Interaction between anthocyanins and carotenoids: effects on bioaccessibility and bioactivities

Minh Anh Thu Phan

A thesis in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Chemical Engineering

Faculty of Engineering

March 2019

Thesis/Dissertation Sheet

Surname/Family Name	:	**Phan**
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Abbreviation for degree as give in the University calendar	:	**PhD**
Faculty	:	**Engineering**
School	:	**Chemical Engineering**
Thesis Title	:	**Interaction between anthocyanins and carotenoids: effects on bioaccessibility and bioactivities.**

Abstract 350 words maximum: (PLEASE TYPE)

The interaction between anthocyanins and carotenoids has not yet been understood. Six different anthocyanins and three carotenoids were mixed in pairs at 1:1, 1:3 and 3:1 ratios (10 μM total) to study their interaction on bioaccessibility and bioactivities by chemical and human Caco-2 cell models. The results showed that anti-oxidative synergy was neither seen in any of the tested carotenoid-anthocyanin mixtures, nor in the models studied. Several combinations such as β-carotene mixed with cyanidin-3-*O*-glucoside or pelargonidin-3-*O*-glucoside showed anti-inflammatory antagonism while other mixtures such as lycopene mixed with malvidin-3-*O*-glucoside (1:1 and 3:1 ratios), or lutein mixed with malvidin-3-*O*-glucoside showed synergy. The impact of anthocyanins (5-7.5 μM) on the cellular uptake of different carotenoids (2.5-5 μM) was varied: increased β-carotene uptake by 68-200%, decreased lycopene uptake by 50-80%, and did not affect lutein uptake.

The effects of co-digestion of vegetables containing anthocyanins and carotenoids on phytochemical bioaccessibility and cellular bioactivities were also investigated to understand the consequences of phytochemical interaction within the food matrix. Red cabbage was co-digested with carrot, cherry tomato or baby spinach (1:1, w:w, total fresh weight of 10 g) with and without added salad dressing. At the end of the digestion, the digestive bioaccessibility of total anthocyanins from the co-digested vegetables increased by 10-15%, but the digestive bioaccessibility of total carotenoids decreased by 21-56% compared to that from the singly digested vegetables. Nevertheless, the intestinal cellular bioaccessibility of total carotenoids from the co-digested vegetables was higher than that from the singly digested vegetables by 46-191%. The digestion of mixed vegetables resulted in an enhancement of the cellular antioxidant activity by 26-31% and the suppression of IL-8 secretion by 27-65%.

The findings of the research indicate the importance of understanding phytochemical interaction and the appropriate combined concentrations that can lead to designing foods or supplements with better-targeted functions and intestinal absorption. The study also unravelled the benefits of vegetable co-ingestion that would lead to better health outcomes. Future studies should focus on understanding the complex interactions on bioaccessibility and bioactivity between multiple phytochemicals and other food components in different food matrices.

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Details of publication #1:

Full title: ***Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability***

Authors: Minh Anh Thu Phan, Janet Paterson, Martin Bucknall, Jayashree Arcot

Journal or book name: Critical Reviews in Food Science and Nutrition

Volume/page numbers: volume 58, pages 1310-1329

Date accepted/ published: published online on 21/07/2017

Status	*Published*	**X**	*Accepted and In press*		*In progress (submitted)*	

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Details of publication #2:

Full title: ***Interactive effects of β-carotene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity in vitro and ex vivo***

Authors: Minh Anh Thu Phan, Martin Bucknall, Jayashree Arcot

Journal or book name: Journal of Functional Foods

Volume/page numbers: volume 45, pages 129-137

Date accepted/ published: published online on 06/04/2018

Status	*Published*	**X**	*Accepted and In press*		*In progress (submitted)*	

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The work is placed in lieu of Chapter 4 and describes the interaction of anthocyanins with the first carotenoid compound (β-carotene) of interest.

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Details of publication #3:

Full title: ***Effect of Different Anthocyanidin Glucosides on Lutein Uptake by Caco-2 Cells, and Their Combined Activities on Anti-Oxidation and Anti-Inflammation In Vitro and Ex Vivo***

Authors: Minh Anh Thu Phan, Martin Bucknall, Jayashree Arcot

Journal or book name: Molecules

Volume/page numbers: volume 23, pages 2035-2046

Date accepted/ published: published online on 14/08/2018

Status	*Published*	**X**	*Accepted and In press*		*In progress (submitted)*	

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Details of publication #4:

Full title: ***Interferences of anthocyanins with the uptake of lycopene in Caco-2 cells, and their interactive effects on anti-oxidation and anti-inflammation in vitro and ex vivo***

Authors: Minh Anh Thu Phan, Martin Bucknall, Jayashree Arcot

Journal or book name: Food Chemistry

Volume/page numbers: volume 276, pages 402-409

Date accepted/ published: published online on 09/10/2018

Status	*Published*	**X**	*Accepted and In press*		*In progress (submitted)*	

The Candidate's Contribution to the Work

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The work is placed in lieu of Chapter 6 and describes the interaction of anthocyanins with the third carotenoid compound (Lycopene) of interest.

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Assoc. Prof. Jayashree Arcot		23/11/2018

Details of publication #5:

Full title: ***Co-ingestion of vegetables affects digestive bioaccessibility of anthocyanins and carotenoids after simulated in vitro gastro-intestinal digestion***

Authors: Minh Anh Thu Phan, Martin Bucknall, Jayashree Arcot

Journal or book name: Food Chemistry

Volume/page numbers:

Date accepted/ published:

Status	*Published*		*Accepted and In press*		*In progress (submitted & under review)*	***X***

The Candidate's Contribution to the Work

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- Designed / set up the methodology in discussion with supervisors
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- Collected, analysed and interpreted experimental data
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The work is placed in lieu of Chapter 7 and describes the effects of co-ingestion of vegetables on the digestive bioaccessibility of anthocyanins and carotenoids.

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Supervisor's name	*Supervisor's signature*	*Date (dd/mm/yy)*
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Details of publication #6:

Full title: ***Effects on intestinal cellular bioaccessibility of carotenoids and cellular biological activity as a consequence of co-ingestion of anthocyanin- and carotenoid-rich vegetables***

Authors: Minh Anh Thu Phan, Martin Bucknall, Jayashree Arcot

Journal or book name: Food Chemistry

Volume/page numbers: volume 286, pages 678-685

Date accepted/ published: 19/02/2019

Status	*Published*	**X**	*Accepted and In press*		*In progress (submitted)*	

The Candidate's Contribution to the Work

- Conceptualised the study in discussion with supervisors
- Designed / set up the methodology in discussion with supervisors
- Conducted all experiments and/or formal analysis
- Collected, analysed and interpreted experimental data
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Location of the work in the thesis and/or how the work is incorporated in the thesis:

The work is placed in lieu of Chapter 8 and describes the effects of co-ingestion of vegetables on the intestinal bioaccessibility of carotenoids and the resultant cellular bioactivities.

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Supervisor's name	*Supervisor's signature*	*Date (dd/mm/yy)*
Assoc. Prof. Jayashree Arcot		28/02/2018

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ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my dearest supervisor Assoc. Prof. Jayashree Arcot for her dedicative guidance and supports during my candidature. She has provided me with direction, encouragement and valuable advices to keep my project always on track. I would also like to send my thanks to my co-supervisor Dr. Martin Bucknall from the Bioanalytical Mass Spectrometry Facility (BMSF) for his encouragement and support.

I am also very grateful to Mr. Camillo Taraborreli for his technical support on ordering research materials and provision of lab consumables during my research at UNSW.

I would also thank Mr. Lewis Adler and Dr. Russell Pickford from BMSF for providing me with their expert advices and technical supports on using mass spectrometry instruments.

I am also sincerely grateful to the Australian Government who provided me with an Endeavour Postgraduate Scholarship to support for my entire study program.

