harvesting proteins in thylakoid membranes resisted hydrolysis by proteolytic enzymes (e.g. pepsin, trypsin), and by gastric and pancreatic juice. Oil emulsified using thylakoid membranes was more resistant towards enzymatic hydrolysis.

A number of papers proposing thylakoids as agents for delaying lipolysis rate, and thus promoting satiety, have also been reported (Köhnke et al., 2009a,

Erlanson-Albertsson and Albertsson, 2015, Köhnke et al., 2009b, Stenblom et al., 2013).

Although PLRP2 was suggested to be the main hydrolysing enzyme of GLs (De Caro et al., 2004, Sias et al., 2004, Andersson et al., 1996), there is a paucity of data on PLRP2 activity against GLs *in vivo*. Ruminants, monogastric herbivores and omnivores consume plants and vegetables daily. However, their intake of galactolipids is quite variable. Therefore, their galactolipase levels during digestion differs considerably. For instance, guinea pigs can eat about 500-900 mg galactolipids per day, whereas, humans only consume approximately 150 -250 mg a day (De Caro et al., 2008, Andersson et al., 1996, Carriere et al., 1993). The pancreases of guinea-pig and coypu produce a classical pancreatic lipase but no detectable phospholipase A₂ activity. As a result, these studies proposed that PLRP2 from guinea-pig and coypu might only hydrolyse phospholipids *in vivo*. Furthermore, Roussel et al. (1998b) reported that PLRP2 from guinea-pig shows preference on phosphatidylethanolamine and phosphatidylglycerol but is also active on phosphatidylcholine.

Bile-salt stimulated lipase (BSSL) known also as carboxyl ester hydrolase (lipase) (CEH) poses similar broad substrate specificity as PLRP2 by hydrolysing TAGs, cholesterol esters; fat-soluble vitamin esters, phospholipids, galactolipids and ceramides (Bläckberg and Hernell, 1983, Hui and Howles, 2002, Nyberg et al., 1998). However, Li et al. (2007), reported BSSL to be the principal enzyme for fat digestion mainly when milk is the principal food,

emphasising that BSSL preferentially facilitates fat digestion in the new-born with lower bile salts concentrations in their intraluminal gut. This enzyme was also shown to have the capacity to hydrolysis galactolipids (Amara et al., 2009, Amara et al., 2013)

On the other hand, galactolipids have recently received attention as health promoters. This was mainly attributed to specific bioactive compounds in the galactolipids of vegetables and fruits mainly, MGDG galactolipid *1,2-di-O- α -linolenoyl-3- O- β -D-galactopyranosyl-sn-glycerol* (DLGG), which was reported to have anti-inflammatory and antitumor effects (Christensen, 2009, Larsen and Christensen, 2007, Murakami et al., 1995). Vegetables including spinach, sugar peas, kale, green beans, heart lettuce and broccoli exhibited greatest DLGG content (316-546 mg kg⁻¹) compared with other vegetables or fruits (Larsen and Christensen, 2007). The higher concentration of DLGG in dark green vegetables is consistent with the fact that chloroplasts are great sources for galactolipids. These studies have not explored whether PLRP2 has influence on the effective release of these bioactive galactolipids for their antitumor effect; and whether or not those animal species ingesting higher galactolipids and consequently secrete higher PLRP2 levels, would also result in health promoting effects in accordance with their lipid hydrolysing enzymes.

5.4. Conclusion

Activity and influence of PLRP2 on galactolipid substrates from commercial (pure Tributyrin and MGDG) or natural (spinach and its CRFs) sources were

carried out *in vitro* to mimic the role of PLRP2 in physiological conditions. A cocktail of porcine pancreatin was also run in parallel with the gPLRP2 and against the same substrates. Results indicated that, on one hand, gPLRP2 was mainly active on pure or naturally occurring galactolipids in spinach and its CRFs with moderate reaction against Tributyrin. On the other hand, pancreatin showed high activity on Tributyrin but minimal reaction against MGDG or those galactolipids from spinach and its CRFs, suggesting different substrate preference of each of these proteins towards the digested materials. Using wet CRF instead of freeze dried, more FFA could also be released by gPLRP2 probably due to the better homogeneity of the wet CRFs as compared to the freeze dried ones.

Chapter Six

Impact of Partial Replacement of Fish Meal With Chloroplast Rich Fractions on Zebrafish Growth and Fish Quality Indicators

6.1. Introduction

Chloroplasts rich fractions (CRF) and their parent whole leaf materials (WLMs) were recovered from spinach. The CRFs and WLMs were used to partially (10%, 20, and 50%) replace standard zebrafish feed, and their impact on a number of general fish performance parameters were evaluated against a control feed containing only standard zebrafish meal. These parameters included, growth rate, feed conversion ratio (FCR) [the relationship between diet input and the weight gain, which is an indicator of how efficient a diet or feeding strategy can be], fish meal ratio (FMR) [indicates the quality of fish meal from aquafeed necessary to produce 1 kg of culture species], fish in fish out (FIFO) [the amount of other fish (wild) it takes to produce farmed fish], specific growth rate (SGR) [the fish natural logarithm of the final fish weight subtracted by the natural logarithm of the initial fish weight over a period of time (days)], protein conversion ratio (PCR) [the amount of feed protein necessary to produce 1 kg of the culture species] and protein efficient rate (PER) [the weight gain of a test subject divided by its intake of particular food protein during the test period].

For growth rate parameters such as percentage fish weight growth and SGR, the higher values the better. Whereas for the rest of parameters such as, FCR, PCR (the closer to 1 the better), PER, FMR and FIFO, the lower the values are, the better the result is. For example, if the FCR value is low, this indicates that low amounts of the test material used for replacement, would yield the desired fish weight.

Ingredient data sheet for fish feeding is given in Table 6.1. Among ingredients of the control is wheat grain; others were confidential.

Table 6.1 Nutritional profile of experimental diets and percentage of fishmeal and experimental material inclusion in each experimental die

Nutrient, % in diet (DM)		Control	#10% FM (CRF)	#20% in FM (CRF)	#50% in FM (CRF)	#10% FM (WLM)	#20% FM (WLM)	#50% FM (WLM)
Crude Protein	% DM	46.56	45.29	45.82	46.38	45.38	45.35	45.63
Crude Lipid		11.30	11.72	12.43	12.09	8.56	8.64	8.79
Carbohydrate		35.06	35.01	33.99	34.19	35.27	34.67	33.28
Crude Ash		7.94	7.79	7.01	5.15	10.82	10.64	10.20
Crude Fibre		0.13	0.11	0.13	0.18	0.11	0.13	0.17
Linoleic Acid	% Lipid	17.88	8.19	8.90	12.33	11.78	13.39	16.78
Linolenic Acid		59.77	48.34	39.28	26.66	67.77	56.77	35.42
Arachidonic Acid		0	0	0	0	0	0	0
EPA		0	0	0	0	0	0	0
DHA		65.17	56.38	45.82	31.08	79.04	66.20	41.31
Arginine	% Protein	4.51	4.67	4.56	4.63	4.66	4.63	4.54
Histidine		1.98	1.77	1.73	1.71	1.82	1.78	1.71
Isoleucine		3.30	2.94	2.88	2.87	3.03	2.97	2.86
Leucine		6.17	6.26	6.17	6.26	6.21	6.22	6.17
Lysine		6.09	6.20	6.12	6.07	6.17	6.11	5.97
Methionine		1.25	1.28	1.27	1.25	1.28	1.28	1.27
Cystine		1.25	1.10	1.16	1.34	1.13	1.20	1.35
Phenylalanine		2.62	2.69	2.66	2.63	2.69	2.69	2.67
Tyrosine		2.62	2.69	2.66	2.63	2.69	2.69	2.67
Threonine		2.92	3.00	2.97	2.93	3.00	3.00	2.98
Tryptophan		0	0	0	0	0	0	0
Valine		3.44	3.53	3.49	3.45	3.53	3.53	3.51
α-Tocopherol	µg/g DM	7.87	90	110	120	10	10	20
Lutein		0	520	620	650	100	130	180
Gross Energy MJ/kg DM		22.55	20.29	20.31	20.66	20.46	20.39	20.33
Ingredient		Control	10% reduction in FM (CRF)	20% reduction in FM (CRF)	50% reduction in FM (CRF)	10% reduction in FM (WLM)	20% reduction in FM (WLM)	50% reduction in FM (WLM)
Fish Meal	% diet	38.74	34.76	29.96	19.77	35.59	30.09	19.10
*Rapeseed Oil		4.37	-	-	-	-	-	-
Vitamin premix		0.31	0.33	0.32	0.33	0.32	0.32	0.32
Mineral premix		0.42	0.43	0.43	0.44	0.43	0.43	0.42
Wheat Gluten		22.91	18.95	22.50	31.33	19.85	23.38	30.95
Corn Starch		31.82	28.04	26.12	25.85	29.97	28.75	26.12
Binder (CMC powder)		0.52	0.54	0.53	0.55	0.54	0.54	0.53
Chloroplast rich fraction		-	15.21	18.19	19.20	-	-	-
Whole leaf material		-	-	-	-	11.77	14.65	20.15
Arginine		-	0.28	0.34	0.55	0.22	0.32	0.51
Leucine		-	0.35	0.35	0.37	0.25	0.29	0.33
Lysine		0.90	1.10	1.26	1.61	1.04	1.21	1.55

* Rapeseed oil was not included in the experimental diets, however, wheat and other ingredients (naturally containing fat) were included. The ingredients were not given for their confidentiality; # the value was the reduction in fish meal (FM), i.e., 10% of the fish meal was, for instance, replaced the either CRF of WLM.

6.2. Growth and Performance

Freeze dried CRFs and WLMs from spinach, were fed to zebrafish as partial fish diet replacement (10%, 20% and 50%), and fish growth judged against a standard fish diet. The impact of CRFs and WLMs on fish growth performance parameters was evaluated.

Results indicated that both CRFs and WLMs had positive impact on the general well-being and performance parameters. No Significant differences ($P \geq 0.05$) were found in growth between the control diet and any of the experimental diets, although the diets with 10% fishmeal reduction, both including the CRF and WLM material, showed improved performance with a significant reduction ($P \leq 0.05$) in FCR, Diets including both CRF and WLM significantly improved FMR and fish in fish out (FIFO) compared to the control with increased fishmeal reduction, however, no such improvements were seen in SGR, PCR or PER. See Fig. (6.1) for in depth results. ta

Significant improvement of FCR, FMR and FIFO over the control diet were achieved through the inclusion of 10% CRF. A number of plants that are not conventionally consumed by human and probably undesirable for fish could be utilized for CRFs preparation, which may act as an acceptable form for food/feed and enriched in valuable nutrients at the same time. Chloroplasts are the site where numerous macro and micro-nutrients including protein (~50-60%), lipid (35-40%), and lipid soluble pigments such as chlorophylls,

carotenoids (including provitamin A and lutein) and tocopherols (5-10%) are formerly synthesised (Gross, 1991, Myers and Graham, 1956). Among the carotenoids and tocopherols are the antioxidant vitamins, provitamin A and vitamin E. Lutein, a xanthophyll carotenoid along with its co-isomer zeaxanthin also received greater attention in the recent decades for its significance in eye health. Consumption of these antioxidant carotenoids are related to a decreased risk of developing age related macular degeneration (AMD) (Seddon et al., 1994). During this trial, using CRFs and WLMs from spinach as partial replacement for fish feed, no fish was found dead. However, if CRFs or WLMs from non-traditional green plants is to be included, toxicity examinations would be necessary.

