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Nutrient value and stability of chloroplast-rich fraction (CRF) material from pea vine haulm, and the role of galactolipolytic enzymes during digestion

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ABSTRACT

Underutilised green plant material is a rich source of chloroplasts, organelles in plants and other photosynthetic organisms, which are responsible for the conversion of light energy into chemical energy. The removal of intact chloroplasts from their cell wall confinement offers a novel way to obtain lipophilic nutrients and galactolipids from green biomass, especially carotenoids, vitamin E, and galactolipids (the main membrane lipids in plants and they represent a major source of the essential α-linolenic acid (18:3, ALA)).

We have developed a physical method of recovering a chloroplast-rich fraction (CRF) from postharvest, pea vine field residue (haulm) by using slow-screw twin-gear juicer without added water. The CRF from pea vine haulm (PVH), like CRF from spinach leaves, is a good source of lipophilic nutrients, including β-carotene (provitamin A), lutein, α-tocopherol (vitamin E), phylloquinone (vitamin K), and essential fatty acids.

Steam sterilisation of pea vine haulm prior to generating CRF was used to inactivate enzymes after harvesting and extend the shelf life of nutrients. The stability of selected nutrients (β-carotene, lutein, and α-tocopherol) in the freeze-dried CRF material was measured over 84 days; the impact of storage temperature (-20°C, 4°C, 25°C and 40°C), light and air on nutrient stability was established. All three nutrients were stable at -20°C and 4°C in the presence or absence of air; this stability was lost at higher temperatures in the presence of air. The extent and rate of nutrient breakdown significantly increased when the CRF samples were exposed to light. β-Carotene appeared to be more susceptible to degradation than lutein and tocopherol at 40°C in the presence of air, but when CRF was exposed to light all three nutrients measured were significantly broken-down during storage at 25°C or 40°C, whether exposed to air

or not.

The bioaccessibility (% nutrient available for uptake) of some essential lipophilic nutrients, contained in a chloroplast-rich fraction (CRF) recovered from post-harvest pea vine field residue (haulm) (PVH), was measured using an *in vitro* gastrointestinal human digestion model with and without additional rapeseed oil. The impact on nutrient stability during digestion (in-digesta) and bioaccessibility of postharvest heat treatment of the biomass (HPVH), or the juice (HJ) derived from the biomass, was determined. The results show that both heat treatments stabilised β-carotene, lutein, α-tocopherol and α-linolenic acid in the CRF material in-digesta. The presence of oil during digestion appears to improve retention of β-carotene, and the bioaccessibility of β-carotene in CRF from HPVH and HJ, except in the case of lutein and α-tocopherol. Conversely, the combination of heat treatment both HPVH and HJ and the presence of oil in PVH CRF enhanced the retention and bioaccessibility of β-carotene, lutein, and α-tocopherol.

The release of nutrients that are embedded into the chloroplast structure is likely to be affected by the extent to which thylakoid membranes are hydrolysed during digestion. The PVH CRF and spinach CRF were therefore measured *in vitro* under simulated gastrointestinal conditions. Using a two-step static model, CRF from both spinach leaves and postharvest pea vine field residue (haulm) were first exposed to enzymes from rabbit gastric extracts and then either to pancreatic enzymes from human pancreatic juice (HPJ) or to porcine pancreatic extracts (PPE). The lipolysis of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) was monitored by thin layer chromatography and gas chromatography of fatty acid methyl esters. For both CRF preparations, MGDG and DGDG were converted to mono galactosylmonoacylglycerol (MGMG) and digalactosylmonoacylglycerol (DGMG), respectively, during the intestinal phase and ALA was the main fatty acid released.

Galactolipids were more effectively hydrolysed by HPJ than by PPE, and PPE showed a higher activity on MGDG than on DGDG. These findings may be explained by the higher levels of galactolipase activity in HPJ compared to PPE, is probably due to presence of pancreatic lipase-related protein 2 (PLRP2) in HPJ. Thus, we showed that CRF galactolipids are well digested by pancreatic enzymes and represent an interesting vehicle for ALA supplementation in human diet.

LIST OF PUBLICATIONS

1. **Wattanakul, J**., Sahaka, M., Amara, S., Mansor, S., Gontero, B., Carrière, F., Gray, D. 2019. In vitro digestion of galactolipids from chloroplast-rich fraction (CRF) of postharvest, pea vine field residue (haulm) and spinach leaves. *Food & Function*, 10 (12), 7806-7817.
2. Sahaka, M., Amara, S., **Wattanakul, J**., Gedi, M., Aldai Elkoro-Iribe, N., Parsiegla, g., Lecomte, J., Christeller, J. T., Gray, D., Gontero, B., Villeneuve, P., Carrière, F. 2020. The digestion of galactolipids and its ubiquitous function in Nature for the uptake of the essential a-linolenic acid. *Food & Function*.
3. **Wattanakul, J**., Syamila, M., Briars, R., Ayed, C., Price, R., Darwish, R., Gedi, M. A., Gray, D. A. 2021. Effect of steam sterilisation on lipophilic nutrient stability in a chloroplast-rich fraction (CRF) recovered from postharvest, pea vine field residue (haulm). *Food Chemistry*, *334*, 127589.

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LIST OF ABBREVIATIONS

α-toc	α-tocopherol
β-car	β-carotene
µg	microgram
µL	microlitre
µm	micrometre (micron)
A	absorbance
A	pre-exponential constant
Ala	Alanine
ALA	α-linolenic acid
AMD	age-related macular degeneration
Amm	ammonia
ANOVA	analysis of variance
ARA	arachidonic acid
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
^{o}C	celsius
C	concentration
Cal	calorie
$CaCl_2.2H_2O$	calcium chloride dehydrate

LIST OF ABBREVIATIONS (cont.)

CCK	cholecystokinin
CEH/BSSL	carboxyl ester hydrolase/ bile salt simulated lipase
$CHCl_3$	chloroform
CH_3OH	methanol
cm	centimetre
CO_2	carbondioxide
conc.	concentration
cont.	continue
CP	purified intact chloroplast
CRF	chloroplast-rich fraction
CRFs	chloroplast-rich fractions
Cys	cysteine
DAG	diacylglycerol
DG	diacyglycerol
DGG	digalactosylglycerol
DGDG	digalactosyl diacylglycerol
DGMG	digalactosyl monoacylglycerol
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
DW	dry weight
E	envelope membrane
E_a	activation energy
EPA	eicosapentaenoic acid

LIST OF ABBREVIATIONS (cont.)

ER	endoplasmic reticulum
FA	fatty acid
FAMEs	fatty acid methyl esters
FAO	food and agriculture organisation of the United Nations
FFA	free fatty acid
FFAs	free fatty acids
FJ	fresh (no-heat treated) PVH juice
FW	fresh weight
g	gram
GC-MS	gas chromatography-mass spectrometry
GI	gastrointestinal tract
GLP-1	glucagon-like peptide-1
Glu	glutamic acid
Gly	glycine
GPLRP2	pancreatic lipase-related protein 2 from guinea pig
GR	grass
HCl	hydrochloric acid
HGL	human gastric lipase
His	histidine
HJ	heated PVH juice
HMF	5-hydroxymethylfurfural
H_2O_2	hydrogen peroxide
HPJ	human pancreatic juice

LIST OF ABBREVIATIONS (cont.)

HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HPVH	heated pea vine haulm
hr	hour
ICP-MS	inductively coupled plasma-mass spectrometry
Ile	isoleucine
IP	inositol phosphate (phytic acid)
IP6	myo-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate
J	joule
K	kelvin
k	the first order reaction rate
KA	kale
kcal	kilocalorie
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
KH_2PO_4	potassium dihydrogen phosphate
KJ	kilojoule
LA	linoleic acid
LAHase	lipid-acyl hydrolase
Leu	leucine
LBF	lipid-based formulation
LOG	lipoxygenase

LIST OF ABBREVIATIONS (cont.)

Lut	lutein
lx	lux
Lys	lysine
M	molar (mol/L)
MAG	monoacylglycerol
MEP	methylerythriol phosphate
Met	methionine
mg	milligram
$MgCl_2(H_2O)_6$	magnesium chloride hexahydrate
MGDG	monogalactosyl diacylglycerol
MGG	monogalactosylglycerol
MGMG	monogalactosyl monoacylglycerol
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
mol	mole
MW	molecular weight
N	normality (mol/L)
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NaOH	sodium hydroxide

LIST OF ABBREVIATIONS (cont.)

NaTDC	sodium taurodeoxycholate
ND	not determined
$(NH_4)_2CO_3$	ammonium carbonate
Nleu	norleucine
nm	nanometre
NT	nettle
PA	palmitic acid
PC	phosphatidylcholine
PCA	principle component analysis
PDA	photodiode array
PE	phosphatidylethanolamine
PFAE	phytyl fatty acid esters
PG	phosphatidylglycerol
pH	potential of hydrogen ion
Phe	phenylalanine
PLRP1	pancreatic lipase-related protein 1
PLRP2	pancreatic lipase-related protein 2
POD	peroxidase
PPE	porcine pancreatic extract
Pro	proline
PTFE	polytetrafluoroethylene
PUFA	polyunsaturated fatty acid
PVH	pea vine haulm

LIST OF ABBREVIATIONS (cont.)

R	gas constant
R^2	coefficient correlation
RCF	relative centrifugal force
RDA	recommended dietary allowance
rDGL	recombinant dog gastric lipase
RGE	rabbit gastric extract
rHGL	recombinant human gastric lipase
RNI	reference nutrient intake
RO	reverse osmosis
ROS	reactive oxygen species
rpm	revolution per minute
Rubisco	ribulose-1,5-bisphosphate carboxylase
s	second
SDS	sodium dodecyl sulphate
Ser	serine
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SLP	spinach leaf powder
SP	spinach
SQDG	sulfoquinovosyl diacylglycerol
SSF	simulated salivary fluid
Std	standard
T	thylakoid membrane

LIST OF ABBREVIATIONS (cont.)

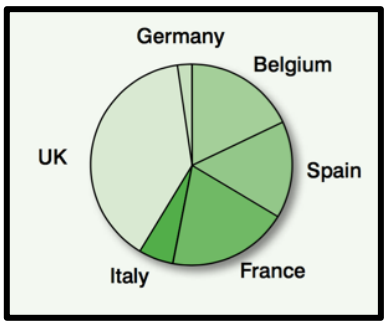
$t_{1/2}$	half life
TAG	triacylglycerol
TEA	triethylamine
TGDG	trigalactosyl diacylglycerol
Thr	threonine
TLC	thin layer chromatography
TMSH	trimethylsulfonium hydroxide
Tyr	tyrosine
U	unit
USP	united states pharmacopeia
UV-Vis	ultraviolet and visible
v	volume
Val	valine
Vol.	volume
vs	versus
v/v/v	volume per volume per volume
W	watt
WHO	world health organisation
WLM	whole leaf material
WLMs	whole leaf materials
WSI	water solubility index
w/v	weight per volume
w/w	weight per weight

CHAPTER I

Introduction

1.1 General overview

The United Kingdom is the largest producer of frozen peas in Europe with large areas given over for pea growing, as shown in **Figure 1.1**; at present, around 31,707 hectares. In 2015, the yield of pea production was 4.69 tonnes per hectares (Processors and Growers Research Organisation, 2016). After 8-10 weeks of pea growing, the peas are harvested, leaving the rest of the plant (stems, leaves, stalks, vines and pods-collectively called haulm (**Figure 1.2**) on the field (Xia et al., 2016). The haulm can be used to feed livestock (Anderson et al., 2002) or is ploughed back to the soil to provide nitrogen (Abi-Ghanem et al., 2011); it is an abundant source of green biomass that could be valorised.



UK Vining Pea Production 2011 - 2015					
	2011	2012	2013	2014	2015
Hectares drilled	32987	32588	31720	32844	34584
Hectares vined	31065	29226	26794	32820	31707
Yield t/ha vined	4.88	3.61	5.01	4.35	4.69

Figure 1.1: Estimation of vining pea production over 5 years (2011-2015) in the UK and leading frozen pea producers.





Figure 1.2: Characteristics of pea plant and pea vine haulm (PVH) **A-B)** morphology of pea plant **C)** during harvesting **D)** postharvest, pea vine field residue (haulm).

Post-harvest, pea vine field residue (haulm) is an underutilised green plant material that is a rich source of chloroplasts, organelles in the plant where photosynthesis occurs converting light energy into chemical energy. The chloroplast (**Figure 1.3**) is an important reservoir of lipophilic nutrients, including carotenoids, vitamin E, vitamin K, and galactolipids enriched in omega-3 fatty acids (Raven and George, 2002, Hincha, 2008, Kelly et al., 2016).

The Lipid Group in the School of Biosciences at University of Nottingham has recovered the chloroplast-rich fraction (CRF) (**Figure 1.4**) from green plant materials, such as spinach, kale, nettle, grass using grinding leaves in 0.3 M sucrose solution (Gedi et al., 2017) and from pea vine haulm (PVH) using a screw-press juicer without added water (Torcello-Gomez et al., 2019).

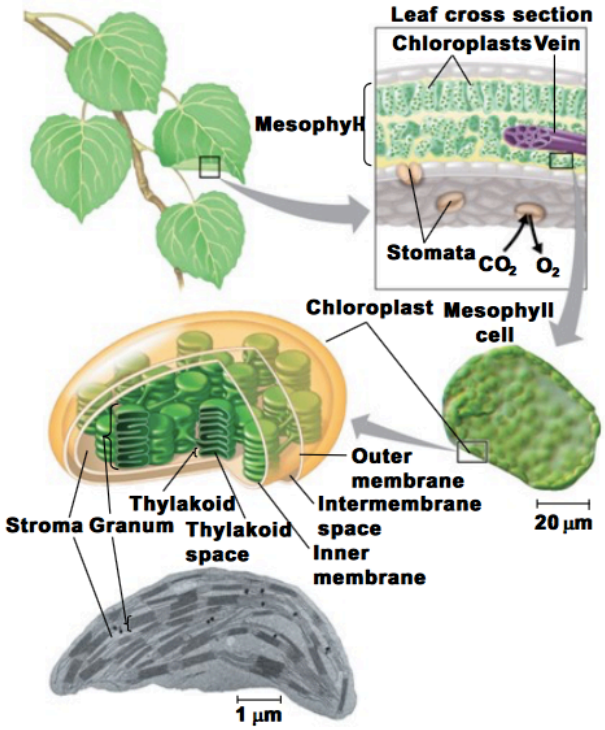


Figure 1.3: Structure of leaves (Audesirk et al., 2013).

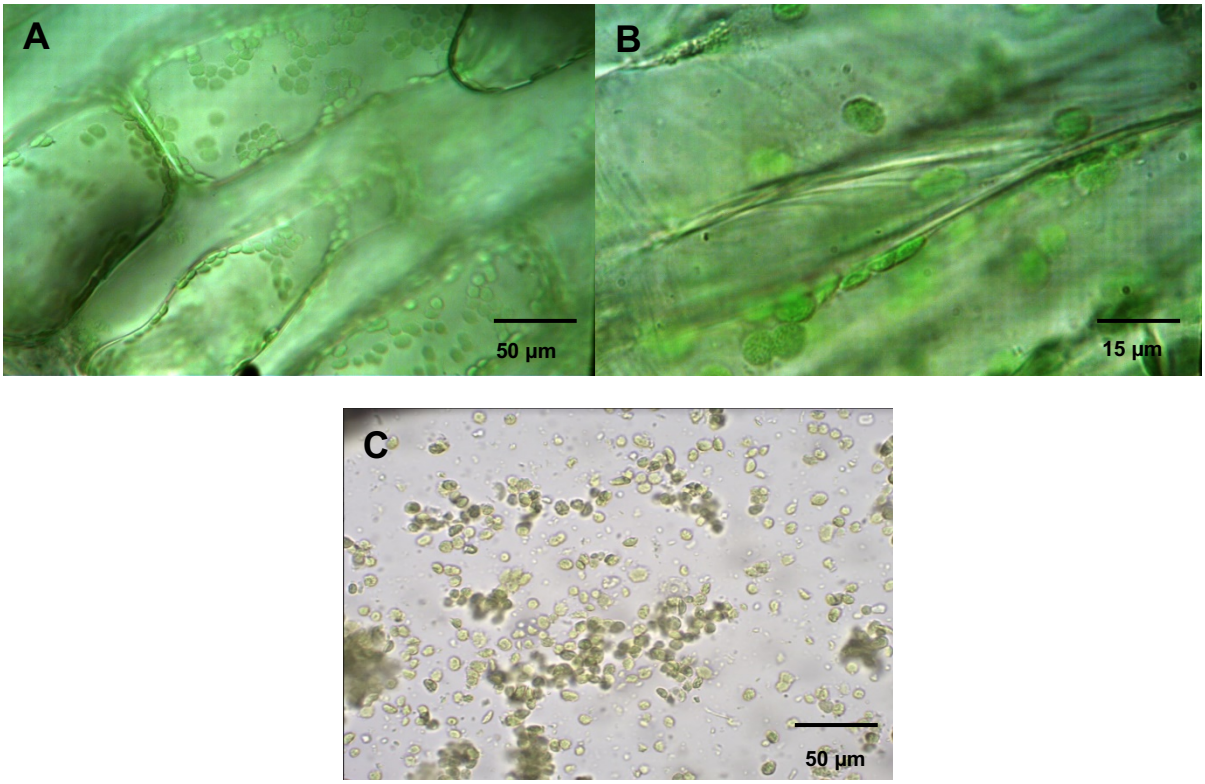


Figure 1.4: Microscope image of spinach leaves **A-B)** cross section of spinach leaves **C)** chloroplast-rich fraction (CRF) of spinach leaves.

1.2. Hypothesis

The removal of intact chloroplast from their cell wall confinement offers a novel way to enrich nutrient from underutilised green plant material. The outstanding nutritional value of a chloroplast-rich fraction (CRF) has been proven by this thesis.

There is interest from industry (confidential) in using this material as a natural cocktail of nutrient in food/feed/supplement formulations. Post-harvest pea vine field residue (haulm) is an abundant renewable biomass, rich in chloroplasts. Pea vine haulm (PVH) is a strong candidate as biomass for a biorefinery; the CRF material has the nutritional and digestibility credentials as an ingredient in the food supplement, and feed industries. Thus, the goal of this work is PVH CRF could be used as a novel food supplement.

However, it must be considered that pea vine haulm is generated only during the harvest season, between July and August. Thus, the valorisation of pea vine should only be processed during these months or alternatively, it should be stabilised and stored in a way that its nutrient content is maintained, such as inactivation of the endogenous enzyme before the storage. In addition, the possibility of scale-up of CRF preparation during this harvest season should be considered. Scale up of this process has been addressed in a separate applied project and is not central to the focus of this work. Suffice to say, the unit operations to increase processing volumes are commercially available.

To achieve this goal, the hypotheses of this work were set out:

Hypotheses:

1. Intact chloroplasts can be released from pea vine haulm (PVH) using a slow-screw twin-gear juicer.
2. PVH CRF has a similar nutrient composition as spinach CRF.
3. Heat treatment of PVH biomass, or the juice derived from PVH, knocks out endogenous enzymes.
4. Nutrients in PVH CRF are preserved in this dehydrated material, but their stability is affected by storage conditions, for example: temperature; light; and oxygen.
5. Chloroplasts released from PVH biomass can be digested by intestinal enzymes that help to release lipophilic nutrients.
6. The addition of human pancreatic juice increases the hydrolysis of galactolipids
7. Heat treatment of PVH biomass, or its juice, prior to CRF production, affect the bioaccessibility of its lipophilic nutrients.

8. The inclusion of oil during digestion improves the bioaccessibility of PVH CRF lipophilic nutrients.

1.3. Objectives

To test the hypotheses, this research is mainly focused on nutrient composition, nutrient stability of PVH CRF during the storage, the digestibility of lipophilic nutrients in PVH CRF using simulated *in vitro* upper gastrointestinal model and improve nutrient available for uptake in PVH CRF.

The objectives of this work were to:

1. Compare the nutrient compositions of CRF from PVH and spinach leaves.
2. Identify heat-processing strategies that improve the stability of nutrients in CRF material.
3. Establish storage conditions that preserve nutrients in CRF material.
4. Measure the release of nutrients from CRF material (bioaccessibility) using an *in vitro* model that simulates human digestion.
5. Determine the impact of heat processing raw material on the bioacccessibility of nutrients from CRF material.
6. Monitor the digestion of galactolipid in an *in vitro* model that simulates human digestion using digestive enzymes from animal and human.

CHAPTER II

Literature Reviews

2.1. Chloroplast and its function

The chloroplast (**Figure 2.1** and **2.2**) is an organelle in photosynthetic organism, which is responsible for the conversion of light energy into chemical energy. It characteristically appears as a lens shape 5 to 10 μm in diameter, and 2 to 4 μm in thickness (Barber, 1976, Mullet, 1988, Hall and Rao, 1999) and its structure consists of three membrane systems: the outer envelope, inner envelope and thylakoid membranes. These enclose three aqueous compartments: the intermembrane space, stroma, and thylakoid lumen (Sakamoto et al., 2008).

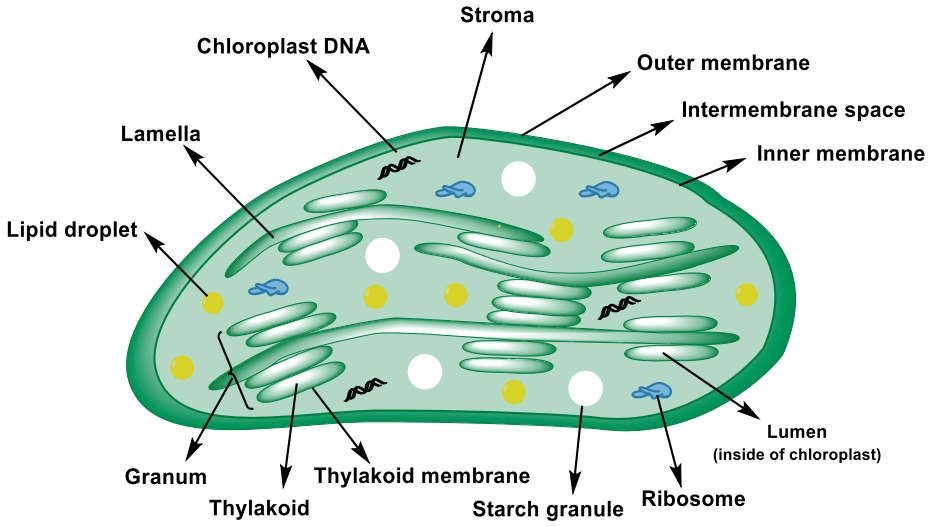


Figure 2.1: Structure of chloroplast (Barber, 1976, Mullet, 1988, Hall and Rao, 1999).

The inside of chloroplasts contains several particles embedded in the stroma, which contains stored photosynthate. The most common of these are starch grains, with a maximum length of 2 μm, and plastoglobuli (lipid pigments) (Barber, 1976). Ribosomes are found in varying abundance in the stroma of higher plant

chloroplasts and play an essential role in the biosynthesis of proteins (Brown and Gunning, 1989).

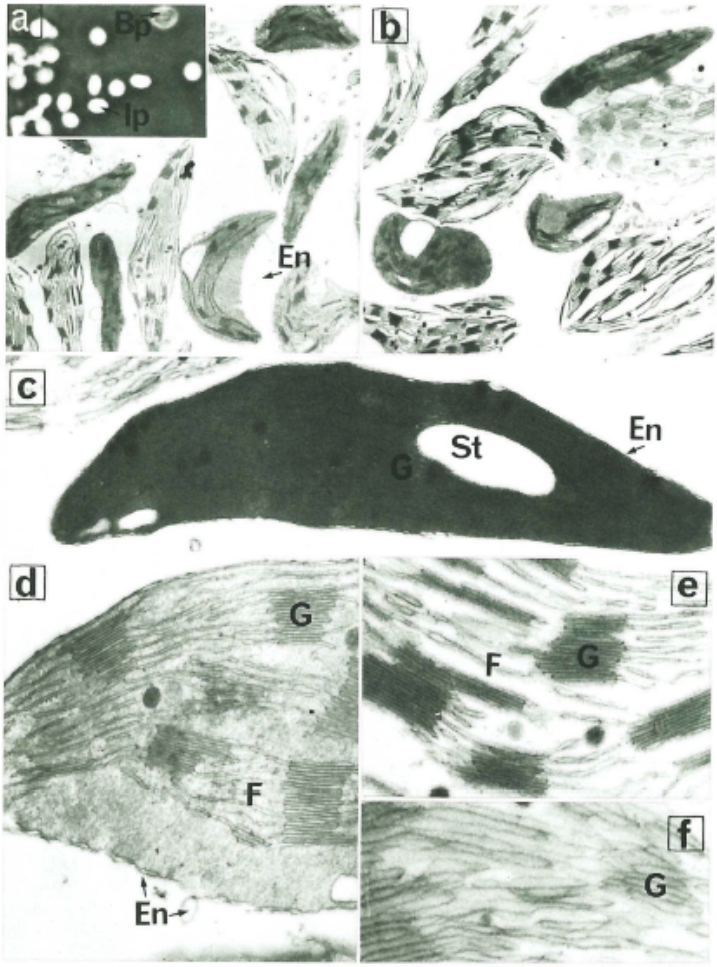


Figure 2.2: Isolated chloroplast from *Spinacia oleracea* **A**) Photomicrograph showing intact (Ip) and broken (Bp) plastid **B**) Field view showing mixed chloroplast type (x 5,000) **C**) Intact chloroplast with dense contents and starch (St) **D**) Apparently intact chloroplast with grana intact (x 20,000) **E**) Envelope-free chloroplast with grana intact **F**) Envelope-free chloroplast with disrupted granular structure (x 20,000) (Barber, 1976).

Photosynthesis is one of the light reactions which occurs at the site of the thylakoid membrane of the chloroplast, refer to **Figure 2.3**. The chlorophyll pigments in the chloroplast absorb the energy from the sunlight and then convert it and store in the

form of chemical energy, such as ATP and NADPH to produce an organic molecule such as glucose and starch from CO_2 in the air via a cyclic metabolic pathway known as the Calvin cycle (Halliwell, 1981, Raven and George, 2002).

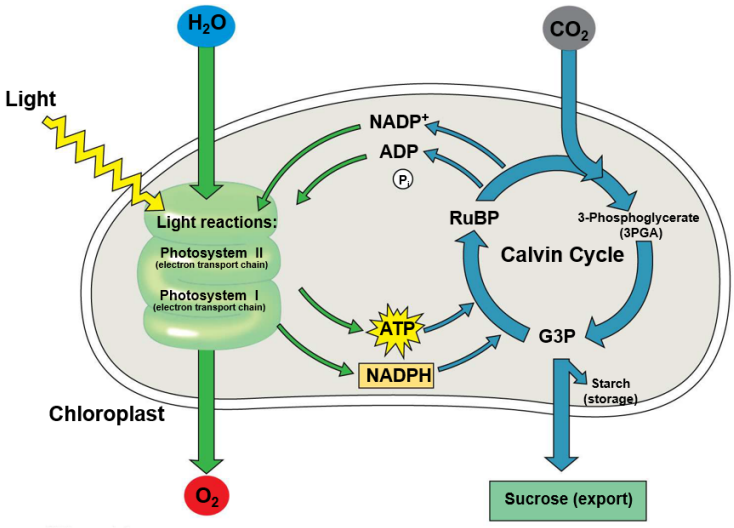


Figure 2.3 Photosynthesis reaction takes place within thylakoid membranes (Halliwell, 1981, Raven and George, 2002).

In addition, it is well known that the chloroplast acts as the site of numerous essential biosynthetic pathways. It synthesises many essential compounds, including plant hormones, fatty acids (C16 and C18 fatty acids), lipids (phospholipids, galactolipids), proteins (amino acids), and secondary metabolites, especially alkaloids and terpenoids (pigments and prenylquinones) (Freeman Allen et al., 1966, Lichtenthaler et al., 1981, Ferro et al., 2000, Lichtenthaler and Buschmann, 2001). Thus, this organelle is a significant source of macronutrients and micronutrients in the plant.

2.2. Chemical compositions in plant chloroplasts

2.2.1. Protein

Proteins are synthesised by ribosomes inside the plant chloroplast (Siddell and Ellis, 1975, Thomson and Ellis, 1972). Thylakoid and envelope membranes contain 40 to 50% of the total protein chloroplast. The thylakoid is a major membrane in the chloroplast; this is where photosynthesis occurs (Wollman et al., 1999). The main protein in the photosynthesis consists of two photosystems (PSI and PSII), cytochrome b6f complex, and ATP synthase complex (Peltier et al., 2000).

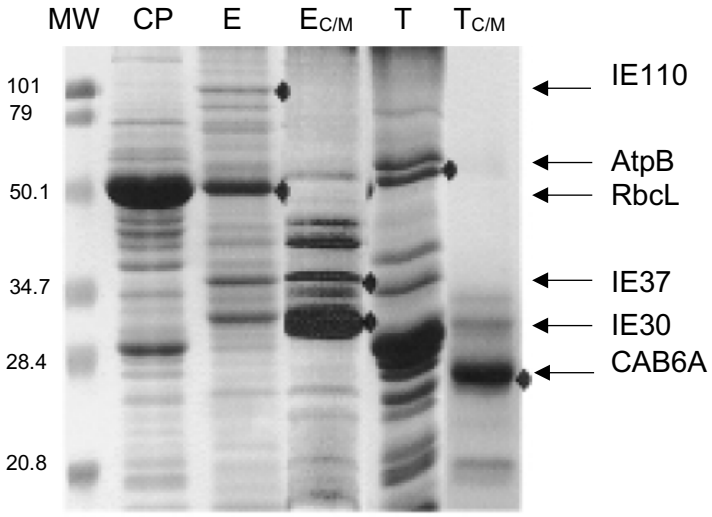


Figure 2.4: Coomassie blue-stained SDS-PAGE gel of chloroplast protein subfractions from spinach leaves; CP (purified intact chloroplast), E (envelope membrane), $E_{C/M}$ (envelope membrane from chloroform-methanol extraction), T (Thylakoid membrane), TC/M (thylakoid membrane from chloroform-methanol extraction); RbcL, 54 kDa rubisco; IE30, 30kDa triose-phosphate/phosphate; IE37, 37 kDa inner envelope membrane protein; IE 110, 110 kDa inner envelop membrane protein; AtpB, β-subunit of thylakoid ATPase; CAB6A, LHC1 chlorophyll binding protein (Ferro et al., 2000).

According to **Figure 2.4**, the major protein in the intact chloroplast and envelope membrane is ribulose-1,5-bisphosphate carboxylase (Rubisco) which is an

enzyme in the Calvin cycle in the stroma of the chloroplast. Polypeptides, triose-phosphate phosphate translocator (IE30), IE37, and IE100 are also found in the inner membrane of the chloroplast envelope. The enriched protein in thylakoid membranes is IE30, LHC1 chlorophyll a/b binding protein (CAN6A) from the light-harvesting complex I, and β-subunit of thylakoid ATPase (AtpB).

Kirk and Leech (1972) reported that glutamate (33%) and aspartate (15%) were the most abundant free amino acids in the intact chloroplast from *Vicia faba* L. The most abundant amino acids biosynthesised during photosynthesis were aspartate (63%) and alanine (23%). In addition, 17 other amino acids: glycine, serine, threonine, lysine, glutamate, valine, arginine, phenylalanine, histidine, isoleucine, proline, ornithine, cysteine, methionine, cysteine, citrulline, and tryptophan, can be synthesised in the intact chloroplast by an aminotransferase reaction from either alanine or aspartate, but no synthesis of leucine from this process.

2.2.2. Lipids

Galactolipids are found in the photosynthetic membranes of plants, especially in the thylakoid and envelope membranes of the chloroplast, and they represent more than 70% of the total membrane lipids (Hitchcock and Nichols, 1971, Douce, 1974, Dörmann, 2013). The chemical structures of these diacylglycerolipids (DAG) are characterised by one, two or more galactose moieties attached to the *sn-3* position of the glycerol backbone. The first galactose is bound in β-anomeric configuration to DAG, while the second galactose is α-anomeric. Oligogalactolipids carrying two or three galactactoses all in the β-anomeric configuration are not observed under normal conditions, but they are accumulated under stress conditions (Van Besouw and Wintermans, 1978). The major galactolipids in plants are monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)

however, there are other galactolipids which are present in lower amounts, such as sulfoquinovosyldiacylglycerol (SQDG) and trigalactosyldiacylglycerol (TGDG) as shown in **Figure 2.5**.

The typical lipid composition of the thylakoid membrane consists of 52% MGDG and 26% DGDG. SQDG is an anionic galactolipid which accounts for approximately 6.5% of thylakoid lipids, and phosphatidylglycerol (PG) is an anionic phospholipid which amounts to approximately 9.5% of thylakoid lipid. The lipid composition of the inner chloroplast envelope approximates to 49% MGDG, 30% DGDG, 5% SQDG and 8% PG, while the outer envelope has a lipid composition of 17% MGDG, 29% DGDG, 6% SQDG, 10% PG, and 32% phosphatidylcholine (PC) (Hölzl and Dörmann, 2019).

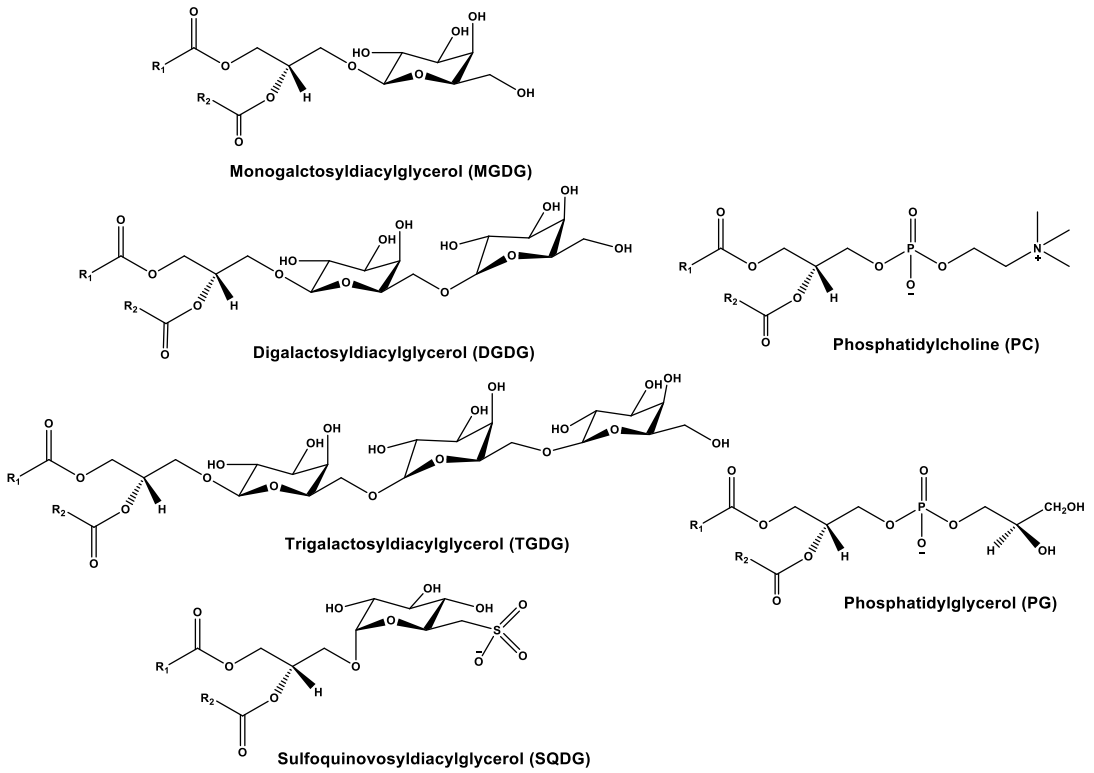


Figure 2.5: Chemical structures of galactolipids and phospholipids found in chloroplasts (Amara et al., 2010, Wattanakul et al., 2019).

Chloroplasts are the major sites for fatty acid *de novo* synthesis in the plant cell (Weaire and Kekwick, 1975, Ohlrogge and Jaworski, 1997). Fatty acids are incorporated into glycerolipids in the chloroplast (prokaryotic pathway) or are exported to the endoplasmic reticulum (ER) for glycerolipid synthesis following the eukaryotic pathway (Heemskerk et al., 1990, Heemskerk et al., 1991). 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 chloroplast. Unsaturated C16 fatty acid, especially hexadecatrienoic acid (16:3, 7-*cis*, 10-*cis*, 13-*cis*) is found in PG and MGDG in 'prokaryotic' plants, such as spinach and Arabidopsis (Mongrand et al., 1998). In addition, chloroplasts contain a large number of several of isoprenoids derived from the methylerythriol phosphate (MEP) pathway, including chlorophylls, carotenoids and prenylquinones (plastoquinone, phylloquinone and tocopherol) (Kaiping et al., 1984, Schultz, 1990, Zhao et al., 2013).

2.2.2.1 Prokaryotic and eukaryotic galactolipids

Most of the membrane lipids in the chloroplast are assembled in the envelope membrane (Li-Beisson et al., 2010). The building blocks for the chloroplast glycerolipids are phosphatidic acid (PA) and diacylglycerol (DAG). PA is produced in both chloroplast and endoplasmic reticulum (ER) depending on the plant species. The DAG backbone for galactolipid synthesis can be derived from two different pathways. First is the prokaryotic pathway, which is localised in the inner membrane of chloroplast. While the another is the eukaryotic pathway, which is localised in the ER (Heemskerk et al., 1990, Heemskerk et al., 1991, Ohlrogge and Browse, 1995). These pathways can be differentiated by the fatty acid specificity of the *sn-2* lysophosphatidic acid-acyltransferase involved. The chloroplast-localised enzyme has a high specificity for C16 fatty acid, while the ER-localised enzyme has a strong

preference for C18 fatty acid. Therefore, C16 fatty acid at the *sn-2* position is a signature for the chloroplastic origin of a DAG backbone. In some higher plants, such as spinach (*Spinacia oleracea*) and *Arabidopsis thaliana*, the chloroplast's galactolipids contain a high proportion of C16:3 fatty acid at the *sn-2* position, which indicates a plastidal pathway for galactolipid synthesis. Thus, these plants are referred to 16:3 plants. While the other plants not using plastidal pathway for galactolipid synthesis such as pea (*Pisum Sativum*) are referred to 18:3 plants. The diacyl precursors derived from ER-localised lipid biosynthesis are transported to the chloroplast envelope by a mechanism that is still not clear (Moreau et al., 1998, A. Sandelius and H. Aronsson, 2008). Typically, in leaves, C18:3/C16:3 MGDG and C18:3/C16:0 DGDG are the predominant molecular species of prokaryotic galactolipids. While C18:3/18:3 MGDG and C18:3/18:3 DGDG are the most represented eukaryotic galactolipids (Heemskerk et al., 1990, Heemskerk et al., 1991).

2.2.3. Chlorophylls

Chlorophylls are widely distributed plant pigment responsible for the characteristic green colour of fruit and vegetables. The major chlorophylls are chlorophyll a, which has a methyl group at the C-3 carbon, and chlorophyll b, which has a formyl group bonded to that carbon atom (**Figure 2.6**). Chlorophylls a and b are found in higher plants and occur in the approximate ratio of 3:1 (Schwartz and Lorenzo, 1990). They are also different colours: chlorophyll a appears blue-green and chlorophyll b is yellow-green. Chlorophyll a has also been reported to be less thermally stable than chlorophyll b.

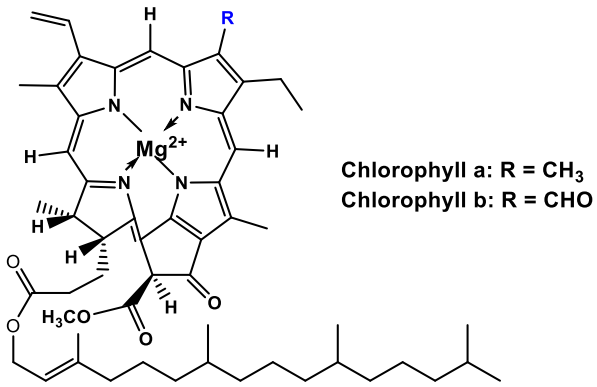


Figure 2.6: Chemical structures of chlorophylls (Schwartz and Lorenzo, 1990).

Chlorophylls are known to be easily degraded by conditions, such as acid, heat, light and oxygen. Heat and light lead to the rapid degradation of chlorophyll products (Mangos and Berger, 1997). During exposure to heat, the chlorophyll pigment can lose its bound magnesium and change from chlorophyll to pheophytin and other derivatives such as chlorophyllide and pheophorbide (Schwartz and Elbe, 1983); the mechanism is shown in **Figure 2.7**.

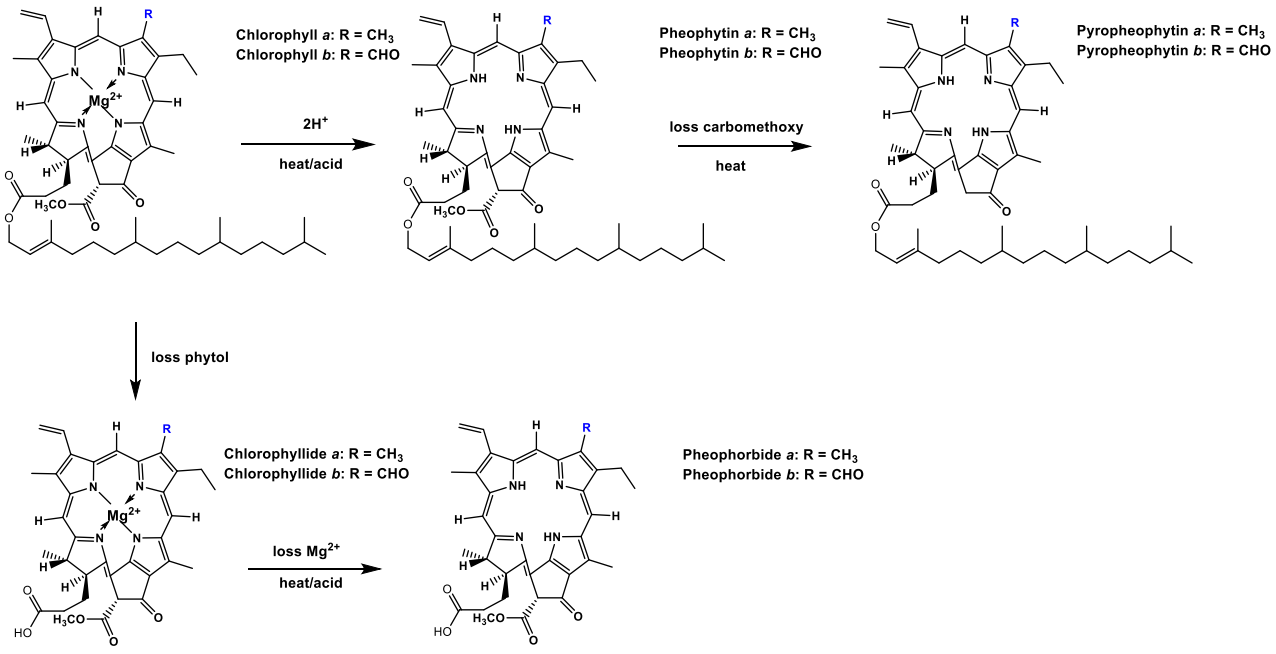


Figure 2.7: Mechanism of chlorophyll degradation (Schwartz and Elbe, 1983, Mangos and Berger, 1997).

Chlorophyll is a source of antioxidant nutrient. It helps to neutralise free radical in the body that can cause damage to healthy cells (Inanc, 2011). Many studies support that chlorophyll and its derivatives have antioxidant properties (Sato et al., 1984, Sakata et al., 1990, Hoshina et al., 1998, Ferruzzi et al., 2002, Mishra et al., 2011, Pangestuti and Kim, 2011). In addition, chlorophyll has also therapeutic properties, such as anti-inflammation, wound healing, anti-mutagen, simulating immune system, detoxification of liver, ability to prevent cancer and normalised blood pressure (Ferruzzi and Blakeslee, 2007, Mishra et al., 2011). Furthermore, chlorophyll concentration is also an indirect indication of the concentration of chloroplast in the plants (Gedi et al., 2017).

2.2.4. Carotenoids

Carotenoids are lipid-soluble pigments, which cannot be synthesised by humans and animals; these pigments are therefore obtained from the diet. Typically, carotenoids are found in all photosynthetic organisms, such as plants, bacteria, algae, and fungi. Carotenoids are divided into two main groups: carotenes or unsaturated hydrocarbons (such as α-carotene, β-carotene, and lycopene) and xanthophylls or oxygenated derivatives (such as lutein, zeaxanthin, and β-cryptoxanthin) as shown in **Figure 2.8**.

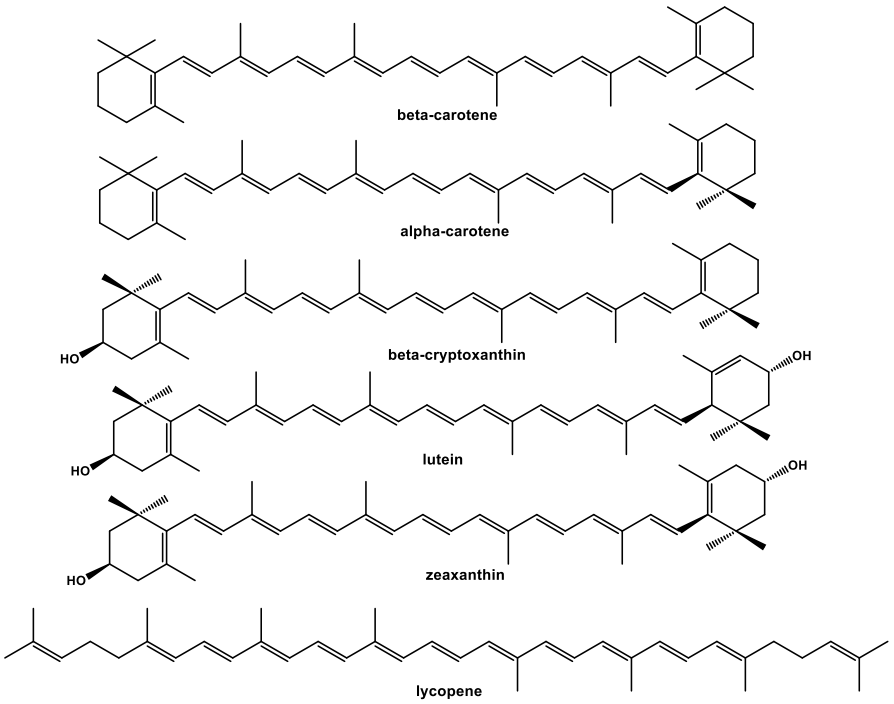


Figure 2.8: Chemical structures of carotenoids (Rodriguez-Amaya, 2015).

2.2.4.1. β-carotene

β-Carotene is a type of carotene and a precursor of vitamin A; it is often known as provitamin A. In the body, it can be broken down by β-dioxygenase in the mucosa of the small intestine into two retinyl molecules, which is later reduced to retinol (vitamin A) (see **Figure 2.9**) (Paik et al., 2001). β-carotene is found in a variety of fruits and vegetables, such as collard, turnip, spinach, lettuce, mango, cantaloupe, melon, pumpkin, carrot, and sweet potato (Holden et al., 1999). Naturally, β-carotene is the most often found as all-*trans* isomers and less often as *cis*-isomers in the following order: all-*trans* >9-*cis*>13-*cis*>15-*cis* (Guo et al., 2008). All-*trans*-β-carotene is unstable and can be easily isomerised into *cis*-isomers when exposed to heat and light (**Figure 2.10**). It has been reported that 9-*cis*-β-carotene is formed when exposed to light while 13-*cis*-β-carotene are formed during storage (Khoo et al., 2011).

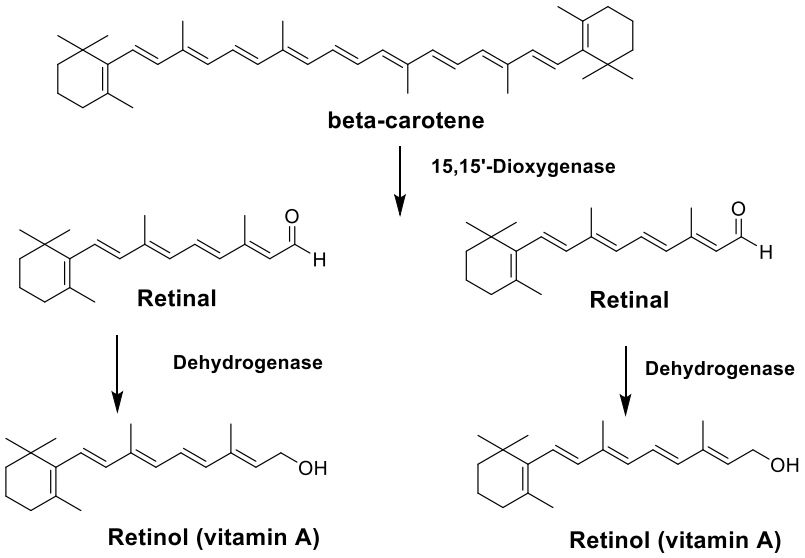


Figure 2.9: Chemical structures of conversion of β-carotene to retinol (vitamin A) (Paik et al., 2001).

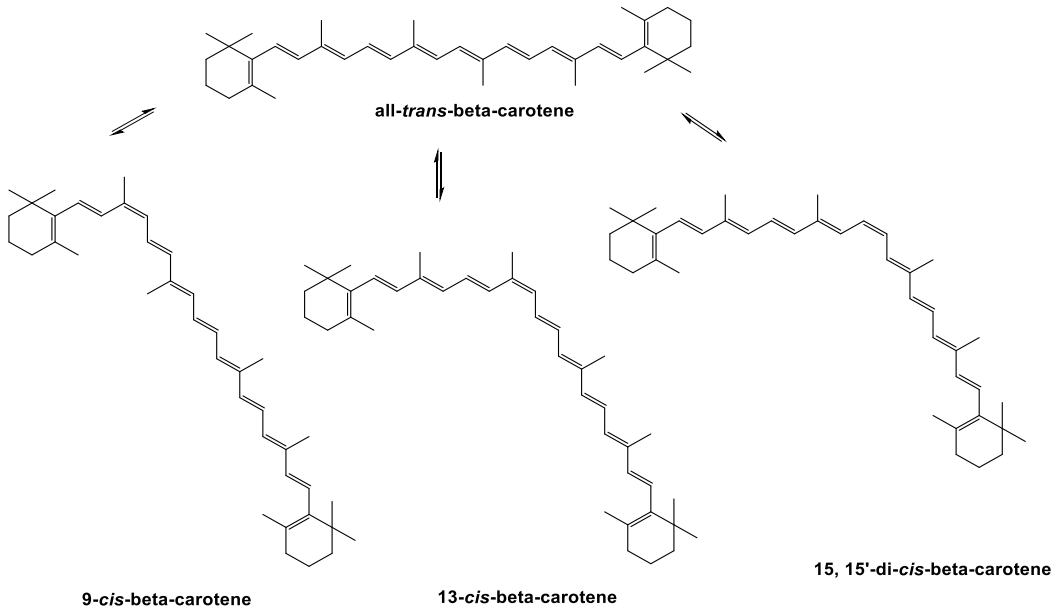


Figure 2.10: Chemical structures of isomers of β-carotene (Pénicaud et al., 2011).

The consumption of β-carotene has been associated with a decreased risk of developing several types of chronic diseases, including cancer (Epler et al., 1993, Ziegler et al., 1996), cardiovascular disease (Kritchevsky, 1999), and age-related macular degeneration (AMD) (Rasmussen and Johnson, 2013). The action of

β-carotene against disease has been attributed to an antioxidant property, especially its ability to quench singlet oxygen and its interaction with free radicals (Palozza and Krinsky, 1992).

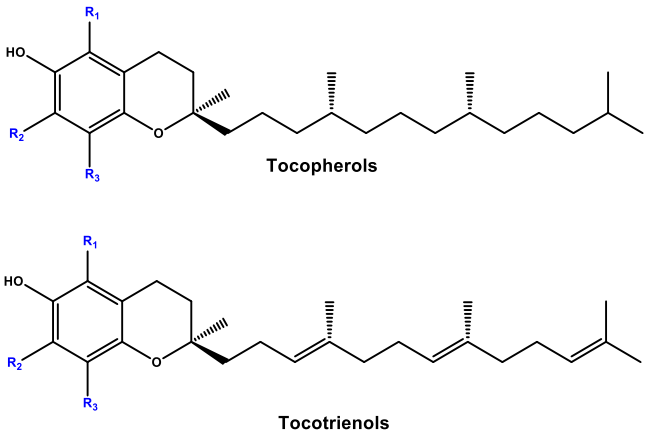
2.2.4.2. Lutein

Lutein is a type of xanthophyll, which is found abundantly in fruits, green leafy vegetables and egg yolks. Its structure is characterised by the presence of a hydroxyl group attached to the 3 and 3' positions of the β-ionone rings in the molecule as shown in **Figure 2.8**. Lutein and zeaxanthin, a structural isomer of lutein, are found in the macular pigment of the human retina (Bone et al., 1988, Sommerburg et al., 1998, Basu et al., 2001, Molldrem et al., 2004). Much research has been directed toward the potential role of lutein in protecting against several age-related diseases, including age-related macular degeneration (AMD) (Sommerburg et al., 1998, Basu et al., 2001, Eisenhauer et al., 2017), cataract, heart disease and stroke, and some forms of cancers (Ribaya-Mercado and Blumberg, 2004). It has been suggested that a dietary intake of 6 mg of lutein per day for adults can reduce the risk of age-related macular degeneration (Rasmussen and Johnson, 2013).

2.2.5. Vitamin E

Vitamin E is a fat-soluble molecule which includes four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ), which differ in their methyl substitution and saturation (**Figure 2.11**). They are synthesised by plants and other photosynthetic organisms. α-Tocopherol is the most important biologically active form of vitamin E (Burton and Traber, 1990, Schultz, 1990, Hincha, 2008, Yang and McClements, 2013, Carbonell-Capella et al., 2014, Reboul, 2017) and it is also localised in the plant

chloroplast (Piironen et al., 1986). β and γ-Tocopherol display a reduced vitamin E activity (15-30%) and δ-tocopherol is almost inactive (Reboul, 2017)



	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 2.11: Chemical structures of tocopherols and tocotrienols (Reboul, 2017).

It has been reported that the main sources of vitamin E are vegetable oils and seeds at around 500-2,000 µg of tocopherol per g of fresh weight. It has also been found in small amounts in some fruits and vegetables at a concentration of 10-50 µg of tocopherol per g of fresh weight (Hirschberg, 1999). The α-tocopherol is found at high concentrations in dark green tissues, moderate in light green tissues and coloured fruits, and low in the roots (Piironen et al., 1986). 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), parley (47.51 µg/g, FW), young tobacco (57 µg/g, FW), old tobacco (180 µg/g, FW), rubber tree *(Ficus elastic)* (300 µg/g, FW), and also found in the leaves of tree, including birch, maple, elder, pine, aspen, and juniper (118-519 µg/g, FW) (Szymanska and Kruk, 2008).

In Europe, the Recommended Dietary Allowance (RDA) for vitamin E is 11 mg per day for females and 13 mg per day for males (Reboul, 2017). Dietary intake of vitamin E has been linked to a reduced risk of different forms of cancer and cardiovascular disease (Borel and Desmarchelier, 2018), the prevention of

degenerative disease, such as arthritis (Hirschberg, 1999), inflammation and immune system disorders (Reboul, 2017), and to antioxidant activity (Hincha, 2008).

2.2.6. Vitamin K

Vitamin K is a lipid-soluble vitamin that comprises two major molecules, including phylloquinone (vitamin K1) and menaquinones (vitamin MKs or vitamin K2) (**Figure 2.12**). Menaquinones are synthesised by bacteria and found in animal products and fermented foods (Booth et al., 1993, Klaczkow and Anuszewska, 2002). In contrast, phylloquinone is synthesised and localised in the green part of the plant, especially the envelope and thylakoid membranes of chloroplast in higher plants (Kaiping et al., 1984). It has been reported that the phylloquinone content is directly related to the strength of the green colour of the plant, especially the leaves, such as spinach (380 µg /100 g, FW), lettuce (127µg /100 g, FW), iceberg lettuce (35 µg/100 g, FW), tomato (2.7 µg /100 g, FW and banana (0.2 µg /100 g, FW) (Damon et al., 2005, Booth, 2012, Jäpelt and Jakobsen, 2016). In the United States, the Recommended Dietary Allowance (RDA) for vitamin K is 65 µg per day for women and 85 µg per day for men in the United States (Joint FAO and WHO expert consultation, 1998). The function of vitamin K is a cofactor for the carboxylation of blood clotting factor (Jäpelt and Jakobsen, 2016). Furthermore, there are many benefits of vitamin K, such as the prevention of osteoporosis and atherosclerosis (Damon et al., 2005).

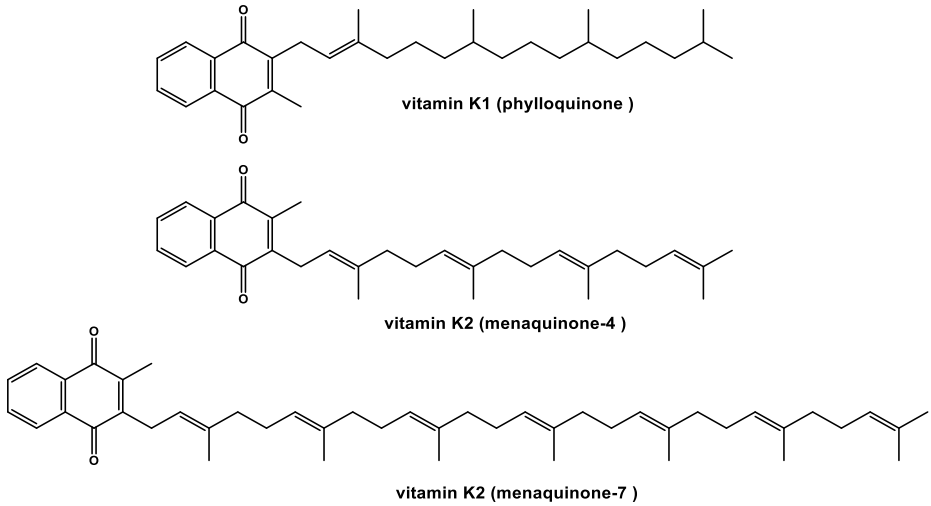


Figure 2.12: Chemical structures of vitamin K (Jäpelt and Jakobsen, 2016).

2.2.7. Minerals

Minerals play an important role in the maintenance of the nutritional and health status of the body. They cannot be synthesised in the body; thus, needs to be taken up through diet. Some minerals are needed in a larger amount, such as calcium, phosphorus, magnesium, sodium, potassium, sulphur and chloride, while others are required in smaller amounts (trace minerals), which include iron, zinc, iodine, fluoride, selenium, manganese, cobalt, and copper.

In plants, magnesium, phosphorus, potassium, calcium and iron are abundant minerals, which are localised in the chloroplasts (Buchanan, 1980, White and Broadley, 2003, Trankner et al., 2018). Magnesium is bound as the central atom in the tetrapyrrole ring of chlorophyll, which is the dominant pigment in photosynthesis (Halliwell, 1981, Barber, 1976, Marschner, 1995) and plays important roles in the metabolic pathways, acting as a cofactor of enzyme activity with ATP and a stabiliser for the ribosomal structure (Marschner, 1995, Tanoi and Kobayashi, 2015). Iron has essential roles in the chlorophyll biosynthesis and the metabolic process, including electron transport chains and redox reactions (Solti et al., 2014). Phosphorous is a

heteroelement in many compounds, including ATP, NADPH, nucleic acids and phospholipids, which have an important role in the photosynthetic system (Carstensen et al., 2018). Calcium can regulate the photosynthetic pathway, which is the main source of energy supply for plant cells, and other enzymes in the chloroplast (Wang et al., 2019). The key Calvin cycle enzyme fructose 1,6-bisphosphatase is activated by low concentration of calcium and inhibited by high concentration. Moreover, high calcium concentration within the stroma tend to inhibit photosynthetic CO_2 fixation (Wang et al., 2016). Potassium plays prominent role in process that is associated with photosynthesis and photosynthate translocation (Jin et al., 2011, Trankner et al., 2018). Potassium influences the process of photosynthesis, such as synthesis of ATP, CO_2 uptake, activation of the enzyme involved in photosynthesis, acting as the counter ion to light-induced H^+ flux across the thylakoid membranes, and balance of the electric charges required for photophosphorylation in chloroplast (Marschner, 1995).

Table 2.1: Major and trace mineral compositions (mg/100g) of the freeze-dried chloroplast-rich fraction (CRF) from green plant materials (Gedi et al., 2017).

	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
Spinach	21 ± 5	337 ± 24	298 ± 42	1196 ± 68	677 ± 15	7.7 ± 0.6	31.4 ± 3.1	2.0 ± 0.1	3.6 ± 0.5
Kale	39 ± 11	322 ± 17	628 ± 111	518 ± 95	920 ± 30	6.5 ± 0.7	25.7 ± 9.1	0.5 ± 0.1	3.6 ± 0.2
Nettles	63 ± 2	396 ± 19	370 ± 54	872 ± 64	1736 ± 11	7.9 ± 0.6	42.1 ± 12	1.0 ± 0.1	3.6 ± 0.2
grass	44 ± 2	232 ± 28	436 ± 94	1195 ± 234	354 ± 4	12.0 ± 3.4	37.0 ± 0.6	1.1 ± 0.4	4.0 ± 0.7

Gedi et al. (2017) has analysed the mineral composition of the chloroplast-rich fraction (CRF) from green leafy plants including, spinach, kale, nettle and grass as shown in **Table 2.1**. It was observed that magnesium, phosphorus, potassium, and calcium are abundant minerals found in the CRF of green leafy plants. In the United

Kingdom, the Department of Health has recommended Reference Nutrient Intake (RNI), as shown in **Table 2.2**.

Table 2.2: Reference Nutrient Intake (RNI) for minerals in the United Kingdom (Department of Health, 1991).

Mineral	Female ($mg.day^{-1}$)	Male ($mg.day^{-1}$)
Ca	700	700
P	550	550
Mg	270	300
Na	1,600	1,600
K	3,500	3,500
Cl	2,500	2,500
Fe	14.8	8.7
Zn	7.0	9.5
Cu	1.2	1.2
Se	0.06	0.075
I	0.14	0.14

2.2.8. Anti-nutrients

Anti-nutrients are compounds which limit the nutritional value of foods by interfering with the absorption of nutrients and cause a risk to the consumer. They include phytate (phytic acid), lectins, phenolic compounds, tannins, saponins, calcium oxalate, and enzyme inhibitor, such as amylase, protease and lipase inhibitors (Astley and Finglas, 2016).

Phytic acid or inositol phosphate (IP) is consisted of an inositol ring with six phosphate ester bonds (IP6) as shown in **Figure 2.13**. It is the main storage of phosphorous in many plant tissues, especially seeds, legumes, cereals, and grains

(Larkins and Vasil, 1997). For decades, phytic acid has been considered as antinutrient, which can inhibit uptake of protein and cations, such as Ca^{2+}, Mg^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} (O'Dell, 1969, Reddy et al., 1982, Nolan et al., 1987, Sandberg et al., 1989, Coulibaly et al., 2011). It works by chelating the substance to form insoluble complexes, which are unavailable to the body under normal human digestive conditions (Cheryan, 1980). It has been reported that the high phytate content results in lower iron absorption (Brune et al., 1989). It decreases the solubility of iron to form low soluble iron-phytate and the iron availability from the chyme is affected and then the intestinal iron absorption is inhibited (Sandberg and Svanberg, 1991).

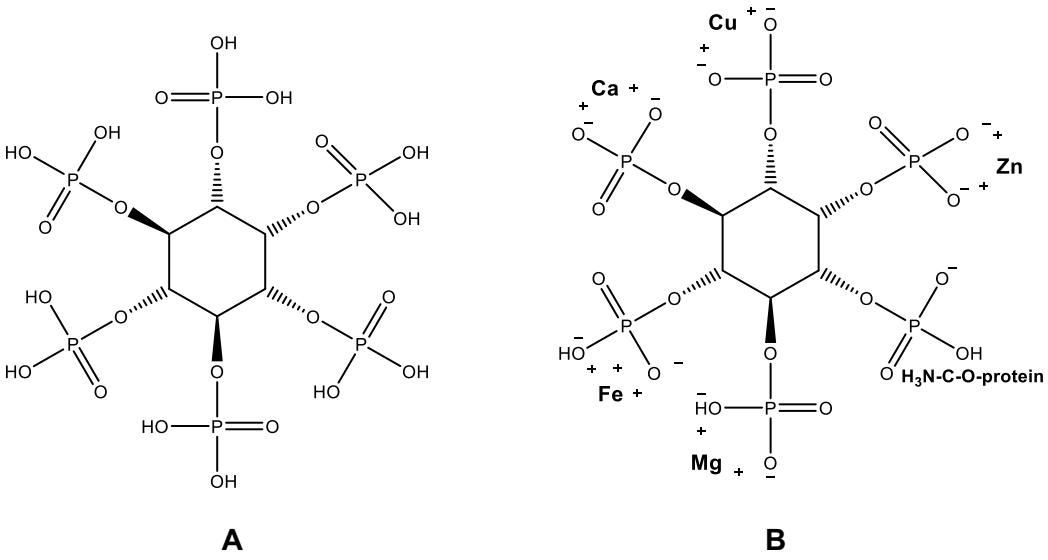


Figure 2.13. Chemical structure of phytic acid or *myo*-inositol-1,2,3,4,5,6-hexakis dihydrogen phosphate (IP6) **(A)** and chemical structure of phytic acid with the different possibilities with metal cations **(B)** (Coulibaly et al., 2011).

However, the consumption of low levels of phytic acid helps to reduce blood glucose, plasma cholesterol and triacylglycerol (Jariwalla et al., 1990, Lee et al., 2007) and cancers (Shahidi, 1997). Phytic acid is predominantly presented in unprocessed food, but it is possible to overcome the antinutrient effect of phytic acid by soaking,

heating, processing, fermenting, sprouting, and adding phytase or agents (i.e. protein, peptide, organic acid, β-carotene, and vitamin C) (Gillooly et al., 1983, Hallberg et al., 1989, Siegenberg et al., 1991, Reddy et al., 1996, Layrisse et al., 2000, Schlemmer et al., 2009). Iron absorption by humans decreases progressively as the phytate content of maize bran increases from 10 to 58 mg. However, the inhibitory effect was overcome by 30 mg ascorbic acid (Siegenberg et al., 1991). It is possible that ascorbic acid stops the oxidation of ferrous to ferric iron and thus preventing the formation of the Fe^{3+} and phytates.

