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Impact of thermal and drying regimes, used to make powders derived from spinach leaves, on their material properties and digestibility

Chao Chi

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Division of Food, Nutrition and Dietetic

School of Biosciences

University of Nottingham

Sutton Bonington Campus

Leicestershire LE12 5RD

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Abstract

Chloroplasts are photosynthetic organelles in plants which are a rich source of lipophilic nutrients like galactolipids, carotenoids, vitamin E and α-linolenic acid (ALA, 18:3). Recent interest in the use of chloroplast fractions as functional food ingredients has led to the development of methods for their isolation from plant tissues. In the current research, the removal of intact chloroplasts from the confinement of the cell wall was achieved using a simple and clean method (slow-screw, twin-gear juicer), without the addition of solvents or chemicals, which is efficient for liberating chloroplasts. This approach was developed in an attempt to avoid excess dilution and contamination as well as maintaining food quality, enhancing shelf-life stability. The extracts were further dried with the use of modern industrial technologies such as spray drying and freeze drying. Thermal preservation technologies such as pasteurisation of spinach juice (70 °C 15s and 90 °C 5min) and blanching of spinach leaves (100°C 30s) were also investigated to inactivate enzymes and eliminate pathogenic microorganisms during storage.

Food processing techniques are designed to improve the quality of products, by promoting physical and chemical changes in tissues but can also lead to losses, especially of micronutrients. Thermal processes, such as pasteurisation and blanching, inactivated endogenous enzymes in spinach extracts, with 95%-99% of peroxidase (POD) activity being reduced. The study on drying techniques showed that both spray and freeze drying retained nutrient levels to a similar level in spinach juice powder; and freeze dried chloroplast rich fraction (CRF) had a higher nutritional value than freeze

dried juice. In addition, significant reductions in moisture content (below 10%) and water activity (below 0.3) in spinach juice/CRF powders are achieved with this suggesting better microbiological safety and enhanced shelf-life stability. However, thermal processing caused a significant quality loss in terms of colour and nutritional value. Key morphology changes of different treated spinach juice/CRF powders were observed by scanning electron microscopy (SEM) and highlighting the impact of processing procedure on organelle structures and providing a means to differentiate between each method used.

In addition, *in vitro* digestion assays, used to mimic the physiological conditions of human digestion, were used to measure the bioaccessibility of nutrients when spinach juice/CRF were processed in different ways. The digestion model consists of three phases (oral phase, gastric phase and intestinal phase), and the results showed that pasteurisation of spinach juice promoted the bioaccessibility of carotenoids and vitamin E, as well as enhanced the digestibility of galactolipids but hindered proteins digestion in spinach juice/CRF compared with fresh samples. The lipolysis of galactolipids, mainly monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) was monitored by high performance thin layer chromatography (HPTLC) during digestion. MGDG and DGDG were converted to monogalactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG), respectively, and ALA was the main fatty acid released. While the major galactolipid lipolysis occurred during the intestinal phase with the presence of porcine pancreatic extracts (PPE), and the protein hydrolysis occurred mainly during the gastric

phase with the addition of pepsin.

List of publications

1. WATTANAKUL, J., SYAMILA, M., DARWISH, R., GEDI, M. A., SUTCHARIT, P., CHI, C., AKEPACH, P., SAHAKA, M., GONTERO, B., CARRIÈRE, F. & GRAY, D. A. 2022. Bioaccessibility of essential lipophilic nutrients in a chloroplast-rich fraction (CRF) from agricultural green waste during simulated human gastrointestinal tract digestion. Food & Function, 13, 5365-5380.

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CONTENT

List of abbreviations

Abbreviation and Meaning

Spinach chloroplast materials

CRF	Chloroplast-rich fraction
SJ	Spray dried juice
FJ	Freeze dried juice
FC	Freeze dried CRF
MJ	Mild pasteurised juice
MC	Mild pasteurised CRF
IJ	Intense pasteurised juice
IC	Intense pasteurised CRF

All abbreviations (samples, chemicals, nutrients, measurements, etc)

α-toc	α-tocopherol
μg	Microgram
μL	Microlitre
μm	Micrometre (micron)
A	Absorbance
ALA	α-linolenic acid
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
β-car	β-carotene

BCA	Bicinchoninic acid
BHT	Butylated hydroxytoluene
C/ conc.	Concentration
Cal	Calorie
CEH/BSSL	Carboxyl ester hydrolase/ bile salt simulated lipase
CHCl3	Chloroform
Chl	Chlorophyll
Chl.a	Chlorophyll a
Chl.b	Chlorophyll b
CH3OH	Methanol
cm	Centimetre
CO2	Carbon dioxide
CRF	Chloroplast-rich fraction
DAG	Diacylglycerol
DGDG	Digalactosyl diacylglycerol
DGMG	Digalactosyl monoacylglycerol
DHA	Docosahexaenoic acid
DW	Dry weight
EPA	Eicosapentaenoic acid
FA	Fatty acid
FC	Freeze dried CRF

FD	Freeze drying
FAMEs	Fatty acid methyl esters
FFA	Free fatty acid
FJ	Freeze dried juice
g	Gram
GC-MS	Gas chromatography-mass spectrometry
GI	Gastrointestinal tract
GPLRP2	Pancreatic lipase-related protein 2 from guinea pig
h	Hour
HCl	Hydrochloric acid
HGL	Human gastric lipase
HMF	5-hydroxymethylfurfural
H2O2	Hydrogen peroxide
HPJ	Human pancreatic juice
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HTST	High temperature short time
IC	Intense pasteurised CRF
ICP-MS	Inductively coupled plasma-mass

	spectrometry
IJ	Intense pasteurised juice
KCl	Potassium chloride
kDa	Kilodalton
kg	Kilogram
KH2PO4	Potassium dihydrogen phosphate
LA	Linoleic acid
LAHase	Lipid-acyl hydrolase
Lut	Lutein
M	Molar (mol/L)
MAG	Monoacylglycerol
MC	Mild pasteurised CRF
mg	Milligram
MGDG	Monogalactosyl diacylglycerol
MGG	Monogalactosylglycerol
MGMG	Monogalactosyl monoacylglycerol
min	Minute
MJ	Mild pasteurised juice
mL	Millilitre
mM	Millimolar
mm	Millimetre
mol	Mole

MW	Molecular weight
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	Sodium hydroxide
NaTDC	Sodium taurodeoxycholate
ND	Not determined
$(NH_4)_2CO_3$	Ammonium carbonate
nm	Nanometre
PA	Palmitic acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
pH	Potential of hydrogen ion
PLRP2	Pancreatic lipase-related protein 2
POD	Peroxidase
PPE	Porcine pancreatic extract
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
R2	Coefficient correlation
RCF	Relative centrifugal force
RGE	Rabbit gastric extract

rpm	Revolution per minute
Rubisco	Ribulose-1,5-bisphosphate carboxylase
s	Second
SD	Spray drying
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Scanning electron microscope
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SJ	Spray dried juice
SQDG	Sulfoquinovosyl diacylglycerol
SSF	Simulated salivary fluid
Std	Standard
TAG	Triacylglycerol
TEA	Triethylamine
TEM	Transmission Electron Microscope
TGDG	Trigalactosyl diacylglycerol
TMSH	Trimethylsulfonium hydroxide
U	Unit
UV-Vis	Ultraviolet and visible
v	Volume

VE	Vitamin E
viz	videlicet
Vol.	Volume
vs	Versus
WLM	Whole leaf material
WSI	Water solubility index

CHAPTER I

1. General introduction

1.1. General overview

Spinach (*Spinacia oleracea* L.) is an economically important leafy vegetable crop in many countries, with around 30 million tonnes of spinach produced worldwide in 2020/2021 (Table 1.1). China is the largest global producer that accounts for more than 90% of the world total spinach production, followed by United States of America, with 367,433 tonnes of spinach produced annually.

Table 1.1: Countries with highest spinach production in 2020/2021 ("Spinach production in 2021; Crops/Regions/World/Production Quantity/Year from pick lists," 2023) (Food and Agriculture Organisation of the United Nations, https://www.fao.org/faostat/en/#data/QCL/visualize)

Rank	Country	Production (Tonnes)	Acreage (Hectare)	Yield (Kg/Hectare)
1	China	28,507,829	724,331	39,357
2	United States of America	367,433	22,743	16,156
3	Kenya	243,336	9,789	24,858
4	Türkiye	231,515	18,482	12,527

In other developed countries, approximately 80% of the available spinach is processed and is available as either canned food or as frozen food, while fresh spinach is usually sold loose, bunched, or packaged fresh in bags (Hodgson et al., 1984). However, in many developing countries like South Africa, farming of vegetables such

as spinach is needed to provide foods that offer opportunities in tackling human nutrients deficiency (Mdoda et al., 2022). Currently, approximately 1 billion people are unable to fulfill their basic energy requirements, and at least 2 billion people are experiencing micronutrient deficiencies (Müller et al., 2005; Berti et al., 2014). According to the available information (Bai et al., 2022), a significant upsurge in nutritional deficiencies and associated health issues has been observed in many countries, and the development of spinach products could aid dealing with this problem. Indeed, researchers have proposed that spinach is a novel non-conventional functional ingredient, with potential to mitigate micronutrient deficiencies in developing populations (Khan et al., 2015).

Indeed, spinach is considered as a functional food due to its nutritional properties, since it contains various macronutrients, such as lipids, proteins, minerals and carbohydrates, as well as micronutrients including carotenoids, chlorophylls, vitamins and phenolic compounds (Bunea et al., 2008). A typical serving of spinach (100 g) contains high levels of iron, potassium and magnesium, that meet 15%, 16% and 20%, of the recommended dietary allowance (RDA). Therefore, the consumption of spinach in the human diet may help in preventing a variety of nutritional related disorders and therefore serves to lower the relative risk of certain types of chronic diseases, such as cancer, diabetes, and cardiovascular disease (Abu Al-Qumboz et al., 2019). The relationship between diet and these physiopathologies has arose interest in design of spinach functional foods as cost-effective means of health requirements.

It is known that spinach is a rich source of chloroplasts. These organelles are found

in photosynthetic organisms such as plants and algae and contain many nutritionally important molecules. Recent research has shown that the chloroplast synthesises molecules, such as omega-3 fatty acids, amino acids (mainly glutamate, aspartate, and threonine), tocopherols, carotenoids, and plant hormones (Gross, 1991). However, despite the well-known nutritional value of spinach, it is a highly perishable crop with a short shelf life, fresh spinach loses most of its folate and carotenoid content over one week refrigerated storage (Batziakas et al., 2020). Chen et al. (1983) reported that folate levels in fresh spinach decreased by 26 % and 27 % after 7 d of storage at 4 °C or 10 h at 20 °C. Pandrangi et al. (2004) reported that total carotenoids retained 54%, 61%, and 44% of initial levels after 8 d (4 °C), 6 d (10 °C), and 4 d (20 °C) of storage. For longer preservation, it is canned, dehydrated or cooked and frozen.

Because of the instability of fresh spinach, the focus of our laboratories (School of Biosciences, University of Nottingham) has been on the extraction of chloroplast rich fractions from green plants including spinach, pea vine haulm, kale, and nettle, to provide a source of essential micronutrients that are shelf stable. Figure 1.1 shows chloroplasts in situ on spinach leaves as well as in an extract.

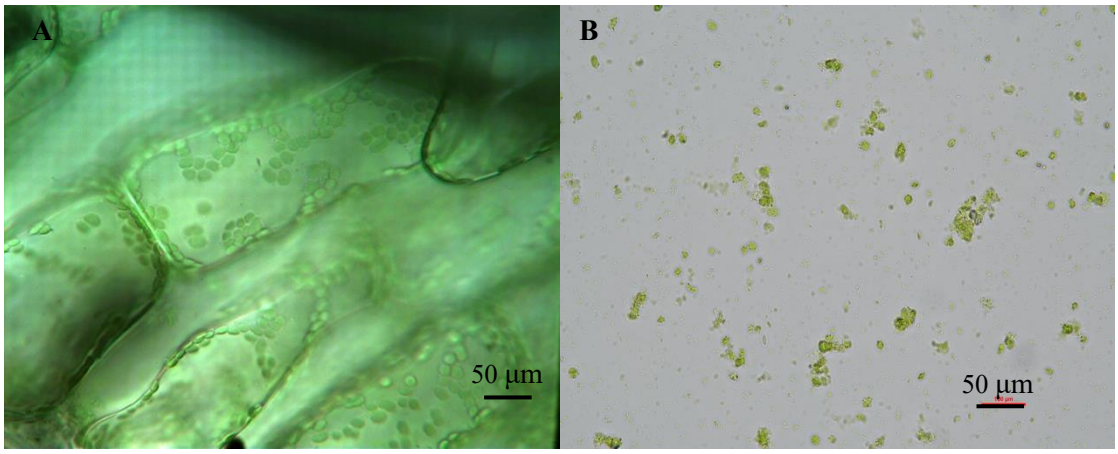


Figure 1.1: Light microscope images of a cross section of spinach leaves (A) and fresh spinach chloroplast rich fraction (B). The scale bar corresponds to 50 μm.

The composition of freshly extracted juice/CRF from green biomass has been measured to determine the stability of micronutrients during processing and storage. In addition, methods to access the bioaccessibility of lipophilic nutrients (carotenoids, fatty acids and alpha-tocopherol) in chloroplast extracts have been developed using *in vitro* digestion models (Gedi et al., 2017; Gedi et al., 2019; Torcello-Gómez et al., 2019; Wattanakul et al., 2019; Wattanakul et al., 2020; Wattanakul et al., 2022).

1.2. Literature review

1.2.1.Chloroplast

Chloroplasts are characteristic organelles in plant and green algae cells (Marchand et al., 2018). Chloroplasts are lens-shaped organelles with a thickness and diameter of roughly 1–4 μm and 5–10 μm, respectively (Barber, 1976; Hall et al., 1999). The chloroplast consists of a double membrane with outer and inner envelopes, separated by the intermembrane space (Figures 1.2 and 1.3) (Karim et al., 2014). The space within the inner membrane is known as the stroma, a matrix containing various enzymes including the key photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), plastoglobulus (pl. plastoglobuli), ribosomes, starch granules, and copies of the chloroplast genome (Staehelin, 2003; Morita et al., 2008). The thylakoid membrane is an additional internal membrane, located in the stroma, and

characterized by its disk like structure. The continuous aqueous phase is enclosed by the thylakoid membrane (Sakamoto et al., 2008). In most higher plants, the thylakoids are arranged in tight stacks called grana, that are connected by the stroma thylakoids/lamella (Pribil et al., 2014; Staehelin et al., 2020).

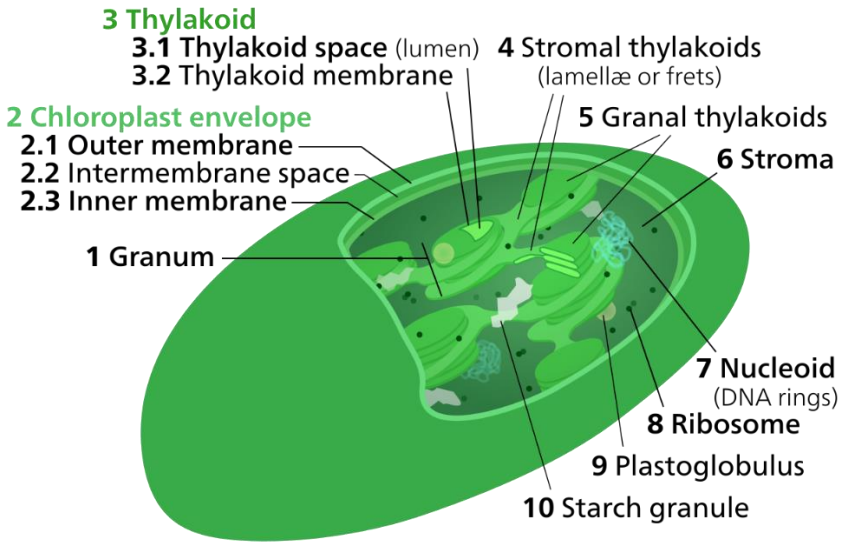


Figure 1.2: Structure of chloroplast.(Chen et al., 2014)

Chloroplasts are the main site of photosynthesis, a process that converts light energy into chemical energy, and then leads to the production of organic molecules such as glucose, sucrose and starch (Staehelin, 2003). Photosynthesis occurs at two sites for light-independent reactions (also called the Calvin-Benson cycle), (Figure 1.4), which takes place in the stroma and the thylakoid membrane. Rubisco is responsible for catalysing carbon dioxide fixation. The series of embedded pigments (chlorophylls and carotenoids) and proteins are essential to perform the light-dependent reactions of photosynthesis. The chlorophylls capture the energy from sunlight and convert it into chemical energy, such as adenosine triphosphate (ATP) and nicotinamide adenine

dinucleotide phosphate (NADPH). The Calvin Cycle uses the ATP and NADPH from the light-dependent reactions plus carbon dioxide to produce organic molecules such as glucose and starch (Raines, 2003; Krupinska et al., 2004) .

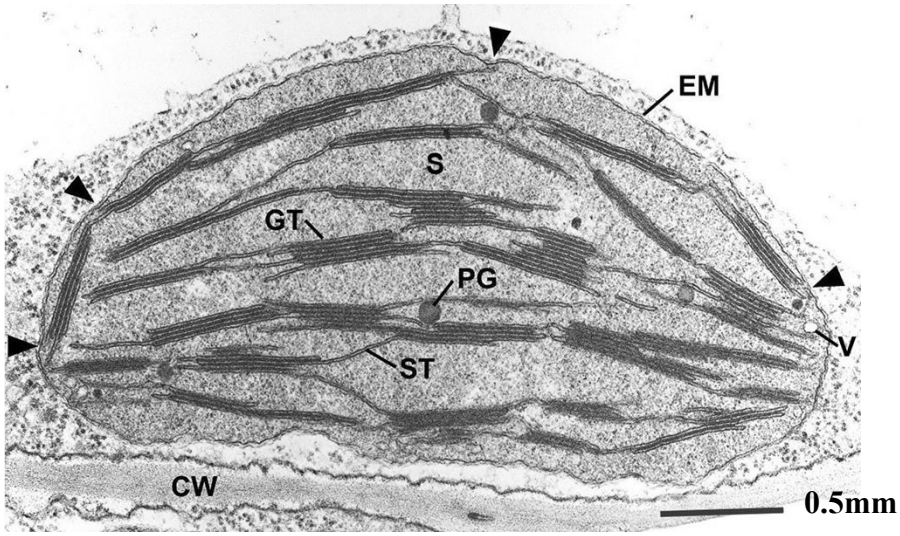


Figure 1.3: Thin section electron micrograph of a chemically fixed chloroplast. The chloroplast lies flat against the plasma membrane and the cell wall (CW) and shows a more or less elliptical structure. The stacked grana thylakoids (GT) are interconnected by non-stacked stroma thylakoids (ST). Stroma (S) surrounds the membranes, and the lightly stained region of the stroma indicates the presence of DNA. A few plastoglobuli lie adjacent to stroma thylakoids. Two envelope membranes (EM) form the boundary layer of the chloroplast. The scale bar corresponds to 0.5mm. (Staehelin & Paolillo, 2020).

In additional to photosynthetic processes, chloroplasts also have other specialized functions crucial for plant growth and development including nitrate and sulphate assimilation, and various biosynthetic pathways linked to plant secondary metabolite metabolism (Jensen et al., 2014). It is well know that chloroplast synthesises numerous

essential compounds including hormones (auxin, gibberellin, cytokinin, ethylene, and abscisic acid), proteins (amino acids), fatty acids (C16 and C18 fatty acid), lipids (galactolipids and phospholipids) and secondary metabolites (alkaloids and terpenoids) (Joyard et al., 1998; Verpoorte et al., 1999). Thus, chloroplast can be considered as a significant source of macro- and micro- nutrients in plants.

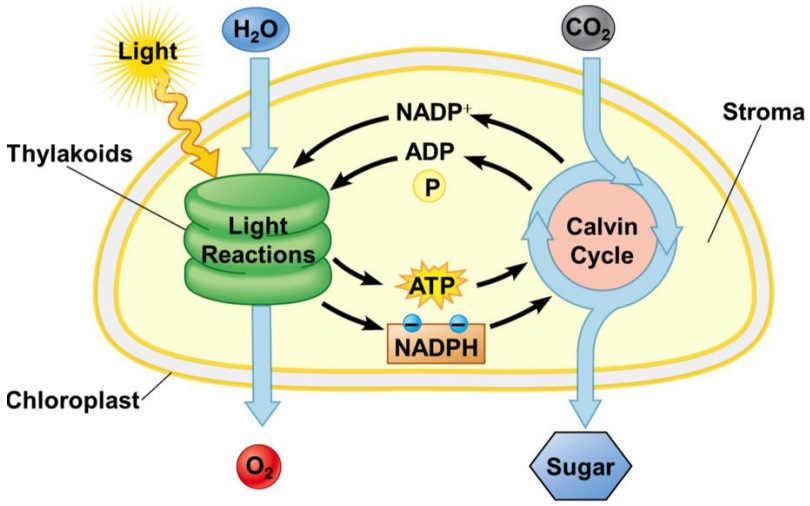


Figure 1.4: Photosynthesis reaction takes place in thylakoid membranes. (Sekar et al., 2015)

1.2.2.Nutrient profiles of plant chloroplast and their importance in human health

1.2.2.1. Lipids

The major lipids contained in the chloroplastic photosynthetic membrane are galactolipids, which account for around 70-80 % of the total membrane lipids (Dörmann, 2013). The lipid bilayer of the thylakoid membrane in plant chloroplasts and cyanobacterial cells is predominantly composed of four unique lipid classes; Their chemical structures are characterized by the number of galactose moieties attached to

glycerol backbone at the SN-3 position. The main form of galactolipids present in the chloroplast are monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)，containing one or two saturated and/or unsaturated fatty acids linked to the glycerol moiety, and account for approximately 50 % and 20-30 % of total glycerolipids respectively (Christensen, 2009). In addition, lower levels of sulfoquinovosyldiacylglycerol (SQDG) and trigalactosyldiacylglycerol (TGDG), a sulfolipid with a sulfoquinovose residue instead of galactose, are found (Amara et al., 2010). Various phospholipids are also contained in the chloroplastic membrane system, in the form of phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylinositol (PI). Furthermore, under stress conditions various neutral lipids (triacylglycerol and fatty acid phytyl esters) accumulate in chloroplasts and are mostly found in plastoglobuli; small lipid droplets in the stroma enclosed by a lipid monolayer membrane (Wijk et al., 2017). These lipids are crucial for maintaining the function of chloroplasts as they consist of a large fraction of the photosynthetic membranes and are believed to play important roles in photosynthesis and thylakoid membrane biogenesis.

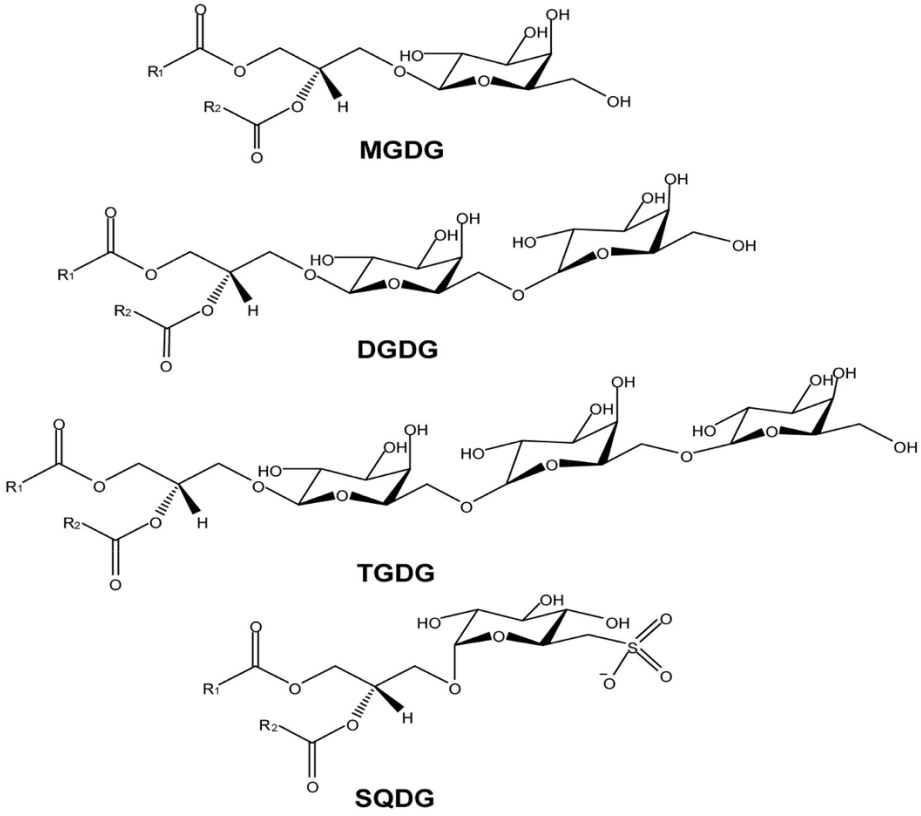


Figure 1.5: Chemical structure of galactolipids in chloroplasts (Wattanakul et al., 2019)

A constant ratio of MGDG to DGDG in thylakoid membranes is essential for maintaining the structure and stability of the photosynthetic membranes (Makshakova et al., 2020). The MGDG/DGDG ratio response under different abiotic stresses contribute to maintain membrane structures and enzymatic activities (Kobayashi, 2016). In spinach, the lipid composition of the thylakoid membrane consists of 53 % MGDG and 27 % DGDG and low levels of anionic galactolipids SQDG (7%) and anionic phospholipid PG (7 %). In the inner envelope membrane, the galactolipids account for 84 % (MGDG 49 %, DGDG 30 % and SQDG 5 %) and 15 % phospholipid (PG 8%, PC 6% and PI 1%). Moreover, the outer envelope membrane has a lipid composition of

52% galactolipids (MGDG 17 %, DGDG 29% and SQDG 6%) and 47 % phospholipid (PG 10 %, PC 32% and PI 5% (Table 1.2), (Block et al., 1983; Dorne et al., 1990).

Table 1.2: Membrane lipid composition of spinach (% fresh weight) (Block et al., 1983; Dorne et al., 1990)

	MGDG	DGDG	SQDG	PG	PI	PC	Others
TM	53	27	7	7	2	0	4
IEM	49	30	5	8	1	6	1
OEM	17	29	6	10	5	32	1

Abbreviations: thylakoid membrane (TM), inner envelope membrane (IEM) and outer envelope membrane (OEM), phosphatidylglycerol (PG), phosphatidylcholine (PC) and phosphatidylinositol (PI).

Chloroplasts are the major site for de novo fatty acid synthesis (Hölzl et al., 2019). Palmitic acid (16:0) and unsaturated C18 fatty acids such as oleic acid (18:1, 9-cis), linoleic acid (18:2, 9-cis, 12-cis) and α-linolenic acid (18:3, 9-cis,12-cis,15-cis) are highly abundant in chloroplasts. Unsaturated C16 fatty acids are found in PG (3-trans-hexadecenoic acid, 16:1, 3-trans) and MGDG (hexadecatrienoic acid, 16:3, 7-cis,10-cis,13-cis) such as *Arabidopsis* and spinach (*Spinacia oleracea)*. A large amount of isoprenoid lipids can also be found in chloroplasts which include chlorophyll, carotenoids, and prenylquinols (plastoquinol-9, phylloquinol, tocopherol) (Schultz, 1990; Zhai et al., 2013).

1.2.2.2. Proteins

In addition to lipids, chloroplasts also contain large amounts of protein. Indeed,

chloroplast proteins are main compounds in plant cells, which represents up to 40 % of total plant cellular protein in leaves. The envelope contains only 1 % of the chloroplast proteins, and the majority of proteins are located in the stroma (40-50 %) and thylakoid membranes (40-50 %) (Bouchnak et al., 2019). The stroma and thylakoid membrane are the main sites for conducting photosynthesis. The thylakoid contains two photosystems which is Photosystem I (PSI) and Photosystem II (PSII). These photosystems are responsible for generating the energy needed to produce carbohydrates in the Calvin cycle. The protein light harvesting complexes LCI and LCII embedded in the photosynthetic membrane are responsible for capturing sunlight. These proteins bind to a network of chlorophyll and carotenoid molecules are the pigment-protein complexes acting as LHCs (light harvesting complexes) that absorb photons of light and effectively leading to the electronic excitation with the excitons eventually arriving at the reaction centers (Lokstein et al., 2021; Zayadan et al., 2021). In addition, the major protein in chloroplast and the envelope membranes is Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) (Figure 1.6). This is a critical enzyme in the Calvin cycle in the stroma for the light-independent reactions (Ferro et al., 2000; Bouchnak et al., 2018).

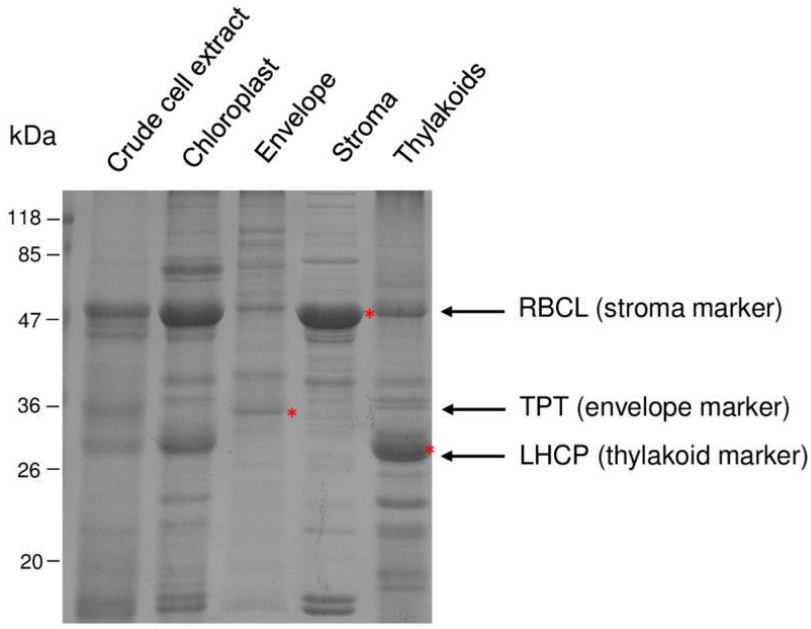


Figure 1.6: Coomassie blue-stained SDS-PAGE gel of chloroplast protein subfractions. Rubisco large subunit (RBCL), Triose-phosphate translocator (TPT), Light harvesting complex protein (LHCP). (Bouchnak et al., 2018)

1.2.2.3. Minerals

Chloroplasts are rich sources of minerals such as magnesium (Mg), calcium (Ca), sodium (Na), phosphorus, potassium (K), sulphur (S), chloride (Cl), and zinc (Zn). These minerals are essential for numerous processes taking place in our bodies. They play essential roles in maintaining a healthy body, including blood, bone and teeth health, and only can absorbed through diet. Mg is bound or incorporated into cellular compartments (Waters 2011), with the highest concentrations in chloroplasts (Karley and White 2009). A significant amount of Mg in leaves is bound as the central atom in the tetrapyrrole ring of chlorophyll a and b molecules, which are the major pigments for photosynthetic light capturing. Both K^+ and Mg^{2+} can facilitate the organization of the grana and stroma lamellae, thus support chloroplast integrity, the efficiency of light

absorption, Rubisco diffusion and carbon assimilation (Tränkner et al., 2018). Calcium (Ca^{2+}) plays a significant role in maintaining both structural and regulatory processes in chloroplast stroma and thylakoids (Miqyass et al., 2007; Wang et al., 2016). Ca^{2+} is a structural component of photosystem II (PSII) and is essential for efficient oxygen evolution. At low concentrations Ca^{2+} can activate fructose 1,6-bisphosphatase, a key enzyme involved in the Calvin cycle responsible for sucrose synthesis pathway (Rakus et al., 2013). Interestingly, high concentration of Ca^{2+} in stroma tends to inhibit CO_2 fixation in photosynthetic reactions (Wang et al., 2019).

The major mineral composition of green leafy plants like spinach, kale, nettles and grass is shown in Table 1.3 (Gedi et al., 2017). It can be found that the CRF from green leafy plants are especially rich in minerals such as magnesium, phosphorus, potassium, and calcium. In the United Kingdom, the reference nutrient intake (RNI) of minerals is shown in Table 1.4.

Table 1.3: Major minerals (mg/100g DW) in freeze-dried chloroplast rich fraction (CRF) of green leafy plants (Gedi et al., 2017)

	Na	Mg	P	K	Ca	Mn	Fe	Cu
Spinach	21±5	337±24	298±42	1196±68	677±15	7.7±0.6	31.4±3.1	2.0±0.1
Kale	39±11	322±17	628±111	518±95	920±30	6.5±0.7	25.7±9.1	0.5±0.1
Nettles	63±2	396±19	370±54	872±64	1736±11	7.9±0.6	42.1±12	1.0±0.1
Grass	44±2	232±28	436±94	1195±234	354±4	12.0±3.4	37.0±0.6	1.1±0.4

Data were expressed as the mean ± SD of 3 separate experiments.

Table 1.4: Reference Nutrient Intake (RNI) for minerals in the United Kingdom (mg/day) (Derbyshire, 2018)

Minerals	Males	Females
Na	1600	1600
Mg	270	300
P	550	550
K	3500	3500
Ca	700	700
Cl	2500	2500
Fe	14.8	8.7
Cu	1.2	1.2
Zn	7.0	9.5
Se	0.06	0.075
I	0.14	0.14

1.2.2.4. Carotenoids

Carotenoids are lipophilic pigments characterized by their diverse colours in plant tissues spanning bright yellow, red and orange colourations. They are highly abundant in plants, algae, photosynthetic bacteria and some fungal species. Carotenoids synthesis in plants and play important function in photosynthesis and biomembrane photoprotection (Swapnil et al., 2021).

In addition, carotenoids are essential components in the diets of humans as the source of antioxidants and vitamins. Commercially, they are used as natural colourants, and nutritional ingredients in food supplements (Li et al., 2012). More than 600 carotenoids are found in natural structural variants which are classified into two main groups: carotenes and xanthophylls (Figure 1.7), (Ellison, 2016). Carotenes, such as α-

carotene, β-carotene and lycopene, are hydrocarbons. Xanthophylls, such as β-cryptoxanthin, lutein, zeaxanthin, are carotenoids containing oxygen atoms (Maoka, 2020). Carotenoids consist of eight repetitive units of isoprene with C40 chain backbone. The cyclic or linear structures at both ends of the carbon chains, resulting in multiple *cis* and *trans* isomers, with the *all-trans* isomeric configuration exist more abundant and stable in nature (Milani et al., 2017). Carotenoids all have antioxidant properties, and some are precursors of vitamin A (e.g. β-carotene, α-carotene and β-cryptoxanthin). Moreover, carotenoids are involved in some beneficial functions including immune system activation, intercellular communication, and disease prevention and therefore enhance human health (González-Peña et al., 2023). The consumption of carotenoid abundant foods is highly recommended since it is linked with the lower risk of chronic diseases. However, according to the existing evidence collected by the European Food Safety Authority (2006), it is insufficient to establish a Recommended Dietary Allowance (RDA) or Adequate Intake (AI) for β-carotene and other carotenoid.

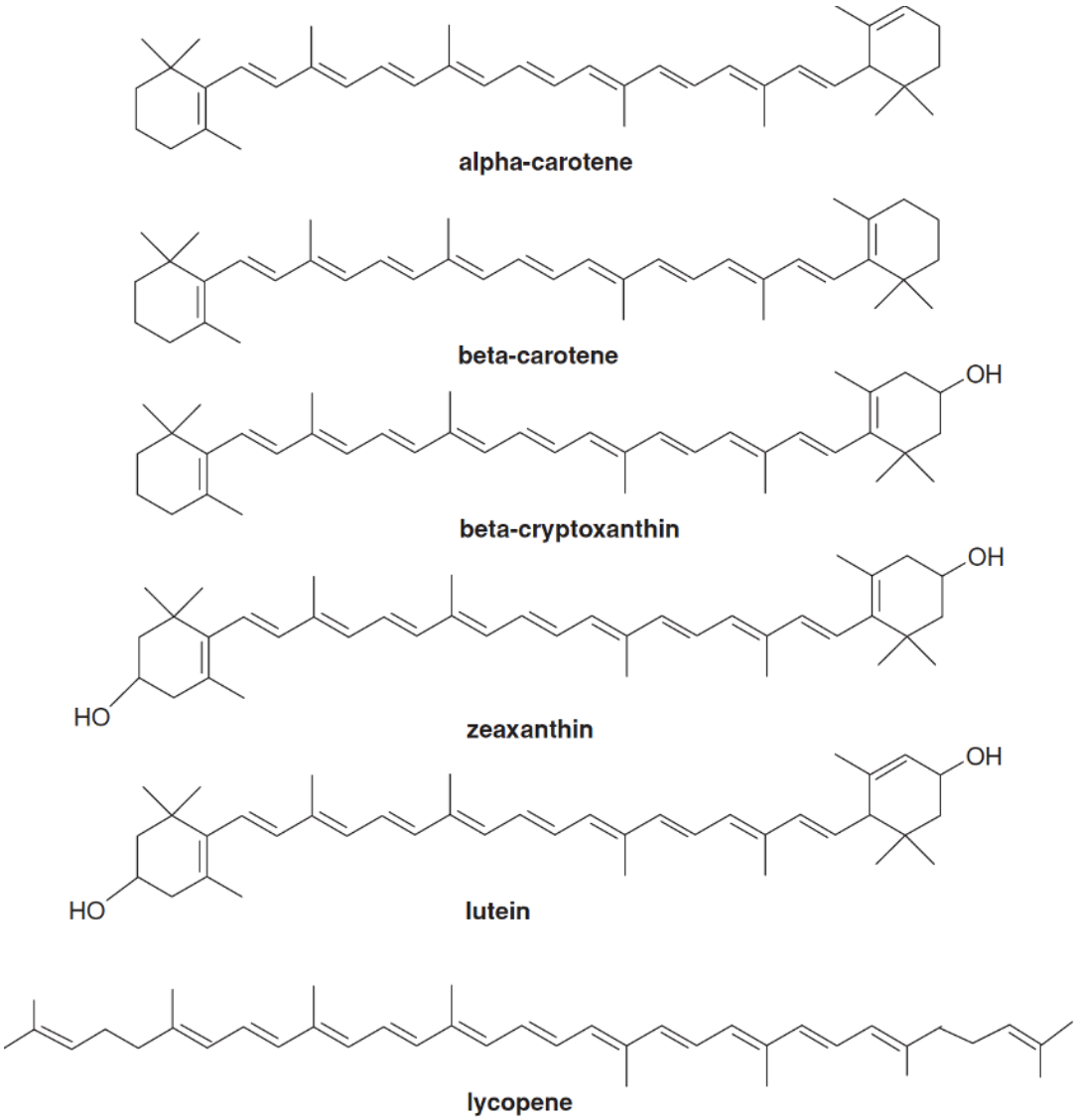


Figure 1.7: Chemical structures of carotenoids (Ellison, 2016)

β-carotene is the most common form of carotenoid found in the diets of humans and is a precursor of vitamin A. In biological systems, the predominant isomer is the all-trans β-carotene (E-isomer) and to a lesser extent, the cis-isomers including 9-cis, 13-cis and 15-cis-β-carotene (Z-isomers) (Figure 1.8) (Grune et al., 2010; Pénicaud et al., 2011). All trans-β-carotene is highly unstable and isomerized into cis-isomers. Trans-β-carotene immediately undergoes thermal and chemical oxidation,

isomerization, and photosensitization when exposed to oxygen, light, and high temperature during processing and storage (Rodriguez-Amaya, 1999). The main sources of β-carotene are fruits and vegetables like tomato, pumpkin, asparagus, carrot, broccoli, cabbage, spinach and lettuce (Weber et al., 2012). β-carotene is believed to have many health benefits due to its anti-cancerous and antioxidant properties. The consumption of β-carotene in the diet of humans has the potential to lower the risk of heart diseases and certain types of cancers, enhancing the immune system, and protection from age-related macular degeneration (Gul et al., 2015).

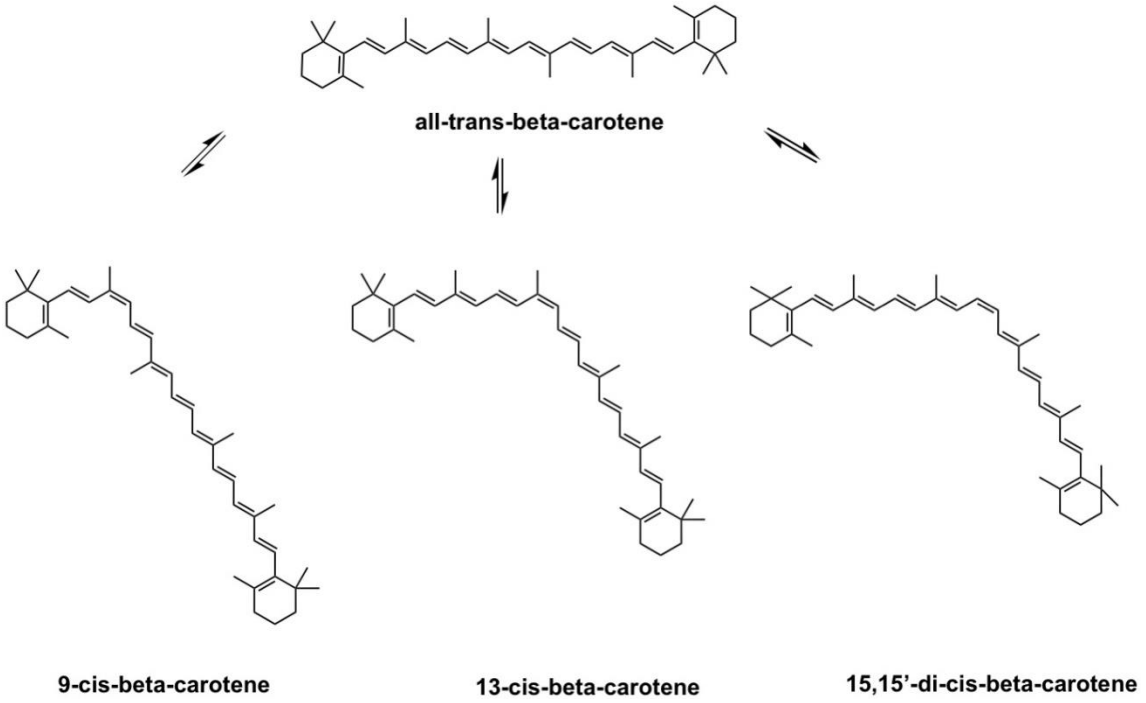


Figure 1.8: Chemical structures of isomers of β-carotene (Pénicaud et al., 2011).

Lutein is a type of xanthophyll carotenoids found particularly in dark-green leafy vegetables (e.g., kale, spinach, broccoli, peas and lettuce), fruits and egg yolks (Perry et al., 2009). Lutein and zeaxanthin are the main dietary carotenoids found in human

retina. Numerous studies indicating that xanthophyll intake or status has been linked with reduced risk of age-related macular degeneration (AMD), cataracts, certain types of cancer, particularly those of the breast and lung, and the prevention of heart disease and stroke (Ribaya-Mercado et al., 2004; Abdel-Aal el et al., 2013). Their beneficial health effects due to the ability to act as scavengers for reactive oxygen species and to bind with physiological proteins in humans (Mitra et al., 2021).

The nutritional credential of chloroplasts recovered from plants are found by (Gedi et al., 2017), that higher concentration of carotenoids is contained in CRF. Therefore, chloroplasts can be potentially used as a source of carotenoid in food formulations.

The composition of different forms of carotenoids in some important vegetables is shown in Table 1.5. These data will be helpful for the selection of high nutritional vegetables for food formula design and diet recommendation (Saini et al., 2015).

Table 1.5: Dietary sources of carotenoids from vegetables (µg/g) (Saini et al., 2015)

Vegetable	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total carotenoids
Broccoli	28.05	-	0.15	11.38	46.46
Kale	65.22	-	0.21	44	132.9
Lettuce	13.5	-	-	14.9	-
Spinach*	775.8	15.1	-	365.3	2386.2

Values are expressed as µg/g fresh weight (*values are expresses as µg/g dry weight).

1.2.2.5. Chlorophylls

Chlorophylls are lipid-soluble green pigments widely distributed in plants; they function in photosynthesis. These molecules are located in Photosystems I and II within the thylakoid membrane in chloroplasts (Mauzerall, 1976; Staehelin, 2003). Structurally chlorophylls are composed of a central magnesium atom surrounded by a nitrogen-containing structure called a porphyrin ring; attached to the ring is a long carbon–hydrogen side chain, known as a phytol chain (Grimm, 2001). To date, several structural variants of chlorophylls have been characterized in higher plants, including chlorophyll a and b (Gaur et al., 2006); the chemical structures are shown in Figure 1.9 (Schwartz et al., 1990). Chlorophyll a appears blue-green in colour and chlorophyll b shows yellow-green (Rodriguez-Amaya, 2019). Chlorophyll a contains a methyl constituent at the C-3 carbon atom, and chlorophyll b this is replaced by a formyl group (Schwartz et al., 2017). Chlorophylls are unstable and readily degrade during thermal treatment (Tan et al., 1962). Indeed, during thermal processing, chlorophyll degradation studies revealed that chlorophyll loses its bound magnesium and transform to other compounds known as pheophytins, chlorophyllides and pheophorbides (Schwartz et al., 1983). The colour changes from bright green to olive brown during this process which ultimately cause the loss of quality in food products. The mechanism of chlorophyll degradation is shown in Figure 1.10.

Chlorophyll and its derivatives can be used as a source of antioxidant nutrients and alternative medicine (Sakata et al., 1990; Hoshina et al., 1998; Ferruzzi et al., 2002). After release from the plant food matrix, natural chlorophyll derivatives are converted

to respective metal-free pheophytins under the acidotic conditions of the gastric phase during digestion. The chlorophyll derivatives are then absorbed by intestinal cells and eventually passes into blood circulatory system (Mishra et al., 2011). Chlorophyll and derivatives have potent antioxidant and radioprotective effects *in vitro* and *in vivo*. They have the ability to inhibit lipid peroxidation, protein oxidation, DNA and membrane damage (Kumar et al., 2001; Taïbi et al., 2016). Several *in vitro* and animal studies have shown that chlorophylls have anti-inflammation, anti-cancer, simulating immune system, normalize blood pressure and wound healing (Gaur et al., 2006; Mishra et al., 2011). With the well-known health benefits of chlorophylls and colour preference in determination of product acceptability, it is important for the food industry to minimise its loss during processing.

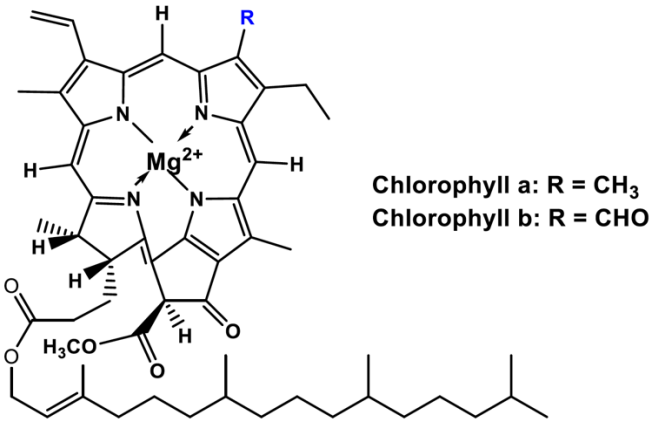


Figure 1.9: Chemical structures of chlorophylls (Schwartz & Lorenzo, 1990)

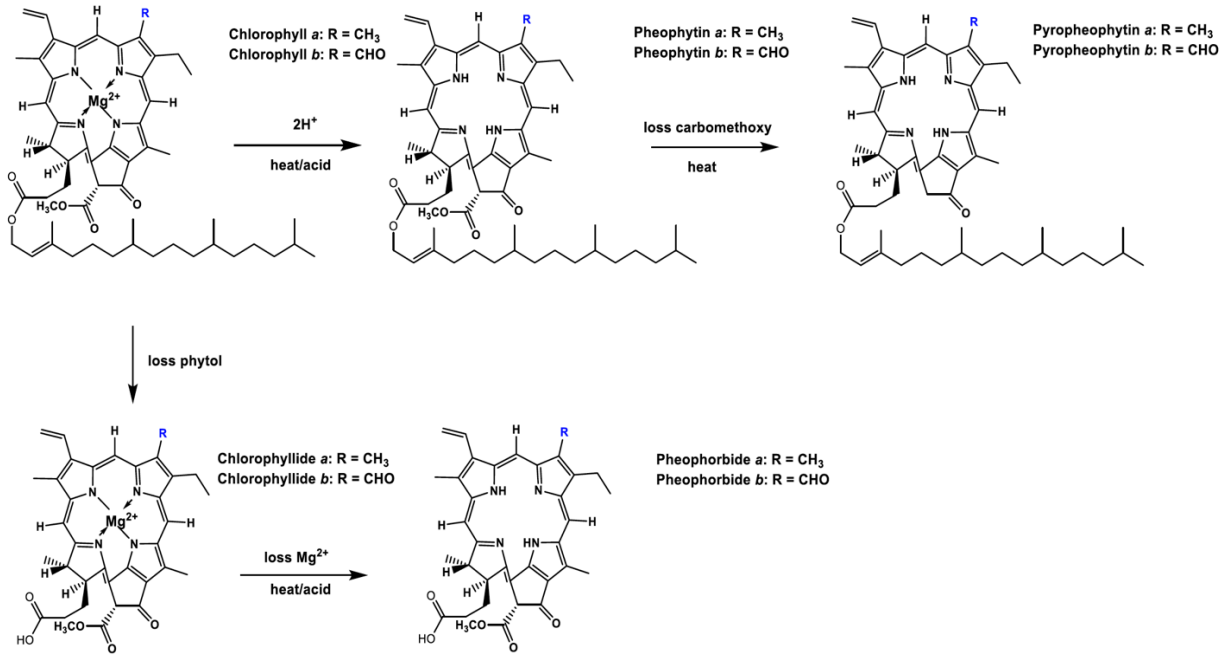
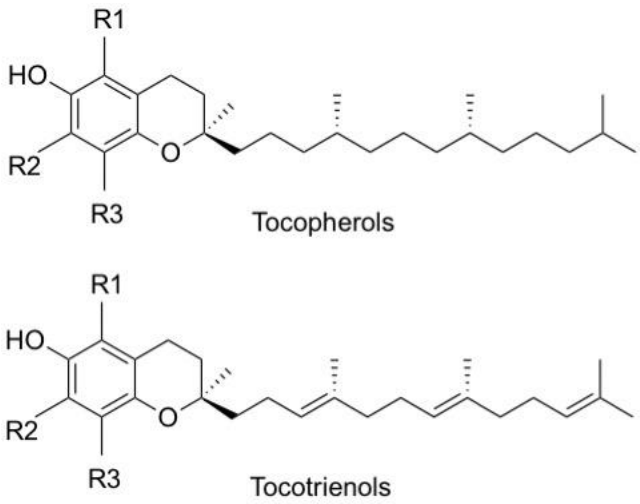


Figure 1.10: Mechanism of chlorophyll degradation (Schwartz & Von Elbe, 1983)

1.2.2.6. Vitamin E

There are eight naturally occurring forms of vitamin E which includes four tocopherols (α, β, γ and δ) and four tocotrienols (α, β, γ and δ), shown in Figure 1.11 (Reboul, 2019b). Alpha-tocopherol is the most abundant form of vitamin E and has the highest biological activity (Burton et al., 1990; Brigelius-Flohé et al., 1999; Reboul, 2017). The antioxidant activity of vitamin E has been linked to numerous health benefits which effectively reduce risk of some diseases, including cancer, ageing, arthritis, oxidative stress, cataracts, immune system disorders, and Vitamin E deficiency (Meydani et al., 1998; Gaziano, 2004; Lippman et al., 2009; Rizvi et al., 2014; Traber, 2014). The Recommended Dietary Allowances (RDAs) for vitamin E (alpha-tocopherol) is 15 mg per day for adults (Kowdley et al., 1992).



	R1	R2	R3
alpha	CH_3	CH_3	CH_3
beta	CH_3	H	CH_3
gamma	H	CH_3	CH_3
delta	H	H	CH_3

Figure 1.11: Chemical structures of tocopherols and tocotrienols (Reboul, 2019b)

Vitamin E is found in various foods and oils. Nuts, seeds and vegetable oils contain high amounts of alpha-tocopherol, which is around 500-2000 μg/g of fresh weight (FW) (Hirschberg, 1999). And significant high amounts of α-tocopherol are also available in green leafy vegetables and fortified cereals (Miyazawa et al., 2011). It has been reported that more than 90% of total tocopherols are found in the form of α-tocopherol in green leaves such as spinach (16.2 μg/g, FW), parsley (47.51 μg/g, FW), and also found in the leaves of tree, including birch, maple, elder, pine, aspen, and juniper (118-519 μg/g, FW) (Szymańska et al., 2008).

1.2.3.Food preservation technologies

Vegetable products consumption is highly depending on changes in pigments, nutritional value, and visual appearance during storage. The fresh vegetables are highly perishable and have a relatively short shelf life. The degradation of vegetable products can be summarised with the following types of reaction: microbiological degradation, hydrolytic reaction, oxidative reaction and enzymatic/non-enzymatic browning reactions (Mounir et al., 2017). As descried earlier, spinach is rich in micronutrients such as carotenoids, chlorophylls, vitamins as well as macronutrients such as proteins and lipids. These nutrients are sensitive to heat, light, oxygen, pH and enzymes (Mehta, 2015). During storage, chlorophylls are susceptible to chemical and enzymatic changes and eventually degrade into olive green catabolism products, including pheophytins, pheophorbides. Major chemical degradation routes are pheophytinization, epimerization, pyrolysis, hydroxylation, oxidation or photo-oxidation (Mangos et al., 1997). Carotenoids also show poor stability in vegetable products. The degradation of carotenoids is accelerated by intermediate radicals in plant as a result of autoxidation and lipid peroxidation (Liu et al., 2015). The isomerisation of carotenoids happens under high temperature and leads to the colourless products (Zeb, 2012). Lipid undergoes lipolysis and enzymatic oxidation reaction during storage and degrades to unsaturated fatty acids (Gyamfi et al., 2019).

Meanwhile, the enzymatic and non-enzymatic browning are also major degradation affecting vegetable product quality. The polyphenol oxidases (PPO) and peroxidase (POD) are main oxidative enzymes in enzymatic oxidation which are

responsible for the changes in flavour, texture, colour and nutritive properties of vegetables (Hamdan et al., 2022). Enzymatic browning occurs in fresh-cut vegetables, where the damaged tissues allow the interaction of phenolic compounds with PPO and exposed to oxygen, consequently results in a rapid browning reaction. So far, various techniques have been developed to control the enzymatic browning of fresh-cut fruits and vegetables, including dips in anti-browning solutions, modified atmosphere packaging (MAP), heat treatment and refrigeration storage (He et al., 2007). Non-enzymatic browning (NEB) occurs in fruits and vegetables due to the interaction of aldehyde and ketone group of reducing sugars with amino acids and proteins (Mitra et al., 2015). The Maillard reaction have been identified as the main non-enzymatic reaction pathways. The NEB reaction is favoured by high heat treatment and water activity. Therefore, it can be inhibited by lowering temperature during the drying process to achieve desired high quality dehydrated food products (Manzocco et al., 2000).

Moisture content and water activity are also crucial factors in microbial growth of food products. Moisture content is simply considered as the total water content in food products. While, water activity is defined as the ratio of the water vapor pressure of a food system to the saturated water vapor pressure at a specific temperature. High value of moisture content and a_w of food products increase the risk of microbiologically growth (Alegbeleye et al., 2022). To avoid microbial growth, the moisture content and water activity must be kept below approximately 10% and 0.60, respectively (Wason et al., 2021). However, fresh foods are commonly high in moisture content and water

activity nearly 0.99 which are particularly prone to microbial growth (Vera Zambrano et al., 2019). Therefore, drying is an ideal food preservation technique which can efficiently remove the total moisture of food products and increase shelf stability (Calín-Sánchez et al., 2020).

Food preservation is defined as the processes or techniques undertaken in order to mitigate internal and external factors which may cause food spoilage (Amit et al., 2017). The principal objective of food preservation is to extend its shelf life retaining original nutritional values, colour, texture, and flavour, and improve consumer acceptability. Different food processing steps are involved to maintain food quality with maximum nutrition values and minimum side effects. Conventional food preservation technologies such as drying, freezing, pasteurisation and sterilization is widely used (Tavman et al., 2019). Recently, food preservation methods include thermal, electrical, chemical and radiation techniques have been emerged in food industry (Sridhar et al., 2021), heat transfer, moisture removal, and prevention of enzymatic and chemical reaction was included. Here, we review the advanced food preservation techniques, which focuses on green leafy plants (i.e. spinach) processed with thermal/nonthermal and spray/freeze drying methods. During which processing, enhances the shelf stability and improves nutritional and physicochemical properties.

1.2.3.1. Spray drying

Spray drying (SD) is a method of particle production which comprises the transformation of a fluid material into dried particles with hot drying medium (Rattes et al., 2007). During the 1880s-1920s, spray drying equipment and techniques was

developed and applied in industry for the need to reduce the transport weight of foods and other materials during World War II (Patel et al., 2009). Nowadays, spray drying is widely applied in food and pharma industries, for the production of food powders, nutraceuticals, aromas, probiotics, enzymes, antibiotics, blood plasma, organic and inorganic chemicals, ceramic powders, detergents and fertilizers (Samborska et al., 2022). Spray drying is a thermally efficient process maintaining quality of a product by rapid dehydration. It provides a large surface area in the form of fine liquid droplets through atomization in the drying chamber, which leads to the production of regularly and spherically shaped powder particles (Fazaeli et al., 2012). Spray drying does have several advantages over other production processes such as freeze drying, since it is faster and cheaper (Sagar et al., 2010; Eun et al., 2020).