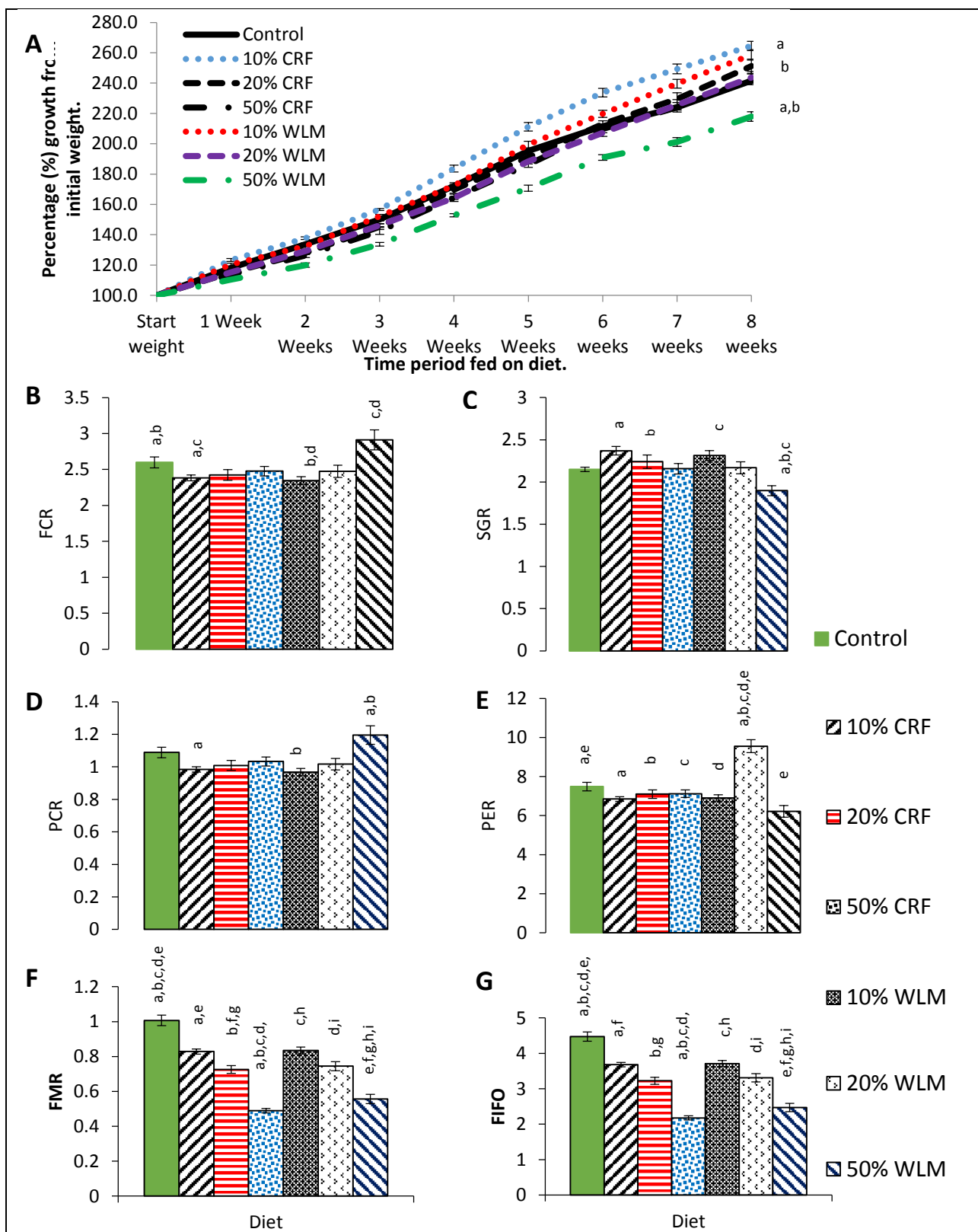


Figure 6.1 Mean ($\pm$ se) values for growth and performance indicators for fish fed with experimental diets (10%, 20% and 50% replacement of the standard fish meal). A) Percentage growth from initial weight. B) Feed conversion ratio (FCR). C) Specific growth rate (SGR). D) Protein conversion ratio (PCR). E) Protein efficiency ratio (PER). F) Fishmeal ratio (FMR). G) Fish in fish out ratio (FIFO). Within each figure section, diets that share a letter are significantly different ($P < 0.05$), for example figure 1B: the control diet is significantly different from the 10% CRF diet, marked with the letter a, and the 10% WLM diet, marked with the letter b.

6.3. Protein and Lipid Content

Protein content of the test trials as well as the control (the protein content measured after trial study of the zebrafish fed with the experimental or control diets was completed) was determined by analysing the composition of nitrogen in both test diets and control as presented in Fig.6.2. No significant difference in protein content ($P \geq 0.05$) between the modified diets and the control was apparent; this allows one to compare protein uptake data between trials (PCR and PER data – see Fig 6.1). In general these values are constant between the trial and control diets. Similarly, the lipid content in the zebrafish fed with different concentrations of feed trials from spinach CRFs and WLMs was presented in Fig (6.2) along with protein. Results indicate that no difference was obvious in lipid content among test trials (CRF and WLM) and between them and the control except for 50% CRFs, which showed significantly higher ($P \leq 0.05$) lipid ratio compared to the rest.

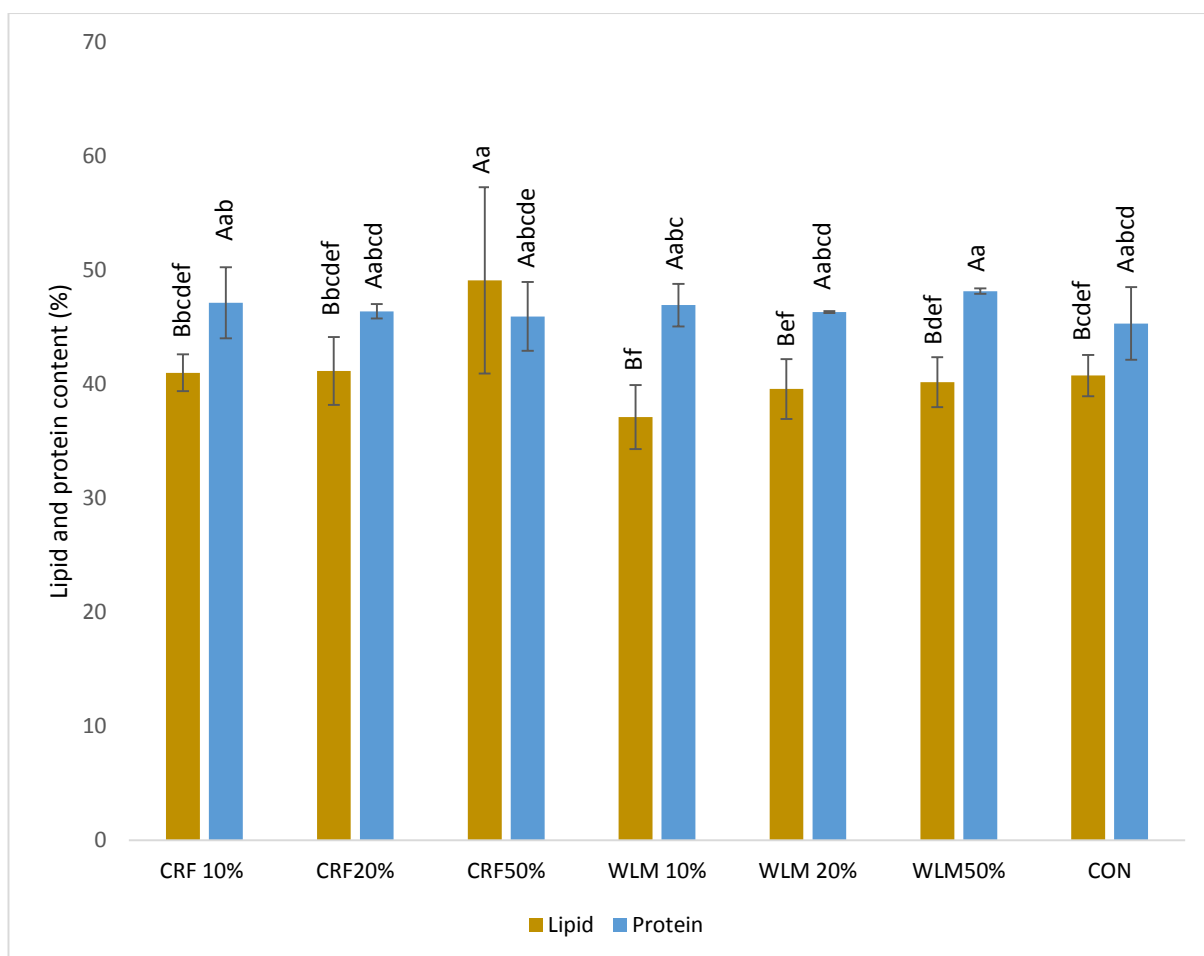


Figure 6.2. Lipid and protein content (freeze dried weight basis) of zebrafish trials compared to their controls. Means that share uppercase letters are not significantly ($P \geq 0.05$) different among each separate treatment (lipid or protein), and means that share lowercase letters are not significantly different between treatments (lipid versus protein).

6.4. Fatty Acid Composition

Fatty acid composition of zebrafish, fed the trial diets, is presented in Table (6.2). Fatty acid composition of fish reflects the fatty acid composition of the diet. For example, those fatty acids that were not originally the principle fatty acids in the CRFs and the WLMs such as oleic and linoleic acids (LA) showed significantly ($P \leq 0.05$) higher proportions in the control compared to the feed

trials. In contrast, major fatty acids in CRFs and WLMs such as palmitic acid (C16:0), hexadecatrienoic acid (C16:3) and α -linolenic acid (C18:n3; ALA) were all significantly ($P \leq 0.05$) higher in the zebrafish feed trials (mainly in CRFs modified diets but also in WLMs test diets) compared to the control. In addition, there is evidence of the conversion of some of the α -linolenic acid (C18:n3), to other very long chain omega-3 poly unsaturated fatty acids (PUFAs) such as, eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA) by the zebrafish (Table 6.2). For this, the content of EPA in the test samples was often greater and distinct from those of the control. Specifically, using 10% and 20% of CRF enhanced the EPA content in the zebrafish and it was significantly ($P \leq 0.05$) higher than the control (Table 6.2). This could be further explained by the EPA and DHA variations in zebrafish fed with test samples and the control as a result of LA to ALA ratios presented in Table (6.2). Studies suggest that in humans (and probably zebrafish, expressing similar functional fatty acyl desaturases, $\Delta 6$ and $\Delta 5$ to those in human, which are essential enzymes for the biosynthesis of PUFAs) 8% and 4% of the essential fatty acids such as α -linolenic acid is converted into EPA and DHA, respectively (Kris-Etherton et al., 2002). For this to take place, the balance between LA to ALA fatty acids in the diets is important (Budowski and Crawford, 1985), as LA and ALA compete for the same elongase and desaturase enzymes in the synthesis of EPA and DHA (Burdge and Calder, 2005). Conversion of ALA to EPA and DHA is disrupted and lowered when the ratio of LA to ALA is high (Stark et al., 2015). Harnack et al. (2009) reported that the rate of EPA and DHA formation was greatly increased to its maximum when the ratio between LA and ALA

was 1:1 or close to this point. The ratio of LA and ALA in CRF diets is, 1.5:1.0, 1.5:1.0 and 1.8:1.0, respectively, for 10%, 20% and 50% modified diets. However, similar modified diets from WLMs resulted in a ratio of 3.5:1.0, 3.3:1.0 and 3.1:1.0, which is not as balanced as those modified diets from CRF, with the control showing the least balanced ratio (5.0:1.0) between LA and ALA in zebrafish.

Therefore CRF is close to the proposed ideal balance between LA and ALA for converting C18 fatty acids to PUFAs; which may explain the relatively higher long chain omega-3 fatty acid accumulation in zebrafish (see Table 6.2).

Table 6.2. Fatty acid composition (mg g⁻¹) of whole body zebrafish fed with various test diets

Fatty acid (mg g ⁻¹ DW)	CRF 10%	CRF 20 %	CRF 50%	WLM 10%	WLM20 %	WLM50%	Control
C14:0	2.2±0.0 ^A	2.1±0.0 ^A	1.8±0.0 ^{BC}	2.1±0.1 ^A	2.1±0.0 ^A	1.7±0.0 ^C	1.9±0.0 ^B
C16:0	24.9±1.8 ^{CD}	27.2±0.3 ^{AB}	28.9±0.1 ^A	23.2±1 ^{DC}	26.3±0.4 ^{BC}	26.2±0.9 ^{BC}	21.5±0.3 ^E
C16:1n-7	2.7±0.2 ^{AB}	2.8±0.0 ^{AB}	2.6±0.0 ^{BC}	2.7±0.1 ^{ABC}	2.9±0.0 ^A	2.2±0.2 ^D	2.4±0.1 ^{CD}
C16:3 n-3	0.9±0.0 ^B	1.1±0.0 ^A	1.1±0.0 ^A	0.3±0.0 ^E	0.4±0.0 ^D	0.4±0.0 ^C	0.03±0.0 ^F
C18:0	4.3±0.6 ^A	4.6±0.1 ^A	4.4±0.2 ^A	4.7±0.3 ^A	4.8±0.2 ^A	4.7±0.3 ^A	3.0±0.0 ^B
C18:1 n-9c	18.4±1.7 ^{AB}	17.8±0.2 ^{BC}	19.7±0.4 ^{AB}	15.7±0.7 ^C	18.0±0.0 ^{AB}	17.6±0.8 ^{BC}	20.2±1.4 ^A
C18:2 n- 6c	9.9±1.3 ^D	11.8±0.0 ^C	13.7±0.0 ^{AB}	11.4±0.7 ^{CD}	12.9±0.0 ^{BC}	12.8±0.8 ^{BC}	15.0±0.5 ^A
C18:3 n-3	5.7±0.9 ^B	7.5±0.1 ^A	7.3±0.0 ^A	3.2±0.1 ^{DE}	3.9±0.0 ^{CD}	4.1±0.2 ^C	3.0±0.0 ^E
C20:4 n- 6	0.55±0.0 ^A	0.5±0.0 ^A	0.5±0.0 ^A	0.5±0.0 ^A	0.5±0.0 ^A	0.6±0.0 ^A	0.5±0.0 ^A
C20:5 n- 3 (EPA)	1.9±0.2 ^A	1.8±0.0 ^A	1.4±0.0 ^C	1.8±0.0 ^A	1.7±0.0 ^{AB}	1.4±0.0 ^C	1.5±0.1 ^{BC}
C22:6 n- 3 (DHA)	6.6±1.4 ^{AB}	7.3±0.0 ^A	6.2±0.1 ^{AB}	6.8±0.2 ^{AB}	6.7±0.0 ^{AB}	5.6±0.3 ^B	6.0±0.1 ^{AB}
ΣSFA	31.4	33.9	35.1	30	33.2	32.6	26.4
ΣMUFA	21.1	20.6	22.3	18.4	20.9	19.8	22.6
ΣPUFA	25.55	30	30.2	24	26.1	24.9	26.03
Σn-6 FA	10.45	12.3	14.2	11.9	13.4	13.4	15.5
Σn-3 FA	15.1	17.7	16.0	12.1	12.7	11.4	10.5
ΣLA:ALA	1.7:1.0	1.5:10	1.8:1.0	3.5:1.0	3.3:1.0	3.1:1.0	5.0:1.0