Last but not least, I would like to thank my family for being with me all the time throughout my PhD journey. Thanks are also sent to my office colleagues and friends who have boosted my mood when I was put down by failed experiments.

LIST OF PUBLICATIONS, SUBMITTED MANUSCRIPT AND PRESENTATIONS

Publications:

Phan, M. A. T., Paterson, J., Bucknall, M., & Arcot, J. (2018). Interactions between phytochemicals from fruits and vegetables: Effects on bioactivities and bioavailability. *Critical Reviews in Food Science and Nutrition*, *58*(8), 1310-1329.

Phan, M. A. T., Bucknall, M., & Arcot, J. (2018). Interactive effects of β-carotene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity in vitro and ex vivo. *Journal of Functional Foods*, *45*, 129-137.

Phan, M. A. T., Bucknall, M., & Arcot, J. (2018). Effect of Different Anthocyanidin Glucosides on Lutein Uptake by Caco-2 Cells, and Their Combined Activities on Anti-Oxidation and Anti-Inflammation In Vitro and Ex Vivo. *Molecules*, *23*(8), 2035

Phan, M. A. T., Bucknall, M. P., & Arcot, J. (2019). Interferences of anthocyanins with the uptake of lycopene in Caco-2 cells, and their interactive effects on anti-oxidation and anti-inflammation in vitro and ex vivo. *Food Chemistry*, *276*, 402-409.

Phan, M. A. T., Bucknall, M. P., & Arcot, J. (2019). Effects on intestinal cellular bioaccessibility of carotenoids and cellular biological activity as a consequence of co-ingestion of anthocyanin- and carotenoid-rich vegetables. *Food Chemistry*, 286, 678-685.

Submitted manuscript (under review):

Phan, M. A. T., Bucknall, M. P., & Arcot, J. (2019). Co-ingestion of vegetables affects digestive bioaccessibility of anthocyanins and carotenoids after simulated in vitro gastro-intestinal digestion – submitted to *Food Chemistry*.

Presentations

Phan, M. A. T., Bucknall, M., & Arcot, J. (2017). Interaction between β-carotene and anthocyanins antioxidant activities in vitro and ex vivo. Presented at the 10th International Conference and Exhibitionon Nutraceuticals and Functional Foods: Platform for Food and Medical Sciences (ISNFF 2017), Gunsan, South Korea, 22-25 October 2017 – poster presentation.

Phan, M. A. T., Bucknall, M., & Arcot, J. (2017). Impact on β-carotene absorption in the presence of anthocyanins using intestinal caco-2 cells. Presented at the 5th International Vitamin Conference: Vitamins and Well-being-from womb to tomb, 8-10 August 2018 – oral presentation.

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABIP	2,2’-azobis[2-(2imidazolin-2-yl)propane] dihydrochloride
ABTS	2,2’-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
AMVN	2,2’-azobis(2,4-dimethylvaleronitrile
ANOVA	Analysis of variance
AOXs	Antioxidants
APCI	Atmospheric pressure chemical ionization probe
ASC	Ascorbic acid
ASTA	Astaxanthin
BAC	Bioaccessibility
BAI	Baicalein
BHT	Butylated hydroxytoluene
CAA	Cellular antioxidant activity
CAG	Chlorogenic acid
CAR	β-carotene
cBAC	Intestinal cellular bioaccessibility
CAT	Catechin
CFA	Caffeic acid
CG	Cyanidin-3-*O*-glucoside (chloride)
CI	Combination index
CNA	Carnosic acid
CTG	Catechin gallate
CUPRAC	Cupric reducing antioxidant capacity

DAI	Daidzein
dBAC	Digestive bioaccessibility
DBPS	Dulbecco phosphate buffer saline
DCFH-DA	Dichlorodihydrofluorescein diacetate
DG	Delphinidin-3-*O*-glucoside (chloride)
DMEM	Dulbecco's modified eagle medium
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EAE	Expected additive effect
ECAT	Epicatechin
ECG	(-)-Epicatechin gallate
EGA	Ellagic acid
EGC	(-)-Epigallocatechin
EGCG	(-)-Epigallocatechin gallate
EPE	Experimental effect
ESI	Electrospray ionization
ET	Electron transfer
FRA	Ferulic acid
FRAP	Ferric ion reducing antioxidant power
FW	Fresh weight
GCG	Gallocatechin gallate
GLB	Glabridin
HAT	Hydrogen atom transfer
HESI	Heated electrospray ionization
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration

IL-8	Interleukin-8
KAEM	Kaempferol
KGP	Kaempferol glycopyranoside
LC-MS	Liquid chromatography-Mass spectrometry
LDL	Low density lipoprotein
LOX-1	Lipoxygenase type I
LUT	Lutein
LYC	Lycopene
MG	Malvidin-3-*O*-glucoside (chloride)
MOR	Morin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYR	Myricitin
m/z	Mass-to-charge ratio
NAR	Naringin
NARG	Naringenin
NCH	Naringenin chalcone
NDCH	Naringin dihydrochalcone
n.d	Not detected
NO	Nitric oxide
HAT	Hydrogen atom transfer
HESD	Hesperidin
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
PC	L-α-phosphatidylcholine
PDA	Photodiode array

PLG	Pelargonidin-3-*O*-glucoside (chloride)
PNG	Peonidin-3-*O*-glucoside (chloride)
4'-PROP	4'-propylpeurarin
PTG	Petunidin-3-*O*-glucoside (chloride)
PUE	Puerarin
Q3G	Quercetin-3-glucoside
QGP	Quercetin galactopyranoside
QRP	Quercetin rutinpyranoside
QUE	Quercetin
RMA	Rosmarinic acid
ROS	Reactive oxygen species
RUT	Rutin
SD	Standard deviation
SRM	Selective reaction monitoring
TAC	Total anthocyanin content
TBARS	Thiobarbituric acid reagent species
TEAC	Trolox equivalence antioxidant capacity
TNF-α	Tumour necrosis factor-α
TIC	Total ion current
TPC	Total phenolic content
TRAP	Total radical trapping antioxidant parameter
UHPLC	Ultra high performance liquid chromatography
UPLC	Ultra performance liquid chromatography
ZEA	Zeaxanthin
v/v	Volume per volume

w:w	Weight: weight ratio
w/w	Weight per weight
α-TOC	α-tocopherol
β-CAR	β-carotene
β-CRYP	β-cryptoxanthin
γ-TOC	γ-tocotrienol

ABSTRACT

The interaction between anthocyanins and carotenoids has not yet been understood. Six different anthocyanins and three carotenoids were mixed in pairs at 1:1, 1:3 and 3:1 ratios (10 μM total) to study their interaction on bioaccessibility and bioactivities by chemical and human Caco-2 cell models. The results showed that anti-oxidative synergy was neither seen in any of the tested carotenoid-anthocyanin mixtures, nor in the models studied. Several combinations such as β-carotene mixed with cyanidin-3-*O*-glucoside or pelargonidin-3-*O*-glucoside showed anti-inflammatory antagonism while other mixtures such as lycopene mixed with malvidin-3-*O*-glucoside (1:1 and 3:1 ratios), or lutein mixed with malvidin-3-*O*-glucoside showed synergy. The impact of anthocyanins (5-7.5 μM) on the cellular uptake of different carotenoids (2.5-5 μM) was varied: increased β-carotene uptake by 68-200%, decreased lycopene uptake by 50-80%, and did not affect lutein uptake.

The effects of co-digestion of vegetables containing anthocyanins and carotenoids on phytochemical bioaccessibility and cellular bioactivities were also investigated to understand the consequences of phytochemical interaction within the food matrix. Red cabbage was co-digested with carrot, cherry tomato or baby spinach (1:1, w:w, total fresh weight of 10 g) with and without added salad dressing. At the end of the digestion, the digestive bioaccessibility of total anthocyanins from the co-digested vegetables increased by 10-15%, but the digestive bioaccessibility of total carotenoids decreased by 21-56% compared to that from the singly digested vegetables. Nevertheless, the intestinal cellular bioaccessibility of total carotenoids from the co-digested vegetables was higher than that from the singly digested vegetables by 46-191%. The digestion of mixed vegetables resulted in an enhancement of the cellular antioxidant activity by 26-31% and the suppression of IL-8 secretion by 27-65%.