The spray drying process consists of four basic steps: (i) atomization of the liquid feed, (ii) droplet-hot air contact, (iii) water evaporation, and (iv) drying gas-powder separation and collection (Sagar & Suresh Kumar, 2010). As shown in Figure 1.12 a schematic is presented for a conventional spray drying process (Patel et al., 2009). Recently, spray drying has been widely applied in the production of fruit and vegetable juice powders for use in food and the pharmaceutical industry (Tontul et al., 2017).

The spray-dried vegetable products are highly stable, because of its low moisture content (2–5%), and water activity (0.2–0.6) (Patil et al., 2014). However, the qualities of spray dried products such as hygroscopicity, morphology, particle size and yield, is highly dependent on the different processing factors during operating. These factors including inlet temperatures, feed flow rate, air dry flow rate, atomizer speed, carried

agent (Phisut, 2012). Moreover, the inlet air temperature appreciably affects the stability of carotenoids in spray dried food products. The reduction of carotenoids content is presumably due to the thermal degradation and oxidation, when expose to oxygen during drying (Goula et al., 2005). Therefore, it is important to optimize the various processing factors prior to spray drying.

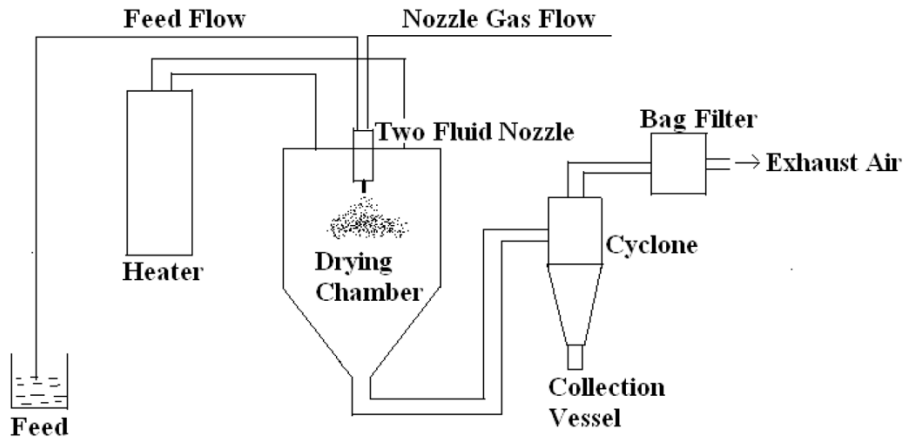


Figure 1.12: Spray dryer configuration (Patel et al., 2009). The laboratory scale spray dryer used in these experiments was FT 80 Tall form with a two-fluid atomizing nozzle.

1.2.3.2. Freeze drying

Freeze drying (FD), also known as lyophilization is a well-known technique for the production of high-quality food powders and solids (Figure 1.13) (Nowak et al., 2020). It is the preferred method for drying foods containing thermally sensitive and oxidative compounds since it operates at low temperatures and under high vacuum (Bhatta et al., 2020). The freeze dried products have many benefits, such as nutrients stability during storage due to low moisture content, reduction of nutrients degradation in heat sensitive products, minimally changed structures by the process (Adams, 2007;

Nireesha et al., 2013) . FD has been applied to various plant-based foods, such as apple, blackberry, coffee, guava, strawberry, pumpkin, garlic, and ginger (Marques et al., 2006; Sablani, 2006; Ciurzyńska et al., 2014; Fissore et al., 2014; Franceschinis et al., 2014; Fante et al., 2015; An et al., 2016). The advantage of FD compared to SD is that it retains nutritional value better while preserves the actual colour and shape of the original raw material, that supporting consumers' demand for whole healthy food (Serna-Cock et al., 2015).

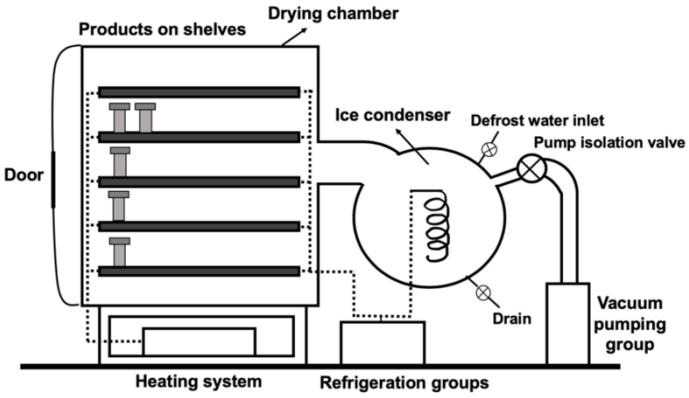


Figure 1.13: Construction of freeze dryer (Garcia-Amezquita et al., 2016)

FD is a process in which water in the form of ice under low pressure is removed from a material by sublimation. It has become an important technology for heat-sensitive drugs and food preservation (eg. fruits, vegetables, meat) (Liu et al., 2022). The FD process can be divided into three steps, (a) freezing, where the sample should be completely frozen, (b) primary drying, when ice is sublimated, usually at sub-atmospheric pressure, and (c) secondary drying, when the remaining unfrozen/bound water is desorbed from the drier food matrix. Water exists in three different states: solid,

liquid, or gas (vapor). As shown in Figure 1.14, a phase diagram of water (pressure versus temperature), where the curve lines show the passage from solid to vapor (sublimation), from liquid to vapor (evaporation), or from solid to liquid (fusion) during freeze drying (Gaidhani et al., 2015; Nowak & Jakubczyk, 2020).

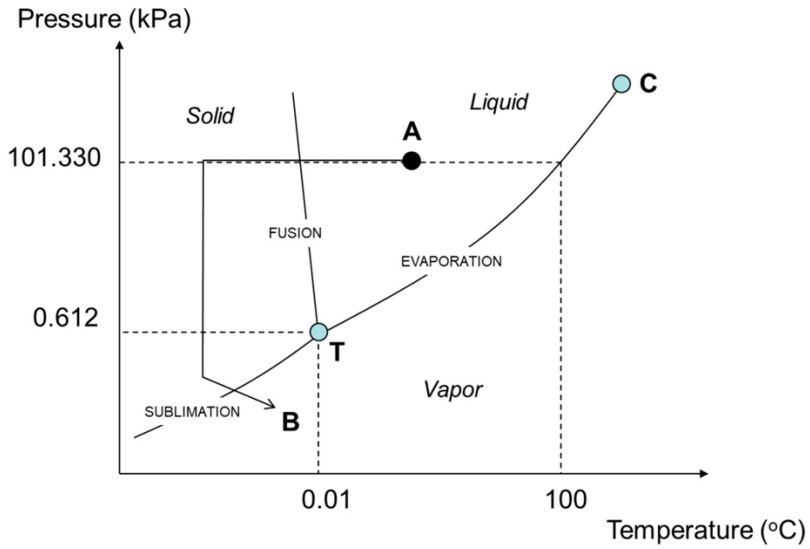


Figure 1.14: Phase diagram of water (T: triple point of water, C: critical point of water). "A" represents the starting point prior to freeze-drying (atmospheric pressure and ambient temperature), while "B", the desired final conditions during sublimation (below the triple point T) (Bhatta et al., 2020).

1.2.4.Thermal pre-treatment

Thermal pasteurisation and blanching are predominantly used in the food industry for inactivation of endogenous enzymes of plants and extend its shelf life after harvesting. However, excessive heat treatment may cause undesirable quality loss of food products, such as protein denaturation, non-enzymatic browning and loss of vitamins and volatile flavour compounds (Torcello-Gómez et al., 2019).

1.2.4.1. Pasteurisation

Pasteurisation is a thermal treatment which widely accepted as an effective preservation method for killing pathogens in food products, with minimal loss of desired food quality (Ramesh, 2020). It usually refers to a mild heat treatment (temperature<100°C), designed mainly for the inactivation of spoilage enzymes and vegetative forms of micro-organisms (bacteria, moulds, and yeasts) (Lund et al., 1988).

However, even a mild thermal process tends to cause a significant loss of quality in terms of colour, texture, flavour, and nutritive value. Thus, it is necessary to optimize a time-temperature combination for thermal processing (Maesmans et al., 1990; Grant et al., 2005). There are two main types of traditional pasteurisation (excluding ultra-high temperature or UHT method) i.e. low-temperature-long time (LTLT) and high-temperature-short-time (HTST) methods (Urquieta-Herrero et al., 2021). Selection of the optimal process is usually dependent upon which will provide the best sensory properties and overall quality for the pasteurised food. There are many factors that need to be considered, such as the nutrients retention, types of the food, type of micro-organisms, and endogenous enzyme activities (Aamir et al., 2013).

Vegetables are generally classified as low acid foods (pH ≥ 4.6), more severe heat treatment is needed to kill spores of pathogenic and spoilage microbes (McGlynn, 2003). However, one consequence of heating is that heat sensitive compounds contained in vegetables such as ascorbic acid, thiamine is reduced during thermal processing (Akinyele et al., 1990; Leistner et al., 1994). Moreover, the presence of residual endogenous enzymes in processed vegetable products may cause further

quality loss during storage and shorten its shelf life. Thus, the complete inactivation of endogenous enzymes is used as a target for an adequate thermal treatment. Major enzymes related to vegetable quality attributes (texture, colour and flavour) are polyphenol oxidase (PPO), peroxidase (POD) and lipoxygenase (LOX) (Silva et al., 2004). Typically, POD is used as an indicator for the effectiveness of heat treatments in fruits and vegetables because this enzyme is the most heat resistant enzyme presented in most plant tissues (Gonçalves et al., 2010; Ercan et al., 2011; Zheng et al., 2011). The assumption is that destruction of POD is accompanied by inactivation of all other enzymes and vegetative micro-organisms (Whitaker, 2018).

1.2.4.2. Blanching

Blanching is a thermal treatment conducted in hot water, steam or microwave, that is commonly used as a pre-treatment prior to further processing (canning, freezing, dehydrating) of many vegetables (Peng et al., 2017). The purpose of blanching is to inactivate endogenous enzymes, preserve colour, flavour, and nutritional value and release the entrapped air within vegetable cells and replaces them with water. Water blanching also involves two process namely, low-temperature long-time (LTLT) or high-temperature short-time (HTST) processing (Aguilar et al., 2004). Typical blanching temperatures ranging from 70 to 100°C depending upon the product and processing conditions (Stanley et al., 1995). POD is chosen as the target for inactivation during the blanching process which indicates if product quality is to be maintained (Ramesh et al., 2002).

Other food quality attributes such as flavour, colour, texture and nutrients are

greatly affected by the type and extent of blanching (De Corcuera et al., 2004). Blanching indirectly and directly affects food flavour by removing bitter flavour and increase flavour retention (Velasco et al., 1989); the colour changes of green leafy plants during blanching is caused by the destruction of chlorophyll and other colouring pigments and Maillard browning due the presence of reducing sugars (Tijskens et al., 2001; Richter Reis, 2017). Blanching can also cause undesirable softening of vegetable tissues and higher leaching of some heat liable vitamins and minerals (Stanley et al., 1995; Severini et al., 2016).

1.2.5.*In vitro* digestion

Food digestion is a complex process and is of direct interest to the food industry since this process dictates the availability of nutrients associated with food quality and health benefits like reduced risk of chronic diseases (Bornhorst, 2016). For the purpose of understanding these changes, *in vitro* digestion methods have been developed. *In vitro* digestion closely simulates the physiological conditions of *in vivo* digestion with either static or dynamic models being commonly used. Several factors are considered in *in vitro* digestion models such as the occurrence and concentration of digestive enzymes, the pH values in gastric and intestinal phases, digestion time and salt concentrations (Minekus et al., 2014). These techniques are widely used in research fields such as nutrition, pharmacology and food chemistry since it is flexible, rapid, inexpensive and reproducible (Calvo-Lerma et al., 2018). To date, *in vitro* digestion models provide a useful alternative to animal and human models without limitations of ethical issues (Egger et al., 2017).

1.2.5.1. Static *in vitro* digestion model: INFOGEST

The INFOGEST *in vitro* digestion model is a standardized model first introduced by Minekus et al. (2014). An updated INFOGEST 2.0 model has been published with some modifications that better reflect *in vivo* digestion processes (Brodkorb et al., 2019). This model consists of three phases that including an oral, gastric and intestinal phase as shown in Figure 1.15. In the oral phase, the food should be diluted at 1:1 (wt/wt) with simulated salivary fluid (SSF), with or without salivary amylase (α-amylase, 75U/ml), incubated for 2 min at pH 7, 37 °C. α-amylase is used for starch digestion, as it can hydrolyse starch molecules into maltose and some glucose molecules. Thus, the α-amylase in the oral phase is selective and sometimes omitted by some researchers which mainly focus on the digestion of other components such as lipids and proteins. Following the oral phase, the gastric phase, the oral bolus is then mixed with simulated gastric fluid (SGF) (1:1, v/v) and gastric enzymes (pepsin 2,000 U/ml and gastric lipase 60 U/ml) and incubated at pH 3.0 for 2 h (37 °C). The addition of gastric lipase (for example, rabbit gastric extracts, RGE) is the main modification based on the INFOGEST 1.0 (Minekus et al., 2014). As lipid starts to digest in the stomach, and gastric lipolysis contributes to the overall digestion of TAGs (10% with a solid–liquid test meal to 25% with an emulsified liquid test meal). Moreover, it also triggers the subsequent action of pancreatic lipase on lipid substrates that may be poorly digested by pancreatic lipase alone (Brodkorb et al., 2019).

Finally, in the intestinal phase, the gastric chyme is mixed with simulated intestinal fluid (SIF) (1:1, v/v), porcine bile (bile salts 10mM) and porcine pancreatic extracts

(PPE) is added and incubated at pH 7 for a further 2 h (37 °C). In each phase of this model, it is possible to collect the processing fluids to assess nutrient liberation and stability.

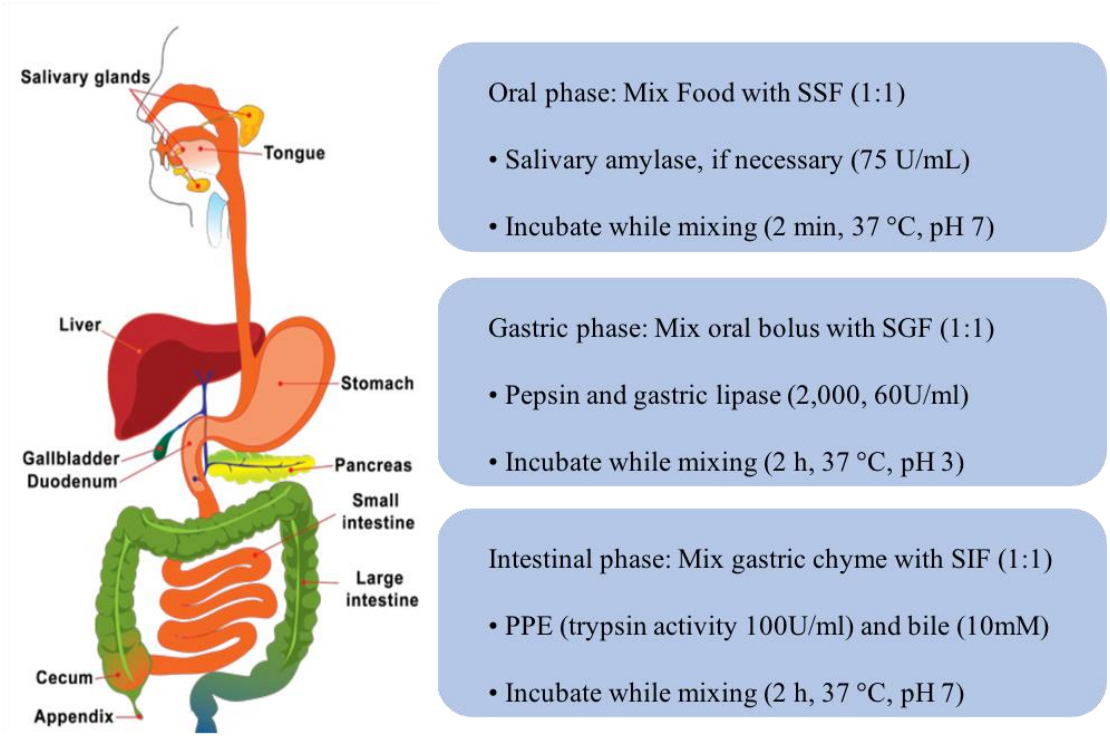


Figure 1.15: Flow diagram of INFOGEST 2.0 digestion method (Brodkorb et al., 2019).

1.2.5.2. Static *in vitro* digestion model from Carriere et al. (2000)

Other static models of digestion have also been developed in recent times. The *in vitro* digestion model simulated meal lipid digestion was based on *in vivo* studies of gastric conditions from a half gastric emptying. As shown in Figure 1.16 and 1.17, the pH in half gastric meal emptying was around 5-5.5 for liquid meal and pH 4.5 for solid-liquid meal (Carrière et al., 2001; Sams et al., 2016). Generally, the use of pH 2-3 for gastric phase is set for most of *in vitro* models and mirrors the recently developed

INFOGEST system (Minekus et al., 2014). While this is more acidic than these in *in vivo* experiments, the pH 2-3 is correspond to approximately 85-95% of meal gastric emptying (Sams et al., 2016). Likewise, the setting of gastric lipase activity to 60 U/ml is correspond to approximately 86% of gastric emptying. In order to accurately mimic the gastric condition, the 50% of gastric emptying is considered, and the pH and gastric lipase will need to be set to pH 5 and 20.4 U/ml respectively.

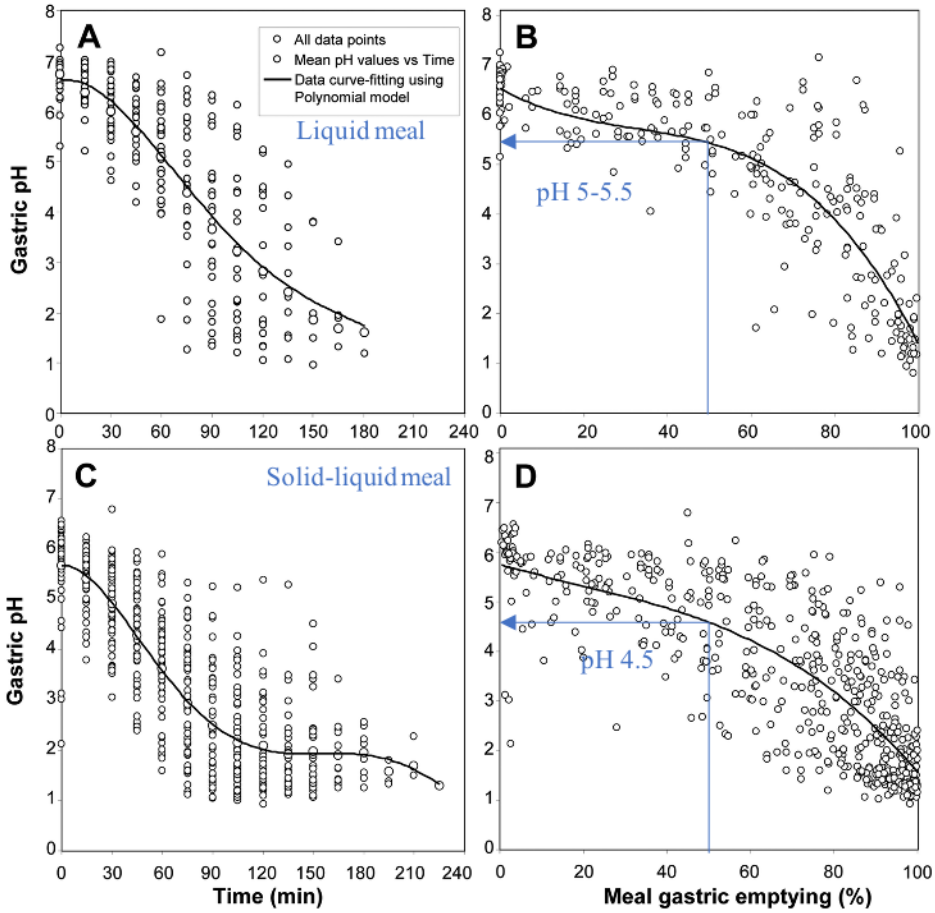


Figure 1.16: Variations in gastric pH during test meal digestion in healthy volunteers. Panel A: pH variation as a function of time during a liquid test meal (256 values from 30 individual experiments); panel B: the same pH values plotted as a function of meal gastric emptying (%); panel C: pH variation as a function of time during a solid–liquid

test meal (545 values from 53 individual experiments); panel D: the same pH values plotted as a function of meal gastric emptying (%) (Sams et al., 2016).

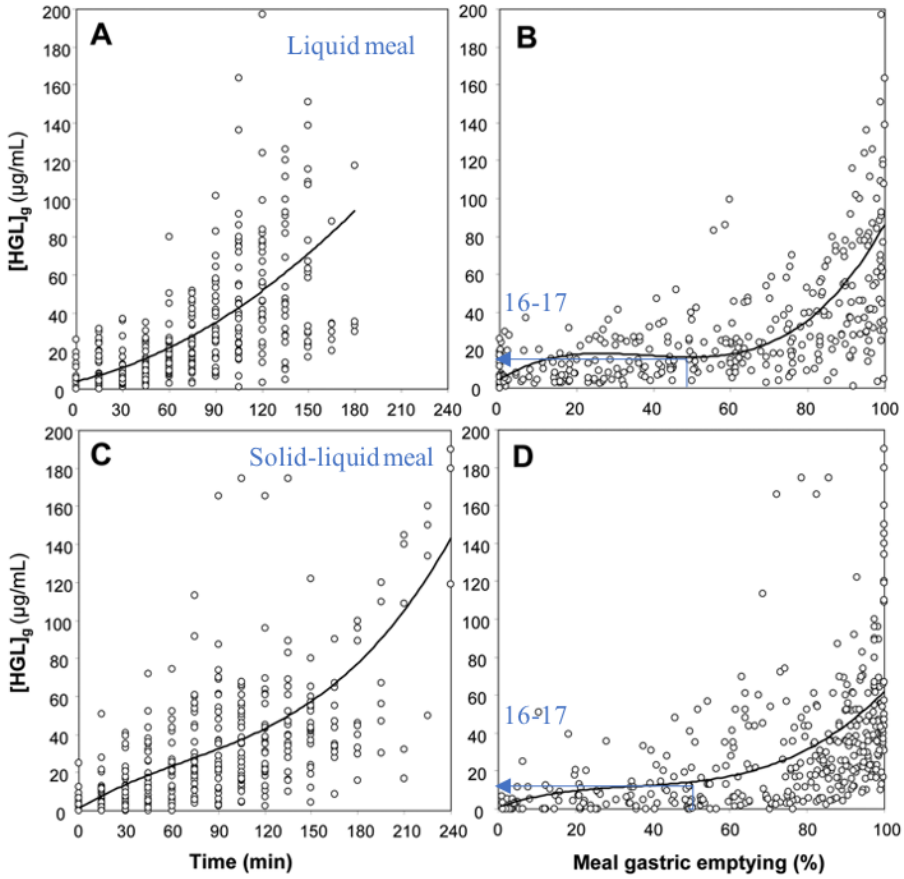


Figure 1.17: Variation of the gastric lipase concentration in gastric contents (Human gastric lipase, [HGL]g) during test meal digestion in healthy volunteers. Panel A: [HGL]g variation as a function of time during a liquid test meal (256 values from 30 individual experiments); panel B: the same [HGL]g values plotted as a function of meal gastric emptying (%); panel C: [HGL]g variation as a function of time during a solid–liquid test meal (545 values from 53 individual experiments); panel D: the same [HGL]g values plotted as a function of meal gastric emptying (%) (Sams et al., 2016).

1.2.5.3. Bioaccessibility of nutrients

Key to our understanding of how to better improve foods and their nutritional

quality is the process of bioaccessibility. Bioaccessibility is a key concept to ascertain nutritional efficiency of food and food formula developed with the aim of improving human health (Fernández-García et al., 2009). It is defined as the proportion of a compound that is released from a food matrix in the gastrointestinal tract, that then becomes available for intestinal absorption and distributed systemically. The aforementioned *in vitro* digestion models are better facilitating our understanding of this process (Hur et al., 2011; Verhoeckx et al., 2015).

Plant based foods including fruits, vegetables are rich source of numerous types of phytonutrients such as vitamins, carotenoids, minerals, polyphenols, polyunsaturated fatty acids, proteins and dietary fibers that have a range of health benefits in humans (Espín et al., 2007). However, phytonutrient compounds must be first released from foods, and effectively absorbed from the gut into the circulatory system and delivered to the appropriate target location to achieve key health beneficial effects (Actis-Goretta et al., 2013). Several factors influence the rates of bioaccessibility of phytonutrients including the food matrix, processing and preservation methods (Thakur et al., 2020). For example, the bioaccessibility of carotenoids is relatively low varying from 5 to 30%, in comparison to other food phytochemicals (Kopec et al., 2018). Moreover, the low bioaccessibility of β-carotene from natural food sources has mainly been attributed to the limited release from food matrix, being bound to proteins, and its restricted release by the cell wall and low efficacy of micellization (Van Loo-Bouwman et al., 2014; Shilpa et al., 2020). Thermal treatment such as cooking, frying and pasteurisation can increase the bioaccessibility of carotenoids like beta-carotene in plant tissues

(Priyadarshani, 2017). Heat treatment can soften and disrupt the cell membranes and plant cell walls, and is known to break protein-carotenoid complexes (Erdman Jr et al., 1988). As a result, this stimulates the release of carotenoids and facilitates efficient digested by digestive enzymes during the digestive tract. Following the release from the food matrix, another major limiting factor is the poor solubilization of carotenoids in the digest. The addition of oil can promote the dispersion of carotenoids by dissolving them and by forming an emulsion in the digestive tract (Nagao et al., 2013). This process aids the incorporation of carotenoids in to mixed micelles and allows for better transport to intestinal epithelia. The bioaccessibility of carotenoids can therefore be improved from plant sources using different cooking methods (Hornero-Méndez et al., 2007; Qian et al., 2012). Reduced particle size has also been evident in several studies that can improve bioaccessibility of carotenoids by increasing the surface area available for enzymes to act (Hedren et al., 2002; Salvia-Trujillo et al., 2013; Lyu et al., 2021). The bioaccessibility of β-carotene from liquefied spinach was higher than that of the whole leaf or minced spinach (Castenmiller et al., 1999). Powder processing is also an effective way to produce small particles and easily cause the cell wall rupture and improve the β-carotene bioaccessibility (Lyu et al., 2021).

1.3. Research within David Gray's group

Our research group has been involved in studies focused on the utilisation of chloroplasts from green plants over a decade. Recent studies have shown interest in the use of chloroplast materials as food supplements, either for animal feed (Gedi et al., 2019) or as functional food ingredient (Torcello-Gómez et al., 2019). Spinach CRFs and whole leaf materials were fed to zebrafish as partial fish diet replacement compared with a standard fish diet, and the fish growth was monitored (Gedi et al., 2019). (!!! INVALID CITATION !!! Syamila et al. (2019)) has investigated a simple way to concentrate the liberated chloroplast materials, and the optimum way of dehydration (spray drying, freeze drying, conventional oven and vauum oven drying) of spinach juice/CRF with enhanced nutrients stability; In addition, Wattanakul et al. assessed the impacts of thermal processing (steam sterilisation, hot water blanching and pasteurisation) on the stability of nutrients (!!! INVALID CITATION !!! (Wattanakul et al., 2020; Wattanakul et al., 2022)). The research present in the current thesis extends our knowledge built on the previous findings. This study mainly tested the optimum processing technologies such as thermal (blanching and pasteurisation) and drying (spray and freeze drying), used to produce nutritionally rich powders derived from spinach leaves, on the material properties and digestibility of powders derived from spinach leaves. The bioaccessibility of essential lipophilic nutrients derived from chloroplast materials was investigated within a modified *in vitro* gastrointestinal human digestion model.

1.4. Aims, hypotheses and objectives.

Aims:

To establish the impact of thermal treatments, used in the production of a chloroplast-rich fraction (CRF) from spinach leaves, on the material properties, nutritional composition, and digestibility of the CRF material.

Hypothesis:

The hypotheses being tested were that a certain degree of thermal treatment and drying process can help to retain nutrients composition, restrict quality loss in the juice/CRF material and can increase their bioaccessibility by altering the material properties of the juice/CRF material at the macro and molecular level.

Objectives:

1. Compare the physical properties of dried spinach juice through spray drying and freeze drying.
2. Compare the nutrients composition of freeze-dried spinach juice and CRF powders.
3. Investigate the effect of pasteurisation and blanching of spinach biomass prior to freeze drying on the physical properties of the derived CRF material.
4. Determine the impact of heat pre-treatment of spinach juice on the bioaccessibility of lipophilic nutrients from the derived CRF material.
5. Monitor the digestion of galactolipids from spinach CRF material using an in-vitro system to simulate human digestion.

1.5. Thesis Structure & Clarification

This thesis consists of eight chapters starting with a general introduction, literature review and materials & methods chapters. It will then go on to five major results chapters, as outlined earlier. Chapter I introduces the background of the current research, raises the aims, hypotheses and objectives. Chapter II provides the explanations and justifications for the research methodology used, explaining the preparation steps and data analysis methods. Chapter III, IV and V compares the effects of different processing procedures such as drying (spray drying and freeze drying) and heat treatment (pasteurisation and blanching) on the physicochemical properties of spinach juice/CRF. Chapter VI presents the digestion of galactolipids and proteins from spinach juice/CRF by using a static *in vitro* digestion model. Chapter VII shows the bioaccessibility of lipophilic nutrients from spinach juice/CRF during *in vitro* digestion. The last chapter summarises the overall findings, conclusions and suggests the prospect of future work.

CHAPTER II

2. Materials and methods

2.1. Materials and chemicals

Fresh baby spinach was purchased from a local supermarket (Tesco, UK). The spinach was stored at 4 °C before using. Different batches of spinach were purchased during 2019-2022, and each chapter uses the same batch of spinach. All solvents (chloroform, methanol, acetonitrile, trimethylethylamine (TEA), ethyl acetate) were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific, UK. All chemicals: butylated hydroxyl toluene (BHT), sodium chloride, trimethyl sulfonium hydroxide (TMSH), triethyl amine (TEA) were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). All standards: β-carotene, α-tocopherol, and methylpentadacanoate were also purchased from Sigma Aldrich (Merck, Darmstadt, Germany), except for lutein which was purchased from Pan Reac AppliChem (Darmstadt, Germany). 10x Tris/Glycine/SDS Buffer, precision plus protein standards, 12% mini-PROTEAN TGX gels were purchased from Bio-Rad, California, USA. InstantBlue coomassie protein stain was purchased from ABCAM, Cambridge, UK. The galactcolipids standards including monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) were purchased from Avanti, Polar lipids, Alabaster, USA. Linolenic acid and lipid standards (mono, di-, & triglyceride mix) were purchased from Sigma-Aldrich, Missouri, USA. High-performance thin layer chromatography (HPTLC) glass plates (10×20 cm) were purchased from Merck Life Science, Gillingham, UK. Other chemicals used were of analytical grade material.

2.2. Enzymes

The following enzymes were used in the current research. Pepsin was obtained from porcine gastric mucosa lyophilized powder, 2500 U/mg protein (P7012), porcine pancreatin extract (PPE) (P7545) and porcine bile extract (B8631), were all purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). Rabbit gastric extract, 15 U/mg, was obtained from Lipolytech (Marseille, France). All enzymes were stored at -20°C before use.

2.3. Methods

2.3.1.Spinach chloroplasts processing

2.3.1.1. Blanching of spinach leaves

Fresh spinach leaves (200 g) were blanched in water at 100 °C for 30 s and then immediately immersed in ice cold water to cool samples to room temperature. Blanched leaves were the spun for 1 min using salad spinner to remove the excess water and then further being juiced. The blanching steps are illustrated in Figure 2.1.

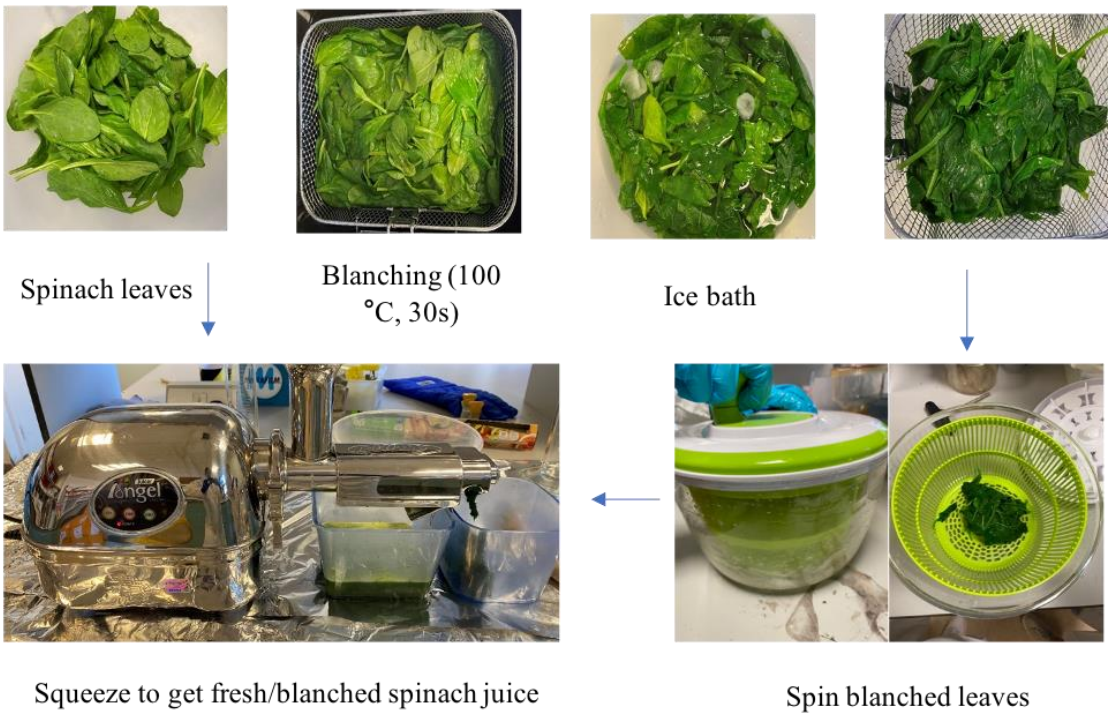


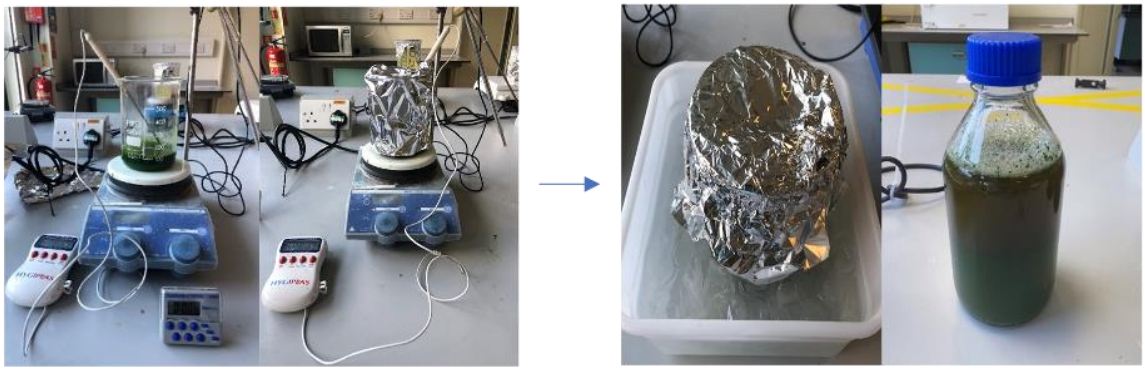
Figure 2.1: Preparation steps of fresh/blanched spinach juice

2.3.1.2. Pasteurisation of spinach juice

Fresh spinach juice (500 mL) was pasteurised at mild (70 °C,15 s) and intense conditions (90 °C, 5 min) in a beaker covered by foil, the temperature was controlled using a water bath with a magnetic stirrer (500 rpm/min), (IKA RCT basic, England). The temperature of the juice was increased gradually and monitored using a thermometer, the average heating time was 4-6 minutes. The conditions were chosen to represent mild and intense pasteurisation respectively. Pasteurised spinach juice was then cooled in cold water (0-4 °C) immediately. All treatments were performed in the dark to avoid any possible interference from light. The chloroplast rich fraction (CRF) was collected by further centrifugation.

Fresh spinach juice/CRF was chosen as control, all the samples were stored at 4 °C

in the dark until required. The pasteurisation steps are shown in Figure 2.2.



Mild pasteurization 70°C15s;
Intense pasteurization 90 °C5min
(stir at 500rpm)

Ice bath

Pasteurized juice

Figure 2.2: Preparation steps of mild/intense pasteurised spinach juice

2.3.1.3. Preparation of spinach juice and chloroplast rich fraction (CRF)

Fresh and blanched spinach leaves were juiced using a twin gear juicer (Angel 7500, Busan, Korea), which separated the fibrous pulp from the nutrient-rich juice. The juice was then filtered through a 75 µm stainless steel mesh sieve.

The resultant juice was then centrifuged at 17,700 RCF (10,000 rpm), using a Beckmann Coulter JS-21M centrifuge with a JA-10 rotor (London, UK) for 10 min at 4 °C. The pellet containing the CRF was retained whilst the supernatant was centrifuged again under the same conditions to obtain another pellet and this was combined with the original pellet (Torcello-Gómez et al., 2019). This method utilises a juicer to mechanically press out fluid from spinach leaves, omitting the need to use harsh physical cell wall disruption steps which could potentially destroy intact chloroplast structures. The whole processing steps are shown in Figure 2.3.

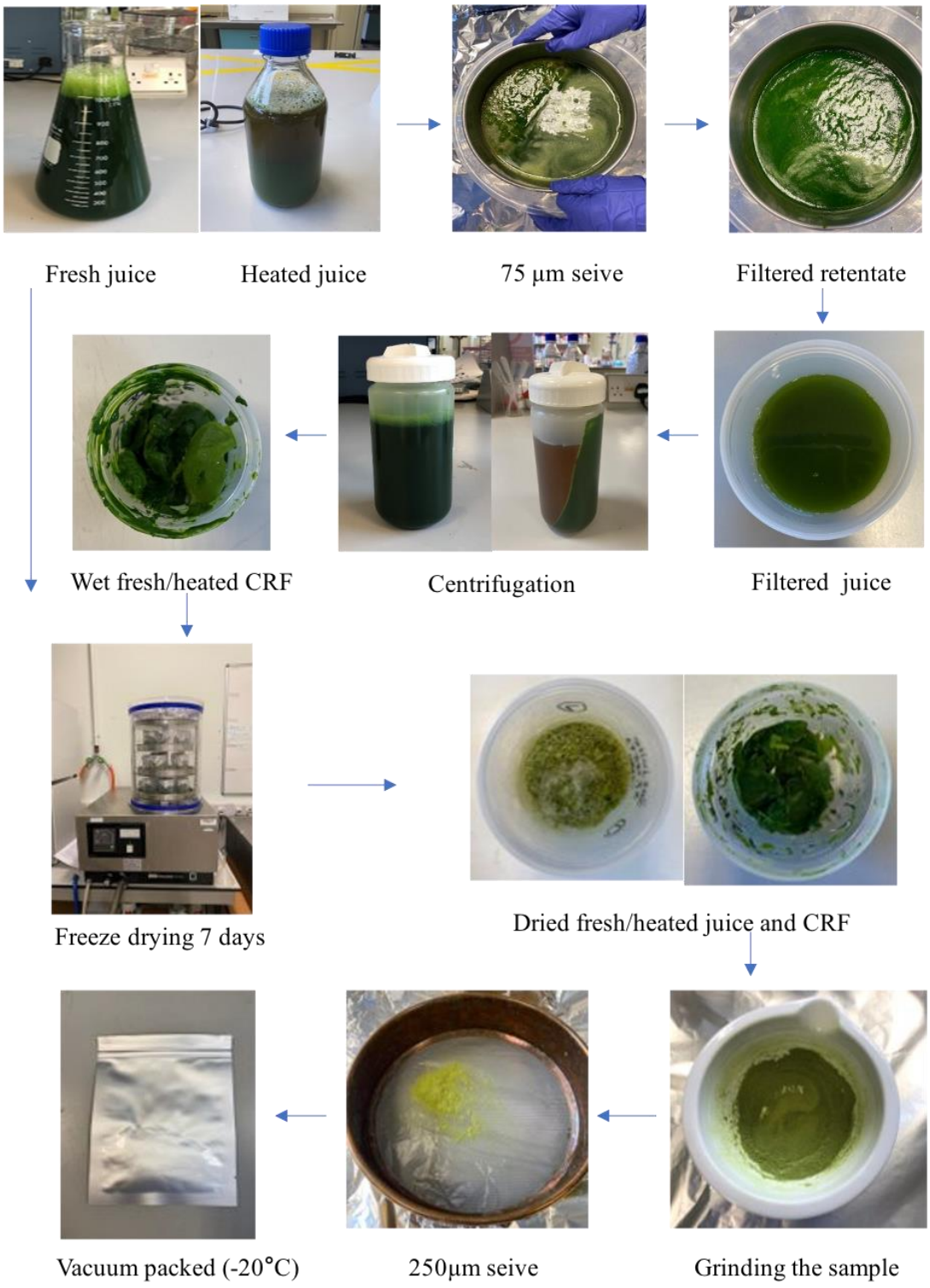


Figure 2.3: Preparation steps for freeze dried spinach juice and chloroplast rich fraction (CRF)

2.3.1.4. Spray drying

Spray drying (SD) was conducted using a spray dryer (Armfield FT80 Tall Form Spray Dryer, Ringwood, UK) using a modified method developed by Syamila et al. (2019). The drying condition was set at 1mm two-fluid atomizer nozzle, inlet air temperature 132 ± 2 °C, outlet temperature of 65 ± 5 °C, feed flow rate (rate of feeding) 420 mL/h, relative humidity range 5-11 %, compressed air pressure of 1-1.5 bar, respectively. The spray-dried samples were then stored in a vacuum-sealed aluminum bag at -20°C for further analysis (Figure 2.4). The principle of spray dryer involves converting a liquid or slurry into dried particles by atomizing the feed solution into droplets and then contacting with hot air.

Figure 2.4: Preparation steps of spray drying spinach juice

2.3.1.5. Freeze drying

Freeze drying (FD) was performed by freeze dryer (Super Modulyo, York, UK) with a modified method of Torcello-Gómez et al. (2019). The filtrated juice and pooled CRF (section 2.3.1.3) were weighed and frozen at -80 °C prior to freeze drying using

the freeze dryer (Super Modulyo, York, UK) for 7 days. Freeze dried juice and CRF were then ground using a pestle and mortar, filtered with 250 µm sieve then stored in a vacuum-sealed foil bag at -20 °C for further analysis. The preparation steps are shown in Figure 2.3. The principle behind freeze dryer is to remove moisture from a substance (solution or a slurry) by freezing it and then sublimating the frozen water under vacuum. Main steps of freeze drying including freezing phase, sublimation phase and desorption phase. When the samples frozen below its freezing point, the water molecules become ice crystals form. The frozen ice is then sublimated to the vaper state in vacuum chamber and accelerated by heating. The residual moisture is further removed during desorption phase, which is a critical for preserving the stability of the product (Ward et al., 2021).

2.3.1.6. Representative freeze dried samples

The morphology and appearance differences of freeze dried spinach chloroplast samples can be observed in Figure 2.5. All the samples were after grinded and filtered with 250 µm sieve, thus the particle size of these samples is lower than 250 µm. This data has been confirmed by the particle size analyser as mentioned in section 2.3.3.3 (f).

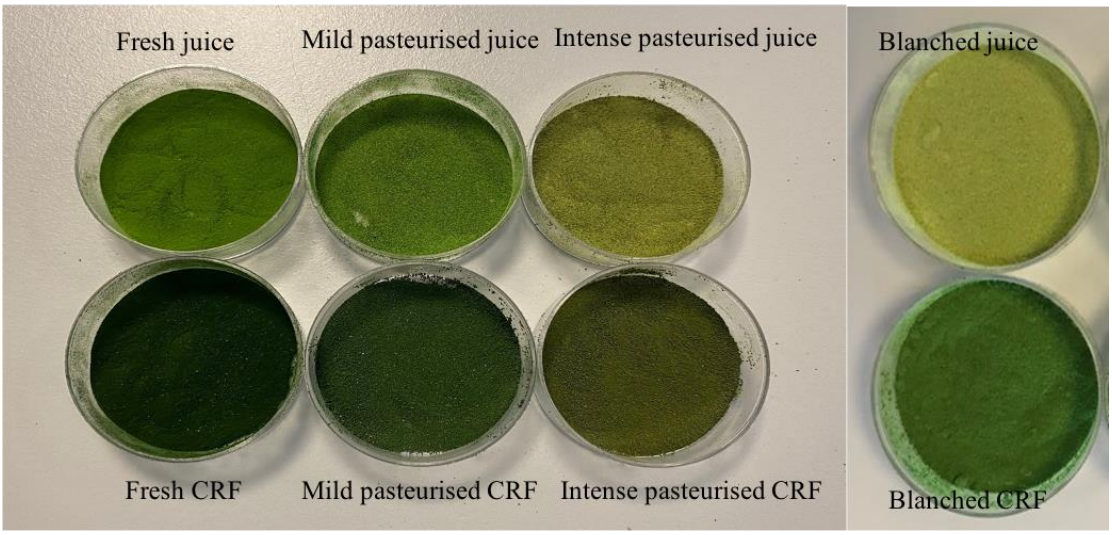


Figure 2.5: Representative freeze dried spinach samples

2.3.2.Spinach juice characteristics during storage at 4 °C for 7 days

2.3.2.1. Total soluble solid content (Brix°) and pH

The total soluble solid content of the spinach juice was determined at 20 °C using a digital handheld refractometer (Pocket PAL-α, Atago, Tokyo, Japan) and expressed as °Brix (which is based on the relationship between refractive index and the total soluble solid content of a pure aqueous sucrose solution). The refractometer measures the refractive index of a liquid sample to determine its sugar content, expressed as Brix°, 1 Brix° equivalent to 1 gram of sucrose per 100 grams of solution. By passing light through the sample, the angle at which the light is bent and determined as the refraction index. The refraction index is proportional to the brix value, higher refractive index indicates higher sugar content of the sample.

The pH of the juice samples was determined by a pH meter (InoLab® pH 7110, German) at 20 °C. The pH meter works by measuring the concentration of hydrogen

ions (H^+) in a solution to determine its acidity or alkalinity, expressed as the pH value. Two or more buffer with different pH values are used to calibrate the meter across the range of interest. The electrodes are immersed in the sample solution, and the voltage difference between the glass electrode and the reference electrode is measured. This voltage difference is converted into a pH value using the calibration curve obtained from the buffer solutions.

2.3.2.2. Determination of POD (peroxidase) reduction rate

POD activity was determined by UV-Vis spectrophotometry (Thermo Scientific, Genesys10S, US) at 20 °C using a modified method of Bahçeci et al. (2005). The reaction assay mixture was prepared with 500 µL guaiacol, 500 µL H_2O_2 diluted to 100 mL with distilled water. Spinach juice (0.1 mL) was then added to 2.9 mL of the assay mixture. The absorbance was measured at 420 nm for 1 min with 2 s interval. The enzyme activity was estimated from the linear portion of the curve of absorbance v/s time. One unit of POD activity was defined as $0.001A_{420}$/min. The activity of the samples was expressed as % POD reduction rate as given in the following equation (Eq. 2.1):

$$\%POD\ reduction\ rate = \left(1 - \frac{enzyme\ activity\ of\ pasteurized\ juice}{enzyme\ activity\ of\ fresh\ juice}\right) \times 100 \quad \textbf{(Eq. 2.1)}$$

The guaiacol-based assay for peroxidase activity is based on the following reaction:

$$2H_2O_2 + \text{guaiacol} \rightarrow 2H_2O + \text{guaiacol oxidation product}$$

The oxidation of guaiacol results in the formation of a colored product, typically a brownish-red or yellow-brown compound. The intensity of the color formed is proportional to the peroxidase activity in the sample. The absorbance of the colored

oxidation product is measured spectrophotometrically at 420 nm. The increase in absorbance over time reflects the rate of guaiacol oxidation, which is indicative of the peroxidase activity in the sample (Zhang et al., 2005).

2.3.3.Physicochemical properties of dried spinach chloroplasts

2.3.3.1. Macronutrients analysis: total lipids, proteins, ash and carbohydrates

Lipids were extracted from dried spinach chloroplasts, using a modified technique of Folch et al. (1957). Samples were dissolved with 1.2 mL (chloroform: methanol solvent at a ratio of 2:1 (v/v), then vortexed for 1 minute. To this 0.5 mL of a 0.9% NaCl solution was added to the mixture. The sample was then vortexed again and further centrifuged using a Thermo Jouan CR3i multifunction centrifuge, 3000 rpm for 10 min at 4 °C. The lowest phase containing the lipids was transferred into another tube and 1.2 mL of chloroform: methanol (2:1) added. The remaining two phases were then vortexed and centrifuged again. The lipid phase was removed, pooled with the original lipid layer, and centrifuged to separate the lipid from residual compounds. The lipid extract was filtrated through 0.45 μm PTFE filter membrane, dried under a flow of nitrogen, and weighed to calculate the total lipid content. Dried lipid extracts were used to measure β-carotene, lutein, α-tocopherol, chlorophyll, and for fatty acid analyses. Total lipid content was calculated using the equation below (Eq. 2.2).

$$\text{Total lipid (\%)} = \frac{\text{dried lipid/g}}{\text{initial sample/g}} \times 100 \qquad \text{(Eq. 2.2)}$$

Proteins were measured using the total nitrogen method (Lynch et al., 2020). In brief, samples (3 mg) were weighed in a tin capsule and two sulphanilamide standards

were also weighed (3 mg). All samples along with the standards (Sulphanilamide) were run on an Organic Elemental Analysis Eager Experience (Flash 200, Thermo Fisher Scientific Inc, 2010). The Nitrogen values were automatically calculated in percentage. The element nitrogen value was converted to protein using 6.25 as a conversion factor.

In preparation for mineral analysis, an acid digestion was conducted by adding 1mL concentrated HNO_3 to 0.2 g of the powdered samples. This was heated at 115 °C for 4 h with occasionally stirring. Then, 1 mL H_2O_2 was added and digested for another 90 min at 115 °C. The flask was then removed and left to cool (in the fume cupboard) for several minutes. Small quantities of deionized 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 deionized 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, UK). 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 (polyether ether ketone) nebulizer (Burgener Mira Mist). Internal standards were introduced to the sample stream using a separate line via the ASX press unit and comprised of Sc (20 µg/L), Rh (10 µg/L), Ge (10 µg/L) and Ir (5 µg/L) 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 µg/L (0, 20, 40, 100 µg/L). 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 µg/L. Phosphorus calibration utilized an in-house KH2PO4 solution standard (10 µg/L). Sample processing was undertaken using Qtegra™ software (Thermo-Fisher Scientific), utilizing external cross-calibration between pulse-counting and analogue detector modes as required. The ash contents of spinach chloroplasts were determined from the total amount of minerals presented in samples.

The percentage of total carbohydrates of spinach chloroplasts were estimated using the following equation (Eq. 2.3).

$$\text{Carbohydrate (\%)} = 100 - (\text{lipid} + \text{protein} + \text{ash}) \qquad \text{(Eq. 2.3)}$$

2.3.3.2. Micronutrients analysis: chlorophylls, β-carotene, lutein, vitamin E (α-tocopherol) and fatty acid composition

The fatty acids of a lipid extract were esterified to fatty acid methyl esters (FAMEs) and analysed using gas chromatography-mass spectrometry (GC-MS) (Thermo Scientific, DSQ), (Gedi et al., 2017). A dried lipid extract was dissolved in 2 ml of chloroform containing 0.1% butylated hydroxyl toluene (BHT). To 1 ml of dissolved lipid, 100 µl of internal standard, methyl pentadecanoate (10 mg/ml), 200 µl of trimethylsulfonium hydroxide (TMSH) were added and filtered (0.45 µm, 13 mm) into amber GC-MS vials. Esterification was achieved through the addition TMSH and the solution was then left, to ensure complete conversion, for a minimum of 10 minutes before GC-MS analysis. The sample was injected at a volume of 10 µl onto a Phenomenex Zebron ZB-FFAP (30 m × 0.22 mm) column using a vaporising injector

with a split flow of 50 ml/min. The oven temperature was maintained at 120 °C for 1 min and then increased to 250 °C at a ramp of 5°/min. The final temperature of 250 °C was held for 2 minutes. Detection was conducted using a mass spectrometer and identification of individual fatty acids was achieved through mass spectrum library and comparison of retention times to FAME standards. The percentage content of each fatty acid was calculated and the concentrations (mg/mL) are determined using the response factor of the internal standard.

GC-MS combines two components, Gas Chromatography (GC) and Mass Spectrometry (MS) to identify and quantify of compounds from complex mixtures. In principle, the targets of interest are volatilized and carried with mobile phase (inert gas normally helium or nitrogen gas) then interact with the stationary phase (often a coated capillary column). As the sample travels through the column, different compounds interact differently with the stationary phase based upon their chemical properties such as boiling point, polarity, and affinity. And thus, being separated by different time that eluted from the column and transferred to MS. The individual compounds are ionised in the MS and characterised by their mass-to-charge ratio (m/z). The identification can be achieved by comparing their retention times and mass spectra with known standards or databases. Quantification can be obtained by correlating the peak areas in the chromatogram with known concentrations or using internal standards (Hussain et al., 2014).

The chlorophyll content was determined using a UV-Vis spectrophotometry (Thermo Scientific, Genesys10S, US). The lipids extract was diluted with acetone to be

a factor of 1:1000. The sample solution was measured at two different wavelengths: 662 nm (chlorophyll a) and 645 nm (chlorophyll b). Pigments concentrations (µg/mL) were calculated as previously described using equation 2.4 and 2.5 (Lichtenthaler et al., 2001). The principle behind UV-Vis spectroscopy involves the interaction of light with matter, particularly molecules, which results in the absorption of specific wavelengths of light.

$$\text{Chlorophyll a} = (11.24 \times \text{A661.6}) - (2.04 \times \text{A644.8}) \quad \text{(Eq. 2.4)}$$

$$\text{Chlorophyll b} = (20.13 \times \text{A644.8}) - (4.19 \times \text{A661.6}) \quad \text{(Eq. 2.5)}$$

The β-carotene and lutein content of dried chloroplast material was analysed by high performance liquid chromatography (HPLC) with photodiode array (PDA) detection (Agilent 1100, Germany) (Wattanakul et al., 2022). B-carotene was detected at 454 nm. The flow rate of the mobile phase (Acetonitrile: Methanol: Ethyl acetate with 0.05% triethyl amine) was set at 0.5 ml/min. Two gradient mobile phases were applied 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. Lipid extract of dried spinach juice and CRF was injected at a volume of 10 µl through a Security Guard guard-column and separated on a Waters Nova-Pak C18 (4µm, 3.9 × 150 mm) column with the column temperature set at 22°C.The concentration of β-carotene and lutein were determined using a linear equation created using a calibration curve produced from a range of external (β-carotene, lutein) standards dissolved in acetone containing 0.1% butylated hydroxytoluene (BHT).

The Vitamin E (α-tocopherol) content of dried chloroplast material was

determined using HPLC detection using an Agilent 1100 series unit with a fluorescence detector (Jasco, FP-920, Japan) as described (Wattanakul et al., 2022). The flow rate of the mobile phase was set at 0.8 ml/min starting with 100% solvent A (Acetonitrile: Methanol: Isopropanol: 1%Acetic acid solution with a ratio of 45:45:5:5, v/v). Lipid extracts of dried spinach juice and CRF were separated following the injection of 10 µl of sample extract a using a Zorbax RX-C18 5 µm (250 × 4.6 mm) column set at 20°C. After 6 min of solvent A running isocratically the mobile phase changed linearly to 100% solvent B (Acetonitrile: Methanol: Isopropanol with a ratio of 25:70:5, v/v) over the following 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 α-tocopherol, at approximately 23.2 min, can be achieved at excitation and emission wavelengths of 298 and 328 nm respectively and quantitation achieved using the linear formula produced from a calibration curve of α-tocopherol standards (4-100 µg/mL) in methanol containing 0.1% BHT.

The injected samples are carried by mobile phase (mixture of solvents) through the column and interacts with the stationary phase to separate the components of the mixture. Separation in HPLC is based on the differential partitioning of analytes between the mobile phase and the stationary phase. Compounds with different affinities for the stationary phase will elute from the column at different rates, leading to separation. HPLC is a technique used for separation, identification and quantification of individual components from mixture, and widely applied in various industries for its high sensitivity and resolution (Ali, 2022).