DW, dry weight; **C14:0**, Myristic acid; **C16:0**, Palmitic acid; **C16:1**, Palmitoleic acid; **C16:3 n-3**, Hexadecatrienoic acid; **C18:0**, Stearic acid; **C18:1n-9c**, Oleic acid; **C18:2 n-6c**, Linoleic acid (LA); **C18:3 n-3**, α-Linolenic acid (ALA); **C20:4 n-6**, Arachidonic acid; **C20:5 n-3**, Eicosapentaenoic acid (EPA); **C22:6 n-3**, Docosahexaenoic acid (DHA). Means that do not share a letter within each row are significantly different; FA: fatty acid; SFA: saturated fatty acids; MUFA: mono unsaturated fatty acids; PUFAs: poly unsaturated fatty acid

In fresh water fish dietary linoleic acid in the phospholipid fraction is elongated and desaturated, and converted to arachidonic acid (ARA) and osbond acid (Docosapentaenoic acid, C22:5 n-6); whereas, linolenic acid is elongated and desaturated to DHA also in the phospholipid fraction (Meinelt et al., 1999). However, not all fish species have the ability to convert C18 fatty acids into PUFAs (Monroig et al., 2013). Zebrafish are among those that are able to elongate and desaturate both n-3 and n-6 FA, as well as the total FA obtained from the diet. They express two functional fatty acyl desaturases, $\Delta 6$ and $\Delta 5$, which are essential enzymes for the biosynthesis of PUFAs (ARA, EPA and DHA), and with similar homology to those of humans (Hastings et al., 2001).

Fatty acids also have an influence on zebrafish embryonic improvement (Meinelt et al., 1999). The impact of different levels of dietary PUFAs on tissue fatty acid and reproductive performance in female zebrafish was conducted; in the study, different PUFAs rich diets (%100 squid, 100% flaxseed oil and 1:1 ratio of squid: flaxseed) was considered (Örn et al., 1998). Highest egg production and hatching rate was found with the diet 1:1 (squid: flaxseed), suggesting the necessity of balanced ratio of dietary n-6 and n-3 (LA: ALA) FA for spawning females.

On the other hand, fatty acids such as C18:3 alpha linolenic acid (omega-3) and C16:3 (omega -3) are predominantly found in green leaves and their chloroplast membranes; to the extent that they are used for the categorization of certain green plants as being C18:3 or C16:3 plants. The significantly ($P \leq 0.05$) lower profile of C18:3 n-3 and probably negligible concentrations of C16:3 n-3 shown by the control compared to the

feed trials may suggest use of C16:3 n-3 as an indicative marker of feed transition into the fish body. This indicates that the diets, notably CRF were digested and the nutrients were absorbed from the feed so contributing to the final fatty acid accumulation in the fish body.

The findings therefore suggests that zebrafish possess lipases responsible for the lipolysis of galactolipids, so PLRP2 could well be present in the zebrafish. 'Gene Tree' (<http://www.ensembl.org>) was used to scan the database for zebrafish to look for genes that might code for PLRP2 protein. The best gene match for the PLRP family of lipases showed 19% homology. Further studies are required to confirm the presence of active PLRP2 in zebrafish.

6.5. Vitamin E Content

The content of vitamin E in zebrafish fed with different diets during 8 weeks trial is presented in Fig. (6.3). Out of four different tocopherol standards analysed for vitamin E (α , β , γ and δ - tocopherols), against test samples, α -tocopherol (α -TOC) was the only component detected in quantifiable amounts in the samples; others were either trace or not detected at all. Since α -TOC is the major form of vitamin E existing in nature, and the most biologically active (Bjorneboe et al., 1990), only α -TOC data was considered in this study. Data in the figures indicates that α -TOC in the 50% diets was significantly greater than those of 10% and 20% feeds; α -TOC in the 50% diets was also slightly greater than that of the control, but not significantly. The 20% replacement by portion in the control was, however, lower than the control

significantly. In general, the best source of plant vitamin E is commonly oil seeds rather than green leaves, so it is not surprising that α -TOC was not significantly enhanced by those feed trials from CRFs and WLMs.

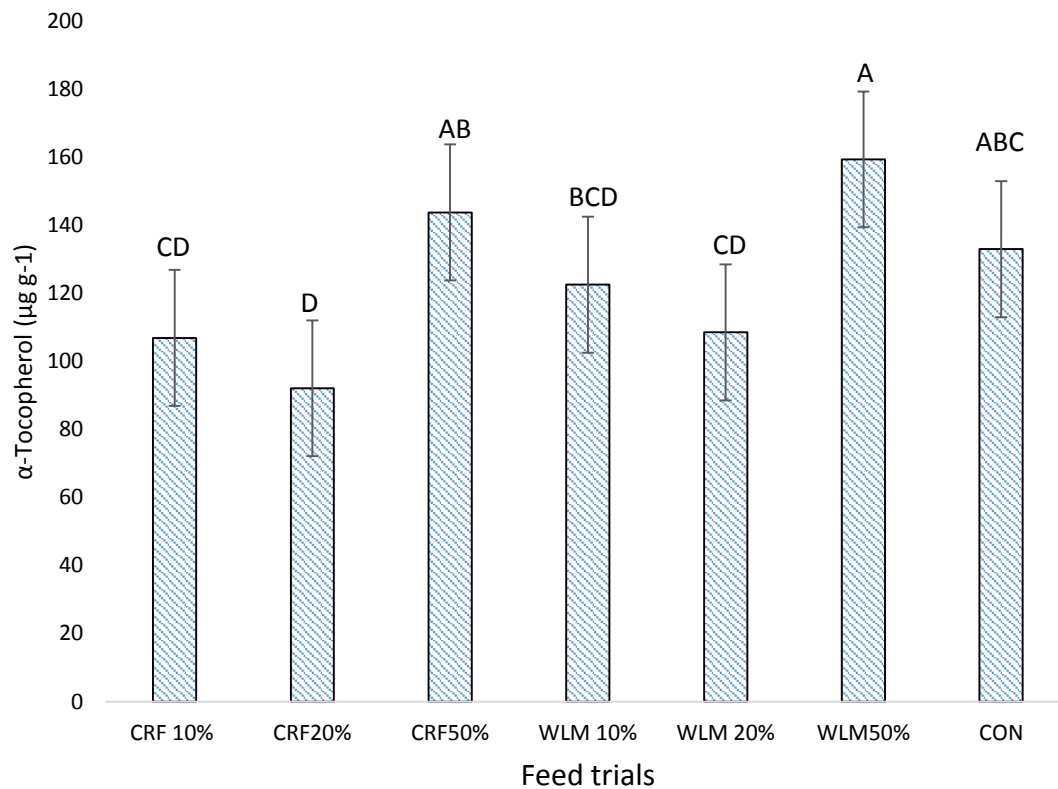


Figure 6.3. Vitamin E (α -tocopherol) content in diet trials fed to zebra fish. Means that do not share a letter between each column in the chart are significantly different.

In vertebrates including fish, α -TOC seems to be preferentially deposited in the body compared to the rest of tocopherols apparently due the existence of tocopherols transfer protein (TTP) in the liver, a protein that discriminates between tocopherol with regard to their methyl group number on the chromanol ring and R-configuration; this protein works to bend the tocopherols of different affinities and brings them back to the circulation i.e. TTP contributes to the secretion of α -TOC from the liver into the blood stream, while major fractions of other tocopherols become lost in the bile

(Hamre, 2011). PUFAs and vitamin E content in fish are inter-related. Lebold et al. (2011) found that zebrafish deficient in vitamin E accumulated lower amounts of the PUFAs (DHA and EPA) and accounted this for three potential factors such as, decreased formation, inadequate availability of essential fatty acids as substrates or increased lipid peroxidation. Authors have ruled out the first and the second grounds in their discussion, and backed the later one (lipid peroxidation) to be the main reason why PUFAs were lowered.

6.6. Carotenoid Content in Zebrafish (Whole)

Beta-carotene and lutein are the major carotenoids in spinach and its recovered chloroplasts, whilst astaxanthin is the prominent carotenoid in many fish species (mainly marine fish) as a result of conversion of dietary carotenoids (from aquafeeds or natural diets e.g. marine algae) into astaxanthin in fish *in vivo*. Beta-carotene was not detected in both the trials and the control; and quite low concentrations of astaxanthin (data not given) was detected only for 10% and 20% CRFs but not for the rest of the zebrafish trials or the control. Lutein, was however, the major carotenoid in the whole body zebrafish; and its concentrations varied depending on the type of the feed and the concentration. Feeds from CRFs initially (in the raw materials) containing higher carotenoids than the WLMs (section 3.7), showed also higher lutein content in the zebrafish (Fig.6.4). It should be noted here that by chloroplast content, 10% CRF was approximately equivalent to 50% WLM.

TanakaYoshito et al. (1976) reported that lutein was the major pigment in salmon and trout if the diet had contained plant carotenoids.

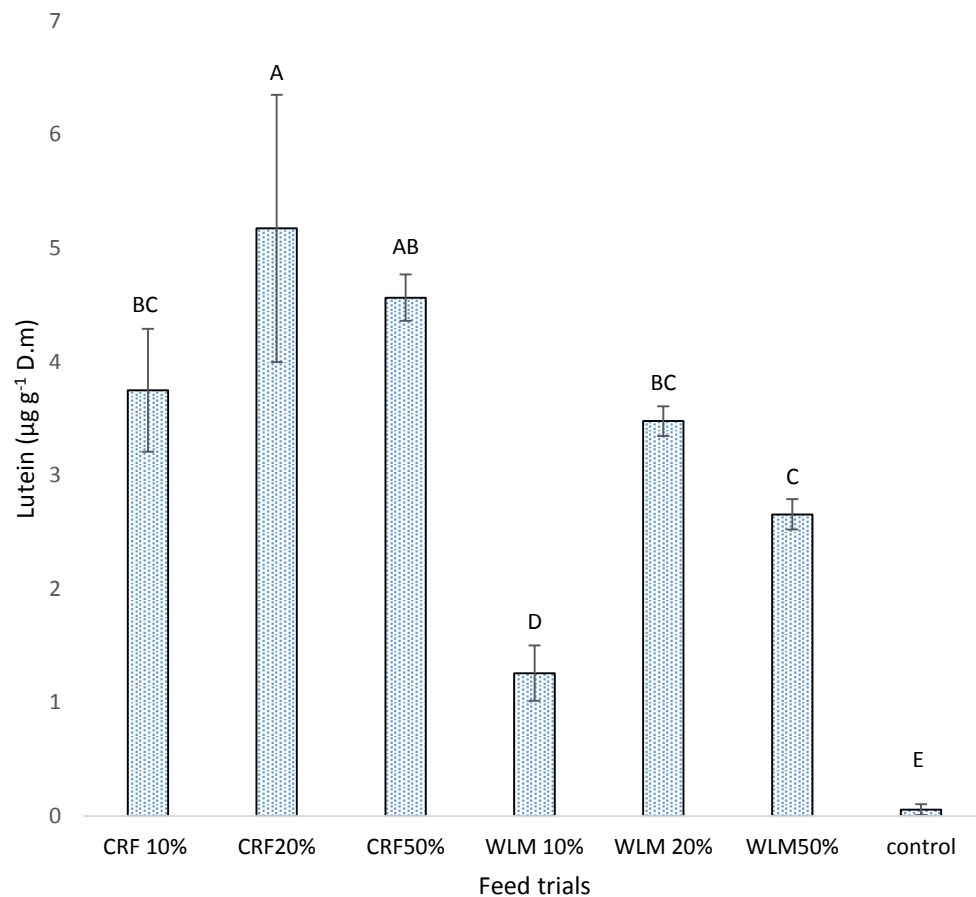


Figure 6.4. Lutein content of zebrafish (whole fish) fed with different concentrations of CRFs and WLMs

In fresh water fish, β -carotene is converted into retinoic acid and in some cases into retinol, whilst lutein is converted into anhydrolutein, which, in turn, is cleaved to dehydroretinol (vitamin A₂), the common form of vitamin A found in most fresh water fish (Barua and Goswami, 1977).

The reason why β -carotene was not detected in zebrafish might therefore be due to its conversion into retinol and retinoic acid.