The findings of the research indicate the importance of understanding phytochemical interaction and the appropriate combined concentrations that can lead to designing foods or supplements with better-targeted functions and intestinal absorption. The study also unravelled the benefits of vegetable co-ingestion that would lead to better health outcomes. Future studies should focus on understanding the complex interactions on bioaccessibility and bioactivity between multiple phytochemicals and other food components in different food matrices.

CHAPTER 1

Introduction

Fruits and vegetables are part of human diets, which provide essential vitamins and functional phytochemicals (Holst and Williamson, 2008). These compounds can interact with each other when they are co-present in foods or co-digested and affect each other's bioaccessibility/bioavailability, which eventually alters the outcome of biological effects (Phan et al., 2018c, Wang and Zhu, 2017). Phytochemical interaction may result in synergy, addition or antagonism in biological effects (Phan et al., 2018c, Wang and Zhu, 2017). It is, therefore, important to understand the interactive effects of food phytochemicals on bioaccessibility/bioavailability and biological activities.

Phytochemicals or phytochemical compounds refer to plant secondary metabolites which protect the plant from a variety of stresses (Holst and Williamson, 2008). When consumed in the diet, they exhibit beneficial effects that promote better health. In addition, unlike nutrients, phytochemicals may not be essential throughout life and cause clinically manifested deficiencies. Nevertheless, they are essential for health and well-being in adulthood and in the elderly population (Holst and Williamson, 2008). Likewise, bioactive compounds are defined as "phytochemicals that are present in foods and are capable of modulating metabolic processes, resulting in the promotion of better health" (Carbonell-Capella et al., 2014). Therefore, the terms "phytochemicals" or "phytochemical compounds" are used interchangeably with the term "bioactive compounds" throughout the thesis.

Several studies have reported about the interaction between bioactive compounds of same polarity (Campbell et al., 2006, Colon and Nerín, 2016, Gonzales et al., 2015,

Hidalgo et al., 2010, Lowe et al., 2009, Rossetto et al., 2002, van den Berg, 1999). Nevertheless, there is limited understanding about the interaction between phytochemical compounds of different polarity. Anthocyanins and carotenoids are ones of the major groups of hydrophilic and hydrophobic phytochemicals respectively in plant-based food materials (He and Giusti, 2010, Saini et al., 2015). They are co-present in some fruits and vegetables, and can be co-digested in diets. Their interaction, however, has not yet been understood.

Traditional definition of bioaccessibility of phytochemicals refers to the fractions of phytochemicals that are bioaccessible from undigested food materials and available for absorption (Cilla et al., 2012). The bioaccessibility and bioactivity of phytochemicals from a singly digested food are often reported in most studies (Boileau et al., 2002, Frank et al., 2003, Charron et al., 2009, Mein et al., 2008) although in fact different fruits and/or vegetables are usually co-consumed in human diets. There are only few studies reporting the effect of food combinations on antioxidant and anti-inflammatory activities *in vitro* using chemical assays (Durak et al., 2014, Durak et al., 2015, Swada et al., 2016, Wang et al., 2011). There is no study investigating the effects of fruit and/or vegetable co-digestion on phytochemical bioaccessibility and bioactivity using a cellular model. When different fruits/vegetables are co-digested, complex interaction between different phytochemicals in the food matrices can occur and result in changes in phytochemical bioaccessibility and bioactivity (Gawlik-Dziki, 2012, Efferth and Koch, 2011, Wang and Zhu, 2017).

In vitro simulated digestion models have been developed and used to predict the release of phytochemicals from food matrix, their bioaccessibility and profile changes prior to absorption (Alminger et al., 2014, Guerra et al., 2012). Most reported models simulate

digestion in oral cavity, the stomach, the small intestine, and in some occasions, the large intestine to include the colonic fermentation. The design of the digestion models depends on the type of phytochemicals studied (lipophilic/hydrophilic) and the purpose of research (screening or studying under close physiological conditions) (Alminger et al., 2014, Guerra et al., 2012). For screening studies with large sample size, static digestion models with fixed conditions (enzyme concentrations, bile salts, pH and so on) should be employed (Alminger et al., 2014). For studies mimicking physiological conditions, dynamic models with varying concentrations of digestive components are more appropriate (Alminger et al., 2014).

The present project aimed to investigate the interactions between some major dietary anthocyanins and carotenoids in pure form and in the food matrix on cellular uptake and biological properties including antioxidant and anti-inflammatory activities.

The main objectives of the present research project are:

1. to investigate how the co-presence of different pure compounds of anthocyanins and carotenoids in pairs at different ratios would affect the antioxidant activities; the anti-inflammatory activities; and the phytochemical cellular uptake.

Different pairs of pure anthocyanins and carotenoids at different ratios were assessed for their combined activity on liposome peroxidation inhibition and lipoxygenase inhibitory activity using chemical models, and cellular antioxidant and anti-inflammatory activities using a human carcinogenic Caco-2 cell model. The interference of anthocyanins on the cellular uptake of carotenoids was also evaluated because hydrophilic bioactive compounds can interfere with the uptake of hydrophobic compounds but not *vice versa*.

2. to investigate how the co-digestion of anthocyanin-containing vegetables and carotenoid-containing vegetables would affect the bioaccessibility of anthocyanins and carotenoids; the cellular antioxidant activity; and the cellular anti-inflammatory activities.

Red cabbage was co-digested with carrot, cherry tomato or baby spinach in pairs and altogether at an equal weight ratio with and without added salad dressing. The digestive bioaccessibility of major anthocyanins and carotenoids from the vegetable mixtures with and without added the oil-based dressing was determined and compared with that from the singly digested vegetables. The digesta of the single and mixed vegetable with and without added the oil-based dressing was examined for their cellular antioxidant and anti-inflammatory activities using a Caco-2 cell model. The intestinal cellular bioaccessibility of carotenoids from singly digested vegetables and co-digested vegetables with and without added the oil-based dressing was also evaluated.

CHAPTER 2

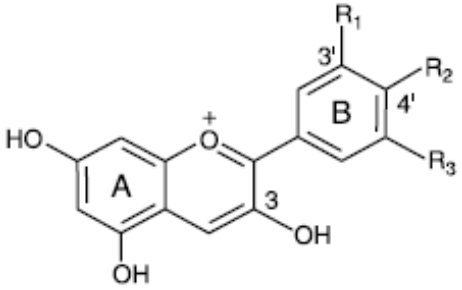
Literature review

2.1. Chemistry of anthocyanins and carotenoids

2.1.1. Anthocyanins

Anthocyanins are referred to as the glycosidic forms of anthocyanidin aglycones. There are six common anthocyanidins, including cyanidin, delphinidin, pelargonidin, malvidin , petunidin and peonidin whose chemical structures are mainly different at the 3' and 5' positions of the B-ring. The first three compounds are non-methylated anthocyanidins, whereas the last three candidates contain methoxy group(s) on their structure (Bueno et al., 2012b). The chemical structure of these aglycones and their corresponding colour are presented in **Figure 1**. These anthocyanidins are rarely found in plants, but instead their glycosylated forms (i.e. anthocyanins) are present abundantly in many fruits and vegetables. Anthocyanins are more stable than their parental aglycones as the glycosylation promotes the formation of intramolecular hydrogen bonds within the anthocyanin molecule (Borkowski et al., 2005). Glycosylation can occur with different sugar moieties including glucose (most predominant), rhamnose, galactose, rutinose, xylose, arabinose, sambubiose or other sugars (He and Giusti, 2010). These sugar substitutes are normally linked to the anthocyanidin aglycone through O-linkages mostly at the 3-OH position, or less common at both 3-OH and 5-OH positions on anthocyanin molecules (Bueno et al., 2012b). They may also be acylated with aromatic acids (e.g. caffeic, p-coumaric, sinapic and ferulic acids), or with aliphatic acids (e.g. acetic, malonic, cinnamic, malic and succinic acids) (Bueno et al., 2012b, He and Giusti, 2010).