2.3.3.3. Physical characterization

a) Moisture content and water activity

The moisture content of fresh CRF and dried chloroplasts powders produced by spray drying and freeze drying was determined according to the method of Syamila et al. (2019). Dried powder samples (0.5 g) were weighed in a tared foil tray and then dried in oven at 105 °C The drying, cooling, weighing processes were repeated until constant weight was obtained. Water activity was measured at 25 ℃ using Aqualab Dew Point Water Activity Meter 4TE (Decagon Devices Inc., Pullman, WA, USA). Samples were triplicate and the mean values ± standard deviation were recorded. The moisture content can be calculate based on the Eq. 2.6.

$$Moisture\ content\ (\%) = \frac{wet\ weight - dry\ weight}{wet\ weight} \times 100 \quad \text{(Eq. 2.6)}$$

b) Solubility at body temperature

Solubility was estimated according to the method proposed by Anderson et al. (Anderson et al., 1970), with some modifications. 0.5 g of samples was added to 14 mL of ultrapure water and vortexed for 1 min, then heated at 37 ℃ incubator which was designed to simulate the human body temperature and shake at 250 rpm for 30 min. The suspension was then transferred to a tube and centrifuged at 3500 rpm at 4 ℃ for 20 min. The supernatant was transferred completely to a pre-weighed aluminium can and dried at 105 ℃ for 4 h. The dried soluble solid after drying was then weighed and used to calculate the solubility as a percentage, according to the following equation (Eq. 2.7).

$$Solubility(\%) = \frac{\text{dried supernatant weight(g)}}{\text{initial sample weight (g)}} \times 100 \quad \text{(Eq. 2.7)}$$

c) Dispersibility

Dispersibility was determined following the method of Jinapong et al. (2008) with slight modification. In brief, 0.5 g dried chloroplast powder/fresh CRF was added to 10 mL distilled water and stirred vigorously for 25 s. Then sieved by passing through 150 μm sieve. A portion of the filtrate (1 mL) was transferred to a pre-weighed aluminum can and then dried at 105 ℃ for 4 h. The dried soluble solid was then weighted and the dispersibility calculated as a percentage.

d) Light Microscope and scanning electron microscopy (SEM)

The morphology of spinach juice/CRF powders was observed under a light microscope (Nikon Microscope Eclipse E400, Nikon Corporation, UK). The dried powdered samples were hydrated in water before loading on the plates for observation. All light micrographs were taken with polarised light technique equipped with 20X or 40X objective lens.

In addition, scanning electron microscopy (JEOL JSM-6060LV Variable Pressure Scanning Electron Microscope (Jeol. (UK)Ltd, Hertfordshire, UK) was used to observe physical changes of the chloroplast structure and the impacts of differing drying methods of organelle integrity. Platinum coating was carried out and analyses were performed with 10 kV accelerating voltage and under a pressure of 40 Pa. SEM was performed/operated with 1000x and 2000× magnification. Meanwhile, the Cryo-SEM was operated with a magnification of 1000× and 2000× for obtaining the micrographs

of fresh CRF. Samples were prepared by high pressure freezing with a Leica ICE and transferred to the SEM with a Quorum Technologies PolarPrep 2000 cryo transfer system. After sublimation at -90 °C for approx. 6 minutes the sample was gold coated before transferring to the SEM chamber for imaging. The sample was maintained at, or below - 160 °C during imaging. Samples were imaged on a JEOL 6060LV SEM operating at 10 kV.

SEM images represent the morphology of a sample and can also reconstruct quasi-three-dimensional views of the sample surface. In SEM, the focused electron beam scans the surface of the sample, and interact with atoms in the sample, producing various types of signals including secondary electrons and backscattered electrons that contain information about the surface topography and composition of the sample (Kannan, 2018).

e) Transmission electron microscope (TEM)

The morphology of fresh and blanched spinach leaves was analysed using a Tecnai Bio-TWIN T12 Biotwin transmission electron microscope (TEM) (FEI Company, Eindhoven, The Netherlands) and run at an accelerated voltage of 120 kV. Images were captured using a MegaView SIS camera.

The fresh and blanched spinach leaves were cut into small pieces around 1-3 mm^2 then immerged into 600 µL 25% EM Glutaraldehyde and 2.5 mL 0.2M Cacodylate buffer and 1.9 mL distilled water overnight at 4 °C. The supernatant was discarded after fixation and then the fixed samples were washed twice and filled up with the 0.1 M Cacodylate buffer. Post fixed samples in 1% osmium tetroxide for 2 h, then washed

twice in distilled water 5 min each time. Then, dehydrated in graded ethanol series in the following sequence: 2×10 min 50% ethanol, 2×10 min 70% ethanol, 2×10 min 90% ethanol, 3×20 min 100% ethanol, 2×10 min 100% propylene oxide (propox). The samples were then infiltrated with resin and immersed in 3:1 propox:resin for 2 h, and then mixed with 1:1 propox:resin overnight at room temperature. Then, the samples were put into the 100% resin 2.5 h for 3 separate times and embedded in an appropriate plastic mould and polymerised. The polymerised spinach leaves were cut into pieces before loading on TEM, and imaging at different magnifications.

TEM creates an image using a broad beam of electrons that transmitted through the ultrathin sample. As a result, TEM offers valuable information on the inner structure of the sample, such as morphology, crystal structure and stress state information (Rao et al., 2010).

f) Particle size measurement

The particle size distribution and mean diameter of fresh CRF and dried chloroplasts powders were determined using LS-13-230 Laser Diffraction Particle Size Analyser (Beckman Coulter, United States). The mean diameter of samples was based on the mean diameter of a sphere of the same volume, the Brouckere diameter ($D_{4,3}$). Fresh CRF was analysed by wet method, with dispersion in RO water. Obscuration values ranged from 1% to 8%, and the refractive index used for dispersed phase was 1.33. Dried powders were analysed by solid mode, with a dry dispersion unit. Obscuration values around 1-6%. The volume-weighted mean diameter $D_{4,3}$ was calculated as follows:

$$D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \quad \text{(Eq. 2.8)}$$

Where D_i is the mean particle diameter and n_i is the number of particles.

The span was calculated according to the equation as follows:

$$span = \frac{d_{90} - d_{10}}{d_{50}} \quad \text{(Eq. 2.9)}$$

Where d90, d50, and d10 are the average volume diameters equal to 90%, 50%, 10% of the cumulative volume, respectively.

The laser diffraction particle size analyser works on the principle that when the laser beam passes through the sample, it interacts with the particles suspended in the medium. The laser light is scattered in different directions by the particles, and the angle of light scattering is inversely proportional to particle size. The angular scattering intensity is subsequently assessed to determine the size of the particles accountable for generating the scattering pattern, with the Mie theory of light scattering (Ryżak et al., 2011).

2.3.4.Static *in vitro* digestion model

An *in vitro* static digestion model was chosen for this study because it is time saving and cost-effective without ethical issues. The static *in vitro* digestion model was based on the method proposed by Minekus and Brodkorb et al. (Minekus et al., 2014; Brodkorb et al., 2019) with some modifications, to encompass oral, gastric, and intestine phases of digestion. The spinach juice/CRF powders (1.5 g) were dissolved in 3.5 mL of ultra-pure water as the starting mixture solution. To the mixture, 5 mL of

simulated salivary fluid (SSF) without α-amylase was added and incubated for 2 min at pH 7 in oral phase. For the gastric phase, 10 mL simulated gastric fluid (SGF) was added, along with the selective enzymes, rabbit gastric extracts (RGE) (20.4 U/ml) and pepsin (2000 U/ml). The pH was adjusted to 5 and the solution and then incubated for 2 h. For the intestine phase, the solution was mixed with simulated intestine fluid (SIF) (1:1), porcine pancreatic extracts (PPE) with final concentration of pancreatic lipase at 2000 U/ml and trypsin around 100 U/ml, in addition, 10 mM porcine bile extracts were also added, then pH of solution was adjusted to 6.5 and incubated for another 2 h. The whole digestion was performed inside an incubator at 37 °C while shaking with the rotator at 20 rpm. The pH of gastric and intestine was set at 5 and 6.5 for mimicking the GI conditions at half gastric emptying time during a meal (Borgstrom et al., 1957; Sams, 2016). The rotating during digestion was in order to gently mix the solution and enzymes. Samples (1 mL) from the digestion mixture were collected at the end of oral, gastric and intestine phase. The samples (1 mL) after oral and gastric phase were mixed with 1 mL $NaHCO_3$ to stop the enzymatic reaction, while for the samples at the end of intestine phase, 1M Orlistat was added to the digesta to stop the enzyme activity. The samples were all kept at -80 °C until further lipid extraction as Section 2.3.4.2.

The preparation of stock solutions for simulated digestion fluids is shown in Table 2.1.

Table 2.1: Volume of electrolyte stock solutions of digestion fluids for a volume of 400 mL diluted with water (1.25× concentrations). The stock solution will be further diluted to the final volume of 500 mL (1× concentrations) for each simulated digestive fluid.

Salt solution	Stock concentrations		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
			Vol. of stock for 0.4L	Final Conc. in SSF	Vol. of stock for 0.4L	Final Conc. in SGF	Vol. of stock for 0.4L	Final Conc. in SIF
	(g/L)	(M)	mL	mM	mL	mM	mL	mM
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaCl [a]	58.4	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

a, $NaHCO_3$ was replaced by NaCl in the electrolyte solutions to avoid unwanted pH drift. Simulated salivary fluids (SSF). Simulated gastric fluids (SGF). Simulated intestinal fluids (SIF). All simulated fluids stock solutions were prepared at x1.25 concentration, and stored at -20 °C.

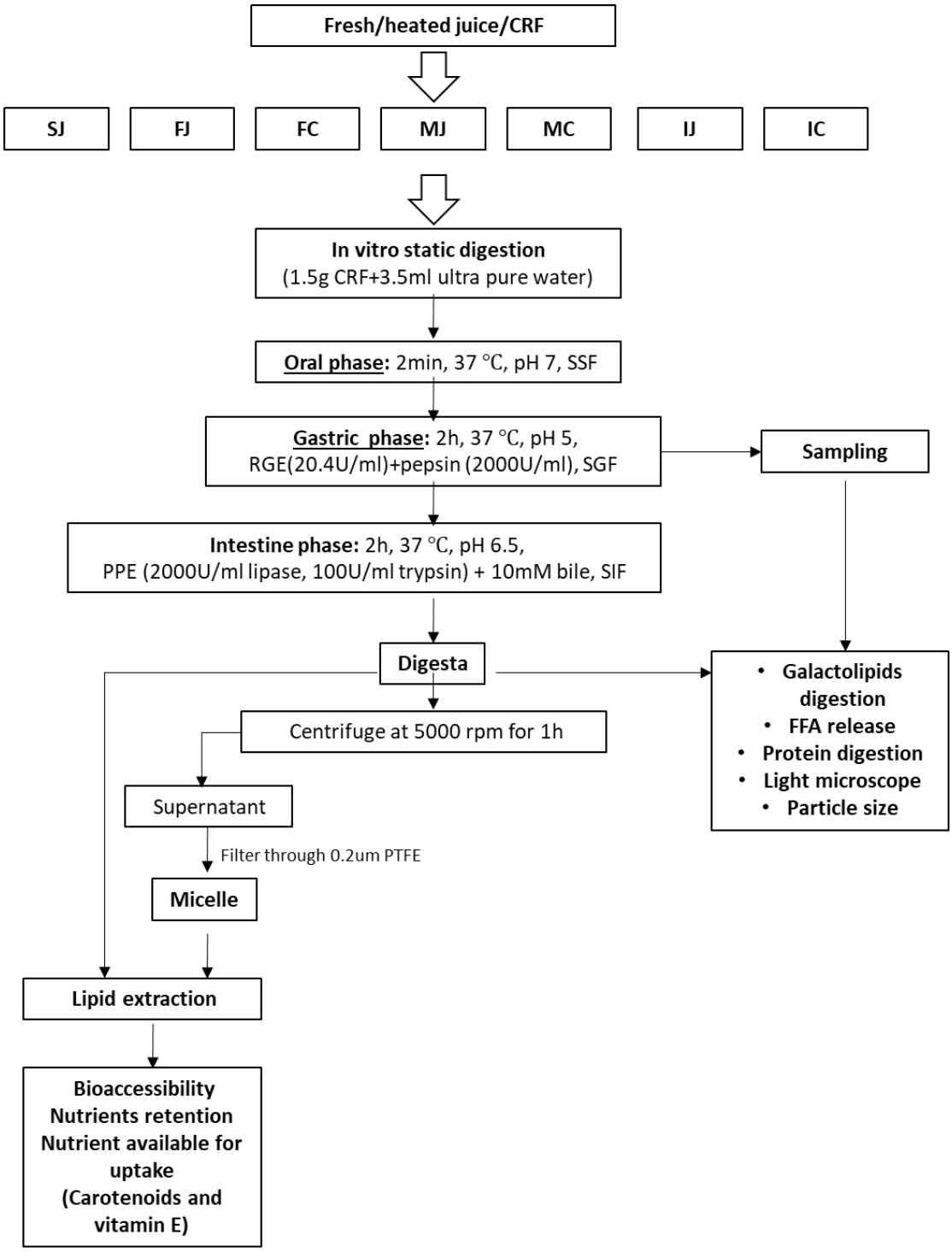


Figure 2.1: Flow chart to measure nutrient bioaccessibility in *in vitro* digestion model.

2.3.4.1. Study of bioaccessibility

The bioaccessibility of carotenoids (β-carotene and lutein) and vitamin E (α-tocopherol) was measured in the current work. The digesta (5 mL), a mixture solution at the end of intestine digestion was centrifuged at 5000 rpm for 1 h at 4 °C, and the supernatant was filtered with a 0.2 μm PTFE filter membrane to obtain the micelle phase. Both the digesta and micelle was further transferred to a lipid extraction vessel (Section 2.3.9.2) and used for nutrients analysis.

The micellarisation rate and bioaccessibility (%) was calculated based on the research conducted by Chen et al. (2019) with the following equations (Eq. 2.10, Eq. 2.11):

$$\text{Micellarisation rate } \% = (\text{C micelle}/\text{C digesta}) \times 100 \quad \text{(Eq. 2.10)}$$

$$\text{Bioaccessibility } \% = (\text{C micelle}/\text{C initial}) \times 100 \quad \text{(Eq. 2.11)}$$

Where $C_{micelle}$ and $C_{digesta}$ is the concentration of the nutrient in micelle and digesta fraction, $C_{initial}$ is the concentration of the nutrient in CRF before digestion.

2.3.4.2. Lipid extraction for digested sample

Lipid extraction was performed using the method of Folch et al. (1957) (Section 2.3.8.1) with slightly modification. Samples (1 mL for HPTLC analysis or 5 mL digesta and micelle for HPLC analysis) were mixed with 1mL or 2 mL of 150 mM NaCl solution, and 1.2 mL or 5 mL of a mixture of chloroform and methanol (2:1,v/v), respectively. The mixture was then vortexed for 1 min and centrifuged (Thermo Electron Corporation, Jounan CR3i multifunction, US) at 3000 rpm for 10 min at 4 °C.

This processing step allowed for the phase separation of the samples. The lowest phase which contained the lipids were transferred and collected and a further 1.2 mL or 5 mL of a mixture of chloroform and methanol (2:1) added. The remaining aqueous phase and the mixture was then vortexed and centrifuged again twice more. The lipid extracts were pooled and dried under gas nitrogen until constant weight and stored at -20 °C until further analysis.

2.3.4.3. High performance thin layer chromatography (HPTLC) analysis

HPTLC technique was used in this study for the analysis of both polar and non-polar lipids of spinach juice/CRF throughout digestion. This analysis can be divided into two separate tests: Polar lipids (Galactolipids: monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)) and non-polar lipids (monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and free fatty acid (FFA)). Separation of individual class of lipids from each other is based on the competition of the solute and the mobile phase for binding places on the stationary phase (normally silica gel) due to polarity differences of the analytes (Hussain et al., 2019). The significant advantage of HPTLC for the analysis of lipids is that lipids can be easily visualised by staining.

The analysed lipids were first extracted from a 1ml sample obtained at the end of the gastric phase (2 h) and intestine phase (4 h) of the digestion process (shown in 2.3.9.2). Then, both the extracted lipids and standards (MGDG, DGDG, mix lipids and α-linolenic acid) were spotted onto a thin-layer silica plate using a Linomat 5 (CAMAG, Swiss) equipped with a 100 µL Hamilton syringe. The plates were then developed in a

TLC developing chamber contained separation mobile phase and dried at room temperature in a fume hood (10 min for polar lipids, 5 min for non-polar lipids).

For polar lipids (galactolipids), the separation mobile phase consists of chloroform: methanol: water (47.5:10:1.25, v/v/v); Then, the polar lipids plate dipped in a thymol solution for derivatisation/staining while avoiding the interference of pigments, like chlorophylls during the densitometric analysis of the plates. The thymol solution was prepared by dissolving 1g of thymol in 190 mL ethanol and then put in the ice bath and gradually added 10 mL 96% sulphuric acid since the mixing reaction is highly exothermic. After staining, the plates were dried and transferred into the oven at 110 °C for 4 min until the bands turn brown-pink and the background turns pink.

For non-polar lipids (MAG, DAG,TAG and FFA), the mobile phase consisting of heptane: diethyl ether: acetic acid (55:45:1, v/v/v). And the non-polar lipids plates dipped in phosphoric acid-copper acetate solution for acyl chain staining. The dipping solution was prepared by mixing a saturated copper acetate solution with 85% phosphoric acid (1:1, v/v). The plates were left in fume hood until dry and transferred in the oven at 180 °C for 6 min.

Densitometry analysis of stained lipids on the TLC plates was carried out by the TLC visualizer 2 (CAMAG) and 'visionCATS' software. Lipid bands were scanned at 366 nm for thymol staining and at 500 nm for copper acetate-phosphoric acid staining, with a 0.5 x 7 mm slit and a speed of 2.5 cm/min. Slit conditions were selected accordingly to band size. The slit should always cover the whole band size. Quantities of the lipids on the TLC plates were estimated from the linear standard curves

established with the pure lipid standards (MGDG, DGDG, mix lipids and α-linolenic acid (C18:3)).

In the field of lipids, HPTLC is typically used for separations, identification of the individual lipids and quantifications. Three main steps are included: sample application, chromatogram development and chromatogram evaluation (Rani et al., 2015). The separation principle relies on adsorption. One or more compounds are applied as spots onto a thin layer of adsorbent material coated on a chromatographic plate. Capillary action, rather than gravitational force, propels the mobile phase (developing solvent) through the system. The compounds migrate based on their affinities towards the adsorbent. Components with greater affinity towards the stationary phase travel slower, while those with lesser affinity travel faster. There are some advantages of HPTLC that makes it widely accepted for lipid analysis in various fields such as lipidomics, food science, pharmaceuticals, and clinical research. Firstly, High quality separations are achievable. HPTLC offers high-resolution separation, allowing for the effective separation of lipid components. Lipid samples often contain multiple lipid classes and subclasses, each with various molecular structures. HPTLC's ability to separate these components efficiently is crucial for accurate analysis. Also, HPTLC can analyse a wide range of lipid classes, including fatty acids, phospholipids, glycolipids, sterols, and triglycerides. This versatility makes it suitable for comprehensive lipid profiling studies (Fuchs et al., 2011).

2.3.4.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)

Protein digestion was characterized using SDS-PAGE on a Mini-PROTEAN Tetra System (Bio-Rad, USA) with 12% Mini-PROTEAN TGX gels. The spinach chloroplasts powder was dissolved in ultra-pure water with the final protein concentration 2 mg/mL, and then mixed with sample buffer (1:1, v/v). The sample buffer was prepared by mixing 2×laemmli with β-Mercaptoethanol (19:1, v/v) the mixture is then heated at 95 °C for 5 min to denature the protein, followed by centrifugation at 3,000 rpm for 5 min. 20 µL of the supernatant were loaded into separated wells on the gel, under this condition, the different preparations of spinach chloroplasts were loaded to constant protein around 20 µg/well, and the precision plus protein standards was loaded 5µg in the first well in order to identify the proteins. SDS gel was ran for 15 min under 80 V and then for 60 min under 160 V in Tris/Glycine/SDS Buffer. Gels were subsequently stained with InstantBlue Coomassie. ChemiDoc^{TM} MP imaging system (Bio-Rad, USA) was used to scan and detect the bands densities and the final analysis was performed by using the Image Lab Software.

SDS-PAGE works on the principle that the denatured proteins can be separated by their molecular weight. Sodium Dodecyl Sulfate (SDS) as a strong ionic detergent along with reducing agent can denaturate proteins by heating, and leading to the unfolding protein structures. The denatured SDS-bound protein with the negative charge migrates towards the electrode with the positive charge when placed in an electric field. The separation of the charged molecules depends on their molecular

weight. The smaller molecules migrate faster due to less resistance during electrophoresis. The proteins can be visualised by specific staining technique, the size of protein can be estimated by comparing with the migration of known molecular weight protein standards (Roy et al., 2014).

2.4. Statistical analysis

All experiments were carried out in triplicate, and the results were expressed as mean ± standard deviations. The statistical analysis was performed by IBM SPSS 25. The comparison of data more than two groups was analysed by one-way ANOVA followed by a post-hoc Tukey test, the statistical significance was set to the $p<0.05$ level, which means a difference of means at $p<0.05$ were considered significant.

Chapter III

3. Effects of drying methods on physicochemical properties of spinach juice/CRF

3.1. Introduction

Spinach (*Spinacia oleracea* L.) is a widely cultivated leafy green vegetable grown all over the world, with around 30 million tonnes of spinach produced worldwide in 2020/2021. Spinach leaves are readily consumed for fresh usage or after processing such as canning, freezing or dehydration. However, current production methods results in approximately 25% waste production even though the waste tissues are abundant in high value chemicals like lutein and chlorophyll (Derrien et al., 2017). Despite the high water content, spinach has a high content of macronutrients, such as lipids, proteins, carbohydrates, as well as micronutrients such as carotenoids (beta-carotene and lutein), chlorophylls (chlorophyll a and b), vitamins (vitamin A, C and E), minerals, phenolic compounds (including flavonoids, phenolic acids and others) (Bunea et al., 2008).

The chloroplast is an organelle abundant in plant leaves; Recent research has shown that chloroplasts contain the majority of plant nutrients with 75–80% of the total leaf nitrogen being found within chloroplastic proteins in C3 plants (most vegetables and trees, and several major cereals) (Ishida et al., 2014). Moreover, the chloroplast-rich fraction (CRF) from green materials, such as spinach, kales, nettles and grass show a larger amount of macro and micronutrients than whole leaf materials (WLM) (Gedi et al., 2017). Spinach is known to have a number of health benefits including anti-

cancer (Abu Al-Qumboz & Abu-Naser, 2019), anti-obesity (Lasya, 2022), hypoglycemic (Park et al., 2012), and hypolipidemic functionalities (Roberts et al., 2016). Therefore, many researchers are interested in developing approaches needed to maintain nutrients levels in spinach products to aid the development of more effective functional food ingredients. Sadly, fresh spinach can only store 2-4 days at room temperature, and nutrient profiles are diminished particularly notable are losses in folate and carotenoid content (Rambuda et al., 2015). It is well known that nutrient contents start to decrease after harvesting due to the processing that expose foods to high levels of heat, light or oxygen and continues during storage. Thus, the development of novel processing methods that extend shelf life is important in the fresh fruit and vegetable industry. One such method is to produce spinach powder as an alternative way to preserve the main bioactive compounds of spinach, and to ensure product stability, while also reducing transportation cost (Daza et al., 2016). Spray drying and freeze drying are used commercially to dry materials, for this reason this we tested the impact of these drying methods on the physico-chemical properties, as well the nutritional content, of the resulting materials.

The current chapter focuses on the characterization of spray dried juice (SJ), freeze dried juice (FJ) and freeze dried CRF (FC) derived from spinach. In all samples, nutrient compositions and physical properties were studied to determine the optimum processing procedure used for spinach chloroplast materials production.

3.2. Sample preparation summary

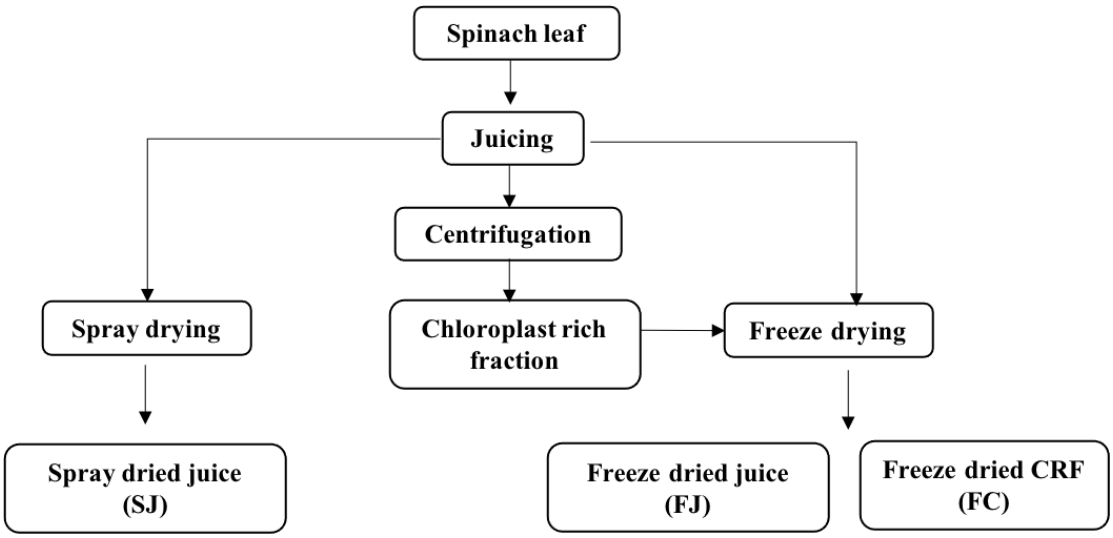


Figure 3.1: Spinach juice/CRF powders preparation steps.

3.3. Results and discussion

3.3.1.Nutrients content

3.3.1.1. Macronutrients content of dried spinach juice/CRF

The macronutrients content of various chloroplast fractions is shown in Table 3.1. The content of macronutrients in dried spinach juice were comparable following both drying methods; slightly higher protein and lipid content were seen in spray dried juice. The major contribution of dried spinach juice was protein (35.5-38.3%), and carbohydrates (33.2-37.1%). While the main contribution of freeze-dried spinach CRF (FC) were proteins (44.6%) and lipids (26.9%), and these nutrients were significantly higher than in both spray and freeze dried juice (SJ and FJ) (Table 3.1).

Our results correlate well with those reported by (Gedi et al., 2017). This research reported that the protein content in CRF and whole leaf material of spinach were 42.6%,

35.3% respectively, and showed close agreement with our data. Similarly, lipid contents were 36.9% and 19.3% respectively, higher than the results observed in this study (Table 3.1). These differences could be mainly due to the batch of spinach used during harvesting rather than preparation methods. Indeed, Gedi et al. (2017) prepared spinach CRF by blending spinach leaves with 0.3 M sucrose solution (ratio of 1:6 (w/v)), however, in our work spinach CRF were prepared with a slow-screw twin-gear juicer. While, no significant difference of nutrients content was observed in same batch of spinach CRF between these preparations as studied by Mansor (2019).

Overall, both drying methods can retain the macronutrients in spinach juice at similar level. Spinach CRF contained higher concentrations of proteins and lipids than juice, due to the fact that CRF samples were more concentrated with thylakoid membranes which are mainly composed of proteins and lipids.

Table 3.1: Macronutrients content of spinach juice/CRF collected by freeze drying and spray drying method

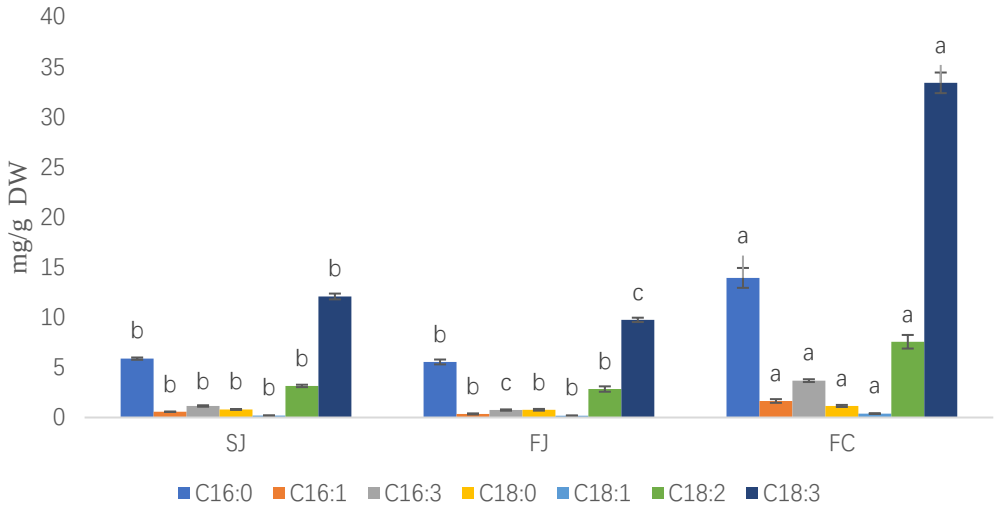
%DW	SJ	FJ	FC
Protein	38.3±0.6b	35.5±0.2c	44.6±0.7a
Lipid	13.0±1.9b	9.7±1.2c	26.9±2.0a
Ash	15.5±0.3b	17.7±0.7a	7.6±0.3c
Carbohydrates	33.2	37.1	20.9

Data was analysed by one-way ANOVA according to Tukey's test ($p < 0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). Carbohydrates were estimated by difference,

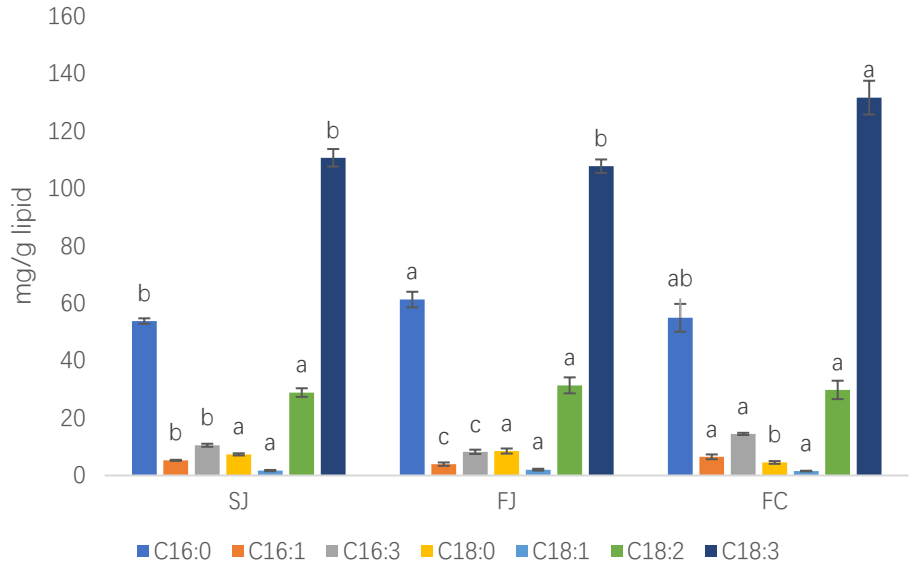
100-(proteins+lipids+ash)/%. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF

3.3.1.2. Fatty acid composition of dried spinach juice/CRF

The effect of different drying methods on fatty acid composition of chloroplasts from spinach is presented in Figure 3.2 (a). Seven fatty acids were detected including palmitic acid (C16:0), palmitoleic acid (C16:1), hexadecatrienoic acid (C16:3), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and alpha-linolenic acid (C18:3). And among which polyunsaturated alpha-linolenic acid (C18:3) (ALA) exhibited the highest amounts in both spinach CRF and juice, followed by palmitic (C16:0) (PA) and linoleic acid (C18:2) (LA). The predominant ALA in chloroplasts is because it is a key component of the thylakoid membranes (Wang et al., 2012a). These fatty acids were also reported as the most predominant in spinach, kale, nettle and grass (Gedi et al., 2017).



(a)



(b)

Figure 3.2: Fatty acid composition of spray-dried juice, freeze-dried juice and freeze-dried CRF Results are expressed on a dry weight (a) and total lipid (b) basis and as means ± SD (n=3). Results are analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF

Comparing the SJ and FJ fractions, there were no significant differences between the different drying methods on fatty acids composition except for hexadecatrienoic acid (C16:3) and alpha-linolenic acid (C18:3). This finding indicated that each drying method retained the natural profile of fatty acids in chloroplast preparations. In addition, fatty acids were more concentrated in the CRF as compared to spinach juice, and critically the total fatty acid content in freeze dried CRF (FC) was 61.8 mg/g DW, being 3 times that of freeze dried juice (FJ) which was 20.2 mg/g DW. These differences could be due to the fact that FJ contains higher ash (15.5%) and carbohydrates (39.3%)

levels than that of lipids. Similar results were found by (Gedi et al., 2017), who also quantified the same fatty acids in spinach preparations and who noted ALA as the most abundant fatty acid. Higher amounts were present in freeze dried CRF compared with freeze dried powdered whole leaf materials (WLM) on a dry mass basis.

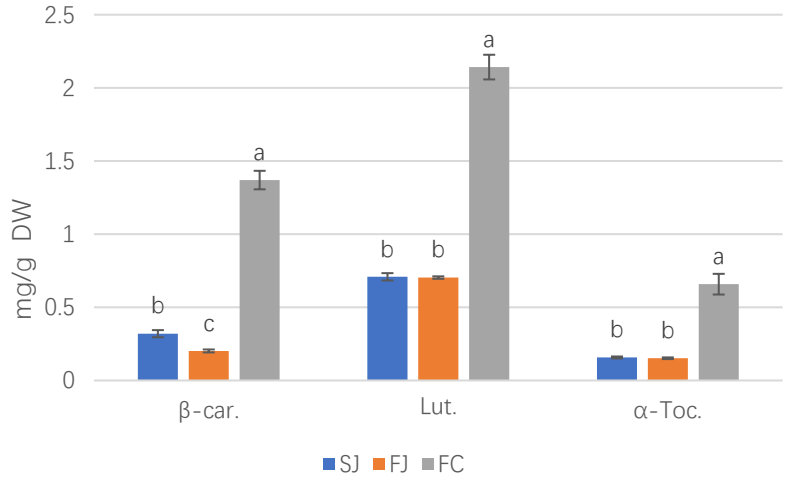
As shown in Figure 3.2 (b), the fatty acids contents (mg/g lipid) were not significantly influenced by drying methods. The levels of alpha-linolenic acid were higher in the CRF (131.6 mg/g lipid) than in juice ([SJ]:110.6 and [FJ]:107.7 mg/g lipid), dry weight material. The chloroplasts contain high levels of lipid so expressing fatty acid per unit mass of lipid tends to result in a convergence of values for all samples.

As reported in previous research, vegetable-based fatty acids are a mixture of unsaturated fatty acids, such as oleic, linoleic and linolenic acid, which is comparable to previous studies (Bajpai, 2014). Alpha-linolenic acid (ALA) is an essential omega-3 fatty acid, which is unable to be synthesised in the human body and must be ingested through diet and is precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). It has been reported to have the function of reducing risk of cancer, cardiovascular disease, inflammation, and various cognitive disorders (MacLean et al., 2006; Rizos et al., 2012). Therefore, the high content of ALA observed in chloroplasts of spinach can be seen as a source of potential nutritional vegetarian ingredient which provides EPA and DHA instead of fish oil.

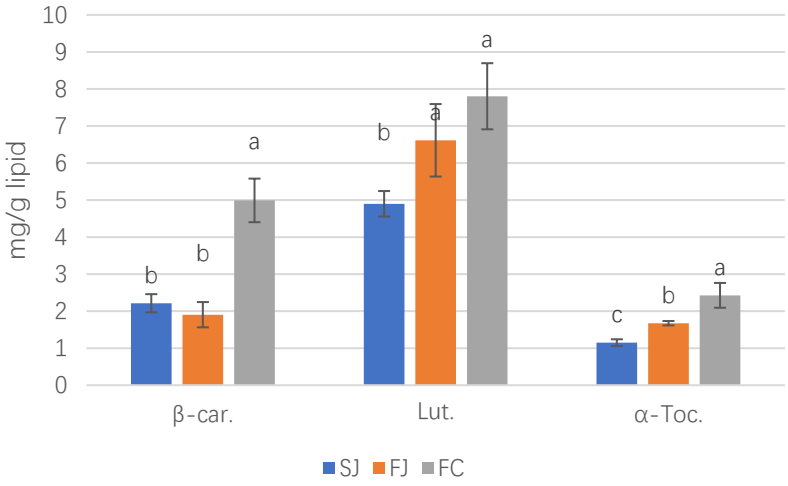
3.3.1.3. Pro-vitamin A, lutein and vitamin E of dried spinach juice/CRF

The content of pro-vitamin A (β-carotene), lutein and vitamin E (α-tocopherol) from dried spinach juice and CRF are presented in Figure 3.3 (a) and (b). In this study,

β-carotene and lutein were the most abundant forms of two categorized carotenoids (xanthophylls and carotenes). α-Tocopherol as the major form of vitamin E existing in nature as well as the most biologically active were considered (Van Bennekum et al., 2005).



(a)



(b)

Figure 3.3: β-carotene, lutein and alpha-tocopherol content of spray-dried juice, freeze-dried juice and freeze-dried CRF.

Results are expressed as means ± SD (n = 3). Results are analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF. *β-car* β-carotene, *Lut* lutein, *α-Toc* alpha-tocopherol.

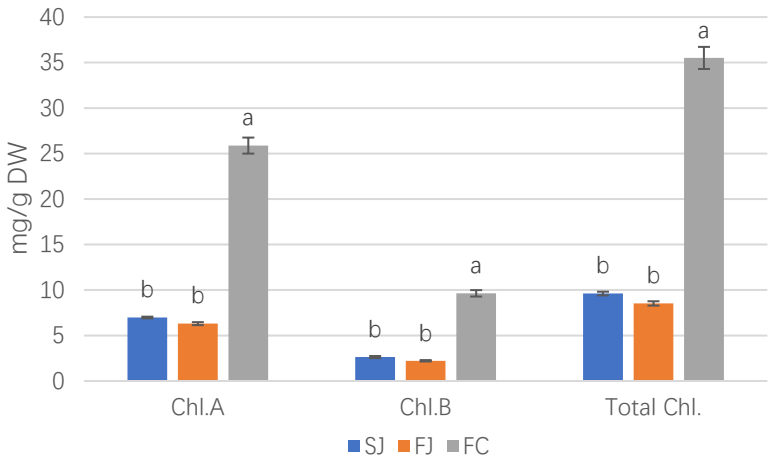
It is known that the majority of leaf pigments (carotenoids) and vitamin E compounds (tocopherols) are found in chloroplast preparations. It is therefore not surprising that the relative concentration of these lipophilic molecules increased in CRF preparations as this action would concentrate the chloroplasts (CRF compared with dried juice). When the results are expressed relative to lipid content it appears that the freeze-dried CRF retains a higher level of carotenoids and tocopherol than when the juice is dried directly. If one assumes that there is no significant loss of the major lipids during drying (Figure 3.2 b), then it appears that the juicing step caused the loss of a measurable proportion of non-chloroplast membrane lipids in dried juice samples compared with CRF. The extent of lutein and tocopherol losses appears to be less if freeze-drying is used by comparing with spray drying. According to the study of Bergquist et al. (2006), baby spinach leaves contained four carotenoids, lutein, violaxanthin, β-carotene and neoxanthin, among which lutein took the major quantity at about 39%, which is in agreement with the results found in this study, the content of lutein was higher than β-carotene and α-tocopherol (Bergquist et al., 2006).

β-carotene is expressed as pro-vitamin A which is rich in green leafy plants like spinach. The carotenoid composition found in this study agrees with that previously reported for spinach, with lutein (50.3-76 mg/kg wet weight) and β-carotene (60.7-83.1

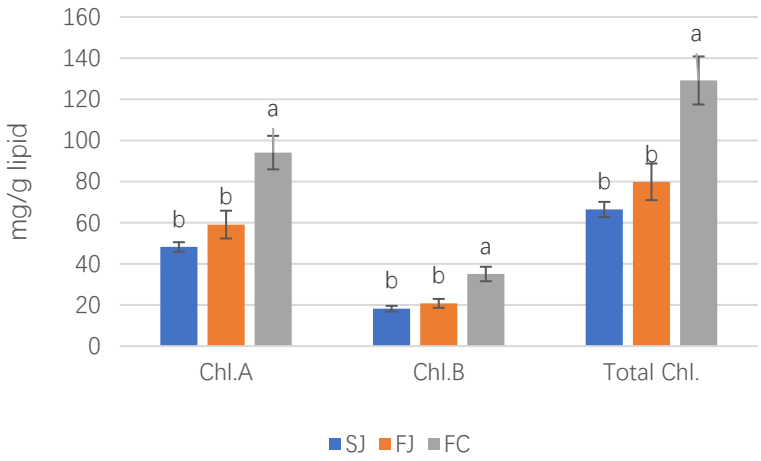
mg/kg wet weight) being the major carotenoids in six spinach genotypes (Kidmose et al., 2001). These molecules are reported to be important for human health. Indeed, lutein is present in high concentration in the retina close to the macula dense, where it protects the eye tissues against damage related to light and the age-related macular degeneration (Carpentier et al., 2009). Another important molecule measured in the current work is the antioxidant, alpha-tocopherol (Vitamin E). Vitamin E is a lipid soluble vitamin with antioxidant and anti-inflammatory effects. In view of the high levels of these compounds in chloroplastic fractions of spinach viz. α-tocopherol and ALA, is seems reasonable to speculate that choroplastic fractions of plants could be used to deliver nutrients that are beneficial to human health.

3.3.1.4. Chlorophyll levels of dried spinach juice/CRF

Chlorophyll a and b levels in dried spinach juice/CRF powders were measured and data are shown in Figure 3.4 (a) and (b).



(a)



(b)

Figure 3.4: Chlorophyll content of spray-dried juice, freeze-dried juice and freeze-dried CRF.

Results are expressed as means ± SD (n = 3). Results are analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF. *Chl.A* chlorophyll a, *Chl.B* chlorophyll b, *Total Chl.* chlorophyll a+b.

If one compares these values with those of carotenoids and tocopherol there is a very similar trend. Syamila et al. (2019) recently compared the chlorophyll content of the freeze dried spinach juice and CRF, with the content of chlorophyll a and b in freeze dried juice being 12.73 and 5.17 mg/g DW. Freeze dried CRF was 45.49 and 20.51 mg/g DW. The results were similar to the current study, the content of chlorophyll a was higher than chlorophyll b in both freeze dried spinach juice and CRF, and the total

chlorophylls were around 4 times greater in CRF. This finding is supported by the study of Fritschi *et al*. (2007) who found that the level of chlorophyll a was higher than chlorophyll b, with the mean chlorophyll a/b ratio of 3.79. The quantity of chlorophyll a is always higher than chlorophyll b in higher plants. In addition, the chlorophyll b is more soluble than chlorophyll a in polar solvents because of its carbonyl group. The highest content of total chlorophylls was found in CRF, then followed by spinach juice (SJ and FJ). This trend is explained by effects of the centrifugation force during sample preparation, which can accumulate as much chloroplasts as possible, due to the fact that chlorophylls concentration is an indirect indicator of chloroplasts (Liang et al., 2017).

3.3.2.Physical properties of dried spinach juice/CRF

Table 3.2: Characterization of chloroplasts from spinach juice and CRF

Property	SJ	FJ	FC	Fresh CRF
Moisture content/%	8.78 ± 0.67^{b}	8.45 ± 0.27^{b}	3.98 ± 0.77^{c}	83.85 ± 0.21^{a}
Water activity	0.26 ± 0.01^{b}	0.18 ± 0.02^{c}	0.24 ± 0.02^{b}	1.00 ± 0.00^{a}
Solubility/%	63.15 ± 0.43^{b}	73.87 ± 1.16^{a}	42.55 ± 2.44^{c}	40.93 ± 4.64^{c}
Dispersibility/%	96.64 ± 1.56^{a}	98.70 ± 1.70^{a}	93.06 ± 1.02^{b}	94.30 ± 3.80^{ab}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF.

The physical properties of dried spinach chloroplast materials are displayed in Table 3.2. Moisture content in food products can significantly affect its appearance, texture and taste, in addition to other impactors that affect shelf life, quality and resistance to bacterial contamination (Isengard, 2001). Fresh CRF had a high moisture content (83.85 ± 0.21 g/100g), which potentially could promote food degradation and growth of microorganisms, and therefore reduce shelf life. This study shows that drying processes can significantly reduce the presence of water in chloroplast fractions. For example, the moisture content of spray dried and freeze-dried juice were around 8%. Moreover, the moisture content of freeze dried CRF was 3.98%, less than 5%. The recommended value for fruit and vegetable juice powder products needed to guarantee reduced microbiological growth and to enhance stability of products for long-term storage is 5% (Tontul & Topuz, 2017). The relatively lower moisture value in FC compared with FJ could be possibly caused by the preparation steps difference. The centrifugation processing that used to collect CRF materials can remove water and accumulate chloroplast membranes which resulted in higher proportion of total lipids and insoluble proteins.

Similarly, water activity is a crucial indicator which generally affects shelf life of dried products. The water activity found in fresh CRF was 1, which agrees with the water activity of distilled water, this high value of water activity supports the growth of bacteria, yeasts, and moulds (Ölmez et al., 2009). The water activity of CRF after freeze drying significantly decreased to 0.24 ± 0.02. There was significant difference between SJ and FJ, with SJ showed higher water activity of 0.26 ± 0.01, and FJ showed the

lowest water activity of 0.18 ± 0.02, this difference could be affected by drying process. This result was in accordance with the water activity of 0.23 for spray dried and 0.18 for freeze dried spinach juice obtained by Syamila et al. (2019). The water activity of the dried chloroplast powders was in the range of 0.2-0.6 as found in dried fruit and vegetables in other studies (Shishir et al., 2017), with values lower than 0.3 commonly regarded as microbiologically and chemically safe, which can be seen as resistant to the microbiological and enzymatic reaction such as browning and oxidative degradation (Tontul & Topuz, 2017).

It is obvious that FC presented higher water activity but lower moisture content than FJ, one possible explanation could be due to the higher proportion of soluble carbohydrates in FJ can reduce the water activity (Sevenich et al., 2015).

Solubility is defined as the ability of powders to form solution or suspension in water, which is a crucial criterion to estimate the behaviour of powder in an aqueous solution (Tontul & Topuz, 2017). Solubility can influence some properties of powder products, such as the reconstitution of the dry extract or the bioaccessibility of nutrients during digestion. In addition, the solubility of powder directly related to the technological application in food industry, i.e. spray dried products used as food ingredients were more widely accepted owing to better stability and enhanced solubility which enable to satisfactory capability of the powders in the desired applications (Lee et al., 2018c). Importantly, solubility can be affected by many factors, such as temperature, pressure, moisture content, particle size, polarity and the physical state of particles (Caparino et al., 2012). Solubility values in dried juice were 63.15 - 73.87 %,

significantly higher than CRF of 40.93 – 42.55 %. The lower solubility of CRF could possibly be due to the state of the powdered CRF is more compact and less easily penetrated by water by gentle mixing to promote material disintegration to form a suspension. From the other way, the relatively higher lipid content contained in CRF could also leads to a lower solubility due to their hydrophobic nature. This also explains the lower solubility of SJ compared with FJ, as higher lipids contained in SJ (data shown in Table 3.1). Solubility in this study was lower than that of Brazilian ginseng roots powders obtained by SD and FD around 90% (Vardanega et al., 2019).

Dispersibility is a key indicator of a powder property in an industrial setting, it represents the ability of powders to separate into individual particles when dispersed in water with gentle mixing (Sharma et al., 2012). High dispersibility indicates less lump formation and agglomerates of powder that fall apart in the water (Laokuldilok et al., 2015). As shown in Table 3.2, all the samples showed great dispersibility with the value higher than 90%, and dried spinach juice exhibit slightly higher dispersibility than CRF. Similar to solubility, dispersibility can be influenced by the properties of the samples, such as particle size and morphology (Ding et al., 2020). Thus, the morphology, particle size, as well as other properties of the spinach samples were investigated in the following study.

3.3.3.Particle size of chloroplast rich powder preparations

Table 3.3: Particle diameter of powders prepared from spinach juice or a chloroplast-rich pellet (μm)

Parameter	SJ	FJ	FC	Fresh CRF
$D_{4,3}$	9.81±0.22^{c}	66.44±0.38^{b}	113.95±4.44^{a}	10.01±0.60^{c}
d_{10}	2.26±0.18^{c}	9.26±0.47^{b}	19.23±1.29^{a}	2.39±0.02^{c}
d_{50}	7.63±0.06^{c}	48.75±0.48^{b}	96.46±2.62^{a}	6.90±0.23^{c}
d_{90}	20.23±1.01^{c}	151.62±1.81^{b}	215.93±2.88^{a}	19.10±1.54^{c}
Span	2.36±0.12^{b}	2.92±0.07^{a}	2.04±0.10^{c}	2.42±0.14^{b}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). $D_{4,3}$: volume mean diameter; d_{10}, d_{50}, d_{90}: median volume diameters of 10%, 50% and 90% of the cumulative volume (μm), respectively; span: polydispersity of the particles. Span: an indication of the width of the distribution. Span is the distance between two points equally spaced from the median. Span= $(d_{90}-d_{10})/d_{50}$. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF.

Particle diameter of spinach juice/CRF powders is influenced by the original characteristics and drying conditions. The volume mean diameter of spinach juice ($D_{4,3}$) was significantly affected by drying techniques, with the value of 9.81 μm for SJ, and 66.44 μm for FJ (Table 3.3). These results are in agreement with the reported for Brazilian ginseng roots powders obtained by spray and freeze drying, with $D_{4,3}$ values of 9 and 207 μm respectively (Vardanega et al., 2019), which suggests that spray dried powders normally produce products with smaller particle size than freeze dried powders. While the $D_{4,3}$ of spinach CRF powders (FC) was nearly twice as FJ. This significant difference is caused by the original characteristics difference between juice

and CRF samples during grinding, that CRF was composed of more compact structures with highly concentrated and thick properties.

The spinach juice and CRF powders used as food ingredient need to be prepared as liquid food before ingestion to perform health benefits. Therefore, the rehydration process in water is worth to be analysed. Particle size is a key parameter influencing the solubility and dispersibility of spinach juice/CRF powders in water. Previous study has shown that superfine powders with particle size in the range of 1-100 μm can increase the dispersibility because of the increased particle surface area and the breakdown of the cell wall of the vegetable materials (Park et al., 2001; Hu et al., 2012).

From another perspective, the reduced particle size can also improve the nutrients bioaccessibility by increasing the surface area accessible for enzymes to act (Priyadarshani, 2017).

3.3.4.Powder morphology

3.3.4.1. Microscope images of spinach juice/CRF

The morphology of the spinach juice/CRF samples were studied under the light microscope at 400× of magnification. As shown in Figure 3.5, individual green organelles or small clusters can be observed in all cases. Some individual chloroplasts were visualised in the picture with the diameter ranged 4-10 μm, which is in agreement with the reported diameters for chloroplasts (Nicholls et al., 2013). Others have reported on similar morphological structures for isolated chloroplasts with observed starch granules in pea vine haulm juice (Torcello-Gómez et al., 2019). Syamila et al.

(2019) found that the spray dried juice contains intact chloroplasts. These findings confirm that intact chloroplasts can be released without the restriction of cell wall by juicing and preserved after both spray drying and freeze drying. In order to better understand how preparative methods, impact on chloroplastic structures various chloroplastic preparations were studied using scanning electron microscopy.

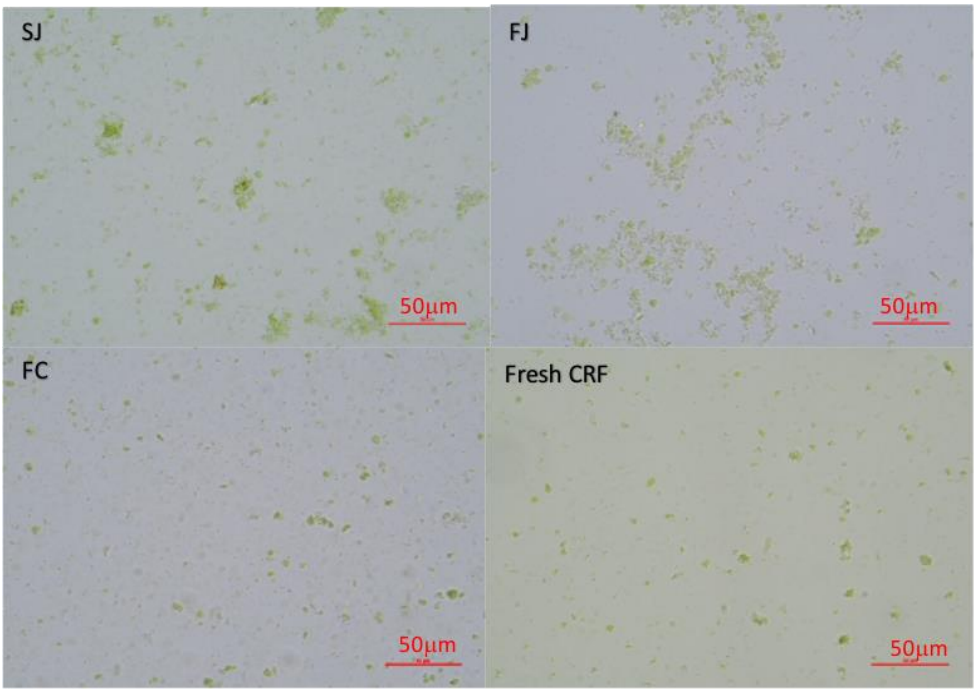


Figure 3.5: The light microscope images of SJ, FJ, FC and fresh CRF at magnification of 400×. *Abbreviations: SJ* Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF.

3.3.4.2. Scanning electron microscope (SEM) images of spinach juice/CRF powders

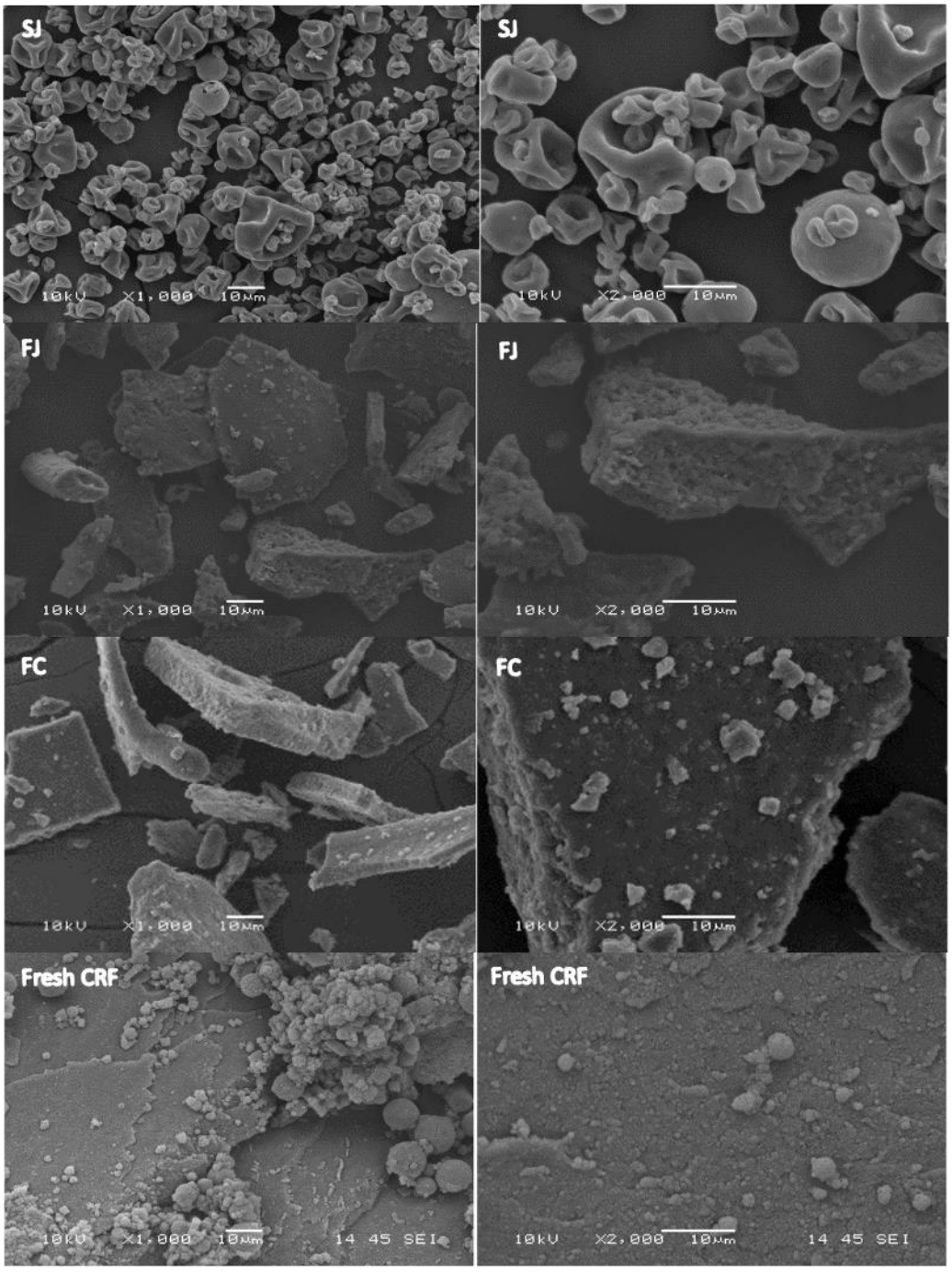


Figure 3.6: Scanning electron microscopy micrographs of spinach chloroplasts

Abbreviations: SJ Spray dried juice, *FJ* Freeze dried juice, *FC* Freeze dried CRF.

The effect of different drying methods on morphology/surface of spinach juice/CRF powders was observed using scanning electron microscopy (SEM) micrographs (Figure. 3.6).

The SEM images of SJ powders indicate irregularly spherical shaped particles with many shrinkages and dents on their surface. Surface dents result from a complex interaction between various factors, such as capillary forces, inlet temperature, drying rate, rapid wall solidification, and uneven shrinkage at early stages of drying. Shrinkage occurs due to the presence of capillary forces. The surface of the droplets dries upon contacting the hot air atmosphere. Then, water diffuses from the inner core towards the surface, which creates sub-atmospheric internal pressures and eventually result in the collapse of the particles (Abdallah et al., 2018). The dented structures can also be affected by the low inlet temperature and insufficient solid content in the feed solution, which cause the deficient drying of the feed droplets therefore leading to the breakage of film or crust (Maas et al., 2011). Spray drying with higher inlet air temperatures caused faster water evaporation and lead to the formation of smooth particle surfaces with a hard crust, which the hollow particles could not deflate when they were cooled (Nijdam et al., 2006). Higher drying rate can also lead to the rapid wall solidification prior to expansion of the particles and forms a strong crust on the particle surface and restricts the complete bubble inflation, thus resulted in caved-in or dented surfaces (Chinnaswamy et al., 2007).