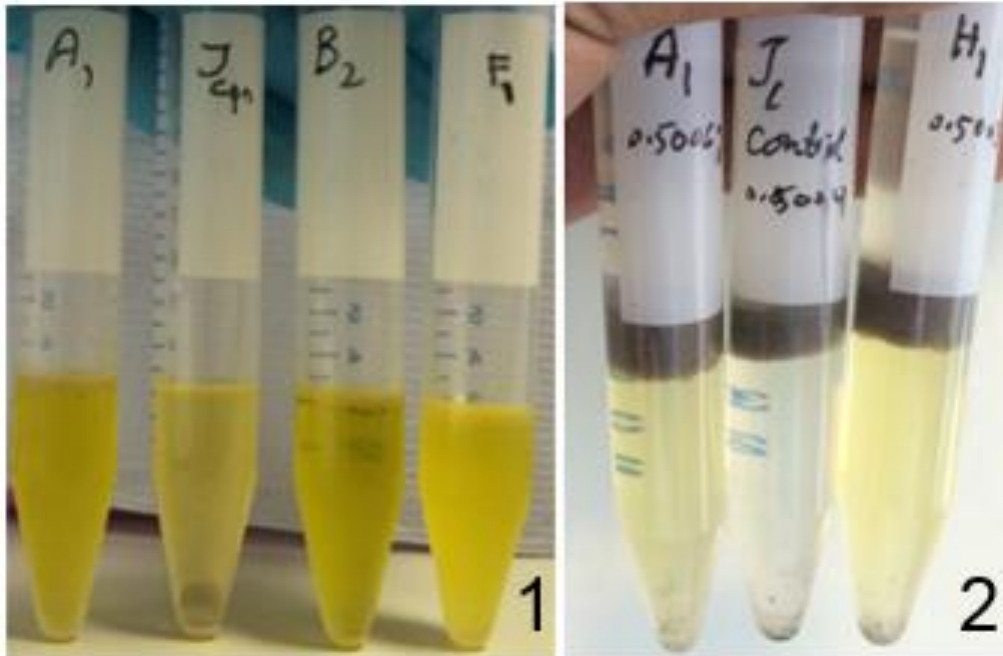


Figure 6.5. First (dense orange) and second (light orange) zebrafish flesh lipid extraction: A1: 10% CRF; J con: control; B2: CRF 20%; F1: 50% WLM

When the lipids were extracted, more intense orange colour in test samples compared to the control was also observed Fig.6.5. As carotenoids are partially lipid soluble pigments mainly orange in colour, it is realistic to obtain more intense yellow/orange colour in test samples compared to the control.

6.7. Zebrafish Egg Carotenoids

During the feeding trial it was noted that feed composition has a significant impact on fish egg colour (Fig.6.6).

The yellow colour in the eggs is presumed to be due to embryonic accumulation of carotenoids by the zebrafish, and as concentration of the carotenoids is increased in the test diet, the intensity of the yellow colours is also increased i.e. CRFs initially containing increased carotenoid content per unit mass showed more intense yellow colour compared with the WLMs. However, the orange colour was not always linear with the carotenoid concentration among CRFs. For instance, the 10% and 20% CRF indicated noticeable more intense orange colours than the 50% CRF and more intense than all WLM enriched feeds. The control samples, on the other hand, showed no visible orange colour; justifying the yellow colour to be completely attributed to the test samples originally rich in carotenoids.

Similar tendency where 10% and 20% display better performance than the 50% in growth rate (Fig.6.1) and in certain fatty acids (Table 6.2) was also observed.

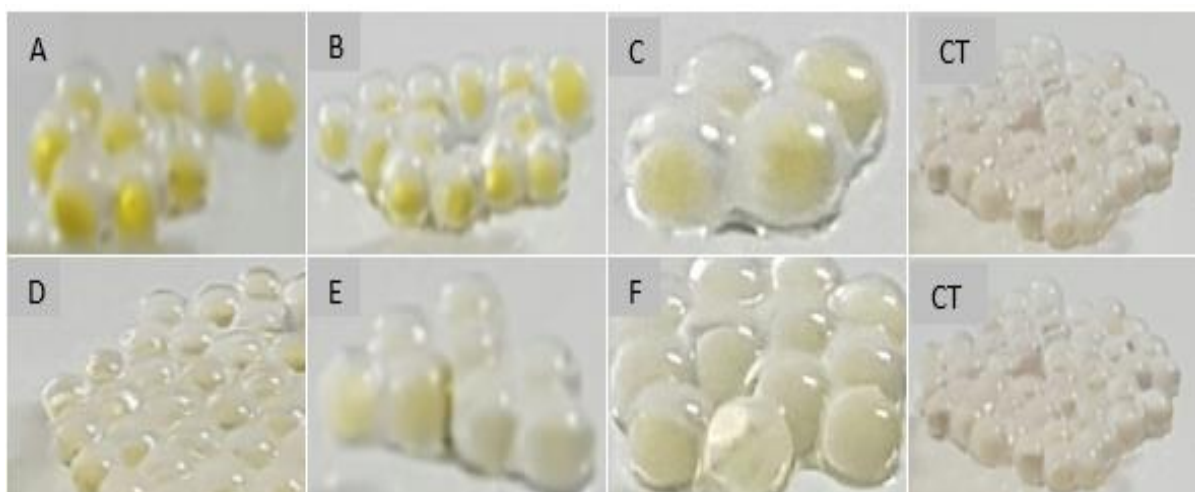


Figure 6.6. Egg colour variations depending on the zebrafish feed type; A: 10% CRF; B: 20% CRF; C: 50% CRF; CT: control; D: 10% WLM; E: 20% WLM, F: 50% CRF. These images were taken with SAMSUNG Galaxy note.3.

Besides the wild zebrafish, experiments and control diets were also fed to a gold zebrafish as shown in Fig.6.7. Although, CRFs diet response to egg colours of gold zebrafish is not as clearly orange in colour and distinguishable as those in Fig 6.6 (the wild zebrafish), the effect of the feed composition is still noticeable as both CRFs and WLM colours are evidently different from the control. Interestingly, the orange colour is clearly accumulated in the fish embryo area (the eggs) compared to the rest of fish body (see female column in Fig.6.7), whereas the pigment accumulates in the flesh of the male fish

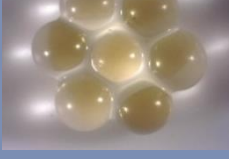
Diet	Male	Female	Embryo
Control			
10% WLM			
20% WLM			
50% WLM			
10% CRF			
20% CRF			
50% CRF			
Scale	 1cm		 1mm

Figure 6.7. Zebrafish (golden) egg pigmentation and colour variation due to carotenoid accumulation in response to carotenoid- rich diets.

In addition to the qualitative investigations of zebrafish eggs, quantitative measurements were also carried out. As expected eggs from fish fed, a diet enriched with green-leaf derivative material had a higher concentration of carotenoids (lutein) (Fig.6.8). HPLC analysis confirmed that out of the three carotenoids analysed (Lutein, β -carotene and astaxanthin) lutein pigments is the major carotenoid present in the eggs ; the latter two carotenoids were at trace levels or undetectable..

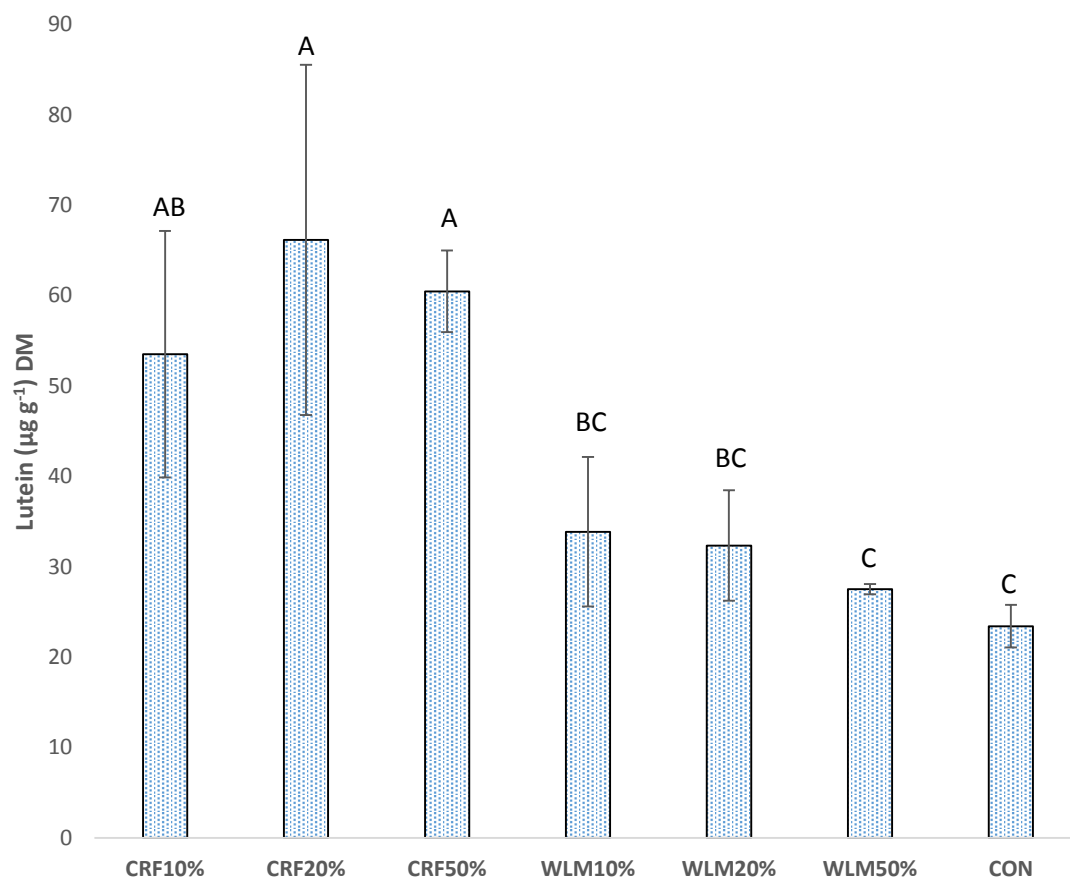


Figure 6.8. Lutein content in zebrafish eggs (freeze dried weight basis) after consuming diets enriched with a chloroplast rich fraction (CRF) or whole leaf materials (WLM)

In contrast to the results from the whole body extract, here both CRF and WLM feed trials showed significant difference ($P \leq 0.05$) as compared to the control (Fig.6.5), it was only the CRF-fed fish that developed lutein-loaded eggs that were significantly distinct ($P \leq 0.05$) from the control (Fig.6.8). It is evident that the concentration of

lutein in the whole body zebrafish is low (from around 0.1-5 mg kg⁻¹ DW) compared to that of zebrafish eggs (23-66 mg kg⁻¹DW (see Figs. 6.5-6.8). This supports the hypothesis that carotenoids, lutein, for example, are released out of their plant matrix and absorbed by the zebrafish to be bioavailable *in vivo*, and that chloroplasts released from the cell matrix (CRF) improves carotenoid uptake by the fish.

Lubzens et al. (2003), attributed the bright yellow or orange colour eggs of salmonids and of other fish to the presence of carotenoids. Lampert et al. (2003b), also reported that provitamin A and non-provitamin A carotenoids exist in the egg yolk of zebrafish. These authors have also found that provitamin A carotenoids in the zebrafish were converted to retinoids (retinal) via β -carotene-15,15-oxygenase (BCO) and existed in the egg yolk but were concentrated mainly in the retina of the eye. The authors revealed that retinoic acid generation, necessary for various developmental processes, depends on local *de novo* formation of retinal from provitamin A via bcov; concluding that carotenoids have essential role in embryonic development.

Although the earlier *in vitro* work indicated that lutein was significantly ($P \leq 0.05$) more bioaccessible from WLMs compared to CRFs (section 4.3), this was not the case when the same samples were fed to the zebrafish. Higher lutein concentrations in fish flesh and eggs were achieved on consuming CRF-enriched feed, suggesting probable limitations of the *in vitro* model. Due to numerous complex physiochemical and physiological factors involving in the animal and human digestive tracts, results obtained using *in vitro* digestion models are often different from those found by *in vivo* models (Hur et al., 2011).

6.8. Retinol Content in Whole Zebrafish

The concentration of all-trans retinol in the freeze dried whole body zebrafish in response to different experimental and control diets was quantified. Results indicate that all test diets increased the retinol concentration by 3-8 x compared with the control, (Fig. 6.9). The test diets showed little significant difference between each other, except for the 20% WLM material which caused the highest increase in fish flesh retinol concentration ($3.5 \mu\text{g g}^{-1}$ DW compared with $0.4 \mu\text{g g}^{-1}$ DW in the control). These results suggest that the provitamin A carotenoids from the diet were converted into retinol *in vivo* by the zebrafish. The reason why 20% WLM supplemented diets result in the highest increase is not clear at this stage.