2.3. Valorisation of pea pod waste and pea vine waste

Valorisation of pea pod waste and pea vine waste have been explored by many researchers. Anwar et al. (2010) utilised carboxylic and phenolic groups in ‘pea pod waste’ as adsorbent for the removal of heavy metal (chromium) from water. Mateos-Aparicio et al. (2010) reported many functional compounds from ‘pea pod waste’, including fatty acids (palmitic acid, oleic acid, linoleic acid, α-linolenic acid), and minerals (iron, potassium, and calcium) and soluble sugars (sucrose, glucose, and fructose), suggesting the added value of pea pod waste and its potential as a source of dietary fibre. Verma et al. (2011) have used 'pea peel waste’ as a cost-effective carbon source for bacterial cultivation (*Trichoderma reesei*) to produce cellulase, an enzyme that is widely used in food processing, detergent and textile industry. Khan et al. (2014) studied on the pea shell waste as an environmental adsorbent for removal of malachite green, which is a toxic cationic triphenylmethane dye with high environmental persistence from the aqueous solutions. In addition, Xia et al. (2016) established the valorisation of pea vine waste as the source of bio-renewable chemicals and potential bioenergy. They reported that the pea vine waste was subjected to pseudo-subcritical water extraction to extract potential molecules, including 5-hydroxymethylfurfural

(HMF), ethanoic acid, sugars (glucose, sucrose, and fructose) and a precipitated biopolymer (starch and pectin). The post-extraction residues of pea vine waste were subjected to microwave pyrolysis to produce a bio-oil and a biochar as potential sources of bioenergy.

2.4. Nutritional benefits and physiological relevance of the CRF

Gedi et al. (2017) reported that the comparison of nutrient composition between whole leave material (WLM) and chloroplast-rich fraction (CRF) from four different plant species, including spinach, kale, nettle, and grass.

Table 2.3: Proximate composition of CRFs and WLMs from selected green leaves (Gedi et al., 2017).

	CRF (% DW)				WLM (% DW)			
	Spinach	**Kale**	**Nettles**	**Grass**	**Spinach**	**Kale**	**Nettles**	**Grass**
Protein	42.6 ± 0.1^{ab}	44.1 ± 2.7^{a}	18.3 ± 1.1^{g}	30.2 ± 0.2^{de}	35.3 ± 4.5^{cd}	36.6 ± 4.8^{bc}	28.0 ± 0.3^{ef}	23.1 ± 1.2^{fg}
Lipid	36.9 ± 1.3^{a}	36.1 ± 1.7^{a}	29.8 ± 1.5^{b}	30.4 ± 2.7^{b}	19.3 ± 1.6^{c}	17.0 ± 0.6^{c}	13.4 ± 0.4^{d}	12.5 ± 0.3^{d}
Ash	4.1 ± 0.0^{d}	2.4 ± 0.0^{de}	2.1 ± 0.0^{e}	1.8 ± 0.0^{e}	18.8 ± 0.2^{a}	14.5 ± 0.1^{b}	16.4 ± 0.0^{b}	8.8 ± 2.5^{c}
Carbohydrate	16.4	17.4	49.8	37.6	26.6	31.9	42.2	55.6

Carbohydrates were calculated by difference (100 - [protein + lipid + ash]). Data was analysed by one-way ANOVA according to Fishers' test with statistical significance at $p \leq 0.05$ to assess differences of the samples. Values with the same superscripts for each row are not significantly different at $p > 0.05$; *CRF* chloroplast-rich fraction; *WLM* whole leave material; *DW* dry weight.

Table 2.3 showed that the CRFs contained more protein and lipid than the WLMs with lower carbohydrate content, except for CRF of nettle which did not follow the trend of the other three plants. On the other hand, it showed lower protein and higher carbohydrate in WLM. In addition, all CRFs showed higher lipophilic nutrients, including α-tocopherol (vitamin E), β-carotene (provitamin A), lutein, fatty acid (mainly

α-linolenic acid (C18:3)) than all WLMs as shown in **Figure 2.14** and **2.15**.

Overall, the data showed that both macronutrient and micronutrient were highly concentrated in CRFs compared with the WLMs of four plant species. Thus, the high value of pro-vitamin A, lutein, and vitamin E, and fatty acids (mainly α-linolenic acid) in these green tissues have the potential to be used as functional food ingredient in human or animal food formulation.

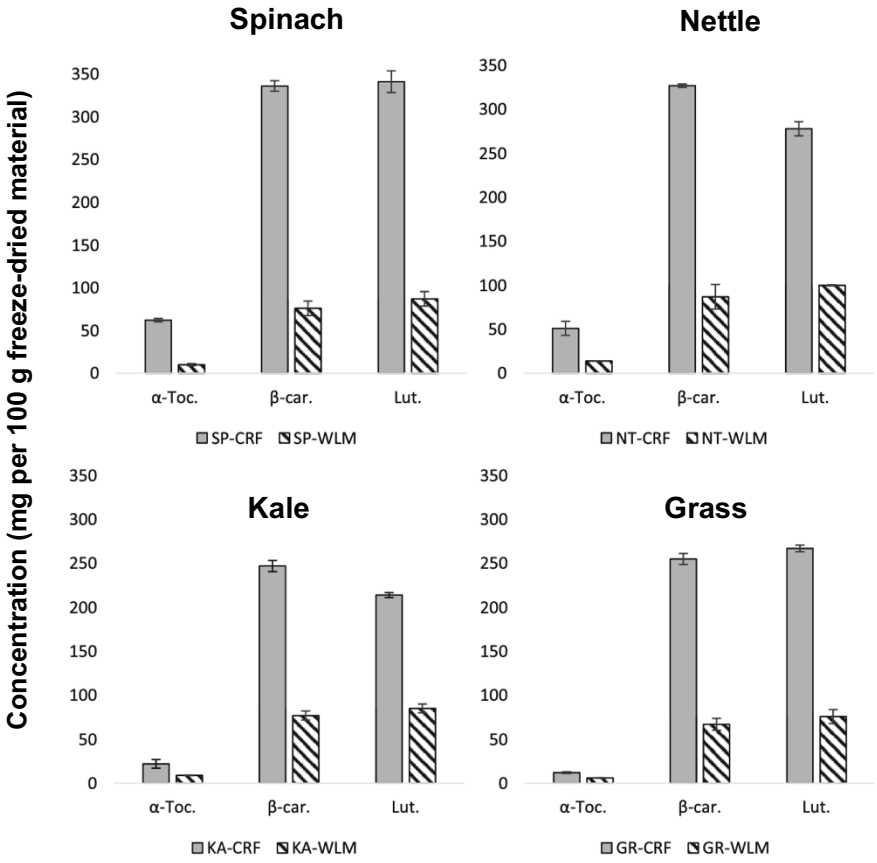


Figure 2.14: α-Tocopherol, β-carotene, and lutein content of freeze-dried CRFs and WLMs from selected leaf tissues. CRF chloroplast-rich fraction; WLM whole leaf material; SP spinach; KA Kale; NT nettles (stinging); GR grass; *α-toc* α-tocopherol; *β-car* β-carotene; *Lut* lutein (Gedi et al., 2017).

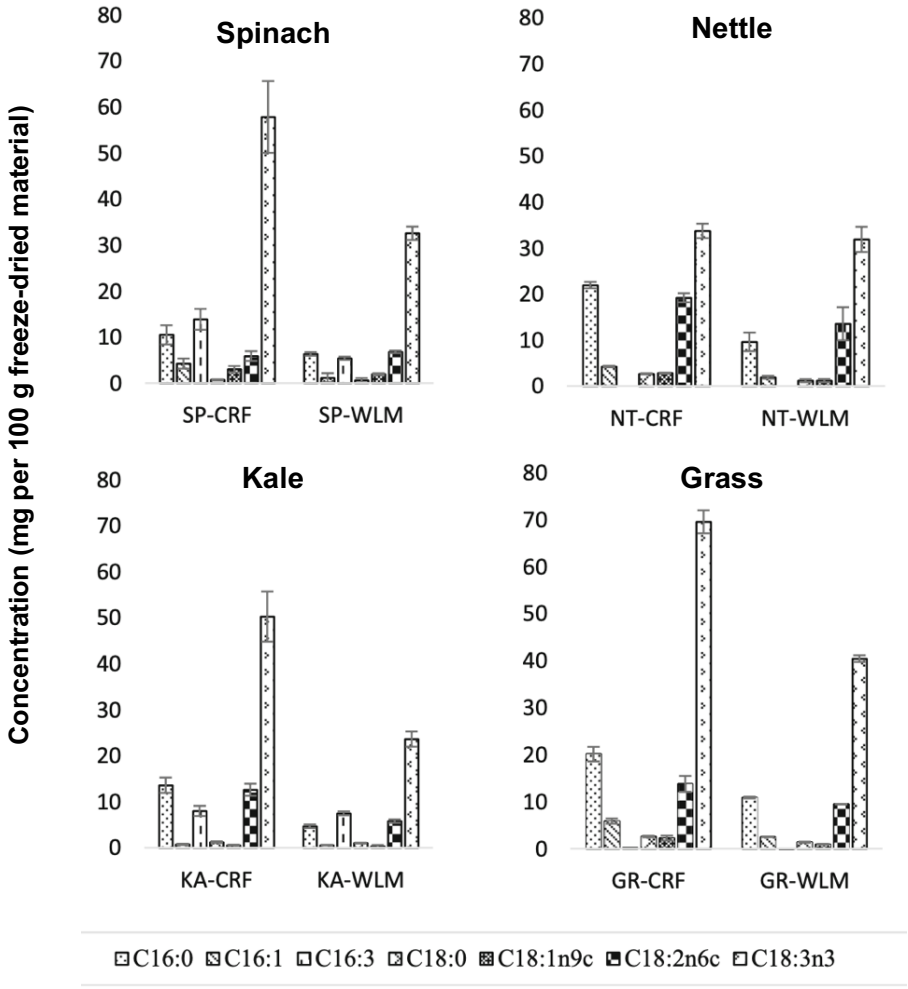


Figure 2.15: Fatty acid composition of CRFs and WLMs from four green plants. Data were the mean ± SD of 3 separate experiments. *CRF* chloroplast-rich fraction; *WLM* whole leaf material; *SP* spinach; *KA* Kale; *NT* nettles (stinging); *GR* grass (Gedi et al., 2017).

There is another study of liberation of chloroplasts from the cell wall matrix to use as an animal food formulation. Gedi et al. (2019) established the digestion of plant chloroplasts incorporated into fish diets. Zebrafish (*Danio rerio*) were fed for 8 weeks either with a standard aquaculture fishmeal or with diets which part of the fishmeal (10%, 20%, and 50% w/w) was replaced either spinach leaf powder (SLP) or spinach CRF. At the end of trial, the zebrafish was euthanised, weighed, and their total

lipids were extracted for the carotenoid and fatty acid analyses. From this result, it showed clearly uptake of lutein in the whole body of zebrafish and its eggs when replacing fish meal with SLP and spinach CRF compared to control (**Table 2.4**). In addition, this study showed that the galactolipids from SLP and spinach CRF were digested by zebrafish and released PUFA that are taken up by the fish. In addition to the uptake of α-linolenic acid, its conversion into longer chain PUFAs (EPA and DHA) was also observed as shown in **Table 2.5**.

Table 2.4: Lutein concentrations in the whole body of zebra fish and its egg (mg/kg, DW) (Gedi et al., 2019).

Diet	**Lutein**	
	Whole fish	**egg**
Control	0.1 ± 0.04^{d}	23.4 ± 2.4^{c}
CRF10	3.8 ± 0.5^{bc}	53.5 ± 13^{ab}
CRF20	5.2 ± 1.2^{a}	66.2 ± 19^{a}
CRF50	4.6 ± 0.2^{ab}	60.5 ± 4.5^{a}
SLP10	1.3 ± 0.2^{d}	33.9 ± 8^{bc}
SLP20	3.5 ± 0.1^{bc}	32.4 ± 6^{bc}
SLP50	2.7 ± 0.1^{c}	27.5 ± 6^{c}

Means that do not share a letter with each column are significantly different; *CRF* chloroplast-rich fraction; *SLP* spinach leaf powder.

Table 2.5: Fatty acid composition of whole body zebrafish fed with various test diets (Gedi et al., 2019).

Fatty acid (mg/g, DW)	Control	CRF10	CRF20	CRF50	SLP10	SLP20	SLP50
C14:0	1.9 ± 0.0^{b}	2.2 ± 0.0^{a}	2.1 ± 0.0^{a}	1.8 ± 0.0^{bc}	2.1 ± 0.1^{a}	2.1 ± 0.0^{a}	1.7 ± 0.0^{c}
C16:0	21.5 ± 0.3^{e}	24.9 ± 1.8^{cd}	27.2 ± 0.3^{ab}	28.9 ± 0.1^{a}	23.2 ± 1^{dc}	26.3 ± 0.4^{bc}	26.2 ± 0.9^{bc}
C16:1 n-7	2.4 ± 0.1^{cd}	2.7 ± 0.2^{ab}	2.8 ± 0.0^{ab}	2.6 ± 0.0^{bc}	2.7 ± 0.1^{abc}	2.9 ± 0.0^{a}	2.2 ± 0.2^{d}
C16:3 n-3	0.03 ± 0.0^{f}	0.9 ± 0.0^{b}	1.1 ± 0.0^{a}	1.1 ± 0.0^{a}	0.3 ± 0.0^{e}	0.4 ± 0.0^{d}	0.4 ± 0.0^{c}
C18 :0	3.0 ± 0.0^{e}	4.3 ± 0.6^{a}	4.6 ± 0.1^{a}	4.4 ± 0.2^{a}	4.7 ± 0.3^{a}	4.8 ± 0.2^{a}	4.7 ± 0.3^{a}
C18:1 n-9	20.2 ± 1.4^{a}	18.4 ± 1.7^{ab}	17.8 ± 0.2^{bc}	19.7 ± 0.4^{ab}	15.7 ± 0.7^{c}	18.0 ± 0.0^{ab}	17.6 ± 0.8^{bc}
C18:2 n-6	15.0 ± 0.5^{a}	9.9 ± 1.3^{d}	11.8 ± 0.0^{c}	13.7 ± 0.0^{ab}	11.4 ± 0.7^{c}	12.9 ± 0.0^{bc}	12.8 ± 0.8^{bc}
C18:3 n-3	3.0 ± 0.0^{e}	5.7 ± 0.9^{b}	7.5 ± 0.1^{a}	7.3 ± 0.0^{a}	3.2 ± 0.1^{de}	3.9 ± 0.0^{cd}	4.1 ± 0.2^{c}
C20:4 n-6	0.5 ± 0.0^{a}	0.55 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.6 ± 0.0^{a}
C20:5 n-3 (EPA)	1.5 ± 0.1^{bc}	1.9 ± 0.2^{a}	1.8 ± 0.0^{a}	1.4 ± 0.0^{c}	1.8 ± 0.0^{a}	1.7 ± 0.0^{b}	1.4 ± 0.0^{c}
C22:6 n-3 (DHA)	6.0 ± 0.1^{ab}	6.6 ± 1.4^{ab}	7.3 ± 0.0^{a}	6.2 ± 0.1^{ab}	6.8 ± 0.2^{ab}	6.7 ± 0.0^{ab}	5.6 ± 0.3^{b}
Σ PUFA	26.03	25.55	30.00	30.20	24.00	26.10	24.90
Σn-6 FA	15.50	10.45	12.30	14.20	11.90	13.40	13.40
Σn-3 FA	10.50	15.10	17.70	16.00	12.10	12.70	11.40

DW, dry weight; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C16:3, hexadecatrienoic acid;C18:0, stearic acid; C18:1n-9, oleic acid; C18:2 n-6, linoleic acid; C18:3 n-3, α-linoleic acid; C20:4 n-6, arachidonic acid (ARA); C20:5 n-3, eicosapentaenoic acid (EPA); C22:6 n-3, docosahexaenoic acid (DHA); PUFA: poly-unsaturated fatty acids; CRF: chloroplast-rich fraction from spinach leaves; SLP: spinach leaf powder. Means that do not share a letter within each row are significantly different.

2.5. Current food-related research on chloroplasts

It has been reported by David Gray group at University of Nottingham that the spinach juice (Syamila et al., 2019) and chloroplast-rich fractions (CRF) from green biomass such as, spinach, kale, grass, nettles (Gedi et al., 2017) and pea vine haulm (Torcello-Gomez et al., 2019) are great sources of macronutrients (protein,

carbohydrate, and lipid) and micronutrients (β-carotene, lutein, α-tocopherol, linoleic acid, α-linolenic acid, and minerals). Thus, they could be used as potential functional food ingredients in human or animal food formulation. Replacing fish meal with spinach leaf powder (SLP) or CRF resulted in excellent growth of zebrafish (*Danio rerio*) and clear uptake of carotenoids and omega-3 fatty acids (Gedi et al., 2019).

2.6. Food preservation technique

Food preservation technologies include procedures that maintain food quality for the longest possible time and ensure that pathogenic microorganisms are not present. Thus, the objectives of food preservation technique are increasing product shelf life, applying technique to achieve inactivation of microorganisms using drying technique, such as spray-drying and freeze-drying.

2.6.1. Freeze-drying

Freeze-drying or lyophilisation (**Figure 2.16**) is a well-known dehydration process in food and pharmaceutical industries that the major applications are the preservation of probiotics and biological materials. Freeze drying is an expensive process compared to other drying, such as spray-drying due to its relatively high investment, operation, and maintenance cost (Brushani and Anandharamakrishnan, 2017). However, the freeze-drying process produces many benefits in the results obtained in terms of viability, quality, and storage time (Sagara and Ichiba, 1994, Nastaj, 1996, Miyamoto-Shinohara et al., 2000, Morgan et al., 2006, Vesterlund et al., 2012). It also has advantages in terms of the characteristics of the final products, such as stability in nutrient compositions, reduction of chemical degradation, control of the final humidity, reduction of nutrient degradation in heat-sensitive products, and

reduction of water content at the end of the process to very low level (GR.Nireesha et al., 2013, Fissore and Pisano, 2015).

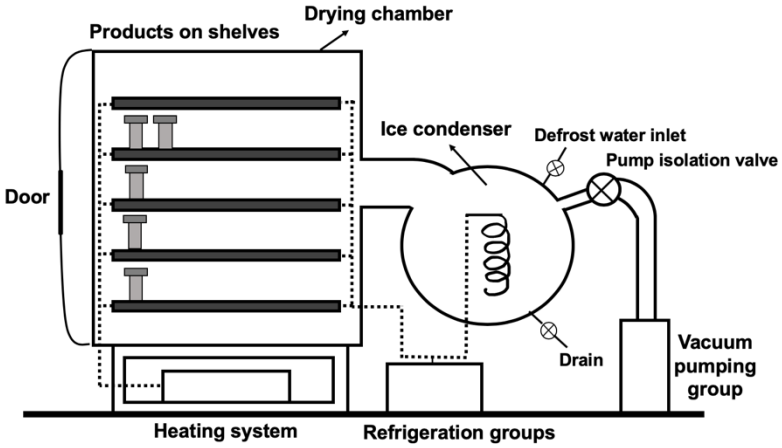


Figure 2.16: Freeze-drying configuration.

(https://tsffarmasiunsoed2012.wordpress.com/2012/06/12/freeze-drying-salah-satu-alat-pengeringan-di-industri-farmasi/comment-page-1/)

Freeze-drying is a process that water is frozen, followed by its elimination from the sample in final steps of the lyophilisation (see **Figure 2.17**). This process consists of three steps. Firstly, the sample is frozen at low temperature. Secondly, the primary drying occurs when the ice is sublimated under reduced pressure. Finally, the secondary drying occurs as a desorption step that the residual moisture is removed or reduced to a low level (Gaidhani et al., 2015).

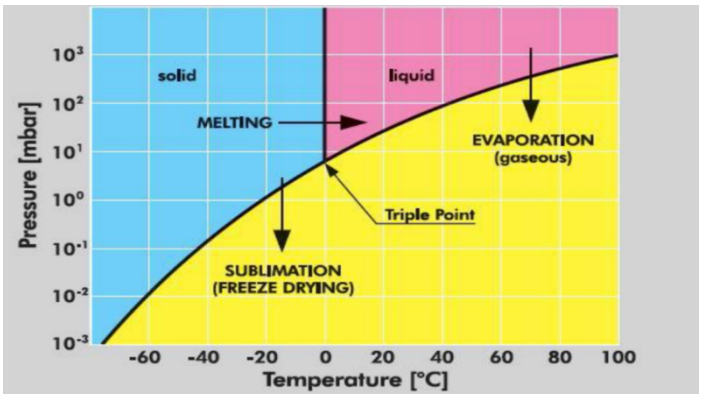


Figure 2.17: Freeze drying process (Gaidhani et al., 2015).

2.6.2. Thermal treatment for food preservation

It is well known that the nutrient content in vegetables and fruits decrease after harvesting the plants mainly due to endogenous enzymes (Kessy et al., 2016). Thermal processing, such as blanching or pasteurisation, is important in order to inactivate these enzymes and extend the shelf life of the nutrients.

2.6.2.1. Blanching

Blanching is a form of thermal treatment conducted in a microwave, boiling water or by steaming (Lin and Brewer, 2005, Kessy et al., 2016, Severini et al., 2016), which not only deactivates the enzymes found in vegetables and fruits, but also reduces microorganisms and stabilises the texture, flavour and nutrient content (Lin and Brewer, 2005).

2.6.2.2. Pasteurisation

Pasteurisation is another form of thermal treatment which can be applied to liquidised vegetables to prevent microorganisms (yeasts, moulds and bacteria) and maintain quality (Peng et al., 2017). It has been reported that pasteurisation extended the shelf life of juice because the heat kills microbes and bacteria that cause spoilage (Rabie et al., 2015).

However, Lešková et al. (2006) reported that loss of vitamins could be experienced during cooking and processing, due to the sensitivity of micronutrients to heat, oxygen and light. Therefore, it is crucial to balance the heat required to cause sufficient enzyme knock-out with the potential for degrading micronutrients and quality through excessive treatment or ‘over-blanching’ (Bahçeci et al., 2005, Gökmen et al., 2005, Andress and Harrison, 2006, Severini et al., 2016).

2.6.2.3. Peroxidase

Peroxidase (POD) is used as an indicator enzyme in the evaluation of the effectiveness of thermal treatments in fruits and vegetables because this enzyme is present in the most plant tissues. POD is more heat resistant than other enzymes responsible for degradation in vegetables and has the benefits of being easy to measure (Thongsook and Barrett, 2005, Severini et al., 2016). Thus, if it can be shown that its activity has been reduced, it can be assumed that the other degradative enzymes have also been knocked out (Severini et al., 2016).

2.7. *In vitro* digestion

In vitro digestion models are widely used today for studying the digestibility and release of nutrient components under simulated gastrointestinal (GI) conditions with either fixed parameters (pH and enzyme concentration in static models) or variable parameters (dynamic models).

A static method is the simplest technique for digestion. There are three digestion steps (oral, gastric and intestine) in the method (Bengtsson et al., 2009, Minekus et al., 2014, Estévez-Santiago et al., 2016), but sometimes just two of these steps are used depending on the digestion break-down profile of the nutrient for instance, gastric and intestinal phases are used to the study of protein and lipid digestions (Garrett et al., 1999, Lemmens et al., 2011). The static method simulates a limited number of parameters, such as pH and enzyme concentration. The advantage of this technique is that it is inexpensive, rapid, consistent and does not have the ethical considerations involved in human or animal studies (Minekus et al., 2014, Calvo-Lerma et al., 2018).

2.7.1. Static *in vitro* digestion model from Minekus et al. (2014) and Brodkorb et al. (2019)

The COST Infogest network propose a generally standardised static *in vitro* digestion method in line with the consensus papers (Minekus et al., 2014, Brodkorb et al., 2019). This model is widely employed in food and nutritional research. It is composed of three stages that mimic oral, gastric and small intestinal phases of the *in vitro* digestion, as shown in **Figure 2.18**.

In the oral phase, α-amylase is used for starch digestion and employs simulated salivary fluid as electrolyte solution in this system. It is recommended that the sample is in contact with the enzyme for 2 minutes at 37^{o}C. For the gastric phase, pepsin is used for protein digestion with simulated gastric fluid as electrolyte solution in this stage at pH 3 for 2 hours. However, Brodkorb et al. (2019) have recently been modified the method from Minekus et al. (2014). They highlight that lipid starts to digest in the gastric phase in the body. Therefore, they recommended that gastric lipase is added to this system when lipid digestion is studied. Pancreatin, bile salts, and simulated intestinal fluid electrolyte solution are then added to the intestinal phase for the digestion of all nutrients, including starch, protein, and lipid with the pH at 7 and further incubated for 2 hours, as if in the small intestine.

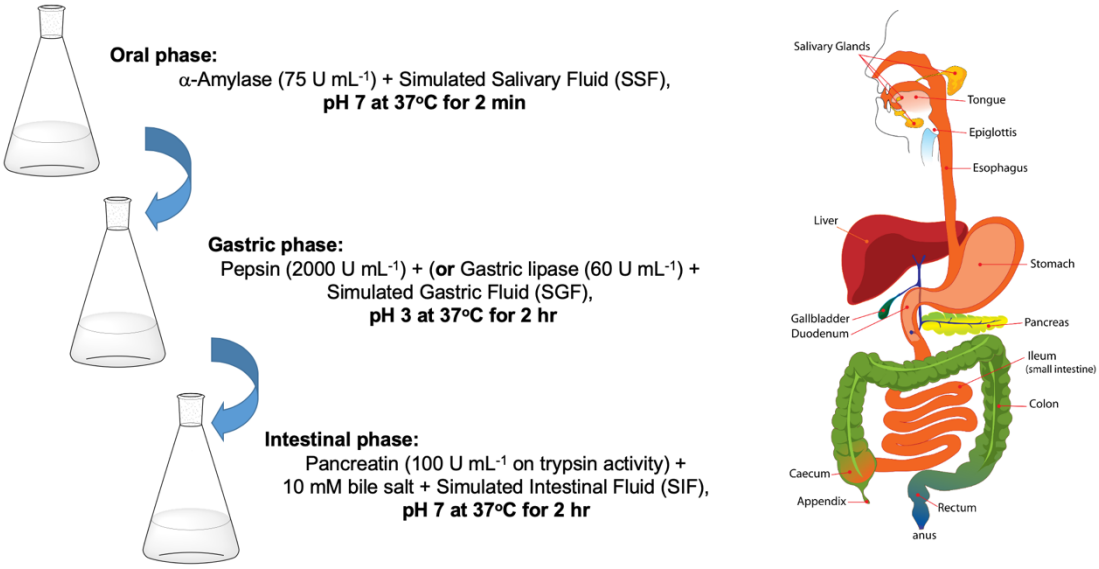


Figure 2.18: Static *in vitro* digestion model of Minekus et al. (2014) and Brodkorb et al. (2019).

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

Another *in vitro* model to simulate meal lipid digestion was based on *in vivo* studies of the gastric conditions from a half gastric meal emptying from previous studies (Carriere et al., 2001, Sams et al., 2016). **Figure 2.19** and **2.20** show that the pH in 50 % of the gastric meal emptying was around pH 5 to 5.5 for the liquid test meal (Carriere et al., 2001, Sams et al., 2016) and pH 4.5 for the solid-liquid test meal (Sams et al., 2016). Normally, the gastric digestion phase in the *in vitro* model is set at pH 2-3, which is more acidic than these *in vivo* experiments show. Based on **Figure 2.19** and **2.20**, the pH 2-3 is actually only found when the meal had almost emptied (around 80-90%). Thus, the *in vitro* digestion model for this current work in **Chapter VII** was set at 37°C, pH 5 for 30 minutes in the stomach phase in order to mimic gastric conditions at half gastric meal emptying time and pH 6 for 1 hour in the small intestine phase as shown in **Figure 2.21**.

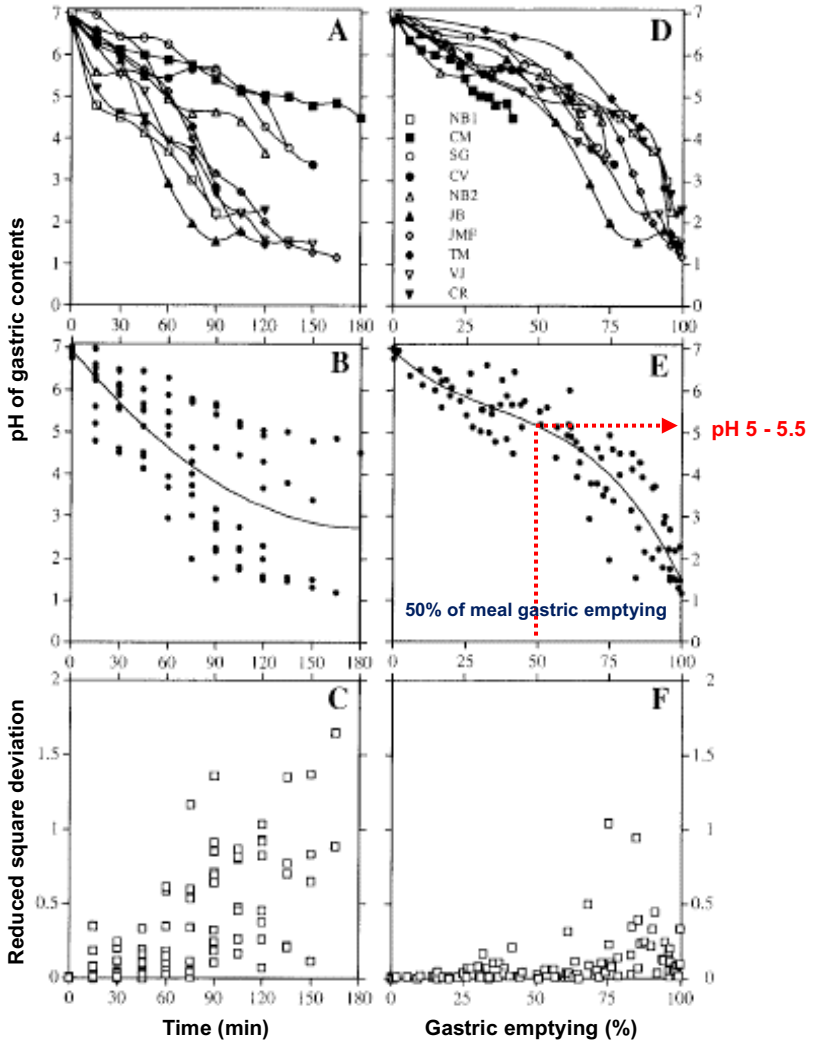


Figure 2.19: Variations in the pH of the gastric contents after the ingestion of the liquid test meal. **A, D** Variations in the pH of the gastric contents as a function of time **(A)** and as a function of the gastric emptying **(D)** in ten individual experiments. **B, E** Model for the variations in the pH of the gastric contents as a function of time **(B)** and as a function of the gastric emptying **(E)** using all the experimental values from the ten individual experiments (Carriere et al., 2001).

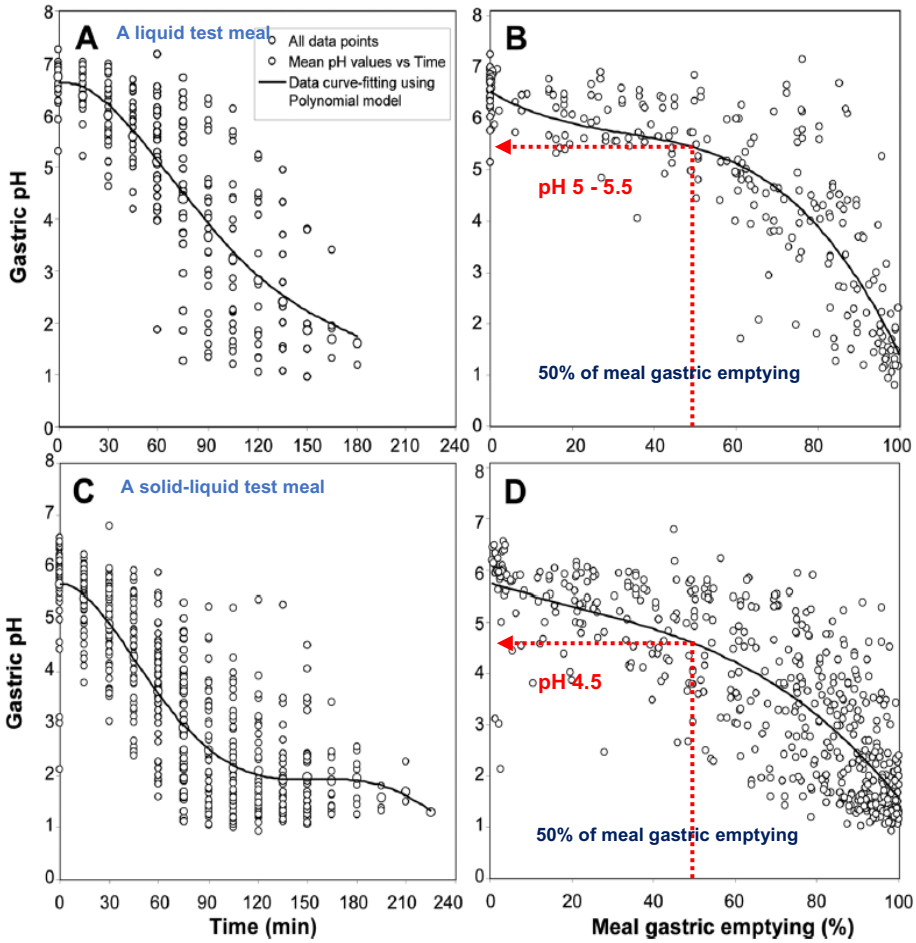


Figure 2.20: Variations in gastric pH during test meal digestion in healthy volunteers. **A)** pH variation as a function of time during a liquid test meal (256 values from 30 individual experiments) **B)** the same pH values plotted as a function of meal gastric emptying (%) **C)** pH variation as a function of time during a solid-liquid test meal (545 values from 53 individual experiments) **D)** the same pH value plotted as a function of meal gastric emptying (%) (Sams et al., 2016).

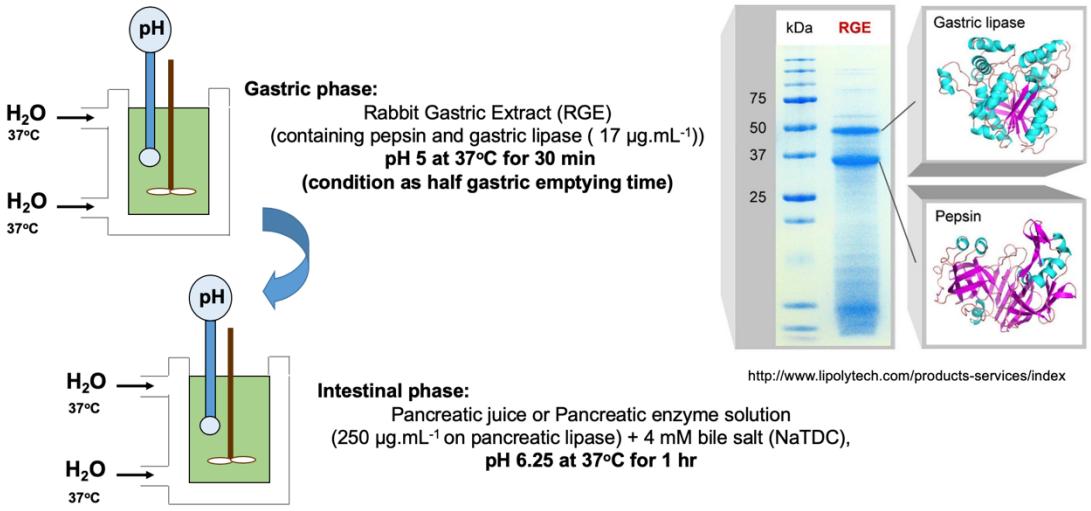


Figure 2.21: Static *in vitro* digestion model of Carriere et al. (2000).

2.7.3. Definition concepts: Bioavailability, Bioaccessibility and Bioactivity

2.7.3.1. Bioavailability

Bioavailability can be defined as the fraction of ingested compound available at the site of action for utilisation in normal physiological function and is determined through an *in vivo* methodology. Overall, the bioavailability includes GI digestion, absorption, metabolism, tissue disruption and bioactivity (Carbonell-Capella et al., 2014, Verhoeckx et al., 2015).

2.7.3.2. Bioaccessibility

Bioaccessibility is defined as the fraction of a compound which is released from its food matrix within the gastrointestinal tract to become available for intestinal absorption and is determined through an *in vitro* procedure (Carbonell-Capella et al., 2014, Verhoeckx et al., 2015).

2.7.3.3. Bioactivity

Bioactivity means the specific effect upon exposure to a substance. It is linked to how the bioactive compound is transported to the target tissue. It is also related to the consequent physiological response, such as antioxidant or anti-inflammation effects. The bioactivity can be determined through *in vitro*, *in vivo*, and *ex vivo* methodologies (Carbonell-Capella et al., 2014, Verhoeckx et al., 2015).

CHAPTER III

Materials and Methods

3.1. Materials

Pea vine haulm (*Pisum sativum* L.), composed of a mixture of vines, stems, leaves, peas, and pods was kindly provided by the Green Pea Company (Yorkshire, United Kingdom). The biomass was collected from the side of the field during the pea harvest (July 2016 for the study of nutrient compositions and July 2017 for the study of nutrient stability, bioaccessibility and *in vitro* galactolipid digestion) and immediately brought to our laboratory facilities in Leicestershire, UK to be processed. Spinach leaves were purchased from a local market (Tesco, Nottingham, UK for the study of nutrient composition and Casino, Marseille, France for the study of *in vitro* galactolipid digestion). Rapeseed oil was purchased from Tesco, UK.

3.2. Chemicals

All standards (β-carotene, α-tocopherol, and phylloquinone) were HPLC grade and purchased from Sigma Aldrich (Darmstadt, Germany), except lutein which was purchased from PanReac AppliChem (Darmstadt, Germany). Thin layer silica gel 60 plates (10 x 20 cm) from Merck Millipore (Darmstadt, Germany) were used to perform the separation of the lipids. Lipid standards (monogalactosyldiacyglycerol (MGDG) and digalactosyldiacyl glycerol (DGDG) were purchased from Avanti Polar lipid (840523P and 840524P) (Alabama, US). Oleic acid, methylpentadecanoate, triethylamine (TEA), trimethylsulfonium hydroxide (TMSH), sodiumtaurodeoxy cholate (NaTDC) were purchased from Sigma Aldrich (Darmstadt, Germany). All solvents for HPLC, GC-MS and HPTLC analyses were HPLC grade and purchased from Fisher

Scientific (Loughborough, UK) and Carlo Erba (Val-de-Reuil, France). Other chemicals used were of analytical grade.

3.3. Enzymes

α-Amylase from human saliva Type XIII-A, 300-1500 U/mg protein (A1031), pepsin from porcine gastric mucosa (P6887), Porcine Pancreatin Extract (PPE) (P7545; 8xUSP specification activity), and porcine bile extract (B8631) were purchased from Sigma Aldrich (Merck, Darmstadt, Germany). Recombinant guinea pig pancreatic lipase-related protein 2 (GPLRP2) was produced in *Aspergillus orizae* and purified as previously described (Hjorth et al., 1993). Human Pancreatic Juice (HPJ) was provided by Prof. R. Laugier, MD (La Timone University Hospital, Marseille, France) and was obtained in 1987 from a patient (36-years-old woman) devoid of pancreatic disease by performing endoscopic retrograde catheterisation on the main pancreatic duct. It was collected on ice, freeze-dried and the resulting powder was stored at -20°C before use. Rabbit gastric extract (RGE) was provided by Lipolytech (Marseille, France).

3.4. Methods

3.4.1. Post-harvest treatment of pea vine haulm (PVH)

The fresh biomass was washed with tap water to remove any soil or stones, and then the excess water was removed using an industrial salad spinner (Sammic ES-200, UK). The haulm was split into two batches for different treatments. One batch (13 kg) was packed into vacuum sealed bags for steam condition (**Section 3.4.2.**). Another batch of haulm (5 kg) was juiced immediately using a twin-gear juicer (Angel 7500, Korea) which separated the fibrous pulp from the nutrient-rich juice. The juice was filtered through a 75 µm stainless steel mesh sieve and processed (with or without pasteurisation (**Section 3.4.3.** and **3.4.4.**) to isolate the CRF (**Section 3.4.6.**).

3.4.2. Steam sterilisation of PVH for the study of nutrient stability and bioaccessibility

The pea vine biomass was packed into vacuum sealed, clear, perforated bags (500 g of pea vine per bag). These bags were placed in the rack of a Retort (Lagarde RP362, Malataverne, France). The chamber was sealed, vented and heated for 5 min 30 sec to reach a temperature of 100°C and 1 bar. These sterilisation conditions were held for 4 min before cooling and depressurising for 5 min 45 sec. The sealed bags of pea vine were plunged into an ice-water bath to rapidly cool. The steam-treated pea vine was immediately juiced using a twin-gear juicer (Angel 7500, Korea) and the juice was filtered through a 75 μm stainless steel mesh sieve and processed to isolate the CRF (**Section 3.4.6.**).

3.4.3. Heated juice extracted from PVH for the study of bioaccessibility

The pea vine juice (500 mL) was placed in a capped amber Duran bottle in a heated water bath with a magnetic stirrer (800 rpm). The temperature of the juice was increased from room temperature (20°C) to 90°C over 20 min; then the temperature was held for 3 min. The heated juice was immediately immersed in an ice-water bath to rapidly cool the juice down to room temperature before further processing to isolate the chloroplast-rich fraction (CRF) (**Section 3.4.6.**).

3.4.4. Heated juice extracted from PVH for the study of the effect of endogenous enzyme in CRF

The pea vine juice (500 mL) was placed in a capped amber Duran bottle in a heated water bath with a magnetic stirrer (800 rpm). The temperature of the juice was raised from room temperature (20°C) to 85°C in 15 min. It was then held at this temperature for 1 min. The heated juice was immediately immersed in an ice-water bath to rapidly cool the juice down to room temperature before further processing to isolate the CRF (**Section 3.4.6.**).

3.4.5. Hot water blanching of spinach leaves

Spinach leaves (100 g) were blanched in hot water at 85°C for 3 min and then immediately immersed in an ice-water bath to rapidly cool to room temperature. Blanched spinach leaves were homogenised in a blender (Waring™) for 30 sec with 0.3 M sucrose solution 1:6 (w/v). The homogenate was then filtered through a double-layered cheese cloth and processed to isolate the CRF (**Section 3.4.6.**).

3.4.6. Isolation of the chloroplast-rich fraction (CRF)

The juice was centrifuged at 17,700 RCF or 10,000 rpm (Beckman Coulter JS-21M with JA-10 rotor, US) for 10 min at 4°C. The pellet containing the chloroplast-rich fraction (CRF) was retained whilst the supernatant was centrifuged again under the same conditions to obtain a further pellet. The chloroplast-rich fractions were pooled, weighed and frozen at -80°C before being freeze-dried (Edwards Freeze Dryer Super Modulyo, UK) for 3-5 days. Freeze-dried CRF was then ground using a pestle and mortar, and stored in a vacuum-sealed foil pouch at -20°C for further analysis. The representatives of isolated CRFs are illustrated in **Figure 3.1**, **3.2** and **3.3**.

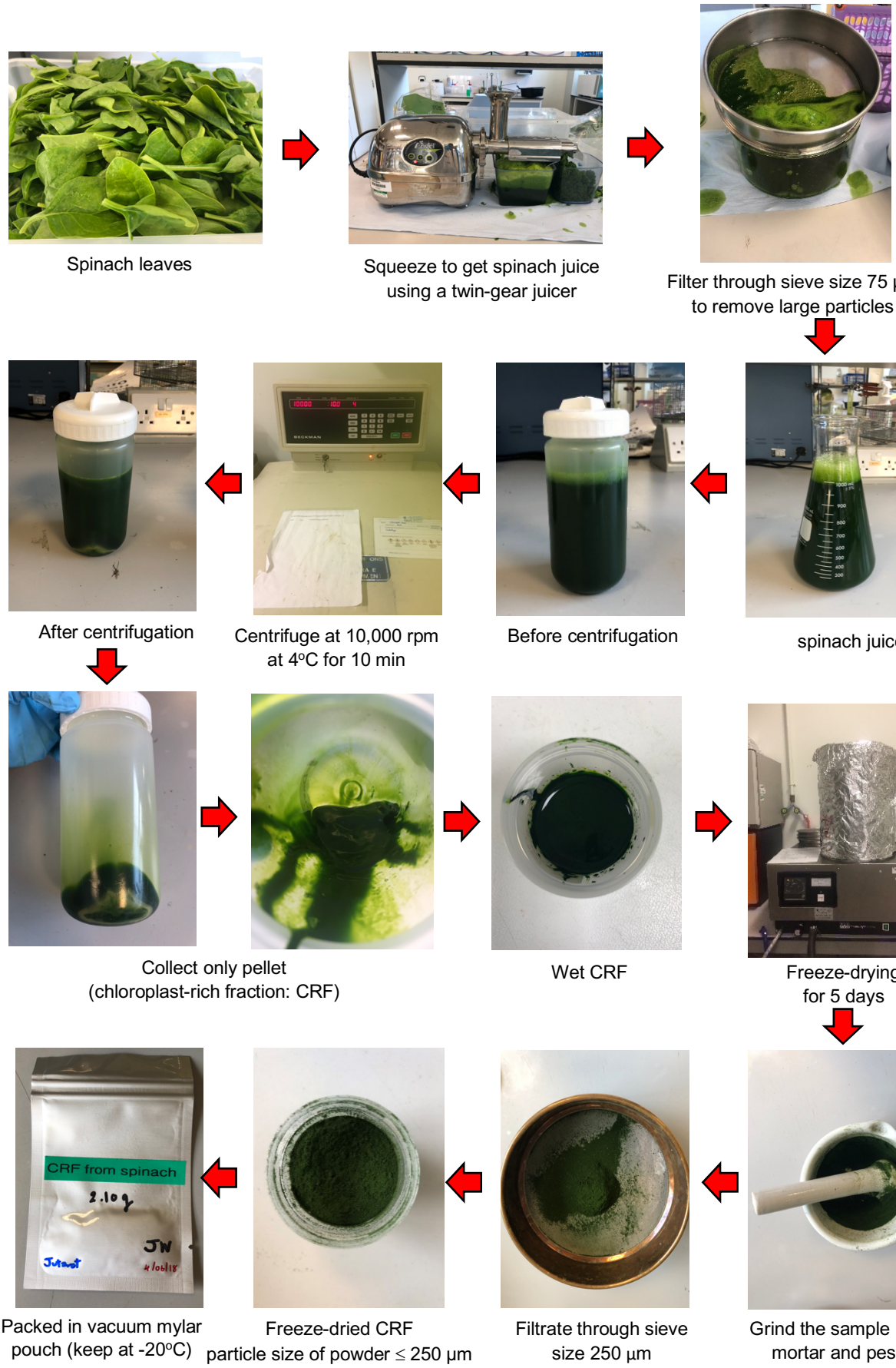
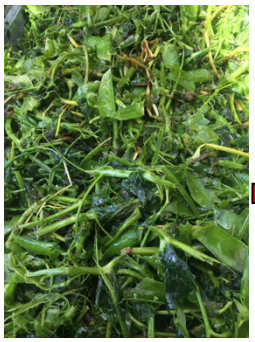


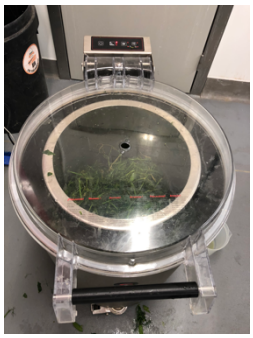
Figure 3.1: Representative of making spinach CRF by using a slow-screw twin-gear juicer.



Pea vine haulm (PVH)



Wash to remove the rock and soil



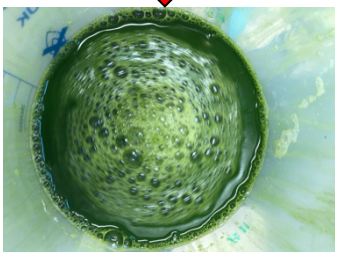
Remove the water using an industrial salad spinner (Sammic, ES-200)



Fibre



Squeeze using a twin-gear juicer



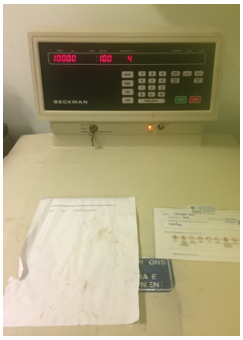
PVH juice



Filtrated through sieve (75 µm)



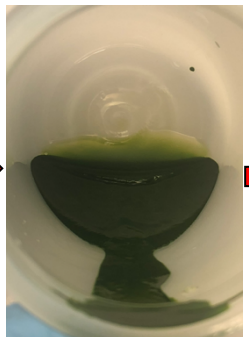
PVH juice after filtration

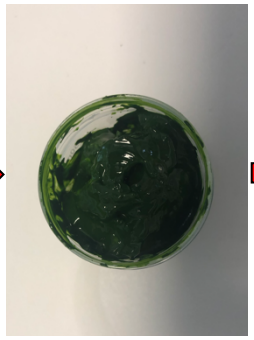


Centrifuge at 10,000 rpm at 4°C for 10 min



Collect only pellet (Chloroplast-rich fraction: CRF)

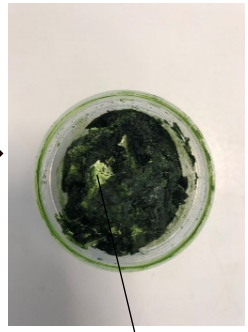




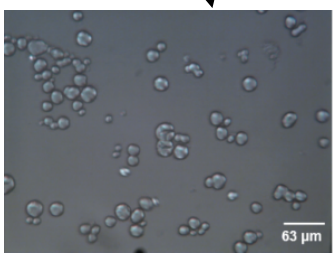
Wet CRF



Freeze-drying for 5 days



Freeze-dried CRF



Starch granule (polarised image)



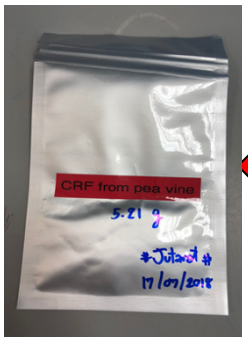
Grind the sample using mortar and pester



Filtrate through sieve size 250 µm



Freeze-dried CRF particle size of powder ≤ 250 µm



Packed in vacuum mylar pouch (keep at -20°C)

Figure 3.2: Representative of making PVH CRF by using a slow-screw twin-gear juicer.

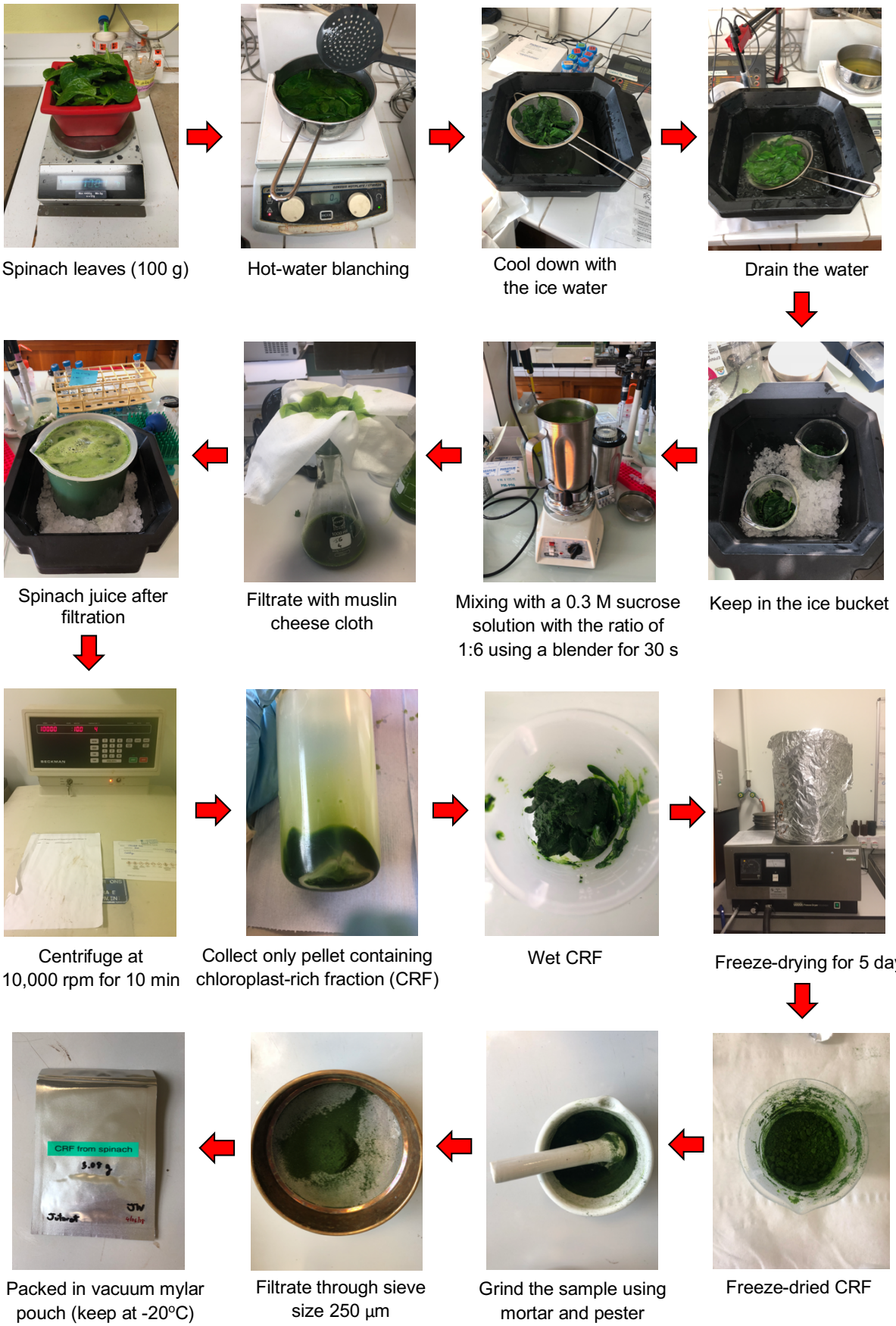


Figure 3.3: Representative of generating spinach CRF by using grinding leaves in 0.3 M sucrose.

3.4.7. Peroxidase enzyme activity assay

The enzyme activity, and therefore enzyme knock-out achieved by the different treatment techniques, was measured through a peroxidase (POD) enzyme assay adapted from Bahçeci et al. (2005) and Gökmen et al. (2005). Samples of juice were diluted 1/10 with deionised water to be added to an assay mix composed of 500 µL of each hydrogen peroxide and guaiacol in a total volume of 100 ml deionised water. The rate of colour change induced by enzyme activity was measured using a spectrophotometer (UV-Vis spectrophotometer, Genesys 10S, US) set at 420 nm. Readings were taken every 2 seconds over a 1.5 min period after 100 µL diluted juice was added to 2.9 mL assay mix. The increase in absorbance in 1 min was considered proportional to the POD enzyme activity and differences in rate of treated to untreated pea vine material were used to determine percentage enzyme activity reduction caused by the treatments.

3.4.8. Moisture analysis

Each of the materials was weighed in aluminium trays and then placed in an oven (Memmert, LN110, Germany) at 105°C and reweighed after 24 hours for 7 days or until constant weight. The moisture content was then calculated gravimetrically using the **Equation 1**.

$$\textbf{Moisture content (\%)} = \frac{(\text{wet sample (g)} - \text{dried sample (g)}) \times 100}{\text{wet sample (g)}} \quad (\mathbf{1})$$

3.4.9. Protein analysis

The protein content was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). 2% of Sodium dodecyl sulfate (SDS) solution (1 mL) was added to 0.1 g of dried sample. The sample was then heated at 60°C in the water bath

for 30 min and vortexed for 1 min. After that the sample was centrifuged (using ROTINA 380R) at 4,000 rpm or 3,112 rcf for 10 min and further diluted 1/100 with 2% SDS solution. A standard working reagent (2 mL) was added to 100 µL of each sample and standard solutions and further incubated at 37°C for 30 min. The standard working reagent was prepared by using 50 parts of reagent A and 1 part of reagent B (supplied in the assay kit). The absorbance was measured at 562 nm using an UV-Vis spectrophotometer (Thermo Scientific, Gensys10S, US) blanked with Reverse Osmosis (RO) water. The concentration was determined using polynomial equation produced from a range of bovine serum albumin (BSA) standards.

3.4.10. Amino acid analysis

Amino acids were determined by oxidising sample with 5 mL of a hydrogen peroxide/formic acid/phenol mixture for 18 hours. Excess oxidation reagent was decomposed with 0.84 g of sodium metabisulphite. The oxidised sample were hydrolysed with 6 M hydrochloric acid for 24 hours in the oven at 110°C ± 1°C. The hydrolysate is adjusted to pH 2.2 with 7.5 N sodium hydroxide solution. The hydrolysed sample was transfer to 200 mL of volumetric flask with 4 mL of norleucine (internal standard) and adjust the volume with tri-sodium citrate buffer, pH 2.2. The hydrolysate (20 mL) was transferred to a centrifuge tube and then centrifuged at 3,000 rpm for 2 min. The sample solution was filtered through 0.22 µm polytetrafluoroethylene (PTFE) filter membrane. The amino acids were separated by ion exchange chromatography (Biochrom) using sodium citrate buffers and determined by reaction with ninhydrin using photometric detection at 570 nm and 440 nm for proline. The representative chromatograms for the separation of amino acid are illustrated in **Figure 3.4**.

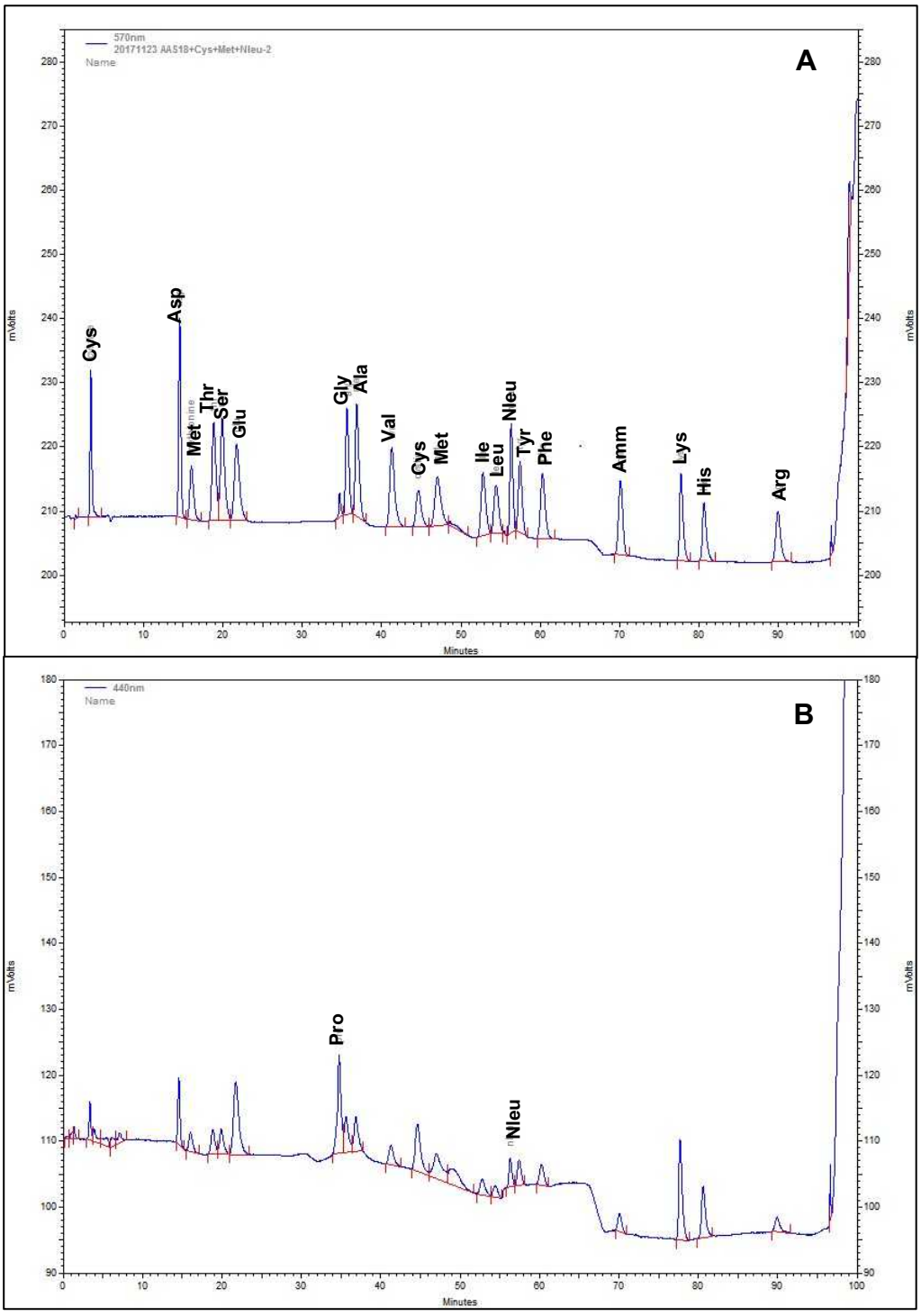


Figure 3.4: Amino acid chromatograms **A)** wavelength 570 nm for Cys (Cysteine), Asp (Aspartic acid), Met (Methionine), Thr (Threonine), Ser (Serine), Glu (Glutamic acid), Gly (Glycine), Ala (Alanine), Val (Valine), Ile (Isoleucine), Leu (Leucine), Nleu (Norleucine), Tyr (Tyrosine), Phe (Phenylalanine), Amm (Ammonia), Lys (Lysine), His (Histidine), Arg (Arginine) **B)** wavelength 440 nm for Pro (Proline).

3.4.11. Lipid extraction for dried powder

Lipid extraction was performed using the method of Folch et al. (1957), modified by Bligh and Dyer (1959). The dried sample (0.1 g) was dissolved in 2:1 mixture of chloroform and methanol (1.2 mL) and mixed for 1 min. 150 mM NaCl solution (1.2 mL) was then added to the mixture, which was vortexed again (1 min) and further centrifuged (using Thermo Electron Corporation, Jounan CR3i multifunction, US) at 3,000 rpm or 1,750 RCF for 10 min at 4°C after which it had separated into 3 phases. The lowest phase which contained lipids, was collected using a pasteur pipette and transferred into a fresh tube. A further 2:1 chloroform:methanol (1.2 mL) was added to the remaining aqueous phases and the mixture was vortexed and centrifuged again according to the same procedure. The lipid was removed and pooled with the original lipid layer. The combined lipid phase was then centrifuged under the same condition to separate the lipids from any residual compounds. The lipid extract was filtered through a 0.45 μm PTFE filter membrane and then dried under a flow of nitrogen. The dried lipid extracts were weighed to calculate the total lipid content as **Equation 2** and subsequently used for the analysis of β-carotene, lutein, α-tocopherol, phylloquinione, and fatty acid. The representative of lipid extraction is illustrated in **Figure 3.5**.

$$\textbf{Total lipid (\%)} = \frac{\text{dried lipid extract (g)} \times 100}{\text{initial sample before lipid extraction (g)}} \quad (\mathbf{2})$$

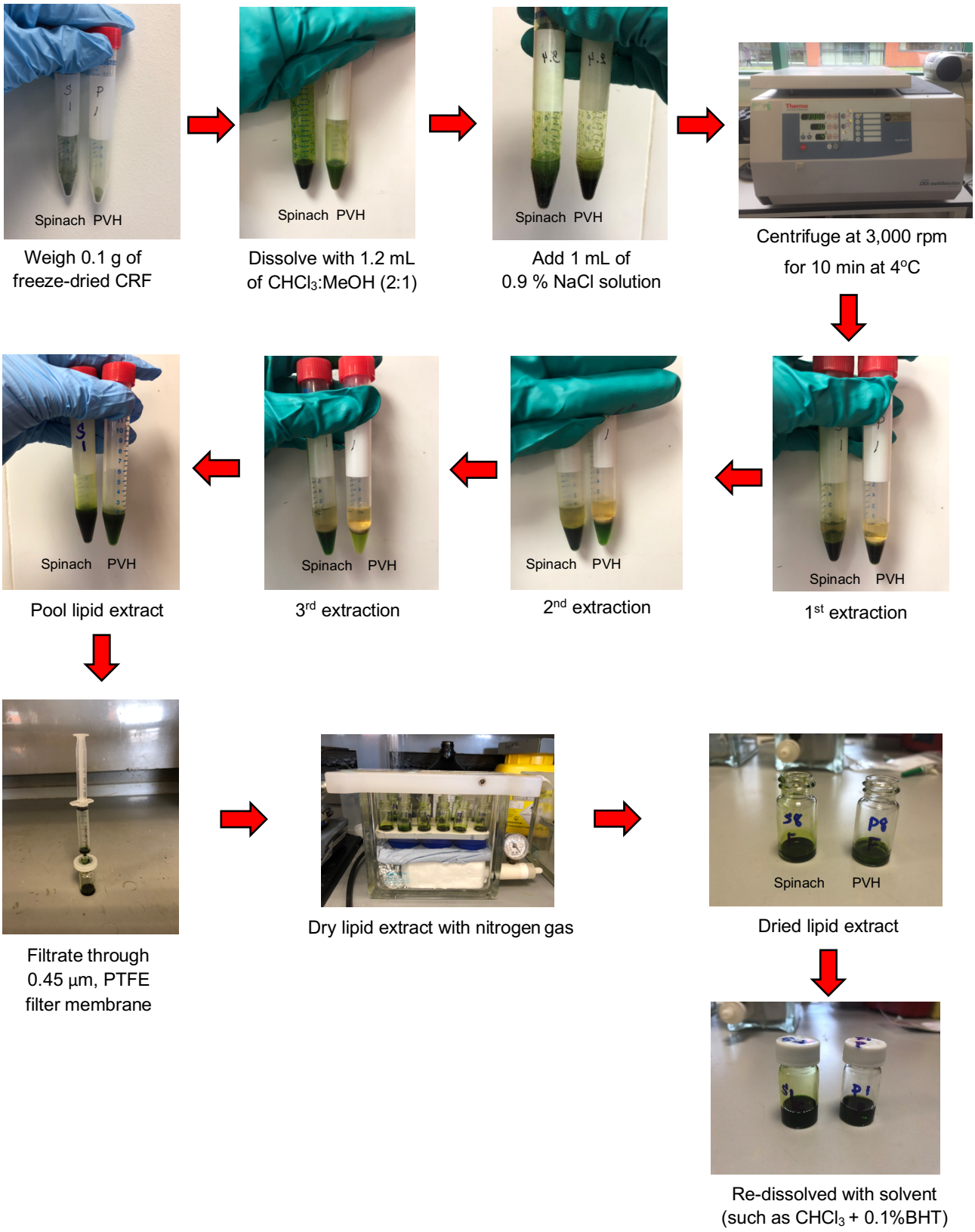


Figure 3.5: Representative of lipid extraction.