The physical and chemical properties as well as the biological activities of anthocyanins are determined by a variety of chemical features including the number of hydroxyl groups, the type and position of the attached sugar residues, as well as the type and number of acylated groups linked to the sugar moieties. These chemical features affect the molecular size, polarity and spatial conformations of anthocyanins, and consequently influence the compound bioavailability (Bueno et al., 2012b).



Anthocyanidins	R1	R2	R3	Colour
Cyanidin	OH	OH	H	Magenta
Delphinidin	OH	OH	OH	Purple
Malvidin	OCH_3	OH	OCH_3	Purple
Pelargonidin	H	OH	H	Red
Petunidin	OCH_3	OH	OH	Purple
Peonidin	OCH_3	OH	H	Magenta

Figure 1. Chemical structure of anthocyanidins (Bueno et al., 2012b, He and Giusti, 2010)

2.1.2. Carotenoids

Around 700 carotenoids have been identified and they all share a common chemical structure, which is characterised by a long chain of conjugated double bonds and a poly-isoprenoid structure (Rao and Rao, 2007, Clinton, 1998, Srivastava and Srivastava, 2013, Young and Lowe, 2001). The conjugated C=C system of carotenoids is accounted for their ability to quench singlet oxygen. Carotenoids are divided into three different groups including carotenes (for example: α-carotene, β-carotene), lycopene and xanthophylls (for example: lutein, β-cryptoxanthin and canthaxanthin) (**Figure 2**) (Young and Lowe, 2001, Clinton, 1998). The first two groups are lipophilic carotenoids, whilst the last one is polar oxycarotenoids (Clinton, 1998). Most

carotenoids found in nature are in all-*trans* configuration, which is a very thermodynamically stable form (Clinton, 1998). The all-*trans* carotenoids can be isomerised to *cis* isomers depending on the environmental conditions (e.g. light, high temperature, chemical reactions) (Clinton, 1998).

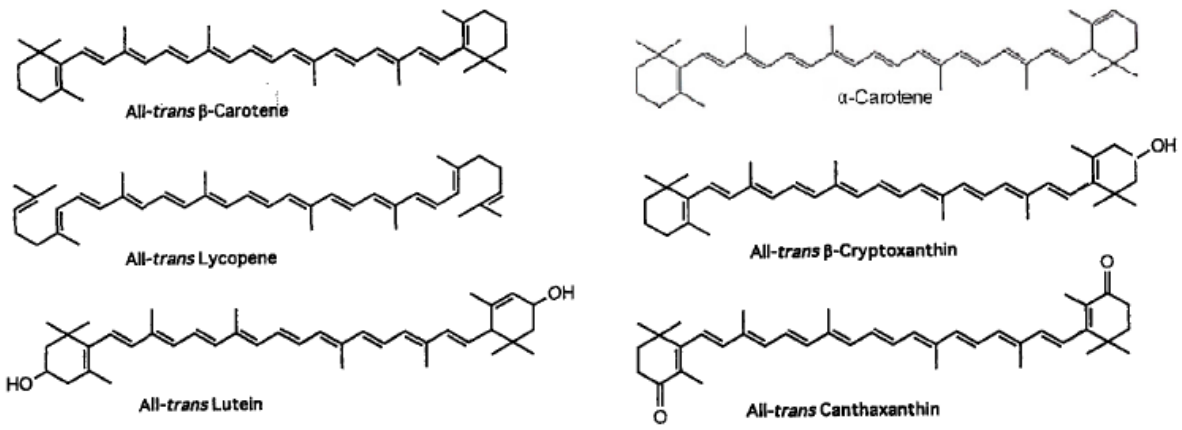


Figure 2. Some major carotenoids in nature (Clinton, 1998)

2.2. Biological activities of anthocyanins and carotenoids

2.2.1. Anthocyanins

Anthocyanins, like most of other polyphenols, are potent antioxidants. Antioxidant activities of a compound are demonstrated by the ability to deactivate reactive oxygen species (ROS) generated in the body, which include free radicals, singlet oxygen and peroxides (He and Giusti, 2010). Several *in vitro* and *in vivo* studies have reported that anthocyanins are very powerful antioxidants whose acting potency is modulated by their chemical structure. The capacity of receiving unpaired electrons from radicals of anthocyanin compounds depends on the types and positions of the chemical moieties that locate in the aromatic ring of anthocyanin molecules (Satué-Gracia et al., 1997, Wang et al., 1997). The free radical scavenging capacity of anthocyanins is equivalent to other synthetic antioxidants such as tert-butylhydroquinone, butylated hydroxytoluene and butylated hydroxyanisole, and far more effective than vitamin E (Galvanoa et al., 2004). Wang et al. (1997) used oxygen radical absorbance capacity

(ORAC) assay to evaluate the antioxidant activities of 14 anthocyanin compounds. They found that cyanidin-3-glucoside provides highest ORAC activity, which is able to quench free radicals at 3.5 times stronger than Trolox (vitamin E analog). The antioxidant strength of cyanidin-3-glucoside is 4 times higher than that of Trolox (Rice-evans et al., 1995). Other anthocyanin compounds such as cyanidin-3-glucosylrutinoside and cyanidin-3-rutinoside also have higher free radical scavenging potency than vitamin E (Seeram et al., 2001).

Anthocyanin aglycones and their glycosides also have the capability of chelating metal ions, inhibiting oxidative enzymes and protecting DNA from oxidative damage (Galvanoa et al., 2004). Some anthocyanin compounds isolated from fruits effectively inhibit lipid peroxidation (Wang et al., 1999) and/or prevent human low density lipoprotein (LDL) from oxidation (Satué-Gracia et al., 1997) by their ROS scavenging and metal-ions chelating properties (Sarma et al., 1997). For instance, the tart cherry anthocyanins and their cyanidin aglycone suppress lipid peroxidation by 39% to 75%, which is similar to the effect of the synthetic anthocyanin compounds and stronger than the effect of vitamin E (Wang et al., 1999). Amorini et al. (2001) reported that cyanidin-3-glucoside is more efficient than resveratrol and ascorbic acid in preventing LDL oxidation. The *in vivo* anti-oxidative effects of anthocyanins are also seen in rat and human models. The results of these studies suggest that the consumption of anthocyanin-rich foods significantly enhances overall antioxidant capacity of the body that decreases LDL oxidation and protects DNA from oxidative damage.

Dietary intake of anthocyanin-containing foods improves overall plasma antioxidant status that contributes to retard LDL oxidation and protects the cells from oxidative stress, and thereby prevents the development of cardiovascular disorders. The development of vascular diseases is, to whatever extent, related to the dysfunction of the

epithelial cells (Lila, 2004). Youdim et al. (2000) reported that cyanidin glycosides from elderberry extract, including cyanidin-3-glucoside, cyanidin-3-sambubioside-5-diglucoside, cyanidin-3,5-diglucoside and cyanidin-3-sambubioside, could incorporate themselves into plasma membrane and endothelial cells and protect them from oxidative damage. This protective effect provided by anthocyanin compounds reduces the risk of cardiovascular diseases. The consumption of anthocyanin-rich food products such as grape juice and wine results in several health beneficial effects including reduced inflammation, improved capillary strength and permeability, decreased platelet formation and decreased nitric oxide release, which would contribute to prevent heart attacks (Folts, 1998). A systemic review from several randomised controlled trials evaluating the effects of purified anthocyanins and anthocyanin-rich extracts on various biomarkers of cardiovascular disease shows that supplementation with anthocyanins significantly improves LDL cholesterol among patients or those with high level of biomarkers (Wallace et al., 2016). Certain anthocyanins provide protection against heart injuries induced by ischemia/reperfusion by activating signal transduction pathways and sustaining mitochondrial functions instead of acting solely as antioxidants (Liobikas et al., 2016). The proposed underlying mechanisms of the cardioprotective activity of anthocyanins include the reduction of cytosolic cytochrome C preventing apoptosis and sustainment of electron transfer between NADH dehydrogenase and cytochrome C supporting oxidative phosphorylation in ischemia-damaged mitochondria (Liobikas et al., 2016).