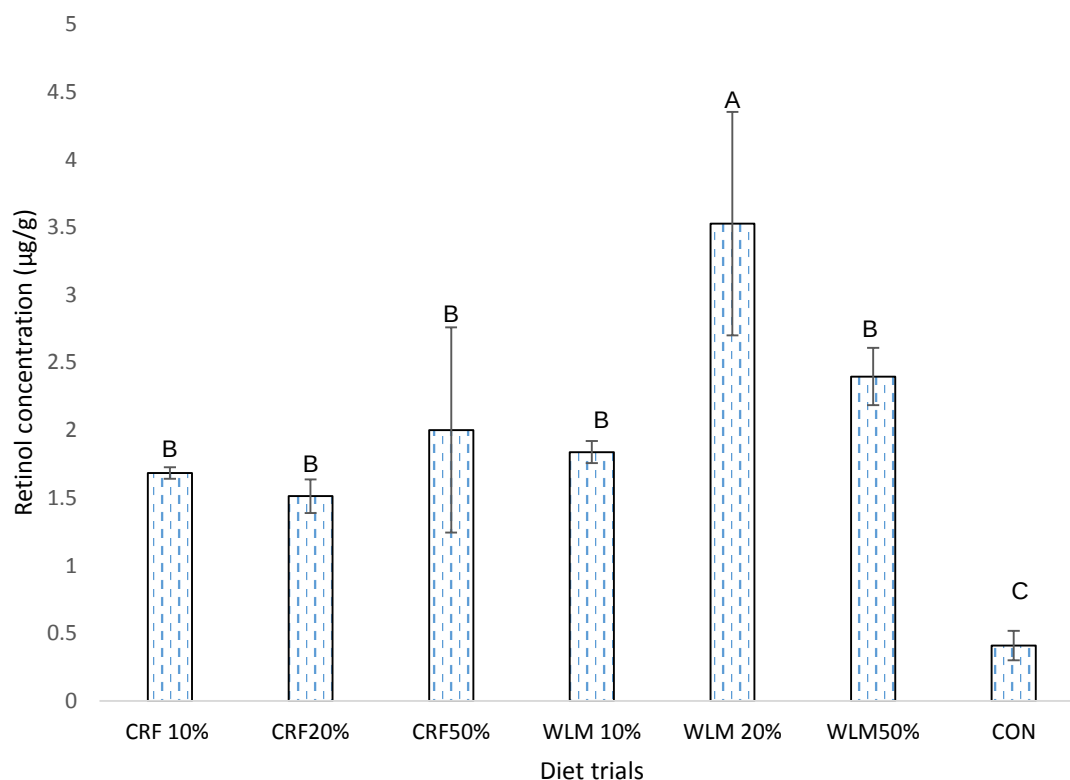


Figure 6.9. Retinol concentration in zebrafish fed with different concentrates of diet trials and control

Two types of carotene oxygenase: β -carotene-15,15-oxygenase (BCO) and β -carotene-9,10-oxygenase (BCO-II) in zebrafish was cloned by von Lintig and Vogt (2004) and confirmed that the existence of these enzymes are not restricted to mammals. Von Lintig and co-authors, further examined the consequence of loss of BCO function during zebrafish development and found malformations in the architecture of the branchial arch skeleton and the eye thus suggesting the significant role of BCO in zebrafish. Similar findings of BCO significance for provitamin A conversion to retinal in zebrafish was published (Lampert et al., 2003b).

Animals cannot synthesize retinoids *de novo* as such, they use plant derived C₄₀ carotenoids as their major source for retinoids by means of enzymatic (BCO BCO-II) cleavage (von Lintig and Vogt, 2004). Dietary carotenoids absorbed through the intestinal epithelia are carried into the circulatory system in association with lipoproteins to turn them into retinoids (vitamin A). Studies suggest that retinoids are essential to fish and their deficiency causes mortality, anemia and reduced growth (Taveekijakarn et al., 1994, Alsop et al., 2008). Besides the other essential roles that retinoids (vitamin A) play in vertebrates, such as vision, development, and genetic functions, Alsop et al. (2008) found that retinoids also have an influence on the reproduction of zebrafish, however, storage levels of retinyl esters was apparently of little significance. Alsop and co-authors examined the importance of retinoids in the reproduction of zebrafish using retinoid rich and deficient diets. Reduction of whole body retinoids by 68% in females and 33% in males was obtained with the retinoid deficient diet. In addition, females fed with the retinoid deficient diets produced significantly fewer eggs than that contained retinoid or the control.

6.9. Conclusion

Fish diets enriched with green-leaf derived material (chloroplasts rich fractions isolated from spinach and its parent powdered 'whole' leaves) at particular inclusion amounts improved growth rates and general performance parameters of zebrafish. Selected markers of chloroplast digestibility (signature fatty acids and carotenoids) were absorbed into the body of zebra fish. α -linolenic acid from the chloroplast appears to

be not only absorbed by the fish, but converted to very long chain poly unsaturated fatty acids mainly EPA; this represents a significant increase in this highly desirable FA compared with fish fed the control diet. Vitamin E content in the 50% test trials (i.e. 50% CRF and 50% WLM) were slightly greater than the control but not distinct. Accumulation of carotenoids mainly lutein was detected in the zebrafish whole body and eggs. CRF trial increased lutein concentration compared with WLM and the control. Similarly, the retinol content in the test samples was greater than that of the control. Findings indicate that chloroplast rich fractions were adequately digested and vital nutrients in the diets converted into potentially important component *in vivo* by the zebrafish. These chloroplast rich fractions are widely available in traditionally and non-traditionally consumed green plants, as well as, waste green materials.

Chapter Seven

General conclusions and suggestions for future studies

7.1. General conclusions

Nutrient composition of chloroplasts rich fractions (CRFs) and whole leaf materials (WLMs) from four green leafy plants including spinach and kale (traditional table vegetables) as well as, stinging nettles and grass (unconventional species for direct human consumption) were characterized for various macro and micro- nutrients. These included total proteins and their amino acids, total lipids and their fatty acids, and total chlorophylls ($a+b$) and carotenoid pigments. Compounds such as, α -tocopherol (vitamin E), β -carotene (provitamin A), lutein (antioxidant and important for eye health), ascorbic acid (vitamin C), and minerals (major and trace) were analysed.

In addition, digestibility of CRFs from spinach and its parent leaves i.e. WLM was examined. The bioaccessibility of β -carotene and lutein from the spinach CRFs and WLM was also determined. This was done either with the addition of oil or without oil treatment. The freeze dried CRFs used in this case were either prepared with sucrose or extracted with juicer.

Further, the impact of pancreatic lipase related protein 2 from guinea pig (gPLRP2) on the hydrolysis of galactolipids (the major form of lipids found in chloroplastic tissues

of green leaves/plants) was assayed. Before, the lipolysis were carried out, composition of glyceroglycolipids i.e. monogalactosyldiacylglycerols (MGDG), digalactosyldiacylglycerols (DGDG) and sulfoquinosyldiacylglycerol (SQDG) were qualitatively and quantitatively determined so that ratio of galactolipid: bile salt (mole to mole) be calculated. Pure MGDG and triacylglycerol rich Tributyrin was also used along with the samples using pH-stat to determine the activity of gPLRP2 and that of pancreatin.

The effect of different concentrations from CRF and WLMs (10, 20 and 50%) reduced from that of fish meal on growth rate and performance parameters as well as, carotenoids uptake by zebrafish trials was carried out. The influence of these materials on egg colour was also examined and control trials run along with the experiment trials.

Compositional analysis of the material indicated various vital compounds present in CRF and WLM. Greater concentrations of total proteins, total lipids, total chlorophylls and carotenoids were present in the CRFs as compared to the WLMs. Higher amounts of α -linolenic acid (ω -3) and the antioxidant vitamins including, α -tocopherol, β -carotene and lutein were also found in CRFs compared to WLM. However, as part of the ascorbates is synthesised in cell compartments other than the chloroplasts (Horemans et al., 2000), ascorbic acid content in CRF was lower than that of WLMs.

Whilst most of major minerals were greater in WLMs, some trace minerals (e.g. Fe) were more concentrated in CRFs. It might be worth to note that Na of spinach WLM (644 mg 100 g⁻¹DW) was 30 times reduced (21 mg 100 g⁻¹DW) after CRFs were prepared from spinach.

Characterizations of these chloroplast rich fractions allows in-depth understanding of which type of nutrients, level and significance of each nutrient, whether it is macro or micronutrient. In the year 2050, world's fast growing population is anticipated to reach 9.1 billion, with shrinking food supply, and nearly all these increases will occur in the developing countries (Abuajah et al., 2015). Although macro-nutrients mainly protein is the principle concern here, child blindness and mortality rate of children and pregnant women in many developing countries due to micronutrient deficiencies is also a global issue (WHO, 2009a). The recovery and concentration of vital nutrients notably pro-vitamin A carotenoids and Iron from underutilized plant species, for example, nettles and grass, thus contributes to the functional food supply in many poorer societies with ever growing populations. As essential nutrients, mainly proteins and lipid soluble molecules, are basically formed in the chloroplasts, it is worth to recover and concentrate them in the form of CRFs for human consumption (especially for sub-optimal populations) or for animal feed.

Graphic micro-structures by light microscopy or by transmission electron microscopy (TEM), used to illustrate digestibility of the material suggested that CRFs were intact and the under lying thylakoid membranes were visible from the TEM. The ultra-structural analysis also revealed break down of both CRFs and WLMs throughout 4 h of static *in vitro* digestion model. Similarly, more organic matter was found with CRF as digestible organic matter via simple centrifugation.

The carotenoids (β -carotene and lutein) differed in bioaccessibility. Oil (olive) treatment enhanced bioaccessibility of both β -carotene and lutein in CRFs but not for lutein bioaccessibility from WLMs. By rate of bioaccessibility lutein showed tremendously higher bioaccessibility than β -carotene. The juicer extracted materials

also showed better carotenoid bioaccessibility even without oil treatment compared to that extracted with sucrose. These outcomes suggested that CRFs are digestible, however, the nature of certain hydrocarbon carotenoids (e.g. β -carotene) in photosynthetic tissues is very complex (embedded with chlorophyll-protein complexes) and was found to be more resistant to digestive fluids and enzymes compared to lutein, an oxygenated (less hydrophobic) carotenoid which an appreciable amounts of it could be released without oil addition.

Subsequently, potentiometric analysis for lipolysis (galactolipid hydrolysis) indicated that gPLRP2 is strongly active on the commercial pure MGDG and that of the samples, specifically on CRFs, whereas pancreatin was greatly active on Tributyrin but not on MGDG. Similarly, for free fatty acids release, materials from CRFs containing more galactolipids were hydrolysed faster than WLMs, highlighting the preference of PLRP2 more on galactolipid rich substrates (CRFs).

Moreover, *in vivo* studies via fish feeding by reducing fish meal with 10% CRFs yielded the best growth rate among treatments. Lutein was detected in both whole zebrafish and its eggs, fed with experimental diets. The concentration of lutein from CRF fed zebrafish trials was dramatically greater than that of the WLM and was enormously higher than the control.

Those zebrafish trials fed with CRF also showed to encompass greater α -linolenic and Hexadecatrienoic acids than those fed with WLMs or control. Long chain omega fatty acids like eicosapentaenoic acid (EPA) was also enhanced due to experimental diets from CRF, specifically, using 10% and 20% of CRF improved the EPA content in the zebrafish and it was significantly ($P \leq 0.05$) higher than the control. Furthermore, CRF

fed zebrafish (notably 10 and 20%) indicated denser yellowed colour compared to WLMs. Both CRF and WLM fed trials were noticeably more orange in colour compared to the control. The lutein concentration of CRF fed zebrafish was again remarkably greater than both WLM and control fed fish trials. These results may suggest that CRFs are a potential alternative feed materials for fish feed. It may also suggest that carotenoids from CRFs are bioaccessible/bioavailable for fish uptake. Since, zebrafish received popularity as model organism for several aspects including developmental biology, genetics, neurobiology, pharmaceuticals and medicine (Bournele and Beis, 2016, Chakraborty et al., 2009, Hölttä-Vuori et al., 2010, Lampert et al., 2003b), results obtained via zebrafish diet trials might also be true for human studies.

Overall, this study laid the ground for recovering nutritionally rich chloroplast fractions from cheaper sources that are widely available in the nature and showed their compositional food/ feed potential and digestibility both *in vitro* and *in vivo* although, the *in vivo* studies were performed with fish instead of human studies.

7.3. Suggestions for future studies

More pure chloroplasts instead of crude preparation could be carried out possibly by density gradient centrifugation

Bioaccessibility/bioavailability of important chloroplasts nutrients (e.g. provitamin A) might be improved by using purified chloroplasts.

The chloroplasts could also be incorporated with other food systems such as whey proteins which may also enhance their carotenoid bioaccessibility.

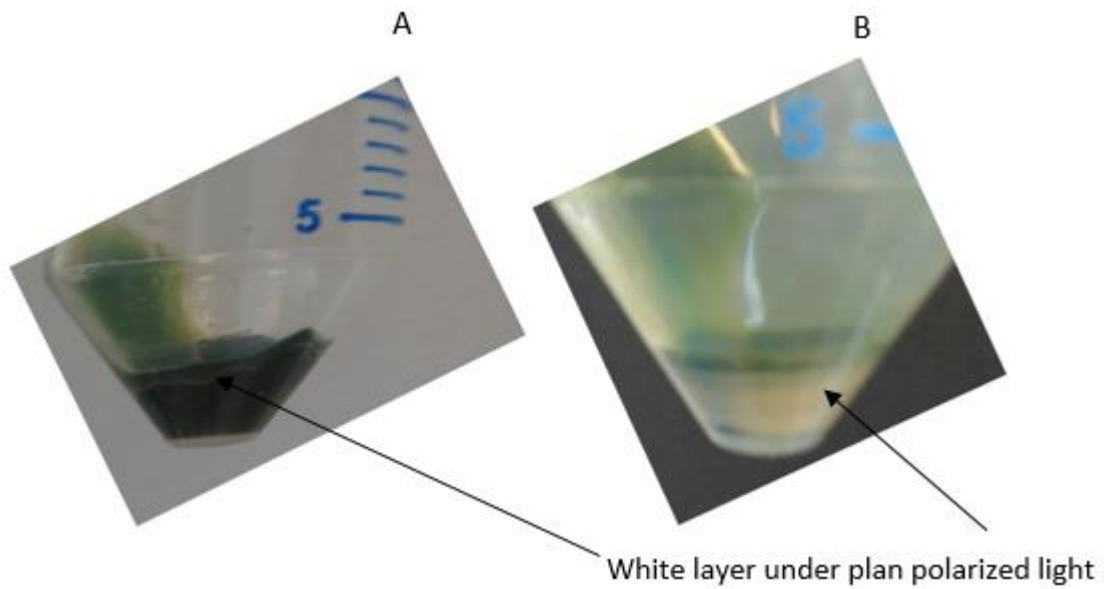
Human feeding trials of chloroplasts may provide more reliable answers for carotenoid bioaccessibility/bioavailability, however, this is ethically restricted, costly expensive and may take long time to get good results.