3.4.12. Starch analysis

The content of native starch was determined by means of enzymatic hydrolysis method using starch (HK) assay kit (SA20 starch assay kit, Sigma-Aldrich). Dimethyl sulfoxide (DMSO) (20 mL) and 8 M HCl (5 mL) were added to dried CRF (0.1 g). This mixture was incubated for 30 min at 60^{o}C in a shaking water bath. Then, deionised ultrapure water (50 mL) was added and the pH was adjusted to pH 5 with 5 N NaOH. The solution was cooled to room temperature and diluted to 100 mL with further addition of deionised ultrapure water comprising the sample to be tested for starch content. The starch assay composed of the addition of starch assay reagent from the kit (1 mL) containing 50 U/mL of amyloglucosidase *Aspergillus niger* and buffer salts to sample (1 mL) in a test tube. Blank was also prepared by mixing starch assay reagent (1 mL) with deionised water (1 mL) (starch assay reagent blank) and sample (1 mL) with deionised water (1 mL) (sample blank) in different test tubes. The test tubes were vortexed and incubated for 15 min at 60^{o}C in a shaking water bath and cooled to room temperature. The glucose assay consisted of the addition of each test tube of starch assay (50 µL) to glucose assay reagent from the kit (1 mL) (containing 1.5 mM NAD, 1 mM ATP, 1 U/mL hexokinase, 1 U/mL glucose-6-phosphate dehydrogenase with sodium benzoate and potassium sorbate as preservatives) in new test tubes. The glucose assay reagent blank was prepared by mixing glucose assay reagent (1 mL) and deionised water (50 µL). These solutions were vortexed, incubated for 15 min at 20^{o}C in a shaking water bath. The absorbance was measured at 340 nm using an UV-Vis spectrophotometer (Thermo Scientific, Genesys 10S, US). The starch content (mg/mL) was determined using **Equation 3** and **4**, respectively.

$$A_{\text{Total Blank}} = (A_{\text{Sample Blank}} - A_{\text{Glucose Assay Reagent Blank}}) + A_{\text{Starch Assay Reagent Blank}} \quad (3)$$

$$\text{Starch concentration (mg/mL)} = \frac{[(\Delta A)(TVSA/SVSA)(TVGA/SVGA)(MW_{starch})]}{[(\varepsilon)(d)(1{,}000)]} \quad (4)$$

Where $\Delta A = A_{\text{Test}} - A_{\text{Total Blank}}$, TVSA is the total assay volume in mL from starch assay (2 mL), SVSA is the sample volume in mL from starch assay (1 mL), TVGA is the total assay volume in mL from glucose assay (1.05 mL), SVGA is the sample volume in mL from glucose Assay (0.05 mL), MW_{starch} is the molecular weight of starch (162.1 g/mole), ε is the millimolar extinction coefficient for NADH at 340 nm (6.22 $mM^{-1}cm^{-1}$), d is the light path in cm (1 cm), and 1000 is the conversion factor for µg to mg.

3.4.13. Mineral analysis

The freeze dried CRF from spinach and PVH were transferred into Pyrex test tubes (16 x 100 mm) and weighted (5-10 mg). The trace metal grade nitric acid Primar Plus (Fisher Chemicals) spiked with indium internal standard was added to the tubes (2 mL per tube). The samples were then digested in dry block heater (DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK) at 115˚C for 4 hours. Once the samples cooled down, 1 mL of hydrogen peroxide (Primar, for trace metal analysis, Fisher Chemicals) was added to the tubes and samples were digested in dry block heater for additional 4 hours at 115˚C. The digested samples were diluted to 20 mL with 18.2 MΩcm Milli-Q Direct water (Merck Millipore). Elemental analysis was performed with an inductively coupled plasma-mass spectrometry (ICP-MS), PerkinElmer NexION 2000 equipped with Elemental Scientific Inc. autosampler, in the collision mode (He). Twenty-two elements (Li, B, Na, Mg, P, S, K, Ca, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Mo, Cd and Pb) were monitored. Liquid reference material composed of pooled samples of the digested CRF powders was prepared before the

beginning of sample run and was used throughout the whole samples run. It was run after every ninth sample to correct for variation within ICP-MS analysis run (Danku et al., 2013). The calibration standards (with indium internal standard and blanks) were prepared from single element standards (Inorganic Ventures; Essex Scientific Laboratory Supplies Ltd, Essex, UK) solutions. Sample concentrations were calculated using external calibration method within the instrument software.

3.4.14. Estimation of carbohydrate content

The percentage of carbohydrate content of freeze-dried CRF was estimated by difference of the other macronutrient and total minerals (ash) using **Equation 5**.

Carbohydrate (%) = 100 – lipid – protein – total mineral (ash) (**5**)

3.4.15. Phytic acid and total phosphorous analysis

The phytic acid (phytate) and total phosphorous were quantified by means of measuring phosphorous released by phytase and alkaline phosphatase (K-PHYT Assay kit, Megazyme). 0.66 M HCl (20 mL) was added to 1 g of freeze-dried CRF. The mixture was stirred at 500 rpm at room temperature for 16 hr under a fume cupboard. The extract (1 mL) was transferred to a microfuge tube and then centrifuged at 13,000 rpm for 10 min at room temperature. The supernatant (0.5 mL) was transferred to a new microfuge tube and neutralised with 0.75 M NaOH (0.5 mL). The representative of sample extraction is illustrated in **Figure 3.6**. For the enzymatic dephosphorylation reaction preparation, the free phosphorous condition was consisted of deionised ultrapure water (0.62 mL), buffer solution at pH 5.5 (containing sodium azide (0.02% w/v)) (0.2 mL), and sample extract (0.05 mL). The total phosphorous was

composed of deionised ultrapure water (0.60 mL), buffer solution, pH 5.5 (0.2 mL), sample extract (0.05 mL), and phytase suspension (0.02 mL). The mixture solutions of both free phosphorous and total phosphorous were vortexed and then incubated at 40°C in a water bath for 10 min. The next reaction was started by addition of deionised ultrapure water (0.02 mL) and buffer solution, pH 10.4 (containing $MgCl_2$, $ZnSO_4$ and sodium azide (0.02% w/v)) (0.2 mL) for the free phosphorous condition and buffer solution, pH 10.4 (0.2 mL) and alkaline phosphatase suspension (0.02 mL) for the total phosphorous condition. Both the conditions were vortexed and then incubated at 40°C in a water bath for 15 min. The reactions were stopped by addition of 50% w/v trichloroacetic acid (0.3 mL). The colour reagent consisted of solution A (containing 10% w/v ascorbic acid in 1 M sulfuric acid) and solution B (containing 5% w/v ammonium molybdate). The colour reagent was mixed in the ratio of 1 to 5 of solution B to solution A. For the colourimetric determination, the sample solution (1 mL) was transferred to a new microfuge tube and followed by addition of colour reagent (0.5 mL). The mixture solution was vortexed and then incubated at 40°C in a water bath for 1 hr. The absorbance of phosphorous was measured at 655 nm using an UV-Vis spectrophotometer (Thermo Scientific, Genesys 10S, US). The phosphorus calibration curve, phytic acid, and total phosphorus contents were calculated using **Equation 6-8**.

$$\mathbf{M} = \frac{P\ (\mu g)}{\Delta A_{phosphorus}} \qquad (\mu g/\Delta A_{phosphorus}) \qquad (\mathbf{6})$$

$$\mathbf{Mean\ M} = (M_{std1} + M_{std2} + M_{std3} + M_{std\ 4}) \qquad (\mu g/\Delta A_{phosphorus}) \qquad (\mathbf{7})$$

$$\mathbf{C} = \frac{\text{mean M} \times 20 \times F \times \Delta A_{phosphorus}}{10{,}000 \times 1.0 \times v} \qquad (g/100\ g) \qquad (\mathbf{8})$$

Where mean M is the mean value of phosphorus standards (($\mu g/\Delta A_{phosphorus}$), 20 is original sample extract volume (mL), F is dilution factor, ΔA is absorbance change of sample, 10,000 is a conversion from µg/g to g/100 g, 1.0 is weight of original sample material (g) and v is sample volume for used in the colourimetric determination step (mL).

It follows for phosphorus using **Equation 9-10**.

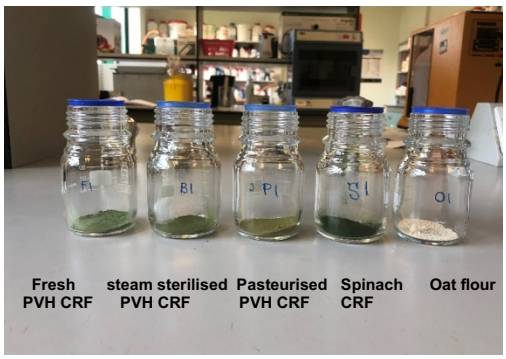
$$\mathbf{C} = \frac{\text{mean M} \times 20 \times 55.6}{10{,}000 \times 1.0 \times v} \times \Delta A_{phosphorus} \quad (g/100\ g) \quad (\mathbf{9})$$

$$\mathbf{C} = \text{mean M} \times 0.1112 \times \Delta A_{phosphorus} \quad (g/100\ g) \quad (\mathbf{10})$$

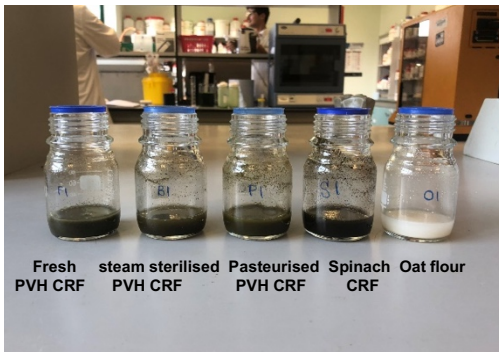
It follows for phytic acid using **Equation 11**.

$$\mathbf{C} = \frac{\text{phosphorus (g/100g)}}{0.282} \quad (g/100\ g) \quad (\mathbf{11})$$

Where 0.282 is the molar ratio of phosphorous present in IP6.



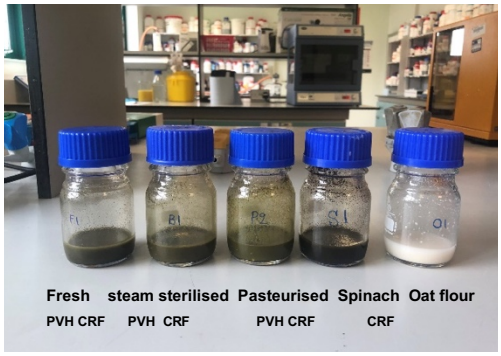
0.1 g dried sample

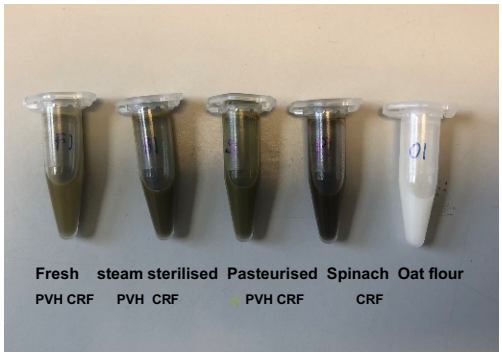


Add 20 mL of 0.66 M HCl

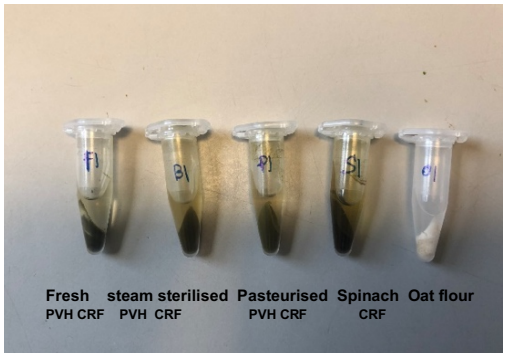


Leave at room temperature and stir at 500 rpm for 16 hr

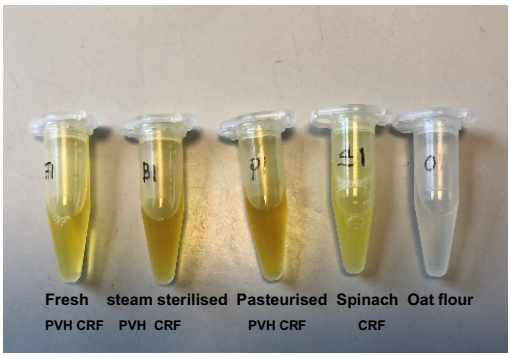




After 16 hours



After centrifugation at 13,000 rpm for 10 min



Supernatant (500 µL) + 0.75 M NaOH (500 µL)

Figure 3.6: Representative of sample extraction for phytic acid/total phosphorous analysis.

3.4.16. Chlorophyll analysis

The pigment content was analysed using an UV-Vis spectrophotometer (Thermo Scientific, Gensys10S, US). The lipid extract was diluted with acetone to be a factor of 1:100 for PVH CRF and a factor 1:500 for spinach CRF. The sample solution was measured at two different wavelengths; 661.6 nm (chlorophyll *a*) and 644.8 nm (chlorophyll *b*). Chlorophyll concentration (µg/mL) was calculated using **Equation 12-14** by Lichtenthaler and Buschmann (2001).

Chlorophyll a:

$$\text{Chlorophyll a } (\mu g/mL) = (11.24 \times A_{661.6}) - (2.04 \times A_{644.8}) \quad (\mathbf{12})$$

Chlorophyll b:

$$\text{Chlorophyll b } (\mu g/mL) = (20.13 \times A_{644.8}) - (4.19 \times A_{661.6}) \quad (\mathbf{13})$$

Total chlorophylls:

$$\text{Total chlorophylls } (\mu g/mL) = \text{chlorophyll a} + \text{chlorophyll b} \quad (\mathbf{14})$$

3.4.17. β-carotene and lutein analyses

The amount of β-carotene and lutein were analysed using High-Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) detection (Agilent 1100, Germany) using a modified method from Kimura and Rodriguez-Amaya (2002). The dried lipid extract was dissolved with acetone (containing 0.1% butylated hydroxytoluene (BHT)) (10 mL) and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter membrane into amber HPLC vials. The flow rate of the mobile phases (acetonitrile: methanol: ethyl acetate with 0.05% triethylamine (TEA)) was set at 0.5 mL/min. A gradient of mobile phases was applied from 95:5:0 v/v/v to 60:20:20 v/v/v in 15 min, and then held for 25 min. The mobile phase was returned to 95:5:0 v/v/v in 5 min and the column re-equilibrated for 15 min. Samples were injected at 10 µL of volume through a guard column (Phenomenex C18, 4 µm, 3.9 x 20 mm) and separated on a Waters, Nova-Pak C18 analytical column (3 µm, 4.6 x 150 mm) with the column temperature set at 22ºC. β-Carotene and lutein were detected at a wavelength of 454 nm. The β-carotene and lutein contents were determined using calibration curves established with reference standards and linear regression. The representative chromatograms for the separation of carotenoids are illustrated in **Figure 3.7**.

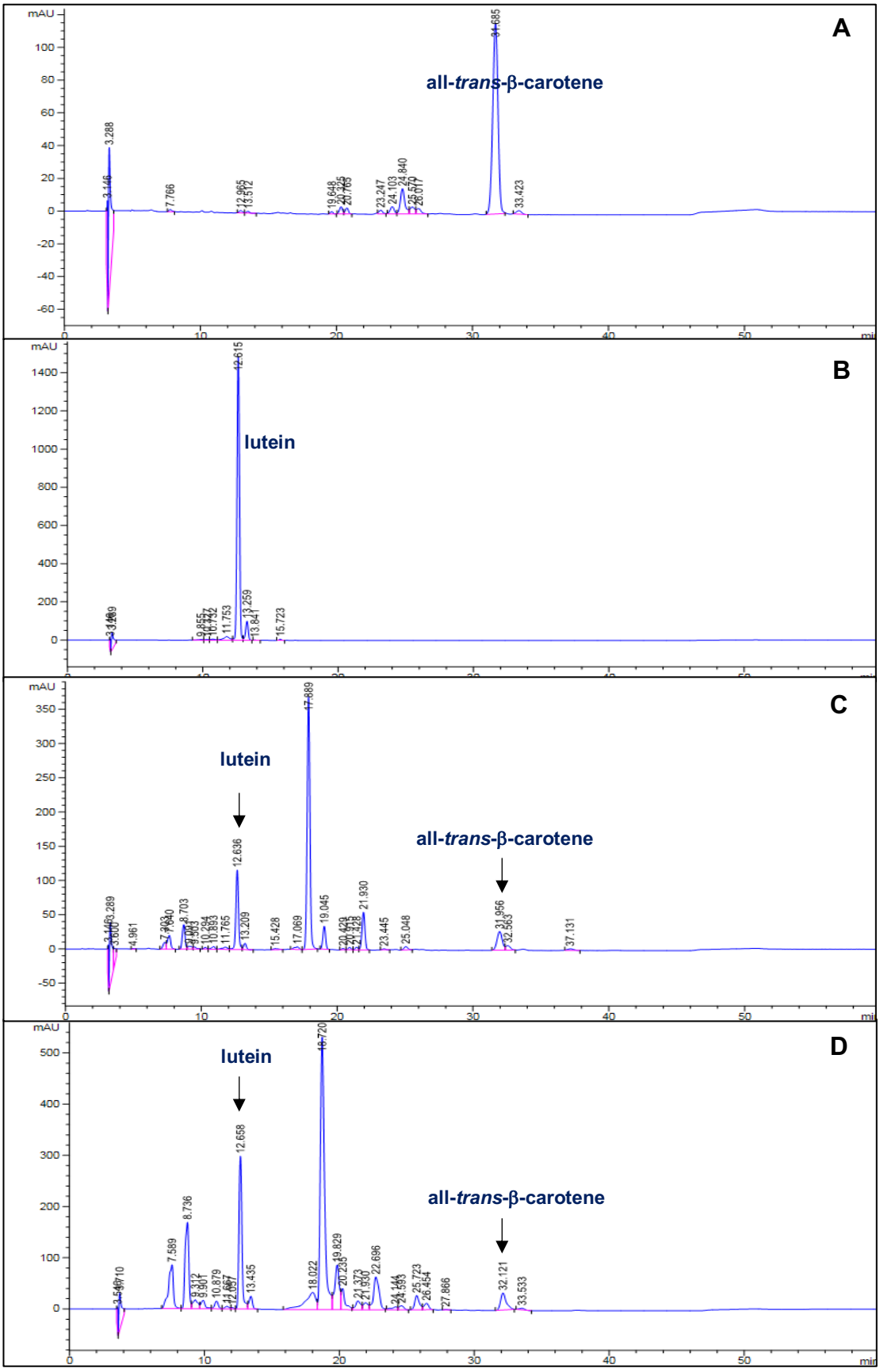


Figure 3.7: Representative of HPLC chromatograms at wavelength 454 nm for carotenoid separation **A)** β-carotene standard **B)** lutein standard **C)** PVH CRF **D)** spinach CRF.

3.4.18. α-Tocopherol analysis

α-Tocopherol content was analysed using high-performance liquid chromatography (HPLC) (Agilent 1100, Germany) with Fluorescence detector (Jasco FP-920, Japan) using a modified method of Rogers et al. (1993). The dried lipid extract was dissolved with methanol (containing 0.1% BHT) (2 mL) and filtered through 0.45 µm PTFE filter membrane into amber HPLC vials. A gradient system of two solvent A (acetonitrile: methanol: isopropanol: 1% acetic acid solution 45:45:5:5, v/v/v/v) and B (acetonitrile: methanol: isopropanol, 25:70:5 v/v/v) was employed. The flow rate of the mobile phase was set at 0.8 mL/min starting with 100% of solvent A for 6 min. Then the mobile phase changed linearly to become 100% of solvent B over the following 10 min. This system was held for 12 min before being returned to the initial conditions with a total run time of 36 min. Samples were injected at a volume of 10 µL through a guard column (Phenomenex C18, 4 µm, 3.9 x 20 mm) and separated on a Zorbax RX, C8 analytical column (5 µm, 250 x 4.6 mm) with the column temperature set at 20°C. α-Tocopherol was detected at the wavelength of 298 nm for excitation and 328 nm for fluorescence emission. The α-tocopherol content was determined using a calibration curve established with a reference standard and linear regression. The representative chromatograms for the separation of α-tocopherol are illustrated in **Figure 3.8**.

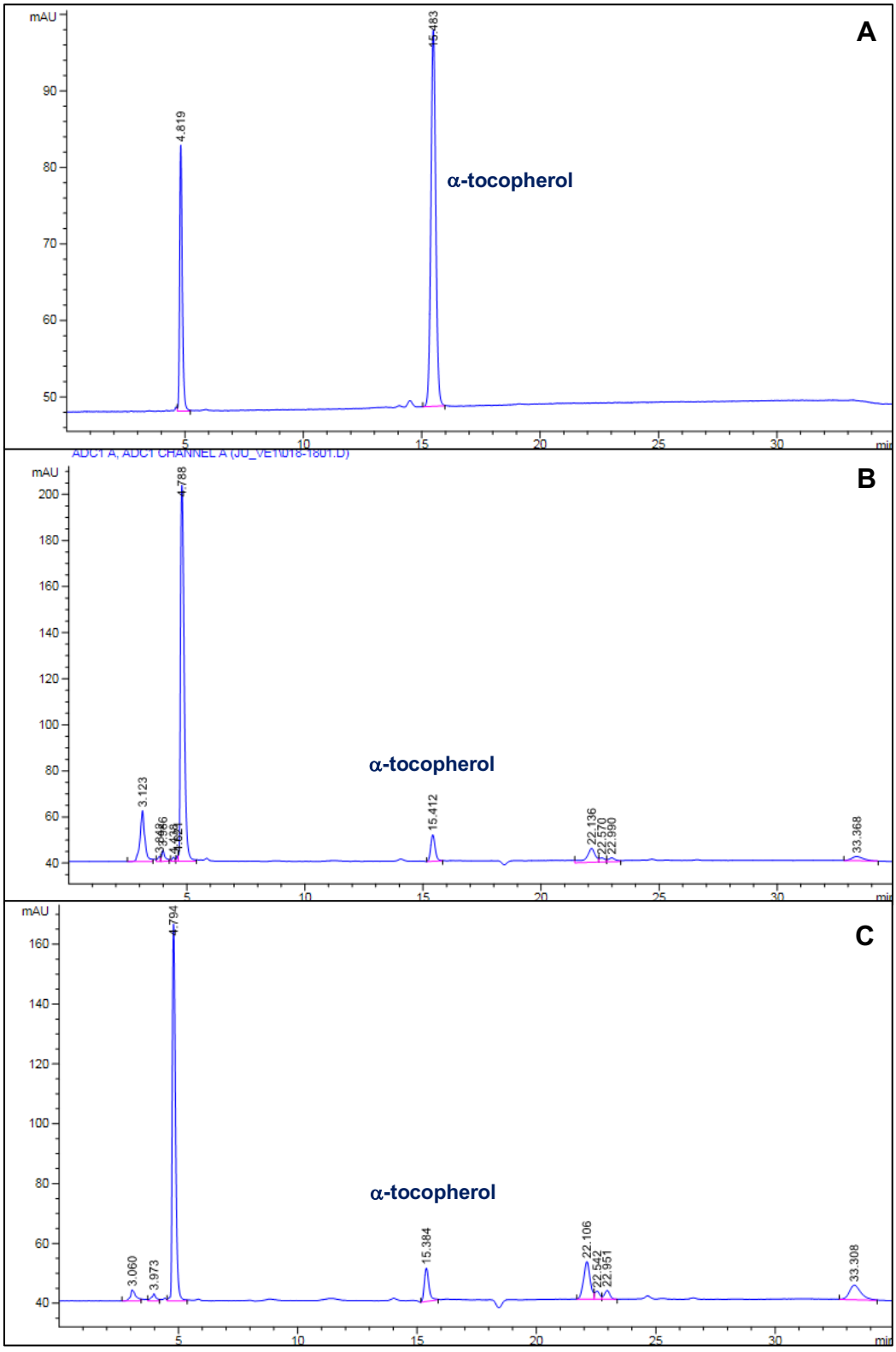


Figure 3.8: Representative of HPLC chromatogram for α-tocopherol separation at wavelength 298 nm for excitation and 328 nm for emission **A)** α-tocopherol standard **B)** PVH CRF **C)** spinach CRF.

3.4.19. Phylloquinone analysis

Phylloquinone was determined using high performance liquid chromatography (HPLC) with Photo Diode Array (PDA) detection (Agilent 1100, Germany) at 255 nm using a modified method of De Orsi et al. (2008). The lipid extract was dissolved with methanol containing 0.1% BHT (1 mL) and filtered through 0.45 µm PTFE filter membrane into amber HPLC vials. The flow rate was set at 1 mL/min starting with 50:37.5:12.5 water:acetonitrile:methanol. After 5 min of running isocratically, the mobile phase was changed using a 5 min linear gradient to 75:25 acetonitrile:methanol and maintained for 20 min. During the following 5 min the mobile phase was returned to 50:37.5:12.5 water: acetonitrile: methanol and the column re-equilibrated for a further 5 min. Samples were injected at a volume of 10 µL through a security guard column (Phenomenex C18, 4 µm, 3.9 x 20 mm) and separated on a Zorbax Eclipse XDB C18 column (5 µm, 4.6 x 150 mm) with the column oven set at 40°C. Phylloquinone content was quantified using a calibration curve established with a reference standard and linear regression. The representative chromatograms for the separation of phylloquinone are illustrated in **Figure 3.9**.

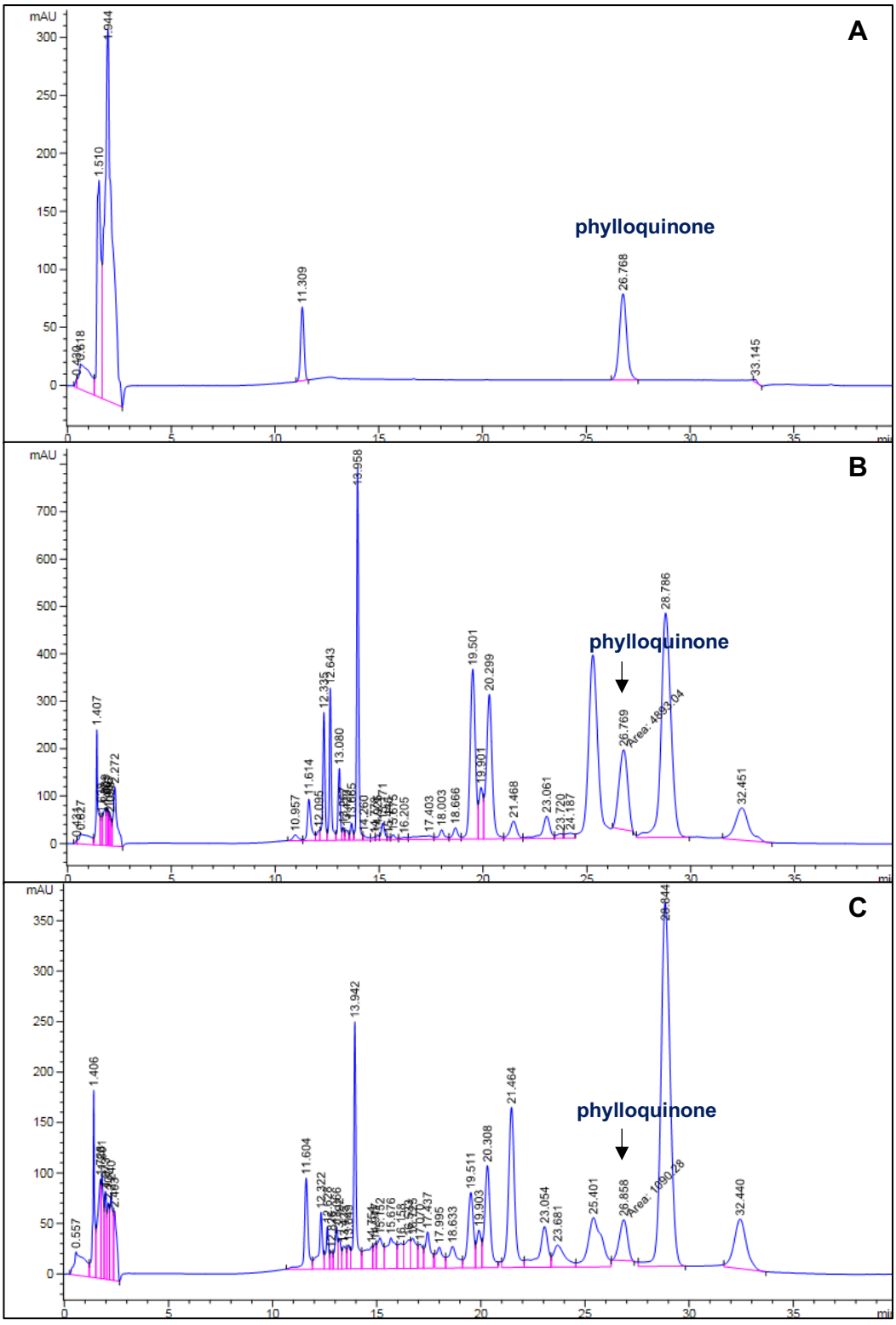


Figure 3.9: Representative of HPLC chromatogram of phylloquinone separation at wavelength 255 nm **A)** phylloquinone standard **B)** PVH CRF **C)** spinach CRF.

3.4.20. Fatty acid analysis

The fatty acids contained within the lipid extract were esterified to fatty acid methyl esters (FAMEs) and analysed using gas chromatography-mass spectrometry (GC-MS) (Thermo Scientific, DSQ, US) using a modified method of Bahrami et al. (2014). Dried lipid extract was dissolved in chloroform containing 0.1% BHT (2 mL) and transfer 1 mL to a new tube. Methylpentadecanoate (internal standard) and trimethyl sulfoniumhydroxide (TMSH) were added to the lipid extract in chloroform to convert both the free fatty acids (FFAs) and esterified fatty acids into FAMEs as shown in **Figure 3.10**. The reaction was performed for at least 10 min to ensure a completed conversion. The mixture (1 mL) was then filtered through 0.45 μm PTFE filter membrane into an amber vial. The sample (10 μL) was injected into a Phenomenex Zebron ZB-FFAP (30 m x 0.25 mm) column using a vaporising injector with a split flow of 50 mL/min of helium. The oven temperature was maintained at 120°C for 1 min and then increased to 250°C at ramp 5°C/min and held for 2 min. Detection was conducted using a mass spectrophotometer and the identification of individual fatty acids was achieved through a mass spectrum library by means of comparison retention time and molecular mass to FAME standards. The representative chromatograms for the separation of fatty acids are illustrated in **Figure 3.11**.

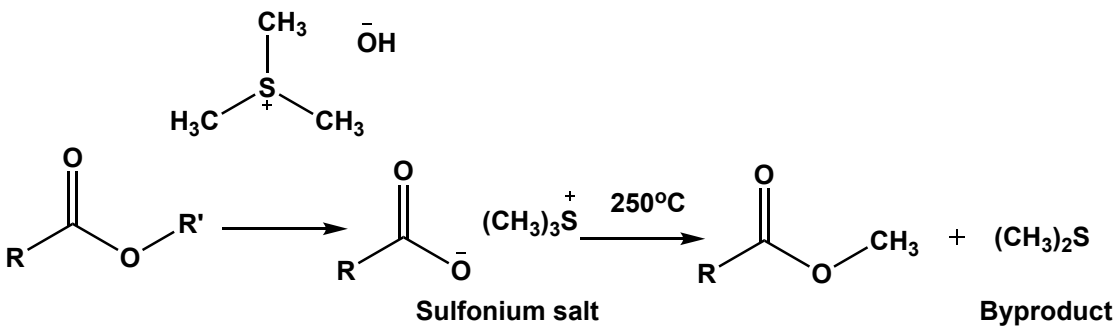


Figure 3.10: Methylation of fatty acid (Christie and Han, 2012).

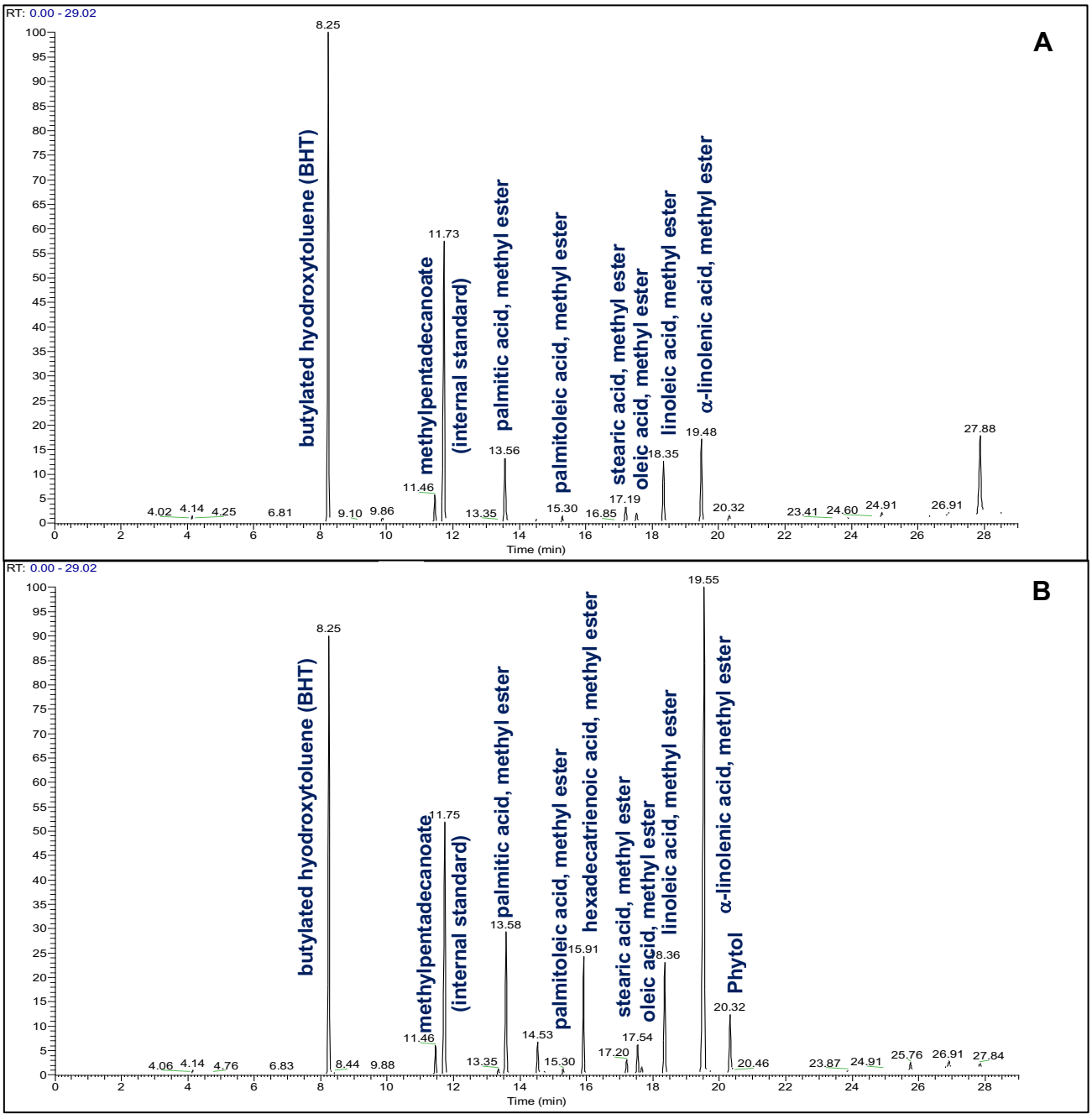


Figure 3.11: Representative of fatty acid chromatogram **A)** PVH CRF **B)** spinach CRF.

3.4.21. Packaging and storage conditions for the study of nutrient stability in CRF from steam sterilised PVH

The freeze-dried CRF was kept in mylar pouches (10 x 15 cm, 0.13 mm thickness, 130 μm mesh, Fresherpack, UK) and transparent bags (10 x 15 cm, Fresherpack, UK) as shown in **Figure 3.12**. Each pouch contained 0.4 g of freeze-dried sample and was sealed either under vacuum or non-vacuum (VacMaster VP215, Kansas, US). Samples were kept in the freezer at -20°C or the incubator (SANYO, MIR-153, Japan) at different temperatures (4°C, 25°C, and 40°C), with or without light (fluorescent lamp (FL15D, 15 W, 30-40 cm distance) at 600-800 lx (VWR, Traceable light Meter, US). The samples were rotated daily to minimise temperature differences and every 14 days one of the packs were taken at random for analysis until 84 days had passed. Their nutrient stabilities of β-carotene, lutein, α-tocopherol, and chlorophyll were analysed at each time point.

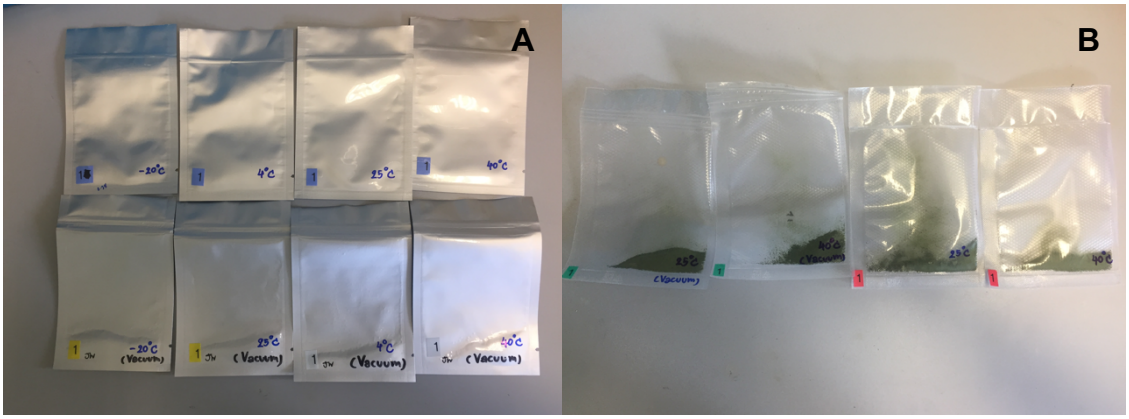


Figure 3.12: Representative of sample packaging **A)** samples in aluminium foil with vacuum and non-vacuum-packed **B)** samples in transparent bags (vacuum and non-vacuum packed).

3.4.22. Degradation kinetic during storage

The kinetic of experimental data were evaluated using first order kinetic **Equation 15** and **16**.

$$\ln C_t - \ln C_0 = -kt \quad (\mathbf{15})$$

$$\ln(C_t/C_0) = -kt \quad (\mathbf{16})$$

Where k is the first order rate constant, t is storage time, C_0 is the nutrient's initial concentration whilst C_t is their concentration at time t. The reaction rate constant was determined by calculating the slope non-linear fit on linear regression plots (GraphPad 8.0) between $\ln(C_t/C_0)$ and storage time.

The half-life ($t_{1/2}$) for the nutrient's concentration in the freeze-dried CRF of steam sterilised PVH to degrade to 50% of their initial concentrations was calculated by **Equation 17**.

$$t_{1/2} = \frac{0.693}{K} \quad (\mathbf{17})$$

The relationship between the rate of reaction storage and temperature was quantified by Arrhenius equation as **Equation 18** and **19**.

$$k = Ae^{-(E_a/RT)} \quad (\mathbf{18})$$

$$\ln k = -E_a/R.(1/T) + \ln A \quad (\mathbf{19})$$

Where E_a is the activation energy of the reaction (KJ/mol) and A is the pre-exponential constant, R is the universal gas constant (8.314 $J.mol^{-1}.K^{-1}$) and T is the absolute temperature of the storage temperature in Kelvin. The activation energy of the reaction over the gas constant was determined by calculating the non-linear slope fit on linear regression plots (GraphPad 8.0) of lnk vs 1/T.

3.4.23. Static *in vitro* digestion

The digestibility of nutrients *in vitro* was determined by the method of Minekus et al. (2014) and Brodkorb et al. (2019) with slight modifications. The preparation of stock solutions of simulated digestion fluids is shown in **Table 3.1**. The CRF (0.5 g) was dissolved in 4.5 mL of ultra-pure water for the condition without added vegetable oil. While rapeseed oil was added at a 1:1 ratio (0.5 g CRF: 0.5 g oil) in 4 mL of ultra-pure water for the condition with the presence of oil. The mixture solution was then mixed to 4 mL of simulated salivary fluid (SSF) electrolyte stock solution. Human salivary α-amylase solution (75 U/mL; 0.5 mL) was added, followed by $CaCl_2.2H_2O$ (0.3 M; 25 µL) and ultra-pure water (0.475 mL). This mixture was thoroughly mixed and shaken (150 rpm) for 2 min at 37°C and then simulated gastric fluid (SGF) electrolyte stock solution (8 mL) was added to the digestion mixture. Pepsin from porcine gastric mucosa (1mL; 2000 U/mL) and $CaCl_2.2H_2O$ (0.3 M; 5 µL) were then added to achieve 2000 U/mL pepsin in the final gastric digestion mixture. The pH was then reduced to 3 using 2 N HCl and the volume was made up to 20 mL with ultra-pure water. The samples were then shaken (150 rpm) for 2 hr at 37°C. The digesta from the gastric phase (20 mL) was then mixed with simulated intestinal fluid (SIF) electrolyte stock solution (11 mL), pancreatin solution (5 mL made up in SIF electrolyte stock solution to reach 100 U/mL of trypsin activity), and bile extract (2.5 mL at 10 mM bile salts) and $CaCl_2.2H_2O$ (0.3 M; 40 µL). The pH was then increased to 7 using 1 N NaOH and the volume made up to 40 mL with ultra-pure water. The intestinal digestion mixture was again incubated in the shaker (150 rpm) for 2 hr at 37°C.

Table 3.1: Preparation of stock solutions of simulated digestion fluids. The volumes are calculated for a final volume of 500 mL for each simulated fluid. It is recommended to make up the stock solution with ultra-pure water to 400 mL instead for storage at -20°C (Minekus et al., 2014, Brodkorb et al., 2019).

Constituent	Stock conc.		SSF pH 7		SGF pH 3		SIF pH 7	
			Vol. of stock	Conc. in SSF	Vol. of stock	Conc. in SGF	Vol. of stock	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
$NaHCO_3$	84	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	0.1	1.1	0.33
$(NH_4)_2CO_3$	48	0.5	0.06	0.06	0.5	0.5	-	-
For pH adjustment								
	M		**mL**	**mM**	**mL**	**mM**	**mL**	**mM**
NaOH	1		-	-	-	-	-	-
HCl	6		0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$ is not added to the simulated digestion fluids								
	g/L	**M**		**mM**		**mM**		**mM**
$CaCl_2(H_2O)_2$	44.1	0.3		1.5		0.15		0.6

SSF: Simulated Salivary Fluid; SGF: Simulated Gastric Fluid; SIF: Simulated Intestinal Fluid.

3.4.24. Study of bioaccessibility

To assess the bioaccessibility of lipophilic nutrients (carotenoids, vitamin E and fatty acids) (see **Figure 3.13**), 10 mL of the intestinal digestion mixture was fractioned by centrifugation at 5,000 rpm for 1 hour and the resulting supernatant was filtered through 0.2 µm PTFE filter membrane to obtain the micellar phase. After that, both the non-centrifuged sample (digesta) and the micellar phase were further submitted to lipid extraction and analysis (**Section 3.4.25**.). The percentages of nutrient retention after digestion, bioaccessibility, and nutrient in CRF available for uptake were calculated using the **Equation 20-22**.

$$\% \text{ Nutrient retention} = (C_{digesta})/(C_{initial}) \times 100 \quad (\mathbf{20})$$

$$\% \text{ Bioaccessibility} = (C_{micellar})/(C_{digesta}) \times 100 \quad (\mathbf{21})$$

$$\% \text{ Nutrient in CRF available for uptake} = (C_{micellar})/(C_{initial}) \times 100 \quad (\mathbf{22})$$

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

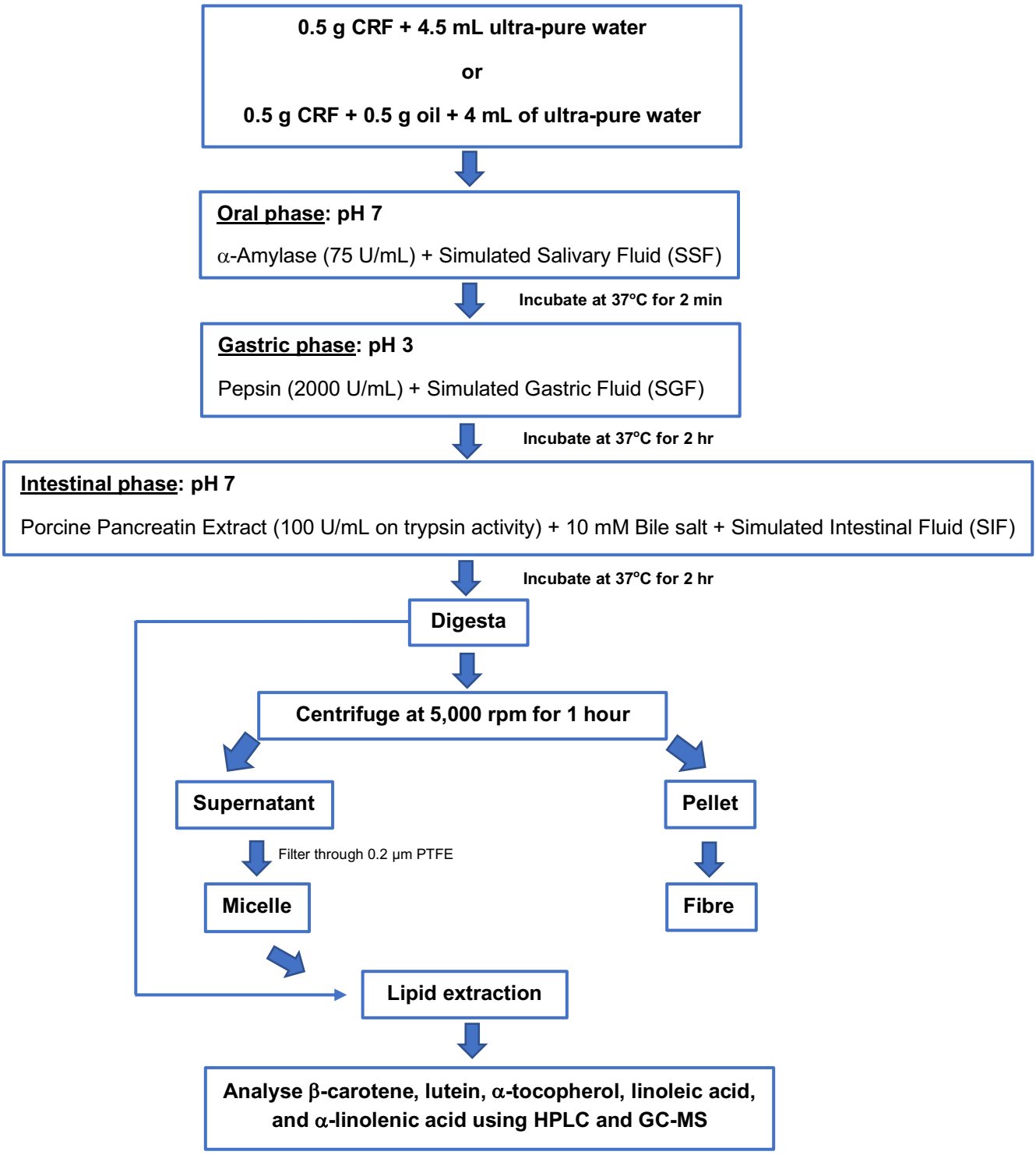


Figure 3.13: Flow chart of the study of nutrient bioaccessibility.

3.4.25. Lipid extraction for digested sample

Lipid extraction was performed using the method of Folch et al. (1957). The micellar phase and total digesta (5 mL each) were mixed with a 2:1 v/v mixture of chloroform and methanol (5 mL) and vortexed for 1 min. Sodium chloride solution (150 mM; 2 mL) was then added to the mixture which was vortexed again (1 min) and centrifuged (using Thermo Electron Corporation, Jounan CR3i multifunction, US) at 3,000 rpm or 1,750 RCF for 10 min at 4°C, after which it had separated into 3 phases. The lower organic phase, which contained lipids, was transferred into another tube. The remaining two phases were then extracted again with 2:1 v/v chloroform: methanol (5 mL), vortexed and centrifuged again. The lower organic phase was collected and pooled with the previous one. The lipid extract was filtered through a 0.45 μm PTFE filter membrane and then dried under a flow of nitrogen. The dried lipid extracts were further used for the analysis of β-carotene, lutein, α-tocopherol, and fatty acids.

3.4.26. Digestion of CRF galactolipid by GPLRP2

Freeze-dried CRF (25 mg) was suspended in 1 mL of buffer solution at pH 8 containing 0.3 mM Tris-HCl, 100 mM NaCl, 5 mM $CaCl_2$ and 4 mM sodium taurodeoxycholate (NaTDC). GPLRP2 was further added at a final concentration of 20 μg/mL and the reaction mixture was incubated at 37°C for 1 hr and compared with a control without GPLRP2. The reaction was stopped by adding 200 μL of 1 N HCl and further extracted lipid (**Section 3.4.27**).

3.4.27. Lipid extraction for High performance Thin Layer Chromatography (HPTLC) analysis

Lipid extraction was performed using the method of Folch et al. (1957) modified by Bligh and Dyer (1959). Sample (25 mg CRF or 1 mL of digestion sample) was mixed with 1 mL of 150 mM NaCl solution and 1.5 mL of a 2:1 v/v mixture of chloroform and methanol, then vortexed for 1 min. The mixture was then centrifuged (using Thermo Electron Corporation, Jounan CR3i multifunction, US) at 3,000 rpm or 1,750 RCF for 10 min at 4°C, which allowed the phase separation. The lowest organic phase, which contains lipids, was collected using a pasteur pipette and transferred into a fresh tube. A further 1.5 mL of 2:1 v/v chloroform: methanol was added to the remaining aqueous phase and the mixture was vortexed and centrifuged again according to the same procedure. The lipid extracts were pooled, and their volumes were measured before the organic phase was dried using magnesium sulphate. After centrifugation, the lipid extract was kept at -20°C until analysis.

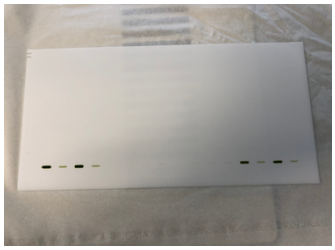
3.4.28. Quantitative analysis of galactolipids and their lipolysis products by High Performance Thin Layer Chromatography (HPTLC)

In order to separate and quantify galactolipids and lipolysis products, 10 to 50 µL of lipid extracts and known amounts (2, 4, 6, 8, and 10 µg) of lipid standards (MGDG, DGDG and oleic acid) were spotted as a 5 mm band onto a thin-layer silica plate using a Limonat IV (CAMAG) equipped with a 100 µL Hamilton syringe. The separation of polar lipids was performed with a chloroform/methanol/water (47.5:10:1.25, v/v/v) elution mixture. The separation of free fatty acids (FFA) on a second silica plate was performed with a mobile phase consisting of heptane:diethyl ether:formic acid (55:45:1, v/v/v) solvent mixture. Following chromatography, the plates for polar lipid/galactolipid analysis were dried at room temperature under a fume hood

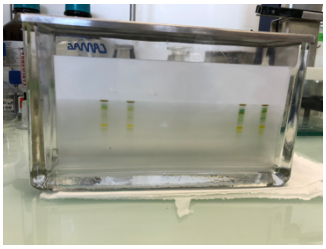
for 15 min and then dipped in a thymol solution prepared by dissolving 1g of thymol in 190 mL ethanol and then addition of 10 mL of 96% sulphuric acid. Since the mixing reaction is highly exothermic, the ethanolic solution has to be placed first in a cold water bath before sulphuric acid is added dropwise. The thymol solution allows the staining of galactolipids while avoiding the interference of pigments, especially chlorophylls, during the densitometric analysis of the plates. After staining with thymol, the plates were dried again in the fume hood for 10 min and then placed in an oven at 110^{o}C for 10 min. The plate for FFA analysis was dipped in a copper acetate-phosphoric acid solution prepared by mixing a saturated copper acetate solution with 85% phosphoric acid in a 1 to 1 volume ratio. The plates were dried for 10 min in the fume hood and then placed in an oven at 180^{o}C for 15 min. Densitometry analysis of the stained lipids on the TLC plate was carried out using a CAMAG TLC scanner II and a D2000+ chromato-Integrator (Merck, Darmstadt, Germany). 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. The densitograms of all tracks were integrated using D2000+Chromator-Integrator. Quantities of the lipids on the TLC plates were estimated from the linear standard curves established with the pure lipid standards (MGDG, DGDG, and oleic acid (18:1)). The representative for the quantification of galactolipid and free fatty acid is illustrated in **Figure 3.14** and the representatives for TLC chromatography of polar and neutral lipids are also shown in **Figure 3.15** and **3.16**.



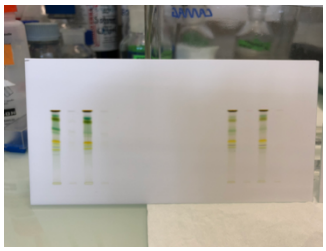
Spot lipid extract on TLC plate using a Limonat IV



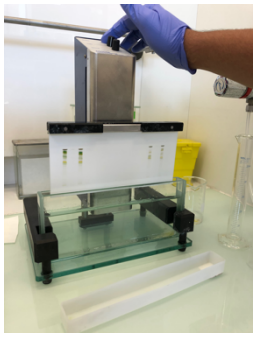
After spotting



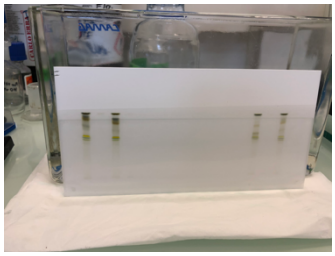
Developing TLC plate with the mobile phase



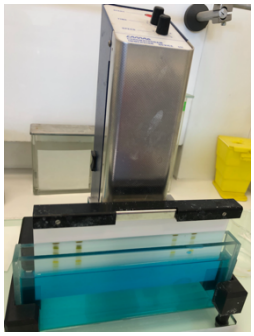
Leave the plate for 10 min under the fume hood



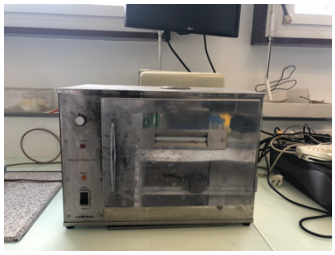
Dip the plate with thymol solution for galactolipid analysis



Leave the plate for 10 min under the fume hood



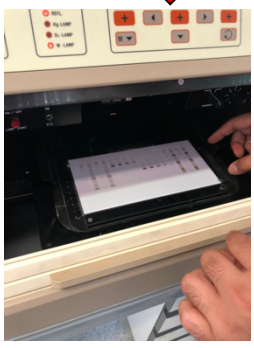
Dip the plate with copper acetate solution for free fatty acid analysis

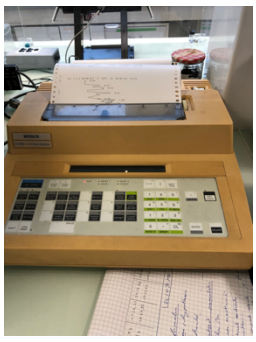


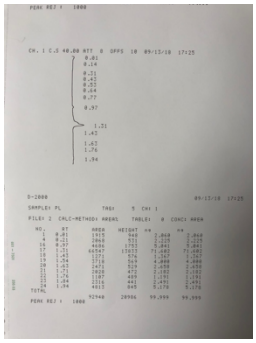
Charing the plate in the oven at 110°C for galactolipid analysis or at 180°C for free fatty acid analysis



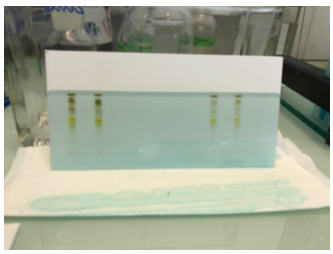
CAMAG TLC Scanner II and a D2000+ chromate-integrator







Quantify the amount of lipid using a densitometer (TLC scanner) to measure the intensity of each band and compare with a standard curve



Leave the plate for 10 min under the fume hood

Figure 3.14: Representative of quantification of galactolipids and free fatty acids using high performance thin layer chromatography (HPTLC).

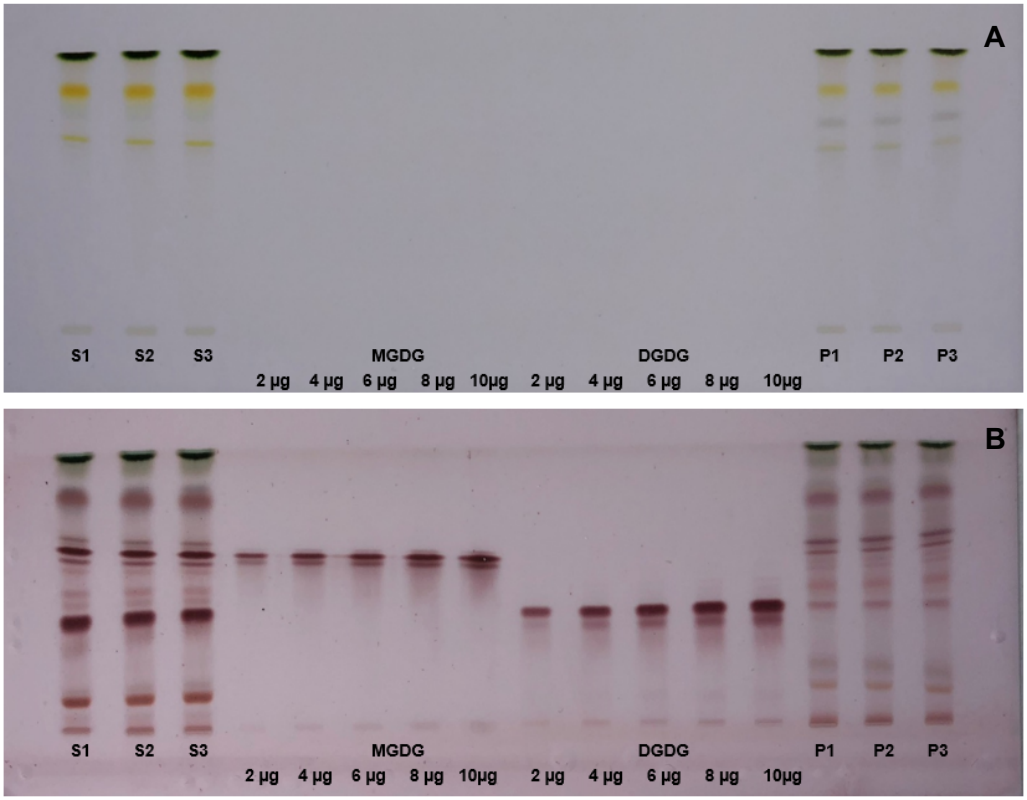


Figure 3.15: Representative of TLC chromatography for the quantification of polar lipid (galactolipid) in spinach CRF and PVH CRF. Lipid was extracted with methanol-chloroform. Polar lipids were separated using chloroform:methanol:water (47.5:10:12.5, v/v/v) elution mixture and were stained with a thymol solution. **A)** TLC plate before staining with thymol solution **B)** TLC plate after staining with thymol solution and charring at 110^{o}C; S: spinach CRF; P: PVH CRF; Loading volume at 20 µL both spinach CRF and PVH CRF.

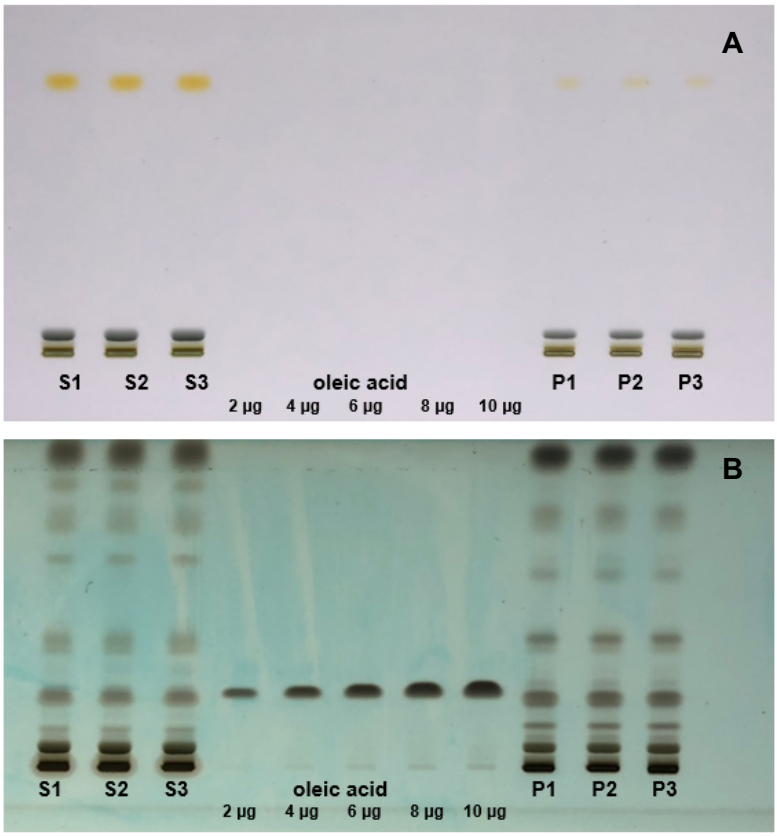


Figure 3.16: Representative of TLC chromatography for the quantification of non-polar lipid (fatty acid) in spinach CRF and PVH CRF. Lipid was extracted with methanol-chloroform. Non-polar lipids were separated using heptane:diethyl ether: formic acid (55:45:1, v/v/v) elution mixture and were stained with a copper acetate-phosphoric acid solution. **A)** TLC plate before staining with a copper acetate-phosphoric acid solution **B)** TLC plate after staining with a copper acetate-phosphoric acid solution and charring at 180^{o}C; S: spinach CRF; P: PVH CRF; Loading volume at 20 µL both spinach CRF and PVH CRF.

3.4.29. Two-step static *in vitro* digestion model

A two-step static *in vitro* digestion model was slightly modified from the procedure of Carriere et al. (2001) and set up at 37°C, pH 5 for the stomach phase and pH 6 for the small intestine phase in order to mimic the GI conditions at half gastric emptying time during a meal (Carriere et al., 2001, Sams et al., 2016). For the gastric phase, CRF (250 mg) was suspended in 10 mL of ultrapure water in a thermo regulated glass vessel (37°C) equipped with a pH electrode. Rabbit gastric extract (RGE; 25 gastric lipase U per mg of powder (tributyrin as substrate (Moreau et al., 1988, Brodkorb et al., 2019), equivalent to 21 µg lipase/mg) was added so that the final concentration of gastric lipase was 17 µg/mL (20 U/mL). The pH was adjusted at 5 and the solution was then incubated for 30 min under gentle magnetic stirring. For the intestinal phase, the solution from the gastric phase was diluted by half using a pancreatic enzyme-bile salts solution, pH was adjusted at 6 and the incubation was continued for 60 min. The final concentration of pancreatic lipase was set at 250 µg/mL or 2000 lipase U/mL and that of bile salt (NaTDC) was 4 mM. Two sources of pancreatic enzymes were tested: (1) freeze-dried HPJ, that contained 228 lipase U per mg of powder or (2) Sigma PPE that contained 67 lipase U per mg of powder. In both cases, lipase units (U) for pancreatic lipase refer to the assay using tributyrin as substrate (Carriere et al., 1993, Minekus et al., 2014). Samples (1 mL) from the digestion mixture were collected at various time points (0, 15, 29, 35, 40, 45, 60 and 90 mins) and immediately mixed with 10 µL of protease inhibitor cocktail (Complete™ from Roche). Each digestion sample was also acidified with 200 µL of 1 N HCl to stop the enzymatic reaction and the lipids were extracted according to **Section 3.4.27**.

3.4.30. Statistical analysis

All experiments were performed in triplicate. The statistical analysis was carried out using IBM SPSS statistics 25. The data for the comparison of two groups were analysed using an independent sample t-test. The data for the comparison of more than two groups were analysed using analysis of variance (ANOVA) followed by a *post-hoc* Tukey test. Statistical significance was set to the $p<0.05$ level and these data were expressed as mean ± standard deviation (SD). A difference of means at $p<0.05$ were considered significant. Principle Component Analysis (PCA) is a method of multivariate data analysis, which uses the spectral decomposition of a correlation coefficient. The use of PCA is to represent a multidimensional data as a smaller set of variables in order to observe trends, jumps, clusters, and outlier (Jolliffe and Cadima, 2016). The PCA analysis was carried out using the software Microsoft® Excel/ XLSTAT© (2019, Addinsoft SARL, 40 rue Damrémont, 75018 Paris, France).

CHAPTER IV

Nutrient composition of the chloroplast-rich fraction (CRF) from post-harvest, pea vine field residue (haulm) and spinach leaves

4.1. Introduction

As covered in the introduction and literature reviews, chloroplasts contain most of the nutrients found in green leaves. By recognising, this fact one can show methods to concentrate the nutrients from green biomass by separating intact chloroplast from the rest of leaf material. Underutilised green plant material is a rich source of chloroplasts. This chapter describes the recovery of a chloroplast-rich fraction (CRF) from postharvest pea vine field residue, or 'haulm' by using a slow-screw twin-gear juicer without added water and compare the nutrient composition of CRF from spinach leaves (*Spinacia oleracea*) that are excellent sources of nutrients. The nutrients of interest include both macronutrient (protein, carbohydrate, and lipid) and micronutrient, such as β-carotene (provitamin A), lutein, α-tocopherol (vitamin E), phylloquinone (vitamin K1), and fatty acid (omega-3 and omega-6 fatty acids). The analytical techniques: high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), UV-Vis spectrophotometry, and high performance thin layer chromatography (HPTLC) were used for these nutrient analyses.

4.2. Results and discussion

4.2.1. Physical characteristics and yield of CRF

The physical characteristics of both spinach leaves and PVH after centrifugation to generate CRF are illustrated in **Figure 4.1**. A large amount of white layer was found in PVH CRF (**Figure 4.1B**), but it was not found in spinach CRF (**Figure 4.1A**). The white layer in PVH CRF was further analysed using a light microscope with the polarised lens. It showed that there was a large amount of visible Maltese cross, which was a characteristic of starch granule under the polarised lens, as shown in **Figure 4.2**. The pellets of both spinach leaves and PVH (**Figure 4.3**) contained intact chloroplast and starch granule found only in PVH. The CRF powders produced by freeze-drying of spinach leaves and PVH are illustrated in **Figure 4.4**.