Chloroplast powders obtained by freeze drying (FJ and FC) had porous and irregular plate shaped particles which are typical characteristics of freeze-dried

products. This phenomenon resulted from ice crystals that formed inside the material during processing, and this helps prevent the shrinkage and collapse of the organelle structure (Chranioti et al., 2016). Similar structures have been reported for freeze dried mango and açaí pulp (Caparino et al., 2012; Lucas et al., 2018). The particle size of FJ and FC as shown in the micrographs was higher than SJ and fresh CRF, which in consistent with the particle size distribution in Table 3.3.

3.4. Conclusion

Overall, the spinach chloroplasts can be released without restriction of cell wall by juicing and sequential centrifugation concentrates the intact chloroplast structures as chloroplast rich fractions (CRF). The quality of spinach chloroplasts can be maintained after drying process, in this study viz. spray and freeze drying. They can potentially be developed as functional food ingredient for the source of macronutrients such as proteins and lipids, as well as micronutrients such as carotenoids, chlorophylls, vitamin E and α-linolenic acid. Both spray drying and freeze drying applied in this study was proved to retain the nutrients in spinach juice at similar level; spinach CRFs contained higher concentration of nutrients (per unit mass) than dried juice. The physical parameters (particle size, solubility, dispersibility etc.) of spinach chloroplast materials after both drying methods will possibly have impact on the nutrients release and delivery in humans. Significantly reduced moisture content (below 10%) and water activity (below 0.3) in spinach chloroplast powders suggest microbiological safe and enhanced shelf stability. Freeze dried CRF (FC) was less soluble and dispersible compared with dried juice (SJ and FJ). The morphology changes of spinach

chloroplasts after spray drying and freeze drying was observed by scanning electron microscopy. Spray dried samples exhibited irregular spherical dented structures, while freeze dried samples showed porous and plates shaped particles. These observations highlighting the impact of each drying method on organelle structures and providing a means to differentiate between each method used.

The bioaccessibility of nutrients and *in vitro* digestion of lipophilic nutrients in these materials during in vitro digestion will further be investigated and discussed in following chapters.

Chapter IV

4. Effect of spinach juice pasteurisation on the physicochemical properties of derived powders

4.1. Introduction

In Chapter 3, it was demonstrated that spinach chloroplasts are rich source of macronutrients, such as proteins, lipids and fiber; as well as micronutrients, such as carotenoids, chlorophylls, vitamins (Pérez-Marín et al., 2019). Sufficient consumption and increased intakes of these compounds is associated with a reduced risk of cardiovascular diseases, cancer, obesity and other chronic diseases (Joseph et al., 1999; Wang et al., 2005; Vázquez et al., 2013; Roberts & Moreau, 2016; Torcello-Gómez et al., 2019). Despite these valuable attributes, the consumption of processed spinach remains low in the general population (Klockow et al., 2009). Therefore, there may be advantages in developing new spinach products or functional ingredients to encourage greater consumption, however, a key challenge here is spinach suffers from high perishability, postharvest (Bassetto et al., 2005). In this context, there is a need for developing novel preservation techniques to ensure the qualities of spinach products are retained during storage and to enhance shelf life.

To date, pasteurisation is considered as the most commonly used pre-treatment for food preservation, since this method inactivates some endogenous enzymes involved in spoilage, and eliminates spoilage microorganisms and pathogens (Peng et al., 2017). Despite its utility, pasteurisation causes some undesirable quality losses in terms of colour, flavour, texture, nutrients degradation and functionalities (sensorial,

physicochemical, nutritional properties and levels of antioxidants). For this reason, it is important to adjust the temperature and time to keep the physicochemical changes to a minimum but sufficient to inactivate the deleterious enzymes (Anthon et al., 2002).

It is known that the presence of endogenous enzymes in raw or processed fruit and vegetable leads to quality loss during storage. Peroxidases (POD) and polyphenol oxidases (PPO) are the main enzymes that contribute to enzymatic browning which can cause both off-flavours and off-colour formation (Lopez et al., 1994; Negri Rodríguez et al., 2021). Therefore, enzymatic browning is regarded as the secondary loss for fruits and vegetables during processing and storage (Thirumdas et al., 2015). Peroxidase (POD) is usually chosen as an indicator enzyme of thermal processing adequacy due to its high concentration in most plant tissues, and the relatively high heat-resistant ability (Barrett et al., 1995). Additionally, the non-enzymatic browning results from pigment degradation (especially chlorophylls and carotenoids), and Maillard browning reactions during thermal processing (Zhu et al., 2009; Jaeger et al., 2010). Several studies have investigated the effect of different processing methods on the level of pigments retention. High temperature short time processing can increase chlorophyll retention while the carotenoids retention varies at different circumstances (Gupte et al., 1964; Finten et al., 2016).

Pasteurisation is a primary step in processing of spinach. In addition, the production of spinach juice powders is also an alternative approach with the advantages of increasing the consumption period and economic potential, reducing cost for transportation (Çalışkan Koç et al., 2017). The dried spinach powders can be used as a

food colourant and nutritious additive in different formulations (Karaaslan et al., 2008; Koç et al., 2018). Spinach powders can be obtained by freeze drying the pasteurised spinach juice with increased stability during storage. Freeze drying is the most efficient drying technique for nutrients preservation in food industry due to the low temperature which stop the microbiological and deterioration activity (Sagar & Suresh Kumar, 2010). Moreover, the powders produced from fruit and vegetable juice extend the shelf life from weeks to months or years depending on packing and storage conditions (Walkling-Ribeiro et al., 2009; Chauhan et al., 2013).

In recent years, there has been an increasing consumer demand for nutritious products with high sensorial quality and increased shelf life. The trend for healthy, minimally processed foods has driven research and development in novel food processing technologies to produce foods with fresh-like quality that are safe for consumers. However, few studies have assessed the combined impacts of thermal pre-treatment with non-thermal drying process on food quality attributes.

The aim of this research is to develop an efficient processing technique to produce dried spinach chloroplast-rich powders that are highly nutritious and retain quality attributes throughout long-term storage. The influence of pasteurisation and freeze-drying processes on the physicochemical properties of dried spinach chloroplasts were investigated and key quality attributes determined including enzymatic inactivation, chromatic parameters and physicochemical characteristics. The importance of this work is to establish the 'novel processing' technique, produce better quality of spinach final products which can be developed as raw material or additive in functional food

formulations for further processing and stored for subsequent use.

4.2. Sample preparation summary

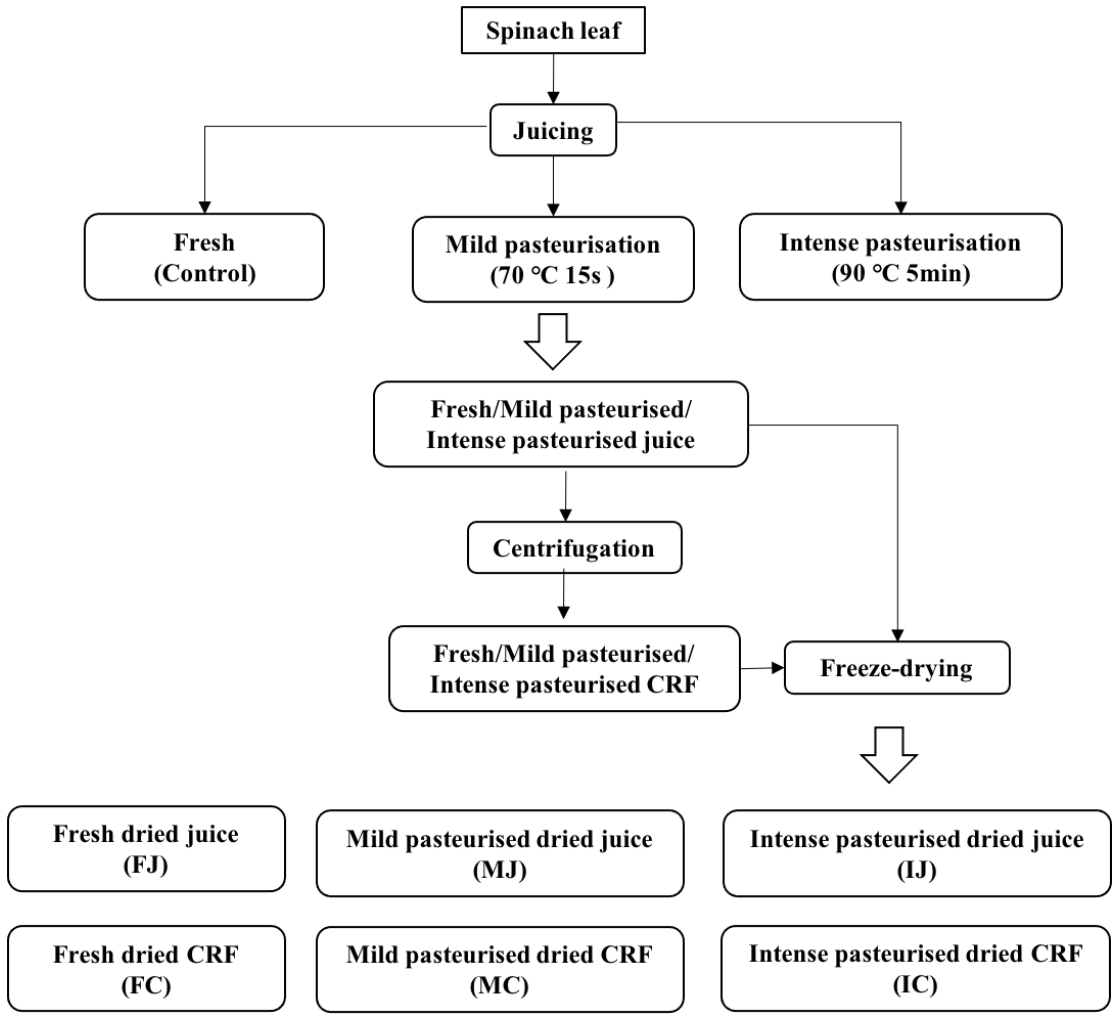


Figure 4.1: Fresh/pasteurised spinach juice/CRF powders preparation steps

Figure 4.1 illustrates the preparation steps of spinach juice/CRF powders. The freeze drying was used in the following studies. As discussed in Chapter 3, both spray drying and freeze drying can retain similar nutrients level in spinach juice powders. Whereas, from technical perspectives, spray drying is limited since only liquid samples with particle diameter lower than 100 μm can be passed through the nozzle of spray dryer (Armfield FT80 Tall Form Spray Dryer, Ringwood, UK) in this study.

4.3. Results and discussion

4.3.1. Quality changes of fresh and pasteurised spinach juice during refrigerated storage

The main goal of pasteurisation of spinach juice is to eliminate microbial contamination, inactivate endogenous enzymes, and to extend shelf life. Some properties may change under thermal treatment which may affect the food quality and sensory characteristics. Food quality and shelf-life safety are crucial criteria when customer select food products. Therefore, the physical-chemical properties of both non-pasteurised and pasteurised spinach juice were monitored under 4°C for seven days (data shown in Table 4.1).

pH plays a key role in food preservation as it determines the extent of microbiological activity. Generally a low pH (pH ≤ 4.6) tends to prevent microbial growth in fresh juices (Tola et al., 2018). In general vegetables are 'low acid' foods (pH ≥ 4.6), and so require stronger thermal treatments to achieve the desirable shelf life. Heat treatments in the range of 82-93°C are commonly used to kill bacteria in low-acid foods (pH ≥ 4.6) (Barrett et al., 2012). In this study, the initial pH value of non-pasteurised juice (NPJ) was pH 7. After pasteurisation this value slightly increased to around pH 7.35-7.59, and was attributed to the release of NH_3 from the breakdown of cell membranes (Kung et al., 1991). The pH of all juice samples significantly decreased to pH 5.75-5.93 over seven days of refrigerated storage. This is probably due to the growth of microorganisms that cause juice spoilage via the fermentation of sugars. In turn, this action produces organic acid hence reduce the pH and changes in the Brix

value. Similar results were also found in carrot and spinach juice which initially showed a neutral pH of 7-8 that significantly decreased to pH 4-6 during refrigerated storage for fourteen days (Khandpur et al., 2016). The decreased pH was also in agreement with the reports for refrigerated passion fruit pineapple and mango juice over the twelve day period (Kaddumukasa et al., 2017). There was no significant difference of pH value in the different treated spinach juices during refrigerated storage. Decreased pH was observed in all cases over the seven days period, while stable pH was only found in pasteurised juice in initial three days of storage (4 °C) (data not shown). Low pH encourages the growth of acid-tolerant bacteria, causing sample deterioration that reduces product storage stability. Both fresh and pasteurised juice after seven-days of refrigerated storage showed unpleasant colour and smell which confirms the microorganisms induce spoilage.

°Brix values showed a significant decrease in spinach juice samples after pasteurisation, decreased from 5.9 to 3.7. This reduction could be due to the reaction between reducing sugar and the alpha amino acids after heating (data shown in Table 4.1). In addition, a further reduction of Brix value was found in fresh juice over seven days storage at 4°C. This decline may probably have been due to microbial metabolic activities which causes sugars in the samples to be converted to organic acids, hence decreased the pH and shortened shelf life. Similar behaviour was also supported by Khandpur and Gogate (2016) who reported a significantly decrease of Brix value ranged from pH 6-8 to 3-5 in both fresh and thermally treated spinach and carrot juice over 10 weeks storage at 4°C.

Thermal treatments like pasteurisation were shown to inactivate enzymes and to eliminate pathogens, which is important for food preservation. Among all endogenous enzymes found in fruits and vegetables, peroxidases (POD) are a particularly heat resistant group of enzymes, which play important roles in quality deterioration (Umair et al., 2022). In this study, POD activity was used as an indicator for the inactivation of endogenous enzymes during spinach juice pasteurisation. It can be observed that 96-99% of POD activity was reduced in pasteurised spinach juice when compared with non-pasteurised juice. In addition, the inactivation of POD activity remained after 1 week of refrigerated storage. Therefore, both mild and intense pasteurisation conditions (70 °C 15s and 90 °C 5min) were sufficient to inactivate spinach POD producing a stable product with improved shelf life. Similar results were reported by Wattanakul et al. (2020) , who found that the reduction of POD activity was around 92.3 - 95% in pea vine haulm juice after hot water blanching (100°C 2 min) and steam blanching (100°C 4 min). It was also reported that spinach leaf blanched at 95°C for 1 min or steamed for 3-6 min was sufficient for reduction of peroxidase activity (Onayemi et al., 1987; Okoli et al., 1988).

The particle size distribution of fresh and pasteurised spinach juice during refrigerated storage was also investigated in this study. The mean volume weighted $D_{4,3}$ value of non-pasteurised juice was around 4.4 μm, and this value significantly increased to 40.55 and 102.97 μm after mild and intense pasteurisation. Moreover, the average particle size ($D_{4,3}$ value) of spinach juice was significantly increased as the pasteurisation level (time and temperature) increased. The larger particles in pasteurised

spinach juice could be caused by the protein denaturation and aggregation events during thermal processing (Bernat et al., 2015; Atalar et al., 2019). Additionally, the high $D_{4,3}$ values suggest that the particles are heavier and tend to settle down rapidly during storage. The sedimentation during the storage further leads to the interactions between particles and aggregation, which explains the increased particle size at the end of the storage (7 days, 4°C).

Table 4.1: Physicochemical changes of fresh and pasteurised spinach juice during refrigerated storage

	Days	pH	°Brix	% reduction of POD activity	Particle size $D_{4,3}$/μm
Fresh juice	0	7.00^{a}	5.9^{a}	0	4.40^{e}
	7	5.78^{b}	4.8^{b}	0	11.82^{e}
Mild pasteurised juice	0	7.35^{a}	3.9^{c}	96^{b}	40.55^{d}
	7	5.93^{b}	3.7^{cd}	97^{a}	61.92^{c}
Intense pasteurised juice	0	7.59^{a}	3.9^{c}	98^{a}	102.97^{b}
	7	5.75^{b}	3.7^{d}	99^{a}	170.78^{a}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means designated with different letters in the same row are significantly different ($p<0.05$). *Mild pasteurised spinach juice (70°C 15s); Intense pasteurised spinach juice (90°C 5min).*

4.3.2.Effect of spinach juice pasteurisation on the nutrient content of derived powders (dried juice and dried pellet)

4.3.2.1. Macronutrients content of fresh/pasteurised spinach juice/CRF

The results of macronutrients content of fresh/pasteurised spinach chloroplasts were presented in Table 4.2. The proteins showed the largest proportion in all samples, with a ratio of 38.5~58.6% approximately. In CRF samples, a significant increase of proteins and decrease of lipids was observed after pasteurisation. While, for juice samples, the proportion of proteins and lipids maintained relatively stable after pasteurisation. This is likely due to the high heat cause the break of lipid-proteins complex in pasteurised spinach juice, while after centrifugation, the protein-rich pasteurised CRFs were collected, the lipids released in the supernatant were discarded.

Significant higher proteins and lipids levels were present in CRFs (FC, MC, IC) compared with juice (FJ, MJ, IJ), which is attributed to the synthesis and concentration of thylakoid membranes, that are rich in proteins and galactolipids, and allied lipid soluble nutrients (carotenoids, chlorophylls and fatty acids) in chloroplasts (Kirchhoff et al., 2002). In contrast, lower ash (total minerals) and carbohydrates were obtained in CRFs, due to the fact that minerals and some low molecular weight carbohydrates are more soluble and therefore abundant in supernatants. The ash (total minerals) content maintained relatively stable after heat processing in both dried juice and CRF, since minerals are resistant to the light, heat and oxygen. As reported by Azima et al. (2020) , who indicated that cooking methods such as boiling, steaming and stir frying have little effect on Fe, Zn and Mg profile of green leafy vegetables (e.g. spinach, pumpkin leaves,

squash leaves and green mustard).

In general, pasteurisation can retain macronutrients in dried spinach juice. The significant changes in terms of the proteins and lipids in CRFs is mainly due to the denaturation by heat and physical separation.

Table 4.2: Nutrients composition of spinach chloroplasts

/%DW	FJ	FC	MJ	MC	IJ	IC
Proteins	39.6 ± 0.4^{de}	50.6 ± 0.5^{c}	41.2 ± 0.1^{d}	58.6 ± 0.2^{a}	38.5 ± 0.9^{e}	54.9 ± 1.0^{b}
Lipids	11.8 ± 0.4^{d}	28.3 ± 0.4^{a}	11.5 ± 0.5^{d}	20.3 ± 0.7^{b}	10.7 ± 0.5^{d}	18.1 ± 0.3^{c}
Ash	20.8 ± 0.2^{a}	7.2 ± 0.1^{d}	18.2 ± 0.5^{b}	7.1 ± 0.1^{d}	18.9 ± 0.3^{b}	7.7 ± 0.1^{d}
Carbohydrates	27.8	13.9	29.1	14.0	31.9	19.3

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n =3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: FJ Fresh freeze dried juice; FC Fresh freeze dried CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.*

4.3.2.2. Fatty acids composition of fresh/pasteurised spinach juice/CRF

The fatty acids (FAs) content and composition of unpasteurised and pasteurised spinach chloroplasts were displayed in Figure 4.2 and expressed as per gram of dry weight and lipid. As can be seen, these samples contain higher polyunsaturated fatty acids (PUFAs), with the most abundant form of fatty acid is the α-linolenic acid

(C18:3n-3, ALA) (n-3), followed by linoleic acid (C18:2n-6, LA) (n-6). These are essential fatty acids (EFAs) as they cannot be synthesised by the human body. This found is in accordance with the fatty acids in pea vine haulm and pea leaves chloroplasts (Dorne et al., 1989; Torcello-Gómez et al., 2019).

On a dry mass basis, the most obvious observation was the significant loss of ALA in pasteurised CRFs, which linked to the significant decrease of lipids in previous section. In contrast, oleic acid (C18:1) and linoleic acid (C18:2, LA) proved to be more heat stable than ALA, with no significant changes after pasteurisation. Moreover, the fatty acids profile as an essential indicator of the nutritional value of the oil (Harhar et al., 2011), did not change after the pasteurisation step (when heating the spinach juice at 70 °C and 90°C). Based on the previous study, fatty acid profile is more affected by heating temperature than time (Liu et al., 2018), olive oil which contains lower PUFAs, visible changes can be demonstrated in ALA when heated at 180 °C or higher at 220°C, since SFAs are more resistant to thermal treatment (Dordevic et al., 2020). Unlike the olive oil, which contains high MUFA, green leafy vegetables such as spinach usually contains higher proportional of PUFAs in the form of ALA (60-70% of total FAs) (Saini et al., 2014; Kim et al., 2018), which oxidize rapidly during storage and heating, therefore, actions should be made in order to maintain the quality of products.

On a lipid mass basis, both dried pasteurised juice and CRFs showed an increasing trend in the levels of α-linolenic acid and palmitic acid content. This could be due to the change of fatty acid profile after heating, that the galactolipids hydrolysis may cause release of FFA (mainly ALA).

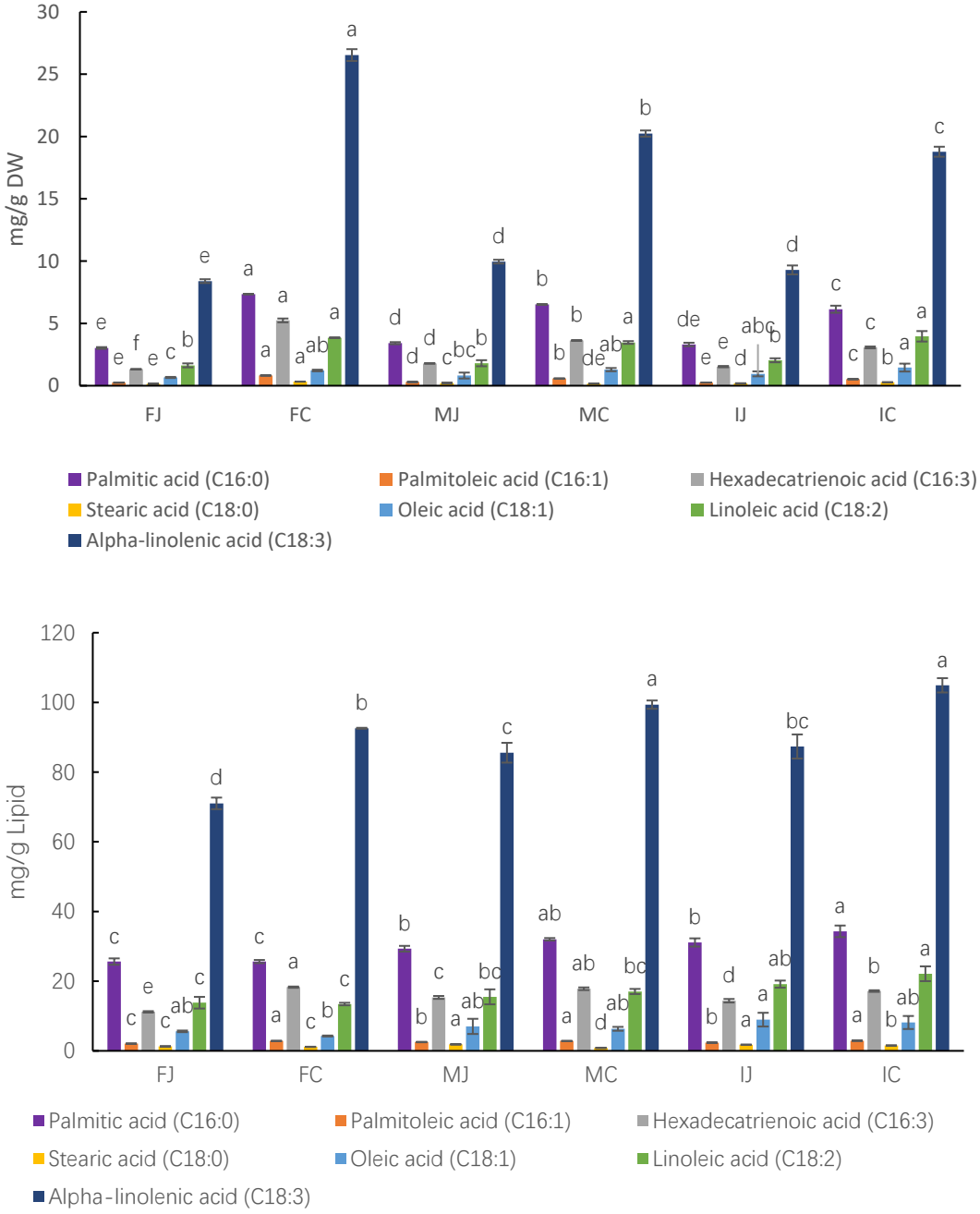


Figure 4.2: Fatty acid composition of fresh/pasteurised dried juice and CRF

Results are expressed as means ± SD (n = 3) and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at $p<0.05$, a>b. *Abbreviations: FJ Fresh freeze dried juice; FC Fresh freeze dried CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense*

pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.

4.3.2.3. Chlorophyll content of fresh/pasteurised spinach juice/CRF

The effect of pasteurisation on colour degradation was evaluated following the analysis of chlorophyll (Figure 4.3) and carotenoids contents (Figure 4.4). Chlorophyll a and b are the two major chlorophylls present in plant chloroplasts (thylakoid membranes). Chlorophyll a is the most abundant form and is typically found at twice that of chlorophyll b in plant tissues. A significant loss of chlorophylls was observed in CRFs after pasteurisation, which is linked to the decrease in total lipid content as their lipophilic nature. Previously studies indicate that chlorophyll retention during processing of vegetables depends upon temperature and length of heat treatment, and that thermal degradation rates of chlorophyll a are two to six times higher than chlorophyll b in spinach puree (Canjura et al., 1991; Schwartz et al., 1991). Thermal degradation of chlorophylls a and b in green peas were studied across the temperature range of 70-100 °C; faster degradation of chlorophylls were detected at temperatures higher than 80 °C (Erge et al., 2009). In this study, intense pasteurisation caused significant ($p<0.05$) degradation of chlorophylls in dried juice. The decrease of chlorophylls is attribute to the high heat treatment induce the loss of central magnesium atom of the chlorophyll porphyrin ring, and converts chlorophylls to pheophytins and other derivatives, such as chlorophyllide and pheophorbide, which also lead to the colour change from green to olive brown (Cubas et al., 2008). It was reported that 1g dried spinach thylakoid powders can provide 0.3 mg chlorophylls (Li et al., 2019a), in

contrast, in this study chlorophylls were found higher with the value of 16.8 and 66.3 mg/g DW in fresh powdered spinach juice and CRF.

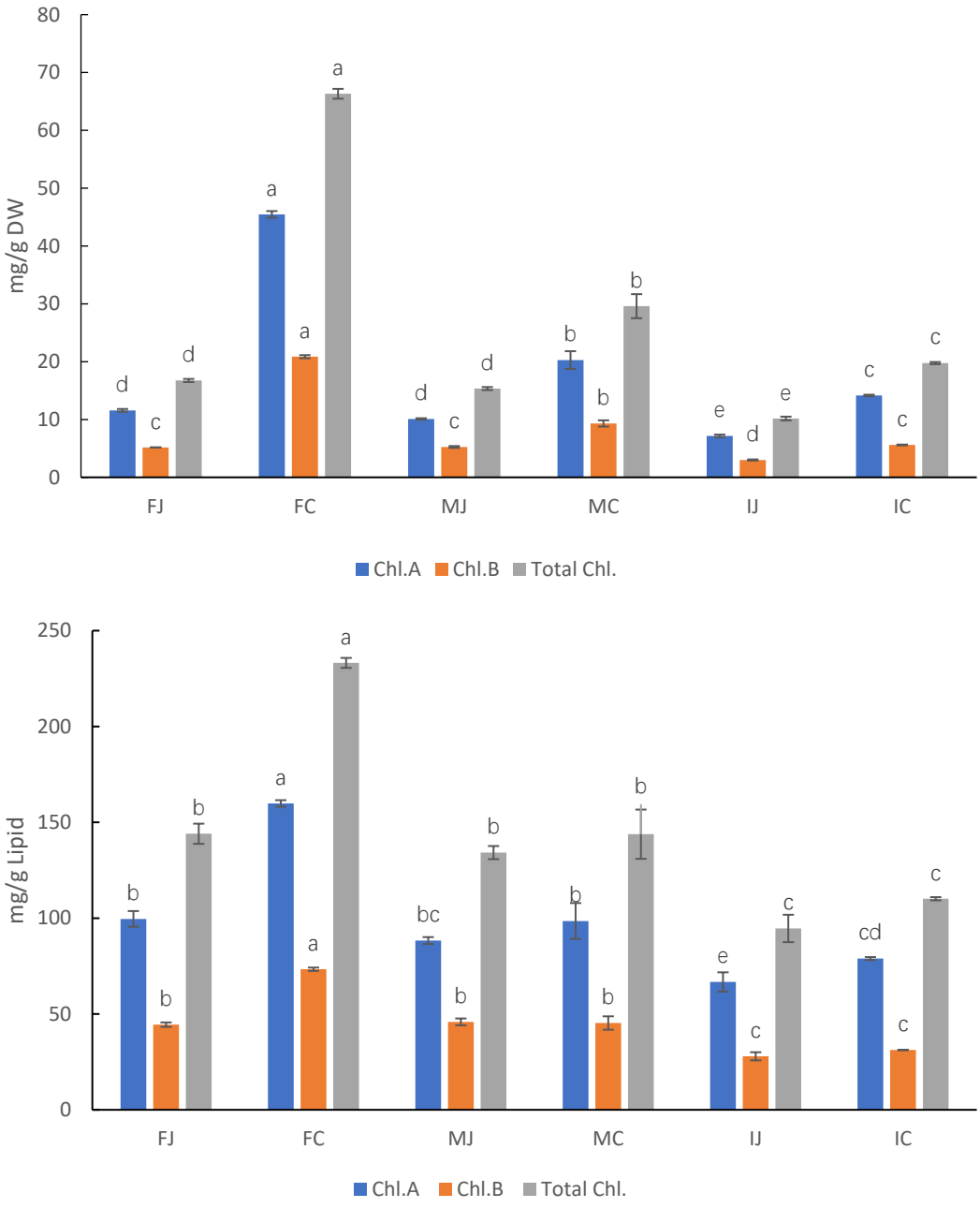


Figure 4.3: Chlorophylls content of fresh/pasteurised dried juice and CRF

Results are expressed as means ± SD (n = 3) and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at $p<0.05$, a>b. *Abbreviations: FJ Fresh freeze dried juice; FC Fresh freeze dried CRF; MJ Mild*

pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.

4.3.2.4. Carotenoids and vitamin E content of fresh/pasteurised spinach juice/CRF

Carotenoids are important pigments with antioxidant properties that are widely found in vegetables. β-carotene and lutein are the predominant carotenoids in plants. Changes in carotenoid levels between unpasteurised and pasteurised dried chloroplasts are presented in Figure 4.4. On a dry mass basis, carotenoids remain stable after both mild and intense pasteurisation in dried juice but remarkably these molecules decreased in CRFs. These changes correlated with a general trend in the loss of total lipids in CRFs after thermal processing (Table 4.2). In addition, the degradation of carotenoids can be also caused by isomerisation of all-trans-β-carotene to cis forms followed by oxidation (Aman et al., 2005; Lemmens et al., 2010). The extend of such degradation is associated with the duration and severity of heat processing (Seybold et al., 2004). Indeed, similar results are supported by Wattanakul et al. (2022) who found 34% and 40% of the β-carotene and lutein losses and increase of cis-β-carotene in heat treated (steam or pasteurisation) pea vine haulm CRF. Reductions in carotenoids levels in heated vegetables have also been reported for boiled cabbage (34.45%) and spinach (24.78%) (Dumbrava et al., 2016). In this instance, the high temperatures induce the breaking of bonds present in the carotenoids and the reformation of single or double bond following isomerisation (Jonsson, 1991).

Similarly, vitamin E is the major lipophilic antioxidant that is responsible for

preventing membrane lipid peroxidation in plants, animals and fungi. This class of molecule consists of four tocopherols (α, β, δ and γ) and the corresponding tocotrienols (α, β, δ and γ) that contain unsaturated side chains. Of this group of compounds α-tocopherol is considered to be the most effective biological antioxidant (Chun et al., 2006; Cruz et al., 2013). The content of α-tocopherol in dried fresh and pasteurised spinach chloroplasts are shown in Figure 4.4. The amount of vitamin E (VE) in FJ and FC was 0.26 and 0.70 mg/g of dry weight, higher VE in FC linked to the higher chloroplasts contained in CRF, since α-tocopherol is mainly situated inside chloroplasts (Barbara et al., 2000). A significant loss of VE was determined in pasteurised juice/CRF compared with fresh samples, the degradation of α-tocopherol could be due to the low heat stability of α-tocopherol from spinach juice/CRF materials (Knecht et al., 2015). However, between mild and intense pasteurisation conditions, it seems like the latter can retain higher VE levels, with 0.13 and 0.53 mg/g DW contained in the IJ and IC while 0.04 and 0.20 mg/g DW found in MJ and MC. This could be due to the increased sufficiency of α-tocopherol extractability following denaturation of proteins and a complete cell disruption as a result of higher temperature and time, which consequently cause more release of α-tocopherol from plastoglobule within the chloroplasts (Wójtowicz et al., 2021). While, as reported by Lee et al. (2018b) α-tocopherol retention varies across heating methods and food matrices. Cooking green leafy vegetables such as broccoli, spinach and perilla leaf leads to a significant higher vitamin E levels, while cooked root vegetables such as potato, sweet potato and carrot lead to lower retention of vitamin E.

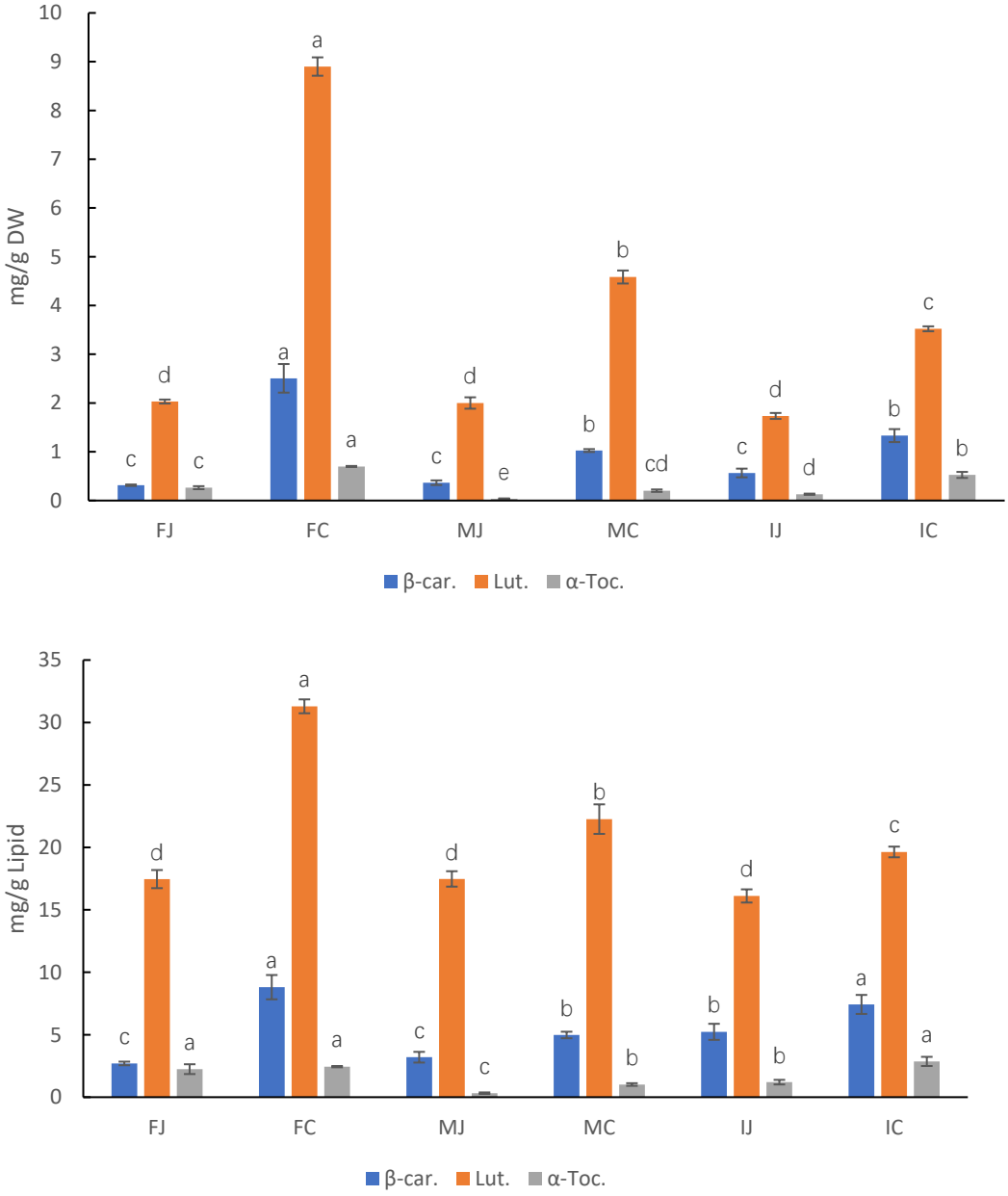


Figure 4.4: Carotenoids and vitamin E content of fresh/pasteurised dried juice and CRF

Results are expressed as means ± SD (n = 3) and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at $p<0.05$, a>b. *Abbreviations: FJ Fresh dried freeze juice; FC Fresh dried freeze CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense*

pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.

4.3.3.Physical properties of fresh/pasteurised spinach juice/CRF

Table 4.3: Characterization of freeze-dried spinach juice and CRF powders

Properties	FJ	FC	MJ	MC	IJ	IC
Moisture content/%	7.7 ± 0.2^{c}	4.7 ± 0.2^{d}	9.3 ± 0.1^{a}	3.8 ± 0.1^{e}	8.3 ± 0.2^{b}	3.8 ± 0.1^{e}
Water activity	0.23 ± 0.01^{c}	0.25 ± 0.00^{b}	0.34 ± 0.00^{a}	0.22 ± 0.01^{c}	0.17 ± 0.01^{e}	0.19 ± 0.01^{d}
Solubility/%	68.6 ± 0.6^{a}	25.3 ± 0.3^{d}	41.3 ± 0.2^{c}	10.1 ± 0.1^{e}	47.0 ± 0.0^{b}	10.2 ± 0.1^{e}
Dispersibility/%	91.0 ± 2.2^{a}	65.0 ± 5.1^{b}	61.0 ± 2.2^{b}	22.4 ± 0.8^{c}	60.9 ± 1.9^{b}	22.9 ± 1.1^{c}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n =3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: FJ Fresh dried freeze juice; FC Fresh dried freeze CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.*

The moisture content of dehydrated materials plays an important role in preservation safety and defines the amount of water in any given food system. High moisture content is indicative of fast food degradation. Thus, producing powders can significantly reduce the moisture content available for chemical reactions and the growth of microorganisms (Mathlouthi, 2001). Higher moisture content was observed in dried spinach juice (7.7-9.3 %) compared to CRF materials (3.8-4.7 %). Similar results were reported by (Koç & Dirim, 2018), with the moisture content of spray dried spinach powders is in the range of 5.56-9.29 %, wet basis.

Water activity is a critical indicator for powders microbiological safety during storage. Different from moisture content, water activity measures the free water available in food which is responsible for the biochemical reactions to occur (Quek et al., 2007). The higher water activity indicates shorter shelf life, since more free water available for deteriorative reactions (Tontul & Topuz, 2017). The moisture content observed in this study was lower than 10% and the water activity was around 0.3. This guaranteed the powders' microbiological safety and stability during long-term storage (Tze et al., 2012a).

Solubility is a fundamental physical property/characterization of powder products which is the evaluation of the behaviour of the powders in the aqueous phase. This indicator is directly linked to the technological application in food products, since powders used as food ingredients must exhibit good solubility which enable the desired functionalities for food design (Bicudo et al., 2015; Vardanega et al., 2019). The solubility of fresh dried juice and CRF (FJ and FC) was 68.6 and 25.3%, and significantly decreased to 41.3-47.0%, and 10.1-10.2% after mild and intense pasteurisation. The solubility may be related to the particle size, smaller particles usually have higher solubility which due to the greater surface area available for hydration (Kuck et al., 2016). The higher solubility of dried juice could be attributed to its lower lipid content but higher ash and carbohydrates which are more solubilised. In addition, thermal treatment and protein denaturation caused a marked and irreversible reduction of solubility. As reported by (Perez-gago et al., 2001)) the solubility of the protein decreased as a function of heating time and temperature (70-100 ℃, 5-20 min).

Dispersibility is the ability of powders to separate into individual particles when dispersed in water with gentle mixing (Laokuldilok & Kanha, 2015). Less lumps and agglomeration fall apart in water when the powder dispersibility is high (Suhag et al., 2021). Table 4.3 shows a significant effect of pasteurisation conditions on the dispersibility of dried spinach juice and CRF, as fresh juice and CRF showed higher dispersibility value of 91% and 65% respectively. Significantly decreased dispersibility of dried juice and CRF was observed after both mild and intense pasteurisation, which was in the range of 60.9-61.0% and 22.4-22.9%. This observation indicates that dispersibility was greatly affected by pasteurisation but no significant difference between mild and intense pasteurisation were seen. This index is important in industrial settings (Sharma et al., 2012).

After pasteurisation and freeze drying, the dried spinach juice/CRF were ground to a fine powder with mortar and pester, and then filtered with 250 μm sieve. After these processing steps, the changes in particle size were noted (data shown in Table 4.4). The mean particle size ($D_{4,3}$) of dried spinach juice was increased from 43.48 μm to 69.53 μm and 101.38 μm after mild and intense pasteurisation, and the dried CRF was increased from 81.91 μm to 182.11 μm and 167.52 μm respectively. Moreover, the span of dried juice and CRF was decreased after both pasteurisation conditions. Spinach powder obtained by freeze drying the spinach leaves in previous studies showed that the $D_{4,3}$ value of 315.3±70.22 μm, D_{50} value of 182.3 μm ± 10.69 μm which is significantly higher than our results. Moreover, micronisation was used to reduce the particle size of freeze-dried spinach leaves. After wet and dry micronisation the $D_{4,3}$

value was decreased to 121.4 μm ± 13.35 μm and 28.1 μm ±1.18 μm respectively (Różyło et al., 2022). Micronisation as a technology that normally used in pharmaceutical industry, which is a mechanical and high shearing operation to downsize the particles of food material to the micron range (Dhiman et al., 2021). It has been recently proved its potential application in food industry.

Table 4.4: Particle size of powdered spinach juice/CRF

/μm	FJ	FC	MJ	MC	IJ	IC
$D_{4,3}$	43.48±2.00^{d}	81.91±1.91^{c}	69.53±3.41^{c}	182.11±9.94^{a}	101.38±4.97^{b}	167.52±7.50^{a}
d_{10}	4.18±0.27^{d}	9.87±1.03^{c}	13.30±1.79^{c}	54.07±4.00^{a}	15.23±0.64^{c}	38.13±1.90^{b}
d_{50}	29.36±1.77^{d}	65.85±2.43^{c}	68.09±3.45^{c}	175.29±6.58^{a}	91.83±6.29^{b}	165.76±2.10^{a}
d_{90}	109.80±4.77^{c}	178.05±3.22^{b}	126.42±6.48^{c}	305.08±14.57^{a}	202.07±7.82^{b}	291.09±14.99^{a}
Span	3.60±0.07^{a}	2.56±0.08^{b}	1.66±0.08^{d}	1.43±0.02^{e}	2.04±0.07^{c}	1.53±0.08^{de}

$D_{4,3}$: volume mean diameter; d_{10}, d_{50}, d_{90}: median volume diameters of 10%, 50% and 90% of the cumulative volume (μm), respectively; span: polydispersity of the particles. Span: an indication of the width of the distribution. Span is the distance between two points equally spaced from the median. Span= (d_{90}-d_{10})/d_{50}. *Abbreviations: FJ Fresh dried freeze juice; FC Fresh dried freeze CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.*

4.3.4.Powder morphology

4.3.4.1. Light microscope images of fresh/pasteurised spinach juice/CRF powders

Light microscope was used to observe the microstructure of the fresh and

pasteurised dried juice/CRF samples (Figure 4.5). All the dried powdered juice/CRF samples were hydrated in RO water as suspension before loading for observation, and then analysed using a light microscope with the polarised lens. In all cases, green organelles which corresponds to the intact liberated chloroplasts were observed. Individual chloroplasts with diameter in the range of 5-10 μm can be found in fresh samples, which is in agreement with the chloroplasts in higher plants (Block et al., 2007). This indicates that the chloroplasts were released from plant cells during the juicing process by disrupting its plant tissue. While, larger and darker green coloured clusters were visualized in pasteurised samples, which could be explained by the aggregation of proteins, and the loss of pigments caused by high temperature during pasteurisation. Chlorophylls are responsible for the green colour in spinach chloroplasts. The colour change from bright green to olive brown during heating is caused by conversion of chlorophylls to pheophytins due to the loss of central magnesium ion (Gaur et al., 2007). The extent of chlorophyll degradation was highly depending on the thermal severity of the process, with intense pasteurised samples showed more loss of chlorophylls and darker olive brown colour than mild pasteurised samples. In order to balance the benefits of thermal processing and the quality of food products (microorganisms, appearance, flavour and nutrients), high temperature short time (HTST) technique can shorten the exposure to heat and preserve green colour in vegetables (Aamir et al., 2013). While the colour differences seems to be more obviously visualised between powdered samples as shown in Figure 2.5. The CRF powders showed darker greenness than juice powders, and pasteurised powders showed

loss of greenness and brightness. This is due to the fact that other than the impact of pasteurisation on chlorophyll degradation, particle size additionally influenced the colour of the grinded powders (Table 4.4). As reported by Haas et al. (2019), increased particle size cause decreased brightness which can be attributed to decreased light scattering of samples. Therefore, the brighter appearance of powders was observed in powders with lower mean particle size.

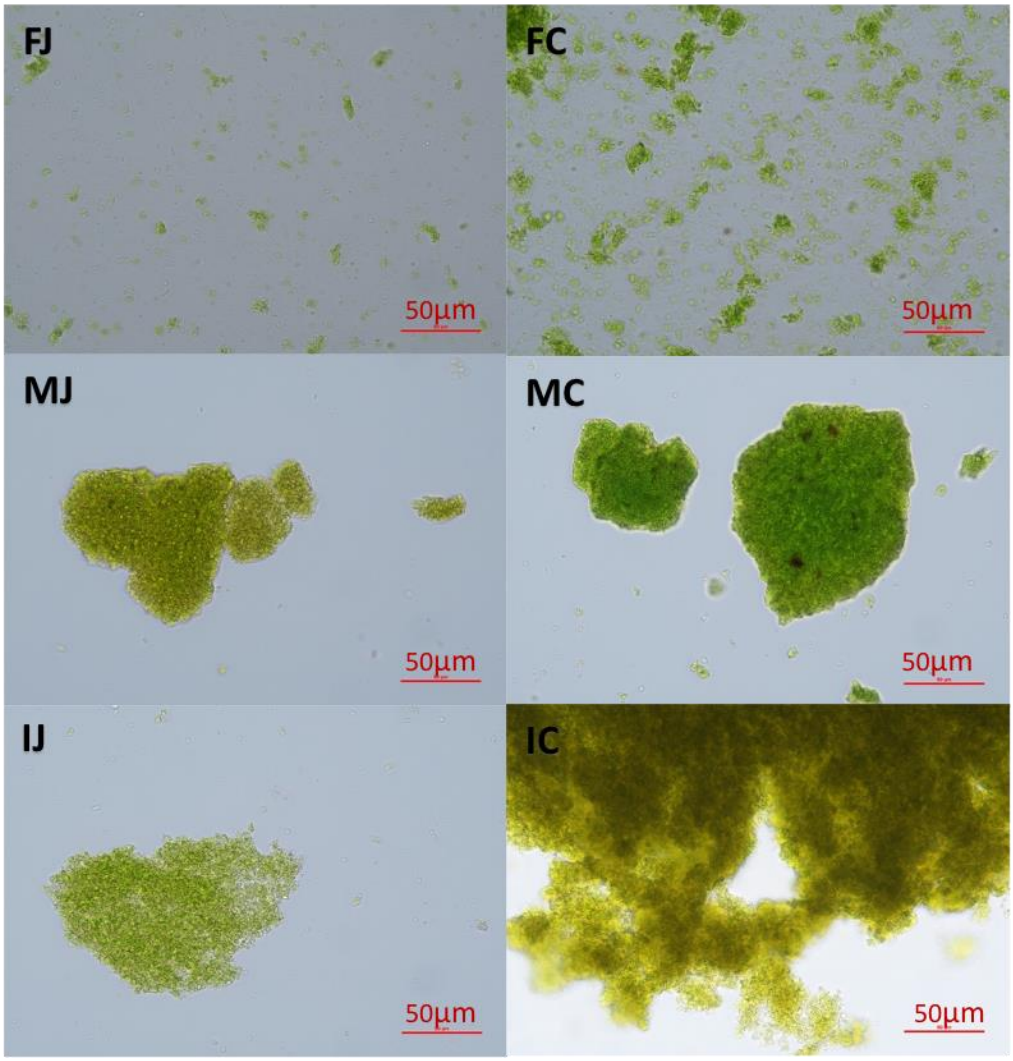


Figure 4.5: Light microscope images of fresh and pasteurised spinach chloroplasts at 400x magnification. *Abbreviations: FJ Fresh dried freeze juice; FC Fresh dried freeze CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF;*

IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF.

4.3.4.2. Scanning electron microscope (SEM) images of fresh/pasteurised spinach juice/CRF

The morphology of freeze-dried spinach juice/CRF samples was examined using a scanning electron microscope (SEM) at 250x and 1000x magnification (Figure 4.6). As can be observed from Figure 4.6, the pasteurisation conditions resulted in powders with different particle morphologies. First of all, the spinach powder samples were all obtained by freeze drying and thus exhibited an irregular and broken glass structure. This has been reported to be typical structure characteristics of freeze-dried products (Amin et al., 2021). Moreover, the intense pasteurisation resulted in largest particles followed by mild pasteurised spinach juice/CRF and fresh juice/CRF (data shown in Table 4.4). In addition, both the fresh/pasteurised dried spinach CRFs exhibited larger particles than juice. The increased size of particles observed after pasteurisation can be explained by the aggregation of proteins in spinach juice during heating. And the extent of increased particle size was depending on the severity and duration of thermal processing. Spinach CRFs were obtained by centrifuging the juice and only collected the pellet which thus were more concentrated than juice. In this consequence, CRFs contained more chloroplasts and showed larger particle size than juice. The SEM morphology of spinach juice/CRF powders were similar to that freeze-dried functional tea powders and blackberry powders (Franceschinis et al., 2014; Vardanega et al., 2019).

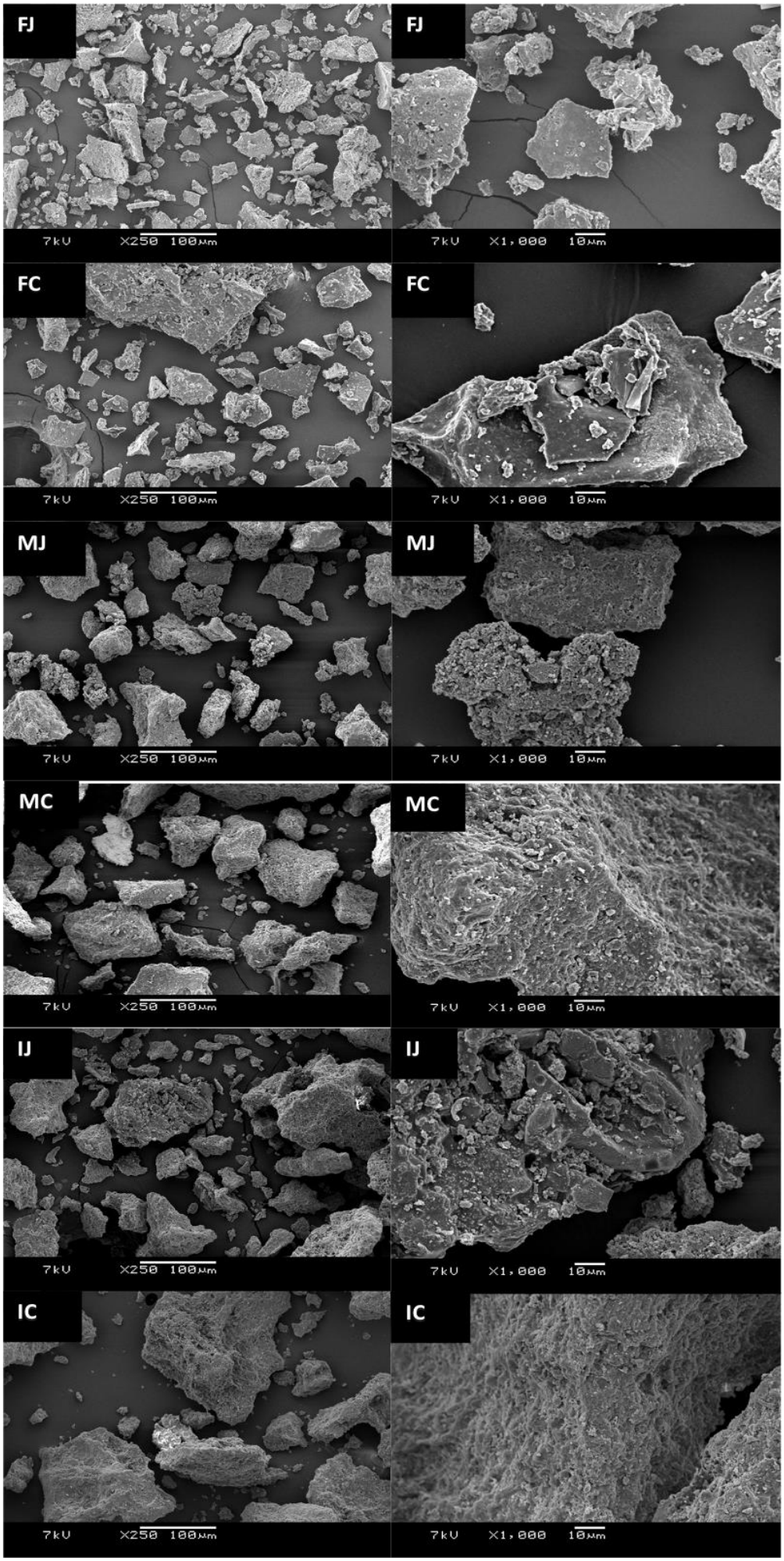

FJ
7kV X250 100µm
FJ
7kV X1,000 10µm
FC
7kV X250 100µm
FC
7kV X1,000 10µm
MJ
7kV X250 100µm
MJ
7kV X1,000 10µm
MC
7kV X250 100µm
MC
7kV X1,000 10µm
IJ
7kV X250 100µm
IJ
7kV X1,000 10µm
IC
7kV X250 100µm
IC
7kV X1,000 10µm

Figure 4.6: Scanning electron microscope images of fresh and pasteurised spinach chloroplasts at x250 and x1000 magnification. Abbreviations: *FJ Fresh dried freeze juice; FC Fresh dried freeze CRF; MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried CRF*.

4.4. Conclusion

The conclusion in this chapter can be summarized as follows:

- Pasteurisation of spinach juice is sufficient to inactivate endogenous enzymes linked to reduced shelf-life. Peroxidase activity after mild (70 ℃, 15 s) and intense (90 ℃, 5 min) pasteurisation was reduced to 96-99% of its activity. Therefore, freeze drying combined with thermal pre-treatment produces spinach chloroplast powders with guaranteed nutrients stability and shelf life.
- Pasteurisation of spinach juice prior to making CRF powders can cause some losses in nutrient concentration, including total lipids (28-36 %) and lipophilic nutrients such as carotenoids (51-58 %), α-tocopherol (29-71 %), chlorophylls (55-70 %), fatty acids especially α-linolenic acid (24-29 %); pasteurised spinach juice powders seems to retain the nutrients level better than CRFs, and only chlorophylls were significantly reduced after intense pasteurisation.
- The moisture content (below 10%) and water activity (0.17~0.34) in all these dried spinach juice and CRF powders were lower than the parent juice. The lowest moisture content was 3.8 % which found in both MC and IC; and the lowest water activity was 0.17 in IJ. The results suggest all these samples would be

microbiologically safe and would have enhanced shelf stability.

- Fresh spinach juice (FJ) showed higher solubility and dispersibility than CRF (FC). Moreover, the solubility and dispersibility was reduced after both mild and intense pasteurisation in spinach juice and CRF samples.
- Both mild and intense pasteurisation of spinach juice affected the morphology of the subsequent freeze-dried samples. The colour loss and aggregation were observed in all pasteurised spinach samples. This quality degradation was highly depending on the thermal severity of the process, with intense pasteurised juice/CRF powders showed olive brown colour and largest particle size.

In this chapter, thermal pre-treatment together with freeze drying was proved to be a method to produce spinach chloroplast powders. In order to balance the benefits of thermal processing and the quality of food products, mild pasteurisation (70 °C 15s) of spinach juice was recommended. While, in the following chapters, it is still worth to investigate different thermal pre-treatment such as blanching on the quality of spinach juice/CRF powders (Chapter 5). Moreover, the pasteurised spinach juice/CRF was selected as samples for determine the impact of heat processing on digestibility of their nutrients using *in vitro* static digestion model in Chapter 6 and 7.