The used WLM materials for the zebrafish feed was ground to a fine powder (~ 1 mm particle size), this is because zebrafish are small in size and require a homogeneous meal of less than 1mm particle size. Therefore, using a larger fish may allow feeding of WLM diet with larger particle size, which in turn, may allow more representative comparisons of feed impact between CRF and WLM on different parameters such as, growth rate performance factors and the subsequent chemical markers.

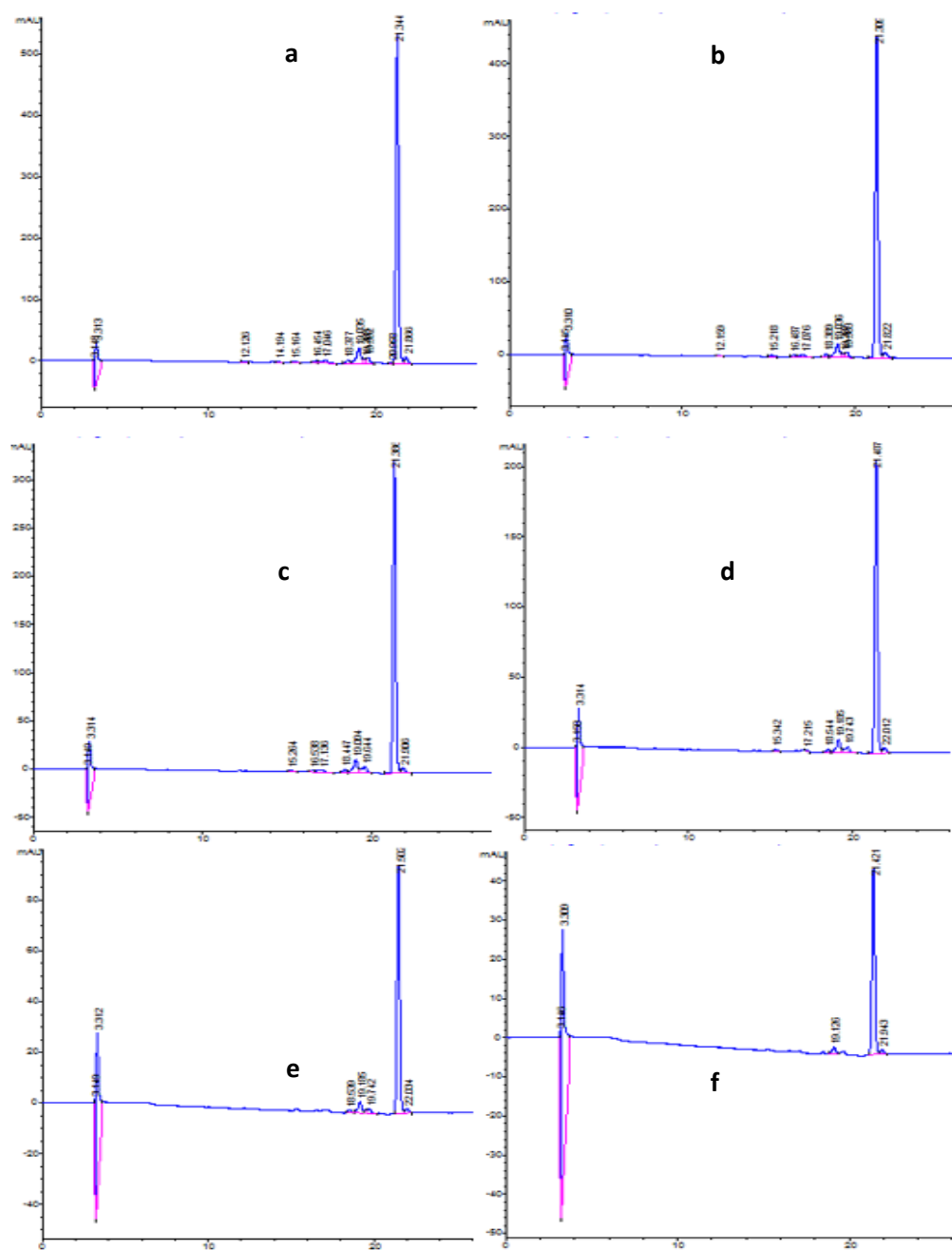
Several unconventional green plant species could be used to formulate chloroplast rich fractions after toxicity issues are resolved, and then could be used for human/animal feed.

Appendences

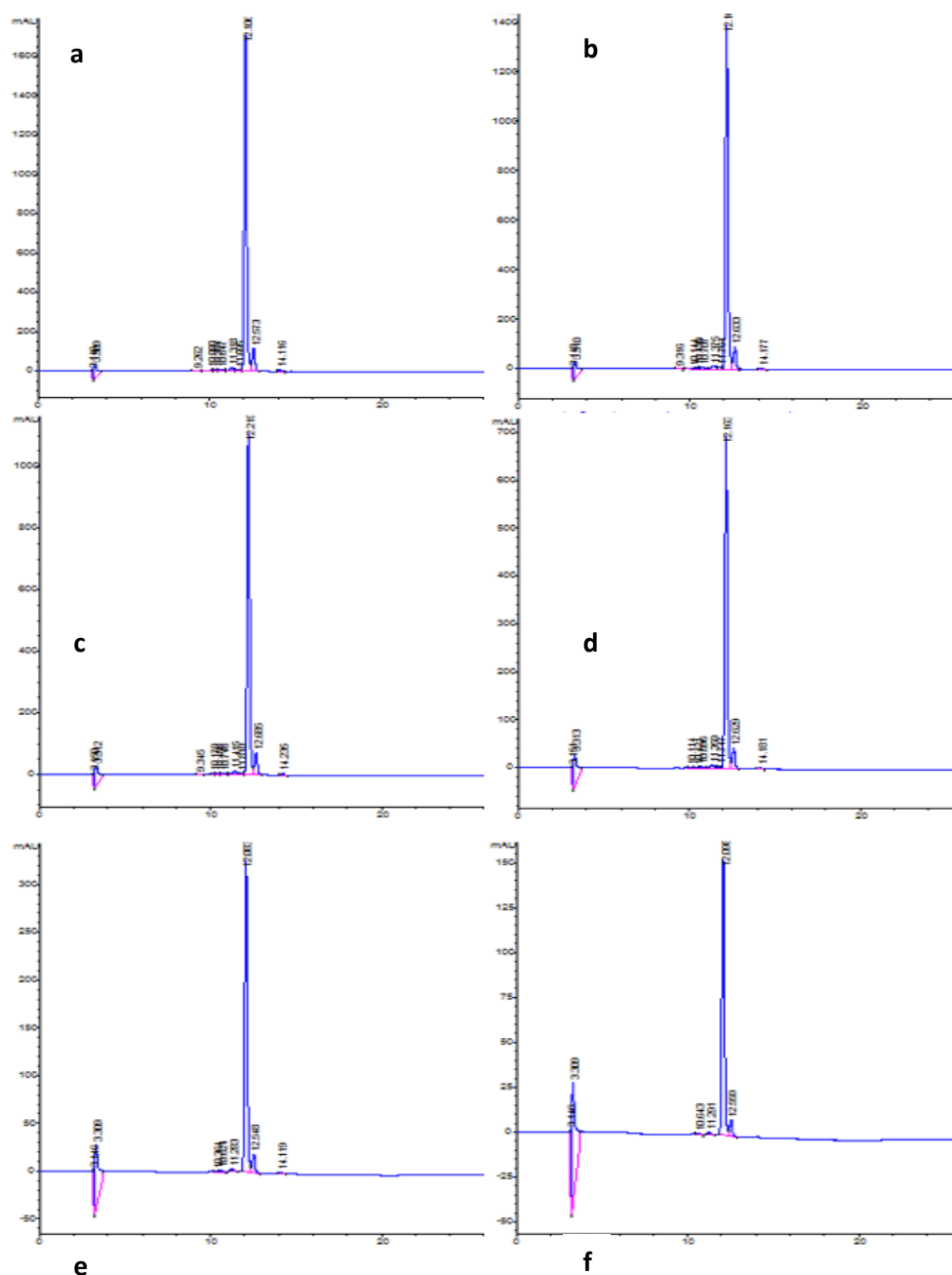
Appendences A. White layers (suspected to be starch) seen contaminated with the chloroplasts pellets



Appendices B1: Different concentrations of β -carotene standard in $\mu\text{g mL}^{-1}$

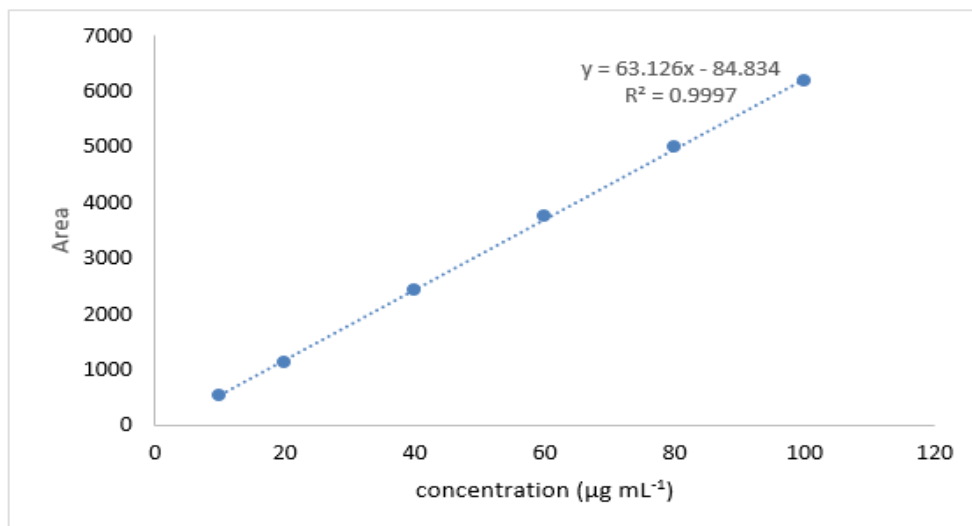


Appendix B2: Different concentrations of Lutein standard in $\mu\text{g mL}^{-1}$

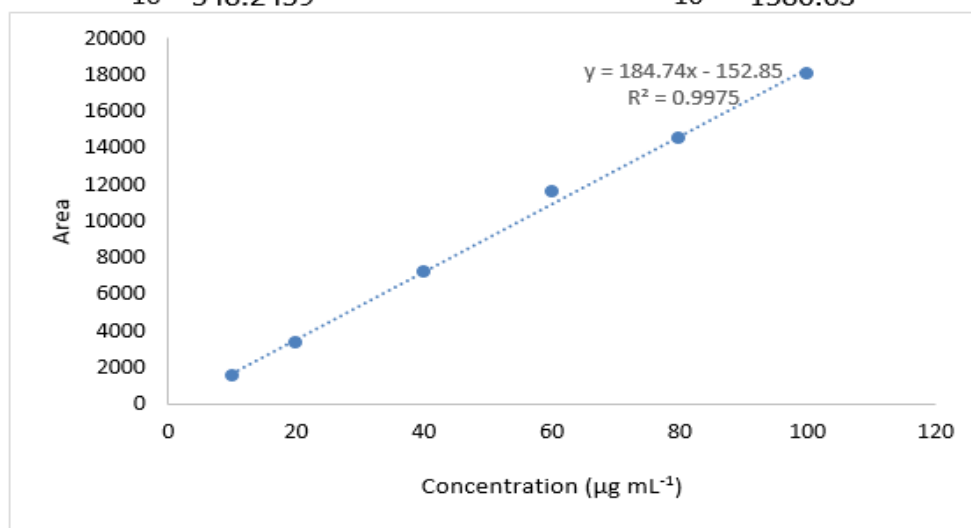


Lutein standard in $\mu\text{g mL}^{-1}$. (a):100, (b): 80, (c):60, (d):40, (e):20, (f):10