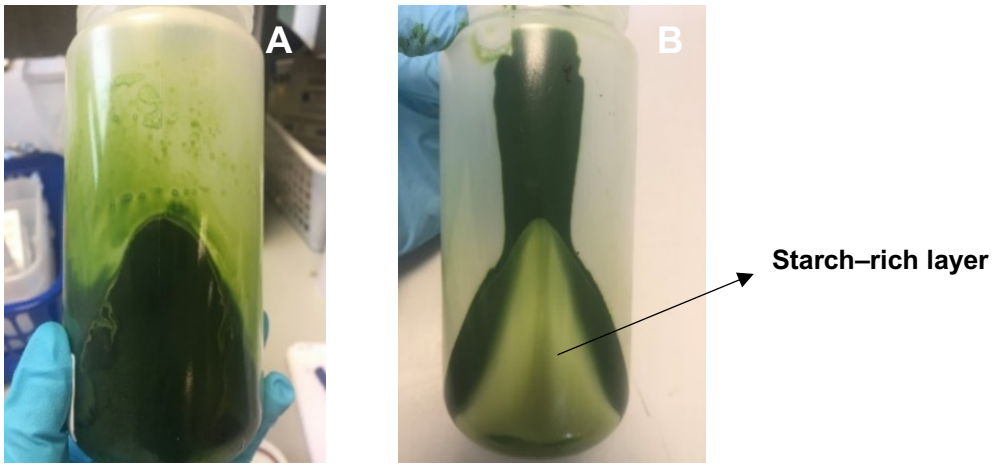


Figure 4.1: Pellet containing CRF after centrifugation **A)** spinach leaves **B)** PVH; The data of PVH in this chapter were PVH that was collected during summer in 2016.

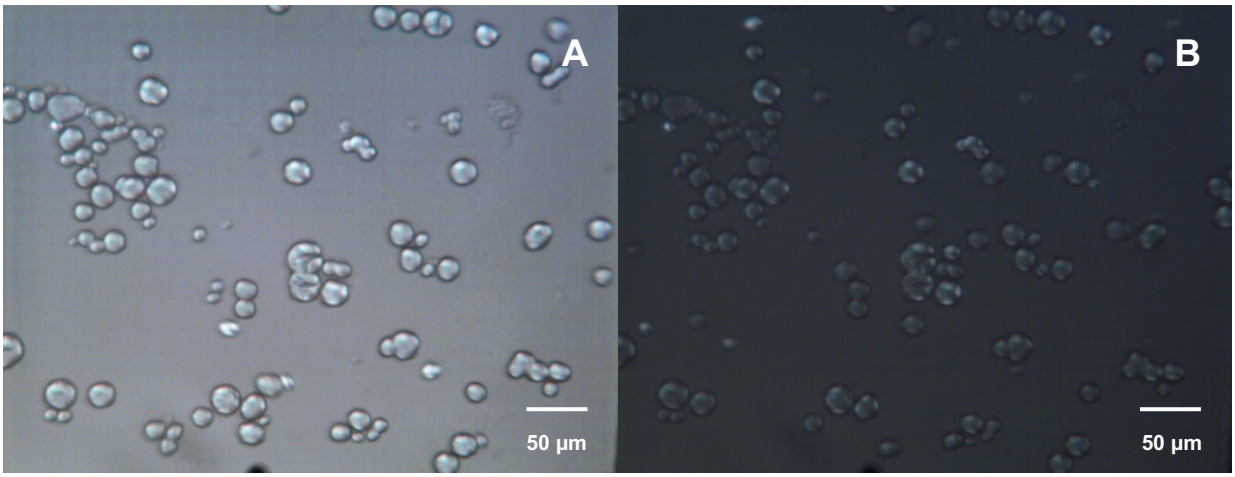


Figure 4.2: Light microscope images of starch granule in PVH using polarised lens **A)** light field image **B)** dark field image.

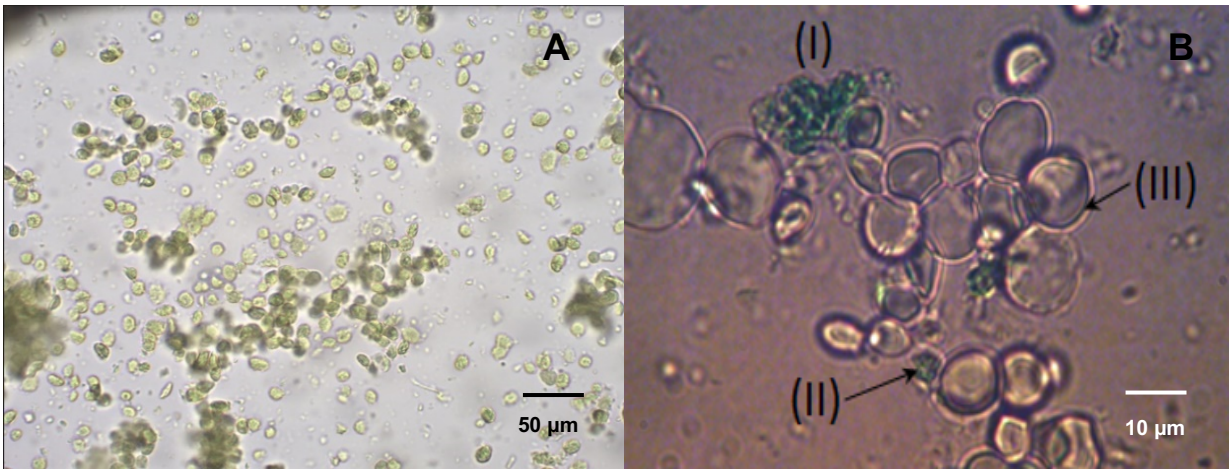


Figure 4.3: Microstructure of intact chloroplast of both CRF materials **A)** spinach leaves **B)** PVH (I: a cluster of chloroplasts, II: individual chloroplast, III: starch grain); The picture of PVH CRF was taken from Torcello-Gomez et al. (2019).

The yields of spinach CRF and PVH CRF after processing were 5.66 g/kg of fresh spinach leaves and 11.86 g/kg of fresh PVH, respectively. The yield of PVH CRF was higher content than spinach CRF. It might be due to starch granule in PVH CRF. The starch granule might promote fast sedimentation, which was observed over time before centrifugation, and also dragging the chloroplasts to the bottom.

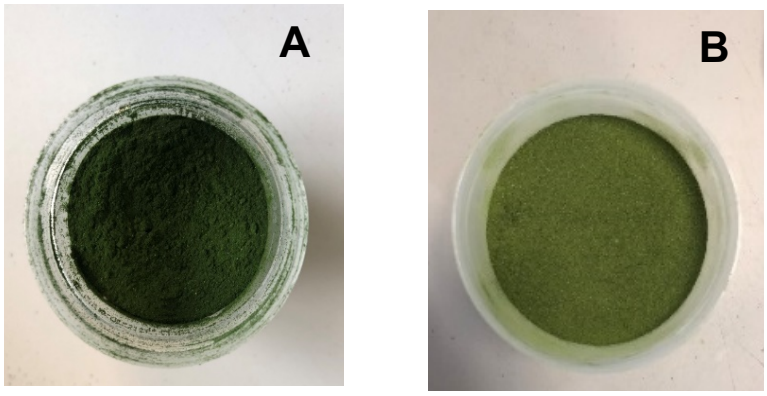


Figure 4.4: Physical characteristics of freeze-dried CRF **A)** CRF from spinach leaves **B)** CRF from PVH.

The freeze-dried CRF from spinach leaves was a darker green powder than CRF from PVH. It might be due to some dilution of starch in PVH CRF (see in **Figure 4.1**). Chlorophyll a and b are the two major structural forms of chlorophyll pigment in plant chloroplasts. This result was supported with the chlorophyll concentration in both CRF materials. The chlorophyll content of CRF from spinach leaves had 15.48 ± 1.86 mg/g CRF of chlorophyll a, 5.69 ± 0.68 mg/g CRF of chlorophyll b, and 21.18 ± 2.54 mg/g CRF of total chlorophyll. Meanwhile, the chlorophyll content of CRF from PVH showed 6.17 ± 0.46 mg/g CRF of chlorophyll a, 3.14 ± 0.15 mg/g CRF of chlorophyll b, and 9.31 ± 0.61 mg/g CRF of total chlorophyll (**Figure 4.5A**). Chlorophyll is one of the lipophilic pigment compounds, which was reported in term of mg/g lipid extract. The chlorophyll a of freeze-dried CRF from spinach leaves (64.54 ± 7.75 mg/g total lipid) was higher content than CRF from PVH (32.15 ± 2.42 mg/g total lipid) (**Figure 4.5B**). The proportion of chlorophyll a of both CRF materials was higher content than chlorophyll b as illustrated in **Figure 4.5C**. This was in agreement with Schwartz and Lorenzo (1990) reported that the level of chlorophyll b was lower level than chlorophyll a and had approximately one-third of chlorophyll a content. The chlorophyll a is the most abundant pigment in the antenna complex and the reaction centre of photosystem

I and II. While the chlorophyll b is only present in the antenna complex (Hall and Rao, 1999, Lichtenthaler and Buschmann, 2001).

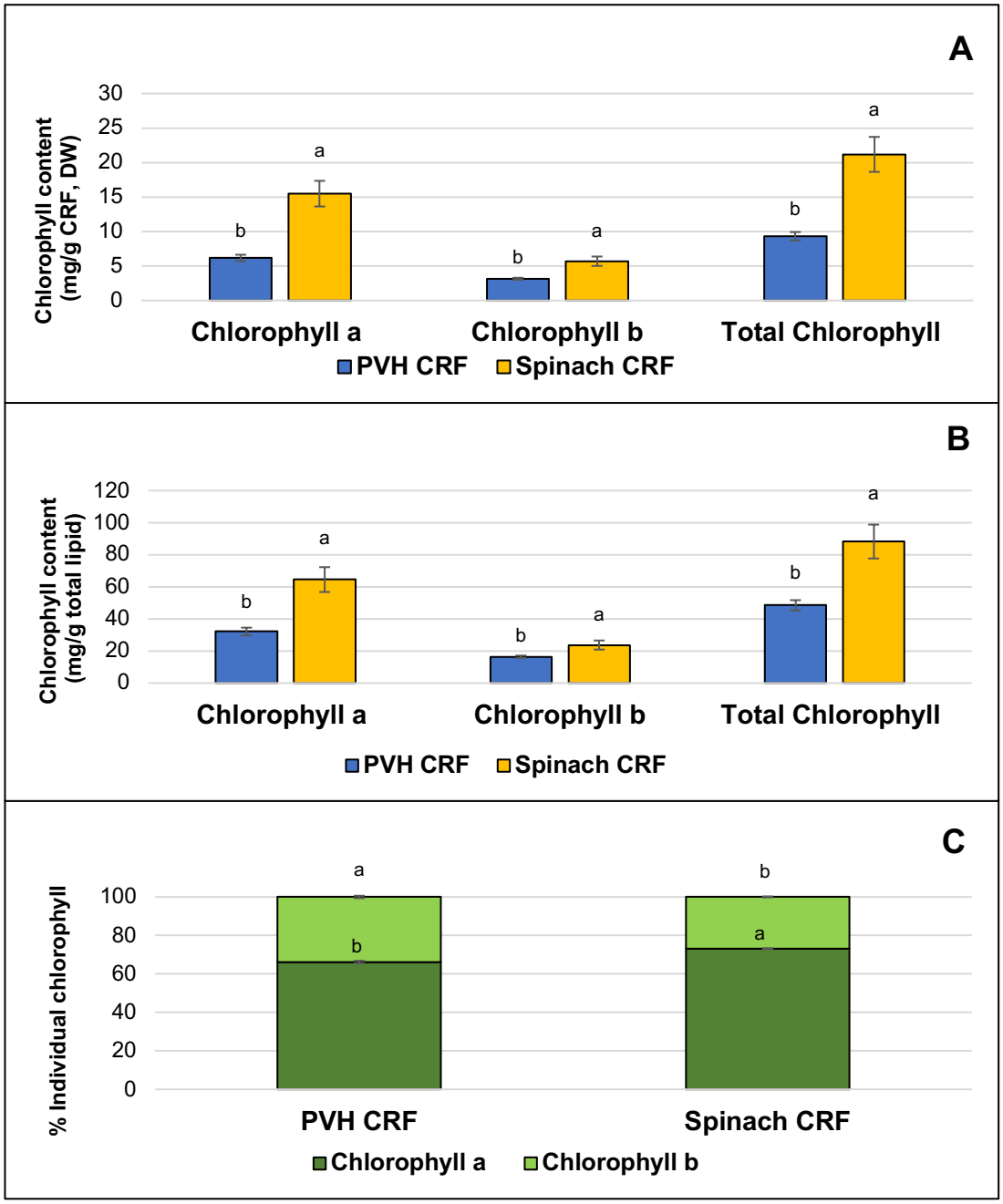


Figure 4.5: Chlorophyll composition of PVH CRF and spinach CRF **A)** mg/g CRF, DW **B)** mg/g lipid extract, DW **C)** percentage (%) of individual chlorophyll; Data were presented as a mean ± SD of 3 separated samples and analysed using an Independent-sample t-test with statistical significance at $p<0.05$, a>b; a means higher value than b; PVH was collected in summer 2016.

4.2.2. Macronutrient composition of CRF

Macronutrients are nutrients that provide calories or energy. They are required in large amounts to maintain body function and carry out the activities of daily life. Macronutrients include protein, lipid, and carbohydrate (Tull, 1996). Macronutrients in the CRF from PVH and spinach leaves were measured (see **Figure 4.6**). The largest contribution of the CRF from PVH came from the estimated carbohydrate (42.8% on a dry-weighted basis), which consisted of 24.2% starch content and 18.6% of sugar and fibre. Next, in decreasing order of content, was soluble protein (38%) and total lipid (19.2%). While Torcello-Gomez et al. (2019) reported that the largest component of macronutrient from PVH CRF was carbohydrate (42%) and followed by soluble protein (24%) and total lipid (22%). The difference of protein and lipid contents between this result and Torcello-Gomez et al. (2019) could be due to the different batch of pea vine harvesting and different types of juicer for making CRF. Torcello-Gomez et al. (2019) collected PVH in August 2015 and extracted CRF using screw-press juicer. While this result, PVH was collected in July 2016 and extracted CRF using slow-screw twin-gear juicer.

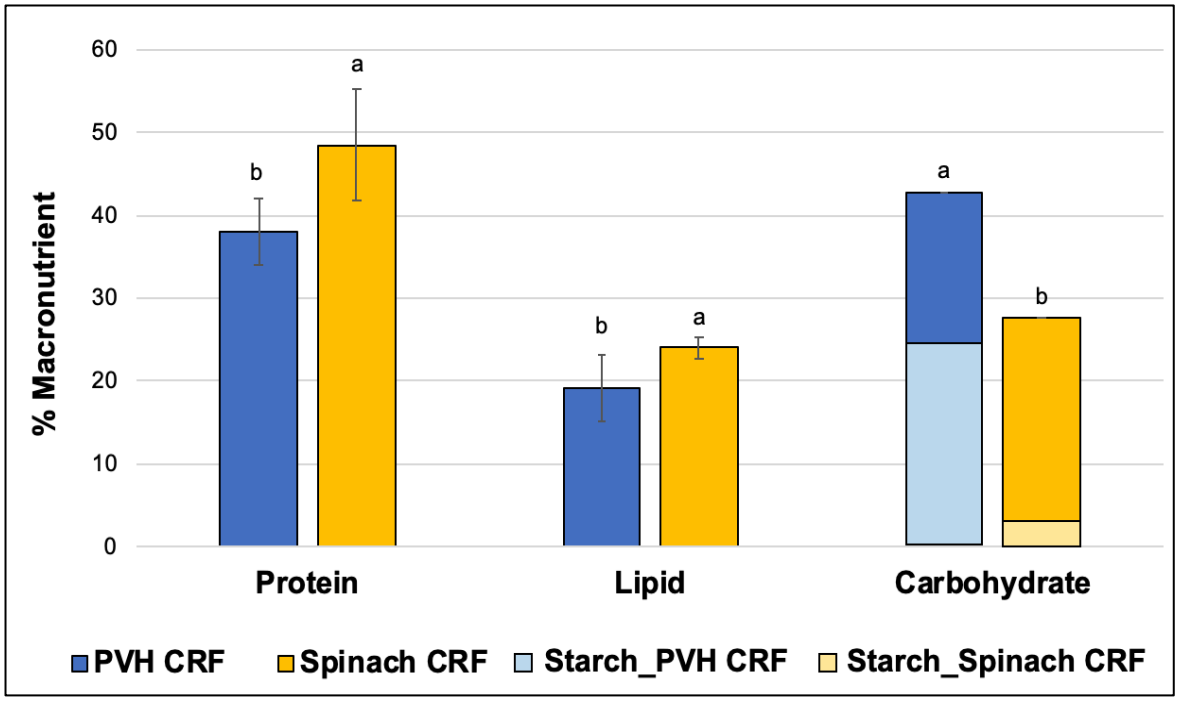


Figure 4.6: Macronutrient compositions of PVH CRF and spinach CRF; Data were presented as a mean ± SD of 3 separated samples and analysed using an Independent sample t-test with statistical significance at $p<0.05$, a>b; a was a higher value than b; PVH was collected in summer 2016.

The major components of macronutrient in CRF from spinach leaves was protein (48.4% on a dry-weight basis), followed by carbohydrate (27.6%), and lipid (24%), respectively. It was observed that the starch content in CRF from spinach leaves was 2.36% and the sugar and fibre was 25.2% from the rest of carbohydrate in spinach CRF (**Figure 4.6**). While Gedi et al. (2017) reported that the major components of macronutrient from spinach CRF was protein (42.6%), followed by lipid (36.9%), and carbohydrate (16.4%), respectively. The difference of macronutrient composition of spinach CRF in this thesis and the result of Gedi et al. (2017) could be due to different batch of spinach leaves and the method of CRF extraction. In this result, spinach CRF was extracted by using slow-screw twin-gear juicer. While Gedi et al. (2017) used 0.3 M sucrose grinding for CRF extraction.

In addition, the caloric contents can be calculated by knowing the composition of protein, lipid, and carbohydrate in the freeze-dried CRF and applying the standard Atwater factor (1 g of protein yields 4 kcal, 1 g of lipid yields 9 kcal, and 1 g of carbohydrate yields 4 kcal). The caloric contents of both CRFs were 4.84 kcal and 4.96 kcal in 1 g of PVH CRF and 1 g of spinach CRF, respectively.

From the thin-layer chromatography (TLC) analysis in **Figure 4.7**, it is illustrated that the major lipid in both CRF materials from PVH and spinach leaves was galactolipids, including MGDG and DGDG and phospholipids, including PE, PG and PC (found only in CRF from spinach leaves). This result was consistent with the previous reports that reported the main lipid composition of the chloroplast contained galactolipids (MGDG and DGDG) and phospholipids, particularly PG (Hitchcock and Nichols, 1971, Douce, 1974, Dörmann, 2013, Hölzl and Dörmann, 2019).

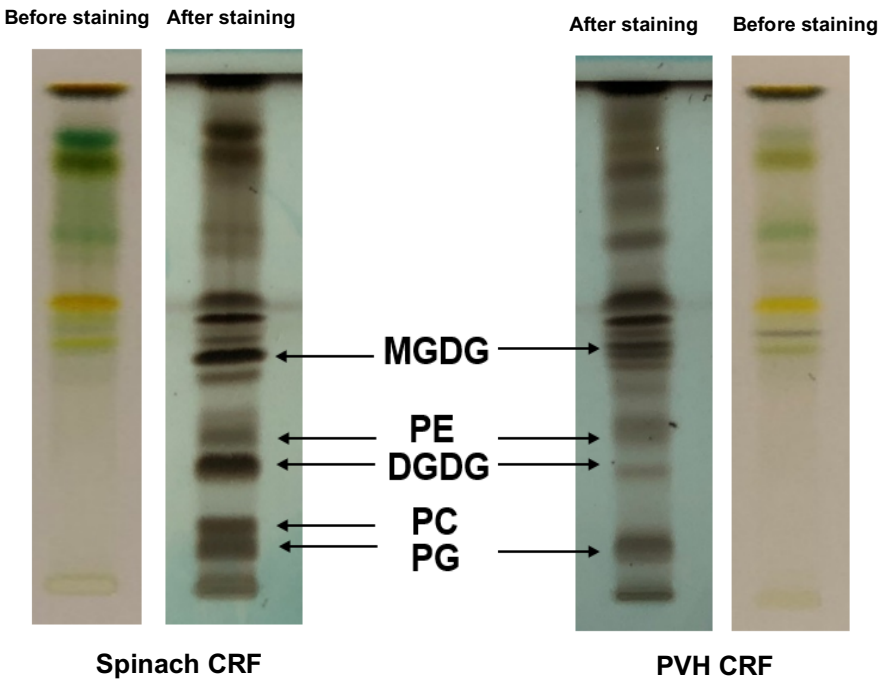


Figure 4.7: Polar lipid compositions in spinach CRF and PVH CRF using TLC analysis with the mobile phase ($CHCl_3$:CH_3OH:H_2O (47.5:10:1.25, v/v/v), staining with copper acetate-phosphoric acid solution, and charring at 180^oC; Loading volume at 20 µL with the concentration at 12.5 mg/mL in both CRF materials; MGDG: monogalactosyl diacylglycerol; DGDG: digalactosyl diacylglycerol; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol.

The amino acid composition of both CRF materials from spinach leaves and PVH was quantified as shown in **Figure 4.8**. Both CRF materials contained 17 amino acids. Eight essential amino acids, including Met, Thr, Val, Ile, Leu, Phe, Lys, and His and nine non-essential amino acids, including Cys, Asp, Ser, Gln, Gly, Ala, Tyr, Arg, and Pro. Both CRF materials and soybean showed lower amounts of all amino acids when compared to albumin from egg white in term of mg/g of dry-weighted basis (**Figure 4.8A**). Meanwhile, some amino acids (Asp, Met, Ile, Leu, Phe, Lys) in both PVH CRF and spinach CRF were higher content when compared to soybean in term of percentage (see **Figure 4.8B**). Seven amino acids (Asp, Gly, Ala, Leu, Tyr, Arg, and

Pro) in both CRF materials showed higher content when compared to albumin in term of percentage (**Figure 4.8B**). Aspartic acid, glutamic acid, and leucine were found as the major amino acids in PVH CRF and spinach CRF. This result was agreed with the previous reports, which reported that glutamic acid was a dominant amino acid in spinach leaves (22.4 % of the total amino acids) (Xbrain et al., 2016). Three major amino acids, including leucine, glutamic acid, and aspartic acid, were found in four different cultivars of a pea, namely Dingi, Gehria, Holland Metor, and P-8 (Jabeen et al., 1988).

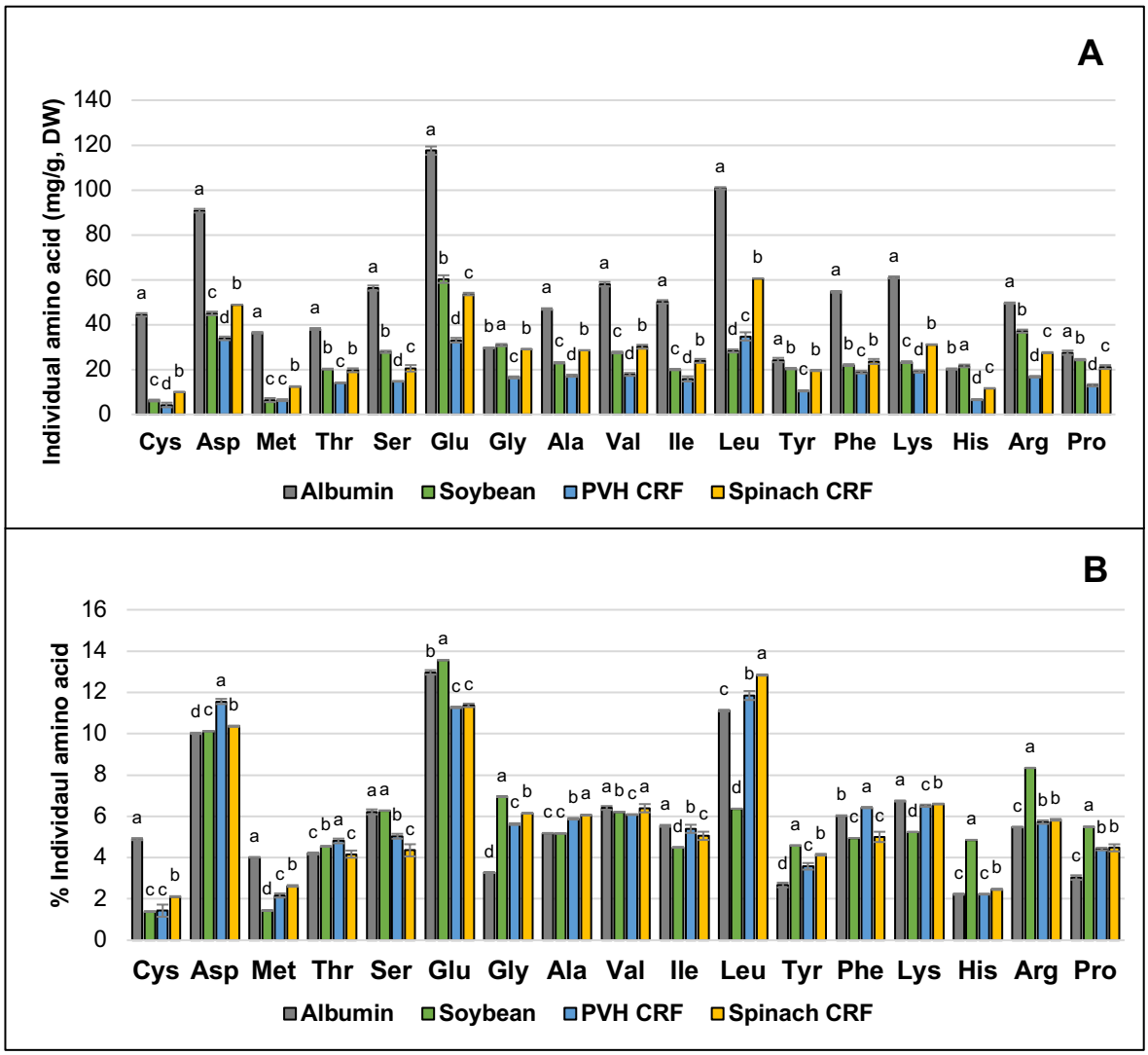


Figure 4.8: Amino acid compositions of albumin, soybean, PVH CRF and spinach CRF **A)** mg/g, DW **B)** percentage (%) of amino acid. Data of soybean was taken from Panthee et al. (2006). Data were presented as a mean ± SD of 3 separated samples and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b; a was a higher value than b followed by c and d, respectively; *Cys* Cysteine, *Asp* Aspartic acid, *Met* methionine, *Thr* Threonine, *Ser* Serine, *Glu* Glutamic acid, *Gly* Glycine, *Ala* Alanine, *Val* Valine, *Ile* Isoleucine, *Tyr* Tyrosine, *Phe* Phenylalanine, *Lys* lysine, *Ile* Isoleucine, *Leu* Leucine, *Tyr* Tyrosine, *Phe* Phenylalanine, *Lys* Lysine, *His* Histidine, *Arg* Arginine, *Pro* Proline.

4.2.3. Micronutrient composition of CRF

Micronutrients are compounds required by the body in small amounts. They are important to facilitate healthy development, disease prevention, and wellbeing. Micronutrients include vitamins, essential fatty acids, and trace minerals (Tull, 1996). The mineral and lipophilic vitamin contents of both CRF materials were measured.

4.2.3.1. Minerals

The mineral compositions of CRF from PVH and spinach leaves were examined as shown in **Figure 4.9**. The mineral profile of both materials was predominantly composed of K, Ca, P, S, Mg and Fe. It was supported with other reports. They found the abundance of Mg, P, K, Ca, and Fe were localised in the chloroplast of plants (Buchanan, 1980, White and Broadley, 2003, Trankner et al., 2018). Magnesium is one of the most important minerals in all green plants, which is generally located in the central atom of the tetra pyrrole ring in the chlorophyll (Barber, 1976, Halliwell, 1981, Marschner, 1995) and plays an essential role for the metabolic pathways (Marschner, 1995). The most abundant of minerals in both CRF materials was potassium as 58% and 33% in spinach CRF and PVH CRF, respectively. Meanwhile, the second most abundant was calcium. There was 31% of calcium in PVH CRF and 17% of calcium in spinach CRF.

The data of CRF from spinach leaves presented in **Figure 4.9** was consistent with that of Gedi et al. (2017). In this result, the mineral values in spinach CRF (mg/100 g CRF, DW) of K (3,563), Ca (1,050), S (549), P (543), Mg (277), and Fe (87) were comparable to the values of CRF from spinach leaves (1196, 677, 337, 298, and 31 mg/ 100 g CRF (DW), for K, Ca, Mg, P, and Fe, respectively), which were reported by Gedi et al. (2017).

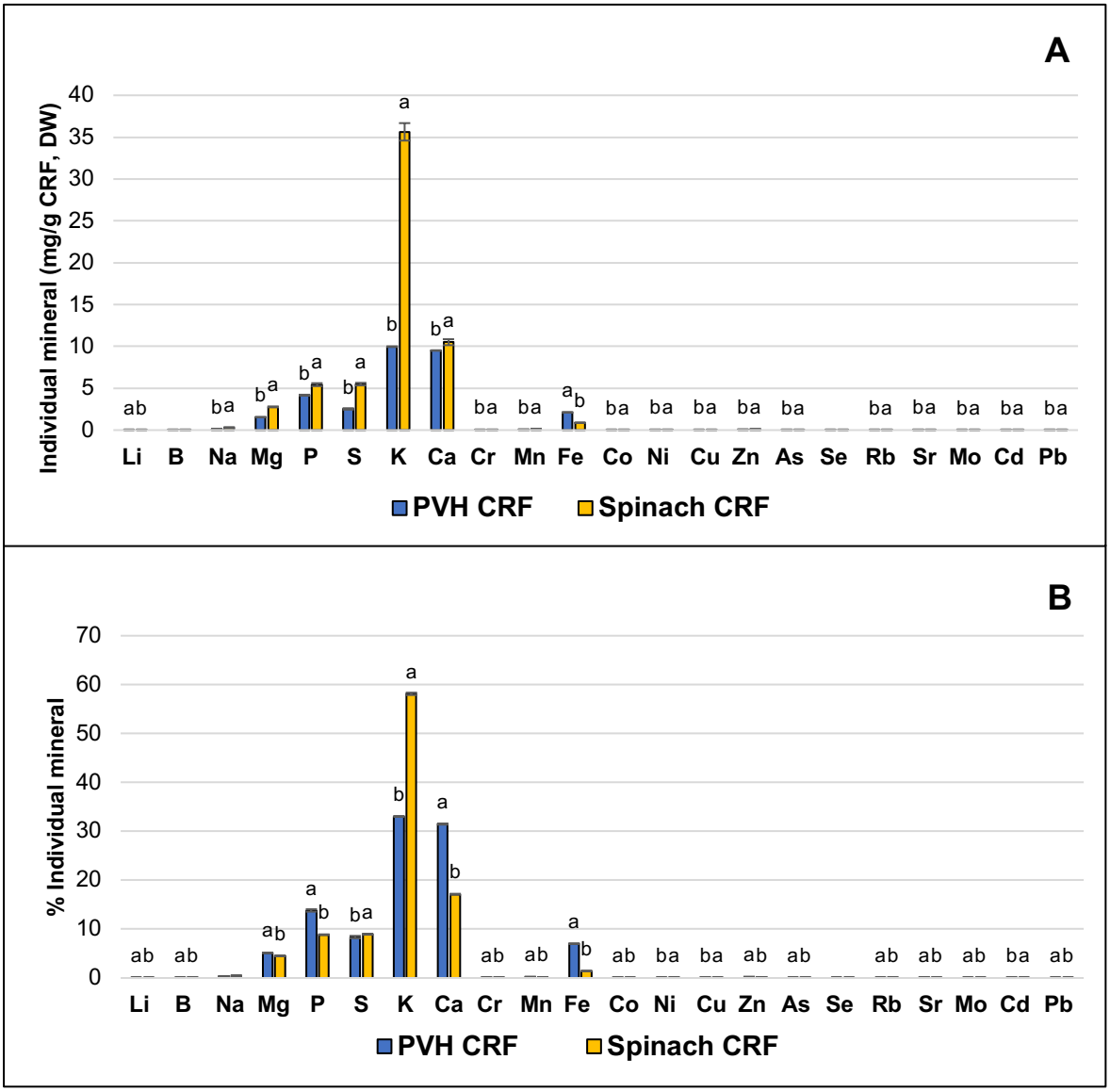


Figure 4.9: Mineral compositions of PVH CRF and spinach CRF **A)** mg/g CRF, DW **B)** Percentage (%) of mineral; Data were presented as a mean ± SD of 3 separated samples and analysed using an Independent-sample t-test with statistical significance at $p<0.05$, a>b; a was a higher value than b; no alphabet means no significant difference at $p>0.05$; DW: dry weight; PVH was collected in summer 2016.

The mineral profile of PVH CRF as expressed in **Figure 4.9** was a similar profile with the pea seed (Harmankaya et al., 2010). However, the content of mineral of CRF from PVH was higher than the seed of pea due to the high concentration of chloroplast in the CRF. In this result, the PVH CRF values (mg/100 g CRF, DW) of K

(997), Ca (950), P (417), S (253), Fe (212), and Mg (155) were comparable to the values of pea seed (758, 303, 145, 84, 82, and 4 mg/100 g, DW for K, P, S, Mg, Ca, and Fe, respectively), which were reported by Harmankaya et al. (2010). Interestingly, the iron was enriched in the CRF more than the seed of pea. Iron has an essential role in the chlorophyll biosynthesis and the metabolic process, such as electron transport chains and redox reactions inside the chloroplast (Solti et al., 2014).

4.2.3.2. Pro-vitamin A (β-carotene) and lutein

The content of β-carotene and lutein in both CRF materials are shown in **Figure 4.10**. The data showed that both β-carotene and lutein are the main lipophilic components compared to other micronutrients in both CRF materials. The level of β-carotene in spinach CRF on a dry-weighted basis (5.30 ± 0.33 mg/g CRF; 22.07 ± 1.36 mg/g total lipid) was higher than β-carotene in PVH CRF (2.83 ± 0.19 mg/g CRF; 14.75 ± 0.63 mg/g total lipid). Conversely, the lutein content in PVH CRF (4.79 ± 0.12 mg/g CRF; 24.97 ± 1.01 mg/g total lipid) was higher than lutein in spinach CRF (3.90 ± 0.39 mg/g CRF; 16.25 ± 1.64 mg/g total lipid). Gedi et al. (2017) found lower concentrations of β-carotene (3.41 ± 0.13 mg/g CRF) and lutein (3.36 ± 0.42 mg/g CRF) in spinach CRF compared to this result. The difference of these carotenoid contents might be due to the different batch of spinach leaves and the CRF preparation as mentioned earlier. In addition, Torcello-Gomez et al. (2019) showed 6.7-times lower β-carotene content in PVH CRF compared to this result. While the lutein content in PVH CRF has never been reported before, except in the result of this chapter. The difference of carotenoid composition in PVH could possibly be seasonal changes of PVH harvesting as described before.

4.2.3.3. Vitamin E (α-tocopherol)

α-Tocopherol is a major type of vitamin E, which is present in the chloroplast of plants (Schultz, 1990). The α-tocopherol content of both CRF plant materials was not significantly different (p>0.05) as shown in **Figure 4.10**. It was 0.46 ± 0.01 mg/g CRF or 2.42 ± 0.05 mg/g total lipid in PVH CRF and 0.52 ± 0.04 mg/g CRF or 2.17 ± 0.19 mg/g total lipid in spinach CRF. This result was consistent with our previous results. We reported that the α-tocopherol content was 0.50 mg/g CRF, DW in PVH CRF and 0.61 ± 0.02 mg/g CRF, DW in spinach CRF (Gedi et al., 2017, Torcello-Gomez et al., 2019).

4.2.3.4. Vitamin K1 (phylloquinone)

The predominant form of vitamin K in plants is phylloquinone or vitamin K1 (Booth et al., 1993). Several studies have reported similar contents of phylloquinone in vegetables with the highest content found in dark green vegetable (Damon et al., 2005, Booth, 2012, Jäpelt and Jakobsen, 2016). It is assumed that phylloquinone content correlates with the strength of green colour (chlorophyll concentration). Phylloquinone content in both CRF materials are presented in **Figure 4.10**. The data showed that the amount of phylloquinone in spinach CRF (2.69 ± 0.11 mg/g CRF; 11.23 ± 0.47 mg/g total lipid) was higher than in PVH CRF (0.64 ± 0.13 mg/g CRF; 3.34 ± 0.66 mg/g total lipid). The data were correlated with the chlorophyll concentration of both CRF as described in **Section 4.2.1**. Spinach CRF was higher chlorophyll concentration than PVH CRF. In addition, this result corresponds with Koivu et al. (1997), who found the phylloquinone contents in spinach leaves (270 µg/100 g, FW) was higher content than seed of pea (28 µg/100 g, FW).

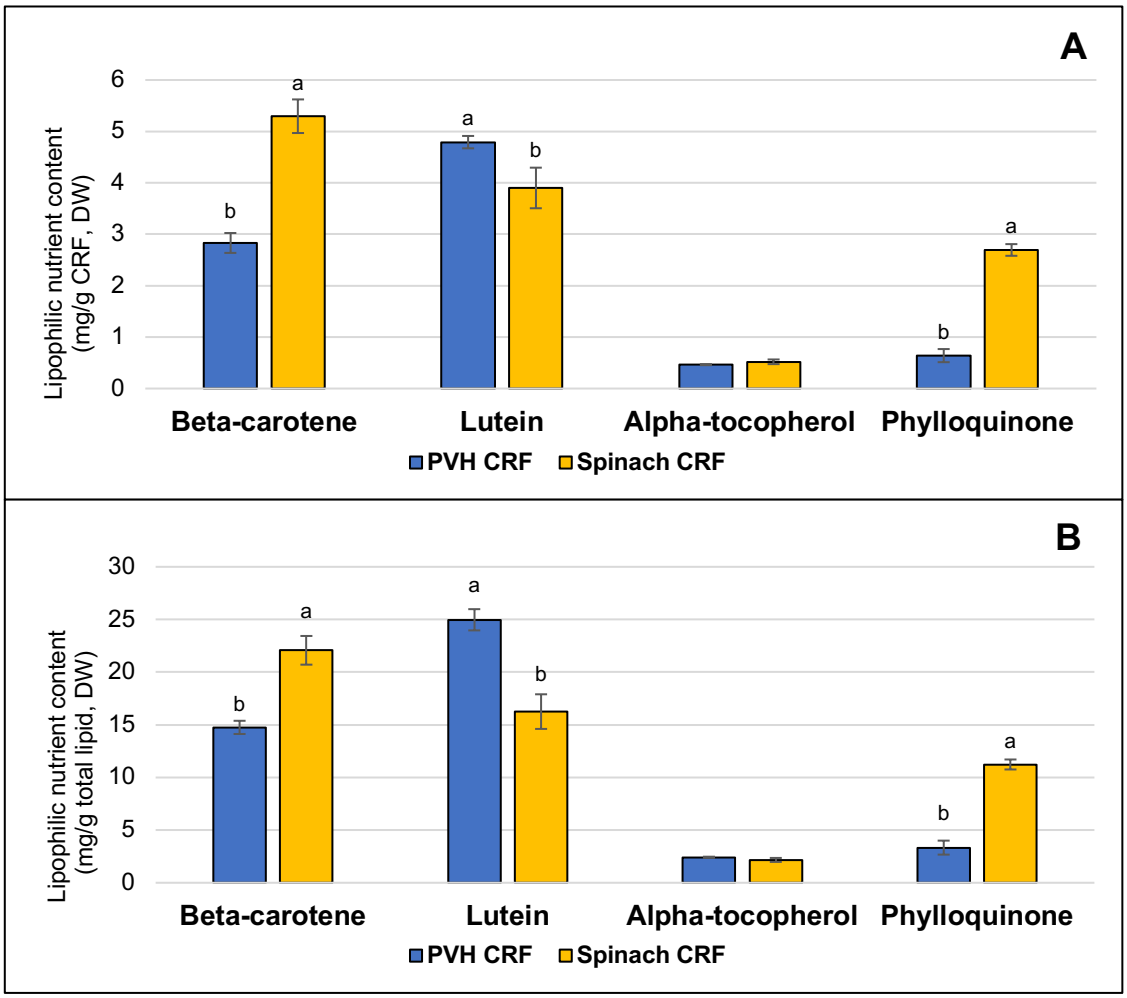


Figure 4.10: Lipophilic nutrients of PVH CRF and spinach CRF **A)** mg/g CRF, DW **B)** mg/g total lipid, DW; Data were presented as a mean ± SD of 3 separated samples and analysed using an Independent-sample t-test with statistical significance at $p<0.05$, a>b; a was a higher value than b; no alphabet means no significant difference at $p>0.05$; PVH was collected in summer 2016.

4.2.3.5. Fatty acid composition in CRF

Fatty acids are building blocks of lipid. They play a number of key roles in metabolism, such as major metabolic fuel (storage and transport of energy) and components of cell membranes (Rustan and Drevon, 2005). The composition of total fatty acids in spinach CRF and PVH CRF are illustrated in **Figure 4.11**. The major fatty

acid in both spinach CRF and PVH CRF was α-linolenic acid (ALA, 18:3 n-3). Its amount in spinach CRF (43.18 ± 3.16 mg/g CRF; 163.7 ± 14.1 mg/g total lipid) was higher than PVH CRF (16.70 ± 3.16 mg/g CRF; 85.08 ± 9.39 mg/g mg/g total lipid). ALA represented 59.95 ± 0.29 % w/w of total fatty acids in spinach CRF, which was consistent with the fatty acid composition of spinach chloroplast (Wolf et al., 1962, Torcello-Gomez et al., 2019). While PVH CRF represented ALA only 37.45 ± 0.15 % w/w of total fatty acids. However, ALA in PVH CRF was higher content than in pea seed (7.3-14.5 % w/w total fatty acids) (Murcia and Rincon, 1991). In addition, ALA is found mainly in the leaves of plants, especially in chloroplast (Wolf et al., 1962, Mackender and Leech, 1974). It is a precursor of essential fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by conversion of ALA to EPA and DHA in the body (Christie and Han, 2012). EPA and DHA (mainly found in fish oil) are important for fetal development, including neuronal, retinal, and immune function (Swanson et al., 2012). Both PVH CRF and spinach CRF showed high amount of ALA. Thus, both materials could be alternative nutritional source of EPA and DHA for vegetarian instead of fish oil. The second most numerous was palmitic acid (PA, 16:0), which is present at similar levels in both spinach CRF (13.88 ± 0.91 mg/g CRF; 52.63 ± 4.25 mg/g total lipid; 59.95 ± 0.29% w/w of total fatty acids) and in PVH CRF (14.72 ± 0.89 mg/g CRF; 75 ± 8.69 mg/g total fatty acid; 19.28 ± 0.1% w/w of total fatty acids). Hexadecatrienoic acid (16:3) was found at a significant level in spinach CRF (7.64 ± 0.17 % w/w of total fatty acid; 5.5 ± 0.41 mg/g CRF; 20.86 ± 2.01 mg/g total lipid), but it was not found in PVH CRF. The absence of 16:3 fatty acid in PVH CRF may also be explained by the classification of pea among “eukaryotic plant”, which preferentially has C18 fatty acid at *sn-2* position in galactolipids while “prokaryotic plant” which preferentially has C16:3 fatty acid at *sn-2* position (Heemskerk et al., 1991). In addition, linoleic acid (LA, 18:2 n-6) is an essential omega-6 fatty acid. LA can be

converted into longer omega-6 fatty acid, such as arachidonic acid (ARA) (Christie and Han, 2012). ARA is an important part of the normal inflammatory process (Christie and Han, 2012). Interestingly, the amount of LA in PVH CRF (9.03 ± 0.43 mg/g CRF; 45.98 ± 4.8 mg/g total lipid) was higher amount than spinach CRF (6.55 ± 0.45 mg/g CRF; 24.81 ± 1.93 mg/g total lipid). It might be due to containing of the seed in PVH. Normally, LA is mainly found in seed more than the leaves of the plants (Murcia and Rincon, 1991).

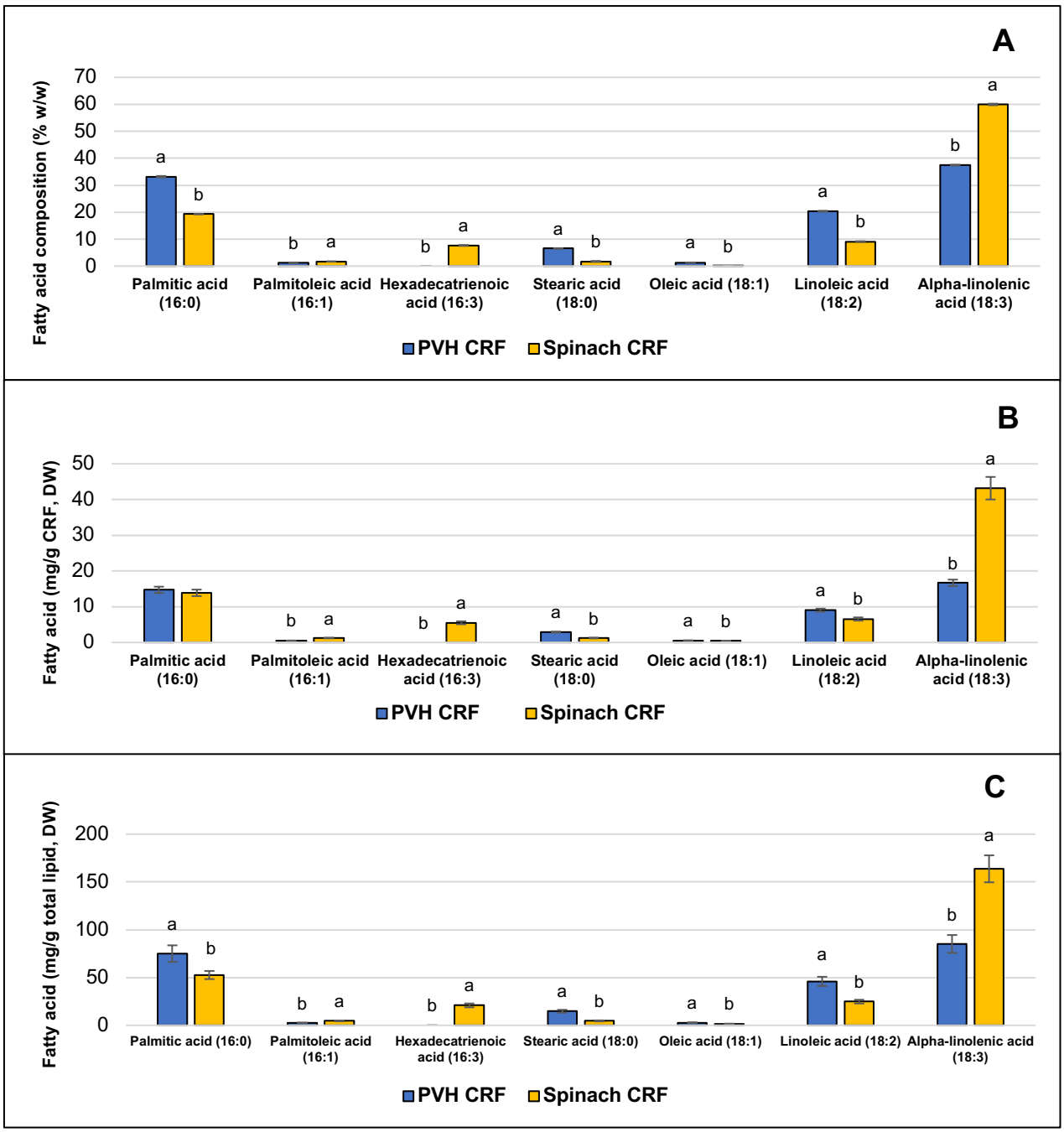


Figure 4.11: Fatty acid compositions of PVH CRF and spinach CRF **A)** percentage (%) of fatty acid **B)** mg/g CRF, DW **C)** mg/g total lipid, DW; Data were presented as a mean ± SD of 3 separated samples and analysed using an Independent-sample t-test with statistical significance at $p<0.05$, a>b; a was a higher value than b; no alphabet means no significant difference at $p>0.05$; PVH was collected in summer 2016.

4.2.4. Comparison with the nutritional reference value

Reference Nutrient Intake (RNI) means the amount of nutrient required to meet the daily needs of 97% of each group in the population (Tull, 1996). **Table 4.1** shows the grams of CRF need to cover the Reference Nutrient Intake (RNI) (Latham, 1997, FAO/WHO, 2002) of lipophilic nutrients on dry-weight basis since the freeze-dried CRF is considered the final product. The values are related to the wet amount of initial fresh PVH and spinach leaves to extract the equivalent CRF. 7.6 kg of fresh PVH and 6.2 kg of fresh spinach leaves are enough to extract CRF using a twin-gear juicer and centrifugation to supply the recommended daily intake of β-carotene, lutein, α-tocopherol, phylloquinone, and α-linolenic acid (see **Table 4.1**). Spinach is known as a good source of lutein (Abdel-Aal el et al., 2013). The consumption of lutein is recommended to help the maintenance of eye vision and reduce the risk of age-related macular disease (Sommerburg et al., 1998, Basu et al., 2001). Interestingly, PVH CRF was 1.2-times higher lutein content than spinach CRF. In addition, **Table 4.1** shows the mineral requirement in human nutrition (British Nutrition Foundation, 2015) and mineral composition in both CRF plant materials. 10 g of freeze-dried PVH CRF is enough to satisfy the RNI for iron while 10 g of freeze-dried spinach CRF supplies 72.5% RNI of iron. Spinach is a well-known as good source of iron and it is recommended for anaemia people and children (Yudhistira et al., 2018). Normally, the iron value of spinach leaves was 0.4-0.6 mg/10 g, DW (Nešković and Ćulafić, 1988). While spinach CRF was higher concentration of iron (8.7 mg/10 g, DW). Interestingly, PVH CRF was 2.4-times higher content of iron than spinach CRF. Thus, PVH CRF can be used as alternative source of iron and lutein instead of spinach leaves.

Table 4.1: Nutritional value of freeze-dried CRF from PVH and spinach leaves.

Micronutrient	RNI (mg/day)	g PVH CRF to meet RNI (DW basis)	g spinach CRF to meet RNI (DW basis)	kg fresh PVH to extract required CRF***	kg fresh spinach to extract required CRF****	PVH CRF		Spinach CRF	
						mg per 10 g dried CRF	% RNI per 10 g dried CRF	mg per 10 g dried CRF	% RNI per 10 g dried CRF
β-carotene	3.3*	1.17	0.62	0.10	0.11	28.3	>100	53	>100
Lutein	2*	0.42	0.51	0.04	0.09	47.9	>100	39	>100
α-Tocopherol	8.75*	19	17	1.6	3	4.6	52.6	5.2	59.43
Phylloquinone	0.06*	0.09	0.02	0.008	0.004	6.4	>100	26.9	>100
α-Linolenic acid	1,500*	90	35	7.6	6.2	167	11.3	432	28.8
Na	1,600**	14,414	5,948	1,215	1,062	1.11	0.07	2.69	0.17
Mg	285**	184	103	16	18	15.49	5.44	27.72	9.73
P	550**	132	101	11	18	41.68	7.58	54.29	9.87
K	3,500**	351	98	30	17	99.70	2.85	356.35	10.18
Ca	700**	74	67	6.2	12	94.97	13.57	105.02	15.00
Mn	6**	86	52	7.3	9.2	0.70	11.67	1.16	19.33
Fe	12**	6	14	0.5	2.5	21.17	100	8.70	72.5
Zn	8.2**	115	80	9.7	14	0.71	8.66	1.02	12.44

*RNI male and female average 19-65 years;

**RNI male and female average 19-50 years;

*** 11.86 g CRF (DW)/kg fresh PVH biomass;

****5.66 g CRF (DW)/kg fresh biomass spinach leaves.

4.2.5. Phytic acid in CRF

It is clear that CRF from both spinach leaves and PVH are alternative sources of macronutrients, micronutrients, and trace minerals. However, the presence of anti-nutrients has the potential to invalidate the benefits of CRF consumption by reducing the biological availability of the nutrients it contains. Phytic acid is a major anti-nutritional compound, which can interfere the absorption of some essential trace elements and minerals (Schlemmer et al., 2009) and negative impacts on the activity of digestive enzymes, such as amylase, pepsin, and trypsin (Hemmige et al., 2017). As it is strongly negative charged under physiological conditions, it has great potential for complexing positive charged multivalent cations, especially Ca^{2+}, Fe^{3+}, Mg^{2+} and Zn^{2+}. These complexes are soluble under acidic conditions in the gastric and precipitate at the neutral pH in the intestine, resulting in decreased absorption of minerals and trace elements (O'Dell, 1969, Reddy et al., 1982, Nolan et al., 1987, Sandberg et al., 1989).

Table 4.2: Total phosphorous and phytic acid contents in PVH CRF and spinach CRF.

Materials	Total phosphorous (g/100 g, DW)	Phytic acid/Phytate (g/100 g, DW)
PVH CRF	0.057 ± 0.003^{c}	0.202 ± 0.001^{c}
Spinach CRF	0.110 ± 0.001^{b}	0.391 ± 0.003^{b}
Oat flour (positive control)	0.510 ± 0.026^{a}	1.796 ± 0.091^{a}

Data were presented as a mean ± SD of 3 separated samples and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b.

In this work, the phytic acid and total phosphorous in both CRF materials and oat flour are expressed in **Table 4.2**. Oat flour with established total phosphorus

and phytic acid values was provided with the total phosphorus/phytic acid assay kit (Megazyme®) (Reason et al., 2015). It was initially used to measure method accuracy. The average values of total phosphorus and phytic acid in this experiment were 0.510 g/100 g and 1.796 g/100 g, respectively. They showed good agreement with the established concentration of total phosphorus and phytic acid as 0.517 g/100 g and 1.790 g/100 g, respectively. In addition, oat flour was reported that it contained a high amount of phytic acid (García-Estepa et al., 1999). Thus, it was used as a positive control in this assay. Both CRF materials contained phytic acid, but they were relatively low in amount. Phytic acid was the lowest in PVH CRF (0.202 g/100 g, DW), followed by spinach CRF (0.391 g/100 g, DW) and oat flour as a positive control (1.796 g/100 g, DW). This result was consistent with Sandberg (2002) and Schlemmer et al. (2009). They reported the phytic acid in the legumes of pea was between 0.06 and 1.22 g/100 g, DW. However, there has been no report the phytic acid content in spinach leaves in the plant species of *Spinacia olerace*s, except spinach leaves in the species of *Basella rubra*, which contained 0.07 g/100 g, DW) (Ravindran, V. et al., 1994). It has been reported that heat processing, such as microwave, cooking, and autoclaving can decrease the amount of phosphorus and phytic acid in cereal brans and seeds of *Vigna aconitifolia* and *Vigna sinensis* (Vijayakumari et al., 1998, Kaur et al., 2012). Similarly, this data showed that the phytic acid content in CRF from pasteurised PVH juice was 30% lower compared to the CRF from non-heated PVH. Meanwhile, the phytic acid content in CRF from steam sterilised PVH biomass at 100ºC for 4 min was 3.6% lower compared to CRF from non-heated PVH CRF as shown in **Table 4.3**. Phytic acid is degraded and changed to another form or molecule during heat processing. Longer heating time can decrease phytic acid. Daneluti and Matos (2013) studied the thermal stability of phytic acid. They found the intermediate products of thermal decomposition of phytic acid using infrared absorption spectroscopy. The infrared spectra of phytic

acid during the heat processing revealed a reduction in the intensity of the absorption band related to O-H stretching as a result of the dehydration process (Daneluti and Matos, 2013). For the condition of steam sterilisation, the PVH biomass was heated for 5 min and 30 sec to reach 100^{o}C and held for 4 min before cooling and depressing for 5 min and 45 sec prior to making CRF. Meanwhile, for the heated juice condition, the PVH juice was heated from room temperature (20^{o}C) to 90^{o}C over 20 min and held for 3 min prior to generating CRF. From this result, it can confirm that heating treatment for longer time can reduce phytic acid as an anti-nutrient.

Table 4.3: Total phosphorous and phytic acid contents in PVH with different CRF treatments.

Materials	**Total phosphorous (g/100 g, DW)**	**Phytic acid/Phytate (g/100 g, DW)**
Non-heat treatment	0.057 ± 0.003^{a}	0.202 ± 0.010^{a}
Heated PVH	0.055 ± 0.004^{a}	0.195 ± 0.013^{a}
Heated juice	0.039 ± 0.004^{b}	0.140 ± 0.001^{b}

Data were presented as a mean ± SD of 3 separated samples and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b.

4.3. Conclusions

Spinach CRF was higher nutrient composition in protein, lipid, pro-vitamin A, vitamin E, vitamin K1, omega-3 fatty acid, and some minerals compared to PVH CRF. However, the PVH CRF was larger amount of carbohydrate, lutein, omega-6 fatty acid, and iron compared to spinach CRF. The phytic acid as an anti-nutrient was detected in this CRF material; it was relatively low in concentration and could be removed using heat processing. Thus, the PVH CRF has the potential to be used as a functional food supplement for the source of carbohydrate, iron, lutein, and linoleic acid (omega-6 fatty acid). The nutrient stability, bioaccessibility, and *in vitro* digestion of lipophilic nutrients in this material were further studied in the next chapter.

CHAPTER V

Effect of steam sterilisation on lipophilic nutrient stability in a chloroplast-rich fraction (CRF) derived from postharvest, pea vine field residue (haulm)

5.1. Introduction

It is well known that the nutrient content from vegetables and fruits starts to decrease after harvesting the plants due to endogenous enzymes (Kessy et al., 2016). In order to inactivate enzyme reactions after harvesting, thermal processes, such as blanching or pasteurisation, are the most practical and efficient methods for inactivating these enzymes and extending the shelf life of the nutrients. Blanching can be conducted in several ways such as microwave processing, placing in boiling water, or by steaming (Lin and Brewer, 2005, Kessy et al., 2016, Severini et al., 2016). Blanching in boiling water can lead to leaching of some nutrients into the water, while microwave blanching is difficult to control as temperature gradients can form, giving an uneven heat treatment to the plant material. Steam blanching minimises leaching and the gaseous steam can easily permeate the plant material and ensure an even heat treatment. So far, there is no published data on the β-carotene, lutein and α-tocopherol stabilities of freeze-dried CRF from steam sterilised PVH upon storage. The deterioration of nutrients in freeze-dried PVH CRF during storage could occur due to common factors, such as exposure to temperature, air, and light. Thus, it is a benefit to determine the stability of freeze-dried PVH CRF as an alternative source of β-carotene (provitamin A), lutein and α-tocopherol (vitamin E). The hypothesis of this chapter is that temperature, light, oxygen have an effect on nutrient stabilities of freeze-dried PVH CRF even though it is inactivated endogenous enzyme prior to CRF

preparation.

Therefore, this chapter aimed to recover the CRF from steam sterilised PVH (retort sterilisation 100°C at 1 bar for 4 min) and investigate storage conditions that preserve nutrient in CRF material to use as food supplement. CRF from steam sterilised PVH was then studied over a period of 84 days in 12 different storage conditions: eight conditions involved stored in the dark at varying temperatures (-20°C, 4°C, 25°C and 40°C) and differing amount of air exposure (vacuum and non-vacuum pouches) and four conditions were stored with light exposure (foil and transparent pouches) and air exposure (vacuum and non-vacuum bags) at 25°C and 40°C. In all samples, the lipophilic nutrients, β-carotene (provitamin A), lutein, and α-tocopherol (vitamin E) were studied.

5.2. Results and discussion

Shelf life is defined as the time span under storage conditions which food products remain acceptable for human consumption in terms of its safety, nutritional value, and sensory characteristics (Corradini, 2018). Both food safety and quality are important aspects of acceptable shelf life. This chapter describes the impact of thermal processing in nutritional quality in freeze-dried CRF derived from steam sterilised PVH. However, it was a lack of study of microorganisms in freeze-dried PVH CRF, which is important for food safety. Thus, it is considered to further study in the future.

5.2.1. Inactivation of the endogenous enzyme prior generating PVH CRF

Steam treatment using a retort is a modified technique based on blanching technique, which allows the inactivation of endogenous enzymes, particularly peroxidase (POD), a relatively stable enzyme on heating Bahçeci et al. (2005), on a large scale to extend the nutrient stability after harvesting the plants. Based on the preliminary results (**Table 5.1**), it can be seen that applying steam treatment using retort at 100^{o}C for 4 min or hot water blanching at 100^{o}C for 2 min decreased the POD activity by 95% and 92.3%, respectively, when compared with non-heat treatment. Thus, in this work, we decided to use steam treatment using a retort at 1 bar, 100^{o}C for 4 min to inactivate POD in the large batch of biomass prior to making PVH CRF. In this study, using a retort on the large scale of PVH can reduce POD activity (66.5%) compared to fresh PVH juice. The POD reduction in the large scale was lower content than that of the small scale due to heat transfer into PVH pouches. It can be explained that in the preliminary study, the bags containing PVH were put in the rack of retort only a few pouches. Meanwhile, applying in the large scale, the pouches containing PVH were put in the rack of retort at least 15 bags. Thus, the efficiency of heat transfer from the

thermal processing into PVH pouches in the preliminary study was higher than the large-scale study.

5.2.2. Degradation of chlorophyll during storage

The chlorophyll content provides an indirect of the concentration of chloroplasts (Gedi et al., 2017). It means that if the sample is higher chlorophyll content, it will be higher chloroplast in the sample. Also, chlorophyll provides the green colour; this colour fades as chlorophyll is lost. Green colour may be an important quality factor for particular applications of the CRF material. In addition, measuring the change in colour of the CRF gives some indication of the chemical degradation occurring in the plant material during storage. Canjura et al. (1991) reported that thermal processing at high temperature could preserve green colour in the plants over a short storage period, but chlorophyll could degrade over longer storage periods. In this work, chlorophyll degradation was studied by storing freeze-dried samples under different conditions (temperatures, air and light exposures) and then analysing chlorophyll content every 14 days up to 84 days of storage.

The initial chlorophyll content was 10.73 ± 0.78 mg/g CRF, dry weight (DW). Storing the samples at -20°C, 4°C, 25°C or 40°C did not affect the total chlorophyll content, when they were stored without air exposure and in the dark over the whole 84-day period. However, the total chlorophyll content decreased significantly when kept at 40°C for 56 days (**Figure 5.1**). The amount of chlorophyll was not affected by non-vacuum storage conditions for the full 84 days at -20°C and 4°C. For 25°C and 40°C, the amount of chlorophyll only remained stable for 42 days; the amount of chlorophyll reduced when stored for 56 days or more at 25°C or 40°C, as shown in **Figure 5.1**. Even if the sample was stored in vacuum-seal packages, exposed to light and stored at 25°C or 40°C, the chlorophylls were destroyed (**Figure 5.2**). This change is likely due

to the degradation of chlorophyll to pheophytin and other derivatives such as chlorophyllide and pheophorbide (Schwartz and Elbe, 1983) (**Figure 2.7**). The chlorophyll loses its bound magnesium ion (Mg^{2+}) and is replaced by two hydrogen atoms, due to heat treatment and increased acidity during storage. These chemical changes affect the chromophores and lead to the brown colour, which also reduces chlorophyll content in the sample (White et al., 1963, Canjura et al., 1991). Thus, from this result, it can be summarised that the storage conditions (low temperatures, without light and air exposures) are important in preventing chlorophyll degradation (Tonucci and Von Elbe, 1992, Mangos and Berger, 1997).

Table 5.1: Percentage (%) of peroxidase reduction in PVH juice with different treatments performed at small scale and large scale.

Treatment	Peroxidase reduction (%)
Hot water blanching at 100°C for 2 min	92.3
Steam blanching at 100°C for 1 min using steamer	52.6
Steam blanching at 100°C for 2 min using steamer	54.9
Steam blanching at 100°C for 4 min using steamer	68.1
Steam blanching at 100°C for 1 min using retort	43.6
Steam blanching at 100°C for 4 min using retort	95.0
Steam blanching at 100°C for 4 min using retort*	66.5

Each treatment contained 200 g of PVH in the preliminary, except steam blanching using retort contain 500 g of PVH in each pouch; * large scale.

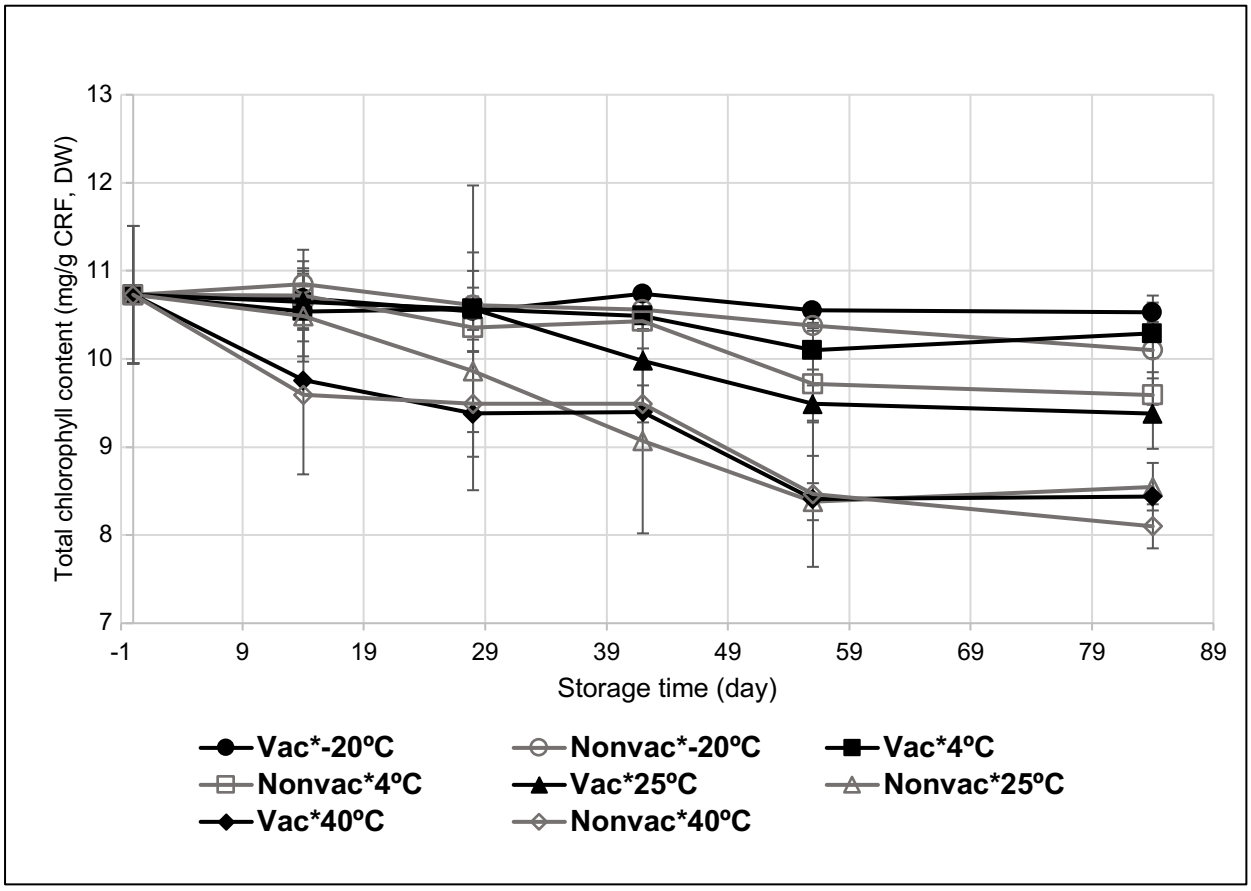


Figure 5.1: The effect of storage temperature in vacuum and non-vacuum conditions on degradation of total chlorophyll in freeze-dried CRF from steam sterilised PVH over 84 days. Both types of packaging excluded light from the samples and D means day.