Apart from their well-known antioxidant activity, anthocyanins also exert other biological activities including anti-inflammatory- , anti-carcinogenic-, anti-mutagenic-, and antimicrobial activities (He and Giusti, 2010, Prior and Wu, 2006, Galvanoa et al., 2004), properties for which anthocyanins have been prescribed as medicines in several

countries for thousands of years (Smeriglio et al., 2016). The consumption of diets rich in anthocyanins is also beneficial for the prevention of obesity and diabetes (Tsuda, 2008, Tsuda et al., 2003) and the improvement of eye vision (Kalt et al., 2008, Nakaishi et al., 2000). In addition, anthocyanin-enriched extract is a promising alternative agent to modulate mitochondrial dysfunctions and redox state of cells that are highly relevant to neurodegenerative diseases such as Parkinson's disease (Neves et al., 2019). Epidemiological investigation and nutritional intervention of anthocyanins have shown that patients with metabolic syndrome related chronic diseases could be beneficial from broad-spectrum biological effects of anthocyanins on adipocytes, endothelial cells, inflammatory cells, hepatocytes, intestinal cells and gut microbiota (Jiang et al., 2019). Furthermore, the lower molecular weight metabolites of the unabsorbed anthocyanins transformed by the colon microbiota in the large intestine may become efficiently absorbed in the colon and exert bioactivities (Williamson and Clifford, 2010). Krga et al. (2018) reported that several anthocyanins and their gut metabolites decreased *ex vivo*-induced platelet activation and reduced platelet aggregation with leukocytes. For instance, cyanidin-3-arabinoside, delphinidin-3-glucoside, and peonidin-3-glucoside decreased agonist-induced P-selectin expression; while the gut metabolites hippuric and protocatechuic acids inhibited P-selectin expression, and 4-hydroxybenzaldehyde affected P-selectin expression, platelet-neutrophil and monocyte aggregation (Krga et al., 2018).

Dietary phytochemicals must be bioavailable to confer health benefits; that is, once administered as components in foods, the digested bioactive compounds must be able to reach target tissues or organs in the human body to elicit an effect (Lila et al., 2016). Anthocyanins are infamous for their low bioavailability ranging from 1-2%, with only trace quantities detected in the expected target organs or in the bloodstream (Lila et al.,

2016). Bioavailability refers to the degree to which an ingested anthocyanin is available to a target tissue (Lila et al., 2016). Pre-systemic metabolism of anthocyanins, however, may cause their bioavailability to be greatly underestimated if only parent compounds and/or phenolic acid breakdown products are targeted in bioassays (Fang, 2014). After the intestinal absorption, anthocyanin glycosides may undergo substantial first-pass metabolism before being circulated in bloodstream and redistributed or excreted in urine or faeces. Using isotopical labeled C^{13}-anthocyanin tracer, de Ferrars et al. (2014) identified 35 metabolites of cyanidin-3-glucoside after the administration of [^{13}C]-cyanidin-3-glucoside in human, of which 17 compounds were detected in the circulation. The cyanidin-3-glucoside metabolites found in serum were mainly comprised of protocatechuic acid (PCA) and phloroglucinaldehyde (PGA), as well as phase II PCA conjugates such as vanillic acid, hippuric acid, ferulic acid, and 4-hydroxybenzaldehyde (de Ferrars et al., 2014). Similar biotransformation of anthocyanin aglycones could also occur at liver. Hepatic microsomal cells can metabolise cyanidin to PCA, which is further glucuronidated to form other conjugates (Woodward et al., 2011). Czank et al. (2013) used isotopical labeling for tracing cyanidin-3-glucoside-derived metabolites and found that the total systemic bioavailability of anthocyanins was up to 12.38% (Woodward et al., 2011). This indicates that these compounds are as bioavailable as other flavonoids such as flavan-3-ols and flavones whose bioavailability ranges from 2.5% to 18.5% (Manach et al., 2005, Williamson and Manach, 2005). Thus, the total bioavailability of anthocyanins is much greater than previously credited when taking into account the unmetabolised parent compounds, phase I and phase II metabolites, conjugated products, and microbe-generated metabolites (Lila et al., 2016). The relative contributions of each of these

classes of metabolites to human health maintenance are still under investigation (Lila et al., 2016).

Anthocyanins, although are eventually extensively degraded to phenolic acids *in vivo*, are persistent in the gastrointestinal tract and can be excreted in faeces in intact form (Lila et al., 2016). They can deposit at a number of tissues and undergo massive enteric and enterohepatic circulation (de Ferrars et al., 2014, Fang, 2014, Lila et al., 2016). This indicates that the gastrointestinal tract and other tissues and organs are chronically exposed to the C6-C3-C6 structured anthocyanins (Lila et al., 2016). Therefore, anthocyanins can be appreciated for the exertion of their well-documented health benefits as intact flavonoids to a far greater extent than originally envisioned and well before ring fission that yields phenolic acid degradation products (Lila et al., 2016).

2.2.2. Carotenoids

A number of human epidemiological studies have revealed that the consumption of diets rich in carotenoids would lower the risks of various chronic diseases resulting from prolonged oxidative stress induced by ROS (Maiani et al., 2009). Carotenoids efficiently quench free radicals and singlet oxygen species to protect cells and tissues against oxidative damage, and prevent LDL oxidation. These antioxidant properties of carotenoids contribute to the prevention of ischemic stroke, heart attacks and other cardiovascular diseases (Perveen et al., 2015). For instance, lycopene reduces cholesterol synthesis by 73%, and increases LDL degradation and LDL removal from circulation by 34% and 110% respectively (Clinton, 1998).. The reductions of cholesterol level and LDL content in blood stream eventually result in lower risk of heart-related diseases for people having lycopene on a regular basis (Clinton, 1998). Carotenoids can exert anti-carcinogenic activity. Epidemiological studies have shown a correlation between a high carotenoid intake in the diet with a reduced risk of breast,

cervical, ovarian, colorectal cancers, and cardiovascular and eye diseases (Milani et al., 2017). β-carotene and lycopene provide protective effects against prostate, lung and stomach cancers (Maiani et al., 2009). The molecular mechanisms behind the anti-cancer property of carotenoids are modulation of gene expression, regulation of cell growth, improvement of gap junction communication, enhancement of immune response and modulation of enzymes of drug metabolism (Bertram, 1999, Rao and Rao, 2007, Milani et al., 2017). In addition, carotenoids have been well studied in relation to their beneficial role in the prevention of preeclampsia (Zielińska et al., 2017). It is currently hypothesised that carotenoids can play an important role in the prevention of preterm birth and intrauterine growth restriction (Zielińska et al., 2017).

These biological benefits are only offered if a reasonable amount of carotenoids is consumed. An overconsumption or overdose of carotenoids in diets or supplements may cause several adverse effects, especially for smokers and alcohol drinkers (Wang, 2012b). In fact, several animal and human studies reported that β-carotene exhibits pro-oxidant properties, which improves health quality if taken at appropriate levels or otherwise causes negative effects if overused (Rao and Rao, 2007). Rats supplemented with high quantity of β-carotene are more likely to suffer from oxidative stress and show increase in phase I enzyme activities in liver, kidney and intestine (Paolini et al., 2001). In humans, the overdose of β-carotene could accelerate lung cancer and cardiovascular diseases in smokers (Omenn et al., 1996).