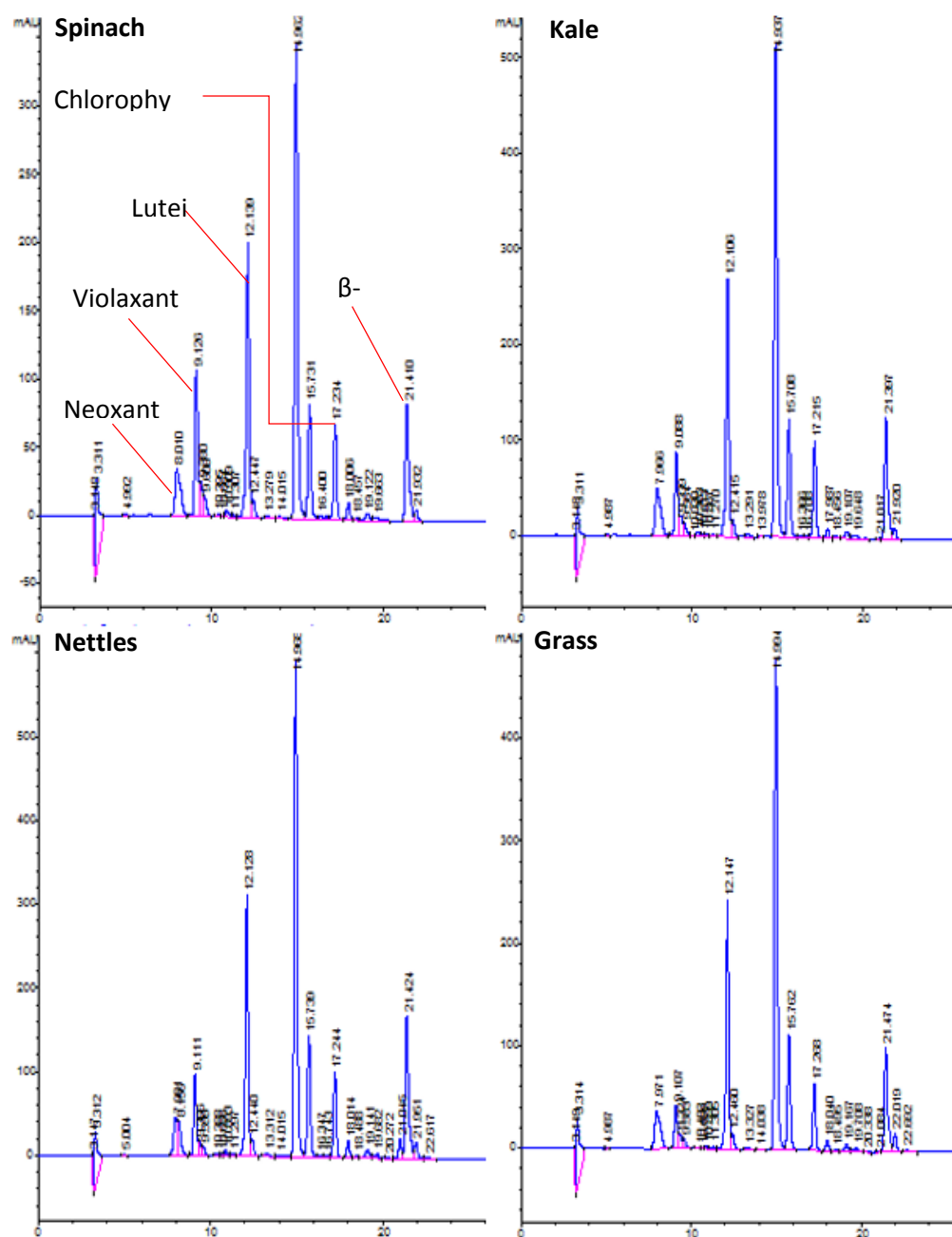
Appendix B3: Standard curve of (A) carotene and (B) lutein for quantitation of the carotenoids



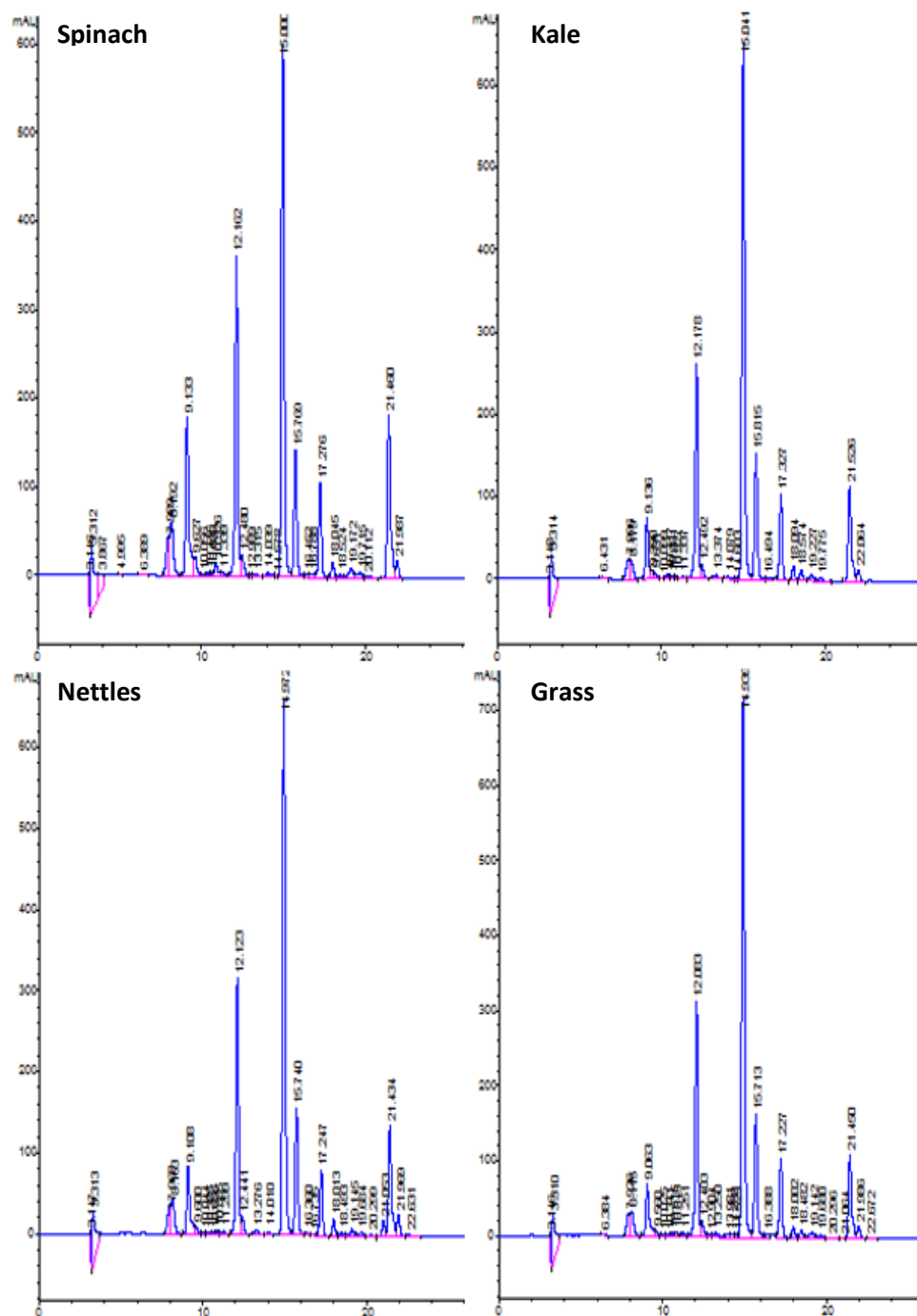
β-Carotene	Area	lutein	Area
100	6181.614	100	18068.9
80	4994.394	80	14528.9
60	3750.74	60	11567.2
40	2442.08	40	7210.042
20	1142.92	20	3389.54
10	548.2459	10	1586.63



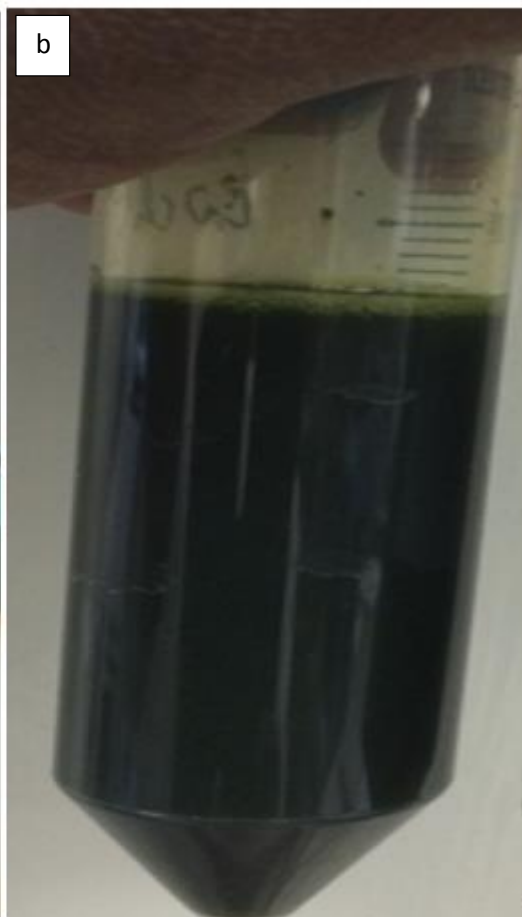
Appendix B4: Representative peak chronograms of carotenoids of WLM



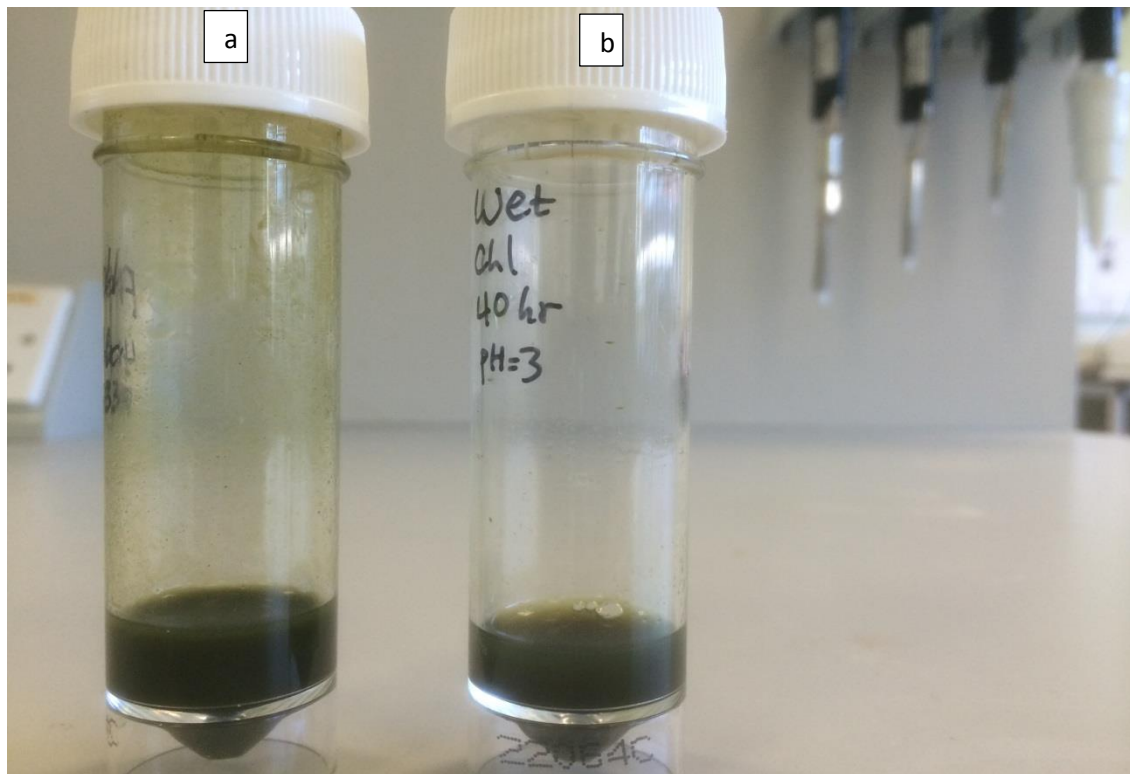
Appendix B5: Representative peak chronograms of carotenoids of CRF



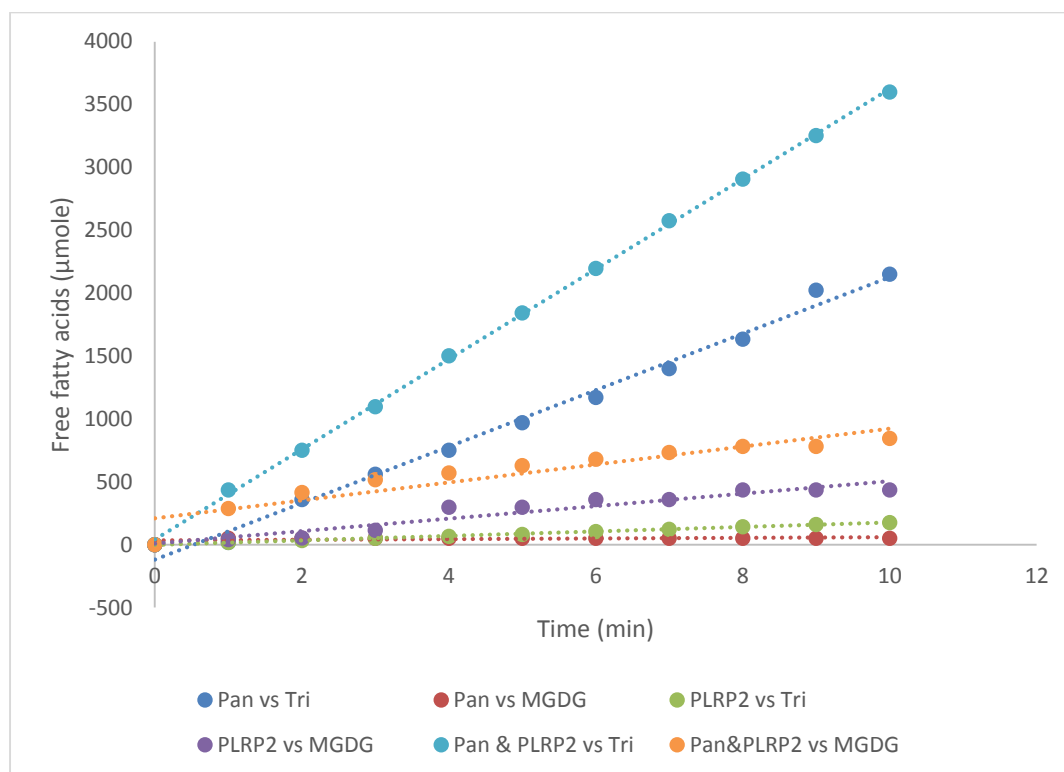
Appendix C1: *In vitro* digested material of freeze dried (a: showing clumping and stack of materials to the bottle walls) and wet (b: showing finely homogenous material) chloroplasts rich fractions from spinach.