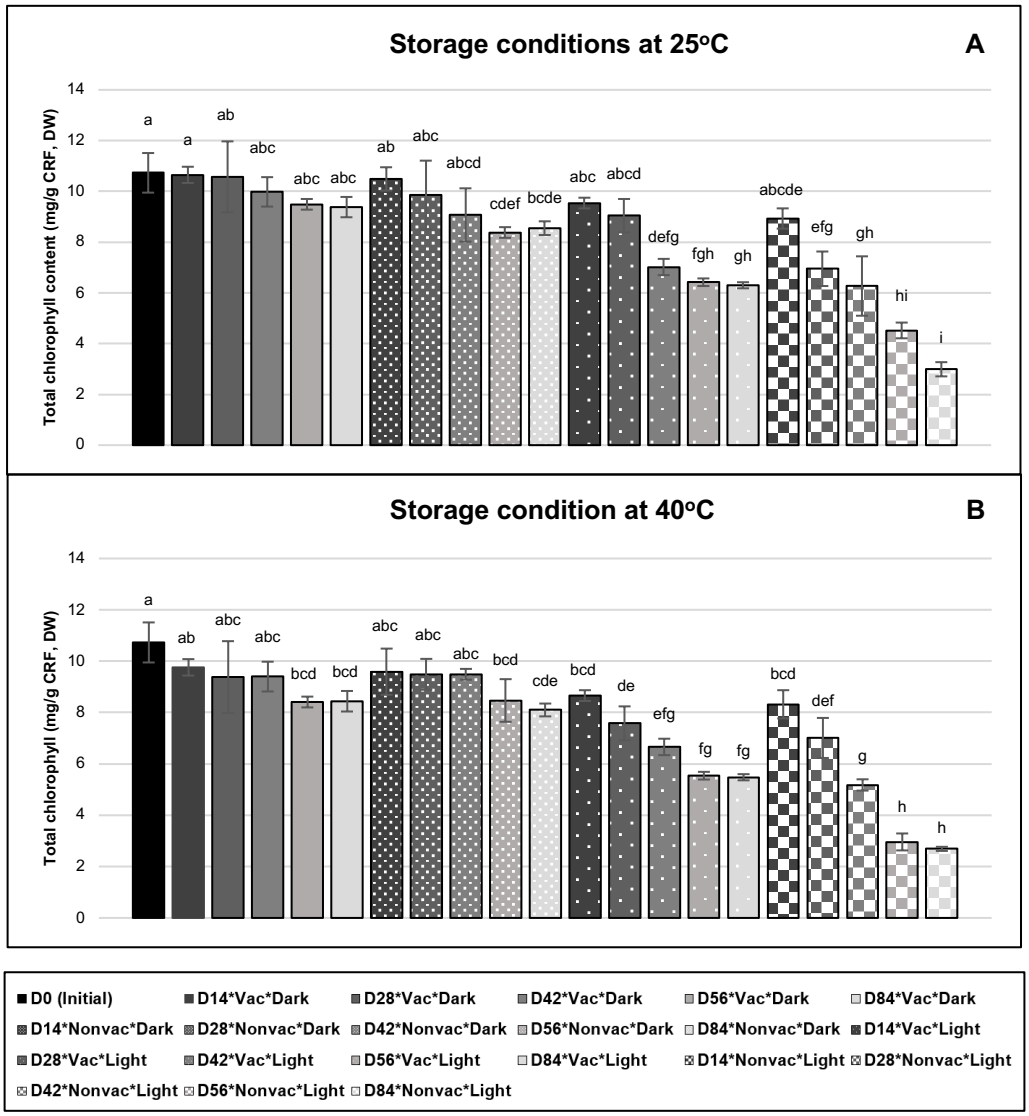


Figure 5.2: The effect of the presence of light on total chlorophyll in freeze-dried CRF from steam sterilised PVH stored at 25°C **(A)** and 40°C **(B)** both vacuum and non-vacuum packages over the duration of 84 days (D: day).

Data were presented as a mean ± SD of 3 separated storage conditions and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at p<0.0.5, a>b.

5.2.3. Kinetic degradation of lipophilic nutrients

The degradation kinetic is useful to predict the quality changes that occur during storage and determine the shelf life of a product. In this work, the initial β-carotene, lutein, and α-tocopherol concentrations were 0.74 ± 0.08, 3.77 ± 0.39, and 0.29 ± 0.02 mg/g CRF, DW, respectively. Carotenoid and vitamin E concentrations decreased with storage time at all storage temperatures under vacuum and non-vacuum packaging conditions (**Table 5.2 and 5.3**). Notably, β-carotene, lutein and α-tocopherol decreased more rapidly at higher temperatures under air packaging and light exposure, which indicated that the degradation degree of the lipophilic nutrients was influenced by storage condition. The degradation of carotenoid and vitamin E in PVH CRF depended on the storage temperature, time duration, and the presence of oxygen and light, which were further discussed in **Section 5.2.4 - 5.2.6**.

Carotenoids, including β-carotene and lutein contain 40 carbons and multiple conjugated double bonds are highly susceptible to degradation. After 84 days of storage, 8.11%, 9.46%, 12.16%, and 14.86% of β-carotene were respectively lost at -20°C, 4°C, 25°C, and 40°C under vacuum-packaged storage conditions and 9.46%, 21.62%, 28.38%, and 70.27% lost, respectively under air packaging (**Table 5.4**). In addition, storage in vacuum and non-vacuum sealed bags at 25°C and 40°C with light exposure had a complete loss of β-carotene, except vacuum condition at 25°C, which showed β-carotene loss of 86.49%. For lutein, a final loss of 2.92%, 5.31%, 18.30%, and 20.69% was measured when stored at -20°C, 4°C, 25°C, and 40°C in vacuum-sealed bags. When the samples had air exposure at -20°C, 4°C, 25°C, and 40°C, the final loss was 2.12%, 13%, 31.03%, and 48.28%, respectively. Where samples were exposed to light, if they were in vacuum-sealed bags the lutein loss was 60.48% at 25°C and 91.78% at 40°C, however when there was no vacuum the lutein

loss at 78.51% at 25°C and 94.43% at 40°C. Similar degradative behaviour of carotenoids in food products during storage was reported previously (Pénicaud et al., 2011, Bechoff et al., 2015, Song et al., 2018).

The α-tocopherol had final loss of 3.45%, 6.90%, 10.34%, and 17.24% when the samples were stored in vacuum-sealed packs at -20°C, 4°C, 25°C, and 40°C, respectively. Loss of α-tocopherol in non-vacuum sealed bags was 3.45%, 10.34%, 37.93%, and 41.38% at -20°C, 4°C, 25°C, and 40°C, respectively. Meanwhile, vacuum and non-vacuum condition at 25°C with light exposure showed the final α-tocopherol loss of 89.66% and 93.10%, respectively. When the sample was subjected to light exposure at 40°C it did not matter whether the bag was vacuum sealed or not: there was a complete loss of α-tocopherol.

Loss of nutrients in our result was not consistent with the result of Syamila et al. (2019), which studied the nutrient stability of β-carotene, lutein, and α-tocopherol in spray-dried spinach juice powder during storage between 4°C and 40°C for 56 days. They found that the loss of carotenoids and vitamin E in spray-dried spinach powder was lower value than that of freeze-dried CRF from steam sterilised PVH. It was possible due to the reason that our sample had inactivation of endogenous enzymes using steam sterilisation before storage. While the experiment of Syamila et al. (2019) did not use heat treatment to suppress endogenous enzyme prior to storage. Generally, enzymatic oxidation can occur in vegetables and fruits because of lipoxygenase. It can thus induce the reaction of loss nutrients (Pénicaud et al., 2011). Heat treatment can disrupt hydrogen bond and non-polar hydrophobic interaction of endogenous enzymes by increasing kinetic energy. It causes the molecules to vibrate and violently that the bonds are disrupted. The enzymes cannot be thus functioned anymore. It was a possible reason why the nutrient in spray-dried spinach was higher loss than freeze-dried CRF from PVH under the same storage temperature. This opinion was supported

by Oruña-Concha et al. (1997). They reported that blanching of the bean before storage at -22°C for one year helped to retard the degradation of β-carotene and lutein. Similar reason with Arya et al. (1982), they reported blanching of carrot before storage helped to inactivate the lipoxygenase and stabilise carotenoids for 8 months compared to non-blanching. Heat treatment could also deactivate the activity of endogenous oxidative enzymes, especially tocopherol oxidase, which was found in all parts of plants, such as leaves, roots, and stems (Lee et al., 2017).

Besides these assumptions, high storage temperatures reduced the energy barrier and induced completely different reaction pathways, such as carotenoid hydroxylation by β-carotene hydroxylase (Davison et al., 2002). In addition, hydrogen peroxide (H_2O_2) is widely used as packaging sterilant in the aseptic processing system and may cause autoxidation. H_2O_2 can decompose and form highly reactive products such as hydroxyl radical (•OH). The presence of H_2O_2 residues (lower than 0.5 ppm) allowed by the FDA in the food packages can cause rapid degradation of anthocyanin in juices (Özkan et al., 2005).

Another reason for the loss of nutrients during storage is the content of water activity or moisture content in dried products. The presence of moisture can accelerate the breakdown of nutrients during storage. The water activity influences the values of the degradation rate constant (k-value). Most of the reaction rate constant increased with a decrease in water activity (Achir et al., 2014). The moisture content of our sample before storage was around 1-2%. During the storage, freeze-dried PVH CRF did not measure the water activity due to the limitation of our samples. Thus, we cannot confirm that the water activity of PVH CRF have an effect on loss of nutrient during storage, or not. However, this issue will be further studied in the future.

Studies describing the kinetics of carotenoid and vitamin E in freeze-dried CRF from steam sterilised PVH is rather limited. However, this information is beneficial

for predicting nutritional changes during its storage. Fitting of the data to a first-order reaction equation between the logarithm of the concentration of the lipophilic nutrients and time was good, with coefficient correlation (R^2) for the linear regression analysis between 0.90 and 0.99, as shown in **Table 5.5**. Several studies have also demonstrated the first-order degradation kinetics of carotenoids. (Tan and Francis, 1962, Wagner and Warthesen, 1995, Weemaes et al., 1999, Pénicaud et al., 2011). For instance, carotenoid degradation in a dehydrated pumpkin was described by a first-order kinetic model, with activation energy (E_a) of 97 KJ/mol (Pérez-Gálvez et al., 2000). Degradation of β-carotene of yellow gari made from cassava also followed first-order kinetics during storage between 19°C and 40°C (Bechoff et al., 2015). Besides, kinetic data indicated a first-order model to describe thermal degradation of carotenoids in dehydrated products like pumpkins (Song et al., 2018), carrots (Lavelli et al., 2007), sweet potato (Bechoff et al., 2010, Haralampu and Karel, 1983). In most cases, the changes in total carotenoids and vitamin E were well fitted by a first-order model. However, the degradation of some individual carotenoids did not fit the first order degradation, such as 9-*cis*-β-carotene, 13-*cis*-β-carotene, and 15-*cis*-β-carotene (Bechoff et al., 2015). It might be due to the reason that the lower concentration of fairly heat-sensitive carotenoids makes the linearisation more difficult.

It was observed that as the storage temperatures increased, the degradation rate constant (k) increased, with a corresponding reduction in the half-life. **Table 5.5** also indicated that the degradation rate of carotenoid and vitamin E degradation of freeze-dried PVH CRF were not significant difference both vacuum and non-vacuum packaging at low temperature (i.e. for β-carotene at -20°C, k = 1.23 x 10^{-3} day^{-1} vs 1.23 x 10^{-3} day^{-1}, respectively). The loss of lutein and α-tocopherol showed a similar pattern. However, the rate constant for lutein degradation was found to be lower than β-carotene, which suggested that lutein had relatively more heat resistance: this

was consistent with the stability of carotenoids found in dehydrated pumpkin (Song et al., 2018) and a light-induced model of pure commercial carotenoids (Xiao et al., 2018). Meanwhile, the degradation rate of β-carotene, lutein and α-tocopherol with air exposure increased with the increase in storage temperature in the range of 25-40°C. This result was consistent with similar studies on the oxidative and thermal degradation of lutein in safflower oils and virgin olive oil, which observed that the loss of lutein fitted the first-order model with a degradation rate, which increased with higher temperatures (Henry et al., 1998, Aparicio-Ruiz et al., 2011).

The half-life ($t_{1/2}$) values for lutein degradation were 2,200, 1,036, 234, and 176 days in vacuum pouches of PVH CRF, respectively (**Table 5.5**). As the temperature increased with the light exposure, $t_{1/2}$ values decreased in consistent with faster reactions accompanied by higher k-values, as shown in **Table 5.5**. Compared to vacuum pouches, the $t_{1/2}$ values for lutein degradation in non-vacuum pouches significantly decreased. Similar results were observed in β-carotene and α-tocopherol. In agreement with the degradation trends shown by the percentage losses in PVH CRF, α-tocopherol had the shortest half-life at 40°C under light exposure either vacuum or non-vacuum packages (12 days). Lutein had the longest half-life at -20°C under vacuum package and without light exposure (2,200 days).

As the storage temperatures between 25°C and 40°C, α-tocopherol had a higher half-life compared to β-carotene and lutein. The lower half-life of β-carotene and lutein might be due to the opening of β-ionone ring in β-carotene and the chromophore extension into apocarotenols, which was more amenable to reaction with the peroxyl radicals ($ROO^{\bullet}$) compared to α-tocopherol (Mordi, 1993, Pénicaud et al., 2011, Rodrigues et al., 2012). In addition to that, the thermal stability of α-tocopherol was due to increasing of the stearic hindrance by the methyl groups, thus reducing the rate of addition and polymerisation reactions (Sabliov et al., 2009, Al-Khusaibi et al., 2012).

To evaluate the relationship between reaction speed and temperature, the Arrhenius equation was computed, and its parameters are shown in **Table 5.5**. The activation energy (E_a) is defined as the minimum energy required to start a chemical reaction and a parameter that indicates the sensitivity of the reaction rate to temperature. For greater activation energy, the value of reaction is more sensitive to temperature change (Ling et al., 2015) and less the nutrient degradation. The activation energy values of β-carotene in freeze-dried CRF from steam sterilised PVH (10.57 to 17.84 KJ/mol) during storage were higher compared with the E_a of β-carotene in non-heat treated spray-dried spinach juice (0.665 to 1.089 KJ/mol) (Syamila et al., 2019). This suggested that the presence of endogenous enzymes in the material during storage could be the effect of kinetic parameters (E_a, k-value, and $t_{1/2}$). The higher activation energy, the slower chemical reaction of nutrient degradation would be. Our sample was inactivated the endogenous enzymes by using heat treatment prior to storage. While the result of Syamila et al. (2019) was not knocked out the endogenous enzymes before storage. Thus, the E_a value of their result was lower than that of our result. This opinion was agreed with Koca et al. (2007). They found the activation energy of β-carotene in unblanched carrot was lower compared to blanched carrots when stored at the same temperature, which confirmed that active enzymes in fruits and vegetables affect the kinetic parameters (Koca et al., 2007). However, our work was similar value of the E_a of β-carotene compared with carrot puree (10.5 KJ/mol) (Lemmens et al., 2010), dehydrated pumpkins (13.82 KJ/mol) (Song et al., 2018) and lower in dried products, such as dried carrot slices (33.33 KJ/mol) (Demiray and Tulek, 2017) and dried sweet potato chips (64.2 KJ/mol) (Bechoff et al., 2010). It indicated that β-carotene in freeze-dried PVH CRF was less stable product than that of carrot slice, sweet potato and potato chip. Meanwhile, the activation energy of lutein in freeze-dried CRF from steam sterilised PVH was 29.67 to 31.07 KJ/mol, which was higher values

than spray-dried spinach juice (2.65 to 4.92 KJ/mol) (Syamila et al., 2019) and dehydrated pumpkin (20.01 to 23.69 KJ/mol) (Song et al., 2018). The activation energy of α-tocopherol in freeze-dried CRF from steam sterilised PVH was 11.15 to 23.12 KJ/mol. Meanwhile, the E_a of spray dried spinach juice was 13.89 to 14.14 KJ/mol (Syamila et al., 2019). The activation energies of both lutein and α-tocopherol in our result were higher than the previously published research. It might be due to the reason that the endogenous enzymes in spinach and pumpkin were still active. It found that spinach did not be heated to suppress endogenous enzymes before storage. While pumpkin was blanched for 40 seconds before storage. However, it might be not sufficient for inactivation of enzymes in pumpkin. It was agreed with Lin and Brewer (2005). They reported that blanching for 1 min was insufficient to inactivate the activity of peroxidase (POD) found in pea. While blanching for 4 mins suppressd 97% of POD in pea. From the assumption, it was possible that the endogenous enzymes could accelerate the chemical reactions of nutrient degradation during the storage. Thus, the E_a values of these materials were lower than that of our result, which PVH was steam sterilised for 4 mins to inhibit POD activity (66%) before storage.

Table 5.2: The interaction among storage temperature, time, and the presence or absence of air in freeze-dried CRF from steam sterilised PVH on β-carotene, lutein and α-tocopherol. All packaging materials excluded light from the samples.

Lipophilic nutrients	Storage times (day)	-20°C		4°C		25°C		40°C	
		Vacuum (mg/g CRF, DW)	Non-Vacuum (mg/g CRF, DW)	Vacuum (mg/g CRF, DW)	Non-Vacuum (mg/g CRF, DW)	Vacuum (mg/g CRF, DW)	Non-Vacuum (mg/g CRF, DW)	Vacuum (mg/g CRF, DW)	Non-Vacuum (mg/g CRF, DW)
β-Carotene	0	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}	0.74 ± 0.08^{ab}
	14	0.76 ± 0.03^{a}	0.75 ± 0.03^{a}	0.75 ± 0.03^{a}	0.69 ± 0.05^{abcd}	0.65 ± 0.01^{abcde}	0.50 ± 0.02^{fg}	0.69 ± 0.03^{abcd}	0.40 ± 0.04^{gh}
	28	0.71 ± 0.03^{abcd}	0.73 ± 0.09^{abc}	0.71 ± 0.08^{abcd}	0.70 ± 0.02^{abcd}	0.68 ± 0.03^{abcd}	0.57 ± 0.04^{def}	0.66 ± 0.07^{abcde}	0.41 ± 0.05^{gh}
	42	0.69 ± 0.01^{abcd}	0.63 ± 0.02^{abcde}	0.66 ± 0.07^{abc}	0.63 ± 0.04^{abcdef}	0.64 ± 0.06^{abcdef}	0.53 ± 0.03^{efg}	0.62 ± 0.01^{abcdef}	0.30 ± 0.02^{hi}
	56	0.64 ± 0.01^{abcde}	0.64 ± 0.09^{abcde}	0.68 ± 0.05^{abc}	0.63 ± 0.02^{abcdef}	0.64 ± 0.01^{abcdef}	0.59 ± 0.00^{cdefg}	0.60 ± 0.00^{bcdef}	0.24 ± 0.00^{i}
	84	0.68 ± 0.00^{abcd}	0.67 ± 0.005^{abcde}	0.67 ± 0.007^{abc}	0.58 ± 0.02^{def}	0.65 ± 0.01^{abcde}	0.53 ± 0.01^{efg}	0.63 ± 0.01^{abcdef}	0.22 ± 0.00^{i}
Lutein	0	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}
	14	3.74 ± 0.33^{ab}	3.79 ± 0.33^{a}	3.75 ± 0.17^{ab}	3.69 ± 0.26^{abc}	3.49 ± 0.18^{abcde}	3.34 ± 0.18^{abcde}	3.55 ± 0.20^{abcde}	3.18 ± 0.20^{abcdef}
	28	3.76 ± 0.04^{a}	3.69 ± 0.04^{abc}	3.68 ± 0.53^{abc}	3.69 ± 0.17^{abc}	3.47 ± 0.52^{abcde}	3.23 ± 0.52^{abcdef}	3.20 ± 0.36^{abcdef}	2.48 ± 0.36^{gh}
	42	3.76 ± 0.15^{ab}	3.77 ± 0.15^{a}	3.71 ± 0.29^{abc}	3.52 ± 0.29^{abcde}	3.18 ± 0.13^{abcdef}	3.07 ± 0.13^{cdefg}	3.09 ± 0.08^{cdefg}	2.49 ± 0.08^{gh}
	56	3.68 ± 0.05^{abc}	3.77 ± 0.05^{a}	3.62 ± 0.10^{abcde}	3.55 ± 0.10^{abcde}	3.09 ± 0.12^{cdef}	3.10 ± 0.12^{defg}	3.02 ± 0.17^{defg}	2.22 ± 0.17^{h}
	84	3.66 ± 0.26^{abcd}	3.69 ± 0.26^{abc}	3.57 ± 0.13^{abcde}	3.28 ± 0.13^{abcde}	3.08 ± 0.07^{cdef}	2.60 ± 0.07^{efg}	2.99 ± 0.05^{efg}	1.95 ± 0.05^{h}
α-Tocopherol	0	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}	0.29 ± 0.02^{abc}
	14	0.29 ± 0.01^{ab}	0.29 ± 0.00^{ab}	0.29 ± 0.01^{abc}	0.28 ± 0.01^{abcd}	0.28 ± 0.02^{abc}	0.28 ± 0.02^{abcd}	0.27 ± 0.01^{abcd}	0.26 ± 0.04^{abcd}
	28	0.30 ± 0.02^{a}	0.30 ± 0.03^{a}	0.30 ± 0.02^{a}	0.28 ± 0.04^{abc}	0.29 ± 0.04^{abc}	0.25 ± 0.02^{abcd}	0.29 ± 0.02^{ab}	0.23 ± 0.02^{cde}
	42	0.29 ± 0.02^{a}	0.29 ± 0.00^{ab}	0.29 ± 0.03^{ab}	0.27 ± 0.01^{abcd}	0.28 ± 0.01^{ab}	0.26 ± 0.01^{abcd}	0.28 ± 0.01^{ab}	0.24 ± 0.01^{abcd}
	56	0.29 ± 0.01^{abc}	0.29 ± 0.01^{ab}	0.29 ± 0.01^{abc}	0.26 ± 0.01^{abcd}	0.26 ± 0.01^{abcd}	0.26 ± 0.001^{abcd}	0.26 ± 0.02^{abcd}	0.22 ± 0.01^{def}
	84	0.28 ± 0.01^{abc}	0.28 ± 0.01^{abcd}	0.27 ± 0.01^{abcd}	0.26 ± 0.00^{abcd}	0.26 ±0.03^{abcd}	0.18 ± 0.00^{ef}	0.24 ± 0.01^{bcde}	0.17 ± 0.01^{f}

Data were presented as mean ± SD of 3 separated and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significance at $p<0.05$, a>b.

Table 5.3: The interaction among storage temperature (25°C and 40°C), time, and the presence or absence of air in freeze-dried CRF from steam sterilised PVH on β-carotene, lutein and α-tocopherol.

Lipophilic nutrients	Storage times (day)	25°C				40°C			
		Vacuum +dark (mg/g CRF, DW)	Non-vacuum +dark (mg/g CRF, DW)	Vacuum +light (mg/g CRF, DW)	Non-Vacuum + light (mg/g CRF, DW)	Vacuum+dark (mg/g CRF, DW)	Non-vacuum + dark (mg/g CRF, DW)	Vacuum +light (mg/g CRF, DW)	Non-Vacuum light (mg/g CRF, DW)
β-Carotene	0	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}	0.74 ± 0.08^{abcd}
	14	0.65 ± 0.01^{abcd}	0.50 ± 0.02^{fgh}	0.46 ± 0.01^{ghi}	0.39 ± 0.02^{ijk}	0.69 ± 0.03^{ab}	0.40 ± 0.04^{hi}	0.30 ± 0.02^{ijk}	0.27 ± 0.01^{lm}
	28	0.68 ± 0.03^{abcd}	0.57 ± 0.04^{def}	0.40 ± 0.07^{ij}	0.21 ± 0.02^{lmn}	0.66 ± 0.07^{abcd}	0.41 ± 0.05^{i}	0.20 ± 0.001^{lmn}	0.17 ± 0.01^{mnop}
	42	0.64 ± 0.06^{abcd}	0.53 ± 0.03^{efg}	0.25 ± 0.02^{lm}	0.14 ± 0.01^{nopq}	0.62 ± 0.01^{bcde}	$0.30 \pm 0.02k^{l}$	0.11 ± 0.01^{opqr}	0.07 ± 0.00^{qrs}
	56	0.64 ± 0.05^{abcde}	0.59 ± 0.001^{cdef}	0.20 ± 0.003^{mno}	0.05 ± 0.001^{qrs}	0.60 ± 0.002^{bcde}	0.24 ± 0.001^{lm}	0.05 ± 0.00^{qrs}	0.04 ± 0.00^{rs}
	84	0.65 ± 0.012^{abcd}	0.53 ± 0.009^{efg}	0.10 ± 0.001^{pqrs}	0.00 ± 0.00^{s}	0.63 ± 0.013^{bcd}	0.22 ± 0.002^{lmn}	0.00 ± 0.00^{s}	0.00 ± 0.00^{s}
Lutein	0	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}	3.77 ± 0.39^{a}
	14	3.49 ± 0.18^{abc}	3.34 ± 0.05^{abc}	3.18 ± 0.03^{bcd}	2.78 ± 0.24^{defg}	3.55 ± 0.20^{ab}	3.34 ± 0.05^{bcd}	2.99 ± 0.05^{cdefg}	2.61 ± 0.26^{defghi}
	28	3.47 ± 0.52^{abc}	3.23 ± 0.21^{abcd}	2.97 ± 0.40^{cdefg}	1.51 ± 0.10^{lmn}	3.20 ± 0.36^{bcd}	3.23 ± 0.21^{fghi}	1.75 ± 0.09^{klm}	1.37 ± 0.16^{mno}
	42	3.18 ± 0.13^{bcd}	3.07 ± 0.04^{bcde}	2.25 ± 0.01^{hijk}	1.41 ± 0.04^{lmno}	3.09 ± 0.08^{bcde}	3.07 ± 0.04^{ghij}	1.21 ± 0.22^{nop}	0.52 ± 0.05^{qrst}
	56	3.09 ± 0.12^{bcde}	3.10 ± 0.28^{bcde}	1.92 ± 0.13^{kl}	0.75 ± 0.03^{pqrs}	3.02 ± 0.17^{bcdef}	3.10 ± 0.28^{ijk}	0.90 ± 0.03^{opq}	0.22 ± 0.03^{st}
	84	3.08 ± 0.07^{bcde}	2.60 ± 0.19^{defghi}	1.49 ± 0.12^{lmn}	0.31 ± 0.02^{rst}	2.99 ± 0.05^{cdefg}	2.60 ± 0.02^{jkl}	0.81 ± 0.07^{pqr}	0.21 ± 0.02^{t}
α-Tocopherol	0	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}	0.29 ± 0.02^{a}
	14	0.28 ± 0.02^{ab}	0.28 ± 0.02^{ab}	0.19 ± 0.01^{efg}	0.11 ± 0.02^{jk}	0.27 ± 0.01^{ab}	0.26 ± 0.04^{abcd}	0.14 ± 0.02^{hij}	0.13 ± 0.02^{ij}
	28	0.29 ± 0.04^{a}	0.25 ± 0.02^{abcd}	0.21 ± 0.02^{defg}	0.06 ± 0.01^{lmn}	0.29 ± 0.02^{a}	0.23 ± 0.02^{bcdef}	0.16 ± 0.02^{ghij}	0.07 ± 0.01^{klm}
	42	0.29 ± 0.01^{a}	0.27 ± 0.00^{abc}	0.11 ± 0.02^{jkl}	0.03 ± 0.01^{mno}	0.29 ± 0.01^{a}	0.23 ± 0.01^{abcd}	0.03 ± 0.01^{mno}	0.01 ± 0.00^{mo}
	56	0.26 ± 0.01^{abc}	0.26 ± 0.01^{abc}	0.04 ± 0.00^{mno}	0.01 ± 0.00^{no}	0.26 ± 0.02^{abcd}	0.22 ± 0.01^{cdef}	0.01 ± 0.00^{no}	0.01 ± 0.00^{no}
	84	0.26 ± 0.03^{abcd}	0.18 ± 0.00^{fgh}	0.03 ± 0.01^{mno}	0.00 ± 0.00^{o}	0.24 ± 0.01^{abcde}	0.17 ± 0.01^{ghi}	0.02 ± 0.00^{no}	0.00 ± 0.00^{o}

Data were presented as mean ± SD of 3 separated and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significance at $p<0.05$, a>b.

Table 5.4: Percentage (%) of nutrient loss after storage for 84 days at different conditions.

Compounds	Temperature (°C)	Vacuum	Light	Nutrient loss (%)
β-carotene	-20	Yes	No	8.11
	4	Yes	No	9.46
	25	Yes	No	12.16
	40	Yes	No	14.86
	-20	No	No	9.46
	4	No	No	21.62
	25	No	No	28.38
	40	No	No	70.27
	25	Yes	Yes	86.49
	40	Yes	Yes	100.00
	25	No	Yes	100.00
	40	No	Yes	100.00
Lutein	-20	Yes	No	2.92
	4	Yes	No	5.31
	25	Yes	No	18.30
	40	Yes	No	20.69
	-20	No	No	2.12
	4	No	No	13.00
	25	No	No	31.03
	40	No	No	48.28
	25	Yes	Yes	60.48
	40	No	Yes	78.51
	25	Yes	Yes	91.78
	40	No	Yes	94.43
α-Tocopherol	-20	Yes	No	3.45
	4	Yes	No	6.90
	25	Yes	No	10.34
	40	Yes	No	17.24
	-20	No	No	3.45
	4	No	No	10.34
	25	No	No	37.93
	40	No	No	41.38
	25	Yes	Yes	89.66
	40	Yes	Yes	100.00
	25	No	Yes	93.10
	40	No	Yes	100.00

Table 5.5: Degradation rate constant (K) and activation energy (E_a).

Compounds	Temperature (°C)	Vacuum	Light	k-value (day^{-1})	R^2	$t_{1/2}$ (day)	E_a (KJ/mol)	R^2
β-carotene	-20	Yes	No	1.23×10^{-3}	0.9983	564	10.57	0.9055
	4	Yes	No	1.59×10^{-3}	0.9983	437		
	25	Yes	No	2.12×10^{-3}	0.9004	327		
	40	Yes	No	3.44×10^{-3}	0.9874	202		
	-20	No	No	1.23×10^{-3}	0.9758	564	17.84	0.7295
	4	No	No	2.46×10^{-3}	0.9924	282		
	25	No	No	2.22×10^{-3}	0.9678	313		
	40	No	No	8.82×10^{-3}	0.9287	79		
	25	Yes	Yes	2.24×10^{-2}	0.9862	31	-	-
	40	Yes	Yes	4.27×10^{-2}	0.9799	17		
	25	No	Yes	4.69×10^{-2}	0.9654	15	-	-
	40	No	Yes	4.73×10^{-2}	0.9682	15		
Lutein	-20	Yes	No	3.15×10^{-4}	0.9734	2200	29.67	0.9560
	4	Yes	No	6.69×10^{-4}	0.9582	1036		
	25	Yes	No	2.96×10^{-3}	0.9888	234		
	40	Yes	No	3.95×10^{-3}	0.9088	176		
	-20	No	No	3.92×10^{-4}	0.9521	1767	31.07	0.9914
	4	No	No	1.18×10^{-3}	0.9594	385		
	25	No	No	3.66×10^{-3}	0.9934	190		
	40	No	No	7.03×10^{-3}	0.9734	99		
	25	Yes	Yes	1.15×10^{-2}	0.9734	61	-	-
	40	No	Yes	2.84×10^{-2}	0.9816	25		
	25	Yes	Yes	3.05×10^{-2}	0.9759	23	-	-
	40	No	Yes	5.99×10^{-2}	0.9941	12		
α-Tocopherol	-20	Yes	No	1.13×10^{-3}	0.9181	614	11.15	0.8770
	4	Yes	No	1.57×10^{-3}	0.9962	442		
	25	Yes	No	1.91×10^{-3}	0.9940	363		
	40	Yes	No	3.48×10^{-3}	0.9838	200		
	-20	No	No	1.13×10^{-3}	0.9181	614	23.12	0.9013
	4	No	No	1.57×10^{-3}	0.9962	442		
	25	No	No	6.18×10^{-3}	0.9965	113		
	40	No	No	7.79×10^{-3}	0.9994	89		
	25	Yes	Yes	2.88×10^{-2}	0.9450	24	-	-
	40	Yes	Yes	6.17×10^{-2}	0.9912	12		
	25	No	Yes	5.63×10^{-2}	0.9793	13	-	-
	40	No	Yes	6.22×10^{-2}	0.9900	12		

5.2.4. Effect of storage temperature

Storage temperature is another most important affecting the stabilities of freeze-dried product. A three-way ANOVA showed that low storage temperatures. (-20°C, and 4°C) with or without air exposure did not significantly impact on the nutrient stability of β-carotene, lutein and α-tocopherol in PVH CRF over 84 days (p>0.05) as shown in **Figure 5.3**. This result was supported by Craft et al. (1993), who reported that there was no significant difference in the stability of β-carotene and lutein when the freeze-dried SRM 1548 typical diet was stored at lower temperatures (-80°C, -20°C, and 4°C) for one year. However, Syamila et al. (2019) found that the nutrient degradation (β-carotene, lutein and α-tocopherol) lose obviously after 28 days when stored at 4°C both vacuum and non-vacuum packages. Chen et al. (1996) also found lutein, α-carotene, β-carotene, and vitamin A in carrot juice decreased after 2 weeks when stored at 4°C. It was possible due to the activation of endogenous enzymes both spinach and carrot during storage. While our sample was steam sterilised to suppress those activities prior to storage. Apart from the presence of endogenous enzymes in the material, a decrease of the collapse temperature in spinach powder might affect the nutrient degradation in spinach powder. Normally, the collapse temperatures of most dried materials are close to or higher than normal environment temperature. When storage temperature is lower than the collapse temperature, the loose material structure cannot be changed. However, if the material absorbs the moisture slowly it corresponding collapse temperature drops gradually. When collapse temperature is lower than storage temperature, several phenomena will appear, such as agglomeration, colour deterioration, and flavour change and cause nutrient degradation (Hua et al., 2010). Thus, these opinions might be reasons why the nutrient degradation in spinach powder decreased rapidly after 28 days even though it was stored at 4°C compared to our results.

However, there was a trend towards a reduction of nutrient stability found in samples stored at higher temperatures (25°C and 40°C). It was observed that β-carotene content in CRF started to degrade significantly different when stored at 40°C in the vacuum bag from day 56 (**Figure 5.3A**). Lutein and α-tocopherol contents started to reduce significantly when stored at 25°C from day 56 (**Figure 5.3B**) and at 40°C from day 84 (**Figure 5.3C**), respectively. Our result was supported by previous studies (Syamila et al., 2019, Koca et al., 2007, Song et al., 2018). They reported that storage at high temperatures increased the degradation rate of vitamin E in spinach powder (Syamila et al., 2019) and carotenoids in dehydrated pumpkin (Song et al., 2018), carrot slice (Koca et al., 2007), and spinach powder (Syamila et al., 2019). A similar trend was also observed in chlorophyll a and b of broccoli juice (Weemaes et al., 1999). It was suggested that the higher temperature could accelerate the chemical reactions, such as protein condensation, melting, polymorphism transform and moisture content reduction, and then caused the degradation of nutrients (Stringer and Denis, 2000). Bechoff et al. (2010) observed that during storage at a higher temperature, the water activity in dried sweet potato decreased, which related to being a faster degradation rate of β-carotene. The higher rate of carotenoid degradation might come from the isomerisation of carotenoids during higher temperature storage. An increase in storage temperature could induce the formation of t*rans* to *cis*-isomer and further reduces the content of *trans*-isomers. This was supported with other published paper that reported the storage at high temperature induced isomerisation and degradation of carotenoids in carrot juice (Chen et al., 1996, Marx et al., 2003). Moreover, a slower decrease in *cis*-isomers compared to all-*trans* isomer, suggesting that *trans*-isomer have been transformed to *cis*-form besides being degraded (Schieber and Weber, 2016). Song et al. (2018) discovered that there was a decrease of *trans*-β-carotene in dehydrated pumpkin. Conversely, *cis*-β-carotene increased significantly during storage at 40°C for

30 days. After storage for 45 days at 40°C, both *cis* and *trans*-β-carotene in dehydrated pumpkin reduced obviously.

An increase of the storage temperature in the presence of air significantly affected on the micronutrient stability (**Figure 5.3D, 5.3E, and 5.3F**). Rapid degradation of β-carotene was clearly observed in the samples which were stored with air exposure and with higher temperatures over time (**Figure 5.3D**). Moreover, it showed that the loss of lipophilic nutrients, including β-carotene, lutein, and α-tocopherol were more rapid at high storage temperatures. Our results were supported by Song et al. (2018). They reported that the storage of pumpkin at high temperature with air exposure increased the rate of loss of carotenoids significantly compared to a lower storage temperature with the presence of air. When carotenoid and vitamin E expose to the air, they can induce the chemical reactions that produce reactive oxygen species (ROS) by autooxidation. Then, the free radical can accelerate nutrient degradation during storage. It was expected that the free radical activity might be more active at high temperature. This finding supported evidence from the previous observation that showed higher temperature resulted in the higher free radical formation in propolis (Olczyk et al., 2016). In addition, increase temperature and the presence of air helped to reduce the activation energy as shown in **Table 5.5**. Thus, this could be a reason why the nutrient decreased rapidly in storage condition under high temperature and air exposure.

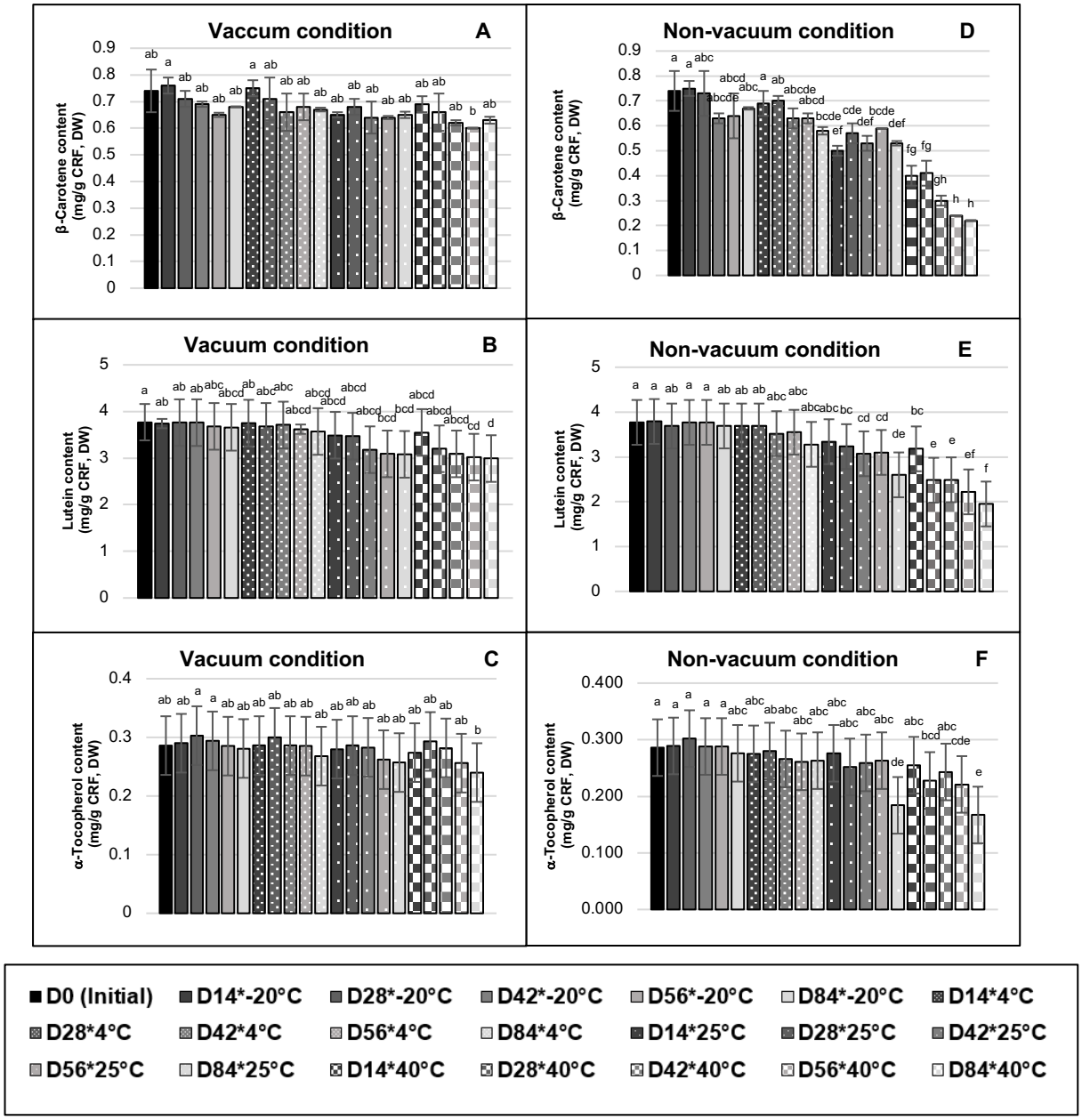


Figure 5.3: The effect of storage temperature (-20°C, 4°C, 25°C and 40°C) in vacuum **(A-C)** and non-vacuum **(D-F)** packaging on the degradation of β-carotene, lutein and α-tocopherol in freeze-dried CRF from steam sterilised PVH for 84 days.

Both types of packaging excluded light from the samples and D means day; Data were presented as a mean ± SD of 3 separated storage conditions and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at p<0.0.5, a>b.

5.2.5. Effect of air exposure

The effect of packaging samples in vacuum and non-vacuum packages over 84 days at temperatures at -20°C, 4°C, 25°C, and 40°C on lipophilic nutrient stability of CRF from steam sterilised PVH was illustrated in **Figure 5.4**. A two-way ANOVA showed that the presence or the absence of air in the storage conditions at -20°C did not give a significant difference in lipophilic nutrient stabilities, including β-carotene, lutein, and α-tocopherol in the samples of CRF from steam sterilised PVH (p>0.05) during storage for 84 days (**Figure 5.4A, 5.4E, and 5.4I**). At 4°C, there was no impact on the degradation of lutein and α-tocopherol either in the presence or absence of air (**Figure 5.4F and 5.4J**). However, β-carotene decreases significantly when stored at 4°C with oxygen exposure at day 84 (**Figure 5.4B**). From this result, it showed that presence of air under low temperature in freeze-dried PVH CRF did not have an effect on the stability of β-carotene, lutein, and α-tocopherol. Our results were not consistent with the previous works. Syamila et al. (2019) observed that β-carotene in spinach powder was stable only 14 days at 4°C either vacuum or non-vacuum packages. After 14 days, it decreased rapidly. Lutein and α-tocopherol were stable only 28 days in vacuum packages and decreased thereafter. A similar trend was observed by Song et al. (2018). They found that the level of β-carotene in dehydrate pumpkin reduced significantly during 15 days at 4°C. It was expected that the endogenous enzymes in spinach and pumpkin were still active. Spray-dried spinach powder was not knocked out enzymes before storage, while the pumpkin was blanched for 40 seconds to repress endogenous enzymes. However, blanching at 40 seconds in pumpkin might not enough for inactivation of these enzymes. The endogenous enzymes might accelerate the reaction of degradation of carotenoid in the plant materials during storage. Meanwhile, our study, pea vine haulm was steam sterilised at 100°C for 4 min

to inactivate the endogenous enzymes before storage at 4°C. From this reason, it was a possible that lipophilic nutrients in PVH CRF was more stable during storage at low temperature than other results even though it was exposed to the air or not.

At the high temperatures (25°C and 40°C), the samples which were not under vacuum had significantly reduced β-carotene, lutein and α-tocopherol levels compared to vacuum packaging during storage for 84 days. To observe the correlation of β-carotene, lutein, and α-tocopherol concentration in the storage condition at 25°C and 40°C in this study, a principal component analysis (PCA) was carried out (**Figure 5.5**). Due to many overlapping data points, which made the score plots difficult to interpret in the storage at 25°C and 40°C, two separate PCA plots were prepared. Both plots, the amount of variation explained by the F2 (axis) was low (<20%) indicating that the factors behind the F1 axis were driving the degradation of nutrient. The PCA analysis showed that storage PVH CRF with air exposure did affect the stability of lipophilic nutrients at high temperatures (**Figure 5.5**). At 25°C and 40°C, the CRF stored in vacuum packs in day 0, 14, 28 and 42 were in the same cluster. It means that there was no significant difference (p>0.05) on the degradation of lipophilic nutrients in these conditions. However, the nutrients started to degrade in the vacuum package of day 56, which was the same cluster of non-vacuum packages, as shown in **Figure 5.5**.

Our study found that the lipophilic nutrients in freeze-dried PVH CRF were not stable over the storage at high temperature with air exposure. Our result was agreed with the previous works (Baiano et al., 2005, Vásquez-Caicedo et al., 2007, Hwang et al., 2012, He et al., 2016). They reported that storage at high temperature with the presence of air had affect loss of lipophilic nutrients. He et al. (2016) reported that increasing temperature was an effect on the rate of oxidation. It has previously reported that high oxygen concentration associated with levels of carotenoid degradation during storage in dried sweet potato flake (Hwang et al., 2012),

pasteurised mango puree (Vásquez-Caicedo et al., 2007), and semi-preserved tomato sauce (Baiano et al., 2005). High temperature might accelerate the oxidative degradation of carotenoid and vitamin E. The highly unsaturated chain of carotenoids makes it react easily with any radical species present (Krinsky and Yeum, 2003); carotenoid fragmentation can be caused by autoxidation (Mordi, 1993); heating (Cremer and Eichner, 2000) and enzymatic activity (Auldridge et al., 2006). The oxidative degradation is the principle cause extensive losses of carotenoids and vitamin E in the presence of molecular oxygen. Carotenoid can react with oxygen to yield carotenoid peroxyl radical, which may react with another radical to give stable products. Then, it attacks another carotenoid molecule to induce autoxidation of carotenoids, or attack a substrate, such as lipid to generate lipid radical and induce chain oxidation. The carotenoid radical may also undergo to give an epoxide and alkoxyl radical, which may continue oxidation (Pénicaud et al., 2011). Also, the oxidation of fatty acids and carotenoids was similarly reported to be a free radical mechanism (Liebler, 1993). It was thus possible that unsaturated fatty acid present in PVH CRF (69% α-linolenic acid (18:3) and 21% linoleic acid (18:2)) might induce autooxidation and further degraded the nutrients.

α-Tocopherol in PVH CRF was the most stable when stored at 25°C either with or without air exposure compared to carotenoids. However, it had started to decrease by day 84 in the non-vacuum package, as shown in **Figure 5.4K**. It was expected that the degree of unsaturated double-bound might have an effect on the stability of nutrient during storage at room temperature with air exposure. Boon et al. (2010) reported that the less number double bond, the less susceptible to oxidation. Carotenoids are highly unsaturated molecules; they are very susceptible to oxidation compared to α-tocopherol. A similar study also found that the degradation rate of α-tocopherol was lower than β-carotene (Pénicaud et al., 2011). Our result was

consistent with the result of Syamila et al. (2019). They found that α-tocopherol in spinach powder showed the most stable over duration of 28 days compared to lutein and β-carotene at 20°C even though the level of α-tocopherol decreased thereafter. Thus, these assumptions might be a reason why α-tocopherol was more stable under storage with air exposure compared to carotenoids.

The mechanisms of lutein and β-carotene degradation under air exposure are illustrated in **Figure 5.6** and **5.7**. The double bond of β-carotene was oxidised to form di-radicals and which resulted in further changes to 5,6-epoxy or 15,15' epoxy β-carotene and other compounds, such as carbonyl, carbon dioxide, and carboxylic acid (**Figure 5.7**) (Mordi, 1993, Boon et al., 2010, Pénicaud et al., 2011). Meanwhile, the mechanism of α-tocopherol degradation in the presence of oxygen is illustrated in **Figure 5.8**. When α-tocopherol is exposed to oxygen, it can generate superoxide radical and a tocopheroxy radical chain. Further interaction of the superoxide radical with initial α-tocopherol could cause a chain reaction and a HO_2 radical interacted with α-tocopherol to degrade it further (Sabliov et al., 2009).

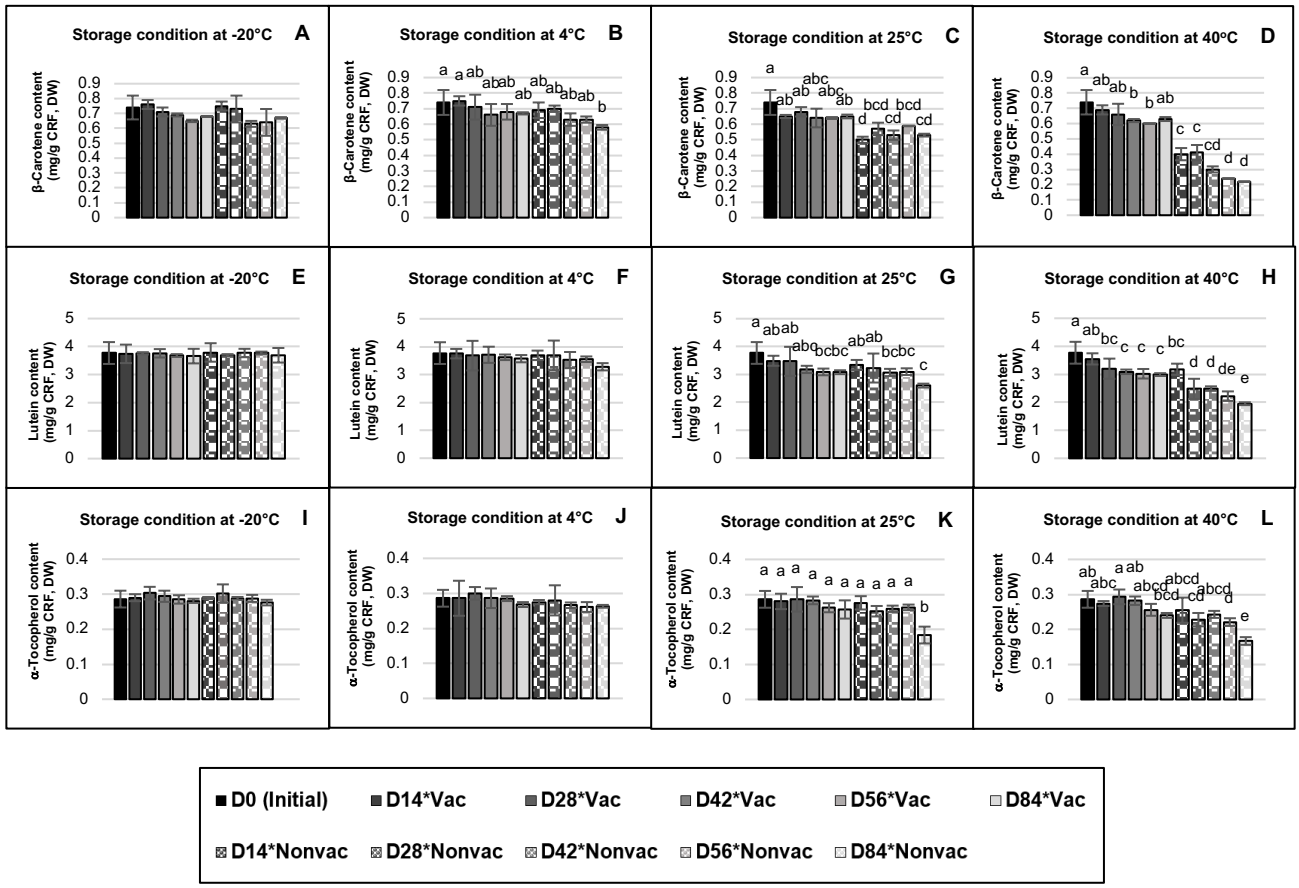


Figure 5.4: The effect of vacuum and non-vacuum packaging on the degradation of β-carotene, lutein and α-tocopherol in freeze-dried CRF from steam sterilised PVH at different temperatures: -20°C **(A, E, I)**, 4°C **(B, F, J)**, 25°C **(C, G, K)** and 40°C **(D, H, L)** over a storage time of 84 days. Both types of packaging excluded light.

Data were presented as a mean ± SD of 3 separated storage conditions and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at p<0.0.5, a>b. (D: day, Vac: Vacuum, Nonvac: Non-vacuum).

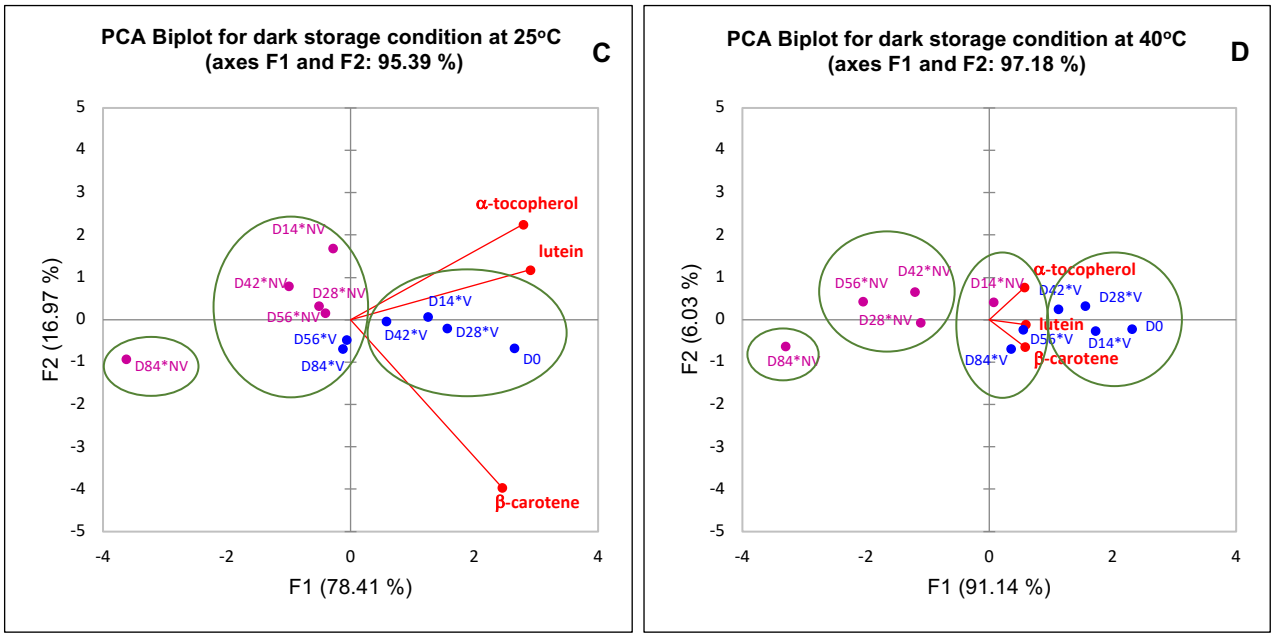


Figure 5.5: PCA plot distribution of freeze-dried CRF from steam sterilised PVH stored at different temperature conditions. **A)** vacuum in dark at 25°C **B)** vacuum in dark at 40°C, along the principal component 1 (F1) and 2 (F2) for β-carotene, lutein, and α-tocopherol; D: Day, V: vacuum, NV: Non-vacuum.

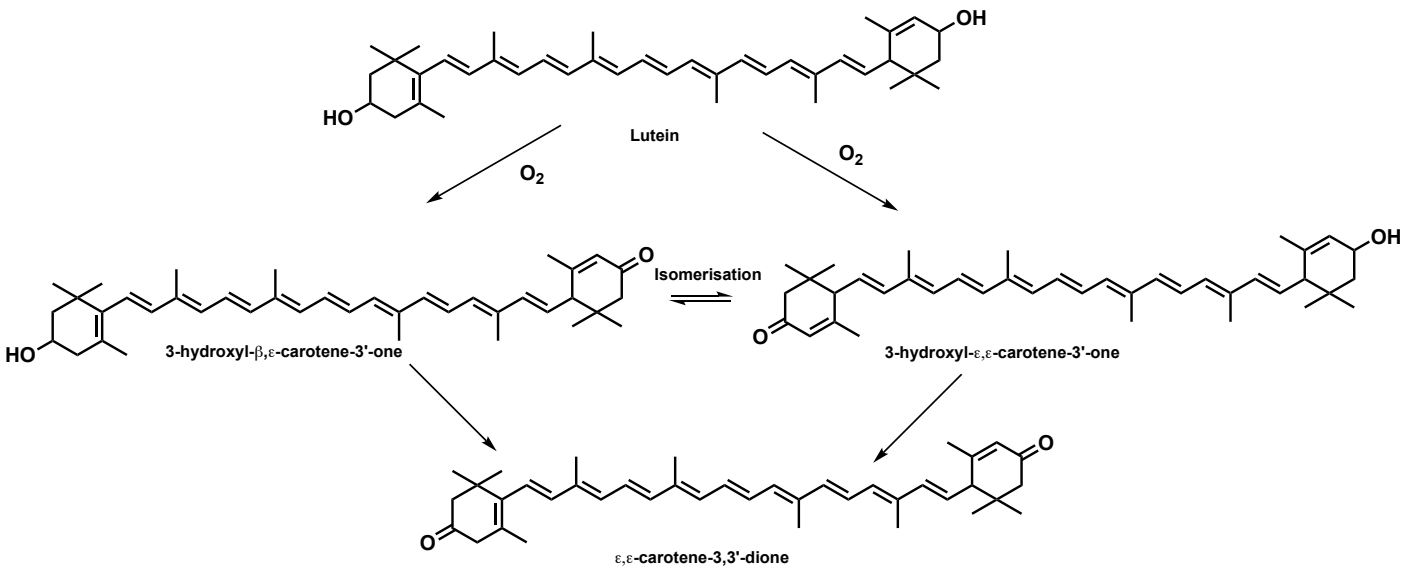


Figure 5.6: Lutein degradation under oxygen exposure.

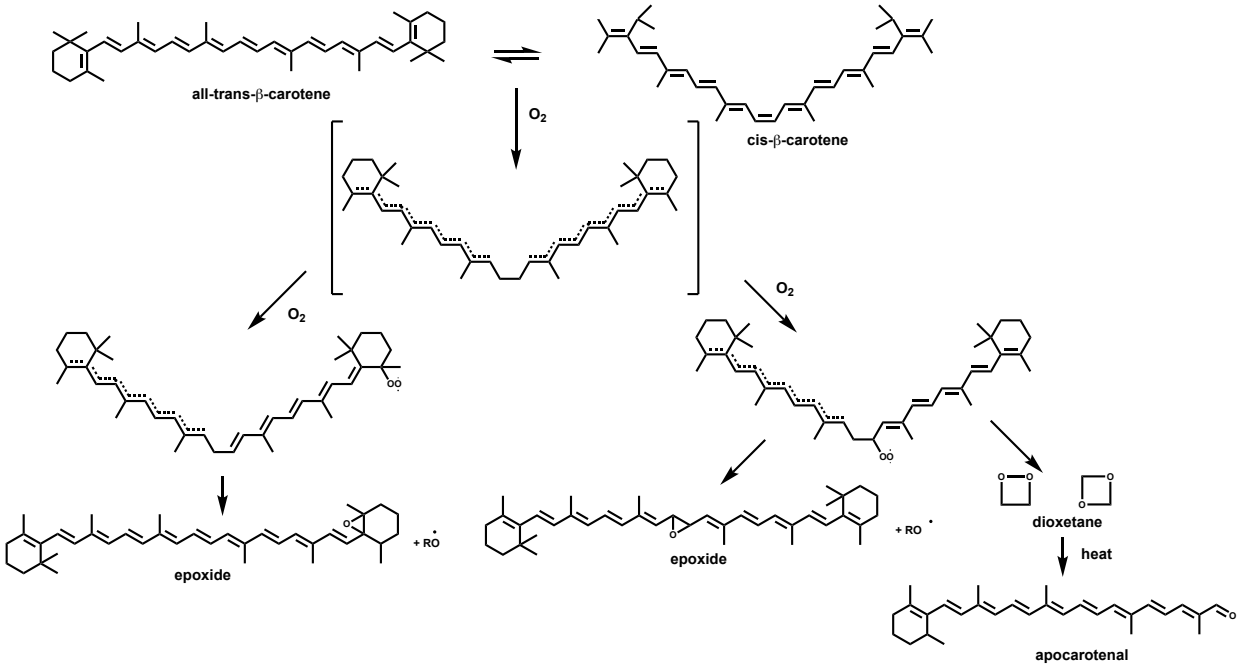


Figure 5.7: β-carotene degradation under oxygen exposure (Mordi, 1993, Boon et al., 2010, Pénicaud et al., 2011).

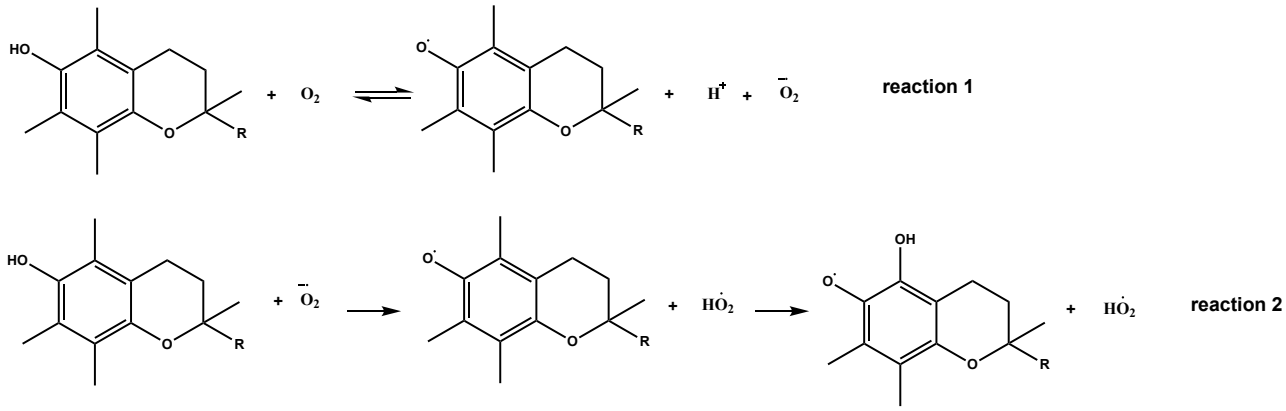


Figure 5.8: Degradation of α-tocopherol under oxygen exposure (Sabliov et al., 2009).

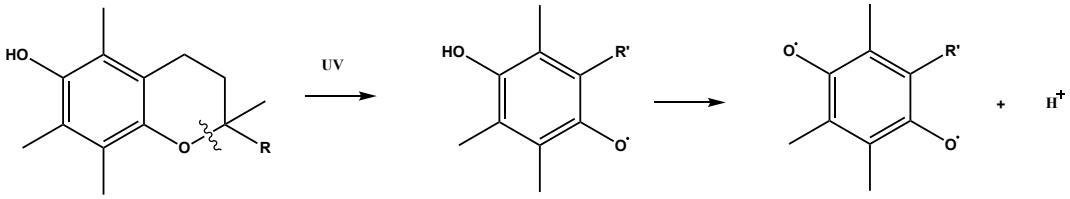


Figure 5.9: Degradation of α-tocopherol under UV light (Sabliov et al., 2009).

5.2.6. Effect of light exposure

In addition to temperature and oxygen, light is also one of the main factors of nutrient degradation. Light promotes isomerisation of the *trans*-form of carotenoids to the *cis*-form and also induces oxidation (photo-oxidation) (Bonnie and Choo, 1999, Pénicaud et al., 2011), it thus causes loss of carotenoids. It was observed that the effect of light exposure at 25°C and 40°C in samples stored in both vacuum and non-vacuum packages had a significant impact on the degradation of lipophilic nutrients (p<0.05) (**Figure 5.10**). A multi-factor ANOVA (**Table 5.3**) showed that the CRF which was exposed to light had a clear reduction of β-carotene, lutein and α-tocopherol (p<0.05) in the high storage temperatures (25°C and 40°C) whether or not there was the presence or the absence of oxygen in the packages. Similar trends were observed by other researchers. Chen et al. (1996) compared the effect of temperature and light on nutrient degradation (α-carotene, β-carotene lutein and vitamin A) in carrot juice at 4°C, 35°C, and 40°C. They found that all nutrients decreased significantly during the light storage compared to dark storage condition. Shi and Chen (1997) also reported that storage of lutein at 25°C was completely degraded 4 days earlier than that in dark condition. It has reported that the degradation rate of lipophilic nutrients (chlorophyll a, chlorophyll b, all-*trans*-β-carotene, 13-*cis*-β-carotene, 9-*cis*-β-carotene, lutein, violaxanthin, neoxanthin) was faster under light storage than the dark storage at 4°C (Kopas-lane and Warthesen, 1995). It was possible due to the reason that light induced oxidation (photo-oxidation) and isomerisation of carotenoids in these plant materials. When the light energy is absorbed, the photosensitiser molecules, such as chlorophylls and carotenoids, are converted into a triplet excited state, which can interact easily with another molecule, producing free radicals or molecular oxygen, leading to the formation of highly reactive singlet oxygen (Limbo et al., 2007). Then, the singlet oxygen can attack double bond of other components in the products directly and it thus causes loss

of nutrients.