2.3. Interactions between phytochemicals from fruits and vegetables: effects on bioactivities and absorption/bioavailability

(This is an updated version of the Accepted Manuscript of an article published by Taylor & Francis in Critical Reviews in Food Science and Nutrition on 21 Jul 2017, available online: https://doi.org/10.1080/10408398.2016.1254595)

Abstract

The combinations of two or more phytochemicals bring about changes in the ultimate biological effects and/or the cellular absorption/bioavailability of each component. A number of mixtures of pure bioactive compounds or phytochemical-containing plant extracts provide synergy with regard to antioxidant status, anti-inflammation, anti-cancer and chemoprevention of several oxidative stress and metabolic disorders *in vitro*. The biological activities of food phytochemicals depend upon their bioaccessibility and bioavailability which can be affected by the presence of other food components including other bioactive constituents. The interactions between phytochemicals during intestinal absorption could result in changes in the uptake/bioavailability of the compounds, which in turn affects the intensity of their bioactivities. This section provides an overview of combined biological effects of phytochemical mixtures derived from fruits and vegetables with a focus on anti-oxidative, anti-inflammatory and anti-carcinogenic activities. The absorption/bioavailability impairment or enhancement caused by the co-consumption of dietary phytochemicals is also discussed. Finally, research gaps for future studies on phytochemical interactions are identified.

2.3.1. Introduction

The risks of many chronic disorders (e.g. cancer, diabetes, or cardiovascular diseases) may be reduced by the regular consumption of fruits and vegetables or other plant-based

foods (Fang, 2014, Wang, 2012a, Graf et al., 2005). A number of bioactive compounds naturally present in fruits and vegetables are effective agents for reducing the onset of oxidation and inflammation (Galvanoa et al., 2004, Hollman et al., 1999, Maiani et al., 2009, Metzler et al., 2013). The combinations of different pure bioactive compounds or their extracts from food sources can enhance the benefits conferred by individual bioactive compounds (Jiang et al., 2015, Hidalgo et al., 2010, Becker et al., 2007, Wang et al., 2011). For instance, α-tocopherol mixed with a flavonol (kaempferol or myricetin) is more effective in inhibiting *in vitro* lipid oxidation induced by free radicals than each component alone (Zhou et al., 2005a), or the mixture of resveratrol, chrysin and curcumin synergistically suppresses the *in vitro* proliferation of colorectal cancer cells (Iwuchukwu et al., 2011). In some cases, however, the combination of phytochemicals may lower the biological effects if they are combined in inappropriate ratios (Hidalgo et al., 2010, Jiang et al., 2015), or if the participant compounds form hydrogen bonds at active hydroxyl groups that decrease their capability to scavenge free radicals (Hidalgo et al., 2010), or they do not have proper orientation /distribution in lipid/water phases to facilitate the interaction (Liang et al., 2009a).

Bioactive compounds may interfere with intestinal absorption of other compounds. There are multiple phytochemicals in a single fruit or vegetable. People usually consume many vegetables in meals or fruits in desserts or drinks. Thus, concurrently consumed bioactive compounds may affect the intestinal absorption of each other. The interactions of phytochemicals may enhance or reduce the bioaccessibility/bioavailability of a given compound, depending on the facilitation/competition for cellular uptake and transportation taking place between them (Claudie et al., 2013, Fale et al., 2013, Reboul et al., 2007a). For example, β-carotene increases the bioavailability of lycopene in human plasma (Böhm and Bitsch, 1999),

and quercetin-3-glucoside reduces the absorption of anthocyanins in rat jejunum mounted in Ussing chambers (Walton et al., 2006).

The interactions of some major classes of food bioactive compounds such as carotenoids and flavonoids with other macromolecules (food matrices, blood proteins, digestive enzymes or intestinal transporters) have been reviewed (van den Berg, 1999, Gonzales et al., 2015, Yang et al., 2011, Parada and Aguilera, 2007). Nevertheless, there is no comprehensive review to date that provides overall understanding of bioactivity and absorption/bioavailability interactions among dietary bioactive compounds. Therefore, the following sections present the interactive effects on bioactivities and absorption/bioavailability of dietary phytochemical combinations derived from fruits and vegetables.

2.3.2. Effects of phytochemical interactions on biological activities

2.3.2.1. Modes of phytochemical interaction

Bioactive compound mixtures may produce a biological effect higher or lower than the summative effects of each single component. The effects of phytochemical interactions can be classified as potentiation, addition, synergy, or antagonism. There is confusion in literature about the difference between potentiation and synergy (Efferth and Koch, 2011). If the phytochemical mixture containing two compounds, in which one is active and the other is inactive, produces a greater effect than that of its single active component, the effect is defined as potentiation: the presence of the inactive compound enhances the potency of the active one (Chou, 2006). If each component of the mixture is active, their mixture can produce an additive, synergistic or antagonistic effect. In additive phytochemical interactions, the combined effect is equal to the sum potency of individual components of the mixture. In synergistic or antagonistic interactions, the

combined effect is analysed by isobologram or combination index that shows greater or less (respectively) than addition (Chou, 2006). These definitions of potentiation, addition, synergy and antagonism will be used in this review.

2.3.2.2. Methods to analyse phytochemical combination data

Because plant phytochemicals are natural drugs, one can analyse results of phytochemical combination studies by using methods used for drug combination analysis. These approaches have been described in detail elsewhere (Bulusu et al., 2016, Chou, 2006, Tallarida, 2001). The following methods have been commonly used to determine the types of interaction of binary mixtures of phytochemicals:

(i) Isobologram (Tallarida, 2001): illustrates an iso-dose effect of two phytochemicals (Efferth and Koch, 2011). A concave isobologram indicates synergy, and a convex one indicates antagonism (**Figure 3**).

(ii) Combination Index (CI) (Chou, 2006): calculation of CI is shown in equation (1) and (2). CI < 1 indicates synergy; CI=1 addition; CI > 1 antagonism.

For binary combination at 50% activity:

$$CI_{50} = \frac{C_A}{IC_{50}(A)} + \frac{C_B}{IC_{50}(B)} \qquad (1)$$

where CI_{50} is Combination Index for the binary mixture at 50% activity; C_A and C_B is the proportional dose of compound A and compound B (respectively) in the mixture that shows 50% activity; $IC_{50}(A)$ and $IC_{50}(B)$ is the single dose of each compound A and B that provides 50% activity.