Chapter V

5. Effects of blanching on properties of freeze-dried spinach juice/CRF powders

5.1. Introduction

Pasteurisation of spinach juice before freeze drying is an efficient method to inactivate endogenous enzymes associated with spoilage, preserves nutrient composition, and prolong shelf life. In addition to pasteurisation, blanching is also used as a practical thermal pretreatment that can be applied on green leafy vegetables before consumption or preparation (freezing, drying or canning). Blanching can be defined as a process of heating vegetables to a desired temperature and maintain for a specific time for the purpose of breaking up enzymes contained in the tissue. The typical temperature and duration time of water blanching is around 70-100 °C less than 10 min (Lin et al., 1995; Njoroge et al., 2015). The benefits of blanching can be the elimination of pathogens, the inactivation of enzymes, preservation of colour, flavour, and nutritional quality. Moreover, the entrapped air and metabolic gases within vegetable cells are removed and replaced by water during blanching, allowing a semicontinuous water phase to form, which promotes more uniform crystal growth during freezing (Aamir et al., 2013). This structure change also increases cell membranes permeability, result in faster drying rate and higher nutrients retention (Severini et al., 2005; Zhu et al., 2021). Blanching can be conducted in several ways, among which hot water and steam are the most traditionally used methods in the food industry. However, food quality is greatly affected by the type and extent of blanching. Water blanching can lead to leaching of

some nutrients (minerals and vitamins), softening vegetable tissues and causing colour loss (chlorophylls and other pigments degradation, Maillard browning) (Severini et al., 2016). Therefore, blanching should be conducted carefully to maximum the benefits of its use to preserve nutrients levels. As mentioned, peroxidases are one of the most heat resistant endogenous enzymes present in vegetables which are associated with food spoilage. Importantly, this class of enzyme can be used as a marker to assess the impacts of blanching on edible plant tissues (Gökmen et al., 2005).

In addition to blanching, drying is a common food processing technique, which is usually applied after blanching to further stabilize vegetables tissues (Mujumdar et al., 2020; Zhu et al., 2021). Freeze drying, as described in Chapter 3 was shown to retain nutrients in both spinach juice and CRF preparations. Indeed, in recent studies, it has been reported that freeze drying of blanched spinach leaves (85 °C 3min) maintained the nutritional content and colour retention of tissues (Joshi et al., 2021).

Therefore, the aims of the current chapter are to determine the impacts of blanching of spinach. To this end, fresh spinach leaves were blanched at 100 °C for 0-90s before juicing, and the physical properties of blanched spinach juice were monitored during refrigerated storage for 7 days. This information was used to determine the optimum blanching condition to preserve spinach juice. The blanched spinach juice/CRF were then freeze dried to stabilize the nutrients composition, to improve quality and extend shelf life.

5.2. Sample preparation summary

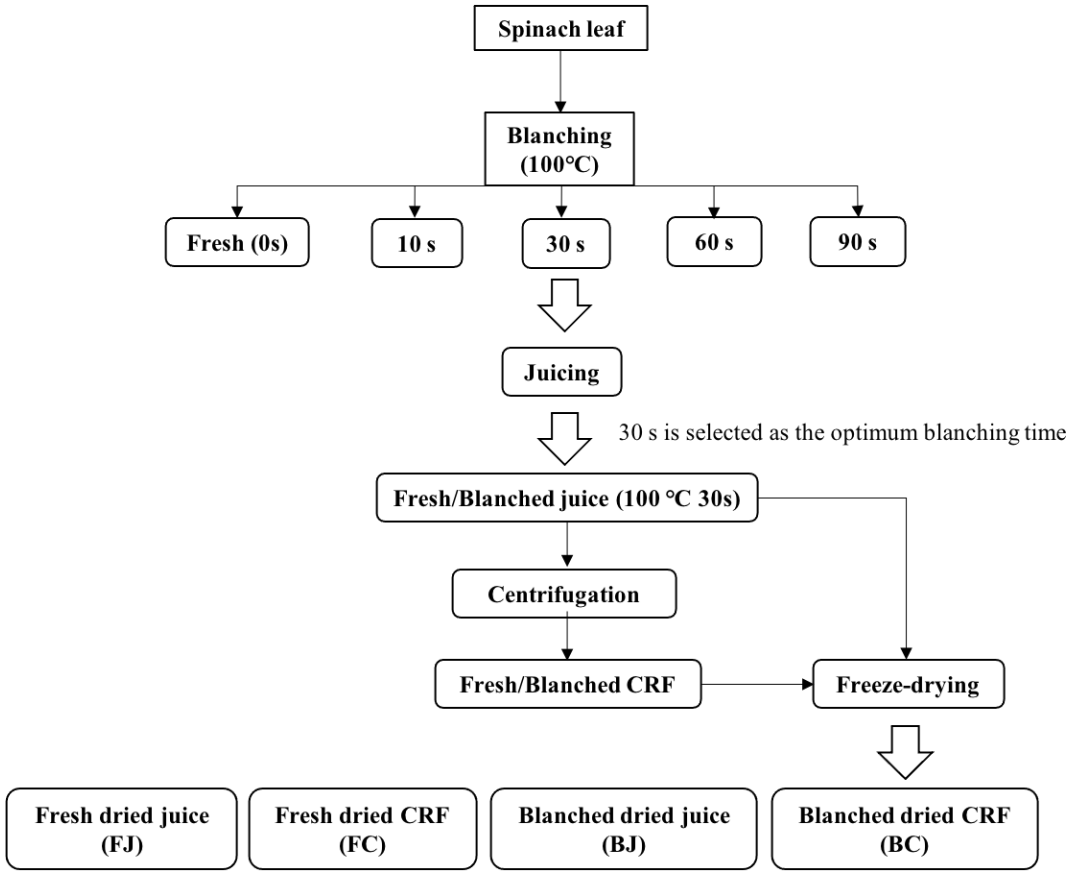


Figure 5.1: Fresh/blanched spinach juice/CRF samples preparation steps

5.3. Results and discussion

5.3.1. Quality changes of fresh and pasteurised spinach juice during refrigerated storage

The blanching process was adopted for use in the inactivation of peroxidase (POD) activity, and for the assessment of the effects of blanching on other quality attributes of spinach samples. In order to balance nutritional quality and acceptable appearance quality of spinach products, spinach leaves were blanched at 100 °C for 0-90s before juicing, the physical properties of fresh/blanched spinach juice were monitored at 4°C for 7 days. The results of this assessment are summarised in Table 5.1.

The pH of fresh/blanched spinach juice was around 6.49-6.96, which indicates a neutral pH. Blanching cause a slightly decrease in pH within 60s heat processing, these changes being time dependent. The changes in pH are associated with the fact that heating causes the release of organic acids that decrease the pH level of the spinach solutions as compared to the fresh samples. These organic acids are further leached out into blanching water after 90s of heating leading to a further decrease in acidity (Arroqui et al., 2001). The increase in pH values were also reported in 'New Zealand spinach' (*Tetragonia expansa* Murr.) blanched at 95-97 °C for 2-3 min and turnip green blanched at 90 °C for 1-2 min (Grzeszczuk et al., 2007; Martínez et al., 2013). According to Severini and colleagues, 47% of the ascorbic acid content in broccoli is reduced after 90s hot water blanching, and the extent of loss in ascorbic acid was associated with blanching time (Severini et al., 2016)

The blanched spinach juice produced a significantly decrease of pH during the 7 days refrigerated storage, reaching values between 5.34-5.81. No significant pH changes were observed in fresh samples during storage under the same conditions. The lower pH values in blanched juice indicated a higher microbially stability than fresh juice during storage (4 °C 7 d). Moreover, the changes in pH also lead to chlorophyll degradation and thus affect the colour of the vegetable samples, higher stability of chlorophylls at alkaline pH levels.

The juice appearance can be seen in Figure 5.2. Browning and turbidity are the major factors which affect quality of fresh and pasteurised spinach juice during storage. Thermal processing is generally applied to improve quality and extend shelf life of juice

products. However, it will also cause the negative effects on the colour and translucency. Indeed, fresh spinach juice had a vibrant green colour and differing changes in the green pigmentation following blanching (Figure 5.2(a)). The loss of green colour (from bright green to olive brown) in the blanched samples could be mainly attributed to pheophytisation, that chlorophyll a and b converted to their corresponding pheophytins under heat treatment (Martins et al., 2002).

It also can be observed that spinach juice became more translucent with increased blanching time. The higher translucency of heated juice maybe due to the inactivation of browning-associated enzymes, and/or reduced phenolic compounds and proteins in the juice (LI et al., 2009).

Meanwhile, the chlorophylls degradation and decrease in translucency also occurs in spinach juice during refrigerated storage. As shown in Figure 5.2(b), the fresh spinach juice still retains a green colour after 7 days of refrigerated storage, due to the low acidic environment (pH 6.07). In contrast, after 7 days storage at 4 °C, the blanched juice tended to show an olive brown colouration under more acidic conditions (pH 5.34-5.81), with the intermediate blanched samples (10-60 s) showing a more brown colour, 90s blanched juice still maintained its green colour. It was reported that high temperature short time (HTST, 150 °C 80s) processing can increase the chlorophyll retention in spinach initially (Gupte et al., 1964). This could explain why spinach leaves blanched at 100°C for 90s in this study is sufficient enough to retain the chlorophylls in juice samples during refrigerated storage within one week. Overall, HTST combined with pH adjustment was proved to have positive impact on chlorophyll retention. The

chlorophyll degradation extent also largely depends on the thermal severity during the processing (Gaur et al., 2007). It is therefore important to investigate the effect of less drastic thermal treatment while maximizing the retention of chlorophyll in thermally processed food.

°Brix values are commonly used to evaluated fruits and vegetables products as it will affect the sensory quality. The brix value of fresh spinach juice was 6.80 with this decreasing to 3.37-4 after blanching. In addition, the brix value was further reduced in all cases over 7 days storage following storage at 4°C. The losses of the soluble solids during blanching could be attributed to the leaching of some sugar into the water. Furthermore, the heat treatment may lead to changes in the physical properties of tissues and therefore result in losses of total soluble solids (TSS) (Quarcoo et al., 2016). Previous research has indicated similar results in turnip greens with 38-50% of TSS reduced after blanching in hot water for 1-2 min (Martínez et al., 2013). In the current work, °Brix values decreased during storage and this could be explained by the microbial metabolic activities that promote sugars in samples to be converted into organic acids. This conversion would decrease the pH of fresh/blanched spinach juice (Khandpur & Gogate, 2016).

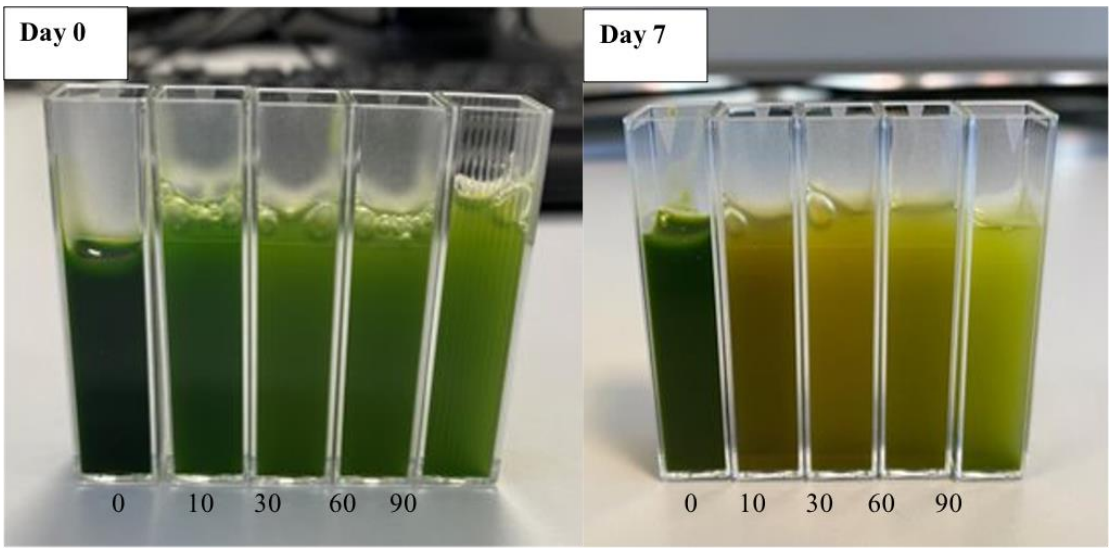
The main purpose of blanching is to destroy endogenous enzymes, which is responsible for food deterioration reactions. As given in Table 5.1, approximately 88.6-98.5% of the POD activity was reduced after thermal blanching for 10-90s. Moreover, there was no significant difference of POD reduction rate after 30s water blanching. Similarly, 90% reduction of POD activity was observed after blanching at 90 °C for 3

min in green beans; 90% of POD inactivation rate ended up in carrots slice when blanching at 70 °C 25 min and 90 °C 1.4 min (Bahçeci et al., 2005; Gonçalves et al., 2010). These observations indicated that inactivation of POD is subjected to blanching temperature and duration time. Therefore, 100 °C 30s as high temperature short time (HTST) blanching condition was identified as the optimum condition in this study to reach a 95.3% reduction of POD activity in spinach leaves. In addition, blanched spinach juice/CRF obtained at this condition (100 °C 30s) was further freeze dried and the physical-chemical properties of these samples were evaluated and discussed in following studies.

Table 5.1: Physicochemical changes of fresh and blanched spinach juice during refrigerated storage

Blanching time (s)	pH		°Brix		reduction of POD activity/%
	Day 0	Day 7	Day 0	Day 7	Day 0
0	6.84 ± 0.05^{ab}	6.51 ± 0.07^{a}	6.80 ± 0.00^{a}	6.07 ± 0.06^{a}	0.00 ± 0.00^{c}
10	6.49 ± 0.03^{d}	5.34 ± 0.07^{c}	4.00 ± 0.06^{b}	3.63 ± 0.12^{b}	88.6 ± 3.9^{b}
30	6.57 ± 0.03^{cd}	5.52 ± 0.16^{bc}	3.70 ± 0.30^{bc}	3.43 ± 0.06^{c}	95.3 ± 1.5^{a}
60	6.71 ± 0.06^{bc}	5.81 ± 0.16^{b}	3.53 ± 0.06^{c}	3.13 ± 0.06^{d}	$95.3\pm0.\ 8^{a}$
90	6.96 ± 0.07^{a}	5.76 ± 0.03^{b}	3.37 ± 0.06^{c}	2.73 ± 0.06^{e}	98.5 ± 0.0^{a}

Figure 5.2: The effect of blanching time on visualized colour difference of spinach juice during storage (4°C 7 days). Spinach juice was obtained after blanching leaves at 100°C for 0-90s.



5.3.2.Morphology of spinach samples

5.3.2.1. Transmission electron microscope (TEM) images of fresh/blanched spinach leaves

TEM was used to determine the ultrastructure changes of spinach leaves before and after the blanching process (100°C 30s) (Figure 5.3).

In blanched spinach leaves there was an obvious collapse of cells as shown in Figure 5.3 d, f and h as compared to fresh spinach leaves (Figure 5.3 c, e and g). In fresh leaves, the intact structures of chloroplasts could be clearly seen, with smooth and defined cell walls, thylakoids, with assembled stacks of grana and plastoglobuli. Chloroplasts in fresh leaves maintained their integrity with some lens shaped chloroplasts (3-10µm in diameter and 1-3µm thick), around the edges of cell, or slightly twisted bands at the cell edges (Burrows et al., 2020). Following blanching, it can be clearly seen that the integrity of the chloroplast fine ultra-structures was largely destroyed, the thylakoids were collapsed and the plastids were dispersed into the intercellular space leaving cell contents indistinguishable and disorganised. Loss of

structural integrity accounts for the low ability of the water blanched samples to retain water during rehydration (Okpala et al., 2014).

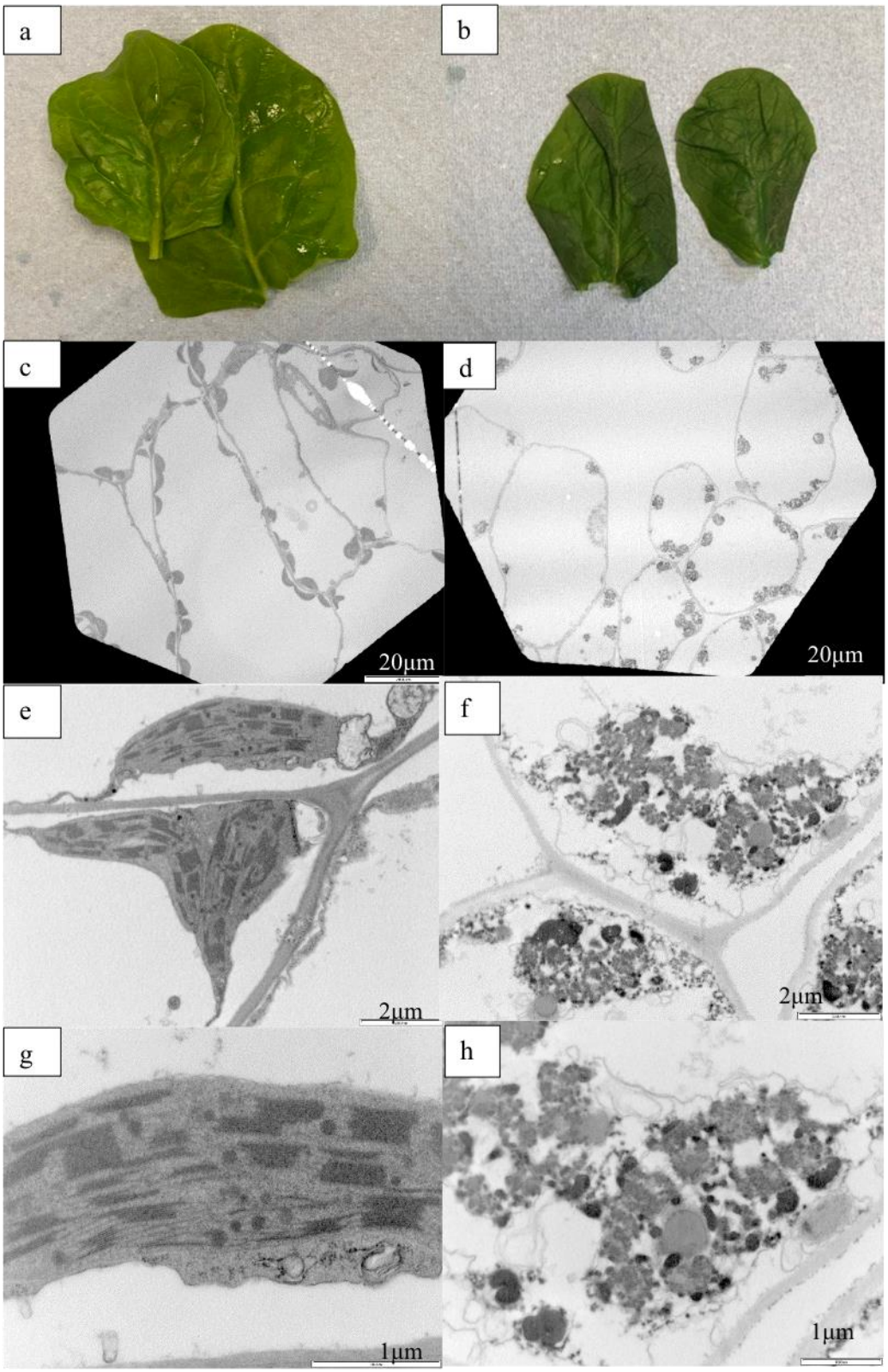


Figure 5.3: TEM images of fresh/blanched spinach leaves at different magnifications

a) Fresh spinach leaves, b) blanched spinach leaves (100 °C 30s), c, e, g) were fresh

leaves at magnification of 800×, 8200× 27000×; d, f, h) were blanched leaves at magnification of 800×, 8200× 27000×.

5.3.2.2. **Microscopic images of fresh/blanched spinach juice/CRF powders**

Figure 5.4 shows the comparison of freeze-dried fresh and blanched (100 °C 30s) spinach samples after grinding and sieving. It can be seen from the image that the samples showed different colour intensities. The green colour comes from the chlorophylls contained in chloroplasts, with the dark green colour of FC samples being associated with a higher concentration of chloroplasts as compared with FJ. The bright green colour of fresh spinach samples turned to olive-green or olive yellow in blanched samples which was ultimately considered as a loss of quality. This loss of greenness was mainly due to chlorophyll degradation or diffusion during blanching treatment. In general, we expect that the chlorophylls were converted into their corresponding pheophytins by losing the magnesium ion in the porphyrin ring when heated (Minguez-Mosquera et al., 1989).

The light microscopic images are present in Figure 5.5 and reveal the microstructural changes in spinach chloroplasts following blanching. All the dried powdered juice/CRF samples were hydrated in RO water before loading for observation, and then analysed using a light microscope with the polarised lens. Therefore, the colour differences between fresh and blanched samples under light microscope was not obvious as shown in Figure 5.4. Comparing with FJ and FC, blanched chloroplasts (BJ and BC) tend to form clumps due to protein denaturation and/or aggregation that occurs

during blanching. Both fresh and blanched CRF showed a higher intensity of chloroplasts as compared with dried juice. The integrity of chloroplasts may vary in all samples as showed in Figure 5.5, and blanched samples appear to contain the most damaged structures.

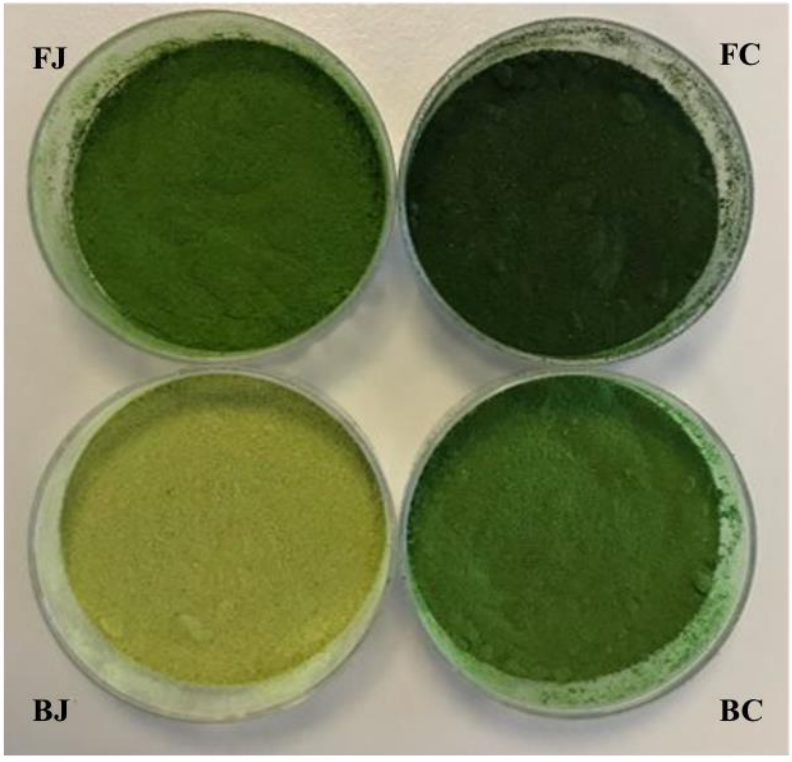


Figure 5.4: Freeze dried fresh/blanched spinach chloroplasts. All samples were frozen at -80 °C overnight and freeze-dried for 7 days. Samples were grinded and passed through 250 μm sieve. FJ Fresh spinach juice; FC Fresh spinach CRF; BJ Blanched spinach juice; BC Blanched spinach CRF.

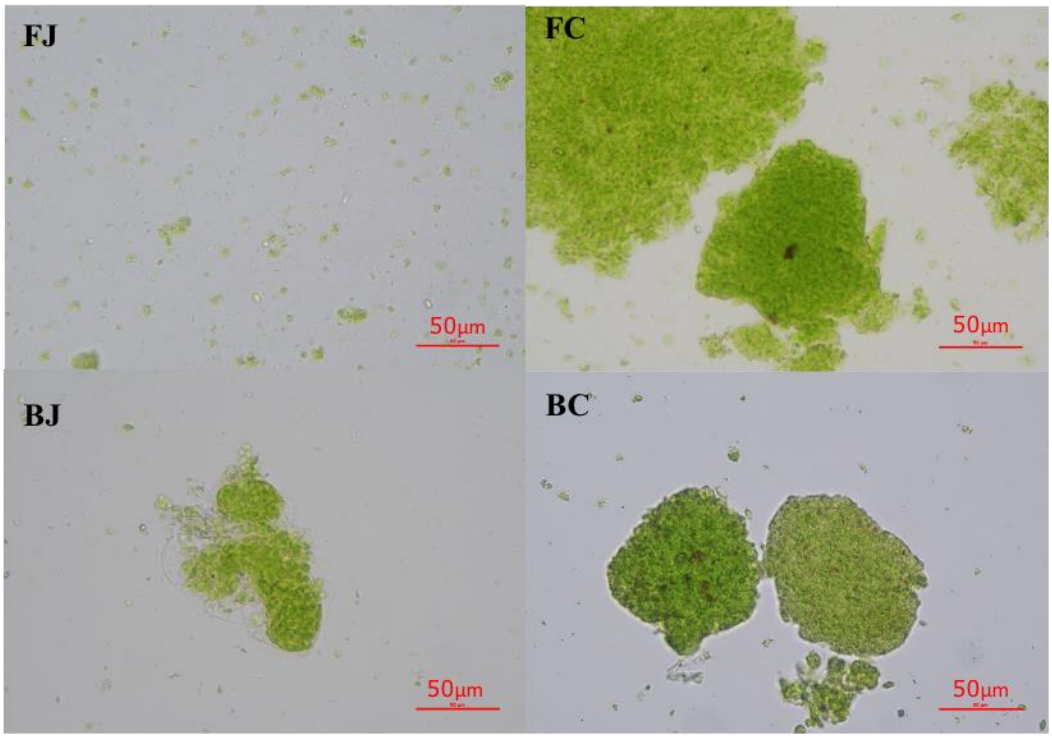


Figure 5.5: Light microscope images at X400 total magnification of freeze-dried fresh/blanched spinach juice and CRFs. The dried powders were diluted (X100) with ultra-pure water before imaging. FJ Fresh spinach juice; FC Fresh spinach CRF; BJ Blanched spinach juice; BC Blanched spinach CRF.

5.3.2.3. Scanning electron microscope (SEM) images of fresh/blanched spinach juice/CRF powders

The impact of blanching on the morphology of freeze-dried spinach tissues is given in Figure 5.6. All the samples showed typical morphology of freeze-dried powders with irregular flake shaped particles with porous structure (Chranioti et al., 2016). Compared with unblanched samples, BJ exhibited a smoother surface than FJ, while BC showed increased porosity than FC. Blanching destroyed the cell wall integrity (Figure 5.3), and the structural changes may result in increased porosity.

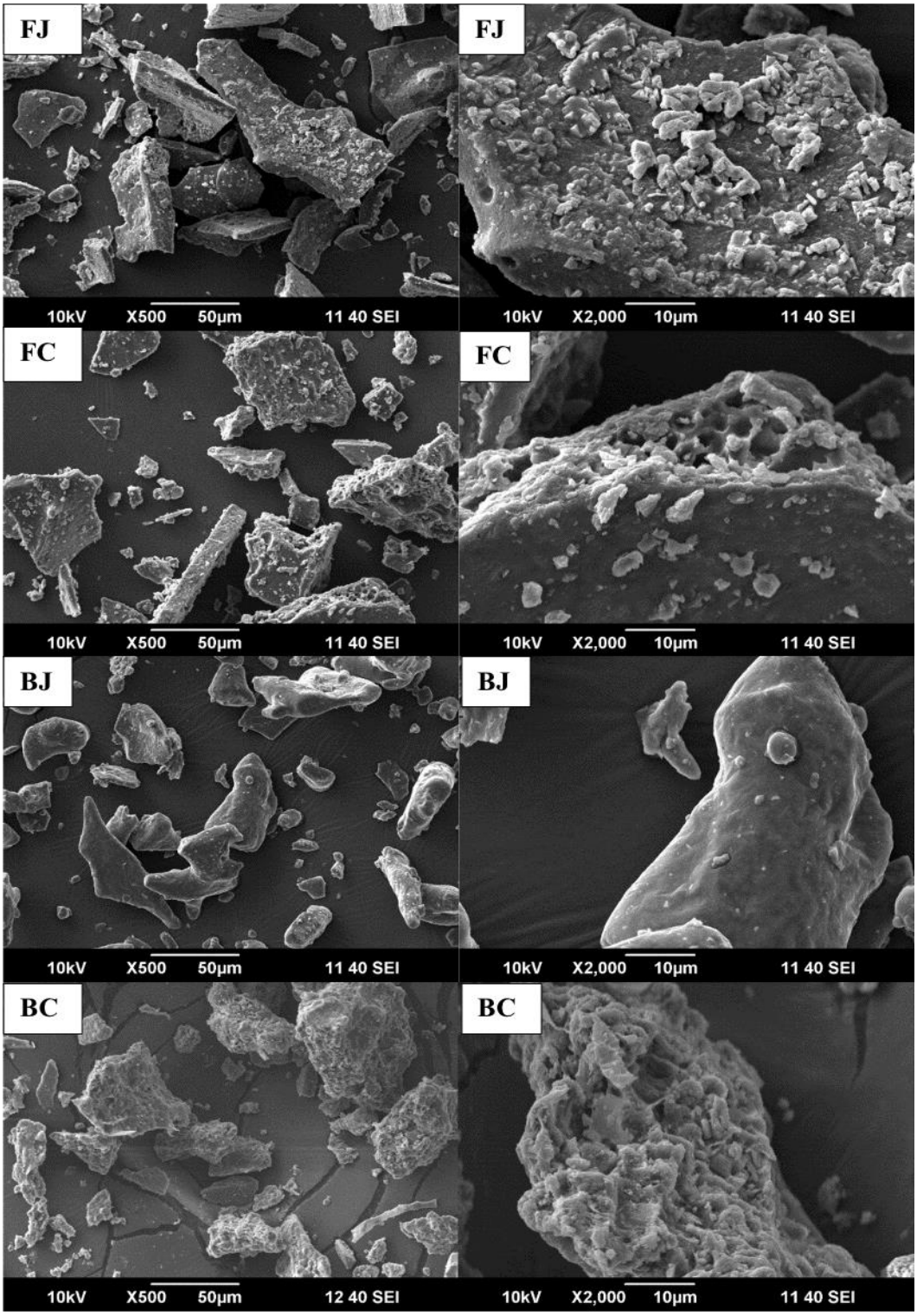


Figure 5.6: SEM images of fresh/blanched spinach juice and CRF samples at X500 and X2000 magnification. FJ Fresh spinach juice; FC Fresh spinach CRF; BJ Blanched spinach juice; BC Blanched spinach CRF.

5.3.3. Macronutrients of fresh/blanched spinach juice/CRF powders

The macronutrient composition of fresh/blanched (100 °C for 30s) spinach chloroplasts powders are documented in Table 5.2. Proteins made up the majority of the spinach CRF samples (FC and BC), whereas estimated carbohydrates made up the majority of the spinach juice samples (FJ and BJ). Significantly decreased proteins (11.9%) and lipids (8.6%) were observed in blanched spinach juice compared with fresh juice; while only significantly reduced proportion of lipids (9%) were found in CRF isolated from blanched spinach leaves (BC). Generally, blanching reduces the nutritional quality of vegetables by causing the leaching or diffusion of important nutrients like minerals (Mukherjee et al., 2007). During blanching the protein-lipids complex are destroyed and protein denaturation occurs. Therefore, a higher proportion of soluble proteins and lipids leached out from plant tissue into the blanching water. Moreover, the loss of lipids could be also due to the loss of membrane lipids such as galactolipids and phospholipids, and some lipophilic nutrients caused by high temperature treatments (Murcia et al., 1999). In addition, the relatively stable protein concentration remained in BC was due to the physical separation that the denatured proteins were all collected in pellet after centrifugation of juice, and lipids phase no longer bond to proteins were discarded in supernatant. The ash (minerals) is not destroyed by light, heat or oxygen and thus being relatively stable maintained during blanching (Bernhardt et al., 2006). In general, blanching may decrease the nutritional

quality of spinach juice/CRF, which is linked to the cellular structure modification and destruction of nutrients at the higher temperatures used during blanching (Philippon, 1984)

Table 5.2: Nutrients composition of fresh/blanched spinach chloroplasts

%DW	FJ	FC	BJ	BC
Proteins	34.2 ± 0.9^{b}	48.3 ± 0.7^{a}	22.3 ± 0.2^{c}	49.1 ± 1.1^{a}
Lipids	11.3 ± 0.8^{c}	27.9 ± 1.3^{a}	2.7 ± 0.4^{d}	18.9 ± 1.3^{b}
Ash	16.8 ± 0.9^{b}	7.4 ± 0.4^{c}	22.8 ± 0.4^{a}	7.5 ± 0.2^{c}
Carbohydrates	37.7	16.4	52.2	24.5

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF.*

5.3.4. Micronutrients of fresh/blanched spinach juice/CRF powders

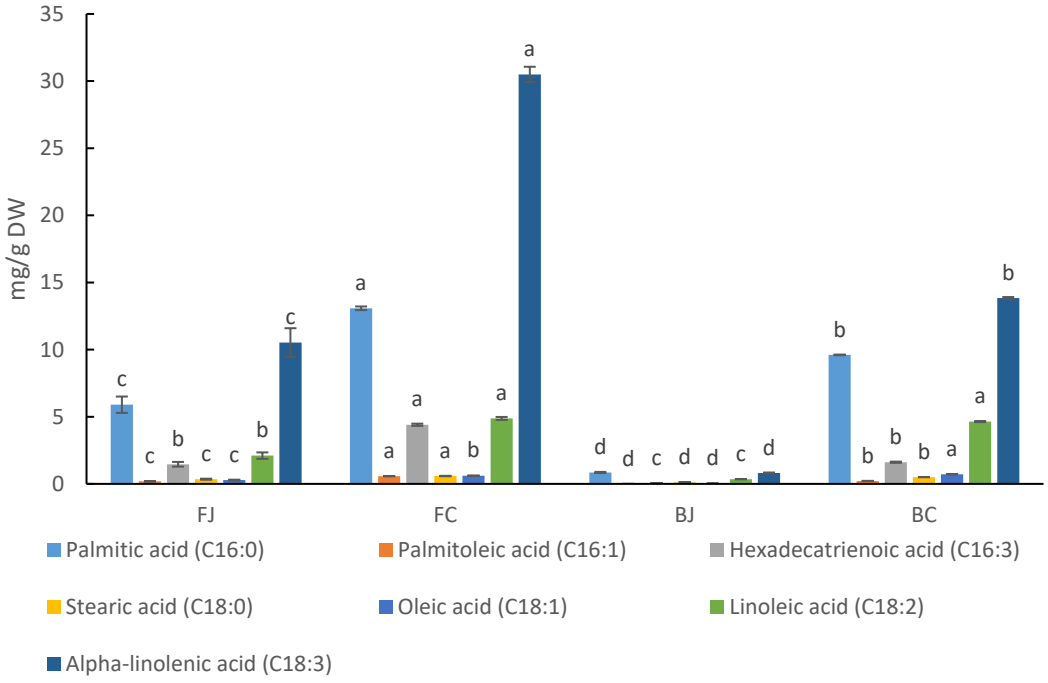
5.3.4.1. Fatty acids composition of fresh/blanched spinach juice/CRF powders

Lipids are most elemental nutrients for humans and fatty acids are building blocks of lipids. The composition of fatty acids (FAs) of fresh/blanched spinach juice/CRF is shown in Figure 5.7 and expressed as mg/g DW and mg/g total lipids. As can be seen, the primary fatty acids identified in all samples were palmitic acid (C16:0), palmitoleic acid (C16:1), hexadecatrienoic acid (C16:3), stearic acid (C18:0), oleic acid (C18:1),

linoleic acid (C18:2) and α-linolenic acid (C18:3). Among which α-linolenic acid (ALA) is the most predominant fatty acid, followed by palmitic acid and linoleic acid. It was established that on a dry mass basis blanching significantly decreased fatty acid levels in both spinach juice and CRFs; the loss of fatty acids was more intense in dried spinach juice compared with CRFs. The levels of the detected FAs in FJ were in the range of 0.19-10.52 mg/g DW and 1mg/g DW in BJ. As for CRFs, the main loss of fatty acid is ALA which showed a 55% decrease than in fresh CRFs on a dry mass and lipid mass basis. The rapid loss of FAs could be attributed to the loss of total lipids, especially membrane lipids, lost during the blanching process. Spinach juice suffered major losses of total lipids as compared to CRFs. On a lipid mass basis, we detected an increase in C16:0, C18:0, C18:1 and C18:2 FA types after blanching, which is probably due to the increased extraction efficiency of FAs in blanched CRFs.

The ratio of unsaturated to saturated fatty acids (UFA/SFA) is considered as an oil degradation indicator (Romano et al., 2012). The ratio of UFA/SFA in fresh juice and CRF was 2.3 and 3.0, which was decreased to 1.3 and 2.1 after blanching. This reduction suggests a decline in the nutritive value of acyl fatty acids of the chloroplast envelope membrane in blanched spinach leaves, which is mainly ALA (C18:3) and palmitic acid (C16:0). Similar results were also found in canned broccoli were the UFA/SFA ratio was reduced to 3.5 from 4.2 (Murcia et al., 1999). Furthermore, linoleic acid is a n-6 polyunsaturated fatty acid (PUFA) and α-linolenic acid is a n-3 PUFA; they are considered as two plant derived essential fatty acids in diet as they cannot be synthesised by human body (Blondeau et al., 2015). The intake of increased level of

ratio of n-3/n-6 fatty acid is associated with lower risk of cancer and chronic diseases (Goodstine et al., 2003; Wijendran et al., 2004). The ratio of n-3/n-6 in fresh spinach juice/CRFs were 5.0 and 6.3, and this was reduced to 2.3 and 3.0 after blanching treatment. Consequently, blanching spinach leaves may cause the negative effect on fatty acid composition and their functional properties.



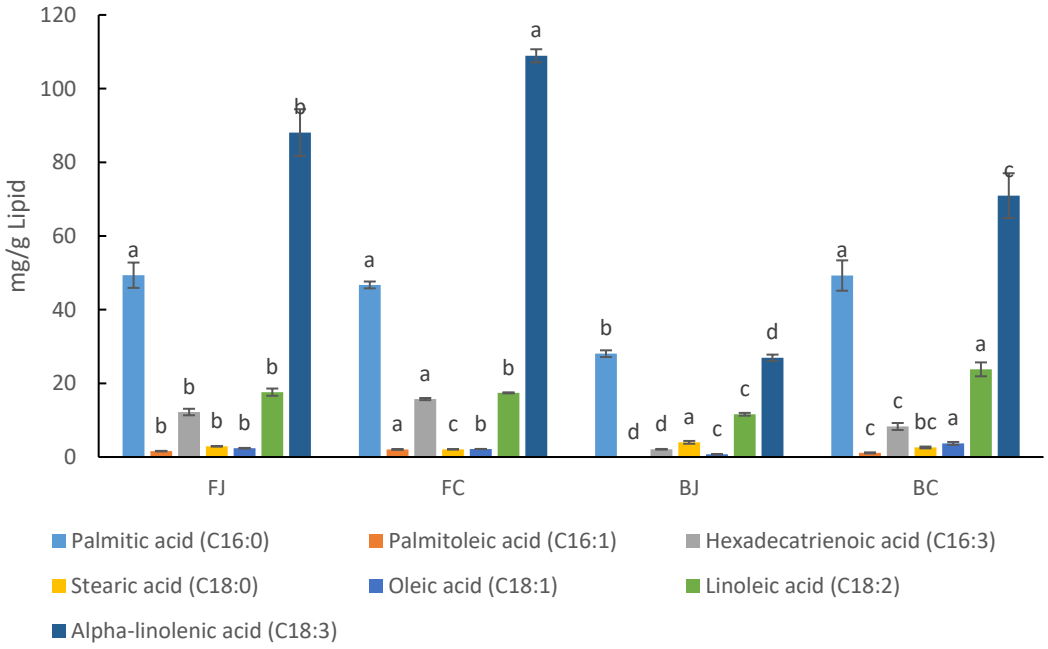


Figure 5.7: Fatty acid composition of fresh/blanched spinach juice and CRF. **a)** mg/g DW **b)** mg/g lipid. Results are expressed as means ± SD (n=3). Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF.*

5.3.4.2. Chlorophyll content of fresh/blanched spinach juice/CRF powders

Colour is considered an important property in the food industry. Chlorophylls are the most abundant pigments in plant tissues, and is heat sensitive (Ghosh et al., 2022). Chlorophylls are an indicator for chloroplasts content and food quality, especially in food items that have been thermally processed. The content of chlorophyll a, b and total chlorophylls in fresh/blanched dried spinach juice and CRF is shown in Figure 5.8 and expressed in mg per g of spinach chloroplasts (DW) and mg per g of total lipids

extracted from spinach chloroplasts. The higher chlorophylls content was observed in fresh juice and CRF samples (FJ and FC) compared with blanched samples, whereas chlorophyll a was higher than chlorophyll b in all samples. The total chlorophylls content decreased after blanching (100 °C at 30s) both in juice and CRF samples. Approximately 93% and 59% of the total chlorophylls were lost in BJ and BC samples on a dry weight basis respectively. In contrast 68.5% and 44% loss on a lipid biomass basis. Researchers have reported that the quality changes of green plants occurring during thermal processing could be attributed to the conversion of chlorophyll a and b to their respective pheophytins, and this was associated with the transition in colour loss from bright green to olive brown (Steet et al., 1996; Teng et al., 1999). The loss of total chlorophylls could be also linked to the loss of total lipids since they are chlorophylls are lipophilic components. The higher loss of chlorophylls and total lipids detected in blanched juice than CRF supports this observation. Previous research has reported that chlorophyll degradation depends on blanching conditions (heating temperature, time and pH) (Van Loey et al., 1998; Rudra et al., 2008). In order to minimise the green colour loss and produce high quality vegetable products, high temperature short time (HTST) blanching combined with pH adjustment is recommended (Belitz et al., 2008). Alkaline pH adjusted with the addition of $MgCO_3$ improves chlorophyll retention in spinach puree when combined with HTST processing (Gupte et al., 1964).

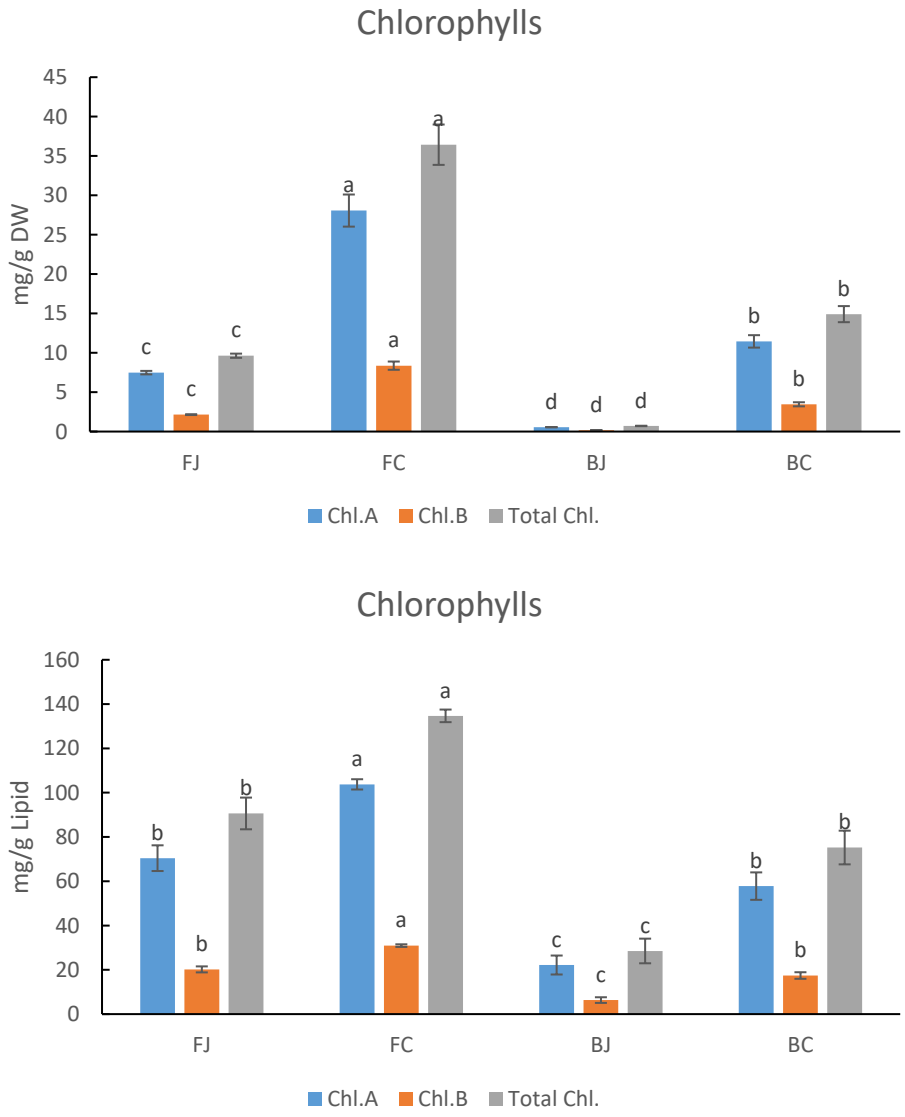
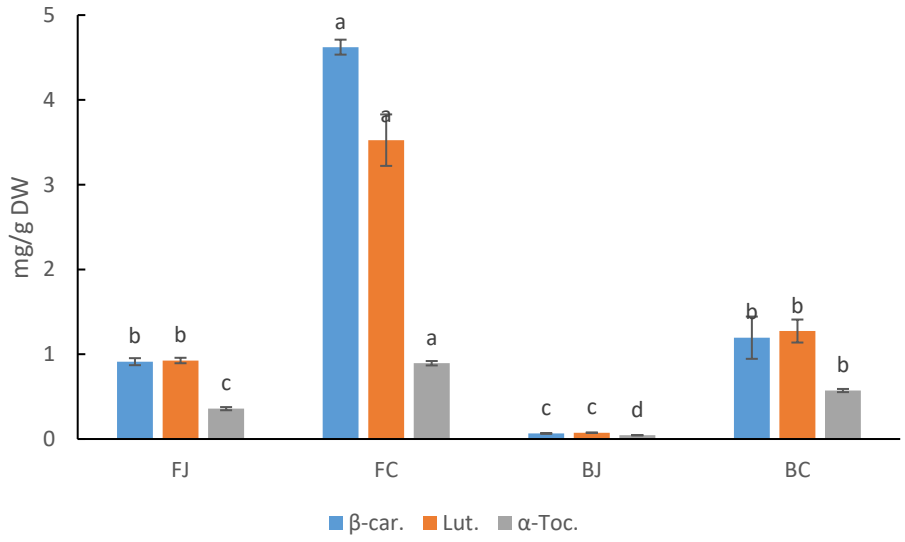


Figure 5.8: Chlorophylls content of fresh/blanched spinach juice and CRF. **a)** mg/g DW **b)** mg/g lipid. Results are expressed as means ± SD (n=3). Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF. Chl.A Chlorophyll a; Chl.B Chlorophyll b; Total Chl. Total Chlorophylls (a+b)*

5.3.4.3. Carotenoids and vitamin E content of fresh/blanched spinach juice/CRF powders

Carotenoids are essential pigments present in fruits and vegetables occurring alongside chlorophylls, that are responsible yellow to red pigmentation (Maoka, 2020). The carotenoid content in fresh/blanched spinach juice and CRF is shown in Figure 5.9, and data are expressed as mg/g DW and mg/g of total lipids. β-carotene and lutein are the predominant carotenoids in spinach, and both occur at greater concentrations in fresh juice/CRF samples than in the blanched samples. Approximately 92% and 69% of the total carotenoid content (beta-carotene and lutein) were decreased in blanched juice and CRF samples on a dry mass basis, whereas 68% and 58% loss on lipid biomass basis. Carotenoid pigments are sensitive to high temperature during blanching, and this can cause isomerisation of carotenoids from trans-isomer to cis-isomers (Lu et al., 2020). In the current work, carotenoid loss was possibly attributed to leaching out into water during blanching and/or degradation during heat treatment. Similar losses are reported in the blanched leaves of both *Hypochaeris* and *Hyoseris*, in which the blanching water had a significantly higher ($p < 0.05$) carotenoid content than the blanched leaves (Sicari et al., 2020). Moreover, the behaviour of carotenoids during thermal processing depends on the food matrix, since evidence shows that some carotenoids like those in carrot are more stable than that of spinach and broccoli (Kopas-Lane et al., 1995; Zhang et al., 2004; Lešková et al., 2006). This should be taken into account that carotenoid stability varies in different types of vegetables due to their different physical state and location in food matrix (Schieber et al., 2005).

Vitamin E was also assessed in the current work. Vitamin E consists of four tocopherols (α-, β-, γ-, and δ-) and the corresponding tocotrienols (α-, β-, γ-, and δ-) which contain unsaturated side chains. α-Tocopherol is considered one of the most important biological antioxidants in cell membranes (Seppanen et al., 2010). Lower levels of α-tocopherol were observed in this work with diminished levels occurring in blanched samples (0.04-0.57 mg/g) compared to fresh ones (0.36-0.89 mg/g) on dry mass basis. As highlighted above, the loss of α-tocopherol could possibility be due to leaching. α-tocopherol seems retained stable in blanched CRF (BC) when normalized to lipid biomass content. The level of α-tocopherol in raw and heat processed vegetables varies in different cases. As reported, higher vitamin E levels are present in boiled broccoli, compared to raw products (Chun et al., 2006).



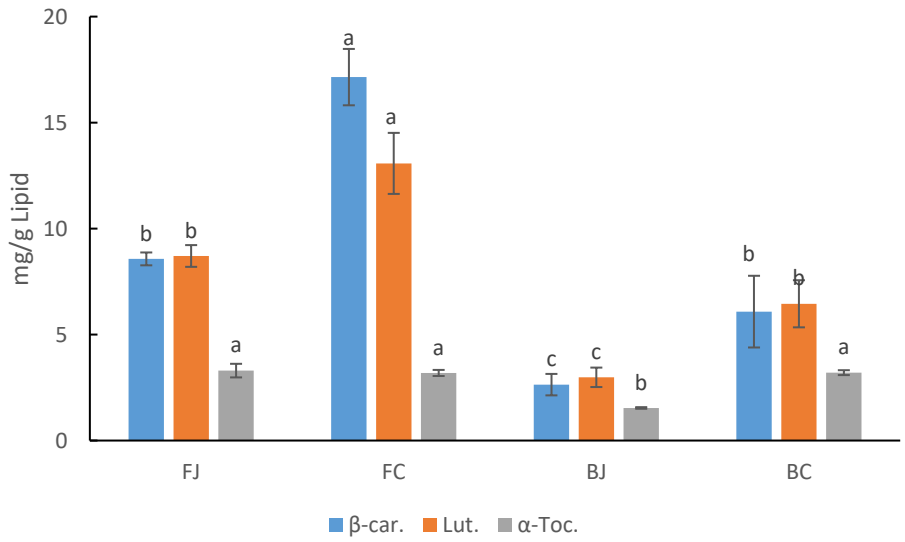


Figure 5.9: Carotenoids and α-tocopherol content of fresh/blanched spinach juice and CRF. **a)** mg/g DW **b)** mg/g lipid. Results are expressed as means ± SD (n=3). Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF. β-car. β-carotene; Lut. Lutein; α-Toc. α-tocopherol.*

5.3.4.4. Galactolipids and free fatty acid content of fresh/blanched spinach juice/CRF powders

The galactolipids are predominant lipids in the thylakoid membrane of plants, and characterised as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) compounds that represent up to 80% of the total lipids in some plants (Block et al., 1983). MGDG and DGDG play major roles in determining the physicochemical properties of the thylakoid membrane, and is responsible for the photosynthetic electron transfer reactions, and stabilization of the

photosystem protein complexes in chloroplasts (Kobayashi, 2016). It appears that maintaining a constant MGDG/DGDG ratio in thylakoid membranes is crucial for the stability and functional integrity of photosynthetic membranes (Rocha et al., 2018). The impact of blanching on galactolipids content and free fatty acid release in dried spinach juice/CRF are shown in Figure 5.10 and Table 5.3. The results are expressed as mg/g of total lipids extracted from dried spinach juice/CRF samples. On average, 0.1g of dried fresh samples contains lipids around 0.0122-0.0297 g, as for blanched samples, lipids content was dropped to 0.0026-0.0185 g (Table 7.3). The blanched spinach juice and CRF (BJ and BC) showed higher MGDG content than DGDG with a ratio of 1.3-2, which is in agreement with a higher proportion of MGDG (50%) than DGDG (30%) in thylakoid membranes, especially in the inner envelope membrane (Seiwert et al., 2017). While the galactolipids in FC showed a different trend, the ratio of MGDG/DGDG was 0.6:1, with higher DGDG being found than MGDG. It seems that MGDG could be degraded by endogenous galactolipase more rapidly than that of DGDG. This has been observed in other reports, in that MGDG disappeared faster than DGDG in barley leaves and tomato leaves (Michalski et al., 1980; Gut et al., 1988). It could be confirmed by the significant high released of free fatty acid (FFA) like α-linolenic acid (ALA) in FC, with the value of 96.91 mg/g lipid. While low levels of FFA (ALA) were found in FJ, BJ and BC with the values ranging from 0-0.02 mg/g lipid. The low amounts of FFA in blanched samples suggests that spinach leaves avoid lipolysis with inactivated endogenous enzymes. This observation is supported by previous reports, that spinach leaves blanched at 100 °C for 3 min prior to storage at

37 °C was able to abolish galactolipase and sulfolipase activities (Hirayama et al., 1969). In contrast, greater amount of ALA occurred in FC as compared with FJ confirmed significant lipolysis occurring during CRF preparation. The fresh spinach CRF were collected by centrifugation of the spinach juice for 10 min at 4°C, (10,000 rpm for 3 times), prior to being frozen at -80°C and subsequently freeze dried. Whereas spinach juice was directly frozen at -80°C before freeze drying. Consequently, this possibly explains the high level of ALA accumulated in FC could originate from the hydrolysis started by activated/liberated endogenous enzymes during fresh CRF preparation (Kaniuga, 2008). As can be seen from Figure 5.10 (a), bands for acyl-MGDG and MGMG with higher intensity was observed in FC. It is known that MGDG can be acylated with a third fatty acid to the galactose head group form acyl-MGDG in plant leaf homogenates (Persson et al., 2000; Nilsson et al., 2015). This enzymatic fatty acid exchange was inactivated by blanching spinach leaves in this study. Moreover, the galactolipids from non-heated samples were more easily to hydrolyse by endogenous enzymes compared with heated samples, thus the MGDG was hydrolysed to MGMG and release FFA in FC samples. Similar results have been in fresh/steam sterilised pea vine CRF, that 3-fold lower FFA and 1.8-3.4 fold higher of MGDG and DGDG levels found in steam sterilised samples (Wattanakul et al., 2022).

In addition, significantly reduced galactolipids content was observed in BJ (44.07 mg/g of MGDG and 22.32 mg/g DGDG) compared with FJ (77.26 mg/g of MGDG and 75.64 mg/g DGDG). This decrease of galactolipids in dried spinach juice could be explained by the loss of total lipids during blanching and possible interaction of the

sugar moiety in galactolipids with blanching water (Cho et al., 2001).

In general, blanching spinach leaves prior to juicing can inactivate endogenous enzymes activity, prevent galactolipids enzymatic change and degradation. While the blanching treatment can also cause significant loss of galactolipids in fresh spinach samples by leaching into hot water.