However, there was another published research that reported the effect of light exposure at 20°C did not have a significant impact on of β-carotene, lutein and α-tocopherol in spray-dried spinach juice during 28 days compared to the sample without light exposure (Syamila et al., 2019). After 28 days, the spray-dried spinach juice decreased rapidly in the storage either the presence or absence of light. While our results found that the storage of freeze-dried CRF from steam sterilised PVH with light exposure had a significant effect on lipophilic nutrients at 25°C and 40°C for 14 days. The difference of both results was expected due to the different temperature storage, even though both experiments used the same intensity of light (600-800 lux). The current work used the storage temperature at 25°C and 40°C. While the experiment of Syamila et al. (2019) stored the spray-dried spinach powder at lower temperature (20°C) than our result. Thus, this might be a reason why the nutrient degradation of our result at 25°C was faster than that of Syamila et al. (2019) at 20°C. Moreover, Rodriguez and Rodriguez-Amaya (2007) did not find a major impact of light exposure on β-carotene degradation during storage for 21 days at room temperature. Compared to our result kept at the same temperature, it might be due to the low light intensity of the fluorescent lamp. The intensity of fluorescent lamp in the storage of PVH CRF was used at 600-800 lux, while the result of Rodriguez and Rodriguez-Amaya (2007) was used at 318 lux. A similar trend with our result, Chen et al. (1996) observed the presence of light at 2000 lux had a major effect on the loss of carotenoids in carrot juice during storage at 25°C for 3 months.

Also, it was observed that the longer the CRF was exposed to light, the more extreme the degradation β-carotene, lutein, and α-tocopherol. This result was confirmed with the PCA plots (**Figure 5.11**) which showed that the major factor of nutrient degradation in CRF from steam sterilised PVH was the light exposure and the

duration of exposed the light. The PCA plot indicated that the CRF stored in the dark condition at 25°C and 40°C appeared in the different cluster with the CRF stored in light condition (**Figure 5.11A - 5.11C**), except the storage in non-vacuum condition at 40°C (**Figure 5.11D**). It showed that the storage in day 14 with light exposure was the same cluster with the storage with the absence of light in day 14, day 28, day 56, and day 84. However, they were not in the same group of initial storage (day 0) (**Figure 5.11D**). It means that light, temperature, oxygen, and storage time had affected the degradation of β-carotene, lutein, and α-tocopherol. This study supported results from the previous observations. For example, storage of dehydrated carrots at 40°C for a month (Lavelli et al., 2007), storage in different packaging materials at room temperature (Singh et al., 2013), and storage of carrot juice in the presence and absence of light (Shi and Chen, 1997) also showed that storage time was the major factor in the degradation of nutrients.

In addition, either high temperature or light exposure could lead to the isomerisation and degradation of carotenoids (Khoo et al., 2011). The presence or increase of *cis*-isomers may occur due to isomerisation of carotenoids' *trans*-isomers during storage. It has been reported in carrot juice and spinach that 9-*cis* carotenoid isomers were the major form found after storage under illumination, whereas 13-*cis* isomers were the most common after storage in dark condition (Chen et al., 1996, Aman et al., 2005). Loss of vitamin E is also greater following storage in the light compared to storage in the dark. α-Tocopherol reacts with light to break an OH bond and the ether linkage and form oxy radical, as shown in **Figure 5.9** (Sabliov et al., 2009).

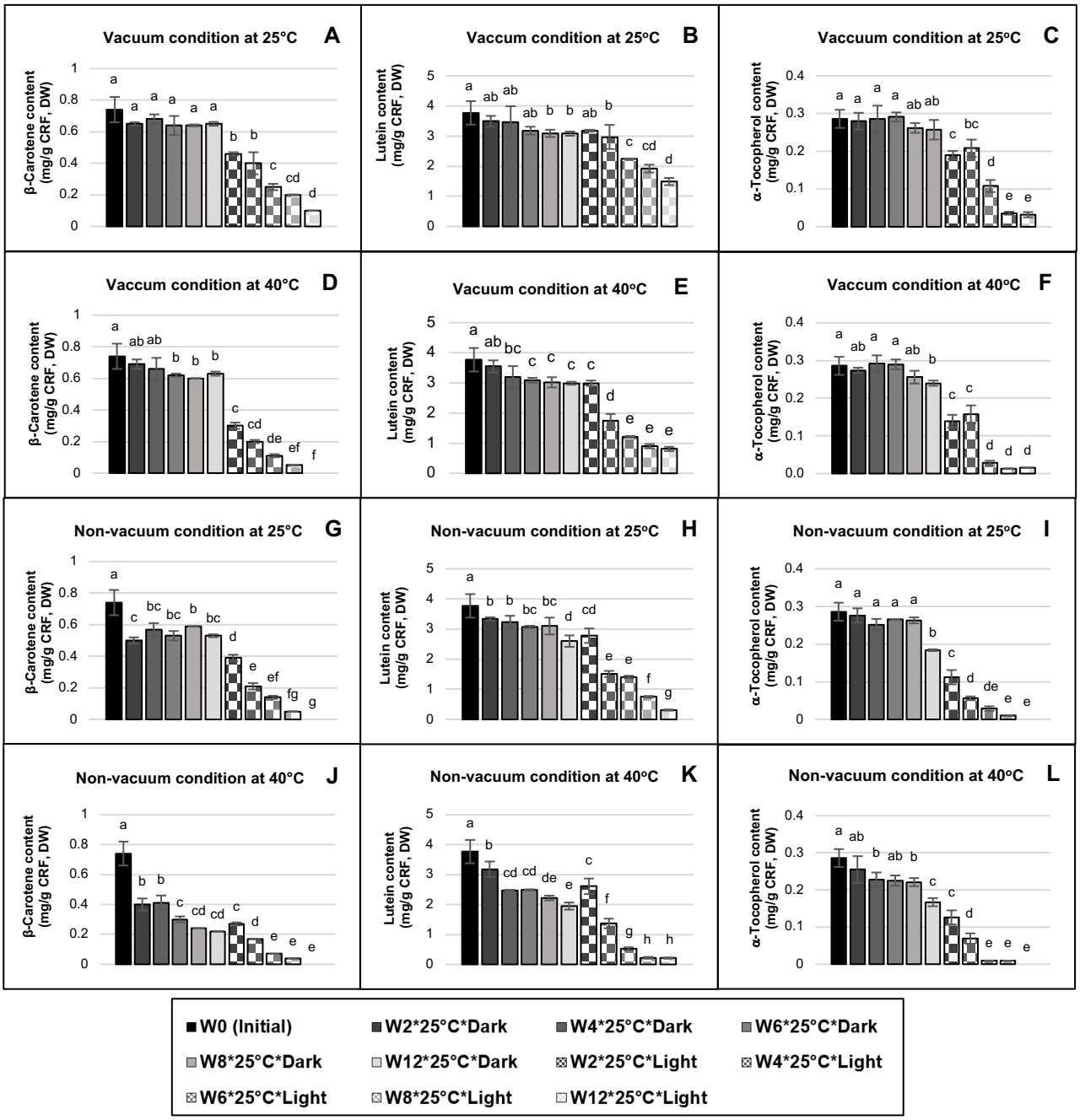


Figure 5.10: The effect of the presence of light on lipophilic nutrients in freeze-dried CRF from steam sterilised PVH stored at 25°C and 40°C both vacuum and non-vacuum packages over the duration of 84 days (D: day).

Data were presented as a mean ± SD of 3 separated storage conditions and analysed using post-hoc analysis of variance (ANOVA) and according to Tukey test with statistically significant at p<0.0.5, a>b.

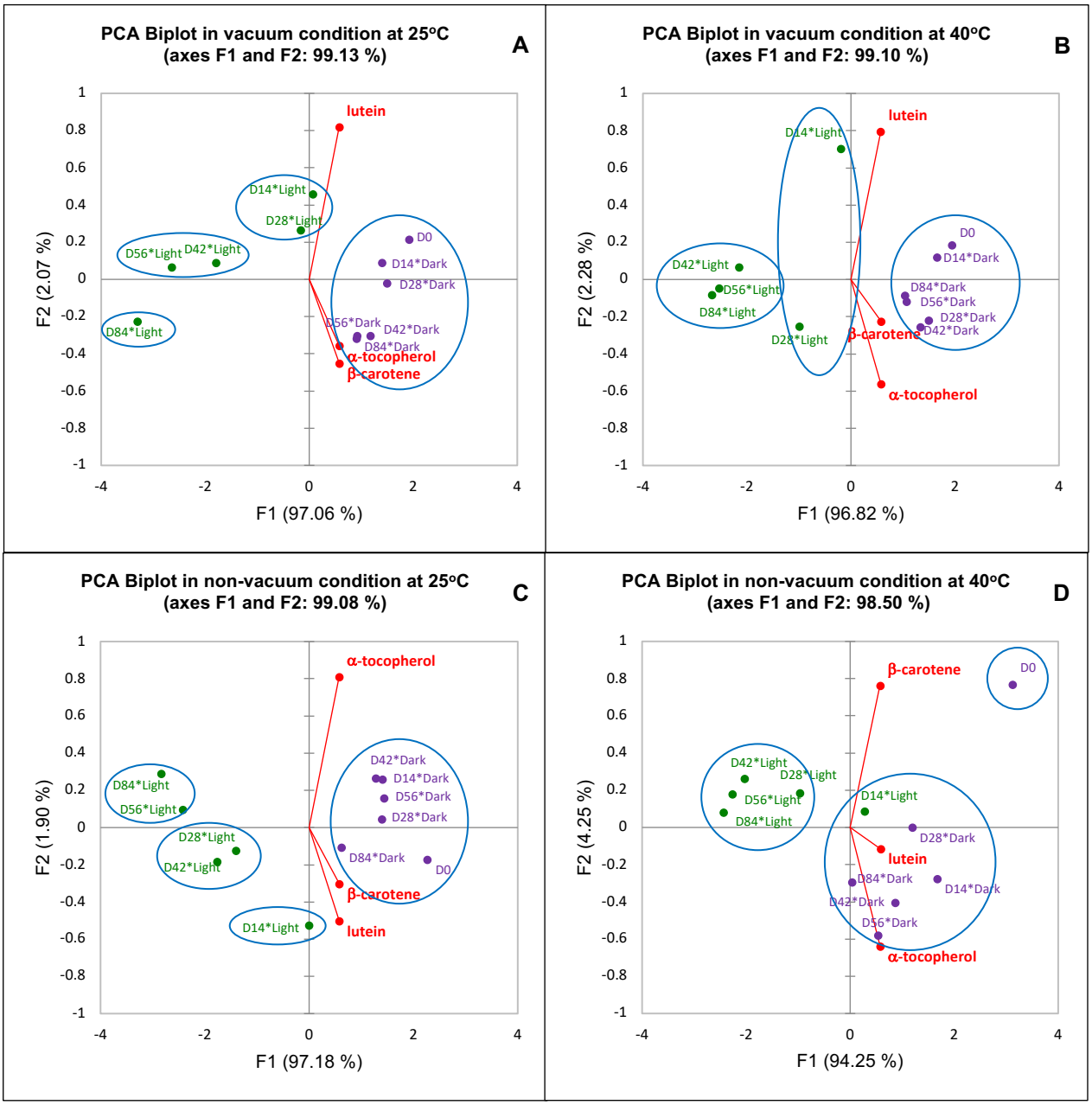


Figure 5.11: PCA plot distribution of freeze-dried CRF from steam sterilised PVH stored at different temperature conditions: **A)** vacuum in dark and light conditions at 25°C **B)** vacuum in dark and light conditions at 40°C **C)** non-vacuum in dark and light conditions at 25°C **D)** non-vacuum in dark and light conditions at 40°C, along the principal component 1 (F1) and 2 (F2) for β-carotene, lutein, and α-tocopherol; D: Day.

5.3. Conclusions

This study showed that using steam treatment could be a method to knock out endogenous enzyme in PVH prior to making CRF. Although endogenous enzymes were inactivated (66% of loss POD activity), temperature, light, oxygen have an effect on nutrient stabilities of freeze-dried PVH CRF. Hence, the CRF from steam sterilised PVH should be stored at the low temperatures, such as -20^{o}C or 4^{o}C ensuring that there is no light exposure. There was no significant difference in the stability of lipophilic nutrients (β-carotene, lutein and α-tocopherol) when the CRF was stored in either vacuum or non-vacuum packages over 84 days. This indicates that the lipophilic nutrients in CRF from steam sterilised PVH are well-protected at low temperatures for long-term storage. However, if storing at higher temperatures, such as 25^{o}C and 40^{o}C, it is recommended that light and air exposure is minimised to avoid these other major factors of nutrient degradation.

CHAPTER VI

Bioaccessibility of lipophilic nutrients in a chloroplast-rich fraction (CRF) recovered from post-harvest, pea vine field residue (haulm)

6.1. Introduction

Measuring the bioaccessibility of nutrients, i.e. their release from a matrix, is an effective way to establish a core requirement if nutrients are to be taken up into the body. This is the first time that the bioaccessibility of nutrients form CRF material has been studied. The impact of heat treatment in the production of CRF on the bioaccessibility of nutrients was included in this study.

In **Chapter IV,** I have shown that CRF from fresh PVH has the nutritional credentials to be used as a food supplement in the form of powder. Heat treatment of the biomass is an effective way of knocking out enzymes that might cause a deterioration in the quality of CRF material during storage (**Chapter V**). Heat treatment of juice recovered from biomass is another way of promoting the preservation of nutrients in the final CRF material/dry powder. There are currently no published studies comparing the quality of a chloroplast-rich fraction derived from biomass or juice derived from biomass.

On July 2017, we collected fresh PVH from a field in Yorkshire. On returning to our labs (a journey time of 2 hours), we immediately separated 30 kg of PVH: one third was used to prepare fresh CRF, another third was steam sterilised at 100ºC for 4 minutes, the other third of the fresh PVH biomass was juiced using the Angel twin-screw juicer. After juicing and filtering, the juice was heated at 90ºC for 3 minutes. CRF was prepared from both the heated biomass and the heated juice as described in **Chapter III**.

All three of these CRF preparations: FJ (fresh juice) CRF; HPVH (heat-treated pea vine haulm) CRF; and HJ (heat-treated juice) CRF were sieved to include particles of 250 μm or less and their composition analysed. These CRF preparations were then used to measure the bioaccessibility of lipophilic nutrients in the presence or absence of oil.

The consumption of carotenoids, vitamin E and essential fatty acids have potential health benefits in humans as they are antioxidants, lowering inflammation in the body, or in the case of the essential fatty acid, must be obtained from food as they cannot be produced by the body. Even though these substance are found in vegetables and fruits, it has been reported that the bioavailability of these carotenoids and vitamin E from vegetables and fruits is relatively low (Brown et al., 2004, Reboul et al., 2006). This has been attributed to several causes, including limited release from plant tissue (Rock et al., 1998, Ryan et al., 2008, Tumuhimbise et al., 2009) and low solubilisation in gastrointestinal fluids (Brown et al., 2004, Nagao et al., 2013). To be available for absorption in the gastrointestinal (GI) tract, nutrients first have to be bioaccessible. Bioaccessibility is thus defined as the fraction of a compound which is released from its food matrix within the GI tract to become available for intestinal absorption and can be determined through *in vitro* procedures (Carbonell-Capella et al., 2014). *In vitro* digestion is a popular method to screen potential bioaccessibility since it is cheap, rapid and gives a preliminary evaluation before performing *in vivo* animal or human studies (Minekus et al., 2014, Brodkorb et al., 2019).

PVH is a by-product in the production of peas and so an ideal candidate for exploitation with no increased cost or requirement for additional land use to produce it. However, before considering in the use of PVH CRF as answer to human vitamin malnutrition, we need to assess how well the body will be able to absorb the nutrients from the PVH CRF. Currently, there are no recorded experiments that measure the

release of carotenoids, vitamin E and essential omega-3 and 6 fatty acids from chloroplasts of PVH that have been liberated from the cell wall confinement during digestion. The bioaccessibility of carotenoids, vitamin E, and essential fatty acids in some plants and algae have been reported to be low (Reboul et al., 2006, Francisco et al., 2020). This study was planned to assess the bioaccessibility of lipophilic nutrients in PVH CRF.

The hypothesis of this study was that 'heat treatment of PVH biomass, or its juice, prior to CRF production, affect the bioaccessibility of its lipophilic nutrients and the inclusion of oil during digestion also improves the bioaccessibility of PVH CRF lipophilic nutrients'. Therefore, the experiments detailed in the study of this chapter aimed to explore the impact of biomass heat treatment, on the quality and quantity of the nutrients under investigation in the CRF recovered from postharvest, pea vine field residue (haulm). The experiment also assessed whether the presence of oil improved the bioaccessibility of β-carotene, lutein, and α-tocopherol from CRF in the course of *in vitro* digestion. The physiological GI conditions were simulated using an *in vitro* gastrointestinal digestion model (Minekus et al., 2014, Brodkorb et al., 2019).

Rapeseed oil was chosen for the study of bioaccessibility of lipophilic nutrients because it is high unsaturated fatty acid and does not contain measurable number of carotenoids. In addition, it has been reported that this oil allowed the highest bioaccessibility of β-carotene from spinach leaves when compared with soybean oil, safflower oil, olive oil, extra virgin oil, sesame oil, palm oil, beef tallow and lard (Nagao et al., 2013). After the digestion step, the determination of lipophilic nutrient retention, bioaccessibility and availability for uptake requires that the isolation of CRF compounds released in the whole digesta and solubilised in the aqueous phase (micellar phase) using lipid extraction of the separated phases. Firstly, centrifugation is used to separate and isolate the aqueous phase from insoluble materials; it is assumed that lipophilic

compounds, including carotenoids, vitamin E and fatty acids are dispersed/solubilised in the aqueous phase in the form of micelles. The resultant aqueous phase was passed through a 0.2 μm PTFE filter membrane (Garrett et al., 1999, Ferruzzi et al., 2006, Schweiggert et al., 2012) to isolate the compounds. It is assumed that particles, which can pass through a 0.2 μm filter membrane, it will also be able to pass through the mucous layer in the small intestine and be available for absorption by the epithelium cells (Yang and McClements, 2013).

6.2. Results and Discussions

6.2.1. Composition of PVH CRF materials before *in vitro* digestion

Three separate CRF materials were produced from pea vine haulm (PVH): fresh (non-heat treated) PVH juice (FJ); heated PVH (HPVH); and heated juice (HJ). The proximate and selected lipid micronutrient composition of these materials was measured (**Table 6.1**). Our result showed that using heat treatment either HPVH or HJ reduced the carbohydrate content in PVH CRF. The carbohydrate content of the CRF in the non-heat-treated material was significantly higher (p<0.05) (1 to 2-fold) than the carbohydrate content of the heat-treated materials. The CRF from FJ contained a large amount of carbohydrate (525.73 ± 0.14 mg/g CRF, DW). The carbohydrate contents in the CRF from HPVH and HJ were 391.11 ± 1.27 mg/g CRF, DW and 289.80 ± 0.62 mg/g CRF, DW, respectively. Our result was consistent with previous study (Ikanone and Oyekan, 2014). It reported that boiling and frying of sweet potato tubers reduced by 3-4% of total carbohydrate content compared to their raw tubers. In addition, the decrease of carbohydrate content in PVH CRF with heat treatment was confirmed by the observation of Maltese crosses using polarised microscopy. The presence of Maltese crosses indicated the presence of native starch in the non-heat treated CRF (FJ) sample. Heat treatment of the PVH, directly (HPVH) or of the juice (HJ), resulted

in the loss of native starch structure (**Figure 6.1B and 6.1C**). The appearance of Maltese crosses was reduced in CRF from HPVH, but some granules with birefringence were still present (**Figure 6.1B**), while in CRF from HJ, the occurrences of both Maltese crosses and birefringence were diminished (**Figure 6.1B**). Other research has shown that heat starch granules broadened the Maltese crosses and reduced birefringence and that upon gelatinisation both of these features were lost (Varriano-Marston et al., 1980, Alam et al., 2013). It appears from these observations that heat treatment of biomass or juice caused gelatinisation of starch and that during the preparation of CRF material, much of the gelatinised starch material likely remains in the supernatant. One needs to allow for this when considering the concentration of nutrients in the CRF materials as the presence of starch will effectively dilute the other nutrients. One way to take this into account is to express the nutrient/pigment concentration values in terms of mass per unit mass of total lipid, as we can assume that most of the lipid extracted from CRF are originating from the chloroplast membranes. This measure will enable a comparison of the nutrient concentrations across the CRF materials. From **Table 6.1**, it can therefore be seen that heat treatment have an effect on the chlorophyll content of PVH CRF. The chlorophyll is significantly lost (about 40%) during the heat treatment of biomass or juice in PVH. This was due to loss of magnesium bound in the chlorophyll causing their degradation (Schwartz and Elbe, 1983). Our result was supported with the previous research (Severini et al., 2016), which studied the effect of different blanching (microwave, boiling water and steaming) on pigment content in broccoli. They found that all thermal treatments decreased chlorophyll content of broccoli compared to the untreated broccoli.

Moreover, using thermal processing also decreases the carotenoid and vitamin E content. However, it depends on the method of thermal treatment. The carotenoids, β-carotene and lutein, appear to be stable in the CRF from HJ compared

to CRF from FJ, but suffer 34% and 40% losses, respectively in the CRF from HPVH. Similar results were also observed by Dutta et al. (2009) in the blanching of pumpkin, Alam et al. (2013) in the blanching of carrot pomace powder, and Siqueira de Oliveira et al. (2013) in the steam blanching of carrots and broccolis. They found that blanching of these vegetables reduced the carotenoid content compared to the vegetables without blanching. It was due to the reason that the heat treatment induced the break the bond of carotenoids and relocation of the single or double bond by isomerisation (D'Evoli et al., 2013). However, Lee et al. (2017) reported that difference in carotenoid retention of cooked vegetables could increase the extraction of carotenoids by softening plant cell walls and disrupting carotenoid-protein complex. For example, microwaved broccoli helped to increase 15% of the retention of β-carotene compared to raw broccoli. Conversely, boiled, blanched, and steamed broccoli decreased 11-21% of β-carotene retention (Lee et al., 2017). In addition, other researchers have noted that lutein is more stable than β-carotene (Bunea et al., 2008, Palermo et al., 2014). The degradation of carotenoids, particular β-carotene, during blanching was thought to be due to oxidation (Bouzari et al., 2015, Junior, 2016) and the isomerisation of *trans* to *cis*-isomer (Chandler and Schwartz, 1988). The extent of degradation due to isomerisation and oxidation was associated with the duration and severity of heat processing (Tumuhimbise et al., 2009).

The concentration of α-tocopherol of freeze-dried PVH CRF appeared to be unchanged after heated PVH (HPVH). Chun et al. (2006) and Lee et al. (2017) also found that heat treatment, such as blanching did not affect the vitamin E content in vegetables, such as broccoli and spinach. Conversely, thermal treating the juice (HJ) prior making CRF seemed to increase the concentration of α-tocopherol; perhaps this is also in part explained in part by heat-induced disabling of tocopherol oxidase that causes loss of tocopherol (Lee et al., 2017). This was supported with other studies that

reported heat treatment, particular heated juice retained higher β-carotene and α-tocopherol than untreated treatment of spinach and broccoli (Chun et al., 2006). In addition, heated PVH showed a 66% reduction of peroxidase (POD) activity, compared to that of untreated PVH. However, heated juiced appeared to show the deactivation of all POD activity, which was presumably due to the prolonged heat-up period, and so this heating may also knock the enzymes that catalyse the breakdown of carotenoids and vitamin E.

Heat treatment also have an effect of fatty acid retention in PVH CRF. The concentrations of linoleic acid and α-linolenic acid was 13% and 14% lower respectively in the heated PVH, compared to the non-heated PVH. These levels in the heated juice decreased by 2% and 14%, respectively, compared to CRF from fresh juice. Our result was supported by Torcello-Gomez et al. (2019). They found the fatty acid retention decreased considerably after blanching PVH prior to CRF preparation. This reduction can be explained by the phenomenon of heat-induced degradation of polyunsaturated fatty acids (PUFAs) that generates free radicals from thermal oxidation and affects the reduction of fatty acid content (Moya Moreno et al., 1999, Grootveld et al., 2001, Tao, 2015).

The ratio (w/w) of protein to lipid in chloroplast material is shown in **Table 6.1**. For comparison, we have included data from CRF made from spinach leaves. Most of the samples on **Table 6.1** show a protein:lipid ratio of 2:1; however the CRF from HJ has a ratio of 2.8:1. It is not clear why this should be; perhaps some cytoplasmic proteins drop out of solution due to heat-induced denaturation and are then recovered in the CRF pellet upon centrifugation. The protein content of all CRF in this study was calculated by the total amino acids. The concentrations of protein of CRF from HPVH and HJ were higher than CRF from untreated PVH. Presumably, heat-induced degradation of peptide bonds in the proteins result in the release of more amino acids

(Lee et al., 2017). The lowest carbohydrate content is found in the CRF material from heated juice suggesting that this juice experiences the highest/longest thermal exposure, this would be consistent with increased protein in the CRF material due to protein denaturation.

Overall, heat treatment prior to CRF preparation reduced the contents of chlorophyll, carbohydrate, fatty acid, and carotenoids. However, it depended on the methods of thermal treatment. In this result, it would appear that the heated juice (HJ) prior to CRF preparation gives the best outcome for carotenoids, vitamin E, omega-3 and omega-6 fatty acids compared to other treatments before *in vitro* digestion. However, PVH CRFs from FJ, HPVH and HJ were assessed how well the body would be able to absorb the nutrients from these materials under simulated *in vitro* digestion. The retention, bioaccessibility, and lipophilic nutrient available for uptake were further discussed in **Section 6.2.3.2 - 6.2.3.4**.

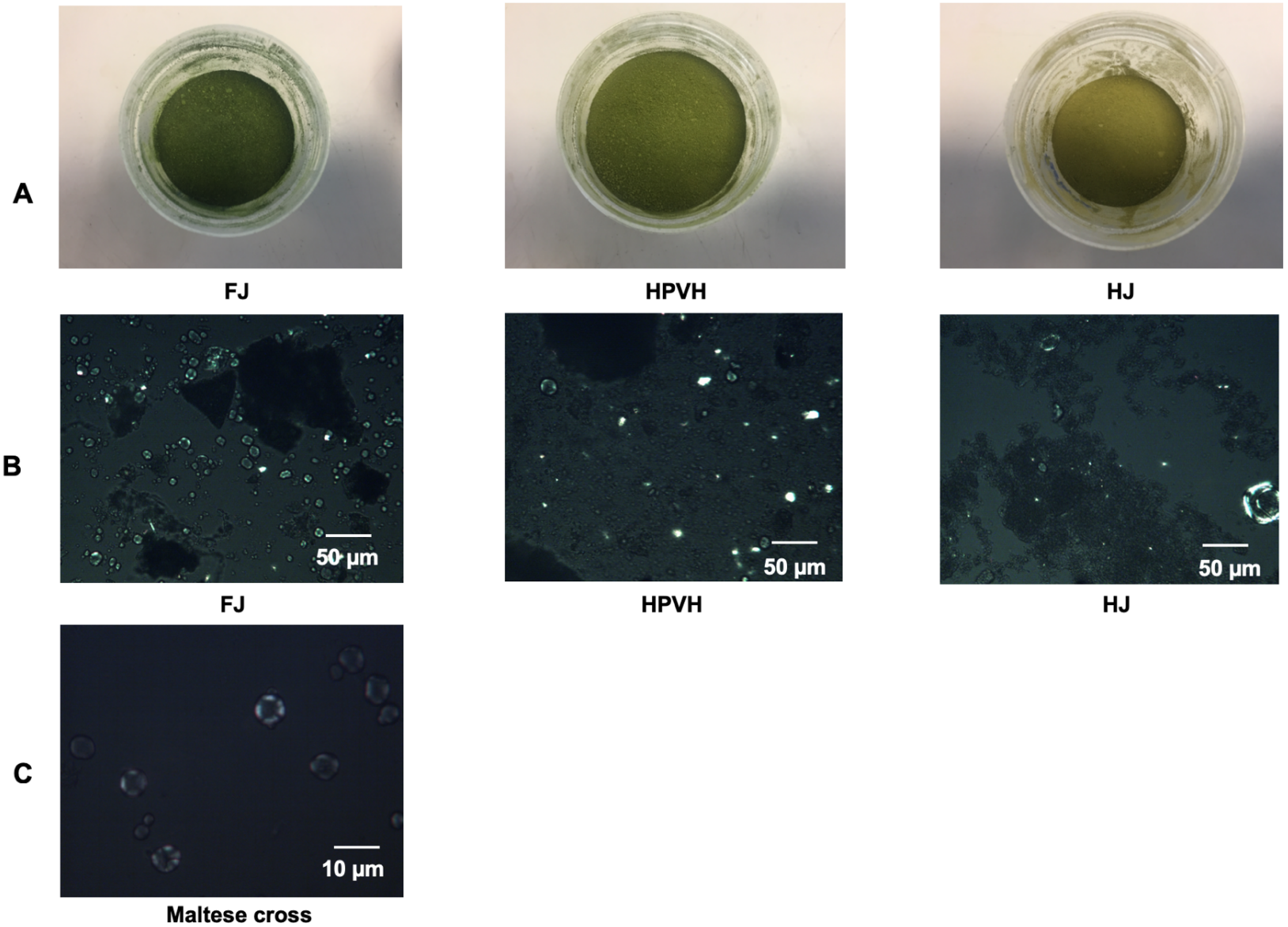


Figure 6.1: Physical characteristics of different freeze-dried CRF from PVH, including non-heat treated CRF (FJ), CRF obtained after heated PVH (HPVH) before juicing and CRF prepared after heated PVH juice (HJ); **A)** physical characteristic of freeze-dried CRF **B)** light microscope polarised image with dark field; **C)** close-up view of the Maltese cross. Maltese crosses can be observed in the non-heat treated CRF sample, which indicates the presence of native starch.

Table 6.1: Nutrient composition of CRF from PVH before digestion.

Nutrient		PVH CRF			Spinach CRF
		FJ	HPVH	HJ	
Protein	mg/g CRF, DW	293.27 ± 10.11^{d}	354.33 ± 10.36^{c}	448.32 ± 3.29^{b}	483.64 ± 6.66^{a}
Lipid	mg/g CRF, DW	150.82 ± 3.78^{b}	173.34 ± 31.23^{b}	160.13 ± 9.87^{b}	239.92 ± 13.12^{a}
Protein to Lipid ratio	ratio	1.94	2.04	2.80	2.01
Carbohydrate	mg/g CRF, DW	525.73 ± 0.14^{a}	391.11 ± 1.27^{b}	289.80 ± 0.62^{c}	
Ash	mg/g CRF, DW	30.18 ± 0.14^{c}	81.22 ± 1.27^{b}	101.75 ± 0.62^{a}	
Total chlorophyll	mg/g CRF, DW	15.99 ± 1.33^{a}	10.73 ± 0.73^{b}	10.44 ± 0.44^{b}	
	mg/g lipid, DW	106.01 ± 8.79^{a}	61.92 ± 4.47^{b}	65.21 ± 2.85^{b}	
All-*trans*-	mg/g CRF, DW	0.99 ± 0.06^{b}	0.74 ± 0.08^{c}	1.21 ± 0.07^{a}	
β-carotene	mg/g lipid, DW	6.53 ± 0.42^{a}	4.29 ± 0.45^{b}	7.55 ± 0.45^{a}	
Lutein	mg/g CRF, DW	4.81 ± 0.29^{a}	3.34 ± 0.39^{b}	4.62 ± 0.34^{a}	
	mg/g lipid, DW	31.88 ± 1.89^{a}	19.30 ± 2.28^{b}	28.83 ± 2.09^{a}	
α-Tocopherol	mg/g CRF, DW	0.29 ± 0.02^{b}	0.29 ± 0.02^{b}	0.45 ± 0.04^{a}	
	mg/g lipid, DW	1.94 ± 0.14^{b}	1.65 ± 0.14^{b}	2.82 ± 0.24^{a}	
Linoleic acid	mg/g CRF, DW	9.03 ± 0.43^{a}	7.88 ± 1.18^{a}	8.85 ± 0.32^{a}	
	mg/g lipid, DW	59.86 ± 2.85^{a}	45.47 ± 6.83^{b}	55.30 ± 1.99^{ab}	
α-Linolenic acid	mg/g CRF, DW	16.70 ± 0.89^{a}	14.29 ± 2.06^{a}	14.89 ± 0.47^{a}	
	mg/g lipid, DW	110.75 ± 5.91^{a}	82.44 ± 11.87^{b}	93.00 ± 2.93^{ab}	

FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; Data were presented as a mean ± SD of 3 separated samples and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at p<0.0.5, a>b; PVH was collected in 2017.

6.2.2. Visual appearance of FJ CRF, HPVH CRF, and HJ CRF before, during and after digestion

The PVH CRF powders produced by freeze-drying are illustrated in **Figure 6.1A**. Thermal processing induces the structural and chemical variations to the tissues of plants that often result in colour changes. The freeze-dried CRF from PVH that had not been heat-treated was a darker green powder than CRF from HPVH or HJ. During exposure to heat, the chlorophyll pigment could lose its bound magnesium and be chemically changed to pheophytin and other derivatives, such as chlorophyllide and pheophorbide (Schwartz and Elbe, 1983). This observation was supported by the chlorophyll concentration results in **Table 6.1**, with higher levels in CRF from FJ (15.99 ± 1.33 mg/g CRF, DW) versus CRF from HPVH (10.73 ± 0.78 mg/g CRF, DW) and HJ (10.44 ± 0.44 mg/g CRF, DW). Our result was also consistent with the study of Erge et al. (2009), which studied the effect of heat treatment on chlorophyll degradation and colour loss in green peas. They found that increasing temperature during thermal processing decreased chlorophyll a and b content and intensity of green colour in green peas. Thus, this confirmed that heat treatments, both heated PVH and heated juice in PVH CRF affected the loss of chlorophyll pigment.

The bioaccessibility of micronutrients from these three CRF powders was measured by using an *in vitro* digestion model. Initially, water (4.5 mL) was added to CRF powder (0.5 g); in all three cases, the water penetrated the powder to some extent, but complete dispersion of the particulate material did not occur (**Figure 6.2**). Over the digestion period, residual CRF material can be seen sticking on the wall of the glass bottle; this material was re-suspended into the incubation mixture at the start of each new phase (oral, gastric or intestinal) by inverting the bottles, but some of the particulate material remained stuck to the glass (see **Figure 6.2**). This is an indirect indicator of the degree of CRF material dispersion in the digestion mixture. It was

expected that the solubility of CRF might be low in the aqueous phases during digestion due to the reason that CRF was prepared from the pellet of centrifugation. A similar trend was observed by Gedi (2017). He found that during *in vitro* digestion, the freeze-dried CRF from spinach leaves showed clumping and some CRF materials also stacked on the bottle wall. Mansor (2019) also studied the *in vitro* digestion of spinach leaves and compared the effect of water solubility in spray-dried spinach juice powder and freeze-dried spinach CRF. She also found some of the particulates of freeze-dried spinach CRF remained stuck to the glass like our result and Gedi's result. However, she did not find this phenomenon in spray-dried spinach juice. It was due to the different sample preparation and water solubility of both treatments. The CRF was prepared by centrifugation from the juice and collected only the pellet (CRF). Then, it was further freeze-dried to remove the water. While spinach powder juice preparation, the juice was direct to spray-dried to remove the water. In addition, Mansor (2019) reported that the water solubility index (WSI) in spinach juice (80%) was higher than CRF (38%). Thus, these assumptions might be the reasons why the CRF showed clumping and stacked on the wall of glass during the digestion.

However, it could be deduced that the dispersion of the CRF material in the digestion mixture is enhanced if the PVH or its juice is heat treated, or if rapeseed oil is added to the incubation mixture (see **Figure 6.2**). It was expected that thermal processing prior to CRF preparation and the presence of oil could increase the solubility in the aqueous phase of enzymes and solutions during *in vitro* digestion. This was supported by our result, which found that the water solubility index of heated samples (HPVH (20%) and HJ (20%)) was a higher value than that of a non-heated sample (FJ (15%)). Thus, it might be possible that the heated samples helped to solubilise the aqueous phase during digestion. It has reported that adding some oil helped to dissolve the hydrophobic compounds in an aqueous phase by the formation of micelle (Hanafy

et al., 2018). Thus, it might be possible that the addition of rapeseed oil helped to increase the solubility of PVH CRF in the aqueous phase during *in vitro* digestion compared to the sample without oil. Hence, these assumptions might be the reasons why the presence of oil in CRF from non-heat (FJ), heated PVH (HPVH) and juice (HJ) did not show clumping and stick on the wall of glass bottle when compared to the PVH CRF without heated sample and added oil.

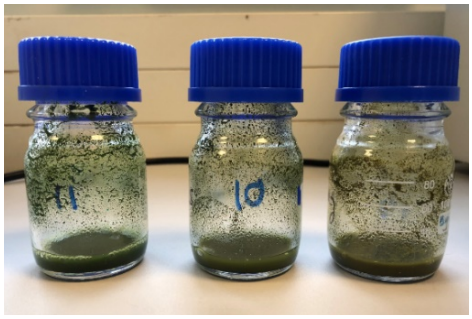
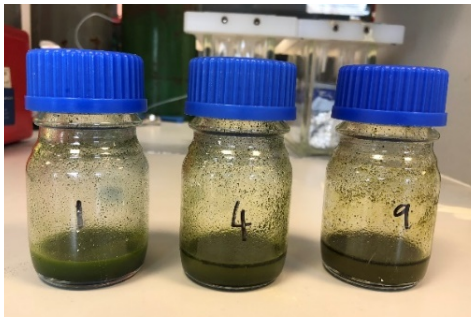
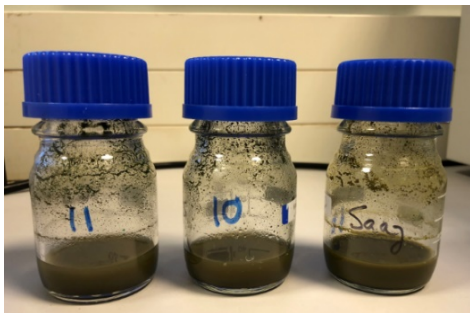
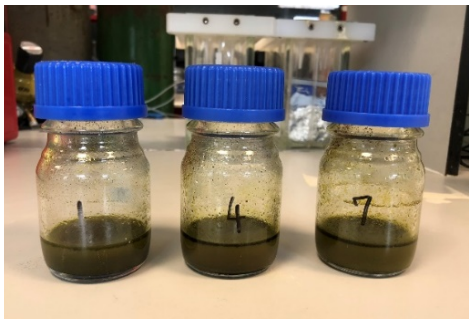
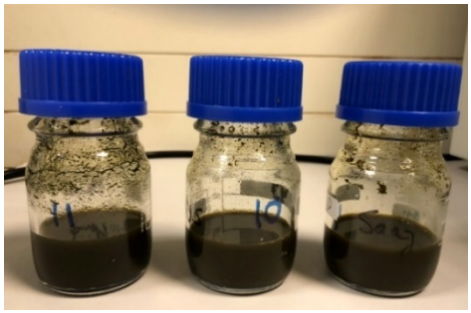
	Heat treatment	Heat treatment + oil
Before digestion	 FJ HPVH HJ	 FJ + oil HPVH + oil HJ + oil
Oral phase	 FJ HPVH HJ	 FJ + oil HPVH + oil HJ + oil
Gastric phase	 FJ HPVH HJ	 FJ + oil HPVH + oil HJ + oil
Intestinal phase	 FJ HPVH HJ	 FJ + oil HPVH + oil HJ + oil

Figure 6.2: Before and after digestion of CRF from PVH in each stage, including oral, gastric and intestinal phases; FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; oil: rapeseed oil.

6.2.3. Bioaccessibiity of lipophilic Nutrients from FJ CRF, HPVH CRF and HJ CRF

In vitro incubations were set up to mimic gastric followed by intestinal human digestion. Bioaccessibility is a measure of the release of nutrients form a matrix, it is therefore necessary to establish loss of nutrients during incubation as this can affect the measurement of bioaccessiblity as described further in **Section 6.2.3.2 - 6.2.3.4**.

The microscope images (**Figure 6.3**) were taken to show the impact of *in vitro* digestion on the different treatment of PVH CRF at each digestion phase. Qualitative data from the microscope revealed that the size of chloroplast and starch granule in freeze-dried PVH CRF gradually decreased over time during four hours of *in vitro* digestion (**Figure 6.3**). In all samples, the reduction of the number of chloroplast and starch granule was due to the dilution effect and addition of enzymes in each stage; the volume of the sample started at 5 mL and increased to 40 mL over the course of the process according to the addition of simulated electrolyte solutions (SSF, SGF, and SIF) and enzymes (α-amylase, pepsin, and pancreatin) during *in vitro* digestion.

The microscope images in the oral phase of the freeze-dried PVH CRF from HPVH and HJ showed a smaller size of chloroplast and a lower amount of starch granule compared to PVH CRF from FJ due to gelatinisation of starch during heat treatment as described earlier in **Section 6.2.1**. In the gastric phase, the starch granules in CRF from FJ were still present although they were partially digested by α-amylase in the oral phase. At the same time, the starch granules of PVH CRF from HPVH and HJ were hardly seen in this phase due to the lower content of carbohydrate in both materials. At the end of the phase, all the CRFs were digested by pancreatic enzymes and bile salt to small particles. Similar trends of structure reduction in spinach CRF and spinach juice powder during digestion were observed by Gedi (2017) and (Mansor, 2019). However, the starch granule was hardly seen in spinach due to the

low amount of starch as described in **Chapter IV**. All samples of PVH CRF were further determined the lipophilic nutrient retention, bioaccessibility and nutrient available for uptake in **Section 6.2.3.2 - 6.2.3.4**.

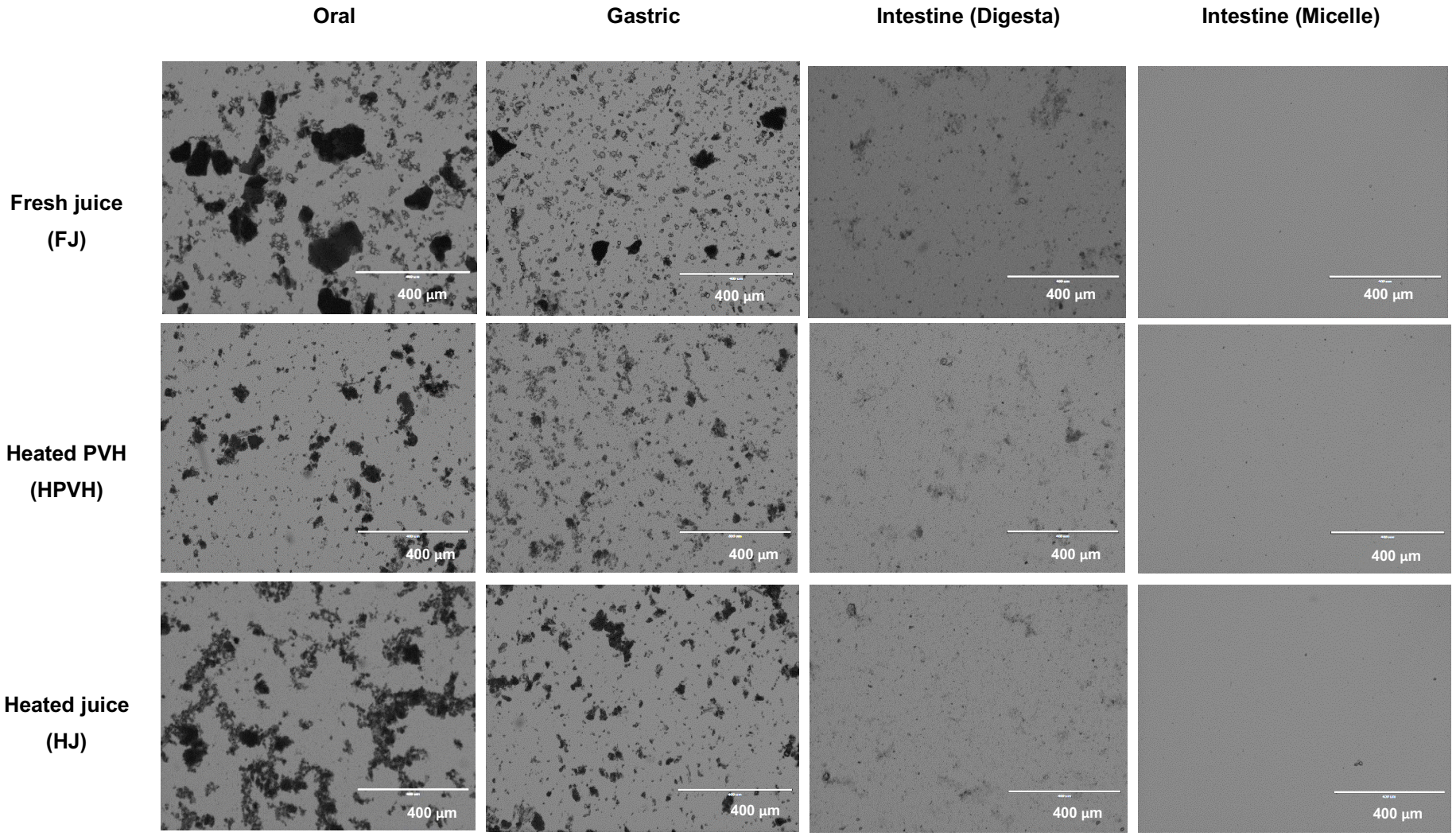


Figure 6.3: Microscope images of CRF with different treatments, including fresh juice (FJ), heated PVH (HPVH) and heated juice (HJ) at different stages: oral (2 min), gastric (2 hr), and intestine (2 hr).

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

A small peak of *cis*-β-carotene in the HPLC chromatogram was present in the heat-treated samples (HPVH and HJ) before the *in vitro* digestion, but it was lost in the non-treated sample as shown in **Figure 6.4**. It can be explained that using the thermal treatment, both heated PVH biomass and heated juice prior to CRF preparation increases the amount of *cis*-β-carotene and decrease *trans*-β-carotene. Similar results were observed by Chandler and Schwartz (1988), Marx et al. (2003), and Lemmens et al. (2010). They studied the effect of thermal processing on the stability of β-carotene in sweet potato, carrot puree and carrot juice. They found that heat promoted changes in the stability of β-carotene of these materials resulting in the conversion into its *cis*-isomers as shown in **Figure 2.10**. Generally, all-*trans*-β-carotene is unstable and can be easily isomerised to heat, light, and acid. Isomerisation energy is involved in the relocation of the single or double bond of one form of carotenoids (Khoo et al., 2011).

After *in vitro* digestion, there was an increasing amount of *cis*-β-carotene (9-*cis* and 13-*cis* forms) in CRF both non-heat (FJ) and heat-treated samples (HPVH and HJ), compared to before the digestion as shown in **Figure 6.4**. The isomerisation of *trans* to *cis*-β-carotene in PVH CRF therefore occurred also during the *in vitro* digestion. Our finding was supported by other studies (Ferruzzi et al., 2006, Mansor, 2019). They also found that there was an increase in the peak of 9-*cis*-β-carotene and 13-*cis*-β-carotene during *in vitro* digestion of spinach powder and test meal compared to before digestion. Moreover, *cis*-β-carotene is reported to be easier to micellarise than all-*trans*-β-carotene during simulated digestion; both isomers are transferred across the brush-border surface of the enterocyte from mixed micelles and absorbed in human similarly by passive diffusion (Ferruzzi et al., 2006). However, α-carotene and *cis*-β-carotene had less than half activity of *trans*-β-carotene (Nagao and Olson, 1994).

These suggested that either heat treatments (i.e. blanching, pasteurisation, and sterilisation) or *in vitro* digestion have an effect on the stability of *trans*-β-carotene. In this work, the study of bioaccessibility of β-carotene from freeze-dried PVH CRF was reported only in terms of all-*trans*-isomer due to the limitation of the standard of *cis*-β-carotene.

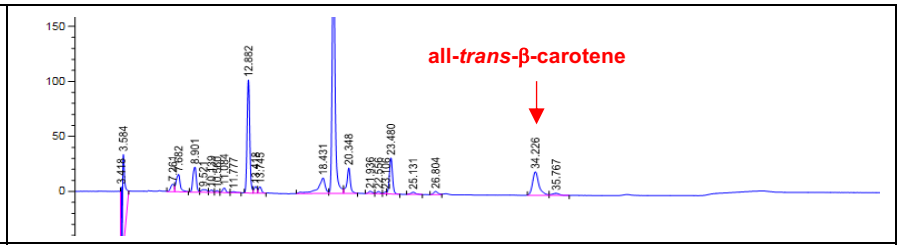
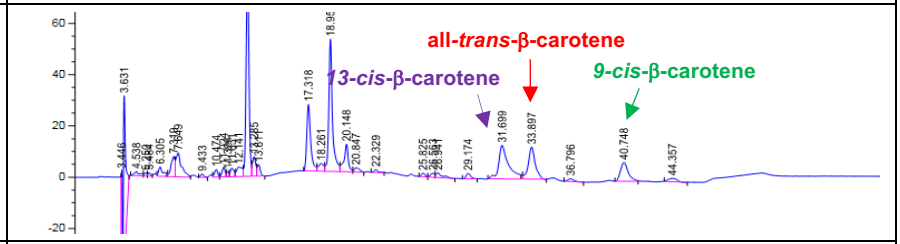
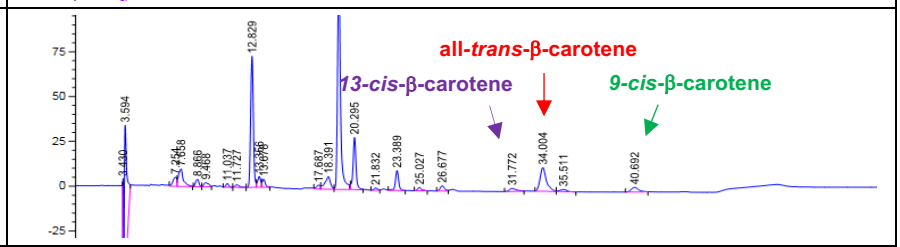
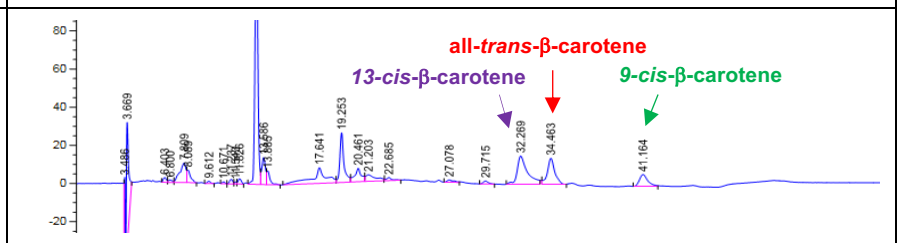
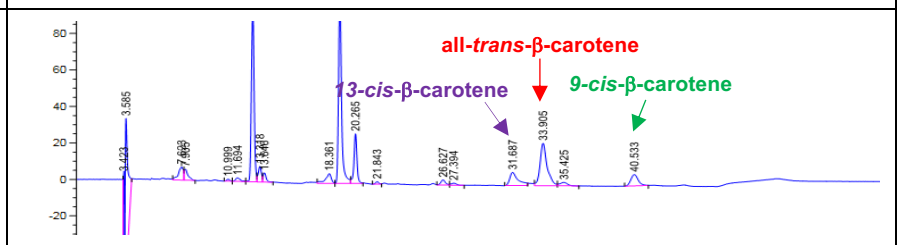
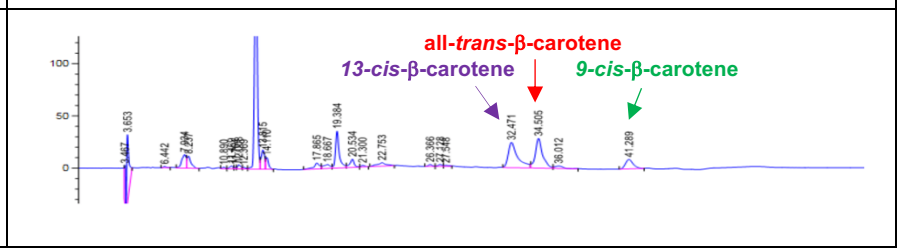
Before digestion	**Fresh juice (FJ)**	
After digestion	**Fresh juice (FJ)**	
Before digestion	**Heated PVH (HPVH)**	
After digestion	**Heated PVH (HPVH)**	
Before digestion	**Heated juice (HJ)**	
After digestion	**Heated juice (HJ)**	

Figure 6.4: Chromatogram of β-carotene of CRF from PVH both before and after digestion. The scale of chromatogram before and after digestion is not consistent and the size of the peak should not be compared directly to each other due to a difference in dilution factor.

6.2.3.2. Nutrient retention

In this work, nutrient retention is defined as the ratio of nutrient concentration in the whole digesta (at the end of the digestion process) to the initial nutrient concentration in the CRF. It reflects the stability of lipophilic nutrients.

Many researchers reported the retention of β-carotene after *in vitro* digestion was 80-90% in broccoli, spinach, lettuce, red pepper, and loquat (Granado-Lorencio et al., 2007) and 65-91% in orange flesh sweet potato (Failla et al., 2009), 71% in spinach (Chitchumroonchokchai et al., 2004), and 1-20% in carrot, tomato, papaya, and mango (Schweiggert et al., 2012). Our study found that PVH CRF from FJ remained only 23% of β-carotene after the *in vitro* digestion compared to initial β-carotene. In addition, many publications reported the lutein retention after *in vitro* digestion was 74-76% in spinach (Chitchumroonchokchai et al., 2004, Courraud et al., 2013) and 23% in carrot (Schweiggert et al., 2012). While our result showed that PVH CRF from FJ remained only 31% of lutein compared to lutein before digestion. The decrease in the amount of β-carotene and lutein after *in vitro* digestion might occur due to their isomerisation/or oxidation within the simulated GI conditions, particularly within the highly acidic environment of the stomach (Failla et al., 2014). This opinion was supported with other studies (Ferruzzi et al., 2006, Mansor, 2019). They found that there was an increase of *cis*-isomers and decrease of *trans*-isomers of carotenoids in spinach powder and test meal during *in vitro* digestion. Moreover, the activation of enzymes during the digestion might induce carotenoid degradation (Davison et al., 2002).

To improve the stability of carotenoid in PVH CRF during the digestion, it was expected that using thermal treatment prior to CRF preparation and the presence of oil could increase the retention of carotenoid during *in vitro* digestion. Our results showed that the nutrient retention of all-*trans*-β-carotene after digestion was improved

by heat-treatment upon CRF preparation and was in the presence of rapeseed oil. The highest retention of all-*trans*-β-carotene was 90% with HJ and oil, followed by HPVH and oil (64%), and FJ with oil (49%), HPVH (46%), HJ (43%), and FJ (23%) (**Figure 6.5A**). From this result, the PVH CRF, which used heat treatment (HPVH and HJ), retained β-carotene more than the PVH CRF without heat treatment (FJ). This was supported by Hedrén et al. (2002). They found that the β-carotene content in cooked carrot (27%) was higher retention than that of raw carrot (21%) after digestion. It was possibly due to heat-induced disabling of enzymes that cause the degradation of carotenoids. It was supported by Koca et al. (2007), who reported that β-carotene in blanched carrot was higher content than unblanched carrot. Also, thermal treatment might bring more efficient release of carotenoids from the food matrix and induced disruption of carotenoid-protein complex. Hence, the digestive enzymes might work more efficiently to carotenoids (Hedrén et al., 2002, Mulokozi et al., 2004).

The presence of oil in PVH CRF increased the retention of β-carotene during digestion compared to the absence of oil. A similar result was observed by Mansor (2019). She found that the addition of oil in spray-dried spinach powder improved the stability of β-carotene during digestion. One can assume that rapeseed oil may be hydrolysed during the *in vitro* digestion and thus generates lipolysis products, which are amphiphiles. They contribute to the formation of mixed micelles under intestinal conditions (Rodriguez-Amaya, 2015). Therefore, it might help to solubilise and protect β-carotene during digestion.

Moreover, the combination of heat treatment and the presence of oil in freeze-dried PVH CRF helped to retain the β-carotene more than PVH CRF without heat treatment and the presence of oil. This was consistent with other published works (Hornero-Méndez and Mínguez-Mosquera, 2007, Bengtsson et al., 2009). Bengtsson et al. (2009) reported that adding 2.5% fat in heated-process fleshed sweet potato

helped improve the stability of β-carotene after digestion. A similar trend with Hornero-Méndez and Mínguez-Mosquera (2007), they found that inclusion of 5% of oil in cooked carrot retained β-carotene after digestion more than uncooked carrot without oil.

On the other hand, after the digestion, lutein retention was the highest in digestions containing CRF material from HPVH (89%), followed by HJ (83%), and the addition of oil did improve retention (74% for the combination of HJ and oil and 80% for the combination of HPVH and oil). Lutein retention with FJ was 36% and 31% with and without oil, respectively (**Figure 6.5B**). Our result showed that heat treatments, either HPVH or HJ, had the same trends with the result of β-carotene as described above. Both heat treatments helped to improve the lutein retention in PVH CRF after digestion compared to PVH CRF without heat treatment (FJ). It was agreed with several researchers, who have studied the effect of different cooking methods on the stability of lutein during digestion. Ryan et al. (2008) reported that the lutein content in courgette and tomato showed higher retention after *in vitro* digestion when applying thermal treatments, such as grilling, boiling, and steaming. A similar trend was observed by Courraud et al. (2013). They found that lutein in cooked spinach was more stable compared to raw spinach after digestion. It was thought to be due to the reason that heating was more efficient release of carotenoids by disruption carotenoid-protein complex (Hedrén et al., 2002, Mulokozi et al., 2004) and inactivation destructive enzymes of carotenoids, which might occur during digestion (as described earlier).

However, the presence of oil in PVH CRF did not affect lutein stability after digestion. Our result was agreed with the result of Mansor (2019). She found that the inclusion of rapeseed oil in spinach CRF did not improve the stability of lutein during digestion. However, it was not clear at this stage why this should be. In the current work, lutein in PVH CRF was more stable than β-carotene after digestion. It was correlated with other published research. It has reported that the stability of lutein was

higher than β-carotene in carrot juice (Schweiggert et al., 2012), tomato paste, kiwi (Granado-Lorencio et al., 2007), and spinach (Chitchumroonchokchai et al., 2004).

α-Tocopherol was the lowest amount of retention compared to other lipophilic nutrients. The decrease in the amount of α-tocopherol may have occurred due to their oxidation within the simulated GI conditions. The retention of α-tocopherol after the digestion was the highest in the system that combined CRF from HJ and oil (52%), followed by the combination of HPVH and oil (43%), HJ (36%), HPVH (31%), FJ with oil (9%) and FJ without oil (5%) as shown in **Figure 6.5C**. The inclusion of heat treatment and oil in PVH CRF seemed to help the stability of α-tocopherol during the digestion. In addition, heat treatment in PVH CRF enhanced the stability of α-tocopherol. It was thought due to more efficient release of vitamin E from plastoglobule by disruption cell membrane within the chloroplast. However, the presence of oil in PVH CRF did not help increase the stability of α-tocopherol during the digestion.

Essential fatty acid showed a lower retention (60-70%) in the absence of heat treatment and the highest retention was in the CRF material from heated juice (80% in linoleic acid and 75% in α-linolenic acid), followed by heated PVH (76% in linoleic acid and 75% in α-linolenic acid) and no heat treatment (69% in linoleic acid and 58% in α-linolenic acid) as shown in **Figure 6.5D** and **6.5E**. These data on essential fatty acids retention may not reflect what happens during the *in vitro* digestion. Indeed, with the non-heat treated CRF materials, polyunsaturated fatty acids mainly present in CRF galactolipids can be released by endogenous lipolytic enzymes prior to digestion (Wattanakul et al., 2019) and they may be further broken down due to oxidation. However, heated PVH prior to CRF preparation increased the stability of α-linolenic acid during digestion. It was possible due to heat-induced disabling enzyme of degradation of fatty acid during digestion (Sahaka et al., 2020). According to the

limitation of the sample in the experiment, this study was a lack of the study of the retention of omega-3 and omega-6 fatty acids in freeze-dried PVH, which included oil during *in vitro* digestion. The sample of PVH CRF included oil was not enough to run GC-MS. However, this is considered to further study in the future.

Overall, using heat treatment during the production of CRF material from PVH helped to improve nutrient retention significantly *($p<0.05$)* during digestion when compared with no heat treatment; this may be due to heat-induced inactivation of endogenous enzymes. The inclusion of oil appears to protect the lipophilic nutrients only β-carotene, but less so in the case of lutein and α-tocopherol, more hydrophilic compounds than β-carotene; this could be explained by their partitioning into a hydrophobic phase that protects the nutrients from enzyme action.

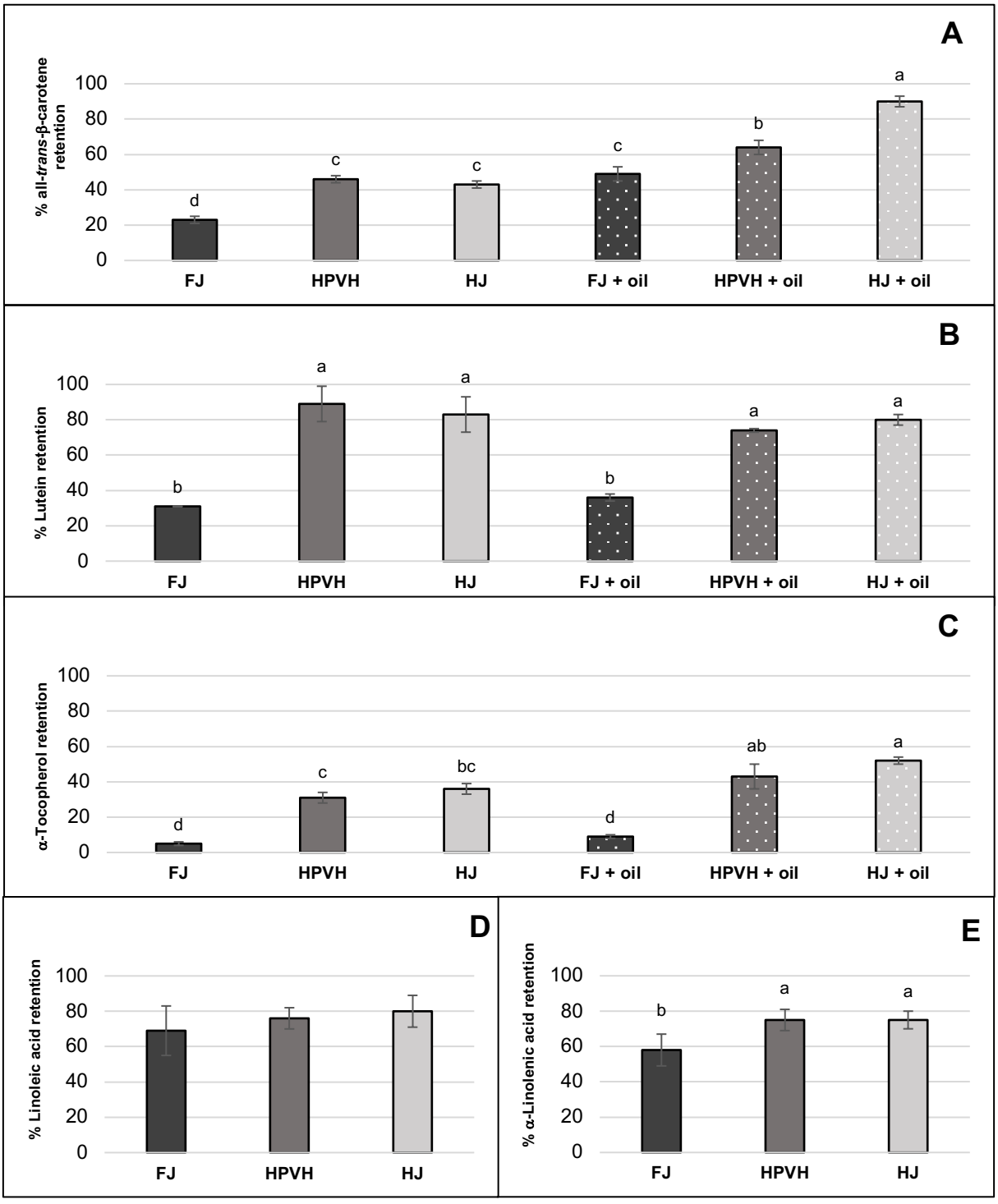


Figure 6.5: Effect of heat treatment and the presence of oil on nutrient retention after *in vitro* digestion in CRF from PVH **A)** all-*trans*-β-carotene retention **B)** lutein **C)** α-tocopherol **D)** linoleic acid **E)** α-linolenic acid; FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; Data were presented as a mean ± SD of 3 separated *in vitro* digestion and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at p<0.0.5, a>b.

6.2.3.3. Nutrient bioaccessibility (micellar solubilisation)

'Bioaccessibility' or 'micellar solubilisation' are defined as the percentage of nutrient present in the whole digesta that is dispersed in solution, and thus presumably micellarised and available for intestinal absorption. Our result showed that the bioaccessibility (micellar solubilisation) of β-carotene was the highest when oil was included in-digesta (during simulation of human digestion) and when CRF material was derived from heat-treated materials: highest bioaccessibility values were 54% for oil and HPVH and 41% for oil and HJ, followed by FJ with oil (28%), HPVH (13%), HJ (11%) and FJ without oil (7%), (**Figure 6.6A**).

The presence of oil in PVH CRF helped to improve the micellarisation of β-carotene. Our result was supported by other researchers. They reported that adding fat or oil (i.e. olive oil, soybean oil, rapeseed oil, palm oil, beef tallow, and lard) increased the bioaccessibility of β-carotene in carrot (Hornero-Méndez and Mínguez-Mosquera, 2007, Nagao et al., 2013), spinach, komatsuna, pumpkin (Nagao et al., 2013), amaranth, cowpea, sweet potato, and pumpkin (Mulokozi et al., 2004). It can be explained that incorporating lipid into the system or lipolysis of fat and oil generate lipolysis products (i.e. free fatty acids and monoglycerides) contribute to the solubilisation of carotenoids during the transfer of gastric chyme to the duodenum (Rodriguez-Amaya, 2015). Also, lipid acts as stimulants for the secretion of pancreatic enzymes and bile salts, facilitating micelle formation (Rodriguez-Amaya, 2015). This process would be similar to what is observed with lipid-based formulations (LBF) for the oral delivery of poorly water-soluble drugs (Carriere, 2016). Moreover, the hydrophobic carotenoids (i.e. β-carotene) have a higher propensity to be localised in the bulk oil phase, and they will require the production of water-soluble lipids in order to be transferred to the micelles (Borel et al., 1996).

Apart from the addition of oil, applying heat treatment to PVH prior to CRF

preparation also increased the solubility of β-carotene in micellar phase. Thus, the micellarisation of β-carotene in the sample via thermal treatment (HPVH and HJ) was higher than that of the sample without thermal treatment (FJ). Similar results were observed by other researchers. They found that the applying thermal treatment (i.e. boiling, steaming, pressure-cooking, stir-frying, baking, or deep frying) enhanced the bioaccessibility of β-carotene in spinach, carrot, courgette, red pepper, tomato, pumpkin, amaranth, fenugreek leaves, and orange fleshed sweet potato (Reboul et al., 2006, Veda et al., 2006, Hornero-Méndez and Mínguez-Mosquera, 2007, Tumuhimbise et al., 2009, Courraud et al., 2013). It was due to the reason that thermal treatment separated carotenoids from protein-carotenoid complex found in the membrane of chloroplasts. Thus, the carotenoids transferred more easily to micelles (Rock et al., 1998, Clevidence et al., 2000, Ryan et al., 2008, Tumuhimbise et al., 2009).