Appendix C2: spinach and spinach chloroplasts (freeze dried versus wet); a: showing material aggregated and stacked to the wall of the bottle and wet (b: showing clear and homogenous mixture) spinach chloroplast rich fractions in acid at (pH= 3).



Appendix D1 Pancreatin and pancreatic lipase related protein 2 (gPLRP2) activities against lipid substrates



Pancreatin and pancreatic lipase related protein 2 (gPLRP2) activities against two substrates:

Triacylglycerol (TAG) rich lipid (Tributyrin) and Monogalactacyldiglyceride (MGDG) as glycerglycolipid. Galactolipase (PLRP2) activity was determined at 37 and pH 8.0 with a bile salt/MGDG ratio of 1.33 using NaTDC under conditions described in the respective materials and methods. Pancreatin activity was determined at 37 and pH 8.0 under conditions described in the respective materials methods. Knowing the volume (μL) of the enzyme added in the pH stat vessel, the activity (A) expressed in U mL^{-1} can be determined as: $A = (x.1000) \text{ v}^{-1}$. **Pan: Pancreatin; PLRP2: pancreatic lipase related protein 2; vs: versus; Tri: Tributyrin; MGDG: Monogalactacyldiglyceride.**

Appendix D2: Rough calculations of chloroplast rich fractions (CRF) galactolipids (GLs)

CRF (dry matter) contains 381.1 mg per g total galactolipid. Given CRF contains 35% lipid of which other pigments represent 10% (chlorophylls, carotenoids etc.), how much galactolipid is going in to the final volume (40 mL) of the *in vitro* model system if 0.5 g CRF (dry matter) was used. According to the *in vitro* model system used (Minekus et al., 2014), 5 mL of the final volume is from pancreatin whereas 2.5 mL comes from bile salt, and so the final volume for the pH-stat becomes 32.5 because the enzyme and the bile salts are added in to the system after pH-stat conditions were made ready.

10% in the 35% lipid is equal to 3.5, so the lipid content in CRF is about 31.5% or 315 mg per g CRF or 0.315 g lipid per g CRF. We used 0.5 g CRF so $0.315/2 = 0.1575$ g lipid per 0.5 g CRF.

If I have 381.1 mg GLs per g lipid

how much xGLs do I have per 0.1575 g lipid
xGLs= 60 mg GL in 32.5mL

For the pure galactolipid (MGDG), 25 mg was mixed with 5 mL of 0.33mM Tris-HCl buffer, containing 0.1M NaCl and NaTDC (a ratio of 1.3 mole Galactolipid/mole NaTDC) and 10 μ l (mg/mL) of PLRP2 used to hydrolyse. (1.3 mole/mole e.g. molecular weight of MGDG is 940 so moles in the 5 ml mixture will be 0.0064×1.3) and then, the mass of NaTDC (MW= 521.69) is calculated from these moles* volume* MW.

As amounts of PLRP2 obtained was not that much, 5 ml from the 32.5 mL above was driven after gastric phase and same volume of PLRP2 was used as active enzyme 10 μ L (mg/g) for pH-stat titration

Therefore if 60 mg GL is to be used for 32.5 mL
how much GLs are in 5 mL
There is 9.2 mg GLs in 5ml

As long as the ratio between GLs to the bile salts is at the optimum how much substrate or how much enzyme used will be normalized when activity is calculated as follows:

Rate of NaOH delivery= x mole NaOH per minute, i.e., μ mole free fatty acid released per minute= yU.

Knowing the volume (v, μ l) of the sample (enzyme) added in the pH-stat vessel, the activity (A) expressed in U/mL of sample can be determined as follows: $A = (x \cdot 1000) / v$ U/mL, which is equal to U/mg if the concentration of the enzyme was made mg/mL initially during preparing.

Appendix D3: Rough calculations of whole leaf materials (WLM) galactolipids (GLs)

WLM contains 262 mg per g total galactolipid. Given WLM contains 19% lipid of which other pigments represent 10% (chlorophylls, carotenoids etc.), how much galactolipid is going in to the final volume (32.5 mL) of the *in vitro* model system if 0.5 g CRF was used.

10% in the 19% lipid is equal to 1.9, so the actual lipid content in WLM is about 17.1% or 171 mg per g WLM or 0.171 g lipid per g WLM. We used 0.5g WLM so $0.171/2 = 0.0855$ g lipid per 0.5g WLM.

If I have 262 mg GLs per g lipid

how much xGLs do I have per 0.0855 g lipid

xGLs= 22.4 mg GL in 32.5 mL

For the pure galactolipid (MGDG), 25 mg was mixed with 5 mL of 0.33mM Tris-HCl buffer, containing 0.1M NaCl and NaTDC (a ratio of 1.3 mole Galactolipid/mole NaTDC) and 10 μ l (mg/mL) of PLRP2 used to hydrolyse. (1.3 mole/mole e.g. molecular weight of MGDG is 940 so moles in the 5 ml mixture will be 0.0064×1.3) and then, the mass of NaTDC (MW= 521.69) is calculated from these moles* volume* MW.

As amounts of PLRP2 obtained was not that much, 5ml from the 32.5 mL above was driven after gastric phase and same volume of PLRP2 was used as active enzyme 10 μ L (mg/g) for pH-stat titration

Therefore if 22.4 mg GL is to be used for 32.5 mL

how much GLs are in 5 ml

There is 3.5 mg GLs in 5 ml

As long as the ratio between GLs to the bile salts is at the optimum (1.3), how much substrate or how much enzyme used will be normalized when activity is calculated as follows

Rate of NaOH delivery= x mole NaOH per minute, i.e., y μ mole free fatty acid released per minute= yU

Knowing the volume (v, μ l) of the sample (enzyme) added in the pH-stat vessel, the activity (A) expressed in U/mL of sample can be determined as follows: $A = (x \cdot 1000) / v$ U/mL

Appendix E: Extra curriculum activities, conferences and publications during the research period.

TRAINING LOG				
TITLE	Presented (P)/ Attended (A)	Location	CREDIT RATING	DATE
Fire safety	A	A03 (LR3) Sutton Boninton campus, University of Nottingham, UK	0.5	04/10/13
A practical look at core teaching skills	A	C1 Burghley Suit, Kings Meadow Campus, University of Nottingham, UK	1.0	16/10/13
Health and Safety Induction	A	Food sciences Building, Sutton Boninton campus, University of Nottingham, UK	0.5	08/10/13
LaTeX for researchers: an introduction	A	A 16 Pope Building, Building 27 on the campus plan, University Park, University of Nottingham, UK	1.0	25/10/13
Nature of the doctorate and the supervision process	A	UP-A11 Highfield House, University Park, University of Nottingham, UK	1.0	30.10.13
Microsoft Excel: Introduction	A	A81 Biology Building, University Park, University of Nottingham, UK	2.0	21/11/13
High Performance Liquid Chromatography (HPLC)Training	A	Lab A6, Food Sciences, Sutton Bonington Campus,	0.5	26/11/13

		University of Nottingham, UK		
Gas Chromatography-Mass Spectrophotometer (GC-MS) training	A	Flavour lab, Food sciences, Sutton Bonington Campus, University of Nottingham, UK	0.5	10/12/13
Agresso Requisitioning (P2P) Training	A	B4 Exchange Building, Jubilee Campus, University of Nottingham, UK	0.5	12/12/13
Statistical Course, Design Expert by Robert Linforth	A	A03 (LR3), Sutton Bonington Campus, University of Nottingham, UK	3.0	20-21 /01/14
Microsoft PowerPoint: Advanced	A	A174, Kings Meadow Campus Lenton Lane, UK	2.0	22/01/14
Shut up and Write	A	Rm C100/1,C Floor, Medical School, UK	0.5	18/02/14
Lipid Course	A	James Hutton Institute, Dundee, Scotland, UK	3.0	27-28/02/14
Division of Food Science Postgraduate Research Presentation (1 st year)	P & A	A03 (LR3) Sutton Bonington Campus, University of Nottingham, UK	3.0	8/07/14
Super Resolution Microscopy Facility Launch	A	LT1 Lecture Theatre, Medical School, QMC, Nottingham, UK	0.5	25/02/15
Academic writing workshops: introduction to scientific writing	A	SBGC The Barn B07, SB Graduate Centre, Sutton Bonington Campus,	1.0	19/01/16

		University of Nottingham, UK		
Academic writing workshops: reviewing literature	A	07 Gateway Building, Sutton Bonington Campus, University of Nottingham, UK	1.0	26/01/16
Getting into the habit of writing	A	BGC The Barn B07, SB Graduate Centre, Sutton Bonington Campus, University of Nottingham, UK	1.0	28/01/16
Division of Food Science Postgraduate Research Presentation (2 nd year)	P & A	Gateway A07, Sutton Bonington Campus, University of Nottingham, UK	3.0	6/07/15
Division of Food Science Postgraduate Research Presentation (3 rd year)	A	<u>Division of Food Science, Room A33 (LR9)</u> Sutton Bonington Campus, University of Nottingham, UK	1.0	11/07/16
Food flavour course (D24AF1)	A	<u>Gate way building, B4,</u> Sutton Bonington Campus, University of Nottingham, UK	10	25-27/4/2017
Chairperson of Postgraduate Symposium 2017	chairman	<u>Lecture Block (BO4),</u> Sutton Bonington Campus, University of Nottingham, UK	5	16/05/2017
Conferences/Symposiums				

IDB- scholars symposium	P & A	Engineering Department Cambridge university, Cambridge, UK	4.0	22/05/14
International Conference on Food Digestion	P & A	University of Naples, Naples, Italy	5.0	17-19/03/15
IDB- scholars symposium	P & A	Sir Alexander Fleming Building (G34), Imperial college London, UK	4.0	26/05/15
International Conference on Advances in Human Nutrition, Food Sciences & Technology	P & A	International Living Learning Centre, Ryerson University, Toronto, Canada	4	26-27/06/16
Publications (title)	Journal	Issue/pages	volume	year
Component analysis of Nutritionally Rich Chloroplasts: Recovery from Conventional and Unconventional Green Plant Species	Journal of Food Science and Technology	Accepted manuscript	Accepted manuscript	Accepted manuscript
Spinach Freeze Dried Materials: Source of Nutrients for Health Benefits	The International Journal of Science and Technology	12/169	3	2015
Optimization of supercritical carbon dioxide (CO ₂) extraction of sardine (<i>Sardinella lemuru</i> Bleeker) oil using response surface methodology (RSM)	International Journal of Fats and Oils (Grasas y aceites)	2/74	66	2015
Under graduate projects (lab. Supervised)				
Project title	Student name	Division /school	University	Year
<i>Chemical Compositions in The Chloroplasts of</i>	Nursyafiqah Abdul latif	Food Sciences, School of Biosciences	Nottingham	2013/14

<i>Different Green Leafy Vegetables</i>				
<i>Nutrient Release from Cell Bound and Liberated chloroplasts</i>	Shirley Lang	Food Sciences, School of Biosciences	Nottingham	2014/15
<i>Impact of Pancreatic Lipase-Related Protein 2 Galactolipase on the Release of β-carotene from a Chloroplast Rich Material</i>	Mira Sethina Mohd Ibrahim	Food Sciences, School of Biosciences	Nottingham	2015/16
Teaching Courses Assisted as a demonstrator				
Course name and (code)	Main lecturer	Division	University	Year
Lipid practical (D21BF1)	Dr. David A. Gray	Food Science	Nottingham	2014/15
Lipid practical (D21BF1)	Dr. David A. Gray	Food Science	Nottingham	2015/16

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