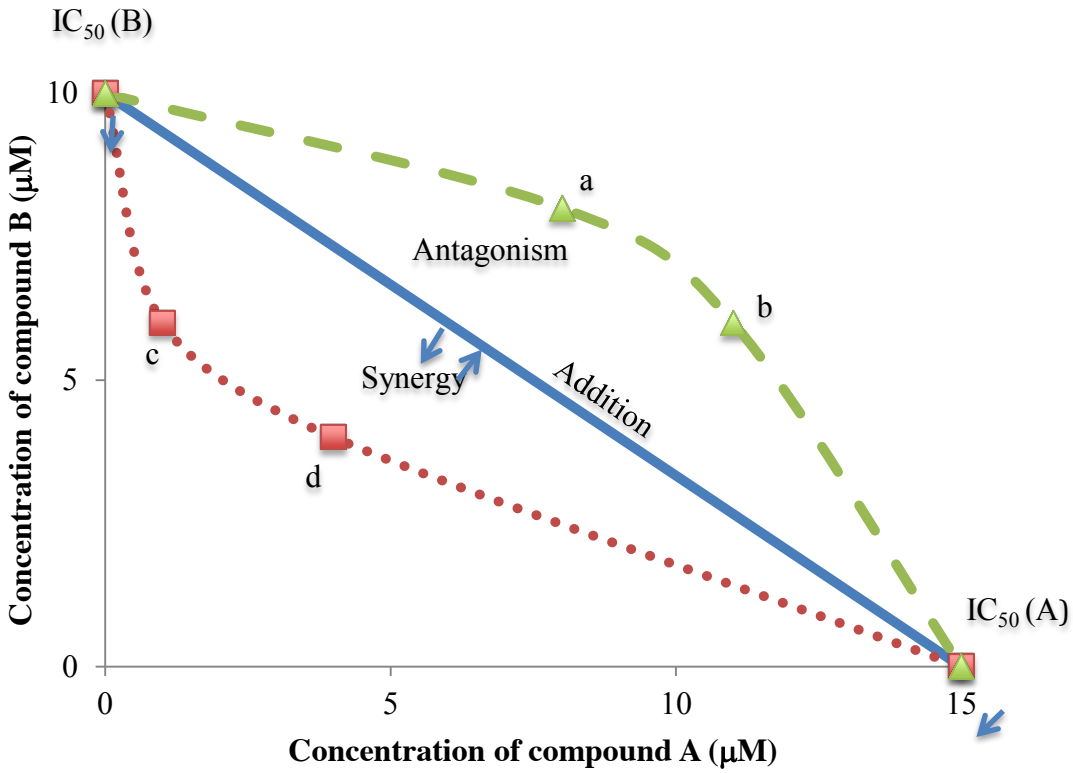


Figure 3. Isobologram of binary mixture at 50% activity (IC_{50}): Addition: a line formed from IC_{50} of compound A and IC_{50} of compound B: any combination points fall in this line indicate additive effect; Synergy: lower left area of addition line: any combination points that fall in this area (e.g., points c and d: form a concave curve) indicate synergistic effect; Antagonism: upper left area of addition line: any combination points that fall in this area (e.g., points a and b: form a convex curve) indicate antagonistic effect.

For n-phytochemical combination at x% inhibition:

$$(CI_x)_n = \sum_{j=1}^{n} \frac{(C)_j}{(IC_x)_j} \qquad (2)$$

where $(CI_x)_n$ is Combination Index for n-compound mixture at x% activity; C_j is the proportional dose of each compound in n-phytochemical mixture that shows x% activity; $(IC_x)_j$ is the single dose of each compound that provides x% activity.

Each of these approaches relies on a dose-effect plot of each component, from which the IC_{50} value (defined as the concentration of a compound that gives 50% activity) is determined and used for determination of interaction mode. These methods are used for assays where the measured activity is a value reversely related to the compound

concentration: a lower IC_{50} indicates a higher activity. Software (CompuSyn, Combenefit, CalcuSyn, SynergyFinder™, Genedata Screener®, Chalice) may be used for analysis of the performance of the mixture (Bulusu et al., 2016). In some studies, the dose-effect curve (IC_{50} value) of each phytochemical component is determined but neither method is used to analyse the interaction mode. The interaction mode in these studies is determined by comparing the experimental effect of the mixture with the sum effects of each component. The latter, in 1:1-ratio mixtures, is calculated by taking the average of the IC_{50} values of the two components. Synergy or antagonism may appear possible, but may not be validly claimed because this calculation is correct only when the components are combined at their equal-potency ratio (IC_{50}-of-compound-1: IC_{50}-of-compound-2) (Chou, 2006). The terms: potential synergy or potential antagonism will be used to describe such results in this review.

When the assessments of biological activities of phytochemicals do not rely on IC_{50} values, the interaction mode is not determined by either isobologram or combination index method, but by comparing the experimental effect of the mixture with the sum of effects of each phytochemical component. The two methods may not be appropriate to assess the contribution of individual phytochemicals to the interactive effects observed in complex mixtures containing multi phytochemical components (such as those occurring in food or herbal extracts) (Efferth and Koch, 2011). In practice, the interactive effects seen in an extract mixture can be compared to that obtained from the mixtures of isolated principle active phytochemicals at concentrations equivalent to those in the extracts (Efferth and Koch, 2011). If these two effects are equal, the ultimate effects observed in the combined extracts are from the interaction between the major active phytochemicals. If they are unequal, there are compounds in the extracts

other than the major active phytochemicals contributing to the effects (Efferth and Koch, 2011).

2.3.3. Interactive effects of bioactive compounds on antioxidant activities

2.3.3.1. Methods for studying antioxidant interactions

It is important to select an appropriate well-developed and validated antioxidant assay that can effectively measure the antioxidant power of bioactive compounds or food extracts. Antioxidant interactions can be studied by either chemical-based or biological-based assays.

Chemical assays

Chemical assays have been extensively used because they are simple, cost less and samples are easy to handle (Niki, 2010, Prior et al., 2005, Schaich, 2005). Chemical assays can be classified into two groups according to their reaction mechanism: hydrogen atom transfer (HAT) or electron transfer (ET). Antioxidant activity refers to the concentration of antioxidants required to provide a specified rate or extent of reaction, whereas antioxidant capacity refers to the total number of electrons donated or target molecules converted per mol of antioxidant at full reaction under given conditions (Schaich et al., 2015). In a HAT-based assay, there is competition between the antioxidant and the target molecular probe (substrate) for the reaction with peroxyl radicals generated by an azo compound (Huang et al., 2005, Özyürek et al., 2008). The peroxyl radicals preferentially abstract hydrogen atoms from the antioxidant resulting in the suppression of the reaction between the radicals and the probe. Oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), inhibition of induced low-density lipoprotein (LDL) autoxidation and crocin bleaching assays are based on the HAT mechanism (Huang et al., 2005). In an ET-based assay, the

antioxidant capacity is evaluated from the changes in the colour of a chromogenic oxidant when reduced by an antioxidant (Huang et al., 2005, Özyürek et al., 2008). The reaction is initiated by the transfer of electrons from the antioxidant to the oxidizing reagent (Huang et al., 2005). An array of ET-based assays has been developed: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) / Trolox equivalence antioxidant capacity (TEAC), Folin – Ciocalteu reagent (FCR), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, ferric ion reducing antioxidant power (FRAP) and cupric reducing antioxidant capacity (CUPRAC) (Huang et al., 2005, Özyürek et al., 2008).

It is essential to consider the solubility of the antioxidants in the reaction media (Özyürek et al., 2008). In most chemical assays, the reaction medium is single-phase water-based or alcohol-water-based. The main assay reagents usually have high affinity towards aqueous solvents (Özyürek et al., 2008). Thus, the majority of these methods study the interactions between hydrophilic antioxidants in homogeneous systems. HAT- and ET-based assays are run in hydrophilic homogeneous media. Some assays, such as ABTS/TEAC and CUPRAC assays are applicable for both hydrophilic and lipophilic antioxidants (MacDonald-Wicks et al., 2006, Özyürek et al., 2008). These methods enable efficient measurement of the total antioxidant capacity of food samples containing both water- and oil-soluble antioxidants, and are often selected for studying the interactions between hydrophilic and/or lipophilic antioxidant mixtures. Like the ET-based assay they use a neutral or univalent-charged chromophore (Özyürek et al., 2008). Other chemical assays use heterogeneous media (multi-phase). These multi-phase systems allow the investigation of phytochemical interactions at a water-lipid interface and avoid the interferences of organic solvents with interactive effects and permit the assessment of the performance or interactions of antioxidants in physiological conditions (Roberts and Gordon, 2003). Some multi-phase assays

investigate oxidative inhibition of emulsions (water-in-oil, oil-in-water) (Becker et al., 2007, Yin et al., 2012), or of artificial biological membranes (liposomes or micelles) (Bermudez-Soto et al., 2007, Dai et al., 2008, Shi et al., 2004, Stahl et al., 1998, Zhou et al., 2005a).