In addition, the inclusion of heat treatment and the presence of oil in PVH CRF was the highest bioaccessibility of β-carotene compared to the other treatments. This result was consistent with other researchers (Hornero-Méndez and Mínguez-Mosquera, 2007, Bengtsson et al., 2009). Hornero-Méndez and Mínguez-Mosquera (2007) reported that the cooked carrots containing 10% of olive oil were the highest micellarisation of β-carotene compared to raw carrot and cooked carrot without adding oil. Bengtsson et al. (2009) discovered that the presence of 2.5% sunflower oil in heated-processed orange-flesh sweet potato by boiling, steaming, and microwave heating was more bioaccessible of all-*trans*-β-carotene in micellar phase than that of non-heated sweet potato with and without oil. It can be explained that the combination of the thermal treatment and addition of oil greatly enhanced the release of lipophilic nutrients and their co-transport, with lipophilic compounds, into micelles.

Similarly, the bioaccessibility of lutein was the highest in the combination of oil and CRF from HPVH (66%), followed by combination of oil and CRF from heated

juice (45%), then FJ with oil (41%), FJ without oil (41%), HJ (34%), and HPVH (30%), respectively (**Figure 6.6B**). Surprisingly, the presence of rapeseed oil alone did not improve the bioaccessibility of lutein in PVH CRF. A similar trend was observed by Nagao et al. (2013). They reported that there was no significant difference (p>0.05) of the bioaccessibility of lutein between the presence and absence of soybean oil in spinach, komatsuna, pumpkin and carrot. Huo et al. (2007) also observed that the addition of canola oil in a salad meal did not help to improve the micellarisation of lutein. It can be explained that more amphiphilic carotenoids (i.e. lutein) have a higher propensity to be localised at the oil/aqueous phase interface, and they could spontaneously transfer to micelles without lipid (Borel et al., 1996). However, Courraud et al. (2013) found that the inclusion of rapeseed oil in carrot juice increased the micellar solubilisation of lutein compared to the exclusion of rapeseed oil. Gedi (2017) and Mansor (2019) also found that the addition of olive oil and rapeseed oil in spinach CRF and whole leaf spinach enhanced the bioaccessibility of lutein. It might be due to the different plants and the different types of oil. The type of lipid was found to influence on carotenoid bioaccessibility. For example, the addition of red palm oil in cowpea, sweet potato, and pumpkin increased twice carotenoid bioaccessibility than that of sunflower oil (Hedrén et al., 2002).

As it was discussed earlier in the result of bioaccessibility of β-carotene, heat treatment helped to improve the bioaccessibility of carotenoids due to the disruption of carotenoid-protein complex in chloroplasts. It has also reported that using thermal treatments (i.e. boiling, steaming, and grilling) enhanced the bioaccessibility of lutein in spinach and courgette (Reboul et al., 2006, Ryan et al., 2008, Courraud et al., 2013). However, our result was not consistent with those studies. It found that using the thermal treatment, either HPVH or HJ, did not improve the bioaccessibility of lutein in PVH CRF. It might be due to the reason that the duration and temperature of heated

PVH or juice might not be enough to break the bond of lutein bound in chloroplasts of PVH. Also, lutein was higher activation energy to degradation than β-carotene as described previously (see **Table 5.5**). Thus, the release of lutein in heat treatment was not different compared to PVH without heat treatment. Neither the presence of oil nor heat treatment did not improve the micellarisation of lutein in PVH CRF. Surprisingly, the combination of oil and thermal treatment only HPVH enhanced the bioaccessibility of lutein in PVH CRF. It was not clear at this stage why this should be.

Our result found that lutein in PVH CRF was a greater bioaccessibility than all-*trans*-β-carotene. It was agreed with other published works. They have reported that lutein in carrot juice and spinach was also more bioaccessible in micellar phase than β-carotene (Chitchumroonchokchai et al., 2004, Courraud et al., 2013, Gedi, 2017, Mansor, 2019). Xanthophylls (i.e.lutein) are more efficiently released by digestion and thus more bioaccessible than carotene (i.e. α-carotene, β-carotene, and lycopene) (Granado-Lorencio et al., 2007), probably due to the lower hydrophobicity of xanthophyll. In addition, oxygenated carotenoids (i.e. lutein) could be incorporated more easily into micelles within the GI tract due to some amphiphilicity. It has reported that compounds containing hydroxyl group are preferentially absorbed in the surface of lipid droplets and more easily facilitate micelle formation (Borel et al., 1996, Werner and Böhm, 2011). Thus, lutein would be more bioaccessible compound than β-carotene.

Although there were a few published works on the bioaccessibility of α-tocopherol, Reboul et al. (2006) reported that its bioaccessibility was highly variable between foods. They found that lettuce and banana had high bioaccessibility (98-100%), while almond, hazelnut, and apple had low bioaccessibility of α-tocopherol (0.5-14%). Nagao et al. (2013) also reported that the bioaccessibility of α-tocopherol of spinach, komatsuna, pumpkin, and carrot was around 50-90%. However, our result found only 25% of α-tocopherol bioaccessibility in PVH CRF. It might be due to the

presence of pectin in our sample. Al-Yafeai and Böhm (2018) reported that pectin has a major effect on the bioaccessibility of vitamin E by increasing the viscosity of intestinal content. Hence, the absorption of vitamin E was reduced due to lower activity of enzymes and increased difficulty in contacting intestinal enterocyte. The bioaccessibility of α-tocopherol in PVH CRF was the highest in the system that combined oil and CRF from heated juice (51%), and in the system that contained oil and CRF from heated PVH (45%), followed by HPVH (35%), FJ with oil (33%), HJ (28%), and FJ without oil (25%) as shown in **Figure 6.6C**. Heat treatments, both HPVH and HJ in PVH CRF, enhanced the bioaccessibility of α-tocopherol compared to PVH CRF without heat treatment (FJ). A similar trend was observed by Cilla et al. (2012). They observed that heat at 90°C for 30 seconds increased α-tocopherol bioaccessibility in milk and soy-based fruit beverage. These results indicated that the thermal treatment applied strongly affects α-tocopherol bioaccessibility compared to the condition without thermal treatment. It was possible that thermal treatment could affect the food matrix, modifying the location of tocopherols, changing their physicochemical state, or altering the amount pf absorption effectors (i.e. fibres, fats, and phytosterols) (Reboul et al., 2006, Cilla et al., 2012). Hence, this compound was more available for incorporation to the micelle after *in vitro* digestion (Reboul et al., 2006, Cilla et al., 2012). In addition, heat treatment might disrupt the chloroplast membrane and enhance the release of plastoglobule (source of vitamin E) to form micelle during digestion. This opinion was supported by Gounaris et al. (1983), who reported that heating induced separation of chloroplast membrane. Also, the heat might inactivate the destructive enzymes of α-tocopherol (Lee et al., 2017).

However, the presence of rapeseed oil in PVH CRF did not significantly affect the bioaccessibility of α-tocopherol. A similar result was observed by Nagao et al. (2013), who reported that the addition of soybean oil in spinach, komatsuna,

pumpkin, and carrot did not improve the bioaccessibility of α-tocopherol compared to the absence of oil. α-Tocopherol was thought to be located at the surface of the oil droplets (Hedrén et al., 2002). Thus, it could spontaneously transfer to micelles without lipid. Studies on the bioavailability of various pharmaceutical formulations of vitamin E have however shown that the plasma concentration of vitamin E can be increased following the oral administration of vitamin E in the form of a self-emulsifying preparation compared to a formulation in pure soybean oil (Julianto et al., 2000). In addition, it was expected that the amount of vitamin E solubilised within micelle phase depended on carrier type of oil and molecular form of vitamin E. Yang and McClements (2013) discovered that the bioaccessibility of vitamin E was around 17% when medium-chain TAG was used as the carrier oil type, but around 39% when long-chain TAG was used. Even though the inclusion of oil in PVH CRF did not help to improve the bioaccessibility of α-tocopherol, the combination of oil and heat treatment enhanced the bioaccessibility of α-tocopherol. It might be the effect of the thermal treatment on enhancing α-tocopherol solubility in micellar phase as describe earlier.

Short-chain and medium-chain fatty acids are absorbed directly by the intestinal mucosa. Long-chain fatty acids, such as linoleic acid (18:2) and α-linolenic acid (18:3) (Cholewski et al., 2018) have to be formed into mixed micelles to be transported for the absorption (Ayala-Bribiesca et al., 2017). The bioaccessibility of essential fatty acids present in chloroplasts was only measured in a system without oil due to the limitation of the sample as described in **Section 6.2.3.2**. For both linoleic and α-linolenic acids, the bioaccessibility was the highest in CRF from HPVH (59% and 62%, respectively); this was followed by the other two systems that were not significantly different ($p>0.05$) to each other (**Figure 6.6D** and **6.6E**). The bioaccessibility of α-linolenic acid in freeze-dried PVH CRF (59%) was higher than that of freeze-dried brown seaweed (*Fucus spiralis*) (11%) (Francisco et al., 2020).

It was due to the reason that the presence of high fibre content in its cell wall of seaweed. The high fibre decreased the bioaccessibility of fatty acid. Meanwhile, freeze-dried PVH CRF was liberated cell wall from the plant cell. Hence, the fatty acid in PVH CRF was more bioaccessible than seaweed (*F.Spiralis*). In addition, the bioaccessibility of linoleic acid in freeze-dried PVH CRF (57%) was higher than almond (10%) (Swackhamer et al., 2019). It was due to the presence of the cell wall in the almond seed, which reduced lipid bioaccessibility by hindering the release of lipid available for digestion (Ellis et al., 2004). Meanwhile, PVH CRF was removed cell wall. It was thus possible that absence of cell wall increased the bioaccessibility of fatty acid. Our result revealed that the degree of unsaturated fatty acid did not have an effect on the bioaccessibility of fatty acid in PVH CRF. The bioaccessibilty of C18:2 and C18:3 fatty acids in PVH CRF was 57% and 59%, respectively. A similar trend was observed by Swackhamer et al. (2019). They found there was not a significant difference between the bioaccessibility of C18:1 and C18:2 fatty acids in almond powder.

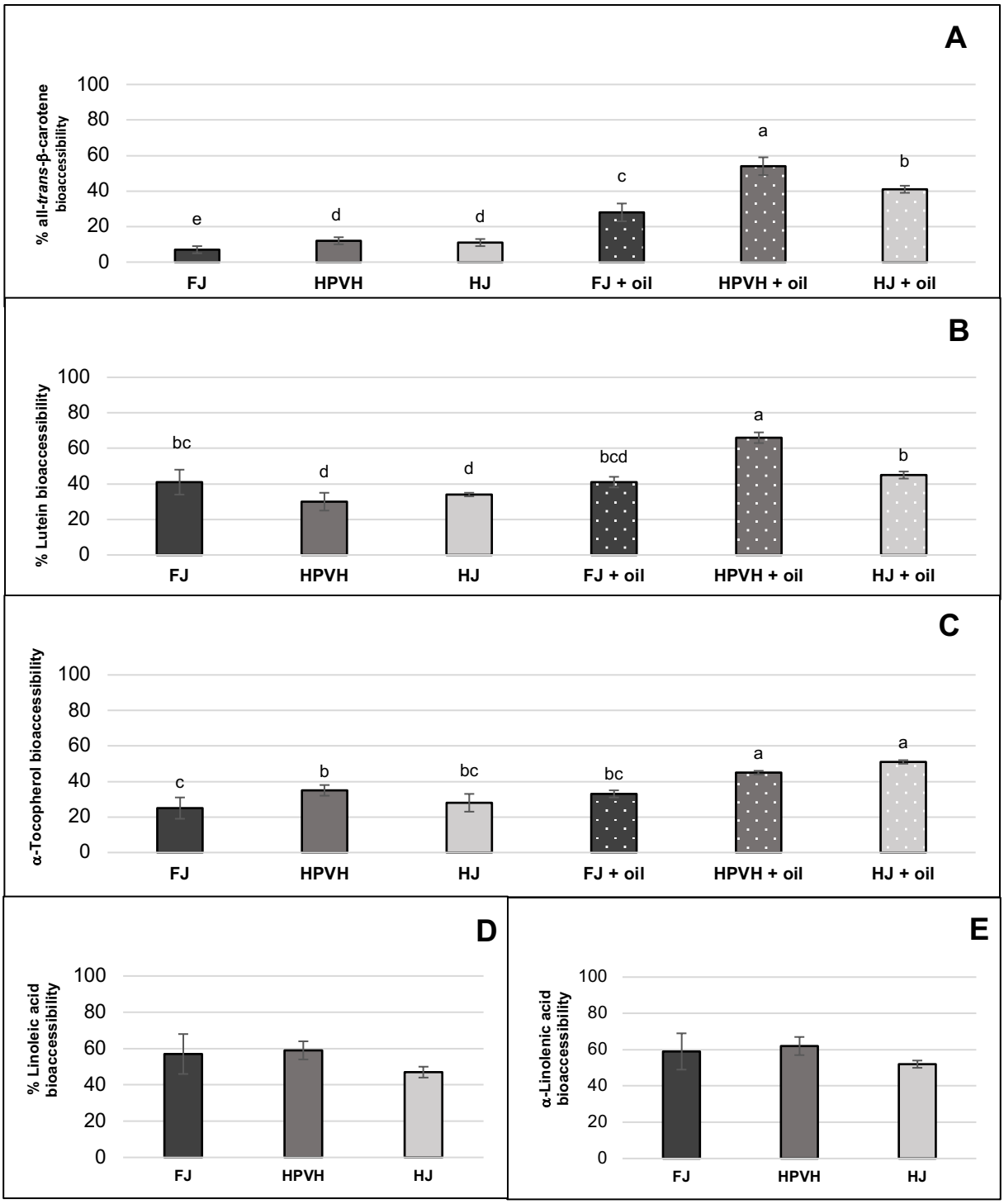


Figure 6.6: Effect of heat treatment and the presence of oil on nutrient bioaccessibility (micellar solubilisation) after *in vitro* digestion in CRF from PVH **A)** all-*trans*-β-carotene bioaccessibility **B)** lutein **C)** α-tocopherol **D)** linoleic acid **E)** α-linolenic acid; FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; Data were presented as a mean ± SD of 3 separated *in vitro* digestion and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at p<0.0.5, a>b.

6.2.3.4. Nutrient available for uptake

The term of 'nutrient available for uptake' is the comparison of nutrient in micelles to the initial nutrient concentration in CRF before digestion. It takes into account the stability of the nutrient during digestion and the liberation of the nutrient into the micellar phase. This is to provide a better understanding on how much nutrient that is available for absorption by the body, relative to the initial concentration of the freeze-dried PVH CRF in term of percentage (%) (see **Figure 6.7**) and mg/g dry weight (mg/g, DW) (see **Table 6.2**). The data of nutrient available for uptake suggests the importance of calculating back the value of bioaccessibility relatively to its pre-digested or initial nutrient content.

After the *in vitro* digestion, the β-carotene available for uptake was the highest in the system that combined oil and heated juice (37%) and oil and HPVH (35%), followed by HPVH (16%), HJ (12%), FJ with oil (12%), and FJ without oil (4%) (**Figure 6.7A**). From this result, either heat treatments (HPVH or HJ) or the presence of rapeseed oil in PVH CRF enhanced the β-carotene available for uptake more than the condition without heat and oil. It was due to the retention and bioaccessibility of β-carotene as described in **Section 6.2.3.2** and **6.2.3.3**. Even though only 23% of β-carotene was retained in PVH CRF from FJ after *in vitro* digestion (**Figure 6.5A**), the nutrient available for uptake from PVH CRF of FJ (4 mg/100 g, DW) (see **Table 6.2**) was better than carrot juice reported by Courraud et al. (2013), which had 100% β-carotene retention. Still only 1.14 mg/100 g, DW is available for uptake. It was due to low bioaccessibility of carrot juice (1.14%). Moreover, Mansor (2019) reported that the amount of β-carotene in spinach CRF had 4.35 mg/g CRF, DW, but only 0.01 mg/g CRF, DW can available for uptake. While our result had β-carotene only 0.99 mg/g CRF, DW, but the β-carotene available for uptake in PVH CRF had 4 mg/100 g, DW,

which was higher than spinach CRF. This might be due to low retention (26%) and low bioaccessibility (0.8%) of spinach CRF. Conversely, PVH CRF had 23% and 7% of β-carotene retention and bioaccessibility, respectively.

On the other hand, the lutein available for uptake was the highest in the presence of oil and after heated PVH (49%), followed by the addition of oil and HJ (36%), HJ (33%), HPVH (27%), FJ with oil (15%), and FJ without oil (11%) (**Figure 6.7B**). Even though heat treatment did not affect lutein bioaccessibility in PVH CRF (see **Section 6.2.3.3**), it improved the stability during digestion as described in **Section 6.2.3.2**. Thus, this was a reason why lutein in PVH CRF from HPVH and HJ was more available for uptake than lutein in PVH CRF from FJ. Although only 31% of lutein was retained in CRF from FJ after *in vitro* digestion, the nutrient available for uptake from CRF of FJ (51 mg/100 g, DW) (see **Table 6.2**) was better than carrot juice reported by Courraud et al. (2013), which had 100% lutein retention. Still only 0.5 mg/100 g, DW is available for uptake. It was due to low bioaccessibility of carrot juice (22%). While PVH CRF had 41% of lutein bioaccessibility. In addition, the lutein available for uptake in PVH CRF (11% or 51 mg/100 g, DW) was higher than the spinach CRF (6.91 % or 32 mg/ 100 g, DW) (Mansor, 2019). It was due to the reason that spinach CRF had 26% and 30% lutein retention and bioaccessilbility, respectively. Meanwhile, PVH CRF had 23 and 41% retention and bioaccessibility, respectively.

Our result showed that vitamin E was the lowest available for uptake compared to other nutrients. It was due to the low stability of α-tocopherol during *in vitro* digestion as described in **Section 6.2.3.2**. However, using thermal treatment or the combination of heat and oil can improve this nutrient available for uptake. Conversely, the inclusion of oil in PVH CRF alone did not enhance α-tocopherol available for uptake. The α-tocopherol available for uptake was the highest in the system that combined oil and heated juice (26%), followed by the inclusion of oil and

heated PVH (19%), HPVH (11%), HJ (10%), FJ with oil (3%) and FJ without oil (1%) (**Figure 6.7C**).

Both linoleic and α-linolenic acids available for uptake were the highest in CRF material from HPVH (80% linoleic acid and 46% α-linolenic acid), followed by CRF from heated juice (66% linoleic acid, and 39% in α-linolenic acid), and no heat treatment (62% linoleic acid, and 33% α-linolenic acid) (**Figure 6.7D** and **6.7E**). Before the digestion, using thermal treatments, either heated PVH or heated juice before CRF preparation reduced the content of essential fatty acids. Conversely, after the digestion, they enhanced fatty acid more available for uptake than PVH CRF without heat treatment, as shown in **Table 6.2**. It was due to the reason that omega-3 and omega-6 fatty acids were more stable during digestion in the thermal treatments than the samples without heat treatment. This was supported by our result in **Figure 6.5D** and **6.5E**. Thus, the fatty acid available for uptake in HPVH and HJ was higher content.

Overall, using either thermal treatment in the process to make CRF or combining this CRF material with oil helped to significantly increase the nutrients in CRF available for uptake, such as β-carotene, lutein, α-tocopherol, and essential fatty acids. In this later case, only heated PVH was significantly efficient to improve PUFA available for uptake, probably because this treatment applied directly to the initial biomass was efficient to inactivate endogenous enzymes, avoid lipolysis and subsequent oxidation (Wattanakul et al., 2019). Interestingly, the presence of oil mixed with CRF helped to increase significantly β-carotene available in CRF for uptake ($p<0.05$), but the presence of oil did not significantly affect the amount of lutein and α-tocopherol available in CRF for uptake ($p>0.05$) when compared with no heat treatment.

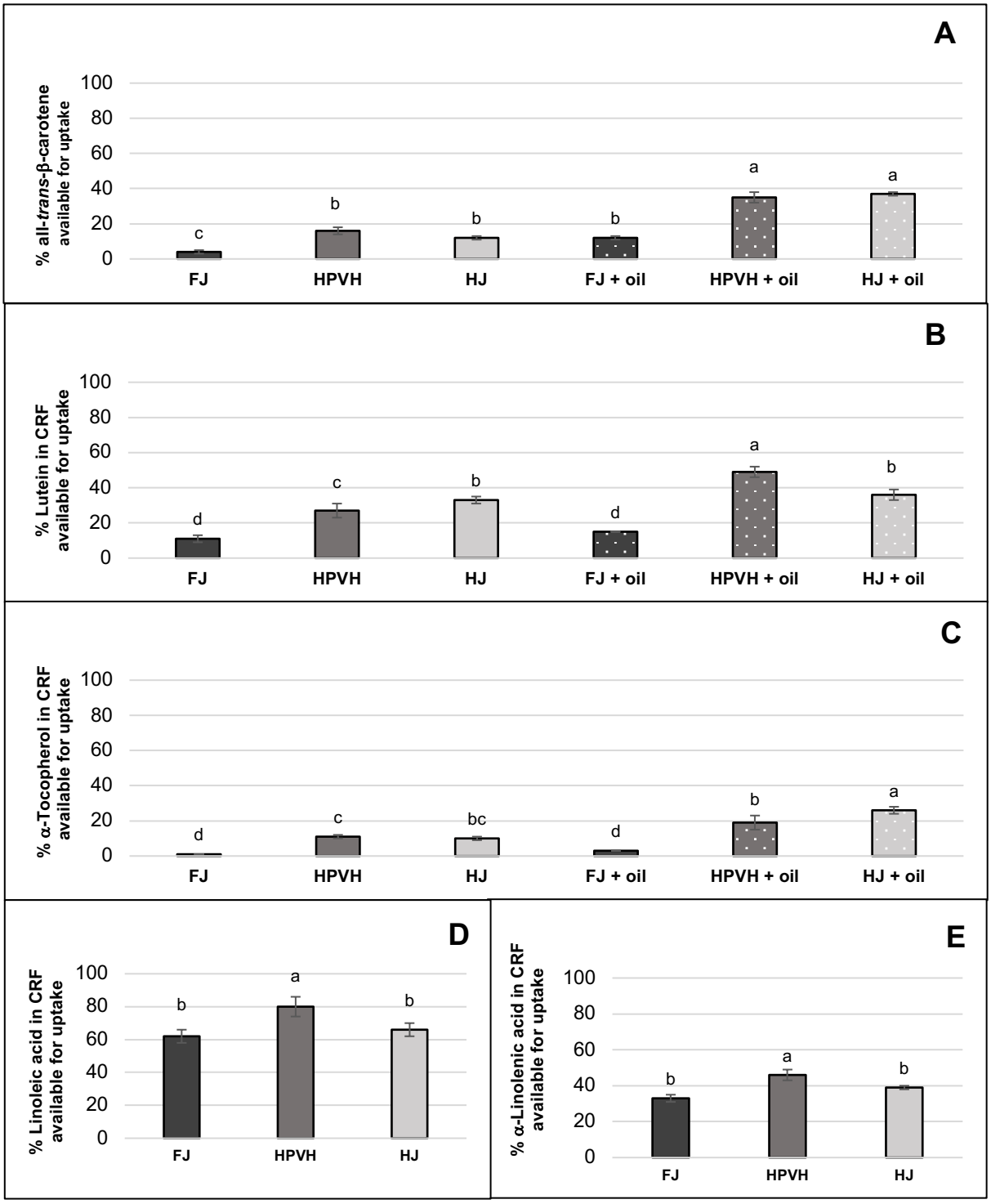


Figure 6.7: Effect of heat treatment and the presence of oil on nutrient in CRF available for uptake after *in vitro* digestion in CRF from PVH **A)** all-*trans*-β-carotene available for uptake after *in vitro* digestion **B)** lutein **C)** α-tocopherol **D)** linoleic acid **E)** α-linolenic acid; FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; Data were presented as a mean ± SD of 3 separated *in vitro* digestion and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.0.5$, a>b.

Table 6.2: Carotenoids, vitamin E and fatty acid contents from PVH both before and after digestion.

	all-*trans*-β-carotene		**lutein**		**α-tocopherol**		**linoleic acid**		**α-linolenic acid**	
	Before digestion	**After digestion**	**Before digestion**	**After digestion**	**Before digestion**	**After digestion**	**Before digestion**	**After digestion**	**Before digestion**	**After digestion**
	all-*trans*-β-carotene (mg/100 g CRF, DW)	all-*trans*-β-carotene in available for uptake (mg/100 g CRF, DW)	lutein (mg/100 g CRF, DW)	lutein in CRF available for uptake (mg/100 g CRF, DW)	α-tocopherol (mg/100 g CRF, DW)	α-tocopherol in CRF available for uptake (mg/100 g CRF, DW)	linoleic acid (mg/100 g CRF, DW)	linoleic acid in CRF available for uptake (mg/100 g CRF, DW)	α-linolenic acid (mg/100 g CRF, DW)	α-linolenic acid in CRF available for uptake (mg/100 g CRF, DW)
FJ	99 ± 6^{b}	4 ± 1^{d}	481 ± 29^{a}	51 ± 8^{c}	29 ± 2^{b}	0.4 ± 0^{d}	903 ± 43	562 ± 40	1670 ± 89	559 ± 40^{b}
FJ + oil		12 ± 1^{c}		71 ± 2^{bc}		1 ± 0^{d}				
HPVH	74 ± 8^{c}	12 ± 2^{c}	334 ± 39^{b}	90 ± 13^{b}	29 ± 2^{b}	3 ± 0^{c}	788 ± 118	628 ± 45	1429 ± 206	660 ± 41^{a}
HPVH + oil		26 ± 2^{b}		162 ± 10^{a}		6 ± 1^{b}				
HJ	121 ± 7^{a}	14 ± 2^{c}	462 ± 34^{a}	154 ± 10^{a}	45 ± 3^{a}	5 ± 1^{bc}	885 ± 32	581 ± 33	1489 ± 47	580 ± 12^{ab}
HJ + oil		45 ± 1^{a}		167 ± 13^{a}		12 ± 1^{a}				

FJ: fresh juice; HPVH: heated PVH; HJ: heated juice; data were presented as a mean ± SD of 3 separated in vitro digestion and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.05$, a>b.

6.3. Conclusions

In conclusion, we measured, for the first time, the bioaccessibility of selected lipophilic nutrients from chloroplasts liberated from the confines of a cell-wall limited cell. The impact of thermal treatment of the biomass (in our case, pea vine haulm), or the juice derived from the biomass, prior to preparing a chloroplast-rich fraction (CRF), appeared to improve the in-digesta retention of β-carotene, lutein, α-tocopherol and α-linolenic acid; this retention was further enhanced, except in the case of lutein and α-tocopherol, when oil was added to the digestion system. No study of the inclusion of oil in PVH CRF helps to improve the retention and bioaccessibility of omega-3 and 6 fatty acids; this is worth to explore further. Although there is little evidence that CRF material from HPVH and HJ has increased bioaccessibility compared with CRF from FJ, the presence of oil increased the bioaccessibility values for β-carotene. It therefore appears that the combination of heat-treatment of biomass or juice, and the presence of oil during digestion, enhances the % of β-carotene, lutein, and α-tocopherol available for uptake from a chloroplast-rich fraction derived from pea vine haulm.

CHAPTER VII

In vitro digestion of galactolipids from chloroplast-rich fraction (CRF) of postharvest, pea vine field residue (haulm) and spinach leaves

7.1. Introduction

Galactolipids are mainly found in the photosynthetic membranes of algae and plants, especially in the thylakoid and the envelope membranes of the chloroplast, and they represent more than 70% of the total membrane lipids (Douce, 1974, Dörmann, 2013). Galactolipids represent the most plentiful lipid class and thus the main sources of fatty acids in the biosphere. They also represent the largest storage form of the essential α-linolenic acid (18:3 n-3; ALA), which represents up to 60% of their total fatty acids. They also contain a large proportion of the shorter omega-3 hexadecatrienoic acid (16:3 n-3). The chemical structures of these diacylglycerolipids are characterised by one, two or more galactose moieties attached to the sn-3 position of the glycerol backbone. The major galactolipids in plants are monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG). However, there are other galactolipids which are present in lower amounts such as trigalactosyldiacylglycerol (TGDG) as well as sulfoquinovosyldiacyglycerol (SQDG), a sulfolipid with a sulfoquinovose residue instead of galactose, as shown in the **Figure 2.5** (Amara, S. et al., 2010). It is therefore worth investigating the digestion of galactolipids in humans and other animal species eating green plant tissues. It was ignored for a long time before a galactolipase activity was discovered in human pancreatic juice and duodenal contents (Andersson et al., 1995). This activity was further associated with pancreatic lipase-related protein 2 (PLRP2) and to a lower

extent to carboxyl ester hydrolase/bile salt-stimulated lipase (CEH/BSSL) (Andersson et al., 1996, Sias et al., 2004, Amara et al., 2009, Amara et al., 2013). Since then, PLRP2 has been found to be present at high levels in monogastric herbivores, which supports its contribution to the digestion of plant lipids (De Caro et al., 2008). Both human and guinea pig PLRP2s were found to display a high activity on DGDG and MGDG from spinach leaves (Amara et al., 2010). These previous studies were mainly dealing with the identification of pancreatic lipase-related protein 2 as a galactolipase and its contribution to the galactolipase activity of pancreatic juice, as well as with the establishment of assay conditions to measure galactolipase activity with both synthetic (radiolabeled and medium chain galactolipids) and natural substrates. Nevertheless, the digestion of galactolipids has been rarely addressed under gastrointestinal (GI) conditions. The novelty of the present study is that we investigated the galactolipase activity of pancreatic juice, pancreatic extracts and a purified PLRP2 (GPLRP2) on chloroplast-rich fractions from whole plant materials under *in vitro* conditions mimicking those found in the GI tract which further support a physiological function for this digestive process and highlights the role of galactolipids in our diet.

In vitro digestion models are widely used today for studying the digestibility and release of nutrient components under simulated gastrointestinal conditions with either fixed parameters (pH and enzyme concentration in static models) or variable parameters (dynamic models). The advantage of this technique is that it is inexpensive, rapid, consistent and does not have ethical restrictions compared to studies involving human or animal (Minekus et al., 2014, Calvo-Lerma et al., 2018). Many publications on *in vitro* digestion use pepsin as the single enzyme in the gastric phase (Garrett et al., 1999, Courraud et al., 2013, Minekus et al., 2014, Rodrigues et al., 2017). However, lipid digestion begins in the stomach with gastric lipase acting on triacylglycerides.

Thus, it is now recommended to add gastric lipase during the stomach phase of *in vitro* digestion (Sams et al., 2018, Brodkorb et al., 2019). Human gastric juice has been used as a source of pepsin and gastric lipase, but its use is limited by the ethical issue. Therefore, native gastric lipases from other mammalian species, such as dogs and rabbits, or from recombinant origin such as recombinant human (rHGL) and dog (rDGL) gastric lipases (Bakala-N'Goma et al., 2015, Sassene et al., 2016, Sams et al., 2017) can be chosen as alternative sources instead of human gastric juice (Brodkorb et al., 2019). Human Pancreatic Juice (HPJ) has been used as a source of pancreatic lipase for the intestine phase of *in vitro* digestion (Carriere et al., 2000, Carriere et al., 2001). However, as for human gastric juice, the use of HPJ has been restricted by ethical constraints. Hence, pancreatin, an extract from porcine pancreas, is widely used for *in vitro* digestion in intestinal phase (Garrett et al., 1999, Courraud et al., 2013, Minekus et al., 2014, Rodrigues et al., 2017).

So far, there is no published work on the digestion of galactolipids in intact chloroplasts by using a two-step static *in vitro* digestion model with relevant and well characterised enzyme sources. Most people studied the lipolysis only the pancreatic enzymes without concerning the presence of lipid digestion in the stomach and used galactolipid standard as a substrate (Amara et al., 2009, Amara et al., 2010, Emek et al., 2011, Salhi et al., 2020). The hypothesis of this chapter was PVH CRF and spinach CRF can be used as substrates for digestive enzymes found in animal and human using two-step static *in vitro* digestion model. Therefore, the aim of this chapter was to test the *in vitro* digestibility of the chloroplast-rich fraction (CRF) from green materials, including PVH and spinach leaves under simulated GI conditions. Our group has recovered intact chloroplasts from spinach leaves using grinding in 0.3 M sucrose solution (Gedi et al., 2017) and from PVH using a slow-screw twin-gear juicer without added water (Torcello-Gomez et al., 2019). Both were exposed first to gastric enzymes

from rabbit gastric extract (RGE) and then either pancreatic enzymes from HPJ or Porcine Pancreatic Extract (PPE).

7.2. Results and discussion

7.2.1. Effect of endogenous galactolipase enzymes and heat-treatment on the CRF galactolipids

As described before in **Chapter V**, inactivation of endogenous enzymes, especially peroxidase by using thermal treatment can extend the nutrient stability of lipophilic nutrients (β-carotene, lutein, and α-tocopherol) during the storage. In this chapter, the action of endogenous enzymes on the galactolipids from pea vine haulm CRF with and without heat treatment was studied (**Figure 7.1**). TLC separations of polar and neutral lipids were analysed first without staining which allowed a qualitative visualisation of carotenoids and pigments, including chlorophylls (**Figures 7.1A** and **7.1C**). No major changes were observed whatever the treatment of CRF. After lipid staining and from the comparison of non-heat treated and steam sterilised or pasteurised CRF, it was clear that galactolipid levels, especially those of DGDG, were lower in non-heat treated samples than in steam sterilised samples and after 1 hr incubation of these samples at 37^{o}C, the band of DGDG has disappeared (**Figure 7.1B**). Without heat treatment, and in contrast to DGDG, the band of MGDG was apparent both before and after incubation. The low levels of galactolipids were associated with high levels of free fatty acids (FFA), which suggests the action of endogenous galactolipases being present in the material (**Figure 7.1D**). In addition to the presence of endogenous galactolipase, the high level of FFA was due to senescence of chloroplasts in plants. Gut and Matile (1988) observed that there was a high accumulation of FFA and breakdown of galactolipids in the barley leaves when induced senescence. It has reported that heat treatment can inactivate lipid-acyl

hydrolase (LAHase), including phospholipase A, C, and D, phosphatase, and galactolipase to prevent loss of lipid during the storage (McCaskill and Orthoefer, 1994). In this study, thermal treatments did inactivate endogenous enzymes as shown in the **Figure 7.1B** where the bands of MGDG and DGDG are visible and remain at similar levels both before and after 1 hr incubation at 37°C, for each treatment. The higher levels of galactolipids were associated with reduced levels of FFA, which indicates that endogenous galactolipases can be heat-inactivated (**Figure 7.1D**). In addition, CRF was also incubated for 1 hr at 37°C after addition of GPLRP2, an enzyme known to display galactolipase activity (Andersson et al., 1996). The galactolipids of both steam sterilised and pasteurised CRF from pea vine haulm were hydrolysed to lysogalactolipids (MGMG and DGMG; **Figure 7.1B**), while FFA levels increased (**Figure 7.1D**). No MGMG and DGMG could be observed however with CRF from non-heat treated pea vine haulm (**Figure 7.1B**), which suggests that endogenous enzymes are also able to hydrolyse MGMG and DGMG. It was established that steam sterilisation at 100°C for 4 min knocks out the endogenous enzymes more thoroughly than pasteurisation at 85°C for 1 min as indicated by a higher intensity of the band of DGDG and a lower amount of free fatty acids (**Figure 7.1B** and **7.1D**). It can be explained that the pasteurisation at 85°C for 1 min prior to making CRF was not efficiently enough to inactivate the endogenous galactolipase activity in this PVH material. While increasing temperature and time by using steam sterilisation at 100°C for 4 min improved the efficiency of enzyme inactivation in PVH. This was agreed with another study, which reported an increasing temperature of blanching can increase the loss of the activity of galactolipase in carrot and spinach (Kim et al., 2001). Our results indicated that endogenous enzymes had an effect on the pea chloroplast galactolipids and this phenomenon was also observed with spinach galactolipids (data not shown). Therefore, heat treatment was applied to all CRF preparations used for *in vitro* digestion

experiments. While CRF from pea vine haulm was steam sterilised, the spinach leaves were first blanched in hot water at 85°C for 3 min to knock out the endogenous enzymes before preparing CRF. It was checked that this treatment allowed the inactivation of the endogenous galactolipase activity of CRF from spinach leaves. A similar trend with Kim et al. (2001) discovered that blanching at 80°C for 6 min could inactivate 90% of endogenous galactolipase in spinach.

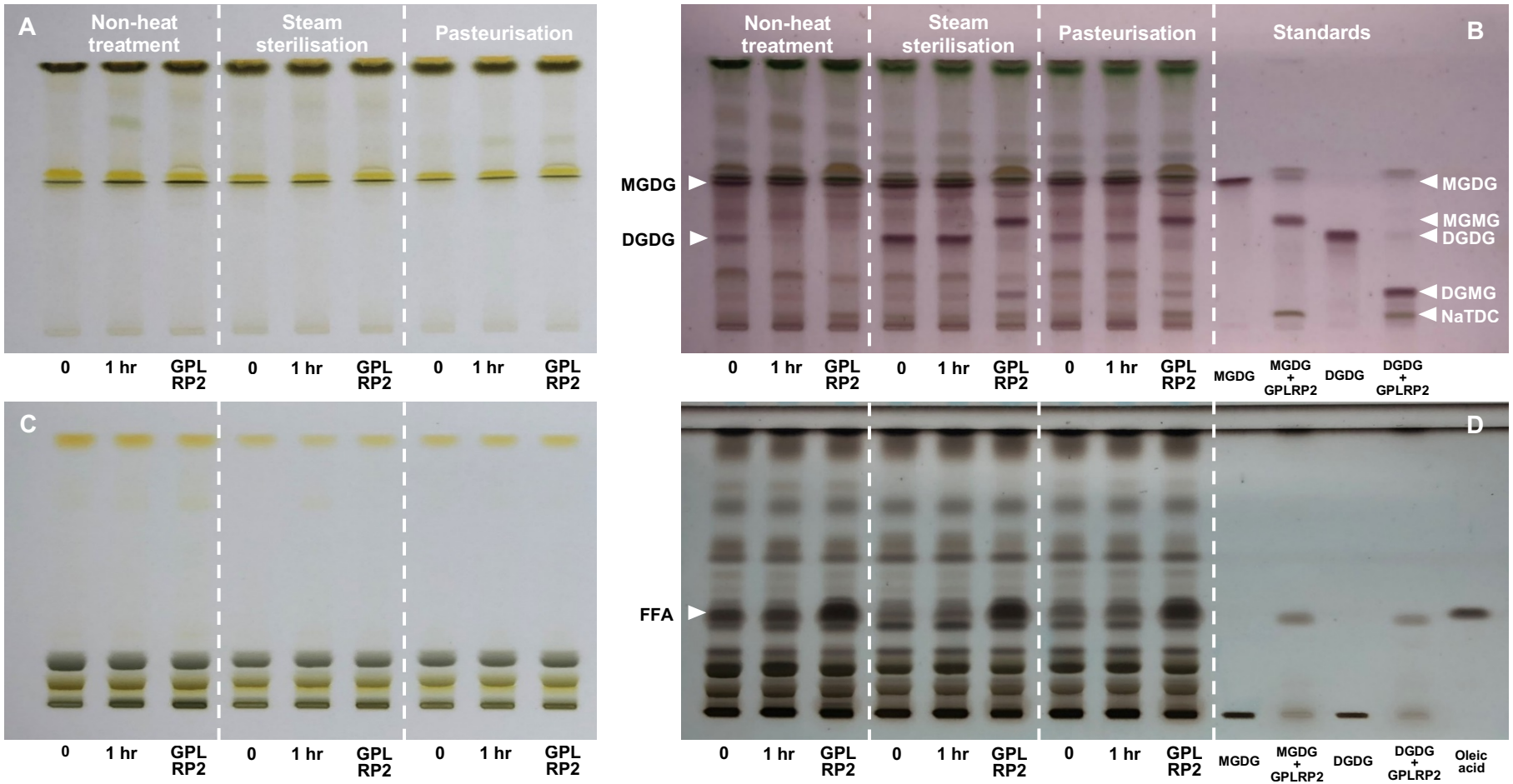


Figure 7.1 TLC analysis of lipid extracts from CRF of PVH showing the effects of endogenous and exogenous (GPLRP2) enzymes on the hydrolysis of galactolipids, depending on CRF pre-treatment. CRF were either non-treated or steam sterilised or pasteurised, and lipids were extracted after 1 hour of incubation with or without GPLRP2. Polar lipid separation was visualised first without any staining (panel A) and then revealed with a thymol solution (panel B). Neutral lipid separation was also visualised first without any staining (panel C) and then revealed with copper acetate-phosphoric acid solution (panel D). As reference standards, pure MGDG, DGDG and oleic acid were used, as well MGDG and DGDG incubated with GPLRP2 in the presence of bile salts (NaTDC), which allowed generating MGMG and DGMG.

7.2.2. Galactolipid and free fatty acid content in CRF before digestion

The amounts of galactolipids and free fatty acids (FFA) in the CRF of blanched spinach leaves and steam sterilised or non-heat treated pea vine haulm were measured (**Table 7.1**). The galactolipids are important role for photosynthetic electron transfer reactions. They are bound via hydrophobic interactions and hydrogen bonds to the polypeptide chains. The galactolipids can thus be considered as cofactors of the photosynthetic complexes, similar to chlorophylls and carotenoids (Dörmann, 2013). The CRF prepared from heat-treated materials showed a higher MGDG content than DGDG. This is in agreement with what has been reported in photosynthetic tissues, especially in the inner envelope membrane and thylakoid membrane of chloroplast where the amount of MGDG is higher than DGDG (Christensen, 2009, Dörmann, 2013). Moreover, Yunoki et al. (2009) observed that the level of MGDG was rich in dark green leafy vegetables, such as spinach, parsley and chive, while crown daisy, green pepper and pumpkin were relatively high in DGDG. The CRF of spinach leaves had more galactolipids per dry mass of CRF than PVH CRF due to a higher lipid content in CRF of spinach leaves. This was supported by the result of Gedi et al. (2019) and Torcello-Gomez et al. (2019), which reported that spinach CRF was 1.7-fold higher lipid than PVH CRF. In addition, spinach CRF (28.59 mg/g of MGDG and 25.81 mg/g of DGDG) was a larger amount of galactolipid than the whole leaf spinach (8.50 mg/g of MGDG and 5.63 mg/g of DGDG), which was analysed by Christensen (2009). The different of galactolipid content in this material was due to the fact that spinach CRF was higher content of chloroplast as a site of biosynthesis of galactolipids (Douce, 1974) than whole leaf spinach (Gedi et al., 2017). The PVH CRF (18.89 mg/g of MGDG and 13.22 mg/g of DGDG) was a larger amount of galactolipid than the seed of pea (0.22 mg/g of MGDG and 6.98 mg/g of DGDG) (Sugawara and Miyazawa, 1999).

It was assumed that this is because the thylakoid membrane with high amount of MGDG in the chloroplast of PVH develops in tissues with active photosynthesis. While the non-photosynthetic tissues, such as seed, lack the large thylakoid membrane system of chloroplast. Thus, the total amount of galactolipid in seed was much lower. Meanwhile, phospholipids are predominant lipid class instead of galactolipids in the seed (Dörmann, 2013). In addition, it was observed that DGDG content was rich in the seed of pea. Conversely, PVH CRF was relatively high MGDG. It was due to the fact that the seed of pea is a non-photosynthetic tissue, which is abundant in DGDG derived from the outer envelope of chloroplasts (Christensen, 2009, Yunoki et al., 2009). The other lipids, including carotenoids, tocopherols, chlorophyll esters, sterols and phospholipids, were not quantified in this experiment due to the lack of standard. The lower relative levels of lipids in pea vine CRF may be due to some dilution by components like starch as described earlier in **Chapter IV**. It was also supported by Torcello-Gomez et al. (2019), who found that carbohydrate was the largest proportion of macronutrient in PVH CRF. The level of free fatty acids in CRF from spinach was quite low 1.30 ± 0.35 mg/g CRF or 5.08 ± 1.35 mg/g lipid extract, in line with the fact that spinach leaves were blanched to avoid lipolysis by endogenous enzymes. This was supported by Hirayama and Oido (1969). They suggested that spinach leaves should be blanched at 100^{o}C for 3 min before the storage at 37^{o}C to repress galactolipase and sulfolipase activities. The CRF of steam sterilised pea vine showed a greater amount of FFA (11.71 ± 0.72 mg/g CRF or 71.95 ± 4.43 mg/g lipid extract), which indicates some significant lipolysis occurring from harvesting and before heat treatment of pea vine haulm. It can be explained that the release of FFA from galactolipids could occur from autoxidation by endogenous lipoxygenase and light-induced activation of endogenous galactolipase (Kaniuga, 2008). However, the determination of the amount of FFA in haulm directly after harvesting was not possible

due to logistical limitation. Nevertheless, the amounts of FFA in the CRF from steam sterilised pea vine was 3-fold lower than those found in CRF from non-heat treated pea vine as shown in the **Table 7.1**. In agreement, MGDG and DGDG levels in CRF from steam sterilised PHV were 3.4-fold and 1.8-fold higher than in CRF from non-heat treated PVH. It was due to the action of LAHase being present in the material without heat treatment (Christensen, 2009). Thus, the galactolipids in CRF from non-heat treated PVH was easy to hydrolyse by endogenous enzymes and release more FFA compared to steam sterilised PVH CRF. This was supported by Yamauchi et al. (1986), who observed that MGDG and DGDG in non-heated spinach leave decreased markedly after storage at 25°C for 6 days. Moreover, Lee and Mattick (1961) found that the level of FFA from non-heat treated pea was a larger amount than blanched pea at 100°C for 1 min during the storage at -17.8°C for 1 year.

The ratio of MGDG/DGDG in PVH CRF without heat treatment (0.7:1) was lower value compared to PVH CRF with heat treatment (1.4:1). It was assumed that the degradation rate of MGDG by endogenous galactolipase might be faster than that of DGDG. This assumption was supported by Gut and Matile (1988), who found that MGDG disappeared much faster than DGDG in barley leaf. Michalski and Kaniuga (1980) also indicated that MGDG was more rapidly degraded than DGDG during the storage at 4°C of tomato leaves due to the presence of endogenous galactolipase enzymes. In addition, it has reported that the nitrogen deficiency in Arabidopsis led to an increase DGDG and a decrease MGDG in the chloroplast, which correlated with an evaluated expression of DGDG synthase gene *DGD1* and *DGD2* (Gaude et al., 2007). The levels of TAG and FFA increased during nitrogen deprivation. Similar to phosphate starvation, lack of water enhanced DGDG biosynthesis in the leaves of cowpea, which indicated that drought induced DGDG accumulation in extrachloroplastic membranes and contributed to the plant tolerate to arid environment (Torres-Franklin et al., 2007).

It is worth noting that the total masses of MGDG, DGDG and FFA are not significant difference ($p>0.05$) in both CRF preparations of PVH CRF (47 mg/g CRF in non-heat treatment and 44 mg/g CRF in steam sterilisation), which confirms that most FFA are generated by the endogenous hydrolysis of galactolipids. A similar agreement with Yamauchi et al. (1986) and Kim et al. (2001), they reported that LAHase and lipoxygenase (LOG) play critical roles in the quality deterioration of galactolipids and phospholipids in carrot and spinach.

Table 7.1: Galactolipids and free fatty acid contents in different samples of CRF from PVH and spinach leaves. The effect of steam sterilisation versus non-heat treatment before CRF preparation was studied with PVH, while spinach CRF were obtained from blanched leaves. For each lipid class (MGDG, DGDG or FFA), concentration was expressed either in mg per g of CRF (DW) or mg per g of total lipid extract from CRF. On average, 1 g of dried CRF from spinach and PVH contains 256.77 ± 9.15 and 162.77 ± 4.63 mg total lipid, respectively. DW, dry weight.

Sample, treatment		**MGDG**	**DGDG**	**FFA**	**Total galactolipids + FFA**
PVH CRF, non-heat treatment	mg/g CRF (DW)	5.50 ± 0.22^{c}	7.29 ± 0.45^{c}	34.46 ± 1.64^{a}	47.25 ± 1.88^{b}
	mg/g total lipids	33.77 ± 1.36^{b}	44.81 ± 2.75^{c}	211.74 ± 10.07^{a}	290.31 ± 11.53^{a}
PVH CRF, steam sterilisation	mg/g CRF (DW)	18.89 ± 1.97^{b}	13.22 ± 0.99^{b}	11.71 ± 0.72^{b}	43.82 ± 2.72^{c}
	mg/g total lipids	116.04 ± 12.08^{a}	81.21 ± 6.08^{b}	71.95 ± 4.43^{b}	269.19 ± 16.71^{b}
Spinach leaves CRF, blanching	mg/g CRF (DW)	28.59 ± 1.12^{a}	25.81 ± 1.12^{a}	1.30 ± 0.35^{c}	55.70 ± 1.96^{a}
	mg/g total lipids	111.35 ± 4.35^{a}	100.50 ± 4.35^{a}	5.08 ± 1.35^{c}	216.93 ± 7.63^{c}

Data were presented as a mean ± SD of 3 separated *in vitro* digestion and analysed using a post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistically significance at p<0.0.5, a>b.

Table 7.2: Total fatty acids in PVH and spinach leave CRF and their release upon digestion. Total fatty acids (FA) in CRF were estimated from GC-MS analysis. FA presents in galactolipids (MGDG and DGDG) and free fatty acids (FFA) initially present in CRF were estimated from TLC analysis and data in **Table 7.1**. Mass amounts (mg/g CRF, DW) of MGDG, DGDG and FFA were converted in µmoles per g of CRF (DW) using average molar masses of 760, 922 and 271 g/mole, respectively, which were estimated from the fatty acid composition of CRF (**Figure 7.7**). FA mole equivalents present in MGDG and DGDG were then estimated. FFA released after 90 min digestion by the combination of RGE and HPJ were estimated from TLC analysis. Values, expressed either in mg/g CRF (DW) or µmole/g CRF (DW), are means ± SD (n=3). Values into brackets are the percentage of total FA in CRF. DW, dry weight.

Sample		Total FA in CRF	FA in galactolipids and FFA	FFA after 90-min digestion by RGE+HPJ
Steam sterilised PVH CRF	mg/g CRF (DW)	37.81 ± 5.67[b]	32.96 ± 2.71[b]	32.65 ± 0.88[b]
	µmole/g CRF (DW)	139.35 ± 20.90[b]	121.46 ± 9.98[b] (87.2%)	120.35 ± 3.23[b] (86.4%)
Blanched spinach leaves CRF	mg/g CRF (DW)	64.16 ± 3.11[a]	36.83 ± 1.81[a]	49.81 ± 2.23[a]
	µmole/g CRF (DW)	237.18 ± 11.51[a]	136.15 ± 6.68[a] (57.4%)	184.13 ± 8.24[a] (77.6%)

Data were presented as a mean ± SD of 3 separated *in vitro* digestion and analysed using an independent-sample t-test with statistically significance at $p<0.0.5$, a>b.

7.2.3. *In vitro* digestion of galactolipids in CRF

According to the TLC analysis of galactolipid digestion (**Figures 7.2 - 7.5**), both the MGDG and DGDG of CRF from blanched spinach leaves and steam sterilised PVH were hydrolysed and converted to lysogalactolipid (monogalactosylmonoglycerol, MGMG, and digalactosylmonoglycerol, DGMG) during the whole digestion process. During the 30-min gastric phase, galactolipids decreased slightly from 25-26 to 22-23 mg MGDG/g CRF and 18-22 to 17-18 mg DGDG/g CRF for spinach leaves CRF (**Figure 7.3A** and **7.3B**) and from 15-16 to 14-15 mg MGDG/g CRF and 9-11 to 8-9 mg DGDG/g CRF for PVH CRF (**Figure 7.3C** and **7.3D**). The weak increase in FFA during the gastric phase (**Figure 7.6B**) confirms that galactolipid hydrolysis both PVH CRF and spinach CRF is not very important under gastric conditions, in line with the fact that gastric lipase has no demonstrated galactolipase activity. Nevertheless, RGE might contain some traces of other enzymes with galactolipase activity. This was supported by Sahaka et al. (2020), who recently reported gastric lipase has no activity of galactolipase. However, gastric lipase shows activity on long chain of triacylglycerol (TAG) at pH 5 (Gargouri et al., 1986). It has also high level of activity on TAG at *sn-3* position, to a lesser extent on diacylglycerol (DAG) and its activity on monoacylglycerol (MAG) is very low in comparison with its activity on TAG (Fernandez et al., 2007, Fernandez et al., 2008).

After adding HPJ and bile salts to the system to initiate the intestinal phase of digestion, both MGDG and DGDG of spinach and PVH CRF were immediately hydrolysed to MGMG and DGMG, respectively (**Figures 7.3A**, **7.3C** and **7.4**), and high amounts of free fatty acids were released (**Figure 7.5** and **7.6**), especially during the first 5 min of the intestinal phase. Because the monogalactosylgalactolipids (MGDG and MGMG) are revealed similarly upon thymol staining (**Figures 7.1B**), and in the absence of a pure MGMG reference standard, the generation of MGMG was tentatively

quantitated by TLC using the calibration curve established with MGDG as reference standard. A good correlation was observed between the appearance of MGMG and the disappearance of MGDG (**Figure7.3**). The band corresponding to DGMG on TLC plates (see **Figure 7.2** and **7.4**) was however too faint and not enough resolved to apply the same method. These results are supported by those of Andersson et al. (1995), who demonstrated that pure galactolipids were hydrolysed by enzymes from Human Pancreatic Juice (HPJ) to lysogalactolipids and FFA. This previous study had led to the characterisation of the galactolipase activity of HPJ and its association with a higher pancreatic lipase related-protein 2 (PLRP2) and to a lower extent to carboxyl ester hydrolase/bile salts stimulated lipase (CEH/BSSL) or known as cholesterol esterase (Andersson et al., 1996, Amara et al., 2009).

Galactolipids from both CRF preparations were hydrolysed at a slower rate when RGE and PPE were combined for *in vitro* digestion (**Figures 7.3B** and **7.3D**). This was agreed with Salhi et al. (2020), who recently reported that PPE has a galactolipase activity (0.28 U/mg on MGDG-C8 at pH 6). Its activity was lower compared to HPJ, which had 8.40 U/mg on MGDG-C-8 at pH 6. PPE is a well-known mixture of digestive enzymes with the broad activities, including protease, amylase, and lipase activities due to the presence of trypsin, chymotrypsin, pancreatic-α-amylase, pancreatic lipase and colipase (Minekus et al., 2014). The activities of galactolipase and cholesterol esterase are not found in pancreatic lipase (Andersson et al., 1996, Amara et al., 2009). PLRP2 is also not found in porcine pancreas (Emek et al., 2011). Recently, Salhi et al. (2020) discovered that PPE has low activity of cholesterol esterase. Our study observed that PPE prefers to hydrolyse MGDG over DGDG, which is not the case for HPJ. Thus, the activity of galactolipase found in PPE could come from CEH/BSSL because this enzyme can hydrolyse both cholesterol ester and galactolipid. This hypothesis was confirmed with the result of Amara et al. (2009).

They reported that CEH hydrolyses MGDG, DGDG, and SQDG, with a hydrolysis rate higher with MGDG than with SQDG and DGDG due to the steric hindrance of the additional galactose at the level of the polar head (Amara et al., 2009). Thus, it is possible that the galactolipase activity found in PPE is CEH/BSSL.

TLC analysis of the fatty acids released during digestion of both CRF from spinach leaves and pea vine (See **Figures 7.5** and **7.6**) showed that the FFA bands obtained using the mixture of RGE and PPE, had a higher intensity than those obtained with the mixture of RGE and HPJ. This was not consistent with the lower hydrolysis of galactolipids by PPE. We then performed control experiments without the CRF substrate and found that FFA could be released during the intestinal phase when PPE was used but not HPJ (**Figure 7.6A**). This finding is probably due to the fact that PPE contains some lipids (U.S. Pharmacopedia) and these lipids can be hydrolysed during the digestion experiment. The FFA analysis by TLC were therefore corrected accordingly by subtracting the FFA levels measured in the controls. This point was never raised however in previous *in vitro* digestion studies, probably because the levels of FFA released from PPE are much lower than those released from dietary triglycerides. But one has to be cautious when low levels of lipids are concerned, as here with CRF galactolipids.

Overall, *in vitro* study on CRF digestion showed that galactolipids from CRF can be rapidly hydrolysed by pancreatic enzymes from human pancreatic juice (HPJ) and porcine pancreatic extract (PPE) during the intestinal phase of digestion. This was supported by published research (Andersson et al., 1995, Andersson et al., 1996, De Caro et al., 2004, Sias et al., 2004) as described earlier. However, our result was inconsistent with previous reports (Albertsson et al., 2007, Chu et al., 2009, Köhnke et al., 2009a, Köhnke et al., 2009b, Chu et al., 2010). Chu et al. have investigated the possible replacement of lecithin by galactolipids as emulsifiers (Chu et al., 2009) and

how the presence of galactolipids at the oil-water can impact the absorption and activity of porcine pancreatic lipase (Chu et al., 2010). They studied the effect of MDGG and DGDG on the *in vitro* digestibility of olive oil under the simulated duodenal condition in the presence of lecithin and bile salts. It was found that olive oil lipolysis started after a longer lag phase and with a decreased lipolysis rate when emulsions were prepared with DGDG instead of MGDG. These effects were amplified by increasing the DGDG to lecithin molar ratio. It was postulated that the larger headgroup and more tightly packed molecular organisation of DGDG at the interface gives rise to a steric hindrance that impairs colipase and lipase adsorption at the oil-water interface and thus delayed and reduced lipolysis. In addition, Erlanson-Albertsson et al. have orally given a preparation of thylakoids from spinach leaves to mice (Köhnke et al., 2009b) and healthy human volunteers (Köhnke et al., 2009a) to slow down lipid digestion and trigger satiety mechanism. They have found that the ingestion of thylakoids from spinach mixed with a high-fat meal could induce the release of gut hormones. Based on *in vitro* digestion experiments, Erlanson-Albertsson and Albertsson (2015) assumed that the mechanism by which thylakoid act is a reduction of the rate of intestinal lipolysis by pancreatic lipase. It allows some lipolysis products to reach the distal intestine and triggers the release of satiety hormone. However, their hypothesis that galactolipids, either purified (Chu et al., 2009, Chu et al., 2010) or originating from thylakoids (Köhnke et al., 2009b, Erlanson-Albertsson and Albertsson, 2015), could slow down intestinal lipolysis was based on *in vitro* digestion studies performed with classical pancreatic lipase alone. The possible hydrolysis of galactolipid by digestive galactolipids PLRP2 and CEH/BSSL was not taken into account like our experiments. Indeed, the effect of galactolipids from green plants could be changed or suppressed under conditions involving all the lipolytic enzymes naturally present in pancreatic secretion.

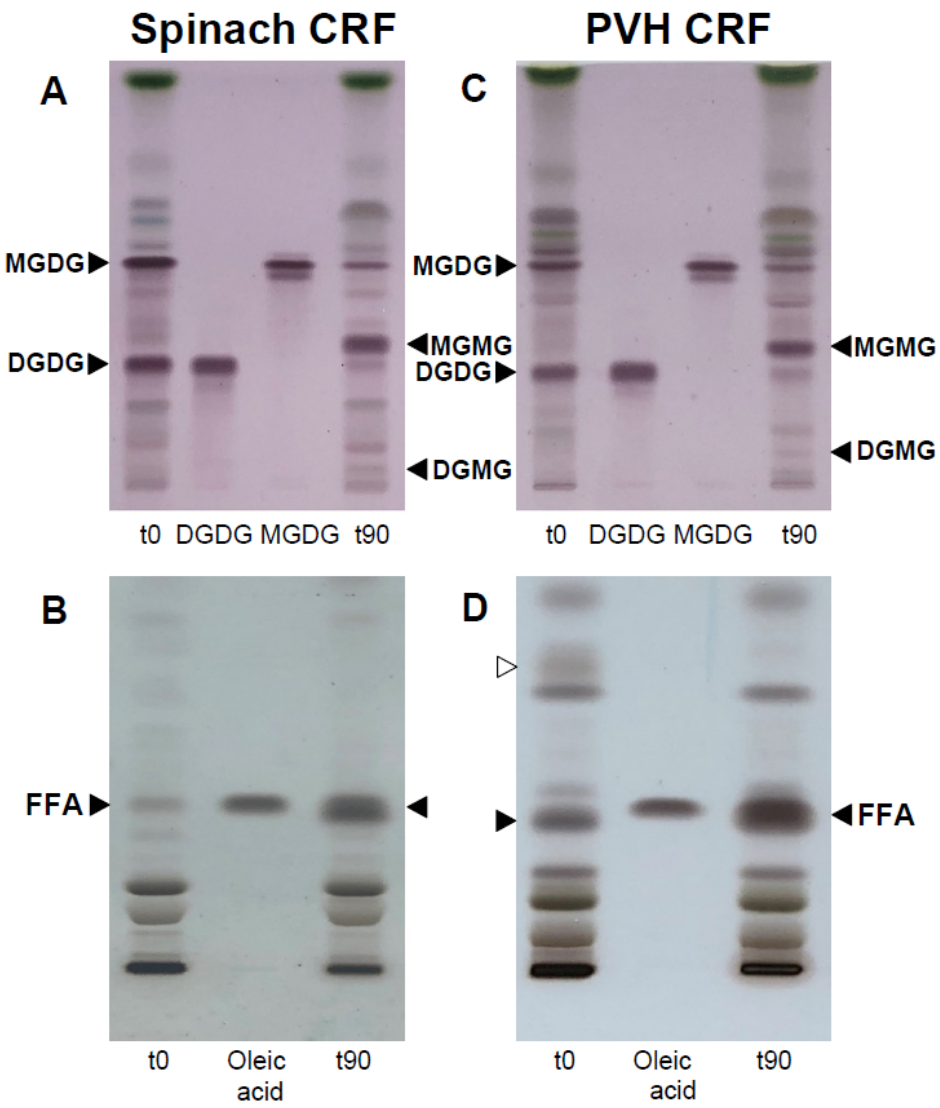


Figure 7.2: TLC analysis of galactolipids and free fatty acids initially present in CRFs (t0) and after 90-min digestion (t90) by a combination of RGE and HPJ. **Panels A** and **B**: CRF from blanched spinach leaves; **Panels C** and **D**: CRF from steam sterilised pea vine haulm (PVH). Polar lipids (**panels A** and **C**) and neutral lipids (**panels B** and **D**) were revealed with a thymol solution and a copper acetate-phosphoric acid solution, respectively. Pure MGDG, DGDG and oleic acid were used as reference standards. The white arrow in panel C indicates a band of apolar lipids present in PVH CRF, which disappears after digestion.

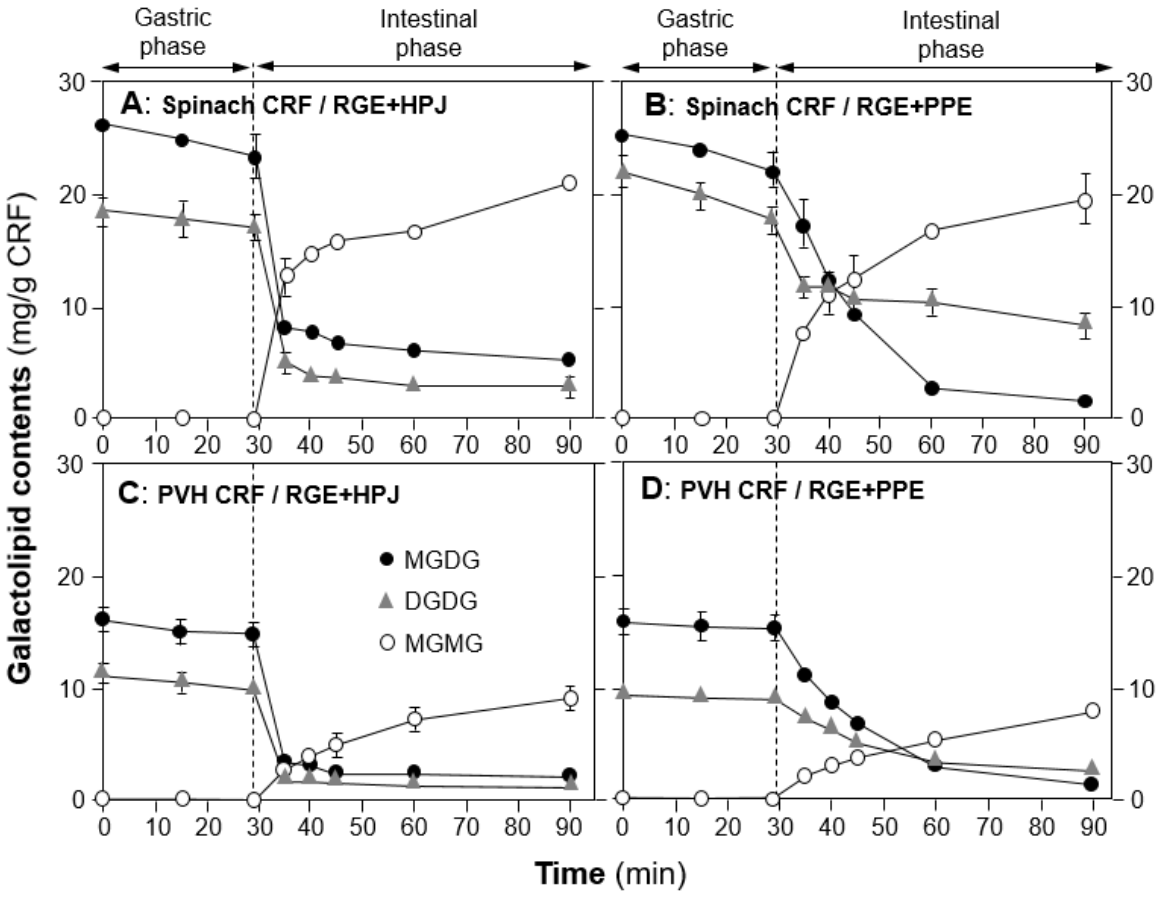


Figure 7.3: Hydrolysis of galactolipids in the course of two-step static digestion of CRFs. CRF from blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric and pancreatic enzymes respectively (**panel A**) or a combination of RGE and PPE as an alternative source of pancreatic enzymes (**panel B**). Similarly, CRF from steam sterilised PVH were digested using a combination of RGE and HPJ (**panel C**) or a combination of RGE and PPE (**panel D**). Symbols: full black circles, MGDG; open circles, MGMG; grey triangles, DGDG. Values (mg of galactolipid per g (DW) of CRF) are means ± SD (n=3).