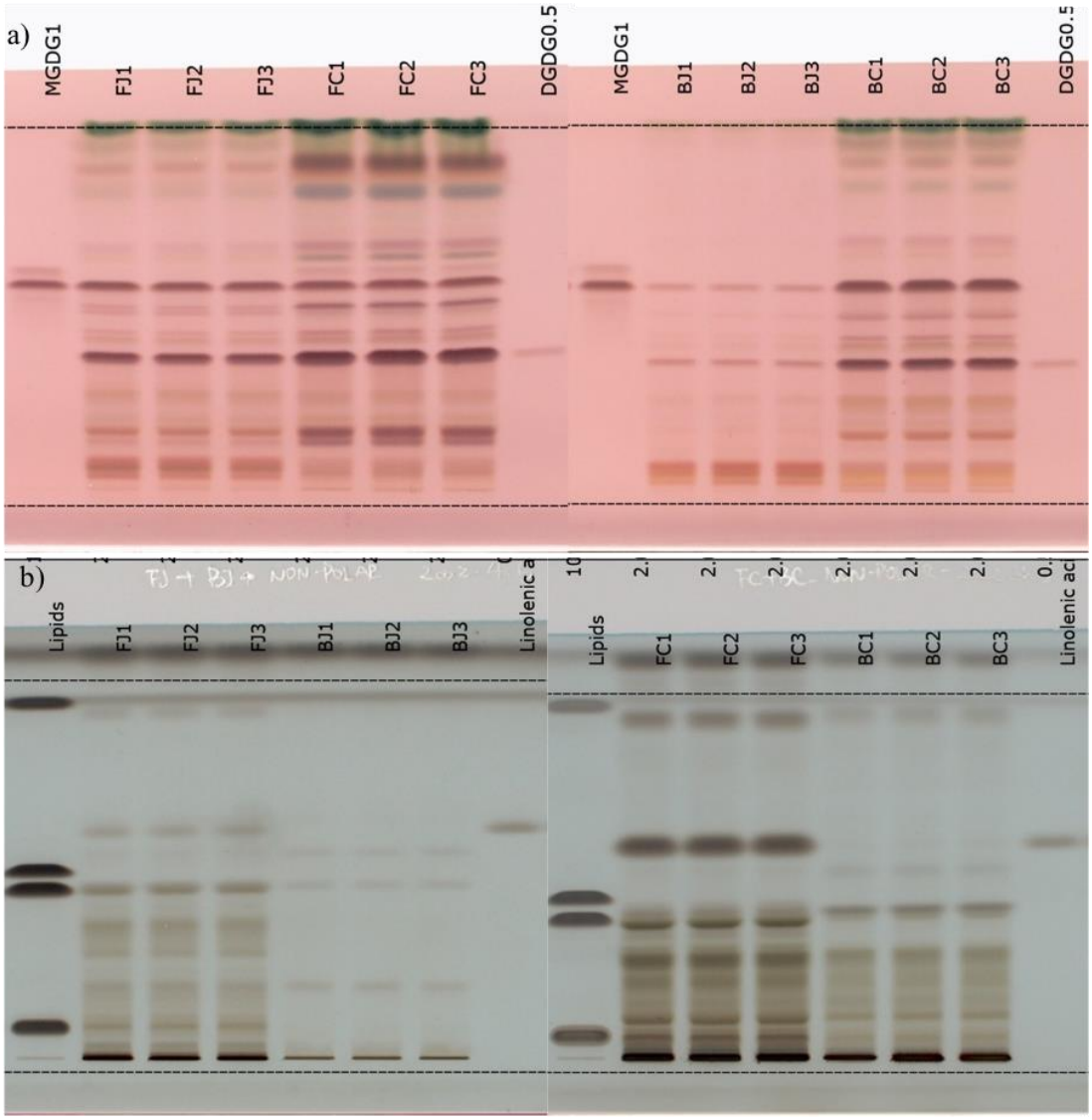


Figure 5.10: HPTLC analysis of lipid extracts from freeze-dried fresh/blanched spinach juice and CRF. Polar lipid separation was visualised in a) and non-polar lipid separation was visualised in b). As reference standards, pure MGDG, DGDG and α-

linolenic acid (ALA) were used. *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF.*

Table 5.3: Galactolipids and free fatty acid contents in freeze-dried fresh/blanched spinach juice and CRF.

mg/g Lipid	MGDG	DGDG	FFA(ALA)	Dried lipid (mg/g DW)
FJ	77.26 ± 3.30^{b}	75.64 ± 5.82^{a}	0.02 ± 0.00^{b}	122 ± 7^{c}
FC	42.33 ± 1.83^{c}	76.01 ± 5.36^{a}	96.91 ± 7.02^{a}	297 ± 18^{a}
BJ	44.07 ± 7.49^{c}	22.32 ± 5.04^{b}	0.00 ± 0.00^{b}	26 ± 6^{d}
BC	91.25 ± 3.12^{a}	67.77 ± 3.35^{a}	0.00 ± 0.00^{b}	185 ± 3^{b}

The concentration of galactolipids were expressed as mg/g of lipid extracted from dried samples. The dried lipid weight extracted from freeze-dried spinach juice/CRF is shown in the table and expressed as mg/g DW. Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF.*

5.3.5.Physical properties of fresh/blanched spinach juice/CRF powders

Table 5.4 provides some physical properties of freeze dried fresh/blanched spinach juice and CRF samples. Freeze drying was found to efficiently reduce the moisture

content and water activity in food products, decreases the perishability, improves value and enhances the shelf-life (Agoreyo et al., 2011). It is noted that all samples showed a low moisture content and water activity with the value in range of 1.71-8.05% and 0.11-0.21 respectively. The recommended moisture content and water activity for food powder products is below 10% and 0.3, our results suggest a guaranteed microbiological safety of spinach samples during storage (Tze et al., 2012b).

The solubility is defined as the maximum amounts of substance dissolved in a given amount of water at specific temperature, it plays a key role in manufacturing food powder products as ingredient for consumers with desired functionalities (Fang et al., 2007). Higher solubility was observed in juice samples (75.70-82.84%) as compared with CRFs (11.32-30.94%). The juice is more soluble than CRF, as the CRF preparation steps was to collect the pellet by centrifuging juice and discard the supernatant, and thus contains higher lipids and proteins.

In addition, blanching pretreatment seems to increase the solubility in juice samples and decrease in CRFs. Solubility might be affected by several factors, such as particle microstructure, particle size and physical-chemical properties of substances (Anandharamakrishnan et al., 2010; Kaptay, 2012). In this study, the higher solubility of dried juice samples was observed compared with CRF samples. Moreover, the blanching process of spinach juice seemed to have increased the solubility of dried juice samples. This is associated with the soluble ash and carbohydrates ratio presented in samples, which are highest in BJ, followed by FJ, BC and FC (data shown in Table 5.2). As for dried CRFs, significantly reduced solubility may possibility be affected by

protein denaturation, and/or aggregation or other structural changes along with increased particle size during blanching and high lipid percentage (Table 5.2), (Borremans et al., 2020). Similarly, water blanching and steam blanching led to significantly reduced solubility in dried cauliflower leaf compared with unblanched samples (Mythili et al., 2021). As reported by Baldelli et al. (2022), powders with high-fat content showed large agglomerates, furthermore, an increase in 10% of fat percentage increased 60 μm of the mean diameter. The relationship between particle size and solubility is that solubility increased with decreased particle size due to enlarged surface area (Sun et al., 2012).

The dispersibility of food powder is dependent on its ability to disperse in solution, this index is important in industrial settings (Sharma et al., 2012). The dispersibility of fresh spinach samples was 85.08-82.14%, which reduced to 64.18-79.39% in blanched samples, all the samples showed a good dispersibility within the range of 67.05–99.98% which is ideal dispersibility for food powders (Jaya et al., 2004). Similarly, dispersibility also related to particle size and the presence of fat, increased particle size and free fat content in the powder further reduces the dispersibility. In this consequence, protein denaturation and/or aggregation or other structural changes during blanching leads to increased particle size in blanched samples and thus reduce the dispersibility (particle size data were not shown). Moreover, lower dispersibility in blanched samples could be linked to their fat percentage (date shown in Table 5.2).

Table 5.4: Physical properties of freeze dried fresh/blanched spinach juice and CRF

Properties	FJ	FC	BJ	BC
Moisture content/%	8.05±0.65^{a}	3.87±1.37^{b}	6.28±0.38^{a}	1.71±0.37^{c}
Water activity	0.12±0.01^{c}	0.18±0.00^{b}	0.11±0.00^{c}	0.21±0.00^{a}
Solubility/%	75.70±1.18^{b}	30.94±0.74^{c}	82.84±17.6^{a}	11.32±0.02^{d}
Dispersibility/%	85.08±0.29^{a}	79.39±3.16^{b}	82.14±2.05^{ab}	64.18±0.62^{c}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). *Abbreviations: FJ Fresh dried juice; FC Fresh dried CRF; BJ Blanched dried juice; BC Blanched dried CRF.*

5.4. Conclusion

A combination of blanching and freezing has been established as the most suitable preservation method for vegetables, therefore, blanching spinach leaves prior to freeze drying was discussed in this chapter in order to make the spinach chloroplast products with minimum quality loss and enhanced nutrients stability.

Overall, blanching spinach leaves (100 °C 30s) before juicing can efficiently inactivate the endogenous enzymes (POD), preventing hydrolysis of galactolipids (MGDG and DGDG) and release of FFA in spinach juice. The blanched juice was then freeze dried to obtain the powdered spinach juice/CRF with enhanced shelf stability. However, significant quality loss was observed in both blanched spinach juice and CRF powders. Blanching is typically an open system, meaning that there is significant loss

of mass or volume as water-soluble compounds, nutrients, and other components leach out into the blanching water. The visual green colour loss and nutrients loss was mostly due to the leaching of water soluble nutritional components to the blanching water and the degradation of thermal sensitive compounds. The total mass/volume losses of blanched spinach leaves/juice compared with control were measured, which was around 7% and 14% respectively (Appendix Table 5.1). Also, the yield of blanched juice was significantly reduced (Appendix Table 5.2). Given this fact, blanched spinach juice/CRF will be not considered for further investigation during *in vitro* static digestion model.

Pasteurisation (chapter 4) primarily focuses on preserving product quality in a closed system, while blanching involves both cooking the spinach leaves and leaching compounds into an open system, leading to quality changes in both the final product and the blanching water. The nutrients loss of freeze-dried spinach juice and CRF after pasteurisation and blanching is shown in Appendix Table 5.3. The foremost reduction in macronutrient content subsequent to thermal treatment occurs in total lipids. Specifically, pasteurized juice exhibits a 3-9% decrease, whereas blanched juice experiences a substantial 76% reduction. Pasteurised CRF displays a loss ranging from 28-36%, while blanched CRF records a reduction of 32%. The micronutrients reduction in terms of total chlorophylls, carotenoids, VE and ALA was also found, and significantly lost obeseved in blanched juice. This study provides impact of technical difference between pasteurisation and blanching on the nutrients loss of spinach juice/CRF products.

Chapter VI

6. *In vitro* digestion of galactolipids and proteins from spinach juice/CRF

6.1. Introduction

Galactolipids are the most abundant lipids on earth since they make up the majority of membranes in chloroplasts which are ubiquitous in the biosphere as key organelles in plants and algae (Hölzl et al., 2006). The lipid bilayer of the thylakoid membrane in plant chloroplasts and cyanobacterial cells is predominantly composed of four unique lipid classes; Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) containing one or two saturated and/or unsaturated fatty acids linked to the glycerol moiety, and account for 70-80% of the total lipids in plant cells (Figure 2.4) (Christensen, 2009). Trigalactosyldiacylglycerol (TGDG) and sulfoquinovosyldiacylglycerol (SQDG) present in lower proportion, a sulfolipid with a sulfoquinovose residue instead of galactose (Figure 2.4) (Amara et al., 2010). MGDG and DGDG are two main galactolipids that essential for facilitating the photosynthesis light reaction in the thylakoid membrane and maintaining chloroplast morphology and for plant survival under abiotic stresses such as phosphate starvation and freezing (Li et al., 2018).

The most abundant and commonly reported galactolipid in plants comprise of large amounts of α-linolenic acid (ALA; 18:3 n-3), which, on a molar basis, represents about 60%-95% of the total fatty acids in some higher plant galactolipids, and hexadecatrienoic acid (16:3 n-3) accounts for 8.5 - 19.2% of total fatty acids found in

MGDG (Whitaker, 1986). However, the digestion of galactolipids in humans has been overlooked since green leaf tissue only contributes a small portion of the total acylglycerolipid (such as triacylglycerol in oils and fats) in the human diet (Sahaka, 2020). Studies addressing the digestion of galactolipids in humans are important, as it is a good source of omega-3 fatty acids and the dismantling of the main lipid in thylakoid membranes should promote the release of these nutrients *viz*. carotenoids and tocopherol.

The digestion of galactolipids are associated with lipolytic enzymes such as pancreatic lipase-related protein 2 (PLRP2) together with pancreatic carboxyl ester hydrolase/bile salt stimulate lipase (CEH/BSSL) (N'Goma, 2012; Sahaka, 2020) . PLRP2 has been identified as an important factor of galactolipid digestion in humans, and also found in monogastric herbivores such as guinea pig, rat, rabbit and sheep (Caro et al., 2008). Both human and animal PLRP2 can hydrolyse MGDGs and DGDGs from spinach leaves to FFAs, MGMGs, DGMGs and water-soluble galactose-containing compounds (Andersson et al., 1995). In addition, the presence of bile salt micelles and colipase with human PLRP2 can hydrolyse long-chain tri-, di-, and monoglycerides (Xiao, 2011). Together, CEH/BSSL and PLRP2 had a synergistic effect, increasing cellular uptake and re-esterification 4-fold compared with the sum of each lipase alone (Andersson, 2011). Human pancreatic juice (HPJ) has been identified as the source of these enzymes (PLRP2 and CEH/BSSL), however its use is restricted due to ethical issues. An alternative to HPJ, has been in the use of porcine pancreatic extracts (PPE). While, PPE has lower galactolipase and cholesterol esterase activities than HPJ, which

indicates a lower level of PLRP2 and CEH/BSSL in this enzyme preparation that can not be determined by mass spectrometry (Salhi et al., 2020).

In addition, lipid digestion starts in the stomach by the action of gastric lipase which contributes about 10 - 25% of the overall total triacylglycerides (TAGs) digested. Interestingly gastric lipase remains active in intestine for a period of time and therefore serves to aid liberation of FFA in the GI tract (Thomas et al., 2012). Fatty acids liberated by human gastric lipase can trigger the subsequent activity of pancreatic lipase on lipid substrates (Brodkorb et al., 2019). Therefore, addition of gastric lipase is recommended in gastric phase during *in vitro* digestion model by INFOGEST 2.0 protocol. Rabbit gastric extracts (RGE) which contain both gastric lipase and pepsin activity is now commercially available, and in combination with PPE appears to be a good substitute for human gastric and pancreatic lipases (Capolino et al., 2011). There is hardly any published work on the digestion of galactolipids in chloroplasts under conditions that mimic the human gastrointestinal conditions (Wattanakul et al., 2019). Wattanakul and colleagues showed for the first time that human simulated digestion of galactolipids in spinach chloroplast obtained by different food processing protocols. The refinement of the INFOGEST *in vitro* digestion model to include RGE was also employed. The rate of lipolysis and the composition of the lipolysis products were assessed as a function of time during digestion after lipid extraction and analysis by high performance thin-layer chromatography (HPTLC).

6.2. *In vitro* digestion model

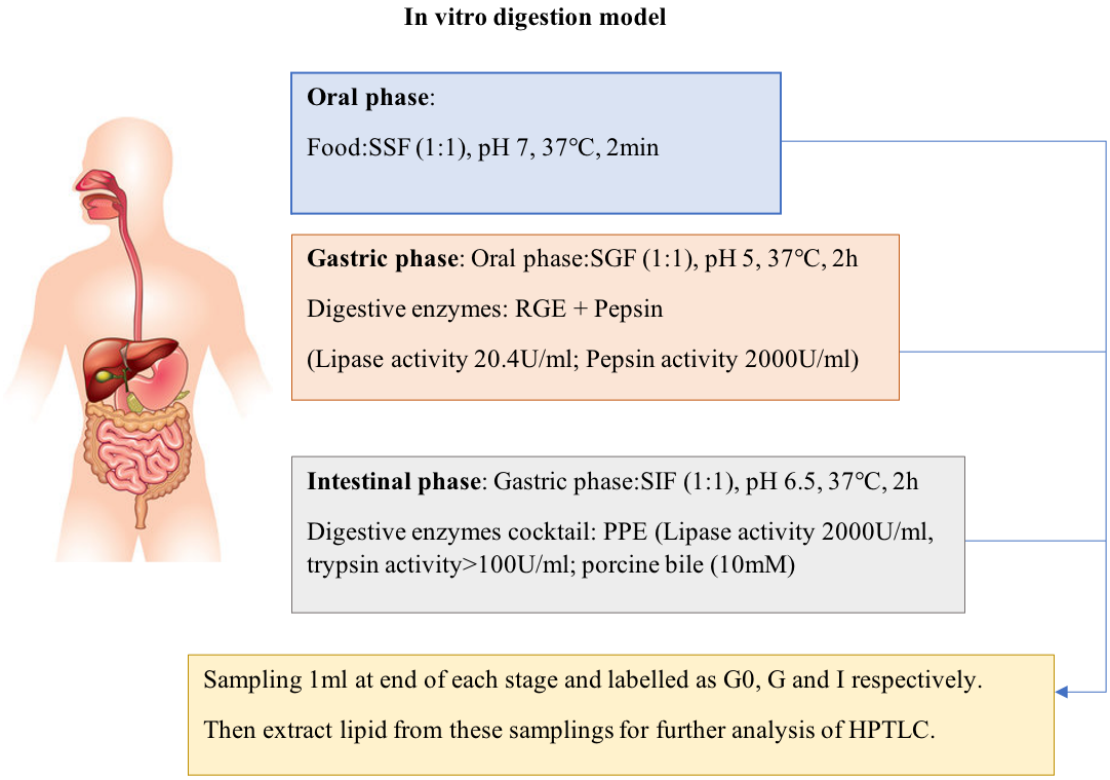


Figure 6.1: *In vitro* digestion model with modifications based on INFOGEST 2.0 (Brodkorb et al., 2019). Simulated Salivary Fluids (SSF). Simulated Gastric Fluids (SGF). Simulated Intestinal Fluids (SIF). Rabbit Gastric Extracts (RGE). Porcine Pancreatic Extracts (PPE).

6.3. Sample preparation summary

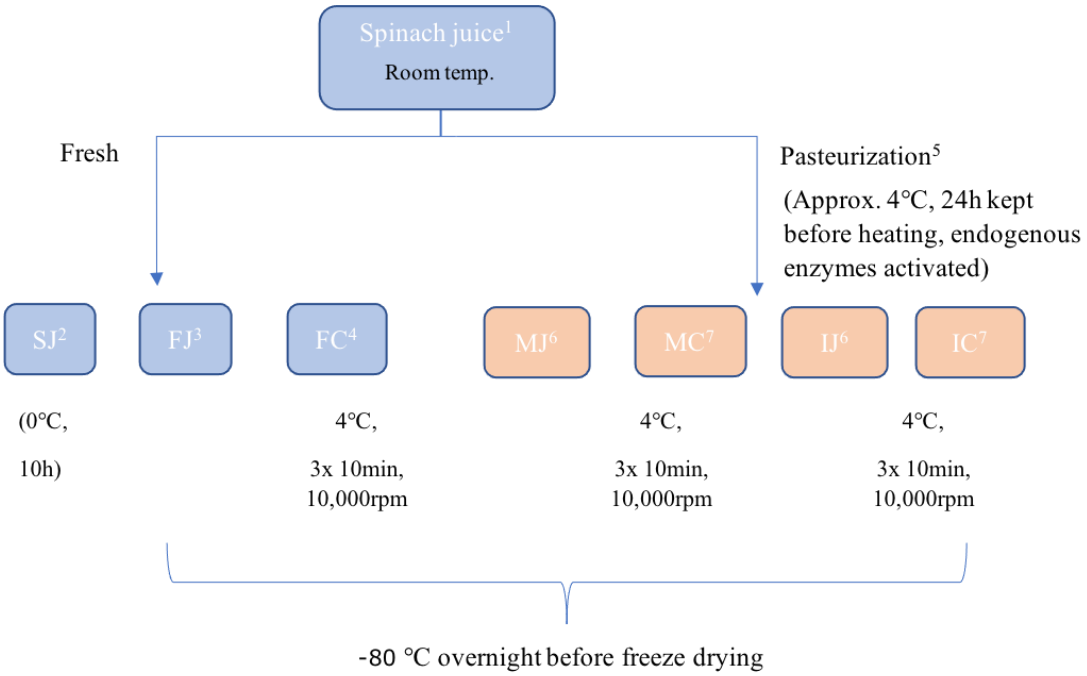


Figure 6.2: Spinach juice/CRF samples preparation steps. (1) Spinach juice was prepared with a twin-gear juicer and held at room temperature before further processing, making 3L spinach juice usually takes 2h at room temperature (RT) and each sample was prepared on a different day but from the same batch of leaves. (2) Fresh juice was kept in an ice bucket (0°C) while spray drying, the whole drying process took roughly 10h. (3) Fresh juice was directly frozen at -80°C overnight before freeze drying. (4) Fresh juice was centrifuged before freezing at -80°C overnight. (5) Spinach juice was pasteurised at mild and intense conditions (70°C 15s and 90 °C 5 min), while the fresh juice was kept in cold room (4 °C 24h) before pasteurisation. (6) Heated juice was directly frozen at -80°C overnight before freeze drying. (7) Heated CRF were collected by centrifuging and then kept in -80 °C freezer overnight before freeze drying. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC),

mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC).

6.4. Lipid extraction and measurement from spinach juice/CRF before and during *in vitro* digestion

The lipids and protein concentration from initial dried spinach juice/CRF powders is shown in Table 6.1. The lipid and protein content of fresh freeze-dried and spray-dried spinach juice are similar. All samples analysed and used in these digestion studies, apart from SJ, were freeze-dried, so we assume a similar degree of lipid extraction in each one to allow comparison across the samples. On comparing the FJ and FC lipid % figures it appears that FC contains approximately 2.5x more total lipid per unit dry weight than FJ; this is to be expected as the centrifugation step concentrates chloroplasts, the major store of lipids in a spinach plant cell and separates them from carbohydrates.

Further interrogation of the data in Table 6.1 reveals no significant impact of heating juice on the lipid and protein concentration as expressed % DW of powder derived from freeze-dried juice. However, in CRF samples, a significant increase of proteins and decrease of lipids (% DW) was observed after pasteurisation. This is likely due to the high heat cause the break of lipid-proteins complex in pasteurised spinach juice, while after centrifugation, the protein-rich pasteurised CRFs were collected, the lipids released in the supernatant were discarded.

Table 6.1: Lipid and protein concentration from spinach chloroplast juice/CRF

powders before digestion

/%DW	SJ	FJ	FC	MJ	MC	IJ	IC
Proteins	38.3±0.6^{e}	39.6±0.4^{de}	50.6±0.5^{c}	41.2±0.1^{d}	58.6±0.2^{a}	38.5±0.9^{e}	54.9±1.0^{b}
Lipids	13.0±1.9^{c}	11.8±0.4^{c}	28.3±0.4^{a}	11.5±0.5^{c}	20.3±0.7^{b}	10.7±0.5^{c}	18.1±0.3^{b}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same row are significantly different ($p<0.05$). Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC).

In order to investigate the galactolipids change and free fatty acid release during digestion, we extracted total lipid from 1ml sampling at end of oral phase (G0), gastric phase (G) and intestinal phase (I).

The quantification of galactolipids and free fatty acid in this study was based on the lipid extracted from the 1 ml sampling during digestion (Table 6.2). However, the 1ml sampling during digestion includes not only the spinach chloroplasts but also the enzymes and other constituent that can carry lipid into the incubation system. Thus, control experiments were carried out by replacing the spinach chloroplast samples with ultra-pure water and undergoing the same digestion process as other samples. The amount of lipids in the sample derived from the chloroplast-enriched powders was calculated by subtracting the control values from the total lipid extracted from each

sample (Table 6.2). Overall, there seems to be little shift in the amount of lipid in the juice samples during digestion, suggesting relatively efficient lipid extraction at each stage. As seen in Table 6.2, the significant reduction of lipid in intestinal phase was observed, which probably due to the sampling issue at the end of intestinal phase; The addition of PPE to the intestinal phase can form clumps and stringy structures which ultimately affect the sampling process and lipid analysis. Moreover, it could also be due to the sampling loss occurs at the end of gastric phase. (There were 20 ml samples left at end of gastric incubation, and 6 ml samples were sampling out for analysis, another 14 ml SIF was added in the digestive tube for intestinal incubation. It is therefore indicate a 30% of total lipid loss in intestinal tube for further sampling).

Table 6.2: Lipid concentration from 1ml sampling during digestion.

Lipid weight	SJ	FJ	FC	MJ	MC	IJ	IC
G0(mg/ml)	3.7 ± 0.5^{b}	4.4 ± 0.4^{b}	16.5 ± 1.9^{a}	5.5 ± 0.2^{b}	9.3 ± 1.0^{b}	4.7 ± 0.2^{a}	11.2 ± 1.5^{a}
G (mg/ml)	5.3 ± 0.2^{a}	5.7 ± 0.4^{a}	18.0 ± 0.5^{a}	6.2 ± 0.2^{a}	12.2 ± 1.1^{a}	5.2 ± 0.4^{a}	9.1 ± 0.6^{a}
I (mg/ml)	4.0 ± 0.3^{b}	4.8 ± 0.5^{ab}	9.3 ± 1.2^{b}	3.8 ± 0.4^{c}	5.4 ± 1.2^{c}	3.6 ± 0.5^{b}	5.7 ± 0.8^{b}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n=3) designated with different letters in the same column are significantly different ($p<0.05$). Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC). G0: End of oral phase, G: End of gastric phase, I: End of intestinal phase. The lipid weight from spinach chloroplasts of G0, G

and I were calculated by subtracting the lipids weight from control.

Figure 6.3 illustrates the lipids content from initial dry powders (%) and from 1ml sampling during digestion at G0 (mg/ml). All the freeze-dried samples showed a similar trend of lipid concentration, which suggests a constant and reliable extraction efficiency. Spray dried juice showed slightly lower extraction from 1ml sampling at G0 compared with initial powders.

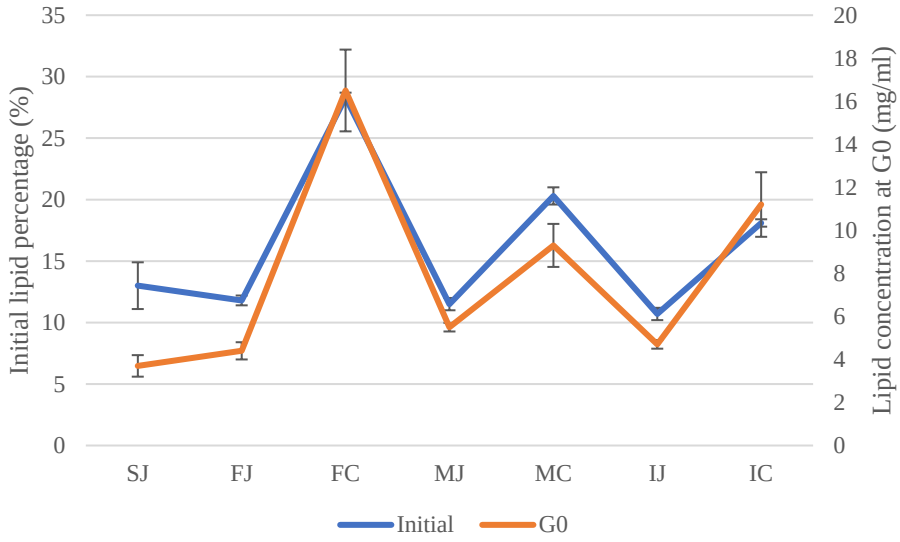


Figure 6.3: The lipid concentration from spinach chloroplast before digestion. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC). Initial: dried spinach chloroplast powders (%). G0: 1ml sampling at the end of oral phase (mg/ml).

6.5. Limitations about HPTLC analysis

The interference, troubleshooting for calculation, and sampling/lipid extraction

efficiency was observed throughout the *in vitro* digestion, and this section will mainly discuss the issues in polar lipids.

The interference in HPTLC analysis may occur when some lipids contained in enzymes. In order to subtract the lane/background noise of MGDG and DGDG, we performed control experiments without the spinach chloroplasts, only water with the addition of digestive enzymes (RGE in gastric phase, porcine bile and PPE in intestinal phase) undergoing the same incubation environments. According to the HPTLC analysis of the control plates (Figure 6.4), a yellow faint band around Rf 0.6 can be observed during gastric phase, this was due to the addition of RGE, since it was the only component contained in the gastric phase in control. As the position of the yellow lipid band did not overlap with the bands for any of target galactolipids, this band 'noise' can be ignored. However, many bands showed up in intestinal phase (Rf 0.23, 0.3, 0.4), which may interfere the quantification of some galactolipids. (The band next to the MGDG at Rf 0.5 was too faint therefore it was ignored in this study). The yellow band next to the DGDG at Rf 0.23 was quite wide and strong and would interfere with the DGDG bands. This yellow bands were partially merged with DGDG and MGMG. Due to the addition of the RGE, PPE and bile extracts in the control experiments, we suspect that the interference bands could result from lipids from these preparations. These interference bands were not observed in Wattanakul's work where RGE, PPE and bile salt (sodium taurodeoxycholate, NaTDC) were used in the control experiments (Wattanakul et al., 2019). The only difference was the source of bile, therefore, the interference bands in intestinal phase could be attributed to the porcine bile extract.

Unlike the NaTDC which is the pure bile salt, the porcine bile extract contains glycine and taurine conjugates of hyodeoxycholic acid and other bile salts, so the interference bands could be caused by the lipid contamination from the bile extract. In consequence, the DGDG band may in effect be partly or wholly composed of contaminating material. The identification and quantification of DGDG is based on the peak integration of the target peak areas (as shown in appendix Figure 6.2). The DGDG peaks are contaminated by bile extract and partially merged with MGMG peaks. Thus, the peak area of DGDG will also include the partially merged interference peak area, in this instance, the subtraction of interference from DGDG is unfeasible, thus the quantification of DGDG is ignored in this study.

The sampling and lipid extraction efficiency was checked in all samples during digestion. The expected lipid content in all samples at each phase is calculated and shown (see appendix Table 6.1). When compared the expected lipid amount with the extracted value, approximately 30% of lipid loss is seen in intestinal phase. This is due to the sampling (6 ml) out from total gastric digestion tube (20 ml), thus 30% of total lipid from gastric phase was lost when entering into intestinal phase. According to this, the galactolipids and FFA content during intestinal phase in all samples will be adjusted to account for its original content. The MGDG and FFA can be calculated and expressed as “mg MGDG/g CRF lipid”, the CRF lipid extracted from 1 ml sampling during digestion is shown in Table 6.2. Meantime, the sampling issues occur at the end of digestion with the addition of PPE, which allows clumps and stringy structures formation. The sampling was done by using the 1 ml pipette, the jelly-like mixture

samplings may be stuck in the tips and cause the underestimate of total extracted lipid and further effect the calculation of galactolipids data.

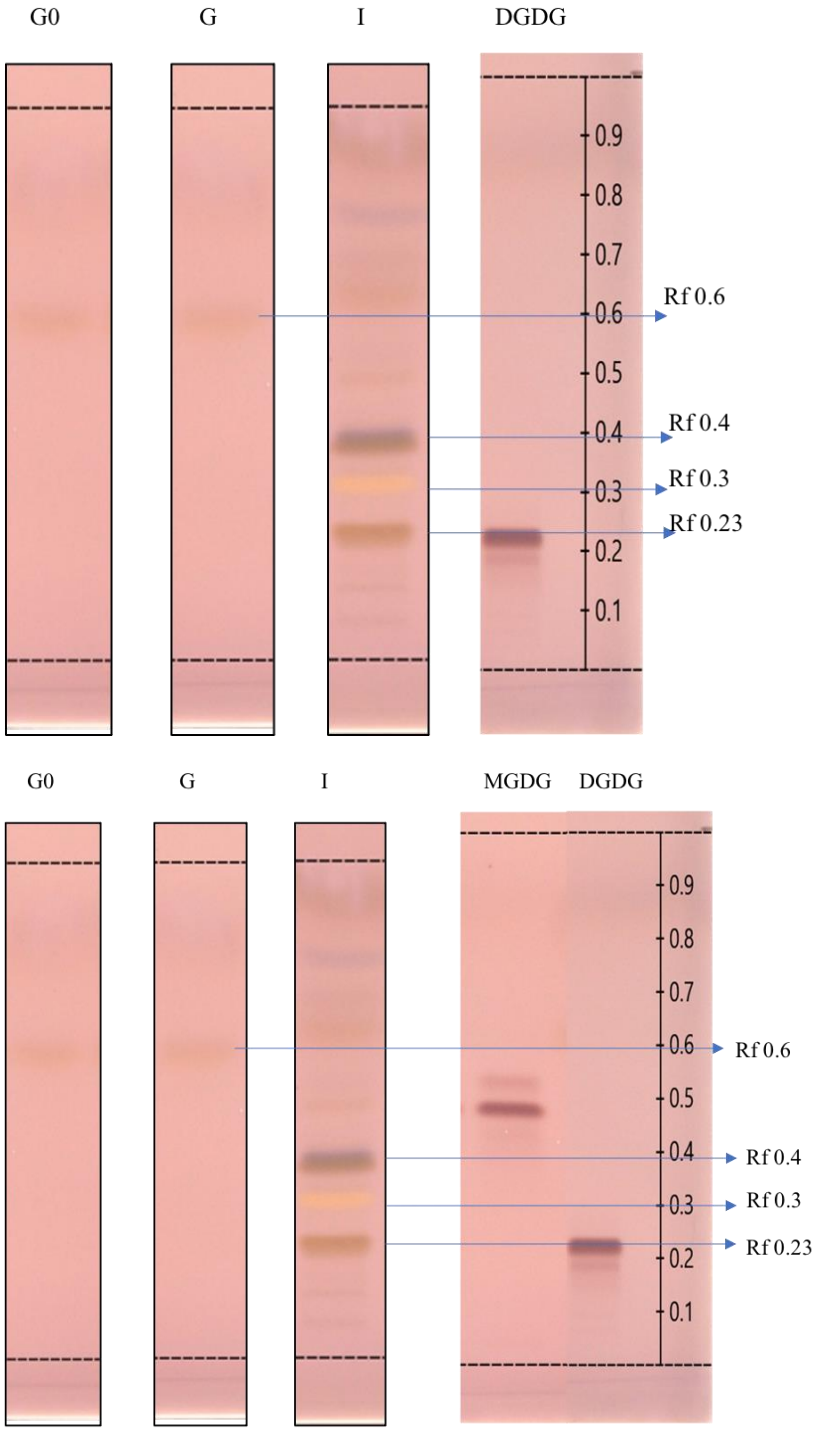


Figure 6.4: HPTLC analysis of control samples during *in vitro* digestion. G0: end of oral phase; G120: end of gastric phase; I120: end of intestinal phase. Polar lipids

standards: monogalactosyl diglycerides (MGDG) and digalactosyl diglycerides (DGDG). The bands from this control plates indicate the interference from the addition of RGE and bile extract.

6.6. Results and discussion

As described in Chapter 3 and 4, different processing procedures were used to produce the spinach chloroplast materials. Both spray drying and freeze drying can be applied to produce dried spinach chloroplast powders with high levels of nutrients retained. Thermal pre-treatment (pasteurisation of spinach juice) combined with freeze drying is able to inactivate the endogenous enzymes, especially peroxidase (POD), which can produce spinach chloroplast materials with enhanced stability. In this chapter, we aim to investigate the effect of spinach chloroplast material processing on the digestion of galactolipids in a simulated human digestion model.

6.6.1. Galactolipid digestion trends in different chloroplast-enriched powders using band intensity from HPTLC separation of lipids

The total lipid extracted from 1ml sampling at the end of each stage along with the standards were transferred to the silica gel thin layer chromatography (TLC) plate. The high-performance TLC (HPTLC) technique was used for the analysis of both polar and non-polar lipids. In this study, polar lipids include monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG), nonpolar lipids include monoacylglycerol (MAG), 1,2 diacylglycerol (1,2DAG) l, 1,3 diacylglycerol (1,3DAG) and triacylglycerol (TAG) and α-linolenic acid (ALA) were used as reference. Figure

6.6 illustrates all the HPTLC analysis of the galactolipids and FFA release in different treated spinach chloroplasts during *in vitro* digestion.

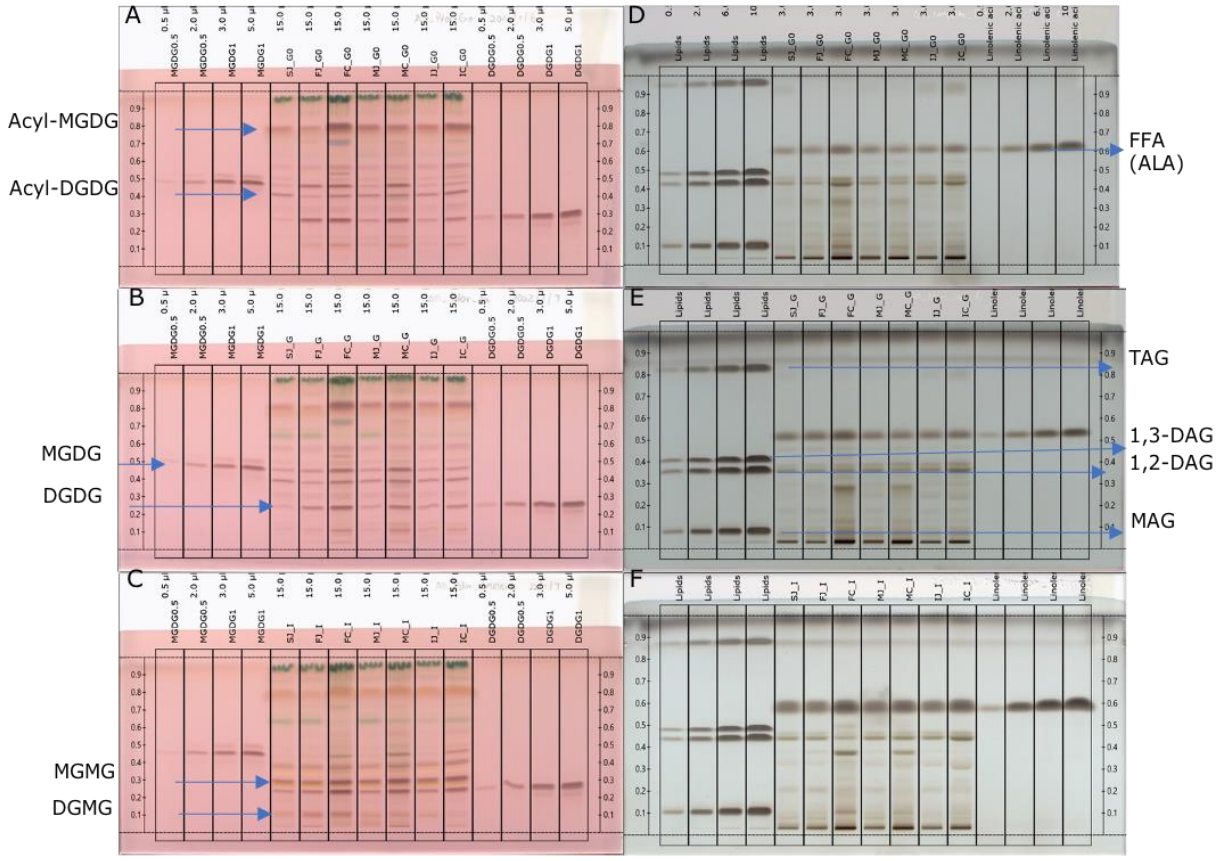


Figure 6.6: HPTLC analysis of the galactolipids and FFA in spinach chloroplasts during *in vitro* digestion. Samples were incubated in oral phase (2min), gastric phase (2 hours) and intestine phase (2 hours) at 37 ℃. Samples were collected at time 0 of gastric phase, G0 (panel A, D), at the end of 2h gastric phase (G) (panel B, E) and at the end of 2h intestinal phase (I) (panel C, F). Panel A-C reveals polar lipids (galactolipids) digestion of spinach chloroplast and control samples during *in vitro* digestion. Panel D-F reveals non-polar lipids (MAG, DAG, TAG and FFA) release of spinach chloroplast and control samples during *in vitro* digestion. Pure monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) standards show bands at Rf 0.48 and Rf 0.28, respectively. Mixed lipid standard (MAG,

1,2-DAG, 1,3-DAG & TAG) show bands between Rf 0.1-0.9; and α-linolenic acid (ALA) standard show band at Rf 0.6.

According to the HPTLC analysis (Figure 6.6 A, B and C), both the MGDG and DGDG from spinach chloroplast materials were hydrolysed and converted to lysogalactolipid (MGMG and DGMG) throughout the whole digestion. In the absence of the pure MGMG and DGMG standards, the bands relating to these products of galactolipid digestion (Figure 6.6 C) were presumed from the work carried out by Wattanakul and co-workers (Wattanakul et al., 2019), we hypothesise that the bands showed at Rf 0.3 and 0.1 are MGMG and DGMG respectively. The decrease of MGDG was in correlation with the increased band intensity of MGMG.

Qualitatively, the MGDG and DGDG remains relatively stable during gastric phase (Figure 6.6 A, B), along with the weakly released FFA (Figure 6.6 D, E), which confirms that the galactolipid hydrolysis is not predominant in gastric conditions. This finding is in line with the fact that gastric lipase in RGE has no galactolipase activity reported by Sahaka (2020). Nevertheless, gastric lipase shows high activity on TAG at sn-3 position at pH 5, and lower activity on DAG and MAG. Also, the acylated galactosyldiacylglycerols (acyl-MGDG and acyl-DGDG) remain relatively stable during gastric phase in all samples, which is in agreement with the minimal hydrolysed galactolipids. Based on other researchers, acyl-MGDG and acyl-DGDG, which is acylated forms of MGDG and DGDG have also been found by thin layer

chromatography in various plants such as spinach (Vu et al., 2014). The band assignment for acylated galactolipids is based on recent work that separate acylated galactosyldiacylglycerol in spinach with mobile phase chloroform: methanol: water (47.5:12:1.25) using HPTLC which is identical to this study (Sutcharit et al., 2023). Therefore, as a reference we hypothesise that the bands at Rf 0.8 and 0.4 represents the presence of acyl-MGDG and acyl-DGDG.

With the addition of bile extract and PPE in the intestinal phase, the galactolipids (MGDG and DGDG) from all spinach chloroplast materials were significantly hydrolysed to MGMG and DGMG, respectively (Figure 6.6 C). High amounts of FFA were also released (Figure 6.6 F). The hydrolysis of galactolipids (MGDG and DGDG) to their galactose constituents such as MGMG and DGMG will result in the release of FFA; ALA was found the most abundant FFA released from sn-1 position of MGDG and DGDG in spinach leaves (Amara et al., 2010). From the HPTLC plates, the bands of MGDG were almost gone, while DGDG remains throughout intestinal phase in all samples. This result clearly shows that the hydrolysis of MGDG is faster than DGDG during the intestinal phase in the presence of PPE. This is confirmed by Wattanakul et al. (2019), that PPE has a higher affinity to MGDG than DGDG. Similarly, a significant decrease in acyl-MGDG and acyl-DGDG were also observed in all samples during intestinal incubation. The acyl-MGDG was completely hydrolysed while the acyl-DGDG remains. The acylation and deacylation reaction is a reversible process. The PLRP2 can deacylated the acylated galactolipids to galactolipids and release FFA

(Sahaka, 2020).

Due to the co-migration of metabolites and the presence of interference bands (Rf 0.27-0.4) during intestinal incubation as described in section 6.5, the measurement of DGDG, MGMG and acyl-DGDG though band intensity is compromised. The DGMG bands were too faint and was excluded for quantification analysis. Therefore, the MGDG and the main released FFA (ALA) are chosen for the markers for the galactolipids digestion in this study. The intensity of the bands for MAG and TAG were too low to be identified or quantified and the band for 1,2-DAG, 1,3-DAG were partially merged together and may comigrate with each other. But the HPTLC analysis does allow qualitative data worthy of comment.

6.6.2. Quantification of galactolipid digestion and FFA release in different chloroplast-enriched powders using band intensity from HPTLC separation of lipids

6.6.2.1. MGDG and FFA content in the oral phase

The galactolipid (MGDG) and FFA changes from different treated spinach chloroplast materials during *in vitro* digestion was quantified and expressed as mg of MGDG/FFA per gram of chloroplast lipid as shown in Figure 6.7 and 6.8 (time 0, 2 and 4h represents G0, G and I respectively). As discussed in section 6.5, the sampling loss happens during the whole digestion process, the reliability of the sampling method may have been compromised by the presence of large particles in some samples, and this will cause an underestimate of the overall data that MGDG and FFA obtained.

Initially, the amount of MGDG in all samples was in the range of 3.3-96.5 mg/g lipid at G0. Since no digestive enzymes were added at G0 (2 min, 37 ℃), hardly any digestion occurred. Thus, we suspect the distinct variation of MGDG and FFA concentrations in spinach juice and CRF samples is mainly caused by its composition difference and the preparation steps (Figure 6.2). The MGDG concentration in FC was significantly lower than FJ despite its higher total lipid content (Table 6.2). This could be attributed to the preparation steps for the spinach materials, spinach juice was directly frozen (-80°C) before drying, whereas the fresh spinach CRF was collected by centrifuging the spinach juice 10 min, 4°C, 10,000 rpm for 3 times, and then frozen at -80°C prior to freeze drying, during which significant lipolysis or acylation of galactolipids seemed to occur, with higher band intensity seen for acyl-MGDG and acyl-DGDG observed in FC (Figure 6.6A). Compared with FJ, SJ showed significantly lower MGDG, since during spray drying the fresh spinach juice (3L) was added to the feed tank gradually which the whole drying process took almost 10h. In this situation, the loss of galactolipids in SJ may arise from the autoxidation reaction started by activated/liberated endogenous enzymes (lipases, lipoxygenases and galactolipase) or the enzymatic transacylation (higher band intensity of acyl-MGDG and acyl-DGDG) (Figure 6.6A) (Duden et al., 1981; Kaniuga, 2008). The relatively low MGDG content at G0 was observed in pasteurised juice (MJ and IJ). This could be also caused by the preparation steps, that the spinach juice was pasteurised 1 day after juicing (kept at 4°C). This gives more time for the endogenous enzymes to act and synthesise acylated form

of galactolipids, and the increased level of acyl-MGDG and acyl-DGDG in pasteurised juice confirmed the enzymatic transacylation. As for the CRF materials, it is worth noticing that MC contains the highest concentration of MGDG (83.6 mg/g lipid) followed by FC (31.6 mg/g lipid) and IC (11.0 mg/g lipid). This is due to the fact that lipid concentration was drastically reduced in pasteurised CRFs (MC and IC) compared with non-pasteurised CRF (FC). As shown in Table 6.2, the lipid concentration from 1 ml sampling at G0 is 16.5 mg in FC and decreased to 9.3-11.2 mg in MC and IC respectively. Moreover, as can be seen in the HPTLC analysis of galactolipids (Figure 6.6A), the MGDG at G0 shows similar band intensity between FC and MC and significantly distinguished in IC. So, when it was calculated by mg/g lipid, the concentration of MGDG in MC was relatively high. One assumes that despite the refrigerated storage overnight, the mild pasteurisation tends to largely retain the GLs levels in the subsequent dried CRF powders and intense pasteurisation seems like decrease their levels. Meanwhile, it is known that pasteurisation process causes the denaturation and aggregation of lipid-protein complex, therefore the pasteurised CRF pellet was able to retain most of the chloroplast membranes with the evidence that the supernatant shows the bright yellow colour. The lipids of the photosynthetic membranes in the chloroplasts are dominated by galactolipids. While the fresh CRF pellet was collected by discarding the green supernatant after centrifugation for 3 times which suggests some loss of photosynthetic membranes which mainly consists of GLs. This could explain the low MGDG in FC powders compared with MC.

As shown in Figure 6.8, high levels of FFA (ALA) can be observed in all cases (G0). The FFA levels in all the initial samples were mainly due to the lipid hydrolysis during preparation with the activate endogenous enzymes. Thermal processing of the green biomass inhibits the activity of lipolytic enzymes and so reduces the release of FFA (Suzuki et al., 2005), and prevent the subsequent fatty acid oxidation and degradation. While, in this study the high accumulation of FFA in pasteurised samples is because of the long storage (24h, 4°C) before heating, during which significantly lipid hydrolysis occurs. This was confirmed in Chapter 5, by blanching spinach leaves prior to juicing, from which step the endogenous enzymes were inactivated immediately and the lipid hydrolysis was prevented in the following process. The FFA(ALA) release between fresh and blanched CRF powders was compared, with the value of 96.91 and 0 mg/g lipid respectively (data shown in Table 5.3), which provides strong evidence that the FFA accumulation can be completely avoided by rapidly heat processing of the biomass.

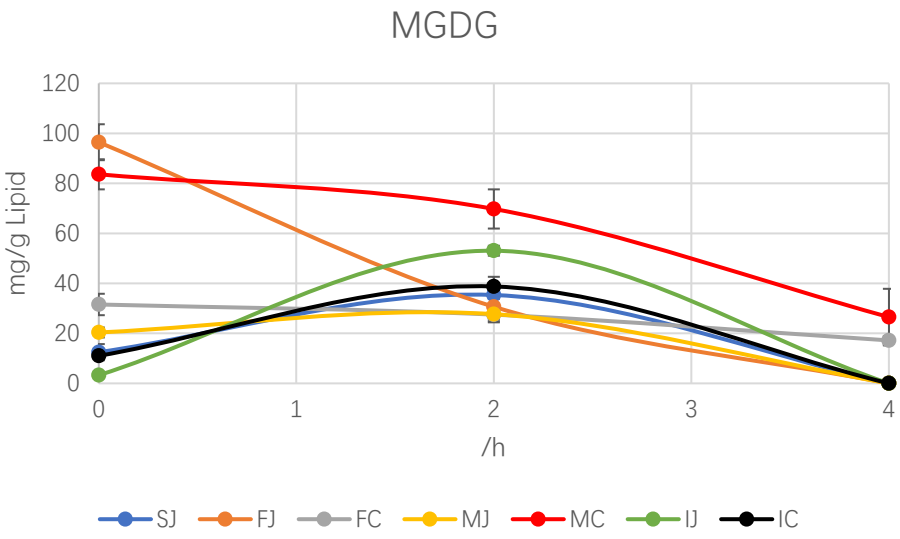


Figure 6.7: MGDG content in different treated spinach chloroplasts during *in vitro* digestion. Time 0 represents G0, the end of 2 min oral phase, without adding any enzyme. In theory, no digestion happens yet at this time point, therefore, it can be seen as undigested CRFs; Time 2 h represents G, end of 2 h gastric incubation; Time 4h represents I, end of 2h intestinal incubation. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC).

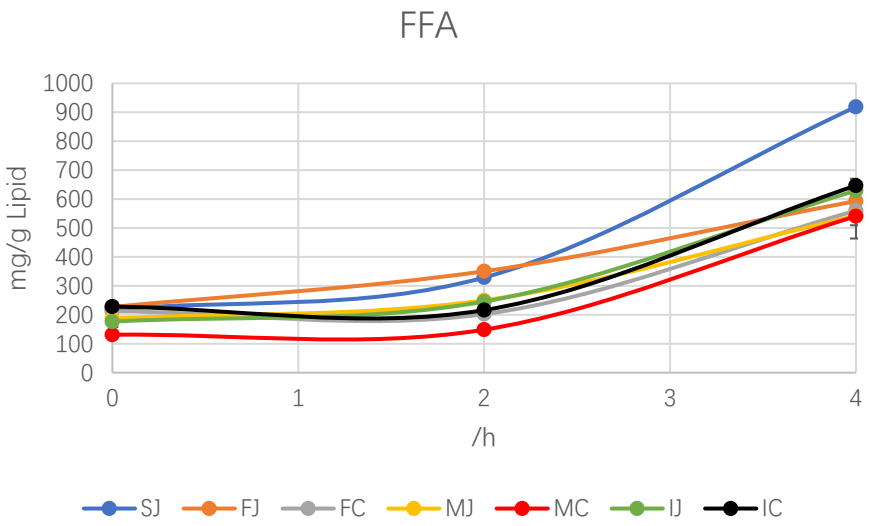


Figure 6.8: Release of free fatty acid (α-linolenic acid, ALA) in different treated spinach chloroplasts during *in vitro* digestion. Time 0 represents G0, the end of 2 min oral phase, without adding any enzyme. In theory, no digestion happens yet at this time point, therefore, it can be seen as undigested CRFs; Time 2 h represents G, end of 2 h gastric incubation; Time 4h represents I, end of 2h intestinal incubation. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild

pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC).

6.6.2.2. MGDG and FFA content in gastric phase (G)

During gastric phase, approximately 68%, 13% and 17% of MGDG have been digested in FJ, FC and MC (Figure 6.7), and about 13-54% of FFA released in all samples (Figure 6.8). Similar results were also reported by other researchers. According to Wattanakul et al. (2019), in freeze dried CRF from blanched spinach leaves in the presence of RGE in gastric phase (30min 37°C), the decline of galactolipids in the gastric phase is low, with MGDG decreasing from 25-26 to 22-23 mg/g CRF and DGDG decreased from 18-22 to 17-18 mg/g CRF. Similarly, Sutcharit found that 15% of MGDG in spinach CRF were hydrolysed during the gastric incubation with RGE (2h 37°C) (Sutcharit et al., 2023). The significant lower digestion of MGDG in FC compared with FJ could possibly be due to its physicochemical and structural difference. It is probably that the aggregated particle structure of FC hindered the hydrolysis of lipid-protein complex during digestion compared with porous particle structure of FJ.

For SJ, IJ and IC treatments, instead of being digested, MGDG in the gastric phase showed a significant increasing trend (SJ [G_0]: 12.5±3.2 → [G]35.3±1.6 mg/g lipid CRF; IJ [G_0]:3.3±0.6 → [G]53.1±2.0 mg/g lipid CRF; IC [G_0]:11.0±1.3 → [G] 38.8±3.9 mg/g lipid CRF). This could be due to the inactivation of acylation enzymes in chloroplast after high temperature during spray drying and intense pasteurisation. And acyl-MGDG contained in initial samples were deacylated to MGDG during gastric

incubation (Figure 6.6 B). As shown in Figure 6.6 A and B, qualitatively, acyl-MGDG is contained in all samples before digestion and decreased diversely after 2h gastric incubation. Interestingly, the slightly higher band intensity of MGDG was observed in SJ, IJ and IC at time point of gastric phase (G) compared with G0, while the anticipated rise in MGDG was not detected in others, which confirms our hypothesis. According to Heinz et al. (Heinz et al., 1978), the acyl-MGDG-forming enzyme was contained in chloroplast envelope membranes, which has activity to convert MGDG to acyl-MGDG. This acylation enzyme activity was activated in disrupted tobacco leaves under homogenization with acidic conditions or in incubation at 40 °C (Matsuzaki et al., 1982). Contrary to tobacco leaves, the acylation in spinach leaves is even stronger, since the acyl-MGDG made up 20% of the leaf lipids after 3 hours incubation at 4 °C (Heinz, 1973). Thus, we suspect that freeze drying or mild pasteurisation will still maintain acylation enzyme activity in spinach chloroplasts, further after incubated at 37 °C for 2 hours at an acidic pH of 5 during the gastric phase, part of the MGDG was hydrolysed to MGMG, while some of the MGDG was converted to acyl-MGDG.

6.6.2.3. MGDG and FFA content in intestinal phase (I)

During the intestinal phase, galactolipids were drastically digested in all samples, and the MGDG were significantly decreased and high amounts of FFA produced (Figure 6.7 and Figure 6.8). However, as mentioned in section 6.5, approximately 30% of lipid loss occurs after sampling (6 ml) from gastric phase (20 ml) once entering into intestinal phase. Thus, the intestinal data listed above (Figure 6.7 and 6.8) was adjusted

to its original content in order to minimise the impact of sampling issue.

Analysis revealed that significant MGDG hydrolysis was found in all samples throughout the 2 hours incubation during the intestine phase for example, 46% and 68% of MGDG were digested in FC and MC at the end of intestinal digestion, and MGDG was fully hydrolysed all other samples. The results indicate that 243-590 mg of FFA/g of lipid CRF were released in all samples at the intestinal phase. Moreover, there seem to be minimal differences in the digestion of galactolipids between the different chloroplast samples. During the whole *in vitro* digestion process, the main lipid digestion of spinach chloroplast materials occurred in the intestinal phase. This was due to the presence of PPE, as previously confirmed by Salhi et al. (2020). This study found that PPE has low galactolipase and cholesterol esterase activities. The low galactolipase and cholesterol esterase activities of PPE indicated that pancreatic lipase-related protein 2 (PLRP2) and carboxyl ester hydrolase/bile salt stimulated lipase (CEH/BSSL) are present at low levels in this enzyme preparation. Importantly, PLRP2 as the essential enzymes for galactolipid digestion was recently found in porcine pancreatic juice and reported to have the highest galactolipase activity. CEH/BSSL also known as cholesterol esterase, which is the dominant lipases in the intestine involved in lipid digestion, can hydrolyse both galactolipids and cholesterol ester. As reported by Minekus (Minekus et al., 2014), PPE is a mixture of digestive enzymes with broad activities which includes protease, pancreatic-α-amylase, pancreatic lipase and colipase. The activities of galactolipase and cholesterol esterase are not found in classic native

human (nHPL) and porcine (nPPL) pancreatic lipase (Amara et al., 2009). Therefore, we hypothesise that the activity of galactolipase found in PPE could originate from both PLRP2 and CEH/BSSL. This hypothesis is also confirmed by the result of Wattanakul et al. (2019), who compared the addition of PPE and HPJ (Human Pancreatic Juice) during the intestinal digestion phase. The observation from their work is that galactolipids of CRF were hydrolysed at a slower rate when RGE and PPE were combined for *in vitro* digestion compared with RGE and HPJ. As the galactolipase activity of HPJ is higher than PPE, with 8.40 and 0.28 U/mg on MDDG-C8 at pH 6 respectively (Salhi et al., 2020). The higher galactolipase activity of HPJ is associated with the higher extent of PLRP2 (Andersson et al., 1996). In addition, PPE has a higher galactolipase activity when MGDG is the substrate compared with DGDG, while the rate of hydrolysis of MGDG and DGDG being similar in HPJ (Wattanakul et al., 2019).

6.6.3.Protein changes during heat treatment and *in vitro* digestion determined by SDS-PAGE

SDS-PAGE was performed to evaluate the protein changes from different treated spinach chloroplasts during *in vitro* digestion, the electropherogram is presented in Figure 6.9. Rubisco protein (54kDa) was chosen as a maker for protein changes as it is the major protein present in chloroplasts. Previous research has reported that heat treatment can break the lipid-protein complex in the food matrix and thus increase the bioaccessibility of lipophilic nutrients (Platel et al., 2016). This study was designed to establish if the method of chloroplast material preparation affects the profile of protein

digestion. Starting materials were dispersed in water then treated as described in the methods (see Section 2.3.4.4); samples from the digestion study were also treated as described. An aliquot equivalent to 20 μg of protein was loaded onto the SDS-PAGE gel in each case.

Before digestion (Figure 6.9A), a gradual decrease in the Rubisco band intensity was observed in pasteurised juice which suggests the denaturation and aggregation of insoluble protein with lower solubility caused by heat treatment. Typically, temperatures higher than 60°C are able to achieve the selective precipitation of proteins including the Rubisco (Opdensteinen et al., 2021). Despite the higher protein concentration in FC compared with FJ (Table 6.1), the band intensity of Rubisco seemed lower. This could be due to the lower solubility of FC (43%) than FJ (74%) powders, see chapter 3, that lowers protein in FC following solubilization. Also, we assume the intense band near the top of the lane (above 250 kDa) in FC is made up of protein aggregates of Rubisco. This band disappeared in MC and IC, this can be explained by the protein dissociated and re-arranged to form aggregates with structure changed (lower molecular mass) when expose to heat (Wang et al., 2017).