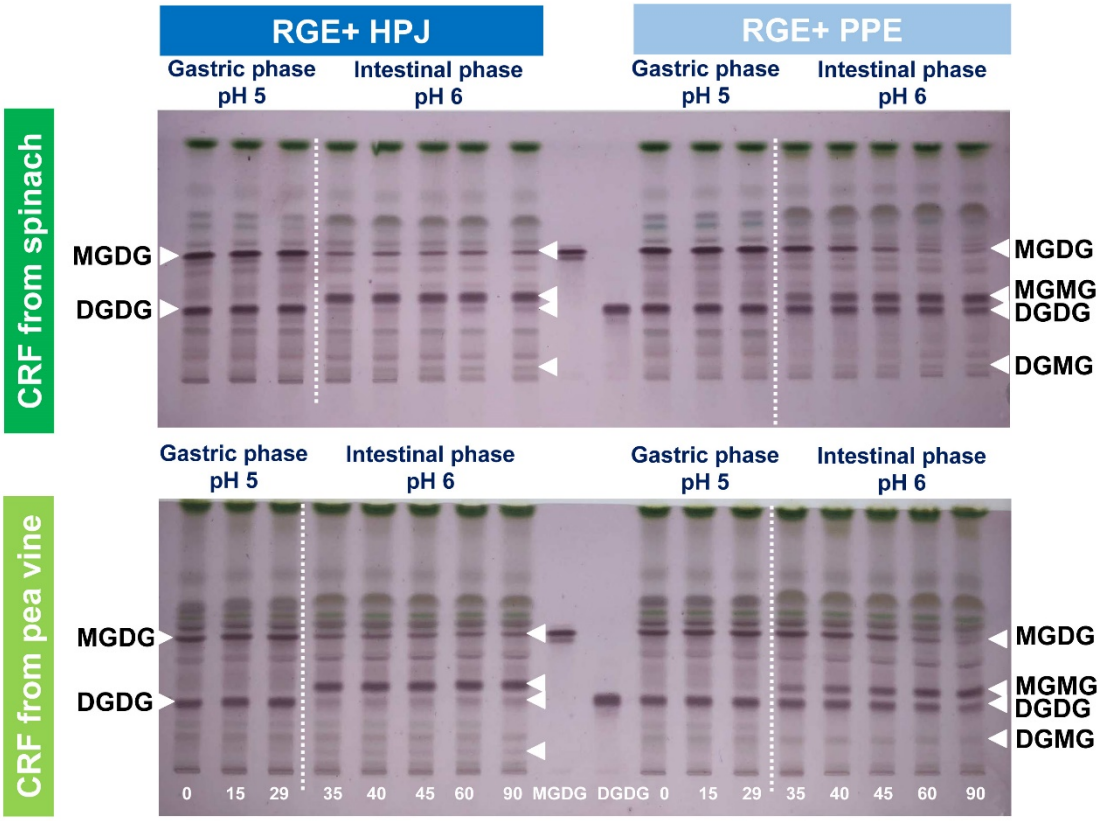


Figure 7.4: Representative thin-layer chromatography analysis of polar lipids in the course of two-step static digestion of CRFs. CRF from blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric and pancreatic enzymes respectively or a combination of RGE and PPE as an alternative source of pancreatic enzymes (upper TLC plate). Similarly, CRF from steam sterilised PVH were digested using a combination of RGE and HPJ or a combination of RGE and PPE (lower TLC plate). Samples were collected at times 0, 15 and 29 min during the gastric phase and at times 35, 40, 45, 60 and 90 min during the intestinal phase. Lipids were extracted with methanol-chloroform as described in the main text. Polar lipids were separated using a chloroform/methanol/water (47.5:10:1.25, v/v/v) elution mixture and were stained with a thymol solution, especially for revealing galactolipids. Pure monogalactosyldiglycerides (MGDG) and digalactosyldiglycerides (DGDG) were uses as reference standards for the identification and quantitation of these lipids in CRFs. The monogalactosylmonoglycerides (MGMG) and digalactosylmonoglycerides (DGMG) generated upon the hydrolysis of MGDG and DGDG are also shown.

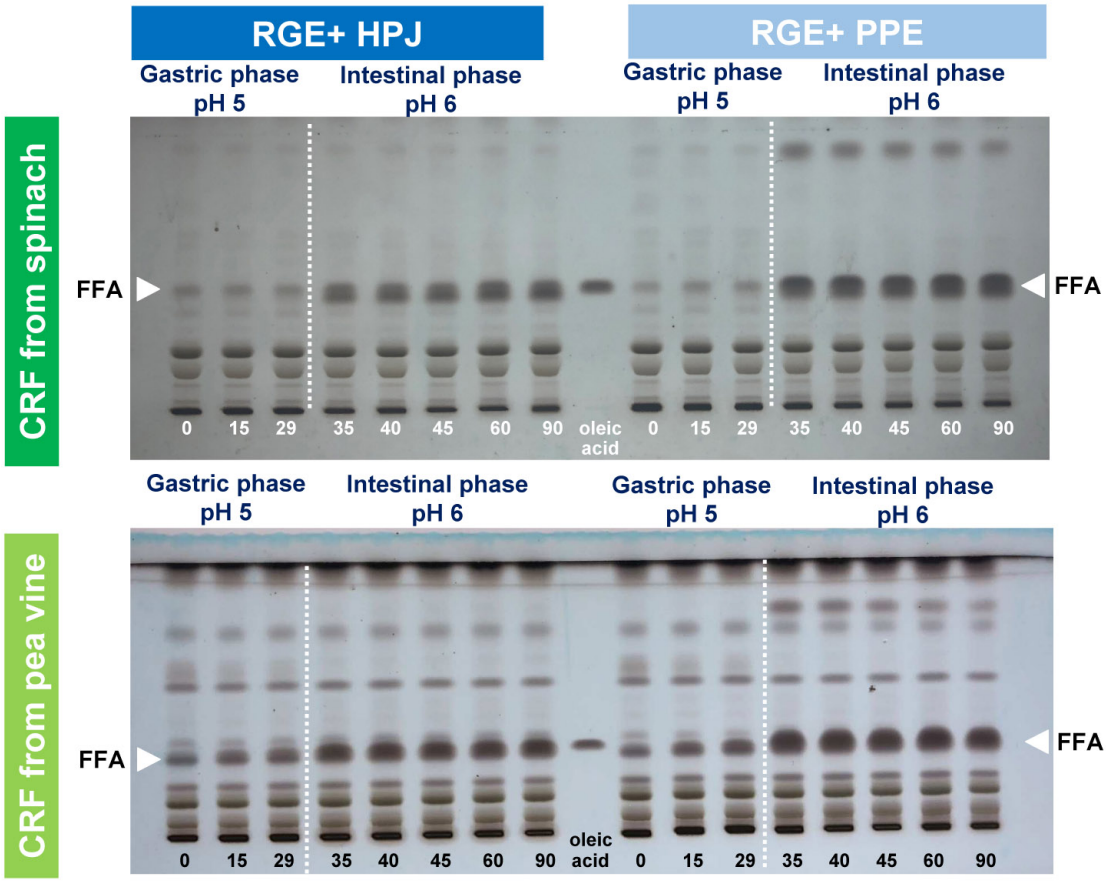


Figure 7.5: Representative thin-layer chromatography analysis of neutral lipids in the course of two-step static digestion of CRFs. CRF from blanched spinach leaves were digested using a combination of RGE and HPJ as sources of gastric and pancreatic enzymes respectively or a combination of RGE and PPE as an alternative source of pancreatic enzymes (upper TLC plate). Similarly, CRF from steam sterilised PVH were digested using a combination of RGE and HPJ or a combination of RGE and PPE (lower TLC plate). Samples were collected at times 0, 15 and 29 min during the gastric phase and at times 35, 40, 45, 60 and 90 min during the intestinal phase. Lipids were extracted with methanol-chloroform as described in the main text. Neutral lipids were separated using a mobile phase consisting of heptane: diethyl ether: formic acid (55:45:1, v/v/v) solvent mixture and they were revealed, especially free fatty acids (FFA), with a copper acetate-phosphoric acid solution. Pure oleic acid was used as reference standards.

7.2.4. Composition and release upon digestion of the CRF fatty acids

The compositions of total fatty acids in spinach leaves and pea vine CRF were analysed using GC-MS and the results are shown in the **Figure 7.7**. The main fatty acid in both spinach CRF and pea vine CRF was α-linolenic acid (ALA; 18:3) but its amounts in spinach CRF (35.56 ± 2.56 mg/g CRF DW) were 2.5-fold higher than in pea vine haulm CRF (14.29 ± 2.06 mg/g CRF DW). ALA represented 55.38 ± 1.33% w/w of the total fatty acids in spinach leave CRF, which is in agreement with the fatty acid composition of chloroplasts obtained from spinach leaves (Wolf et al., 1962, Gedi et al., 2017) and isolated galactolipids from spinach leaves (Yamauchi et al., 1986, Amara et al., 2010). In pea vine CRF, ALA represented only 37.82 ± 0.31% w/w of the total fatty acids. This result was consistent with the fatty acid composition of chloroplast from pea vine haulm (Torcello-Gomez et al., 2019). However, this result was inconsistent with published research (Murcia and Rincon, 1991) that reported the seed of pea was high level of linoleic acid (LA; 18:2) and low level of ALA. It was due to the fact that LA is rich in seeds, while ALA is a major fatty acid in leaves (Andersson et al., 1995). The second most abundant fatty acid was palmitic acid (PA; 16:0), which was found at similar levels in spinach CRF (13.31 ± 0.24 mg/g CRF; 20.77 ± 0.76% w/w of total FA) and pea vine CRF (12.35 ± 1.86 mg/g CRF; 32.65 ± 0.12% w/w of total FA). This result was agreed with the previous research of fatty acid composition in PVH CRF and spinach CRF, which reported PA was the most abundant saturated fatty acid in both plant materials (Gedi et al., 2017, Torcello-Gomez et al., 2019). Hexadecatrienoic acid (16:3) was found at significant levels in spinach CRF (5.98 ± 0.12 mg/g CRF; 9.34 ± 0.29% w/w of total FA) but was not found in pea vine CRF. Therefore, the levels of polyunsaturated omega 3 fatty acids were globally reduced in pea vine haulm CRF compared to spinach CRF. This may result from a preferential oxidation of these fatty acids by endogenous lipoxygenases from harvesting to steam sterilisation of pea vine

haulm. Moreover, this oxidation is known to be coupled to and favoured by the release of FFA by endogenous galactolipase activity as often seen in plant response to stress (Kaniuga, 2008). The absence of 16:3 fatty acid in pea vine haulm CRF may also be explained by the classification of pea among “eukaryotic plants”, i.e. plants which preferentially have C18 fatty acids at *sn-2* position of the glycerol backbone in galactolipids, while “prokaryotic” plants like spinach preferentially have C16 fatty acids at *sn-2* position (Heemskerk et al., 1991). Indeed, the predominant galactolipids in “prokaryotic” plants are MGDG (18:3/16:3) and DGDG (18:3/16:0), while MGDG (18:3/18:3) and DGDG (18:3/18:3) are the predominant ones in “eukaryotic” plant (Heemskerk et al., 1990). This distinction in galactolipids comes from their biosynthesis pathways. Galactolipid synthesis in higher plants consisted of two pathways (Whitaker, 1986, Ohlrogge and Browse, 1995). The fatty acids are synthesised in the chloroplasts and they can then enter the “prokaryotic” chloroplastidial pathway giving “prokaryotic” MGDG and DGDG, or they can be exported to the endoplasmic reticulum where they are incorporated into lipid through the “eukaryotic” pathway. The prokaryotic pathway in chloroplasts forms acyl lipid with a C16 fatty acid at the *sn-2* position of the diacylglycerol backbone. The alternative eukaryotic pathway gives acyl lipid only C18 fatty acid at the *sn-2* position (Edman and Ericson, 1987, Li-Beisson et al., 2010). Galactolipids of spinach are possibly derived from both prokaryotic and eukaryotic pathways (Hirayama and Oido, 1969, Douce, 1974), while pea is only derived from eukaryotic pathway (Heemskerk et al., 1990).

After 90 min of digestion by RGE and HPJ, the composition of FFA generated from CRF hydrolysis was similar to the composition of total FA in both spinach and PVH CRF (**Figure 7.7**), with ALA representing the most abundant FFA, followed by PA. This result was consistent with Amara et al. (2010), who found ALA was the most FFA released from the *sn-1* position of MGDG and DGDG in spinach

leaves, followed by PA. Conversely, Lee and Mattick (1961) reported that LA was the main FFA, which was released from galactolipids in the seed of pea, followed by PA during *in vitro* digestion. With the combination of RGE and PPE, some changes were observed in the relative distribution of fatty acids, which certainly reflects the contribution of fatty acids present in PPE (see **Figure 7.7** and **Table 7.3**). Therefore, we only discuss here the data obtained with RGE and HPJ. We observed that the amounts of total FFA released during the CRF digestion were higher than those expected from the complete lipolysis of MGDG and DGDG, both for spinach leaves and pea vine haulm CRF (**Figure 7.6B** and **Table 7.3**). It was expected that some FFA were certainly released from other acyl lipids present in CRF preparations. This hypothesis is supported by the levels of total fatty acids present in CRF and quantified by GC-MS (**Table 7.2**). **Figure 7.2D** on TLC separation of neutral lipids shows that some apolar lipids present in PVH CRF at time 0 and migrating like triglycerides or sterol esters are no more present at time 90 min. In addition, phospholipids, mainly PG, are also present in chloroplast membranes (Dörmann, 2013, Hölzl and Dörmann, 2019) and can be degraded by the phospholipase activity of HPJ (De Caro et al., 2004, Salhi et al., 2020). Moreover, we have seen that galactolipid lipolysis was not complete, with some accumulation of MGMG (**Figure 7.3**) and DGMG (**Figure 7.2** and **7.4**), in line with the preferential hydrolysis by PLRP2 of the ester bond at the *sn-1* position of galactolipids (Amara et al., 2010). It was also agreed with the previous *in vitro* study by Andersson et al. (1995) on the galactolipase activity in human duodenal contents and in pancreatic juice. They found that DGDG was hydrolysed to DGMG during incubation with duodenal contents and the lysogalactolipid was not hydrolysed further after the 2-hour incubation period. However, it should be noticed that both MGDG and DGDG from wheat flour were found to be rapidly converted into fully deacylated products (monogalactosylglycerol (MGG) and digalactosylglycerol (DGG)) and FFA during

in vivo studies in the intestinal tract of rat (Sugawara and Miyazawa, 2000). This result suggested that other enzymes were involved in the catabolism of galactolipid *in vivo* (Amara et al., 2010).

During stress, such as drought, metallic stress, and nutrient deficiency or senescence, the degradation of chlorophyll and galactolipid from thylakoid membranes in chloroplasts can also lead to the conversion of a large proportion of phytol and fatty acids into phytyl fatty acid esters (PFAE) and triacylglycerol (TAG) (Lippold et al., 2012, Gaude et al., 2007). We did not search nor analyse these various lipids here, but it would be worth identifying these other sources of fatty acids in future studies in order to better describe the lipid composition of CRF. At this stage, it is important to keep in mind that 87.2% of the total fatty acids of pea vine haulm CRF are present in galactolipids and FFA, and that 86.4% of total fatty acids are released upon *in vitro* digestion, while the corresponding values for spinach CRF are 57.4% and 77.6%, respectively (**Table 7.2**). The proportion of fatty acid sources other than galactolipids is therefore higher in spinach CRF than in PVH CRF. In both cases, a large proportion of the total fatty acids can be converted to FFA upon digestion, including ALA as the main FFA.

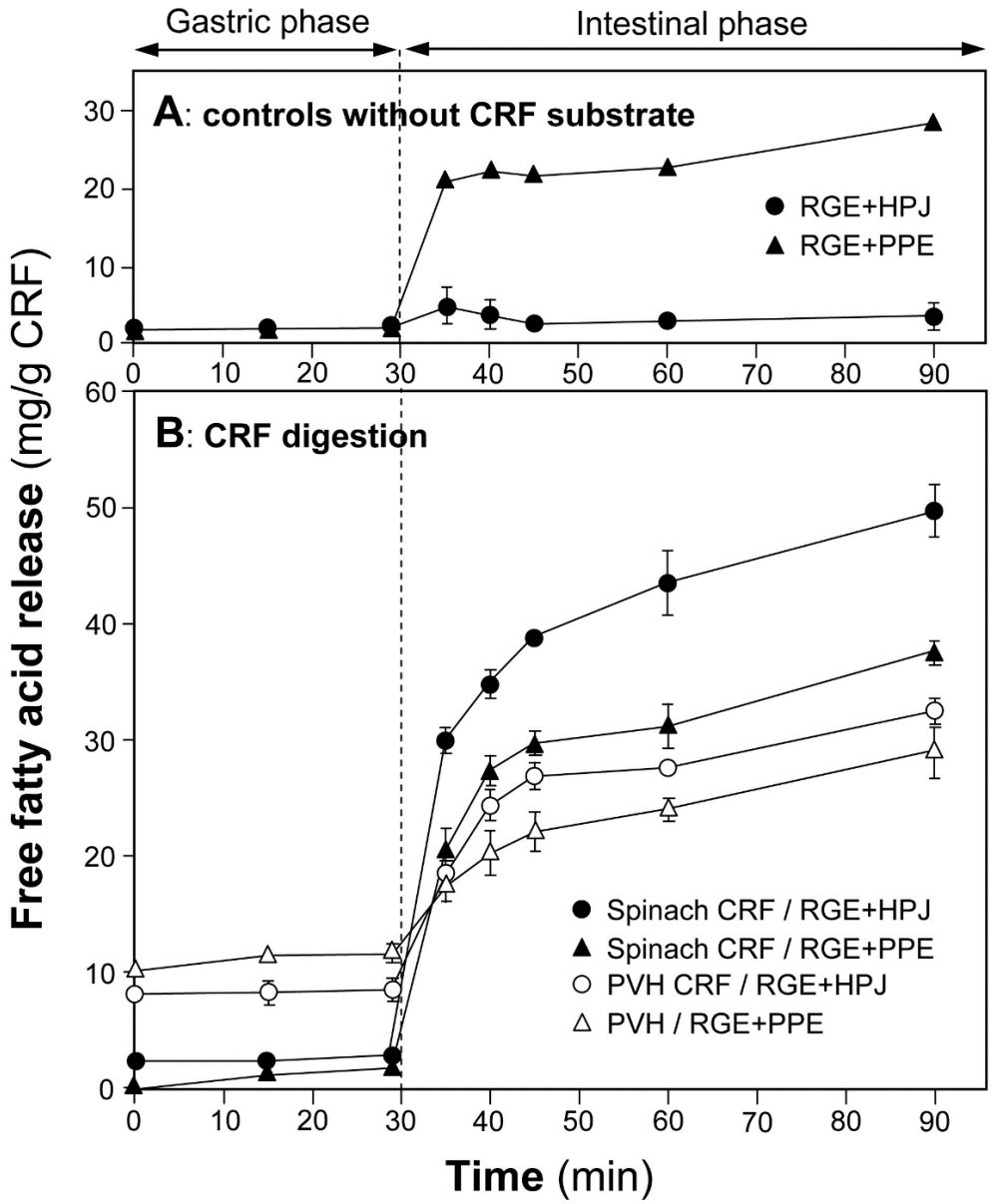


Figure 7.6: Free fatty acid release during the two-step static digestion of CRFs **A)** blank without CRF substrate showing that some FFA can be released from PPE. **B)** Digestion of CRFs from blanched spinach leaves and steam sterilised PVH, incubated with either a combination of RGE and HPJ as sources of gastric and pancreatic enzymes or a combination of RGE and PPE as an alternative source of pancreatic enzymes. Values (mg of FFA released per g (DW) of CRF) are means ± SD (n=3).

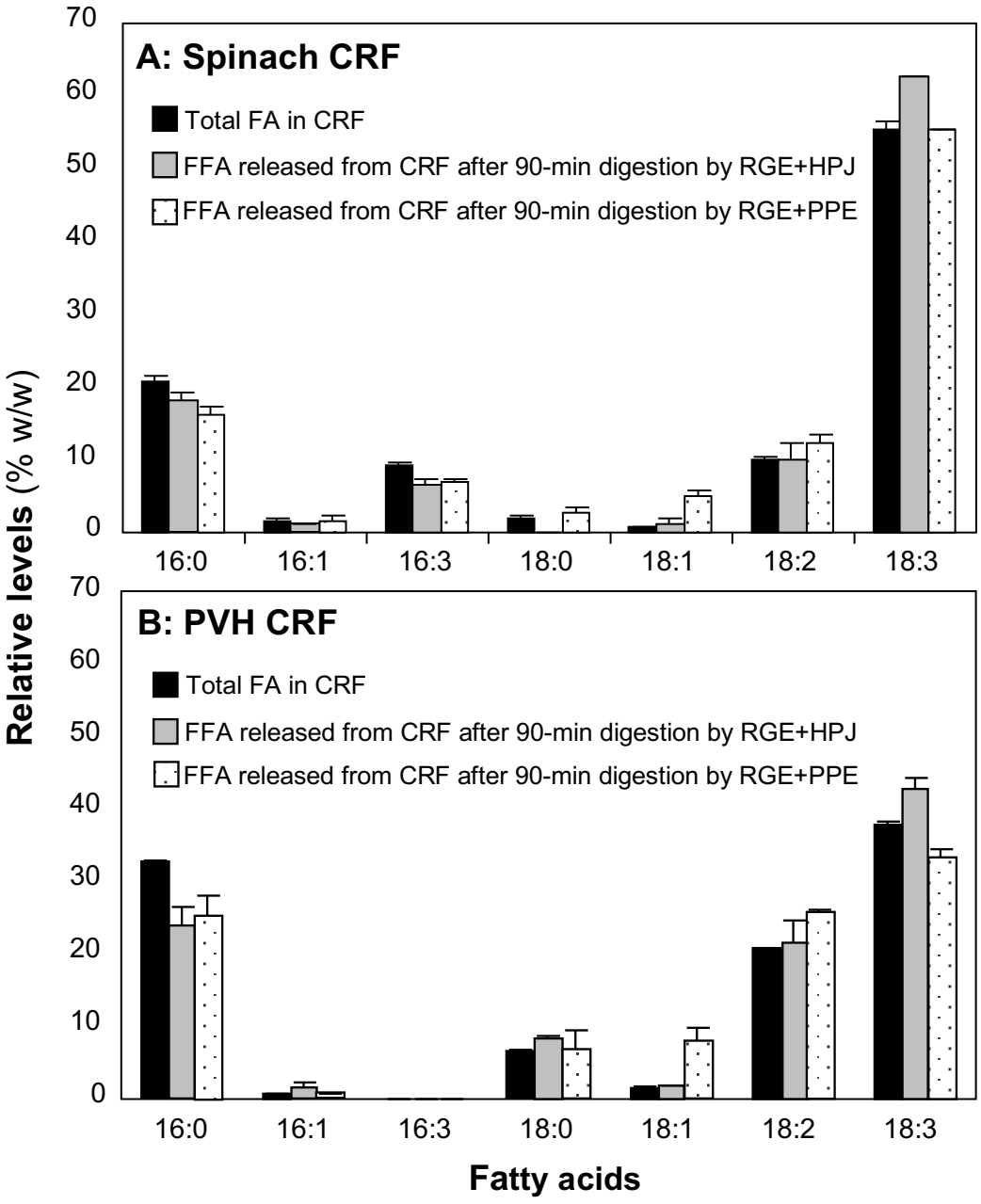


Figure 7.7: Composition of total fatty acids initially present in blanched spinach and steam sterilised pea vine haulm CRFs (**panel A**) and corresponding free fatty acids released after 90-min digestion by RGE and HPJ (**panel B**). Data are expressed in mg per g of CRF dry weight and presented as mean ± SD (n=3).

Table 7.3: Composition of total fatty acids in CRF and free fatty acids released from CRF after 90 minutes of *in vitro* digestion. Fatty acid levels are expressed in mg/g of CRF dry weight, in % w/w, in µmole/g of CRF dry weight and mole %. Values are means ± SD (n=3).

CRF	Sample	Fatty acid composition (mg/g CRF)															
		16:0		16:1		16:3		18:0		18:1		18:2		18:3		Total FA	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Spinach	Total FA in CRF	13.31	0.24	1.08	0.12	5.98	0.12	1.33	0.05	0.54	0.03	6.35	0.17	35.56	2.56	64.16	3.16
	FFA released at 90 min / RGE+HPJ	19.00	1.96	1.02	0.36	4.20	0.31	3.92	1.64	0.88	0.35	6.75	1.31	40.14	0.02	75.92	5.90
	FFA released at 90 min / RGE+PPE	16.39	1.88	0.90	0.36	4.15	0.19	1.56	0.35	3.08	0.03	7.42	0.70	33.52	0.18	67.02	0.77
Pea vine haulm	Total FA in CRF	12.35	1.86	0.33	0.05	0.00	0.00	2.45	0.45	0.51	0.07	7.88	1.18	14.29	2.06	37.81	5.67
	FFA released at 90 min / RGE+HPJ	17.37	2.52	1.14	0.40	0.00	0.00	3.65	0.84	0.90	0.10	9.81	1.21	18.95	0.64	51.81	4.23
	FFA released at 90 min / RGE+PPE	17.01	2.46	0.20	0.06	0.00	0.00	2.79	1.88	3.28	1.05	10.41	0.26	13.11	0.94	46.80	6.52
CRF	**Sample**	**Fatty acid composition (% w/w total FA)**															
		16:0		**16:1**		**16:3**		**18:0**		**18:1**		**18:2**		**18:3**		**Total FA**	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Spinach	Total FA in CRF	20.77	0.76	1.68	0.11	9.34	0.29	2.08	0.10	0.85	0.05	9.91	0.32	55.38	1.33	100.00	0.00
	FFA released at 90 min / RGE+HPJ	25.00	0.64	1.33	0.37	5.54	0.03	5.10	1.76	1.14	0.37	8.86	1.04	53.03	4.16	100.00	0.00
	FFA released at 90 min / RGE+PPE	24.44	2.52	1.34	0.56	6.19	0.35	2.33	0.49	4.60	0.10	11.08	1.16	50.02	0.84	100.00	0.00
Pea vine haulm	Total FA in CRF	32.65	0.12	0.88	0.01	0.00	0.00	6.45	0.21	1.35	0.01	20.85	0.14	37.82	0.31	100.00	0.00
	FFA released at 90 min / RGE+HPJ	33.43	2.13	2.18	0.60	0.00	0.00	7.00	1.05	1.76	0.34	18.89	0.79	36.74	4.24	100.00	0.00
	FFA released at 90 min / RGE+PPE	36.32	0.19	0.43	0.19	0.00	0.00	5.74	3.21	6.92	1.28	22.43	2.58	28.15	1.91	100.00	0.00

Table 7.3 (continued): Composition of total fatty acids in CRF and free fatty acids released from CRF after 90 minutes of *in vitro* digestion. Fatty acid levels are expressed in mg/g of CRF dry weight, in percentage (%) w/w, in µmoles/g of CRF dry weight and mole percentage (%). Values are means ± SD (n=3).

CRF	Sample	Fatty acid composition (µmol/g CRF)															
		16:0		16:1		16:3		18:0		18:1		18:2		18:3		Total FA	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Spinach	Total FA in CRF	51.91	0.94	4.43	0.49	23.90	0.48	4.68	0.17	1.92	0.09	22.64	0.61	127.71	9.20	237.18	11.51
	FFA released at 90 min / RGE+HPJ	74.09	7.66	4.18	1.48	16.79	1.22	13.79	5.75	3.10	1.23	24.09	4.68	144.16	0.09	280.20	21.93
	FFA released at 90 min / RGE+PPE	63.91	7.33	3.67	1.49	16.57	0.74	5.50	1.21	10.91	0.11	26.46	2.48	120.39	0.65	247.41	3.08
Pea vine haulm	Total FA in CRF	48.15	7.27	1.36	0.20	0.00	0.00	8.60	1.58	1.80	0.25	28.11	4.22	51.33	7.39	139.35	20.90
	FFA released at 90 min / RGE+HPJ	67.73	9.82	4.67	1.65	0.00	0.00	12.82	2.96	3.20	0.35	34.96	4.31	68.05	2.30	191.43	16.09
	FFA released at 90 min / RGE+PPE	66.32	9.59	0.80	0.25	0.00	0.00	9.82	6.60	11.62	3.72	37.13	0.91	47.09	3.39	172.77	23.96
CRF	**Sample**	**Fatty acid composition (mole % total FA)**															
		16:0		**16:1**		**16:3**		**18:0**		**18:1**		**18:2**		**18:3**		**Total FA**	
		mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Spinach	Total FA in CRF	21.91	0.79	1.86	0.12	10.09	0.31	1.98	0.09	0.81	0.04	9.55	0.30	53.80	1.33	100.00	0.00
	FFA released at 90 min / RGE+HPJ	26.42	0.66	1.48	0.41	5.99	0.03	4.86	1.67	1.09	0.35	8.56	1.00	51.61	4.07	100.00	0.00
	FFA released at 90 min / RGE+PPE	25.82	2.64	1.49	0.62	6.70	0.38	2.22	0.46	4.41	0.10	10.70	1.14	48.67	0.87	100.00	0.00
Pea vine haulm	Total FA in CRF	34.55	0.13	0.98	0.01	0.00	0.00	6.15	0.20	1.29	0.01	20.17	0.14	36.85	0.30	100.00	0.00
	FFA released at 90 min / RGE+HPJ	35.29	2.16	2.41	0.66	0.00	0.00	6.65	0.99	1.68	0.33	18.23	0.72	35.73	4.20	100.00	0.00
	FFA released at 90 min / RGE+PPE	38.37	0.23	0.48	0.21	0.00	0.00	5.47	3.06	6.64	1.23	21.66	2.48	27.38	1.84	100.00	0.00

7.3. Conclusions

We have shown that heat treatments by both steam sterilisation (at 100°C for 4 min) of postharvest, pea vine field residue (haulm) and hot water blanching (at 85°C for 3 min) of spinach leaves knock out endogenous galactolipase activity inside the plant materials. Based on our results, it is recommended that samples should be treated in this way to avoid the loss of galactolipid content, the release of FFA and further oxidation of polyunsaturated fatty acids.

We have also shown that the galactolipids from both PVH and spinach leaves CRF could be digested *in vitro*, mostly during the intestinal phase of digestion by pancreatic enzymes. PLRP2 and CEH/BSSL, are the main enzymes found in pancreatic secretion that can digest galactolipids and indeed, we showed that CRF galactolipids, as well as some other acyl lipids, are digested by the enzymes present in human pancreatic juice.

Pancreatic extracts can also be used to show this digestion, but lipolysis rates are slower due to a lower galactolipase activity compared to HPJ. In addition, it can be seen that the enzyme(s) with galactolipase activity in PPE hydrolyses MGDG more extensively than DGDG compared to HPJ. After digestion, α-linolenic acid (18:3) is the main fatty acid from CRF of both spinach leaves and in post-harvest, pea vine field residue.

Overall, this work indicated that chloroplasts liberated from their cell wall-bound environment acted as substrates for digestive enzymes with galactolipase activity. Spinach CRF prepared under controlled laboratory conditions are the most enriched in the essential α-linolenic acid, but it is worth noting that PVH CRF produced from a waste from agriculture still contain a large amount of ALA and their post-harvesting heat treatment paves the way to their use as dietary supplement.

CHAPTER VIII

Main Findings, Conclusion and Suggestion for future work

8.1. Thoughts on Main Findings

Thus far, only one published work has reported the digestion/uptake of chloroplasts in juvenile *Elysia timida* or sea slug (Laetz and Wägele, 2017); no research has been published for chloroplast digestion in human. This was the first time that anyone has studied chloroplast/galactolipid digestion in a human model. The novelty of this work was that we investigated the galactolipase activity of pancreatic juice, pancreatic extracts, and purified PLRP2 (GPLRP2) on chloroplast-rich fraction from whole plant materials under *in vitro* condition mimicking those found in the GI tract which further supported a physiological function for this digestive process and highlighted the role of galactolipids in our diet. Previous research studied the bioaccessibility of carotenoids and vitamin E in vegetables and fruits (Clevidence et al., 2000, Goñi et al., 2006, Veda et al., 2006, Ryan et al., 2008, Veda et al., 2008, Nagao et al., 2013) or even in pasta (Werner and Böhm, 2011). However, no research has reported the study of the bioaccessibility of lipophilic nutrients in the chloroplast. Recently, our research group at the University of Nottingham revealed that replacing fish meal with spinach leaf powder (WLM) or chloroplast-rich fraction (CRF) resulted in excellent growth of zebrafishes and cleared uptake of carotenoids and omega-3 fatty acids (Gedi et al., 2019); this thesis described for the first time the bioaccessibility of essential lipophilic nutrients in chloroplast, where we investigated the nutrient retention, the bioaccessibility, and the nutrient available for uptake in chloroplast-rich fractions under *in vitro* simulated GI digestion. Besides the study of chloroplast in the green plant materials of this thesis, Erlanson-Albertsson's group at Lund University in Sweden

studied the impact of thylakoid membrane from spinach leaves on the digestive process. They discovered that thylakoid membrane could be used as both an emulsifier (Rayner et al., 2011) and a functional food ingredient for appetite regulation (Albertsson et al., 2007). It slowed down the digestion process by inhibiting the pancreatic lipase/co-lipase activity, which prompted the investigation of their interfacial properties (Albertsson et al., 2007, Ostbring et al., 2015, Östbring et al., 2018). In addition, thylakoid induced the release of several gut hormones, such as cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and ghrelin. In this way, satiety was promoted, and hunger suppressed, leading to loss of body weight and body fat. The mechanism was a reduced rate of intestinal lipid hydrolysis, allowing the lipolytic products to reach the distal intestine and released satiety hormones (Albertsson et al., 2007, Stenblom et al., 2016). Montelius et al. (2014) revealed that ingesting thylakoids before breakfast can reduce subjective hunger and promote satiety, which could affect body weight when given long-term. The study involved 38 overweight women and ran for three months. Every morning before breakfast, the participants consumed a green drink. The control group lost an average 3.5 kg of body weight while the treatment group (given thylakoid) lost 5 kg. Thus, this study showed the addition of green-plant membrane as dietary supplementary, once daily, had positive effects for body weight and appetite regulation and could thus be used as an effective tool for novel treatment of overweight, obesity, and the metabolic disease.

Our work challenged the assumption that thylakoid membranes, rich in galactolipids, remain intact during transit in the small intestine. Perhaps the association of thylakoid membranes with oil droplets alteres their susceptibility to galactolipase action, and/or perhaps the dose levels of thylakoid membranes in these human feeding trials are excessive compared with normal consumption levels and so lead to substrate

saturation allowing the survival of some thylakoid membranes that have an impact on oil digestion.

In addition, the digestive systems of carnivores and herbivores are different. Typically, carnivores have only one stomach chamber and a simple digestive system. While herbivores (i.e. ruminants) have multiple stomach chambers and much longer digestive tract. Since the plants/grass contain cellulose, which is hard to digest, the digestive tract of herbivore is adapted thus, the food may be digested properly. Once the plants are chewed by ruminants (i.e. cow, deer, sheep), they enter to the first compartment of stomach (rumen). The rumen contains symbiotic bacteria within their guts to assist with the breakdown of cellulose. Then, the ruminants regurgitate the materials from the rumen back to their mouths. They chew their materials again to further break down the cellulose. After that they are swallowed again and re-enter to the rumen. This cycle repeats until the material is broken far enough to pass into the true stomach (Reece et al., 2014). Digestion of grass/chloroplasts by herbivores has taught us there is something that is still hidden and needs to further investigate. It is interesting to speculate what might happen to residual chloroplast material that makes it to the large intestine.

8.2. General conclusion

Overall, this thesis presented a valorisation of the underutilised green plant materials, particularly post-harvest, pea vine field residue by recovering the chloroplast rich in nutrients using a simple physical process without adding any solvents or chemicals. The main findings of this thesis were summarised that:

- Twin-screw extrusion juicing of green biomass was an effective way to liberate/recover chloroplasts without the need to use a grind medium.
- Concentrated/enriched chloroplast material from pea vine haulm (PVH) and spinach leaves contained significant amount of lutein, α-tocopherol, linoleic acid (omega-6), and iron compared to CRF from spinach leaves.
- Freeze-drying the chloroplast-rich fraction (CRF) was an effective way to preserve nutrients.
- Cool storage of freeze-dried CRF in lights impervious foil was effective at retaining nutrient of β-carotene, lutein, and α-tocopherol, even if air is present.
- Heat treatment of green biomass or fresh juice helped to retain nutrients (β-carotene, lutein, α-tocopherol, and α-linolenic acid) during digestion.
- Lipophilic nutrient, especially β-carotene, was released into the micellar phase when oil is present in an *in vitro* digestion model, except in lutein and α-tocopherol.
- The inclusion of thermal treatment prior making CRF and the addition of oil in simulated i*n vitro* digestion helped to increase the nutrient retention, the nutrient bioaccessibility, and the nutrient available for uptake of carotenoids and vitamin E.

- Heat treatment both steam sterilisation and pasteurisation appeared to inactivate endogenous lipolytic activity of plant material.
- The digestibility of galactolipids in PVH CRF and spinach CRF occurred mainly in intestinal phase of *in vitro* digestion by pancreatic enzymes in both human pancreatic juice (HPJ) and porcine pancreatic extract (PPE) and release free fatty acids, especially α-linolenic acid.
- Both HPJ and PPE could digest the CRF galactolipid; the rate of lipolysis in PPE was slower due to a lower galactolipase activity compared to HPJ that contained main enzyme of galactolipase acivity, particularly pancreatic lipase related protein 2 (PLRP2) and carboxyl ester hydrolase/ bile salt simulated lipase (CEH/BSSL).
- The galactolipase activity in PPE preferred to hydrolyse monogalatosyl diacylglycerol (MGDG) more than digalactosyldiacylglycerol (DGDG) compared to HPJ.

Although the nutrient composition of PVH CRF was not exactly the same as spinach CRF, it was enriched in carbohydrate, lutein, α-tocopherol, iron, and essential fatty acids (mainly linoleic acid (LA) and α-linolenic acid (ALA)). Heat treatment following harvest and the addition of oil to a supplement of freeze-dried PVH CRF would increase the amount of lipophilic nutrient available for uptake by the body. Furthermore, PVH CRF could be used as a substrate for the digestive enzymes in animals and humans, to release lipophilic nutrients, such as β-carotene, lutein, α-tocopherol, omega-3 and omega-6 fatty acids. This information showed that PVH CRF could be used as a novel food supplement with the benefit of recovering galactolipid PUFA and other nutrients from green waste created in agriculture. In European countries, the current recommendation for adults was to decrease their intake of omega-6 and increase intake of omega-3 fatty acid to limit cardiovascular disease and other chronic

diseases (Simopoulos et al., 2000). Galactolipids from PVH CRF contained high levels of ALA (omega-3) and low levels of LA (omega-6), which satisfied these criteria. Presently, ALA supplementation is mostly available to consumers in the form of ALA-rich vegetable oil. However, research into this oil has shown that ALA-enriched emulsions have a high susceptibility to oxidation, the results of which could be harmful to human health, and, in any case, the incorporation of this oil into functional food was still limited (Arab-Tehrany et al., 2012). Even though the stability of ALA in PVH CRF has not yet been extensively studied, the high antioxidant content (β-carotene, lutein, and α-tocopherol) in the chloroplasts of PVH CRF might prevent oxidation occurring in the ALA, resulting in a more stable product.

8.3. Suggestion for future work

There are some suggestions to improve further storage trial study. Firstly, the study of the microbial load of the dried sample during storage should be investigated to ensure food safety. Secondly, nitrogen flushing prior to vacuum packaging or containing the oxygen absorber inside the packages should be done to minimise the presence of oxygen in the packaging. Thirdly, the presence of moisture in the sample might accelerate the chemical breakdown of some lipophilic compounds. Thus, it is recommended to determine the moisture content and water activity inside the non-vacuum packages.

The static *in vitro* digestion model uses just a single set of parameters through the course of the experiment; maintaining the pH, concentration of enzymes, and bile salts. In reality, the biochemical environment of the digestion process changes over time and includes mechanical forces to aid the disintegration of the food material. Therefore, it would propose further research using a semi-dynamic (Mulet-Cabero et al., 2020) or dynamic *in vitro* digestion models (Dupont et al., 2019) to study the

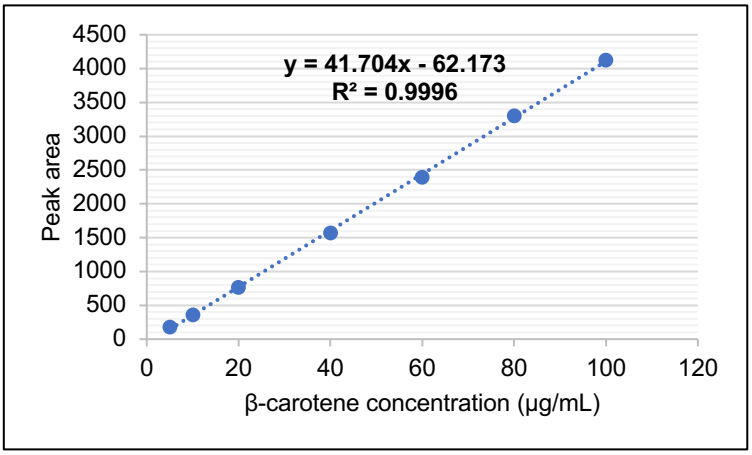
breakdown of chloroplasts and the release nutrients they contain and also compared with the current method used (Minekus et al., 2014).

Furthermore, it would recommend using rabbit gastric extract (RGE), which contains both pepsin and gastric lipase, instead of using only pepsin during *in vitro* digestion of the gastric phase. Also, RGE has a similar range of activity as the human gastric lipase (HGL) (Sams et al., 2018). Thus, using this extract would give more pertinent data compared to using only pepsin during digestion.

This thesis demonstrated that endogenous enzymes in the plant materials could affect the hydrolysis of galactolipids and release of free fatty acids. It is recommended that the sample from plant materials should be treated using heat treatments either heated biomass or heated the juiced biomass prior to making CRF to inactivate the activity of endogenous galactolipase and avoid loss of nutrients. However, it is beneficial if we can further study the effect of endogenous galactolipase enzymes during green biomass processing and its impact on the quality of the liberated chloroplasts in the CRF material.

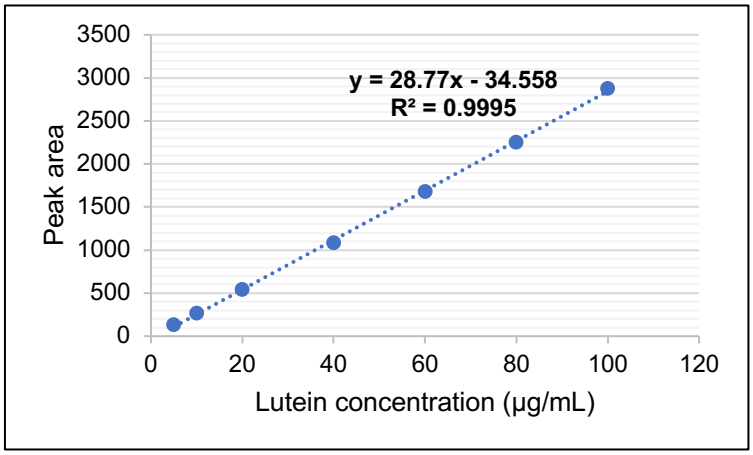
Moreover, several underutilised green plant materials distributed in each country and over the world could be used to generate chloroplast-rich fraction (CRF) after toxicity issues are resolved, and then it could be used for a food supplement.

APPENDICES



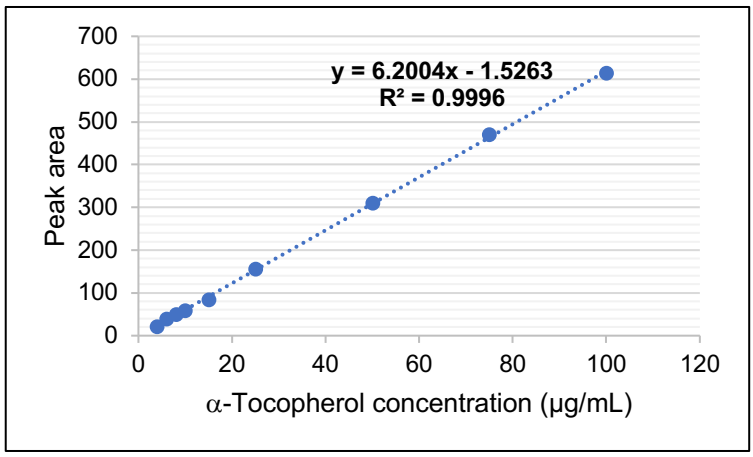
β-Carotene concentration (µg/mL)	Peak area
100	4123.63
80	3307.13
60	2393.37
40	1567.85
20	765.53
10	365.48
5	178.43

Appendix figure 1: Representative of standard curve of β-carotene for quantitation of β-carotene.



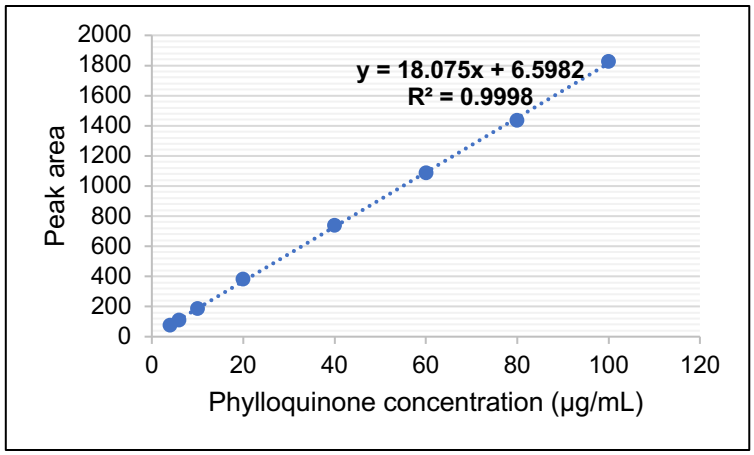
Lutein concentration (µg/mL)	Peak area
100	2876.90
80	2249.31
60	1676.84
40	1083.67
20	537.846
10	265.482
5	130.693

Appendix figure 2: Representative of standard curve of lutein for quantitation of lutein.



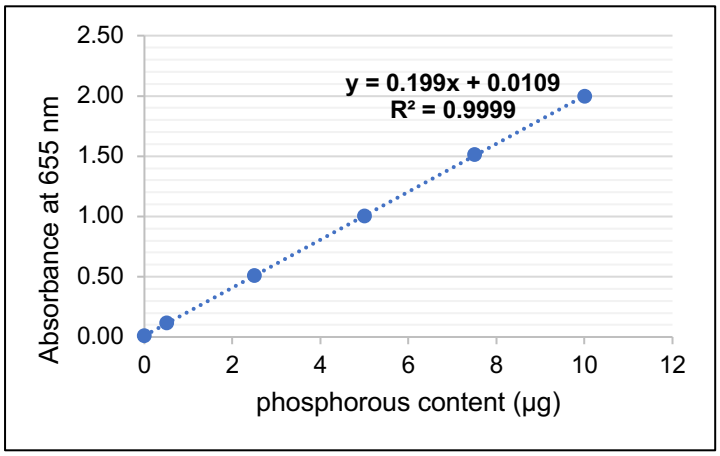
α-Tocopherol concentration (µg/mL)	Peak area
100	613.26
75	469.89
50	310.21
25	155.59
15	84.26
10	59.20
8	49.72
6	39.11
4	21.72

Appendix figure 3: Representative of standard curve of α-tocopherol for quantitation of α-tocopherol.



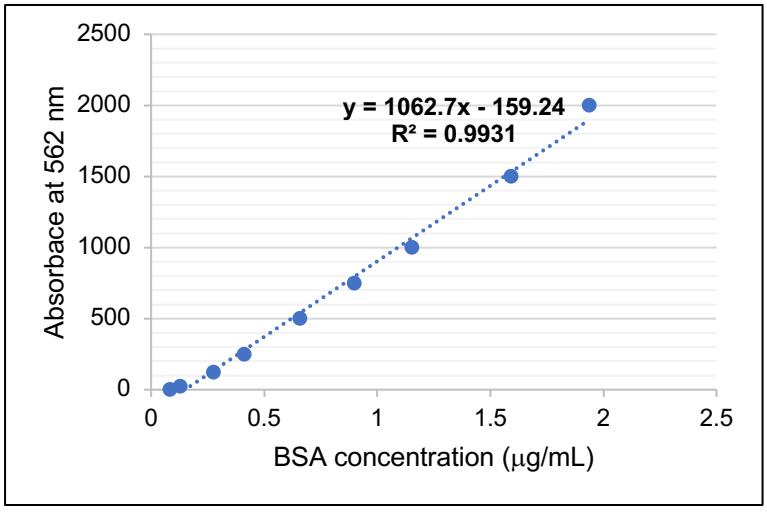
Phylloquinone concentration (µg/mL)	Peak area
100	1824.26
80	1436.91
60	1085.60
40	738.27
20	382.26
10	184.77
6	110.96
4	73.61
2	33.22

Appendix figure 4: Representative of standard curve of phylloquinone for quantitation of phylloquinone.



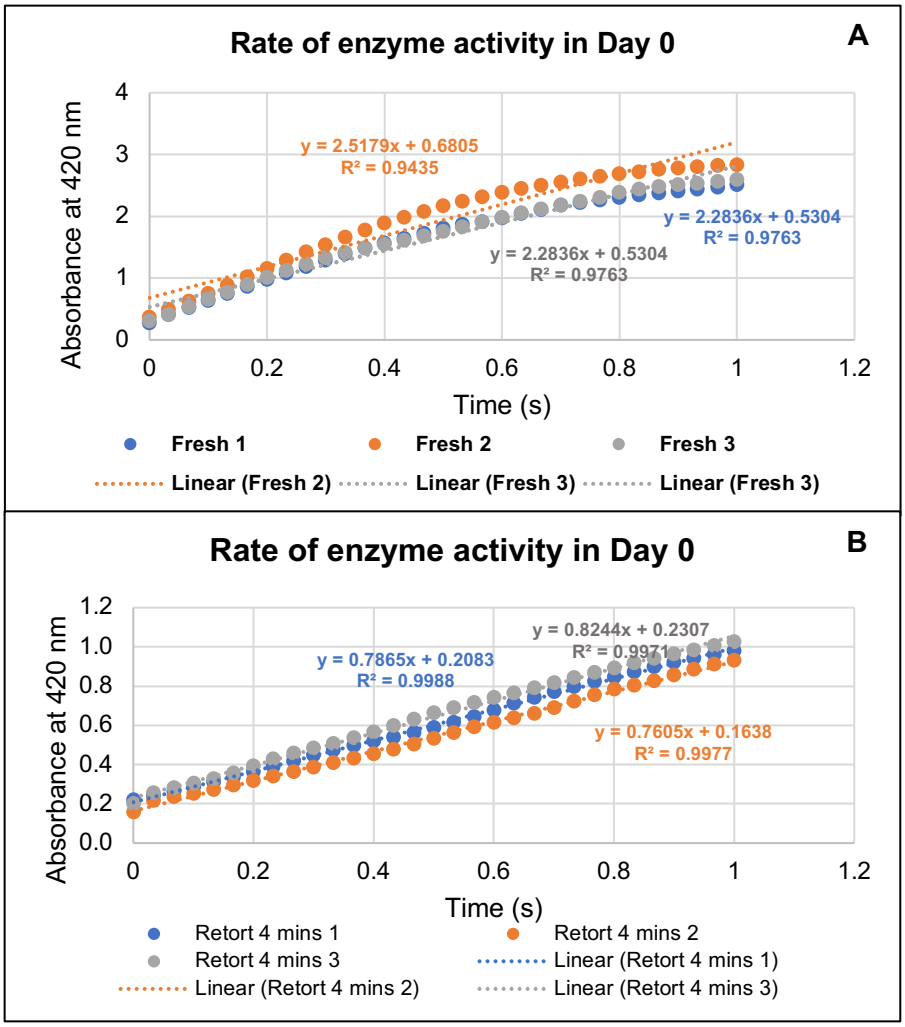
Phosphorous (µg)	Peak area
0	0.008
0.5	0.114
2.5	0.5082
5.0	1.0005
7.5	1.514
10	1.996

Appendix figure 5: Representative of standard curve of phosphorous for quantitation of phytic acid and total phosphorous.



BSA concentration (µg/mL)	A_{562}
0	0.085
25	0.128
125	0.275
250	0.411
500	0.657
750	0.898
1000	1.153
1500	1.592
2000	1.937

Appendix figure 6: Representative of standard curve of bovine serum albumin (BSA) for protein analysis.



Appendix figure 7: The graph of rate of enzyme activity in day 0 **A)** pea vine juice (non-heat treatment) **B)** steam sterilised pea vine juice (retort 4 min).

Appendix table1: Percentage (%) of reduction of enzyme activity in day 0.

	slope 1	slope 2	slope 3	average	% enzyme activity	% enzyme reduction
Fresh (control)	2.2836	2.5179	2.2836	2.3617		
Retort 4 min	0.7865	0.7605	0.8244	0.7905	33.47	66.53

Appendix table 2: Nutrient composition of CRF from spinach leaves using difference CRF preparations between sucrose solution and juicer.

Nutrient	Spinach CRF (mg/g CRF, DW)	
	Grinding in 0.3 M sucrose solution	**Slow-screw twin-gear juicer without added water**
Lipid	305.27 ± 17.34[a]	289.10 ± 7.50[b]
Chlorophyll a	14.89 ± 1.03	15.48 ± 1.86
Chlorophyll b	5.69 ± 0.68	5.71 ± 0.44
Total chlorophyll	21.18 ± 2.54	20.61 ± 1.47
β-Carotene	5.56 ± 0.18[a]	4.74 ± 0.13[b]
Lutein	4.75 ± 0.25[a]	4.38 ± 0.22[b]
α-Tocopherol	0.46 ± 0.03[b]	0.64 ± 0.01[a]
Ascorbic acid	2.60 ± 0.16[b]	2.81 ± 0.16[a]
Phylloquinone (vitamin K1)	1.12 ± 0.06[b]	1.54 ± 0.11[a]
α-Linolenic acid (18:3)	33.74 ± 2.09[a]	31.18 ± 5.11[b]

Data were presented as a mean ± SD of 3 separated samples and analysed using an independent-sample t-test with statistical significance at $p<0.05$, a>b; no alphabet means no significant difference at $p>0.05$.

Appendix table 3: The variation of nutrient composition of PVH in different years.

Nutrient	PVH CRF (mg/g CRF, DW)		
	Summer 2016	**Summer 2017**	**Summer 2018***
Lipid	250.01 ± 19.80^{a}	191.88 ± 38.91^{b}	124.15 ± 6.19^{c}
Chlorophyll a	6.17 ± 0.46^{b}	10.64 ± 0.86^{a}	5.53 ± 0.40^{b}
Chlorophyll b	3.14 ± 0.15^{b}	5.35 ± 0.46^{a}	2.18 ± 0.15^{c}
Total chlorophyll	9.31 ± 0.61^{b}	15.99 ± 1.33^{a}	7.71 ± 0.45^{b}
β-Carotene	2.83 ± 0.12^{a}	0.99 ± 0.06^{b}	0.33 ± 0.01^{c}
Lutein	4.79 ± 0.19^{a}	4.81 ± 0.29^{a}	0.62 ± 0.01^{b}
α-Tocopherol	0.49 ± 0.02^{a}	0.29 ± 0.02^{c}	0.36 ± 0.01^{b}
Phylloquininone	0.64 ± 0.13	0.69 ± 0.08	ND
α-Linolenic acid	15.46 ± 1.03^{a}	16.70 ± 0.89^{a}	6.42 ± 0.42^{b}

**The weather is very hot and dry, no raining during the harvesting.*

Data were presented as a mean ± SD of 3 separated samples and analysed using post-hoc analysis of variance (ANOVA) and according to a Tukey test with statistical significance at $p<0.0.5$, a>b; no alphabet means no significant difference at $p>0.05$; ND: not determined.

Training record during research period

General Training Course			
Course name	**Course location**	**Duration**	**Training point**
Introduction to Academic Writing: Grammatical	D137 Portland Building, University Park	10, 17, 24 Oct 2016	-
Academic Writing Grammatical accuracy	D137 Portland Building, University Park	6 days (31 Oct, 7, 14, 21, 28 Nov, 5 Dec 2016)	-
Grammar Review	B21 Maths & Physics Building, University Park	9 days (25 Jan, 1, 8, 15, 22 Feb, 1, 8, 15, 22 March 2017)	-
Academic Writing: Planning an Assignment	UP-A02 Highfield house, University Park	6 days (14, 21, 28 Feb, 7, 14, 21 March 2017)	-
Getting into habit writing	UP-A11 Highfield house, University Park	21 March 2017	1
Impact workshop for 1st year doctoral student	A29, Vet School, Sutton Bonington campus	30 March 2017	1
Nature of the doctorate and the supervisor progress	B38a, Trent Building, University Park	2 May 2017	1
Planning your research	A01 Highfield house, University Park	4 May 2017	1
Academic Writing: Achieving Clarity	ESLC Room B13, Engineering& Science Learning Centre	6 days (14, 11, 18, 25 May, 1, 8 June 2017)	-
Drafting a chapter of your Thesis	UP-A09 Highfield House, University Park	5 Jun 2017	-
Introduction to the literature review process	Moderated online learning course	(10 days): 8-19 May 2017	3
Understanding your research degree	Standalone online learning course	1 Oct 2016	2
Drafting a chapter of your Thesis	UP-A09 Highfield House, University Park	5 Jun 2017	1
Research Integrity	Standalone online learning course	13 Jun 2017	2

Course name	Course location	Duration	Training point
Fire Safety Training	A17 Plant Science, Sutton Bonington Campus	11 Jul 2017	-
Microsoft Excel: Essentials	LG101, Hallward Library, University Park	25 Jul 2017	2
Microsoft Word: Essentials	LG101, Hallward Library, University Park	3 Aug 2017	2
Unconscious Bias	Standalone online learning course	1 Oct 2017	-
Microsoft PowerPoint: Essential	LG101, Hallward Library, University Park	5 Oct 2017	2
CELE-Improving Pronunciation	B38a Trent building, University Park	9 days (13, 20, 27 Oct, 3, 10, 17, 24 Nov, 1, 8 Dec 2017)	-
CELE-Sutton Bonington Language Support	B07-SR7 Seminar Room, Lecture Bloch, Sutton Bonington	9 days (18, 25 Oct, 1, 8, 15, 22, 29, Nov, 6, 13 Dec 2017)	-
Microsoft Power Point: Creating a Research Poster	Room LG101, Hallward Library, University Park	24 Oct 2017	1
Microsoft: Charts	LG101, Hallward Library, University Park	24 Oct 2017	1
CELE-Academic Writing: Synthesis sources	LG101, Hallward Library, University Park	6 days (30 Oct, 6, 13, 20, 27 Nov, 4 Dec 2017)	-
CELE-Academic Writing: Sciences & Engineering	LG9, Trent Building, University Park	6 days (31 Oct, 7, 14, 21, 28 Nov, 5 Dec 2017)	-
Working with Microsoft Office 365 Online	LG101, Hallward Library, University Park	23 Nov 2017	-
CELE-Effective Academic Reading	LG9, Trent Building, University Park	9 days (26 Jan, 2, 9, 16, 23 Feb, 2, 9, 16, 23 March 2018)	-

Course name	Course location	Duration	Training point
CELE-Effective Academic Reading	LG9, Trent Building, University Park	9 days (26 Jan, 2, 9, 16, 23 Feb, 2, 9, 16, 23 March 2018)	-
CELE-Improving Intonation	LG9, Trent Building, University Park	6 days (13, 20, 27 Feb, 6, 13, 20 March 2018)	-
Microsoft Word: Collaborating	LG101, Hallward Library, University Park	21 Feb 2018	1
Microsoft Excel: Using as a Database	LG101, Hallward Library, University Park	22 Feb 2018	2
Structuring Your Thesis	UP-A09, Highfield house, University Park	6 March 2018	1
Creative Problem Exploration in Research	C09, Engineering & Science Learning Centre	10 May 2018	2
Drafting a Chapter of your thesis	UP-A09, Highfield house, University Park	15 May 2018	1
Where to publish: tools to help you choose (Sciences, Engineering and MHS)	Computer room A13, George Green Library, University Park	17 May 2018	1
Microsoft PowerPoint: Advanced	LG101, Hallward Library, University Park	6 Aug 2018	2
Introduction to Endnote for Researchers	A07 Gateway Building, Sutton Bonington	15 Nov 2018	1
Research ethics for SB doctoral students	B13 Main Building, Sutton Bonington	25 Feb 2019	1
Editing Academic Writing	B2, The Hemsley Building, University Park	18 April 2019	0.5
Total			**32.5**

Disciplinary Training Course			
Course name	**Course location**	**Duration**	**Training point**
Intro to SPSS	Standalone online learning course	1 Oct 2016	2
Statistics and Experimental design for Bioscientists	Gateway Building, Sutton Bonington Campus	5 Oct-14 Dec 2016	10
Nuclear magnetic resonance (NMR) course	Chemistry building, University campus	Dec 2016-Jan 2017	-
Statistic course by Rob Linforth	Lecture block, Sutton Bonington campus	16-18 January 2017 21-23 January 2018	6
GE Protein Workshop (Protein purification workshop)	Gateway building, Sutton Bonington campus	20 Feb 2017	1
Mylnefileld lipid course (Fatty acids and lipid analysis)	The James Hulton institute, Dundee, Scotland	23-24 Feb 2017	2
Food Waste Utilisation& alternative proteins workshop	Committee room, Main building, SB	28 Nov 2017	2
Electron Microscopy: Introduction to Scanning Electron Microscopy (SEM)	A05, Cripps South Building (nmRC)	28 Feb 2018	1
Electron Microscopy: Introduction to Transition Electron Microscopy (TEM)	A05, Cripps South Building (nmRC)	14 March 2018	1
Electron Microscopy: Introduction to SEM and TEM Sample Preparation	A05, Cripps South Building (nmRC)	21 March 2018	1
Conference attendance Manufacturing Food Futures Conference 2018 (attending)	School of Vet, Sutton Bonington Campus	26-27 March 2018	2
Food Flavour course	Gateway Building, Sutton Bonington Campus	10-12 April 2018	6

Course name	Course location	Duration	Training point
Lipid digestion training	CNRS (Bioenergetics and Protein Engineering laboratory), Aix-Marseille university, France	3 June- 2 Nov 2018	-
Total			**34**

Conferences/Symposium (Poster presentation)			
Title	**Location**	**Duration**	**Training point**
31st EFFost conference: "Nutrient composition of chloroplast rich fraction (CRF) from pea vine haulm and spinach leaves" **(Reference number: P1.223)**	Melia Hotel, Sitges, Spain	13-16 Nov 2017	3+2
Postgraduate Symposium: "Effect of steam treatment on nutrient stability of chloroplast-rich fraction (CRF) derived from postharvest, pea vine field residue (haulm)"	The Barn, SB campus	16 May 2018	2+2
14th GERLI International Lipidomics meeting: "Bioaccessibility of lipophilic nutrient in chloroplast-rich fraction (CRF) from postharvest pea vine field residue (haulm)" **(Reference number:** **Poster 23)** **Award:** the best poster	St. Maxima, France	30 July- 3 Oct 2018	4+2

Title	Location	Duration	Training point
6th International Conference on Food Digestion (Infogest) "*In vitro* digestion of galactolipid from chloroplast-rich fraction (CRF) of spinach leaves and postharvest pea vine field residue" **(Reference number: 378/047)**	Granada, Spain (not attended; the poster was presented by Prof. Dr. Frédéric Carrière)	2-4 April 2019	-
9th European Symposium on Plant Lipids "*In vitro* digestion of galactolipid from chloroplast-rich fraction (CRF) of spinach leaves and postharvest pea vine field residue" **(Reference number: PO-38)**	Marseille, Spain (not attended; the poster was presented by Prof. Dr. Frédéric Carrière)	7-10 July 2019	-
17th Euro Fed Lipid Congress and Expo "*In vitro* digestion of galactolipid from chloroplast-rich fraction (CRF) of spinach leaves and postharvest pea vine field residue" **(Reference number: HENU-015)**	Seville, Spain	20-23 Oct 2019	4+2
Total			**21**

Conferences/Symposium (Oral presentation)			
Title	**Location**	**Duration**	**Training point**
Postgraduate Symposium: "Nutrient composition of chloroplast rich fraction (CRF) from pea vine haulm and spinach leaves"	Committee room, main Building, SB campus, UoN	16 May 2017	2+2
4th International Conference on Food Chemistry and Technology (FCT-2018): "Effect of steam treatment on nutrient stability of chloroplast-rich fraction (CRF) derived from postharvest, pea vine field residue (haulm)"	Sheraton Berlin Grand Hotel, Esplanade, Berlin, Germany	5-7 Nov 2018	3+2
Postgraduate Symposium: "*In vitro* digestion of galactolipid from chloroplast-rich fraction (CRF) of spinach leaves and postharvest pea vine field residue"	Lecture Block, SB campus, UoN	26 Jun 2019	2+2
Total			**13**

Supervision (Undergraduate final year project and summer intern)			
Project Title	**Student name**	**Division, University**	**Year**
Bioaccessibility of carotenoid from spinach leaves compared with a chloroplast-rich fraction derived from spinach	Olivia Cox	Food, Nutrition and Dietetics, University of Nottingham	2018
The impact of oil on carotenoid bioaccessibility from a chloroplast-rich fraction from spinach leaves	Victoria King	Food, Nutrition and Dietetics, University of Nottingham	2018

Project Title	Student name	Division, University	Year
Bioaccessibility of mineral from chloroplast-rich fraction of spinach leaves	Beatrice Browning	University of Birmingham	2018
Effect of oil body preparation on digestion; an *in vitro* lipolysis study	Raymond Kato	Food, Nutrition and Dietetics, University of Nottingham	2019
Enzymatic determination of phytic acid in spinach leaf extracts	Katherine Holloway	Food, Nutrition and Dietetics, University of Nottingham	2019
Monitoring leaf galactolipase activity during the liberation of chloroplasts using high performance thin layer chromatography (HPTLC)	Marie Mohammadi	Food, Nutrition and Dietetics, University of Nottingham	2020

Teaching Course Assisted as a demonstrator			
Course name	**Main lecture**	**Division, University**	**Year**
Lipid practical (D21BF1)	Dr. David A. Gray	Food Sciences, UoN	2016/2017
Lipid practical (D21BF1)	Dr. David A. Gray	Food Sciences, UoN	2017/2018
Lipid practical (D21BF1)	Dr. David A. Gray	Food, Nutrition, and Dietetics, UoN	2018/2019
Lipid practical (D21BF1)	Dr. David A. Gray	Food, Nutrition, and Dietetics, UoN	2019/2020

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