During digestion (Figure 6.9 B, C and D), most of the visible bands were noted below a molecular weight of 20 kDa at the end point of each gastric and intestinal phase. This indicates that most of the digested protein can be attributed to protein hydrolysis by pepsin and pancreatic enzymes (Rao et al., 2020). Qualitatively, larger amount of digested protein below 20 kDa was detected in pasteurised samples (Panel C and D),

since the Rubisco was denatured and more resistant to hydrolysis (Wang et al., 2014). This finding concludes that heat treatment may inhibit the digestibility of proteins from spinach juice/CRF materials.

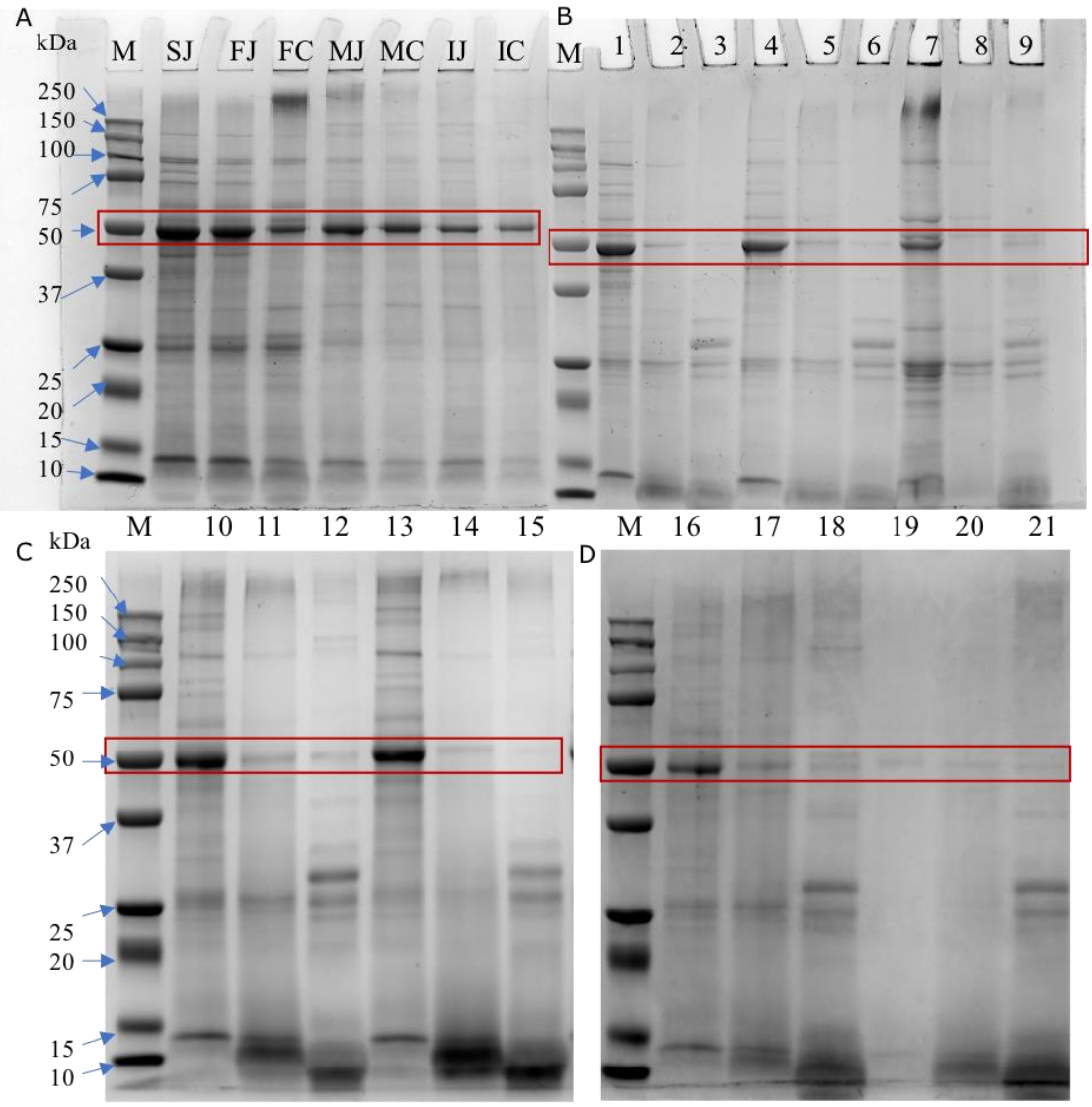


Figure 6.9: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of spinach chloroplast protein during *in vitro* digestion (M: Standard molecular mass; Panel A: initial samples before digestion. Panel B, C and D: spinach chloroplast samples during digestion. Lane 1-3, SJ, SJ-G, SJ-I; Lane 4-6, FJ, FJ-G, FJ-I; Lane 7-9,

FC, FC-G, FC-I; Lane 10-12, MJ, MJ-G, MJ-I; Lane 13-15, MC, MC-G, MC-I; Lane 16-18, IJ, IJ-G, IJ-I; Lane 4-6, IC, IC-G, IC-I. 54kDa, Rubisco)

6.6.4.Overall discussion

Several investigations in our group have focused on the process of galactolipids digestion in chloroplasts rich fraction (CRF) from spinach during *in vitro* digestion. Wattanakul and colleagues used a two-step static *in vitro* digestion model for studying the galactolipids digestion of blanched spinach CRF (Wattanakul et al., 2019). The model is maintained at 37 °C and starts from stomach phase at pH 5 with the presence of RGE (20 U/ml) incubated for 30 min, and then followed with an intestinal phase at pH 6, with the addition of human pancreatic juice (HPJ) or PPE and bile salt (NaTDC 4mM) incubated for 60min. The results have shown that blanching spinach leaves (85 °C, 3min) can knock out endogenous galactolipase activity inside the plant materials, avoiding the loss of galactolipids and the release of FFA.

Sutcharit's research also focuses on the galactolipids digestion of fresh freeze-dried spinach CRF and use the same digestion model as this study with slightly difference of gastric pH (half gastric emptying pH 5.5 for liquid sample) (Sutcharit et al., 2023). The main findings are identical to this study, that the MGDG, DGDG, acyl-MGDG and acyl-DGDG were detected in spinach chloroplast samples before digestion. And the major hydrolysis of both non-acylated and acylated galactolipids occurred in the intestinal phase, which accompanied by a significant increase in MGMG and DGMG values.

Despite the rapid advances achieved in our group, there are still significant gaps in our understanding of how various foods with different compositions, physical properties and structures prepared with different processing procedures are affected during gastric-intestinal (GI) tract. Therefore, the key purpose of this study was to establish the impact of spinach chloroplast processing or preparation procedures in the digestion of nutrients (galactolipids and proteins). As the various spinach chloroplast structures caused by different food processing technologies (spray drying vs freeze drying, heat treatment vs non-heat treatment) may be disintegrated dissimilarly during gastric-intestinal tract. Understanding the influence of food processing procedures on the digestion of nutrients in spinach chloroplast samples within the *in vitro* digestion model will assist the development of new products with enhanced functionalities. As well known, the complex food structure is a major limiting factor for the digestion of nutrients (Chen, 2009). Thermal treatment can stimulate the breakdown of protein-lipid complex and enhance the release of lipophilic nutrients (Gomes et al., 2021). In this context, the production and processing steps for spinach chloroplast in this study was aim to preserve their quality, address their limitations and promote their functionality for health attributes.

6.7. Conclusion

In this study, we have shown that both mild and intense pasteurisation (70 ℃ 15s and 90 ℃ 5min) of spinach juice inactivate endogenous enzyme activity. Therefore, it is recommended that thermal treatment should be applied in spinach juice/CRF

powders production to avoid lipid hydrolysis, accumulation of FFA and further oxidation of polyunsaturated fatty acids.

During the simulated *in-vitro* digestion, the galactolipids from spinach juice/CRF could be digested. Moreover, thermal pretreatment can enhance the digestibility of GLs from spinach juice/CRF with higher digestion rate especially found in intense pasteurised samples. GLs maintained relatively stable in gastric phase with the presence of RGE, and predominately digested in small intestinal phase with the main enzyme PLRP2 and CEH/BSSL which is found in PPE. Furthermore, PPE hydrolyses MGDG more extensively than DGDG. We have also shown that the acylated galactolipids (acyl-MGDG and acyl-DGDG) were also digested throughout the digestion and mainly digested by the enzymes in PPE. After digestion, the main FFA released from spinach chloroplast samples during is ALA, and the increasing rate is low in gastric phase, but considerably raising upon entering the intestinal phase.

In addition, it can be seen that proteins in spinach juice/CRF samples were digested by pepsin and PPE during digestion, and majority of protein hydrolysis occurred in gastric phase. However, heat pretreatment was found inhibit the digestion of proteins with larger amount of digested protein below 20 kDa was detected in pasteurised samples during digestion compared with fresh samples. This suggests that heat treatment could lead to a reduction of protein digestion due to their denaturation. Overall, this work indicated that thermal pretreatment can stimulate the digestion of galactolipids from spinach juice/CRF but hinder the digestion of proteins.

Chapter VII

7. Bioaccessibility of lipophilic nutrients from spinach juice/CRF during *in vitro* digestion

7.1. Introduction

Spinach chloroplasts are rich in proteins and lipophilic nutrients such as carotenoids (β-carotene and lutein), α-tocopherol (vitamin E), chlorophylls (a and b), and omega-3 fatty acids (constituents of galactolipids). The consumption of these nutrients is associated with health benefits in the human body, as they can decrease oxidative stress, lower the relative risk of cancer and chronic diseases, and promote brain, cardiovascular, and eye health (Roberts & Moreau, 2016; Lasya, 2022). It is therefore important to research how the digestive process impacts on these nutrients derived from chloroplast products. In this chapter, the release of carotenoids, vitamin E and galactolipids from spinach chloroplast-rich materials, produced by different food processing technologies, during simulated human digestion was evaluated.

Bioaccessibility is a definition used to describe the availability of nutrients from foods needed for use by the cells and tissues of our bodies. An understanding of bioaccessibility is useful as it allows researchers a means to ascertain nutritional efficiency of food and food formula in the development of products aimed at improving human health (Fernández-García et al., 2009). In essence, bioaccessibility relates to the proportion of a compound that is released from a food matrix in the gastrointestinal tract, that then becomes available for intestinal absorption and distributed systemically.

Currently, this process has been investigated using several validated *in vitro* models (Brodkorb et al., 2019).

Interestingly, it has been reported that the bioaccessibility of carotenoids and vitamin E from fruits and vegetables are generally quite low (Reboul et al., 2006; Lemmens et al., 2014). This is mainly attributed to two main causes, the limited release from food matrix (Aguilera, 2019; Vitali Čepo et al., 2020) and the low solubility in gastrointestinal fluids (Savjani et al., 2012). During food preparation, processing and/or mastication the fractured plant cell can promote the release of carotenoids from food plant tissues. Following release from the food matrix, the major limiting factor is solubility of carotenoids/vitamin E in digesta given their hydrophobic character (Faulks et al., 2005). In this situation, improving carotenoids and vitamin E bioaccessibility of fruits and plants food by specific methodologies and technologies has becoming a worldwide topic (Failla et al., 2005). In Chapter 3 and 4, we recovered intact chloroplasts without the confinement of cell wall by juicing spinach leaves using a twin-gear juicer, and then produce dried powders with different processing protocols, spray drying or freeze drying; with or without pasteurisation. The bioaccessibility of carotenoids and α-tocopherol from these materials was investigated in this study. The impact of food processing, e.g., drying techniques and thermal pre-treatment on the bioaccessibility of these lipophilic nutrients was measured and compared.

It is known that various factors influence nutrient bioaccessibility including dietary factors such as fat, fibre, dosage of carotenoid, location of the carotenoids in the

plant tissue, heat treatment, particle size of food, carotenoid species, interactions among carotenoids, isomeric form and molecular linkage and subject characteristics. (Priyadarshani, 2017). In this study, we focused on the impact of the food matrix and its microstructural characteristics on the release of selected nutrients. The microstructure characteristics are a result of the application of specific unit operations such as thermal processing, mechanical processing, etc (Rodríguez-Roque et al., 2016; Sun et al., 2018). Thus, the hypothesis of this study was that the thermal pre-treatment e.g. pasteurisation of spinach juice prior to CRF production will improve the bioaccessibility of lipophilic nutrients. Also, the spray drying as a cheaper, faster and more efficient alternative to freeze drying was also used to produce dried spinach juice fine powders. The experiment will also investigate whether the drying method will modulate bioaccessibility of spinach bioactive compounds without the confinement of plant cell wall during digestion. All these seven spinach chloroplasts productions, SJ (Spray dried juice); FJ (Freeze dried juice); FC (Freeze dried CRF); MJ (Mild pasteurised juice); MC (Mild pasteurised CRF); IJ (Intense pasteurised juice); IC (Intense pasteurised CRF) was sieved to include 250 μm or less and their composition before and during digestion were measured and compared with an *in vitro* digestion model as described in section 6.2. After digestion, centrifugation was used to separate the micelles from total digesta, and the micelles phase need to be passed through a 0.2 μm PTFE filter. It is assumed that the lipophilic nutrients are solubilised in aqueous phase of micelles and only the filtered micelles can be considered being able to pass

through the mucous layer in the small intestine and absorbed by the epithelium cells (Yang et al., 2013).

7.2. Results and discussion

Samples preparation steps are shown in Section 6.3.

7.2.1. Lipophilic nutrients of spinach chloroplasts before *in vitro* digestion

The concentration (mg/g DW and mg/g lipid) of lipid micronutrients in the chloroplast samples were measured before digestion (Figure 7.1). The initial samples before digestion were prepared as liquid samples (juice/CRF powders mixed with water 1.5:3.5, w/w). The addition of water makes it a consumable food as suggested by the INFOGEST method in 2019: high-solid foods must be reconstituted in liquids to reflects real food or meal (Brodkorb et al., 2019).

Spinach CRF contained higher lipophilic nutrients content (carotenoids and α-tocopherol) than juice, and pasteurisation can significantly reduce these compositions in CRFs but maintained relatively stable in juice (Figure 7.1a). The highest nutrients content can be found in FC with β-carotene and lutein was 3.05 and 4.29 mg/g DW, and α-tocopherol was 0.41mg/g DW. While, more than 50 % of these nutrients were lost in mild and intense pasteurised CRFs. The explanations were discussed in section 3.2.1.3 and 4.2.2.4 in detail.

The lipophilic nutrients content (wet extraction) shown in Figure 7.1 differs from

the previous results reported for the dry extracted material described in chapter 3 and 4, showed an overall decreasing trend in wet system. This was suspected to be caused by differences in the extraction efficiency between solid and liquid samples. Since the addition to water in dried samples prior to extraction may decrease the extraction efficiency and will lead to the reduced amount of lipophilic nutrients including carotenoids and α-tocopherol. Based on the research by Balasubramanian et al. (2013), a biomass moisture content higher than 5% reduces the lipid extraction efficiency. It is hypothesized that water molecules in wet biomass form a hydrophilic outer layer of chloroplast material prevents the penetration of non-polar solvents from reaching lipids, and further hinders the lipid extraction process. The efficiency of extraction, measurements, and identification is crucial in carotenoids and α-tocopherol analysis. The method used to extract carotenoids in this study includes extraction with chloroform and methanol from dry powders and liquid samples (with or without addition of water in initial samples), drying under gas nitrogen, dissolving with acetone, separating on HPLC, identification and quantification. Identification is a prerequisite step for accurate quantification, it can be achieved by the retention times attained through co-chromatography with authentic carotenoid standards (Butnariu, 2016).

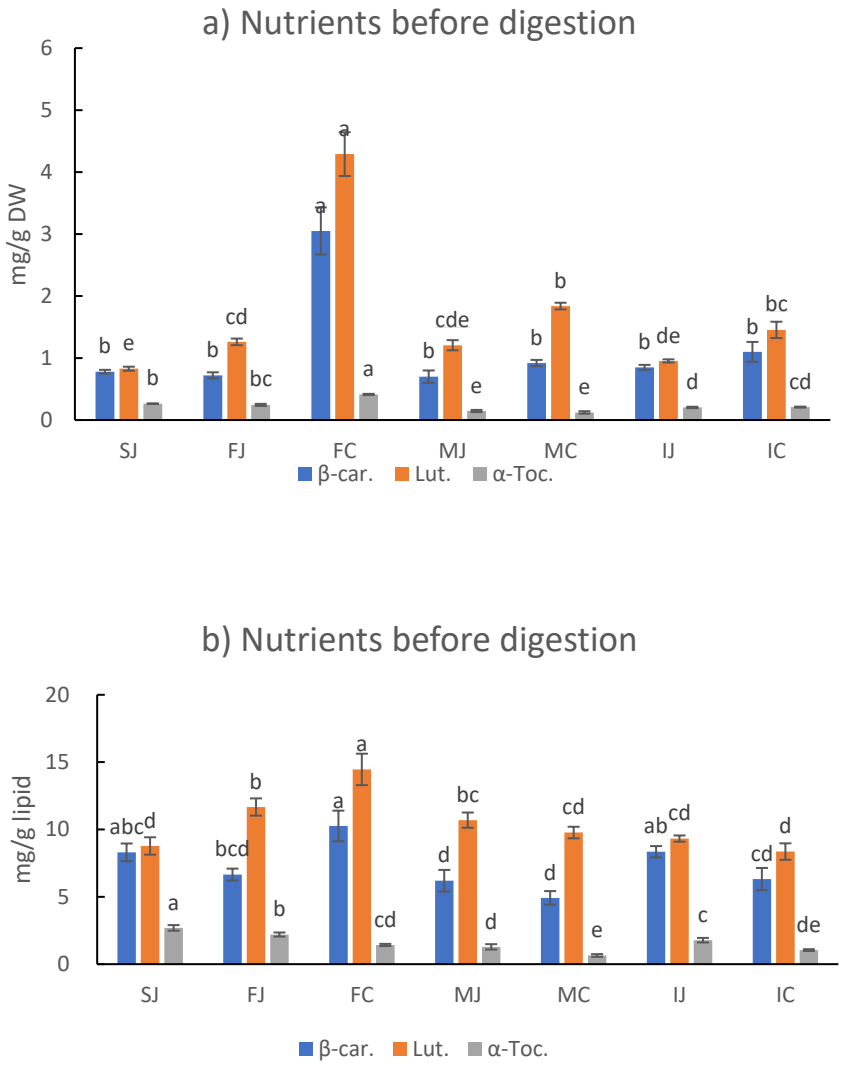


Figure 7.1: Lipophilic nutrient composition of spinach juice/CRF before digestion. Data were expressed as a MEAN±SD of triplicate independent experiments. Different letters indicate significant statistical differences according to one way ANOVA and Tukey test ($p<0.05$). Samples include spray dried juice (SJ), freeze-dried juice (FJ), freeze-dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze dried juice (IJ), intense pasteurised freeze dried CRF (IC).

7.2.2. Behaviour of spinach juice/CRF during *in vitro* static digestion model

The *in vitro* static digestion was performed in an incubator at 37 ℃ containing the digestion tubes that were rotating at a speed of 20 rpm. Rotation was used to provide a continuous force allowing for the samples to mix with the digestive fluids and enzymes. This process was set to mimic the oral, gastric, and intestinal digestion in human. The transformation/behaviour of spinach chloroplasts during in-vitro static digestion model was imaged by light microscope at ×40 magnification, the images were taken at the end of oral phase (2 min), gastric phase (2h) and intestinal phase (2h). The impact of *in vitro* digestion on the different treated spinach chloroplasts at each digestion phase was revealed from the following pictures shown in Figure 7.2.

For all samples, the initial 1.5g weight of spinach chloroplast powders were mixed with 3.5 mL of water to the final volume of 5 mL, added 5 mL SSF incubate for 2 min and then 10 mL SGF (without enzymes), the oral phase sampling was taken at this time in order to make the dilution factor same as the gastric phase. As for the gastric and intestinal phase, the sampling was taken after another 2 and 4 hours' incubation. Then 1M $NaHCO_3$ was added to the sampling of both oral and gastric phase separately (1:1, v/v), to raise the pH to 8. The gastric lipase activity was stopped and the samples were diluted by half, which in agreement with the dilution factor of the intestine phase (Egger et al., 2016). Therefore, in all of the samples, the dilution factors are the same, the reduction in number of chloroplasts and reduced particle size was not due to the dilution

effect only the addition of enzymes at each stage.

In the oral phase, the samples were incubated at a short time (2 min) without adding enzyme, which indicates undigested status as initial samples. For un-pasteurised samples, the SJ showed smaller particle size of chloroplasts compared to FJ, and FJ was smaller than FC. This confirms that spray drying usually produces particles with a mean particle size below 10 μm (Daniel et al., 2017), in this study, the mean particle size of spray dried juice was 9.81±0.22 μm. In contrast, the freeze-dried samples showed larger particle size (FJ 66.44±0.38 μm and FC 113.95±4.44 μm), this was influenced by the material structure and grinding. As for pasteurised samples, the particle size increased with the heating temperature and time in both juice and CRF samples (data shown in Table 4.1 and Table 4.3). Moreover, the pasteurised samples showed darker brown colour due to the transformation of chlorophylls (green) to pheophytins (olive brown). The formation of pheophytins is initiated by the release of cellular acids and synthesis of new acids during heating, the magnesium atom in the chlorophyll porphyrin ring was replaced by two hydrogen ions (Erge et al., 2008).

In the gastric phase, all dried juice samples (SJ, FJ, MJ, IJ) were digested to significantly smaller particles by the addition of RGE and pepsin, while CRFs (FC, MC, IC) still maintain most of its large particles/aggregates. At the end of the intestine phase, with the addition of porcine pancreatin extracts (PPE) and bile salts, all the samples were digested to small particles (in digesta). The digesta was centrifuged and filtered through 0.2μm PTFE filter to obtain the micelle. The micelle is formed from bile salt

and lipid digestion products (free fatty acids, monoglycerides and fat-soluble substances such as vitamins and cholesterol). The micelles are water soluble small fat droplets and enable the transportation of lipid digestion productions to small intestinal epithelium for absorption (Cheng et al., 2020). Therefore, the formation of micelles is a critical step which can affect digestibility.

The same structure reduction of spinach juice and CRF powders during digestion was also be found iby Authors in our group (Gedi et al., 2017) (Wattanakul et al., 2019).

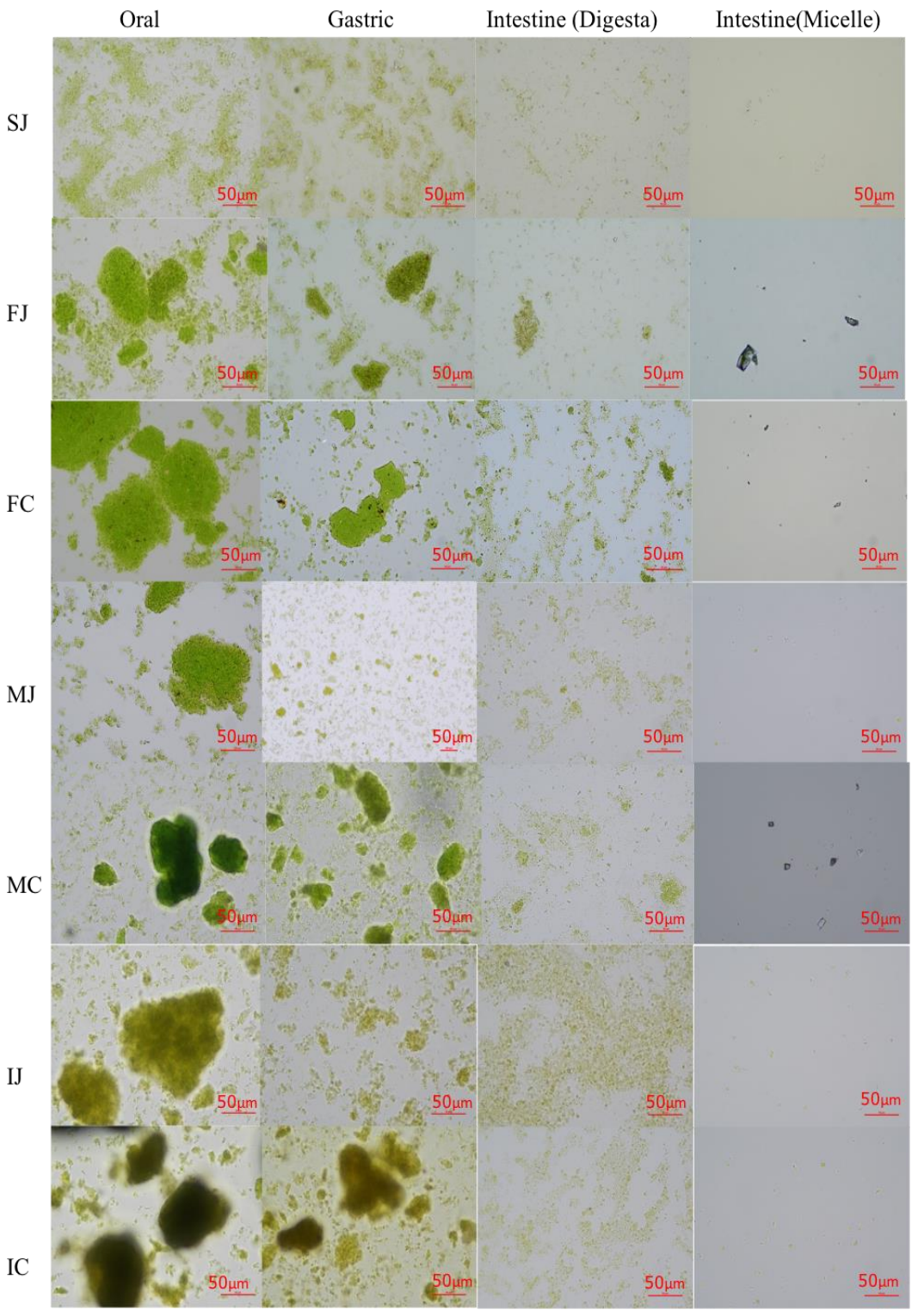


Figure 7.2: Microscope images of spinach juice/CRF with different treatment during *in-vitro* static digestion model, includes spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze- dried juice (MJ), mild pasteurised

freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC). The spinach chloroplast powders were incubated in oral (2min), gastric (2 h) and intestinal (2 h) phase with *in vitro* static digestion model.

7.2.3.Carotenoids release from chloroplast material during simulated human digestion

7.2.3.1. Isomerisation of β-carotene both before and after digestion

The carotenoids in all samples before and after *in vitro* digestion (both total digesta and micelle at the end of intestinal phase) were analysed by HPLC. Two standards were used in this experiment, β-carotene (all-trans/all-E) and lutein respectively. Naturally, the majority β-carotene exists in the all-trans form (Hiranvarachat et al., 2008). However, the trans-to-cis isomerisation of β-carotene was found occur in both food processing especially thermal treatment and during digestion. The major cis-form of β-carotene is 15-15'-di-cis-β-carotene, 13-cis-β-carotene and 9-cis-β-carotene (Marx et al., 2003; Pénicaud et al., 2011).

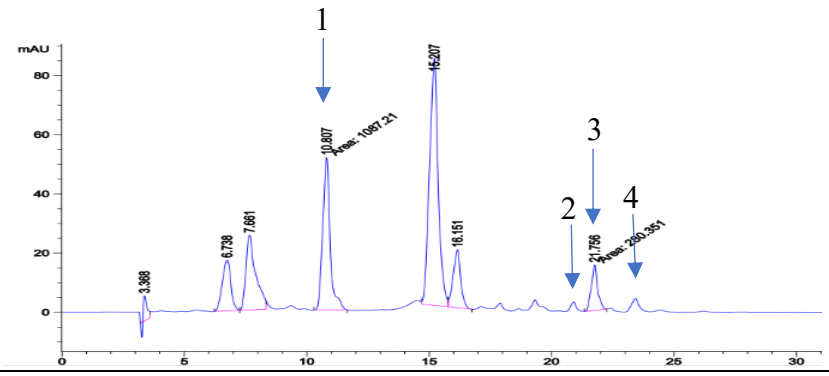
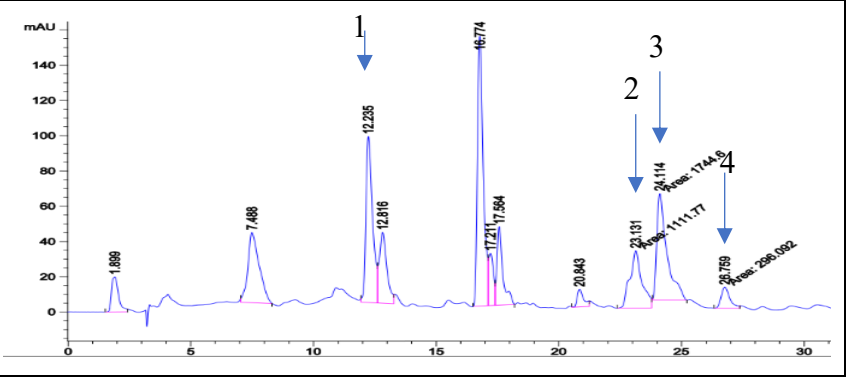
In this study, prior to the *in vitro* digestion, all samples showed two small peaks of cis-β-carotene (around RT 21 and 23 min) which represents 13-cis-β-carotene and 9-cis-β-carotene respectively (Figure 7.3). The isomers of β-carotene were identified by comparison with Wattanakul's publication (Wattanakul et al., 2022). This isomerisation is mainly caused by the processing procedure, apart from heat treatment, β-carotene can also be affected by mechanical treatment. Firstly, the juicing step in preparation can cause the loss of tissue integrity by disrupting cellular structure and the activated

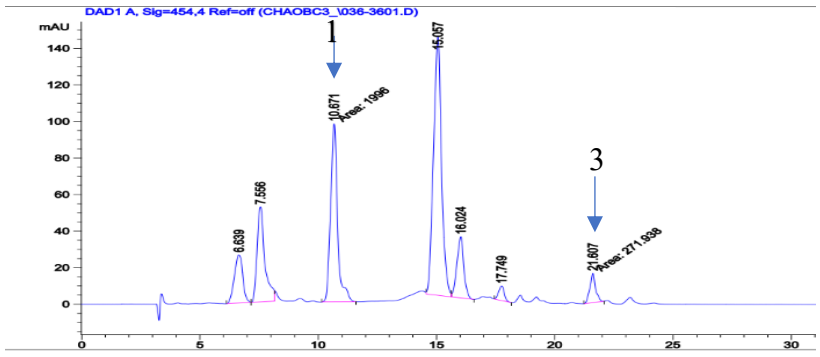
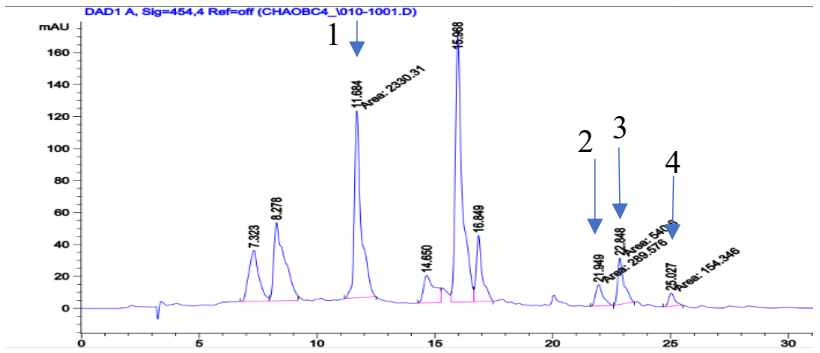
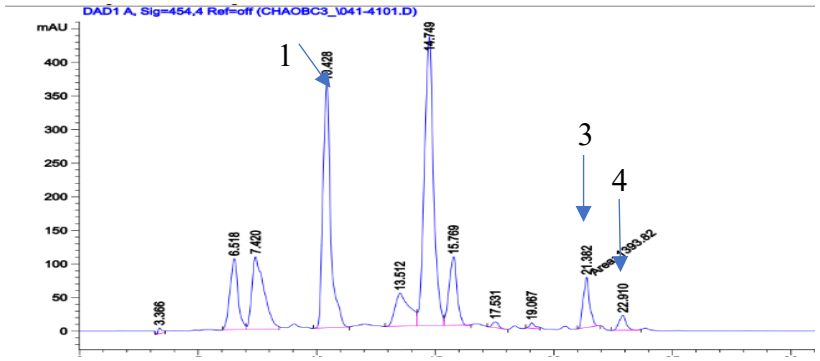
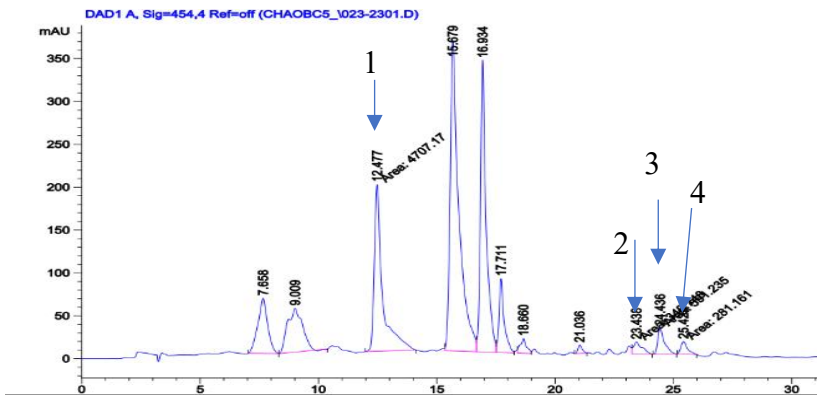
endogenous enzymes can promote the release of organic acids (e.g. oxalic acid), which will result in the formation of cis-form β-carotenes. Secondly, the spinach juice is exposed to heat and oxygen during pasteurisation which will further stimulate the trans to cis (E-Z) isomerisation process (Pénicaud et al., 2011). The level of isomerisation is increased with the heating temperature and time as shown in Figure 7.3. The isomerisation or degradation of carotenes can both occur during processing and storage, therefore, in order to minimise non-enzymatic oxidation on storage, the CRFs were vacuum packed in aluminum bags and kept in -20 ℃. Any nutrient losses in pre-digested samples discussed in this work are likely due to the processing steps prior to freezing.

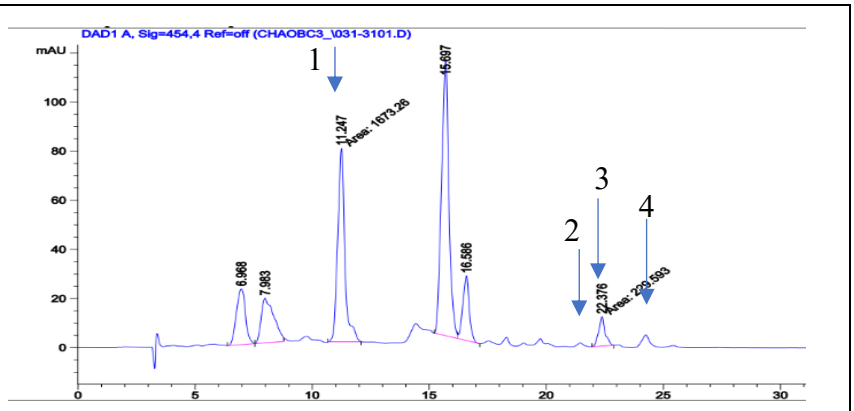
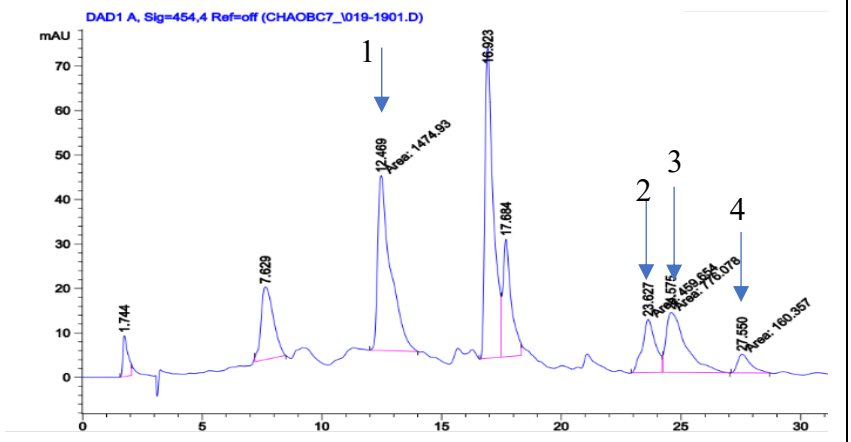
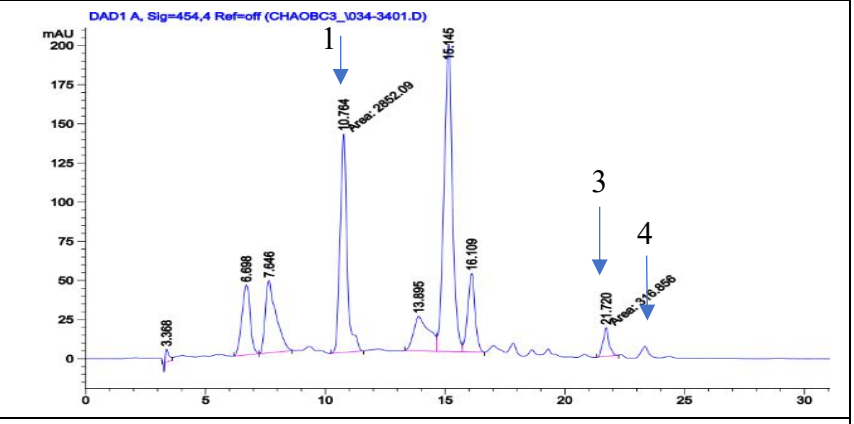
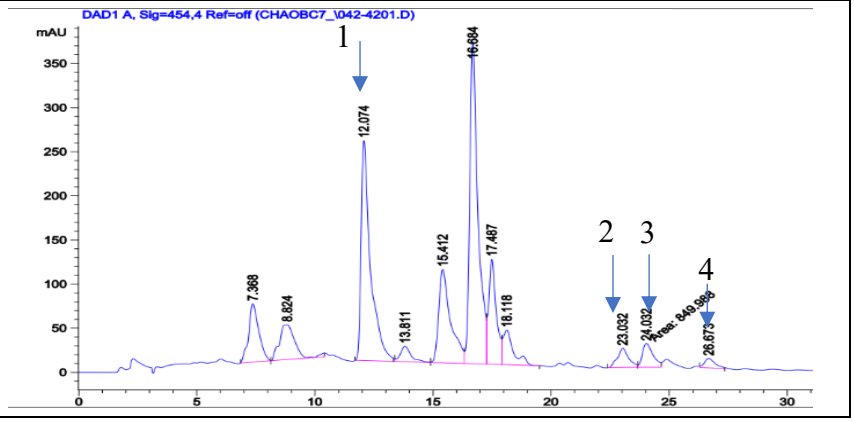
After *in vitro* digestion, there was an increasing trend of cis-β-carotenes levels observed in all samples, comparing their peak area and dilution factor (data not shown). This suggests that the isomerisation of trans to cis-β-carotene also occurs during *in vitro* digestion. Moreover, greater levels of cis-isomers were found in pasteurised samples after digestion. In addition, the food matrix and degree of processing play important role in carotenoid isomerisation and bioaccessibility (Aherne et al., 2010). An increase of cis-isomers and decrease of trans-isomers of carotenoids in carrots during *in-vitro* digestion has been reported by other researchers; Tao et al. (2021) found that all trans-β-carotene reduced 75% and degraded to 15-cis- and 9-cis-β-carotene during *in vitro* digestion and the predominant degradation occurs in intestinal phase compared with oral and gastric phase. Moreover, recent studies have proved that cis-β-carotene is

preferred to be micellarised than all-trans-β-carotene during the small intestinal phase; while cellular uptake appears higher for the all-trans-form (Bohn et al., 2019). Both all trans and cis isomers of β-carotene are transferred across the brush-border surface of the enterocyte from mixed micelles and absorbed in humans by passive diffusion (Ferruzzi et al., 2006).

In conclusion, these findings confirmed that the trans-to-cis isomerisation of β-carotene from spinach juice/CRF occurs during both thermal treatment and simulated digestion.

SJ	Before digestion	
	After digestion	

FJ	Before digestion	
	After digestion	
FC	Before digestion	
	After digestion	

MJ	Before digestion	
	After digestion	
MC	Before digestion	
	After digestion	

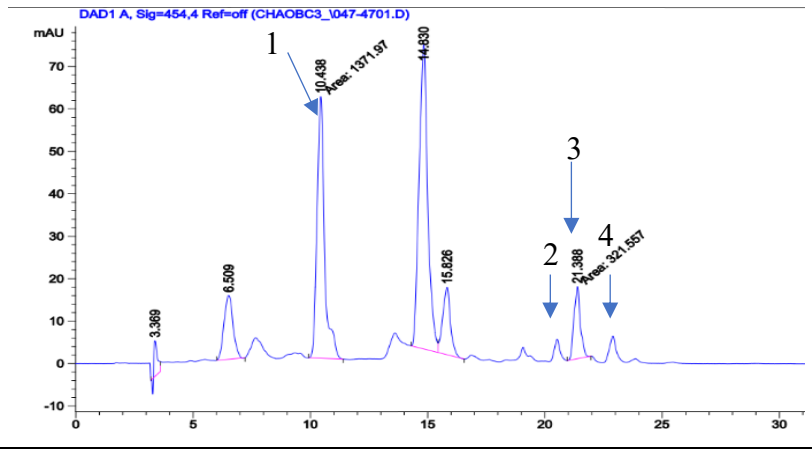
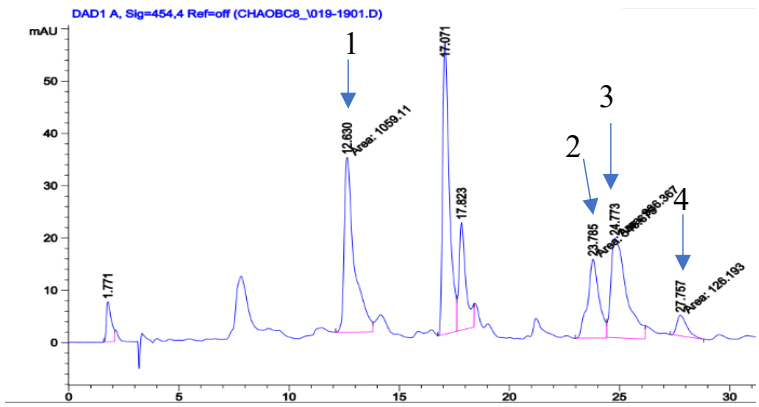
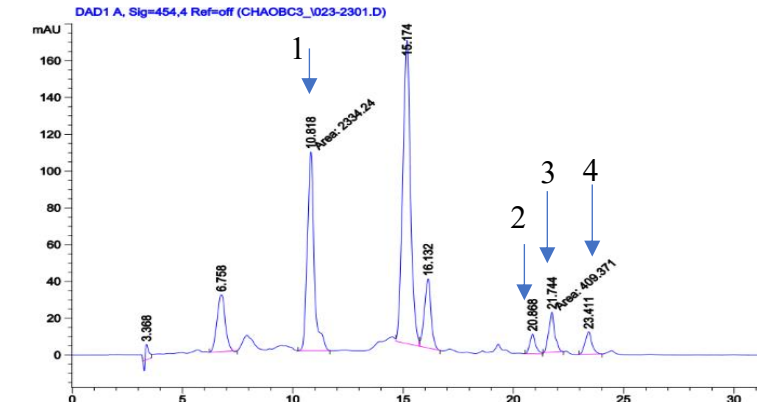
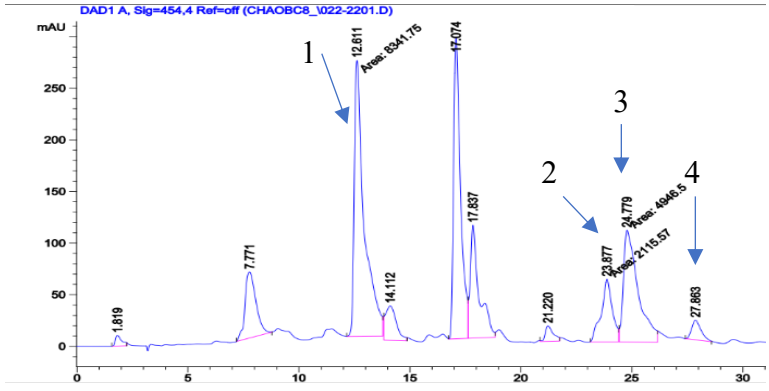
IJ	Before digestion	
	After digestion	
IC	Before digestion	
	After digestion	

Figure 7.3: Chromatogram of β-carotene in spinach juice/CRF before and after

digestion. Peak 1:lutein; Peak 2:13-cis-β-carotene; Peak 3: all-trans-β-carotene; Peak 4: 9-cis-β-carotene. The scale of chromatogram is not consistent and the peak areas should not be compared directly to each other due to different dilution factors. The slight shift in retention time of all β-carotenes before and after digestion in this study were due to the retention time drift in HPLC overtime, and the standards were run in between every five samples in order to confirm the target compound.

7.2.3.2. Micellarisation rate of carotenoids after *in vitro* digestion

In this study, micellarisation rate after *in vitro* digestion shows the ability of nutrients from spinach juice/CRF to be incorporated into mixed micelles and potentially absorbed by intestinal cells.

The micellarisation rate of carotenoids from fresh and pasteurised spinach juice/CRF after *in vitro* digestion is shown as concentration of β-carotene/lutein in micelle in relative to their concentration in total digesta in terms of percentage (%) (see Figure 7.4) and mg/100g DW (see Table 7.2). In fresh samples, higher β-carotene and lutein micellarisation rate was obtained in juice compared with CRF, with 11% and 30% in SJ, 12% and 13% in FJ, while only 3% and 7% in FC. The data also suggests that spray drying of spinach juice resulted in higher lutein micellarisation rate than freeze drying. In addition, even though carotenoids were found to be higher in FC than SJ and FJ (Figure 3.3) , their micellarisation rate was relatively lower.

It is known that multiple factors interfere with the carotenoids micellarisation rate, including food matrix (particle size, location of carotenoids in tissue), carotenoids type

(species, interactions among carotenoids, isomeric form, molecular linkages), heat pre-treatment, digestion environment (Priyadarshani, 2017). In this study, we suspect the reason could be the particle size and morphology differences between samples, CRF showed more compacted structure with the largest particle size, and spray dried juice showed more porous structure with the lowest particle size. According to Lemmens et al. (2010), the significantly reduced particle size for both raw and cooked carrots showed enhanced ability of carotenoids to be incorporated into the micelle phase during digestion. On the other hand, dried juice contained lower lipids (%) which makes it more easily to be solubilised in GI tract (Table 3.1 and Table 4.2).

In addition, pasteurisation prior to freeze drying in this study was found to promote the micellarisation rate of carotenoids (β-carotene and lutein) in both dried juice and CRF after digestion. Pasteurised CRF still showed lower carotenoids micellarisation rate than juice. It is worth noticing that micellarisation rate of lutein significantly increased in pasteurised juice and β-carotene significantly increased in pasteurised CRF. Micellarisation depends on the degree of food processing, high processing degree can maximise the amount of compound which is soluble from the matrix (Fernández-García et al., 2012). Several researchers have shown that β-carotene is generally less efficiently transferred to the micelles than lutein (Ryan et al., 2008), however the transfer efficiency varies among foods (Granado-Lorencio et al., 2007). The type of food matrix and the composition of final digesta can enhance or inhibit its transfer efficiency. It is also known that carotenoids interact during incorporation and absorption and then type

and the relative amounts of carotenoids present in the final digesta (i.e., free versus ester forms) may become relevant (Tyssandier et al., 2001). The transfer of carotenoids may be prevented because they are deeply embedded in the food tissues and because of interfacial characteristics (i.e., surface-active proteins) or electric charges (ζ-potential) (Rich et al., 2003).

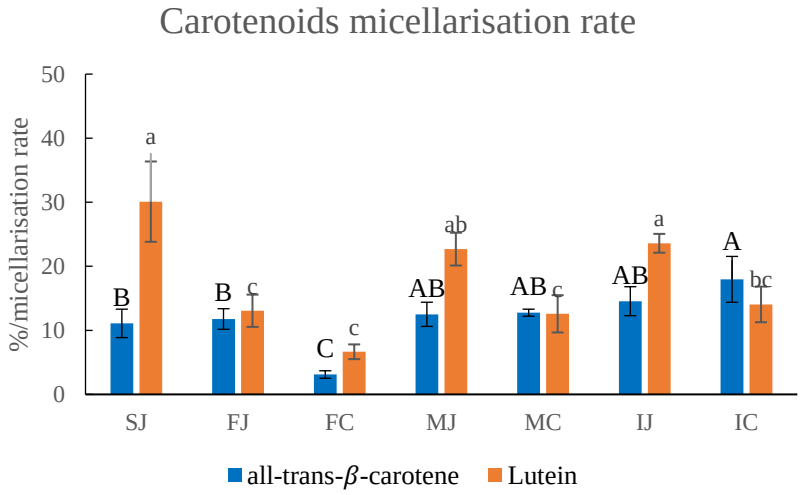


Figure 7.4: Carotenoids micellarisation rate of different processed spinach juice/CRF during digestion. Data were expressed as a MEAN±SD of triplicate independent experiments. Different letters indicate significant statistical differences according to one way ANOVA and Tukey test ($p<0.05$). Two separate groups of tests were conducted, with upper case letters represent group "all-trans-β-carotene" and lower case letters represent group "lutein". Samples include spray dried juice (SJ), freeze-dried juice (FJ), freeze-dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze dried juice (IJ), intense pasteurised freeze dried CRF (IC).

7.2.3.3. Carotenoids bioaccessibility

In this study, "bioaccessibility" was defined as the proportion of nutrient concentration in micelle phase compared to the initial nutrient concentration in samples before digestion. This factor reflects how much nutrients is available for absorption by the human body, and the stability of the nutrient during digestion and liberation of the nutrient into the micelle phase. It provides a valuable indication to help select food source and dosage to ensure nutritional efficiency of food products as their bioactive compounds claiming health benefits (Chen & Roca, 2019).

The carotenoids bioaccessibility from the spinach juice/CRF during *in vitro* digestion is shown in Figure 7.5 expressed as % and in Table 7.2 expressed as dry weight (mg/100g, DW). In fresh samples, higher carotenoids bioaccessibility was found in dried juice (SJ and FJ) than CRFs (FC). The lowest carotenoids bioaccessibility was obtained in FC (β-carotene 0.5% and lutein 2%). In addition, both mild and intense pasteurisation conditions can enhance the carotenoid bioaccessibility in freeze dried spinach juice and CRF during digestion. While the most significant increase was observed after intense pasteurisation, with the highest carotenoids bioaccessibility was observed in the IC (β-carotene 44% and lutein 10%), followed by IJ (β-carotene 21% and lutein 8%). This result suggests that intense pasteurisation was sufficient enough to break the bond of carotenoids in spinach chloroplasts, consequently enhance the micellarisation of carotenoids.

It is widely believed that thermally processing can enhance the carotenoids

bioaccessibility, this improvement could be due to the thermal treatment separating the carotenoids from its complex with proteins, thus the carotenoids transferred more easily to micelles (Palmero et al., 2014). The enhanced bioaccessibility of β-carotene and lutein after thermal treatment was also found in cooked carrots by some researchers (Hornero-Méndez & Mínguez-Mosquera, 2007; Lemmens et al., 2011b; Knockaert et al., 2012). Furthermore, *in vivo* studies conducted by Rock et al proved that β-carotene plasma levels in subjects were 3 times higher after consuming of thermally processed carrot and spinach compared with fresh ones (Rock et al., 1992; Rock et al., 1998).

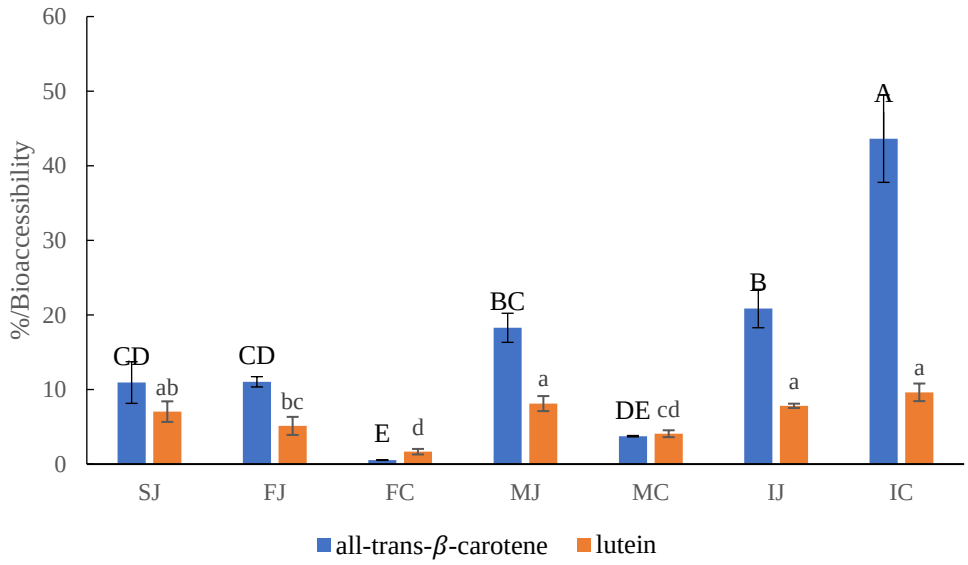


Figure 7.5: Carotenoids bioaccessibility of different processed spinach juice/CRF during *in vitro* digestion. Data were expressed as MEAN±SD of triplicate independent experiments. Different letters indicate significant statistical differences according to one way ANOVA and Tukey test ($p<0.05$). Two separate groups of tests were conducted, with upper case letters represent group "all-trans-β-carotene" and lower case letters represent group "lutein". Samples include spray dried juice (SJ), freeze-dried juice

(FJ), freeze-dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze dried juice (IJ), intense pasteurised freeze dried CRF (IC).

7.2.4.α-Tocopherol release from spinach juice/CRF during simulated human digestion

The micellarisation rate and bioaccessibility of α-tocopherol in spinach juice/CRF during *in vitro* digestion is shown in Table 7.1 and 7.2 (expressed as % and ug/g DW). From our results, the level of α-tocopherol retained in micelle or digesta phase after digestion in some samples might be too low to be detected (ND), therefore, these individual data were not shown.

In this study, SJ showed more efficiently micellarisation during digestion compared with FJ. As can be seen, micellarisation rate and bioaccessibility of α-tocopherol in SJ was 20.5% and 10.5% respectively, and no data detected in FJ. This is probably due to the porous structure and significantly reduced particle size of spray dried samples in comparison to FJ. Moreover, spray drying as a food processing technique has been proved to both preserve the nutrient over time and enhance its stability, bioaccessibility and release of these bioactive compounds. So, it is considered as a suitable technique for encapsulation which may result in a stable powder (Ezhilarasi et al., 2013; Katouzian et al., 2016; Mujica-Álvarez et al., 2020).

Among freeze dried spinach juice/CRF samples, the micellarisation rate and bioaccessibility of α-tocopherol was found to be enhanced in pasteurised samples. The

values of micellarisation rate and bioaccessibility in MJ was twice as IJ (no data in FJ). In contrast, the micellarisation rate and bioaccessibility was only detected in IC with the value of 7.8% and 15.1% respectively (no data in FC and MC). This indicates that the release and micellarisation of α-tocopherol in spinach juice/CRF during digestion can be promoted by pasteurisation. As previous studies have demonstrated that thermal processing by disrupting the food matrix can enhance α-tocopherol transfer into digesta and micelles (Gounaris et al., 1983).

Apart from the food matrix, another important factor for α-tocopherol bioaccessibility is the amount and type of oil/fat in a meal. Wattanakul et al. (2022) compared the α-tocopherol bioaccessibility in freeze-dried fresh pea vine haulm CRF with thermal processed or the addition of oil, and the results revealed that both of these methods can promote its bioaccessibility but more conspicuous with the presence of oil.

Table 7.1: α-tocopherol digestibility of different processed spinach juice/CRF during *in vitro* digestion

/%	SJ	FJ	FC	MJ	MC	IJ	IC
Micellarisation rate	20.5 ± 1.2^{a}	ND	ND	10.0 ± 2.9^{b}	ND	5.0 ± 1.8^{b}	7.8 ± 1.3^{b}
Bioaccessibility	10.5 ± 0.8^{ab}	ND	ND	8.9 ± 2.9^{ab}	ND	5.0 ± 2.3^{b}	15.1 ± 3.2^{a}

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n =3) designated with different letters in the

same row are significantly different ($p<0.05$). ND means not detected. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze dried juice (MJ), mild pasteurised freeze dried CRF (MC), intense pasteurised freeze dried juice (IJ), intense pasteurised freeze dried CRF (IC).

Table 7.2: Carotenoids and α-tocopherol content of different processed spinach juice/CRF during in vitro digestion

	All-trans-β-carotene			lutein			α-tocopherol		
mg/100g DW	undigested	micelle	digesta	undigested	micelle	digesta	undigested	micelle	digesta
SJ	78±3b	9±2cd	74±5cd	83±3e	6±1c	20±2d	26±0b	3±0a	13±1c
FJ	72±5b	8±1cd	68±9d	126±5cd	6±2c	49±5c	24±2bc	ND	ND
FC	305±38a	2±0d	54±5	429±35a	7±2bc	107±9a	41±1a	ND	ND
MJ	70±10b	13±0bc	103±5	121±8cde	10±1b	43±2bc	15±2e	1±0b	13±1c
MC	92±5b	3±0d	27±2e	184±5b	8±1bc	61±12b	12±2e	ND	1±0d
IJ	85±4b	18±2b	123±14b	95±3de	7±0bc	32±3c	20±1d	1±0b	20±2b
IC	110±16b	48±7a	270±21a	145±13bc	14±2a	101±10a	21±1cd	3±0a	40±3a

Data was analysed by one-way ANOVA according to Tukey's test ($p<0.05$) to access the differences of the samples. Means (n =3) designated with different letters in the same column are significantly different ($p<0.05$). Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze dried juice (MJ), mild pasteurised freeze dried CRF (MC), intense pasteurised freeze dried juice (IJ), intense pasteurised freeze dried CRF (IC). DW means dry weight.

7.3. Conclusion

Overall, in this chapter, the powdered spinach juice/CRF obtained through different

food processing technologies (spray drying vs. freeze drying, pasteurisation) were used during *in vitro* digestion model. The impact of food processing on digestion of lipophilic nutrients including carotenoids and α-tocopherol within simulated GI tract were discovered. It appears that enhanced bioaccessibility of lipophilic nutrients in spinach juice can be achieved by spray drying rather than freeze drying. Another aspect is that combination of pasteurisation of spinach juice prior to freeze drying can efficiently break the lipid-protein complex and stimulate the release of lipophilic nutrients, which further leads to higher proportion of micellisation during *in vitro* digestion. Given this context, the optimum pasteurisation conditions for spinach juice and CRF is mild pasteurisation (70 ℃ 15s) and intense pasteurisation (90 ℃ 5min) respectively. This study provides insight of production of spinach juice/CRF powders with the aim of achieving better bioaccessibility of lipophilic nutrients during simulated human GI tract.

Chapter VIII

8. Main Findings, Conclusion and Suggestion for future work

8.1. Thoughts on main findings

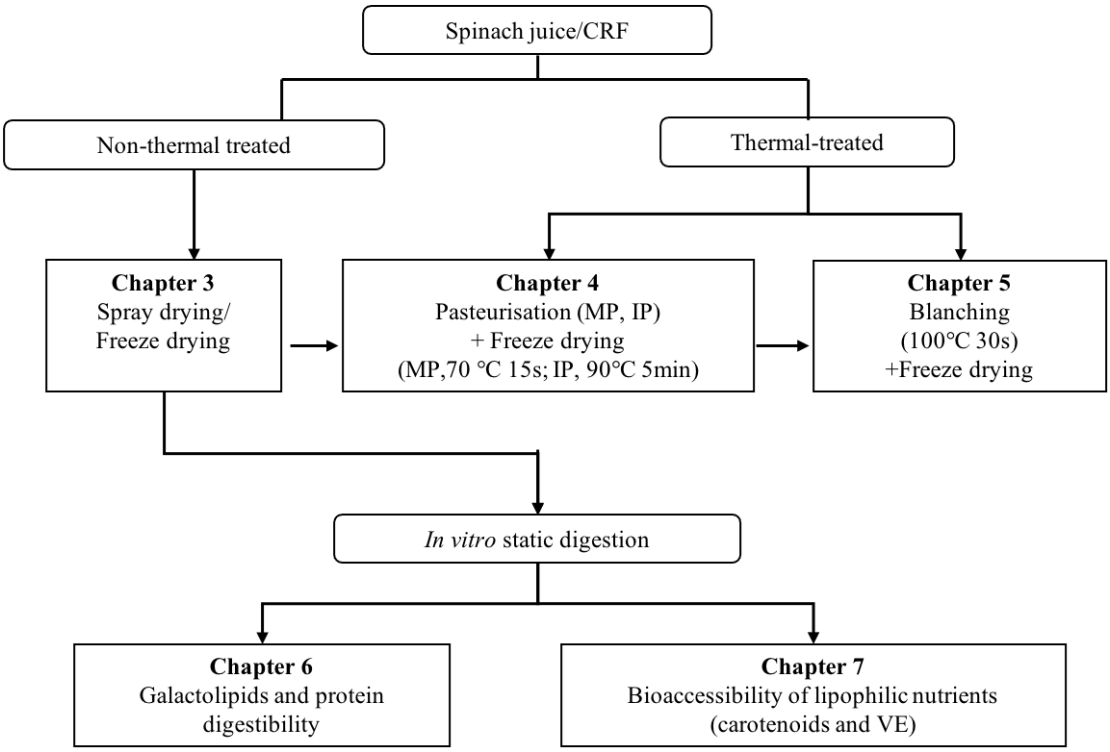


Figure 8.1: Flowchart of experimental design

Tests on the nutritional credentials of spinach juice/CRF powder products confirmed that they contain various macronutrients including proteins, lipids, minerals, carbohydrates, and micronutrients such as carotenoids, chlorophylls, α-tocopherol and essential fatty acids. Not surprisingly, spinach CRF contains higher levels of lipophilic nutrients compared with spinach juice. Moreover, both spray drying and freeze drying procedures retain the nutrients of spinach juice to a similar extent. Further, thermal

preservation techniques such as pasteurisation of juice and blanching of leaves which inactivate endogenous enzyme, eliminate/reduce the potential for pathogen contamination and growth all prevent quality loss during storage. However, thermal treatment of leaves prior to juicing, or of juice from fresh leaves causes some loss in nutrients in spinach juice/CRF powders to different extents. Significant loss of total lipids (28-36%) and lipophilic micronutrients was observed only in pasteurised CRF, but relatively stable in pasteurised juice. On the other hand, hot water blanching of spinach leaves (100°C 30s) causes 76% and 32% loss of total lipids in both freeze dried juice and CRF powders, along with the decrease of lipophilic nutrients. From these observations it seems that pasteurising juice is better than blanching leaves in terms of preparing spinach leaf-derived powders that are stable and rich in nutrients.

As a matter of fact, healthy food design should not only consider the composition, but also food structure, texture, shelf stability, dynamics and absorption of food components during digestion (Mengucci et al., 2022). Food structure can be a key indicator when developing food products that control the extent and rate of food lipids release during the digestion process (Guo et al., 2017). Whereas, it has always been overlooked in previous studies. For this reason, the correlations between the microstructure of powdered spinach juice/CRF samples and their nutrients digestibility was studied. The *in vitro* digestion model had recently been developed by researchers across Europe (Quadram Institute), and this new INFOGEST 2.0 was adopted in the current work (Sams, 2016; Brodkorb et al., 2019). It allows researchers to follow

structure changes, nutrients release and digestion, solubilization of food components along the different phases of GI tract (mouth, stomach and small intestine). Following the *in vitro* digestion model, the investigation was conducted to test the impact of processing procedure on the digestibility of nutrients from chloroplasts.

The structure of spinach juice/CRF samples in this study is the result of combinations of extrusion, heating and drying preparations. The liberation of nutrients from the food matrix and the intestinal micellization are main factors that affect bioaccessibilities during simulated digestion model (Eriksen et al., 2017). It is assumed that the liberation of chloroplasts from their cell via juicing removes one major barrier to digestion of chloroplasts and the release of their nutrients, that is the cell wall (Li et al., 2019b). In addition, the morphology and particle size of dried spinach juice/CRF powders is also an important food matrix factor for their digestibility. Several studies suggested that reduced particle size improves bioaccessibility of nutrients by increasing the surface area available for enzymes to act and thus stimulate release of bioactive compounds from food matrix (Hedren et al., 2002) . Meanwhile, the heat treatment of plant biomass can soften the plant tissues, disrupt cell membranes and cell walls, more importantly break the protein-carotenoid complex (Priyadarshani, 2017). From the results reported in this thesis, the spray dried juice (SJ) showed porous spherical structures and small particle size ($D_{4,3}$, 9.81μm), and freeze dried juice (FJ) and freeze dried CRF (FC) showed smooth and compact plate shaped structures with larger particle size ($D_{4,3}$, 66.44 and 113.95 μm). A higher bioaccessibility of carotenoids

from SJ was observed compared with FJ, and lowest bioaccessibility was in FC despite its high nutritional value. In addition, pasteurised spinach juice/CRFs powders obtained by freeze drying had larger particles ($D_{4,3}$, 69.53-182.11 μm) with rougher surface compared with fresh samples. The results reveal that higher bioaccessibility of carotenoids was found from pasteurised samples. Overall, these findings confirmed that food matrix did play a vital role in releasing and uptake of nutrients in GI tract to different extents and from different aspects.

In addition, thermal treatment enhanced the digestion of galactolipids but hindered proteins digestion derived from spinach chloroplasts. The majority of these galactolipids, MGDG and DGDG respectively, were digested in the small intestinal phase. This finding was confirmed by the increase in MGMG and DGMG bands intensity and released FFA (ALA) throughout the digestion process. In contrast to lipid digestion, the majority of spinach chloroplast protein hydrolysis occurred in the gastric phase in the presence of pepsin. Moreover, a higher amount of digested proteins (peptides below 20 kDa) was detected in pasteurised samples compared with fresh samples during digestion. This reveals that heat treatment impede the protein hydrolysis. These findings are closely linked to the bioaccessibility results, that thermal treatment of plant-based food can increase permeability of plant tissues and enhance the hydrolysis of nutrients (Pallares et al., 2018). It has been suggested that protein-rich food may cause slower rates of lipid digestion (Salentinig, 2019). The protein digestion occurs mainly in gastric and intestinal phase, facilitating the subsequent release of fat

aggregates from the degraded juice/CRF bolus (Žolnere et al., 2019).

The absorption of lipophilic nutrients (i.e., carotenoids) is affected not only by the disruption of the food matrix that stimulate their release, but also by the emulsification into lipid droplets and incorporation in mixed micelles (Wang et al., 2012b) . During the intestinal stage of digestion, lipophilic bioactive compounds are incorporated with other lipids (i.e., cholesterol, phospholipids) and lipid digestion products (i.e., free fatty acids, monoacylglycerols, lysophospholipids) into mixed micelles, then becoming available for uptake in the epithelial cells of the small intestine (Reboul, 2019a). That is the reason why Wattanakul et al. (2022) added rapeseed oil during digestion with the purpose of improving the bioaccessibility of lipophilic nutrients in pea vine haulm CRF. In this present study, porcine bile extracts were used in small intestinal phase during simulated GI tract for the formation of mixed micelles where the digestive enzymes can act on. The accumulation of FFA was incorporated into mixed micelles at the start of small intestinal phase which facilitated carotenoid micellarisation.

Overall, the findings of this study support the concept of preparing nutrient-rich powders from underutilized green biomass and highlight the impact of processing parameters on the materials properties, and hence digestibility, of these powders.

8.2. Conclusions

Overall, this thesis was set out to investigate the impact of food processing procedures on the nutritional and functional properties of spinach chloroplast materials.

The static *in vitro* digestion model was also developed to gain a detailed insight into the influence of food processing on the digestibility of lipophilic nutrients and proteins in chloroplast materials. The main findings are as follows:

1. Chloroplast can be liberated from the confinement of the plant cell wall by disrupting the green biomass using a twin-gear juicer. This offers a simple, clean and effective procedure that circumvents the need for solvents or chemicals.

2. Both spray drying and freeze drying methods produce dried spinach chloroplast materials which efficiently remove the moisture and stabilise the nutrients level for a long period of storage until further utilisation.

3. Spinach chloroplasts contain high levels of various macro and micronutrients, including total proteins, lipids, carotenoids, chlorophylls, α-tocopherol and α-linolenic acid. Chloroplast rich fractions (CRF) contain higher total lipids and their lipophilic nutrients than spinach juice.

4. Heat treatment of green biomass or fresh juice such as blanching and pasteurisation can effectively inactivate endogenous enzymes and prevent the free fatty acid release due to the inhibition of endogenous galactolipases.

5. Blanching spinach leaves promotes a significant loss in lipophilic nutrients in both juice and CRF. However, pasteurisation of spinach juice from fresh leaves retains the levels of these nutrients in spinach juice, but not in CRF.

6. Compared with fresh spinach juice/CRF powders, both mild (70℃ 15s) and intense (90℃ 5min) pasteurised spinach samples have improved bioaccessibility of lipophilic nutrients (carotenoids and α-tocopherol) as determined using *in vitro* digestion model.

7. Pasteurisation of spinach juice prior to making juice/CRF powders enhances the digestibility of galactolipids but hinders protein digestion.

8. The majority of the galactolipids and acylated galactolipids digestion occurs in the intestinal phase during simulated digestion with the galactolipase active enzymes PLRP2 and CEH/BSSL which is found in PPE. High levels released free fatty acids especially α-linolenic acid, were noted in the current work. Moreover, the galactolipase activity in PPE hydrolyses MGDG more extensively than DGDG.

9. The majority of protein in spinach juice/CRF hydrolysed in gastric phase with the addition of pepsin during *in vitro* digestion. Compared with fresh spinach juice/CRF powders, larger amount of digested protein below 20 kDa was detected in pasteurised samples during digestion.

8.3. Suggestion for future work

Several limitations associated with the current study can be overcome. These optimization procedures are beyond the scope of the current project but will be detailed below for future reference.

As shown in previous studies, thermal processing has been widely used for food

preservation, and the nutrients bioaccessibility in thermal processed foods are positively promoted (Lemmens et al., 2011a; Barba et al., 2017). Therefore, pasteurisation and blanching of spinach biomass were compared in this study. The pasteurisation steps followed in this study involved samples being left in a cold room for a period of time (roughly 4 ℃, 24h) prior to undergoing the heating steps. Even at 4ºC, it is anticipated that undesirable accumulation of FFA due to the hydrolysis of galactolipids by endogenous enzymes in fresh spinach juice occurs. This problem can be overcome using blanched spinach leaves prior to juicing. The loss of galactolipids in blanched samples was merely due to leaching into the boiling liquor. However, the yield of blanched CRF is extremely low. In this consequence, the future work for the preparation of thermal processed chloroplast materials could be directly pasteurising fresh juice.

Despite the benefits of thermal treatment on plant-based foods, the product quality and the micronutrients content can be negatively affected. Therefore, the development of non-thermal technologies should be considered in future studies. The development of these approaches will be critical and needed to facilitate the production of extracts that contain thermally labile bioactive compounds (Jayathunge et al., 2017; Lorenzo et al., 2019; Ozkan et al., 2022). Recently, pulse electric fields (PEF), ultrasounds (US), high pressure processing (HPP) and high pressure homogenization (HPH) have been proposed for the purpose as a means to preserve the levels of thermally labile compounds found in plant tissues (López-Gámez et al., 2021). Moreover, emerging

evidence suggests that some of these technologies can enhance plant bioactive nutrients bioaccessibility as a result of modifying the structure of plant tissues.

Critical in the development of any food-based product is the confirmation that any nutrient present is bioaccessible, and available for cellular uptake. To this end, the bioaccessibility of micronutrients in this study was investigated using an *in vitro* digestion model according to the INFOGEST protocol (Sams, 2016; Brodkorb et al., 2019).There is a major limitation in interpreting galactolipids digestion data by using HPTLC analysis as described in section 5.5. The interference bands on HPTLC plates next to target galactolipid will hinder the integration of their peak area, and cause troubleshooting for calculation. The interference bands in this study could be originated from the lipid contamination from the porcine bile extract. These inference bands were not observed in a previous study reported from our research group (Wattanakul et al., 2019). The key difference here being that in the work of Wattanakul, the porcine bile extract was replaced by the bile salt sodium taurodeoxycholate (NaTDC). Consequently, the inference bands (yellow/grey colour) were partially or wholly merged with the DGDG bands which leads to the difficulty in the identification and quantification of DGDG. Another major problem is the sampling efficiency during the intestinal phase. The addition of PPE within the intestinal phase step caused clumps to form, and gelatinization of the reaction fluid inside the digestion tube. The formation of clumps and gelatinization of the mixture hindered the sampling efficiency and made analysis of samples difficult. This phenomenon happens due to the mechanical forces generated

by the rotating tubes, as these forces are not strong enough to break the PPE and mix the cocktails homogenously. This step clearly needs some improvement and possibly the use a propeller inside of the tube revolver to facilitate. The propeller would provide a more evenly and thoroughly mix reaction fluid to aid interactions between the food matrix with the PPE. This hypothesis was confirmed in the work of Sutcharit et al. (2023) who developed a modified digestion device containing an installed propeller, pH-stat and thermometer. In addition, it is worth considering an alternative to the PPE in future work to develop a more reliable analytical model. PPE is commonly used and recommended for use in the INFOGEST model. However, as reported by Salhi et al. (2020), PPE is not an ideal substitution of the lipolytic enzymes contained in human pancreatic juice (HPJ). Indeed, as compared galactolipase activities of PPE with human (HPJ) and porcine (PPJ) pancreatic juice, it was found lowest in PPE (0.31± 0.01 U/mg) and higher levels in PPJ (1.78±0.02 U/mg) then HPJ (7.73±0.2 U/mg) at pH 8 (Salhi et al., 2020). The galactolipase activity relies on the pancreatic lipase-related protein 2 (PLRP2) and carboxylester hydrolase/bile salt-simulated lipase (CEH/BSSL) (Sias et al., 2004; Kergomard et al., 2023). The low galactolipase activities of PPE indicate that the PLRP2 and CEH/BSSL are still contained but in low levels and were not detected by mass spectrometry (Salhi et al., 2020). Given this evidence, PPJ or HPJ should be considered in future digestion studies for better testing the bioaccessibility of the dietary lipids.

In this study, we have used *in vitro* static model, and the enzyme selection and

incubation conditions are adapted by the study objective. Whilst the static model is widely used with the advantages of simple and cost-effective to operate, the model can only maintain initial conditions at each phase in GI tract, it is therefore not a comparable simulation of complicate *in vivo* conditions (Thuenemann, 2015). Dynamic models are more complicated but more physiologically relevant than static models (Dupont et al., 2015). Recently, several *in vitro* dynamic digestion models have been developed, such as the TIM-1, DIDGI and ESIN, they are able to closely mimic the food digestion during the GI tract (Dupont et al., 2019). These models can reproduce the dynamic transit times, digestive enzymes secretion rate, dynamic pH changes, and peristaltic movements such as shearing and grinding which can largely affect the breakdown of large particles and release of nutrients of food matrix during digestion process (Sensoy, 2021). The data obtained from these models are validated against *in vivo* data, which showed that they accurately reproduced the gradual transition of ingested food through the GI tract (Barroso et al., 2015). Until now, only a limited number of studies have focused on the comparison of static and dynamic *in vitro* digestion models on the digestibility of carotenoids and proteins. Lee et al. (2018a) compared the static digestion model and dynamic model as a means to estimate the bioaccessibility of lutein in high-fat foods. Similarly, Egger et al. (2019) determined the milk proteins hydrolysis in skim milk powders with both static (INFOGEST) and dynamic (DIDGI) models. The results from both studies exhibited strong similarities to *in vivo* data from the pig. However, the dynamic model showed better physiological situation at the level of free

amino acid release. Nonetheless, *in vitro* digestion protocols have their limitations to completely mirror the complexity of the *in vivo* situation, which leaves vast potential for future studies (Bohn et al., 2018). An *in vivo* study has been conducted in our group by Gedi et al. (2019), by including the dried spinach leaf powders and chloroplast rich fraction (CRF) partially as fishmeal content in a fish feeding trail for their growth and nutrients uptake. The results showed that the zebrafish fed with CRF showed higher lutein and PUFA (alpha-linolenic acid, C18:3 n-3 and hexadecanoic acid, C16:3) which is in agreement with *in vitro* data.

Furthermore, the recovery of total lipids and biomolecules from spinach could be an important challenge for sustainable resource exploitation. This gives insight for the utilisation of natural green plants as nutritional supplements, has an additional advantage in terms of environmental protection through food processing waste minimization. For this purpose, a mass balance of the processing could be carried out determining the mass of lipid and lipophilic nutrients recovered from the fresh/heat treated spinach and comparing to the total dry starting mass.

Overall, there is growing interest in the development of plant-based food materials which contain high nutritional quality coupled with the presence of functional food ingredients. Future research may explore emerging technologies (non-thermal treatments) for the production of quality stable spinach chloroplasts. The parameters of static *in vitro* digestion model could be further updated in accordance with the specific research target for better mimicking the GI environment, including the transition time,

digestive enzymes, pH, mechanical forces, etc. Furthermore, both dynamic *in vitro* digestion model and *in vivo* digestion is worth to be conducted for understanding the food digestion behaviours during human GI tract in order to design healthier food products. Despite the expensive cost and ethically limitations of human feeding trials, it is still worth to be conducted by consuming chloroplasts during daily meal. This may provide more reliable data for the bioacessibility of chloroplast nutrients and comparable with the fish feeding trial data and test the accuracy of *in vitro* studies.

IMPACT OF THE COVID-19 PANDEMIC

The first national lockdown (March 2020- September 2020) occurred when I was in 2nd year of my research. I went back to China during May-August 2020, and returned back to lab in December 2020.

Following nearly a year of complete closure, the laboratories in my building remained at minimum capacity (25%) until 01/06/21. The chemistry laboratory (where I conducted around 90 % of my practical work) was limited to a maximum occupancy of 2 people at this time. Given that I was one of around 20 lab users, this significantly limited the amount of practical work I was able to complete. After 01/06/21, the maximum occupancies of some labs in my building increased, but my primary laboratory remained at a maximum occupancy of 2 persons.

On 27/07/21 our safety team announced plans for a phased approach, planning to return to 100% occupancy before the start of term. The building did not resume 100% capacity until 30/09/21. This disrupted my work further. Consequently, closures resulting from the pandemic forced me to push back experimental work. This compounded with supply chain delays, which repeatedly delayed experiments.

In September, the lab reopened with more restrictions, only 1 working slot (8:30am – 12:30pm or 13:00pm – 17:00pm) can be booked per day and maximum 3 days week per person. A lot of paper work are required to be completed and approved, including procedure risk assessments (PRA, for every lab-related activity), activity risk assessment (ARA, for every lab-related activity), permission to work (PTW), moodle

induction course, moodle quiz (covid-19), guide to working during covid-19 pandemic.

The pandemic also affected me personally on my mental health. During March and April 2020, I was isolated in my flat, experiencing daily anxiety, stress and weight-loss. May - August 2020, upon returning to China, I endured a five-week quarantine in a hotel. During this period, I can only read papers, write paper manuscripts and have online meetings with my supervisor and colleagues. Concerns about my PhD studies weighed heavily on my mind. September 2020, I went back to campus to get prepared for laboratory work. However, after the 2nd year viva, my internals doubted my ability to complete PhD study with suggestions for a shift to an MPhil study. Seeking another chance to prove myself, I got approval from both my supervisor and internals, with the final decision deferred until a re-viva in May 2021. I retuned back to my lab experimental work in December and since that day I worked at least 5 days a week in the lab until June 2022. Since 2021, regular consultations with a therapist became necessary, and I grappled with persistent anxiety, depression, and insomnia, a struggle that persists to this day.

Appendices

Chapter 5:

Table 5.1: Yield of fresh and blanched spinach juice (ml/kg)

	Spinach (g)	Spinach after blanching (g)	Juice (ml)	Sieved juice[1] (ml)	Yield (ml/kg)
Control	500	N/A	350	300	600
Blanched[2]	500	465	300	235	470

1: Both fresh and blanched spinach juice was filtered through a 75 μm sieve.

2: Blanching condition (100 ℃ 30s)

Table 5.2: Yield of freeze dried fresh and blanched spinach juice/CRF powders（% DW）

Yield/% DW	Juice	CRF
Control	4	0.74
Blanched	1.1	1.1

The yield of freeze dried fresh and blanched spinach juice and CRF powders was calculated by the mass of powders passed through 250 μm sieve compared with raw spinach.

Table 5.3: Main nutrients loss of spinach juice/CRF under pasteurisation and blanching conditions

Nutrients loss/%	Juice			CRF		
	MJ	IJ	BJ	MC	IC	BC
Total lipids	3	9	76	28	36	32
Total chlorophylls	8	39	93	55	70	59
Total carotenoids	/	1	92	51	57	70
VE	85	50	89	71	24	36
ALA	/	/	92	24	29	55

Abbreviations: MJ Mild pasteurised freeze dried juice; MC Mild pasteurised freeze dried CRF; IJ Intense pasteurised freeze dried juice; IC Intense pasteurised freeze dried

CRF. BJ Blanched dried juice; BC Blanched dried CRF. Total Chl. Total Chlorophylls (a+b); Total carotenoids: beta-carotene and lutein.

Chapter 6:

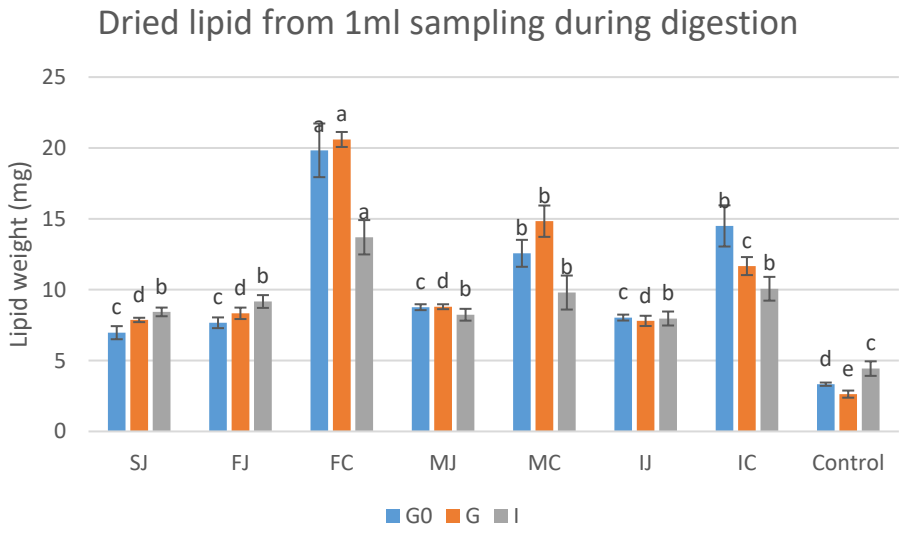


Figure 6.1: The total lipid content extracted from 1 ml sampling during digestion. Samples include spray dried juice (SJ), freeze dried juice (FJ), freeze dried CRF (FC), mild pasteurised freeze-dried juice (MJ), mild pasteurised freeze-dried CRF (MC), intense pasteurised freeze-dried juice (IJ), intense pasteurised freeze-dried CRF (IC). Control experiments are spinach chloroplasts replaced with pure water by mass and undergoing the same digestion process as other samples. G0: End of oral phase, G: End of gastric phase, I: End of intestinal phase.

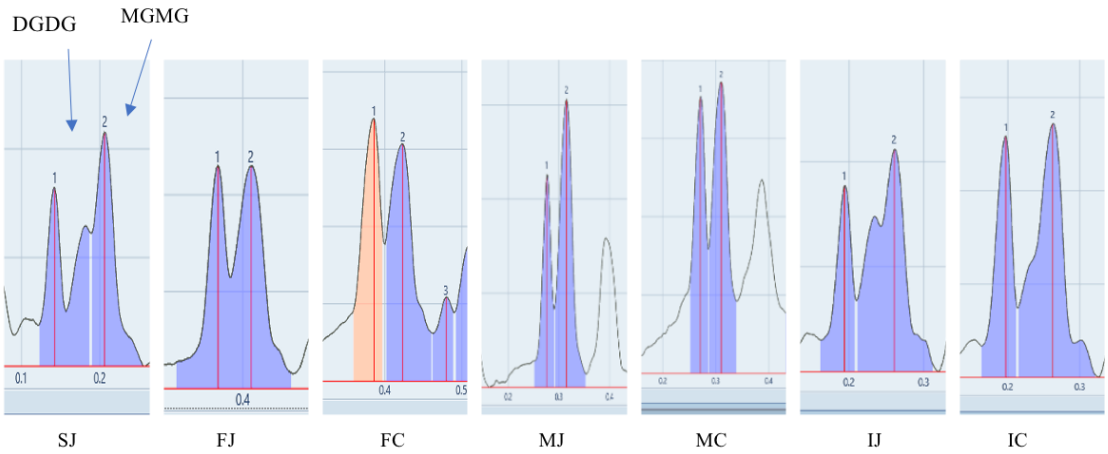


Figure 6.2: Band intensity of different treated spinach chloroplasts at the end of *in vitro* digestion. Peak 1 stands for the area of DGDG, peak 2 stands for the interference band overlapped with the bands for DGDG and MGMG.

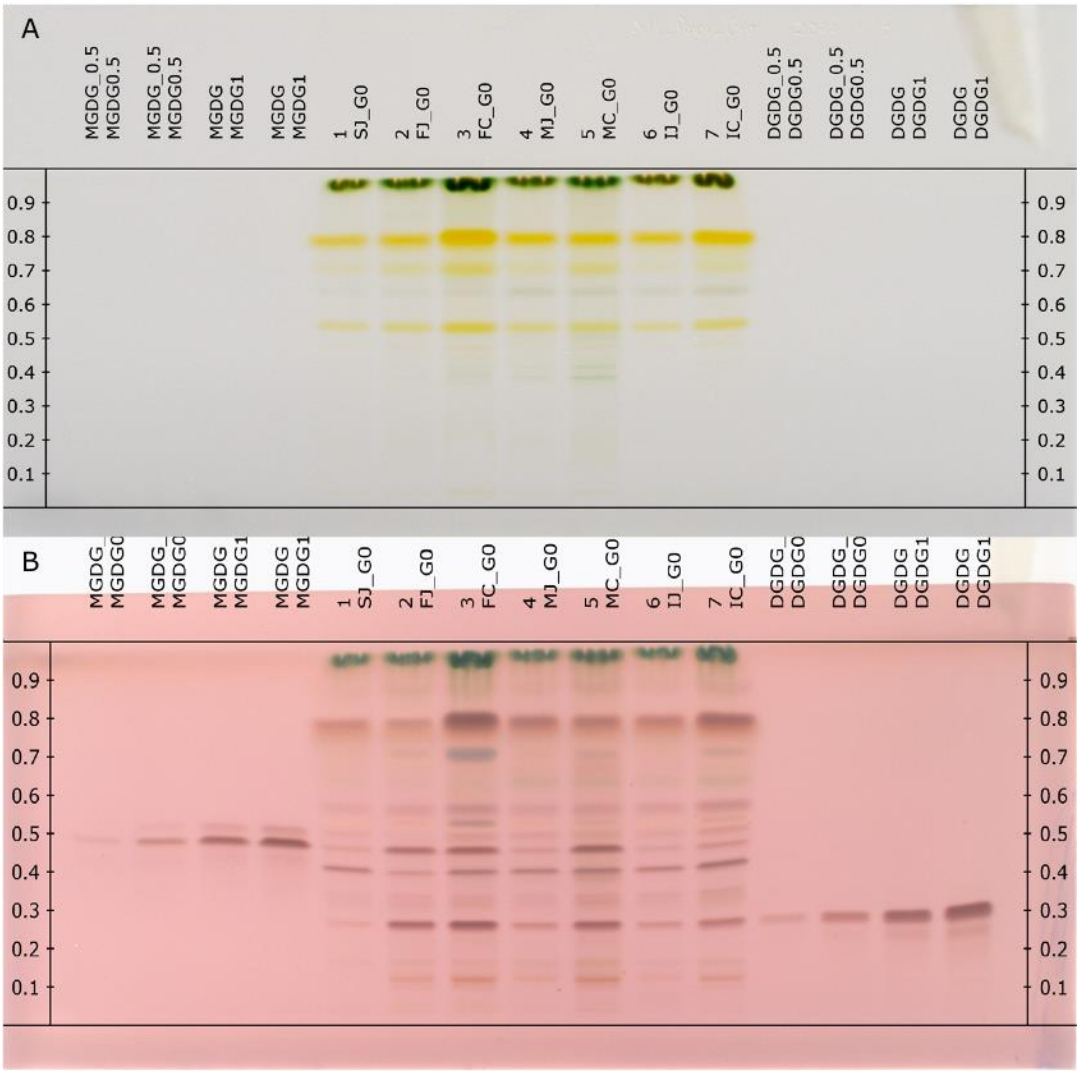


Figure 6.3: Representative of HPTLC chromatography for the quantification of polar lipid (galactolipid) in spinach chloroplast materials. A) HPTLC plate after developing and before staining with thymol solution B) HPTLC plate after staining with thymol solution and charring at 110 ℃ 10min. Samples loading volume at 15 µL. Polar lipids standards: monogalactosyl diglycerides (MGDG) and digalactosyl diglycerides (DGDG).

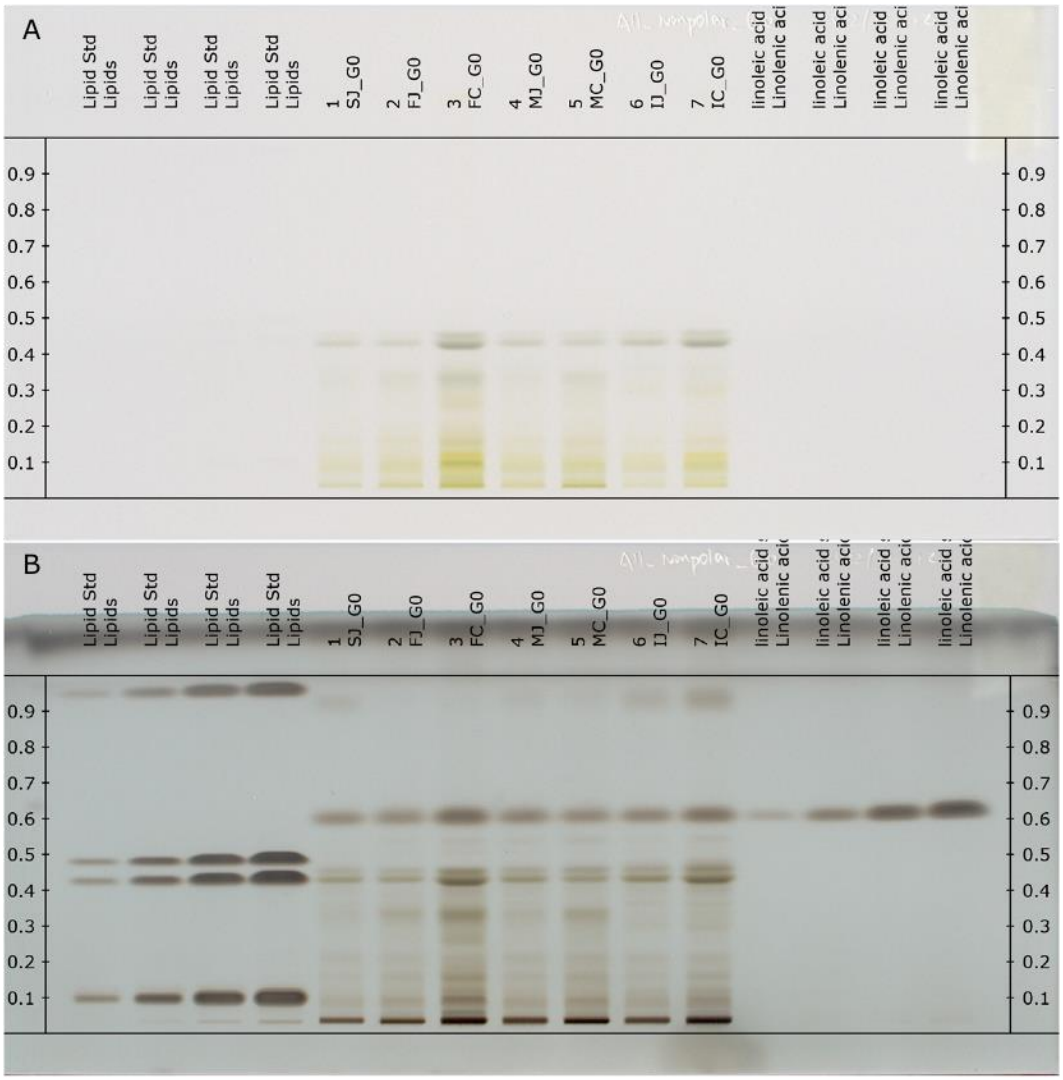


Figure 6.4: Representative of HPTLC chromatography for the quantification of non-polar lipid (fatty acid) in spinach chloroplast materials. A) HPTLC plate after developing and before staining with copper acetate-phosphoric acid solution B) HPTLC plate after staining with copper acetate-phosphoric acid solution and charring at 180 ℃ 15min. Samples loading volume at 3 µL. Nonpolar lipids standards: mix lipid standard and alpha-linolenic acid. Mix lipid standard consist of monoacylglycerol (MAG), 1,2 diacylglycerol (1,2DAG) l, 1,3 diacylglycerol (1,3DAG) and triacylglycerol (TAG).

Both polar and non-polar lipids were analysed first without staining which allowed qualitative visualisation of pigments such as carotenoids and chlorophylls. (Figure 6.3 A and Figure 6.4 A).

HPTLC separation coupled to derivatization by thymol-sulfuric acid reagent and scanning densitometry was applied in this experiment for the quantification of polar lipids (galactolipids) present in spinach chloroplasts and galactosylated lipolysis products resulting from *in-vitro* digestion (Figure 6.3 B). Thymol solution is used to selectively stain the galactolipids (MGDG & DGDG) avoiding the interference of other pigments, such as chlorophylls that may occur during the densitometric analysis by the HPTLC. Initially, thymol solution was used for detection of carbohydrates, that a sugar solution turns carmine red when mixed with a solution of thymol with 15–20% alcohol and excess sulphuric acid (Molisch, 1886). In 2021, Sahaka and co-workers used the thymol-sulfuric acid reagent for the qualitative analysis of galactolipids (Sahaka et al., 2021). In this experiment, the galactolipids was detected on thin layer chromatography (TLC) plates by charring in the presence of thymol solution at 110 ℃ for 10 min, after which the bands of samples and standards started to turn brown with higher sensitivity. MGDG and DGDG was used as markers to monitor the breakdown of chloroplast membrane during the *in vitro* digestion.

As shown in Figure 6.3 A, there are some yellow pigments showed on the plate before staining with thymol staining and charring. We suspect these yellow pigments are carotenoids in different forms (i.e. beta-carotene or lutein). The carotenoid content

in different samples is shown in Figure 4.4, CRFs contain higher carotenoid concentrations than juice; significant apparent losses of carotenoids is only observed in CRFs after pasteurisation (48-61% losses, DW) but remained relatively stable in juice. This may be due to real losses of carotenoids, but we have already observed the dilution of lipids in CRF samples due to the increase of proteins in the CRF pellet caused by precipitation of denatured proteins after heat treatment. The intensity of these yellow bands follows the same trend, which confirms our hypothesis.

Carotenoids will stay yellow and not interact with thymol solution which stains galactose rings in galactolipids and appears burgundy red after heat treatment. However, some of these carotenoid pigments commigrated with the acyl-MGDG bands at Rf 0.8, which will still influence the band intensity quantification of acyl-MGDG. Therefore, with the lack of pure standard of acyl-MGDG and difficulty in its band intensity quantification, we can only do qualitative analysis.

As for non-polar lipids, copper acetate-phosphoric acid solution was utilised for the quantitative analysis of free fatty acid release from spinach chloroplast materials during *in-vitro* digestion (Figure 6.4 B). This method is able to quantitatively detect unsaturated lipids, including phospholipids, neutral lipids, fatty acids, and hydrocarbons (such as squalene)(Churchward et al., 2008). The use of copper acetate-phosphoric acid solution as a fluorometric staining reagent offers reproducible improvements over existing fluorescent reagents such as Nile Red or molybdenum blue reagent after charring, at the same time provides more significantly enhanced sensitivity

of detection, ease of use, and lower cost (Fewster et al., 1969). Copper acetate in phosphoric acid is able to develop a strong intensity of staining regardless of the degree of unsaturation, the higher unsaturation result in higher staining intensity. In this experiment, the lipids were detected on thin layer chromatography (TLC) plates by charring in the presence of copper salts at 180 ℃ for 15min, after which the bands of samples and standards started to turn into black with higher sensitivity.

Table 6.1: Calculation of the expected amount of lipids in spinach chloroplast samples during digestion.

G0	Sample/g	Lipid/%	Expected lipid/g	Extracted lipid/g in G0	Volume/ml	Total (g lipid/20ml)	Apparent extraction efficiency/%
SJ	1.5	13	0.195	0.0037	20	0.074	38
FJ	1.5	12	0.18	0.0044	20	0.088	49
FC	1.5	28	0.42	0.0165	20	0.33	79
MJ	1.5	12	0.18	0.0055	20	0.11	61
MC	1.5	20	0.3	0.0093	20	0.186	62
IJ	1.5	11	0.165	0.0047	20	0.094	57
IC	1.5	18	0.27	0.0112	20	0.224	83

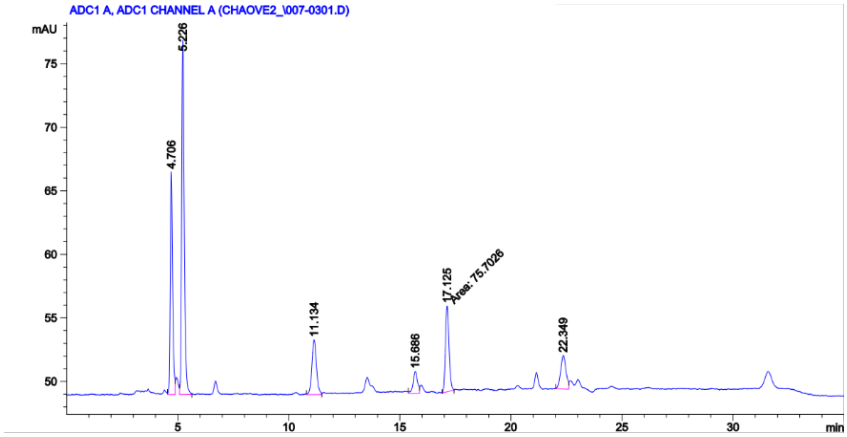
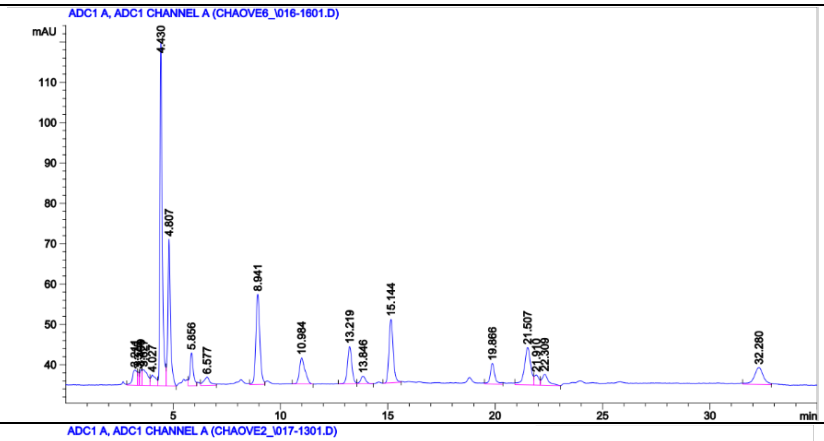
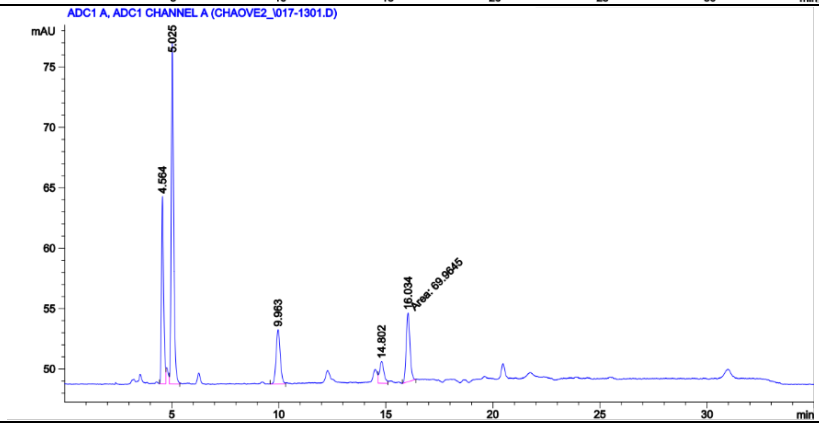
G	Expected lipid/g in G	Extracted lipid/g in G	Volume/ml	Total (g lipid/20ml)	Apparent extraction efficiency/%
SJ	0.195	0.0053	20	0.106	54
FJ	0.18	0.0057	20	0.114	63
FC	0.42	0.018	20	0.36	86

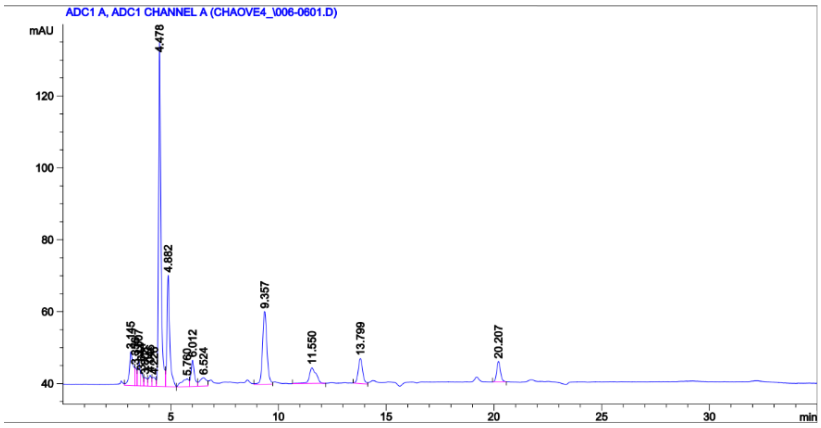
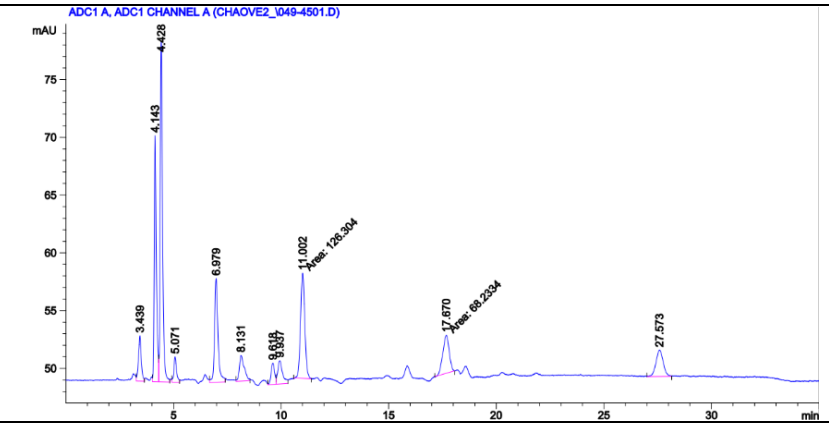
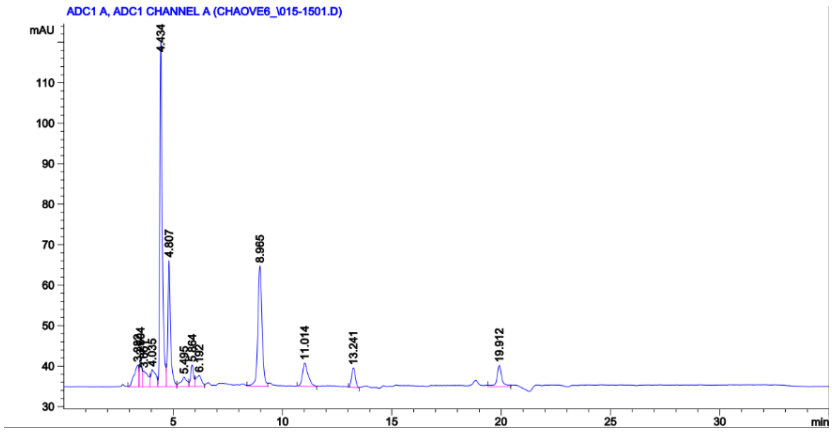
MJ	0.18	0.0062	20	0.124	69
MC	0.3	0.012	20	0.24	80
IJ	0.165	0.0052	20	0.104	63
IC	0.27	0.0091	20	0.182	67

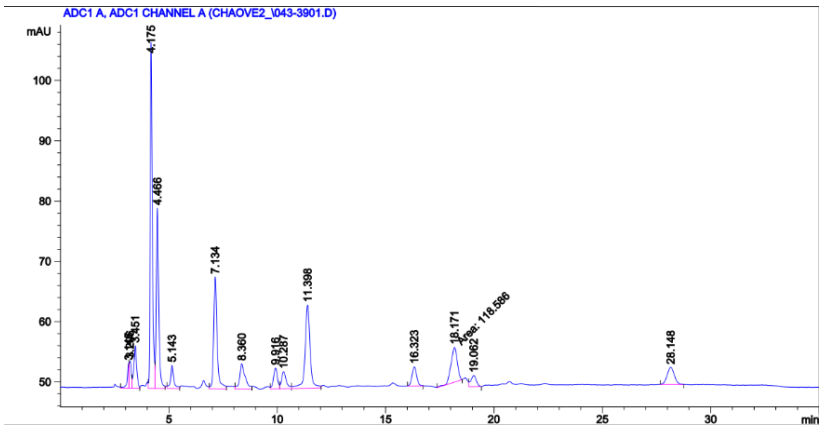
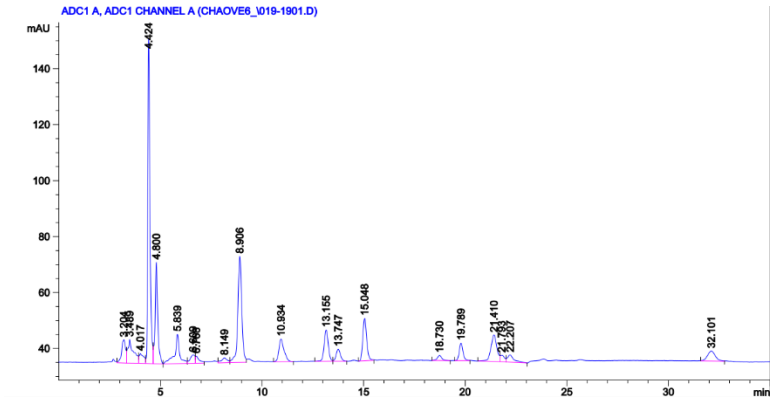
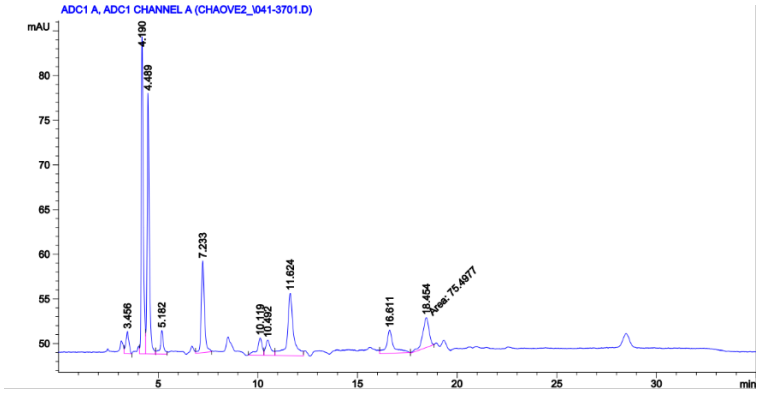
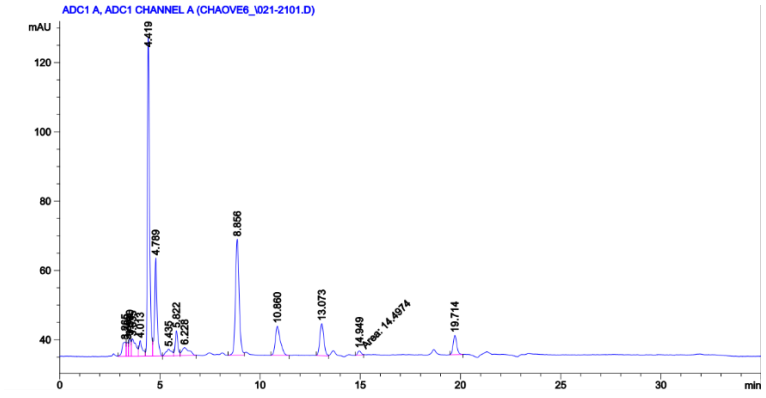
I	Expected lipid/g in I	Extracted lipid/g in I	Volume/ml	Total (g lipid/28ml)	Apparent extraction efficiency/%
SJ	0.1365	0.004	28	0.112	82
FJ	0.126	0.0048	28	0.1344	107
FC	0.294	0.0093	28	0.2604	89
MJ	0.126	0.0038	28	0.1064	84
MC	0.21	0.0054	28	0.1512	72
IJ	0.1155	0.0036	28	0.1008	87
IC	0.189	0.0057	28	0.1596	84

(The expected lipid amount in intestinal phase is 30% lower from G, as 6ml sampling took out from 20ml of gastric tube.)

Chapter 7

SJ	Before digestion	
	After digestion	
FJ	Before digestion	

	After digestion	
FC	Before digestion	
	After digestion	

MJ	Before digestion	
	After digestion	
M C	Before digestion	
	After digestion	

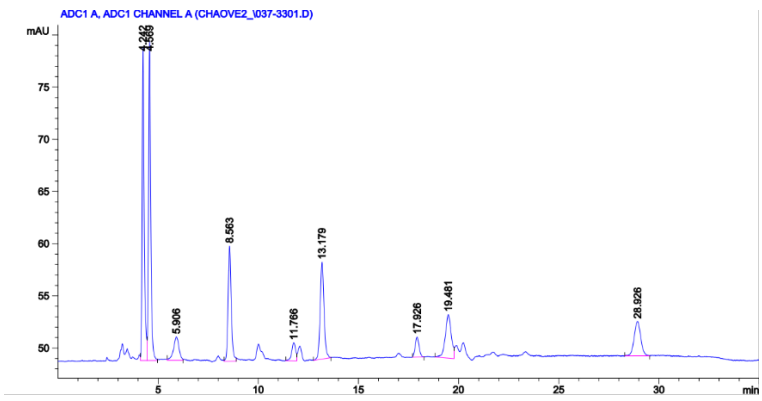
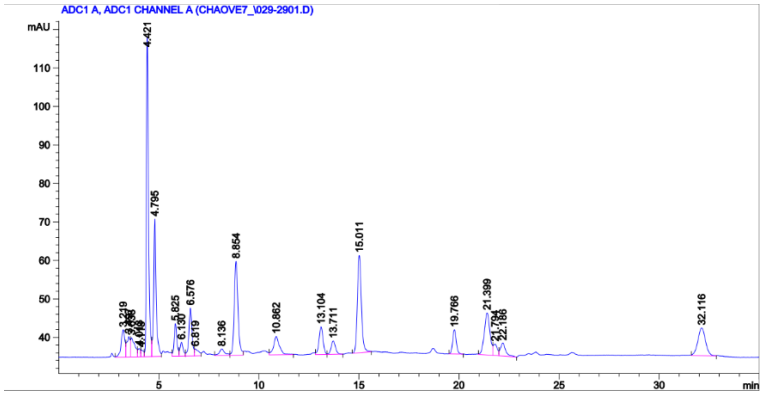
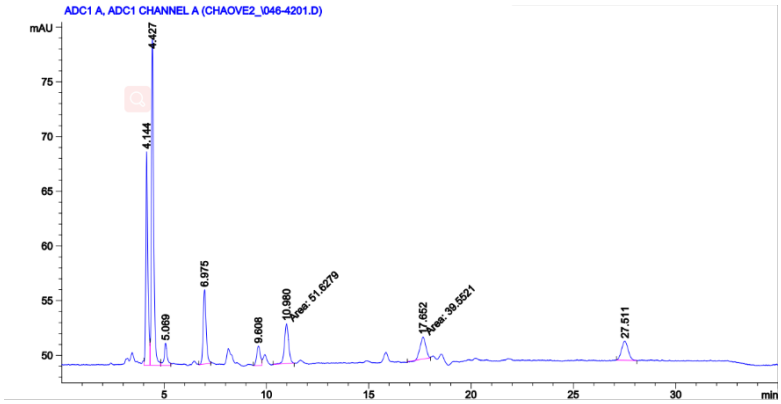
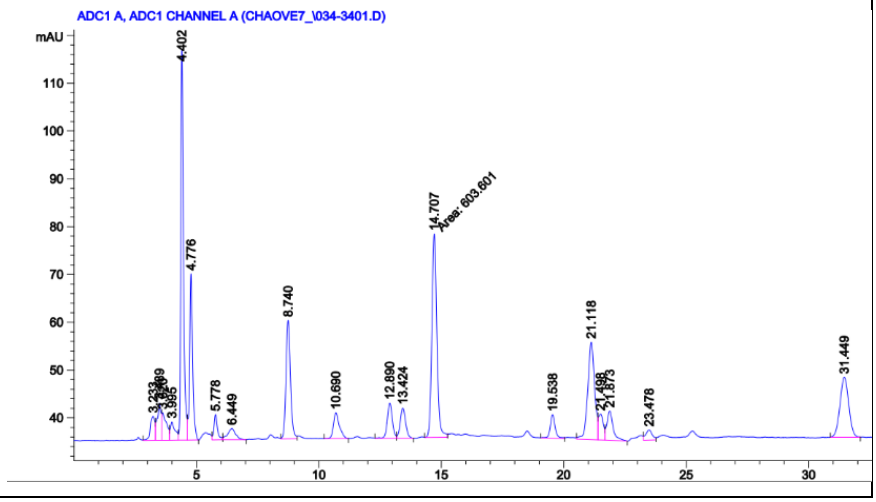
IJ	Before digestion	
	After digestion	
IC	Before digestion	
	After digestion	

Figure 7.1: Chromatogram of α-tocopherol in spinach chloroplasts before and after digestion. The scale of chromatogram is not consistent and the peak areas should not be compared directly to each other due to different dilution factors. The slightly shift retention time of α-tocopherols before and after digestion in this study were due to the retention time drift in HPLC overtime, and the standards were set in between every five samples in order to confirm the target compound. (The initial/undigested samples, the α-tocopherol peak is around 17-18 min, while the digested ones are around 14-15 min).

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