Biological assays

In vitro biological models: food products (bread, chicken breast, and turkey meatball), cultivated living cells, human plasma and human low-density lipoprotein, and *in vivo* biological models: animals and humans have been used to examine phytochemical interactions (Ajuwon et al., 2013, Bruno et al., 2006, Fuhrman et al., 2000). A recent review by Wang and Zhu (2015) discussed antioxidant synergy in these systems. Although *in vitro* biological assays are more effective than heterogeneous chemical assays in mimicking *in vivo* conditions, their uses in phytochemical interaction studies are less common because of the high cost and the complexity of the analysis. *In vivo* assays are even more costly and complicated. To date, there is no model optimal for the study of antioxidant interactions *in vivo* (Wang and Zhu, 2017).

Table 1. List of mixtures of bioactive compounds that exert antioxidant antagonism.

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
Combination of hydrophilic antioxidants				
Interaction among five major anthocyanin (ACNs) compounds (CY, DP, MV, PE, PG):				
PNG + DG	1:1, 200 μM	DPPH, FRAP	Antagonistic interactions could be explained by the formation of intermolecular hydrogen bonds between two different compounds that reduce the availability of the active hydroxyl groups for radical scavenging activities. The interactive effects could also depend upon the compatibility of the compounds to the reaction mechanism of the antioxidant assays	Hidalgo et al. (2010)
PNG + CG		FRAP		
PNG + MG				
ACNs and flavan-3-ols:				
PNG + ECAT	1:1, 200 μM	DPPH, FRAP		
PNG + CAT		DPPH		
CG + CAT				
DG + CAT				
DG + ECAT				

Table 1. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
MG + CAT	1:1, 200 μM	DPPH,	Antagonistic	
PLG + CAT		FRAP	interactions could be	
PLG + ECAT			explained by the	
ACNs and flavonols:			formation of	
PNG + MYR		DPPH	intermolecular	
PNG + QUE			hydrogen bonds	
PNG + Q3G	1:1, 200 μM		between two different	
DG + QUE			compounds that	
DG + Q3G			reduced the availability	
MG + KAEM			of the active hydroxyl	
PLG + Q3G			groups for radical	
CG + Q3G		DPPH	scavenging activities.	
MG + QUE			The interactive effects	
MG + Q3G			could also depend upon	
PLG + QUE			the compatibility of the	
PNG + KAEM		FRAP	compounds to the	
Flavan-3-ols and Flavonols			reaction mechanism of	
MYR + QUE	1:1, 200 μM	DPPH,	the antioxidant assays	
MYR + Q3G		FRAP		
		DPPH		
QUE + Q3G		DPPH		
Flavan-3-ols and Phenolic acid				
CAT + EGA	1:1, 5 μM	Copper-catalysed human LDL oxidation	The ability of catechin to donate hydrogen is disabled by the formation of hydrogen bonds between the carbonyl groups in ellagic acid and the *o*-dihydroxyl groups in catechin	Meyer et al. (1998)
Combination of hydrophilic antioxidants				
Ascorbate and Flavonols				
ASC + RUT	2.9 μM + 4.1 μM	DPPH	Not reported	González and Nazareno (2011)
ASC + HESD	2.9 μM + 400 μM	DPPH	Not reported	González and
ASC + NCH	2.9 μM + 25.9 μM			Nazareno
ASC + NDCH	2.9 μM + 29.2 μM			(2011)
ASC + NARG	2.9 μM + 672 μM			

Table 1. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
Combination of lipophilic antioxidants				
ASTA + ZEA	1:1, 9 μM		Similar orientation in the membrane, so not able to synergize.	Liang et al. (2009b)
LYC + γ-TOC	1:1, 10 μM	- Human LDL oxidation is initiated by copper ions ($CuSO_4$) at 37°C and assessed by the formations of TBARS - AAPH-induced LDL oxidation	LYC may cause pro-oxidative effect to γ-tocotrienol	Fuhrman et al. (2000)
Combination of lipophilic and hydrophilic antioxidants				
Carotenoid and Flavones				
β-CAR + DAI β-CAR + BAI	1:1, 3 μM 1:1, 3 μM	Liposome oxidation is initiated by AMVN and assessed by the formation of conjugated dienes at 43°C	β-CAR radicals react with the flavones to form β-CAR/flavone adducts leading to decreases of active antioxidant concentration in lipid phase	Liang et al. (2010)
Carotenoid or α-tocopherol and Flavan-3-ols				
β-CAR + ECAT β-CAR + EGC β-CAR + ECG β-CAR + EGCG	1:1, 3 μM	Liposome oxidation is initiated by AMVN and assessed by the formation of conjugated dienes at 45°C	β-CAR radicals react with the flavan-3-ols to form β-CAR/flavan-3-ols adducts leading to decreases of active antioxidant concentration in lipid phase	(Song et al., 2011)
α-TOC + CGA	1:1, 5 μM		Chlorogenic acid has a steric structure and low affinity to membrane lipids, so low concentration presenting in the membrane does not show synergy with α-TOC	Neunert et al. (2015)

CG: Cyanidin-3-glucoside; DG: Delphinidin-3-glucoside, MG: Malvidin-3-glucoside, PNG: Peonidin-3-glucoside, PLG: Pelargonidin-3-glucoside; CAT: catechin; ECAT: epicatechin; MYR: myricitin; QUE: quercetin; Q3G: quercetin-3-glucoside; KAEM: kaempferol; EGA: ellagic acid; RUT: rutin; ASC: ascorbic acid; HESD: hesperidin; NCH: naringenin chalcone; NDCH: naringin dihydrochalcone; NARG: naringenin; ASTA: astaxanthin; ZEA: zeaxanthin; LYC: lycopene; γ-TOC: γ-tocotrienol; DAI: daidzein; BAI: baicalein; β-CAR: β-carotene; ECAT: epicatechin; EGC: (−)-epigallocatechin, ECG: (−)-epicatechin gallate; EGCG: (−)-epigallocatechin gallate; α-TOC: α-tocopherol; CGA: chlorogenic acid; AMVN: 2,2'-azobis(2,4-dimethylvaleronitrile; TBARS: thiobarbituric acid reactive substances; LDL: low-density lipoprotein; DPPH: 2,2-Diphenyl-1-picrylhydrazyl assay; FRAP: ferric ion reducing antioxidant power.

Table 2. List of mixtures of bioactive compounds that exert antioxidant synergy.

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
Combination of hydrophilic antioxidants				
ACNs and Flavonols			Synergistic effects have resulted from the cooperative interactions between individual compounds, in which one could act as a hydrogen/electron donor to regenerate the partner. The interactive effects could also depend upon the compatibility of the compounds to the reaction mechanism of the antioxidant assays	Hidalgo et al. (2010)
CG + Q3G	1:1, 200 μM	FRAP		
MG + QUE				
MG + Q3G				
PLG + QUE				
Flavan-3-ols and Flavonols				
ECAT + MYR	1:1, 200 μM	FRAP		
ECAT + QUE				
ECAT + Q3G				
CAT + QUE				
CAT + Q3G				
Flavonols and Flavonols				
KAEM + MYR	1:1	DPPH		
KAEM + QUE				
KAEM + Q3G				
QUE + Q3G	1:1	FRAP		
QUE + RUT	1:1, 0.15 mol%	- Liposome oxidation is initiated by AAPH and assessed by the formation of conjugated dienes - Oxidation of methyl linoleate emulsion		Becker et al. (2007)
ACNs and Flavan-3-ols				
MG + CAT	1:1, 12 μM	Linoleic acid oxidation initiated by AIBP	CAT recycles MG and PE	Rossetto et al. (2002)
PNG + CAT				
Tea polyphenols				
GCG + EGCG;	1:1	DPPH	The galloyl fragment on the C rings of these compounds has been suggested to play an important role on the antioxidant synergy	Colon and Nerin (2016)
GCG + ECG;				
CTG + GCG;				
CTG + ECG;				
EGCG + CTG;				
EGCG + ECG				
Ascorbate and Flavonols				
ASC + QUE	2.9 μM + 3.8 μM	DPPH	QUE or NAR reacts with dehydroascorbate (DHA) to regenerate semiascorbyl radical ($SAsc^{\bullet}$) as the following equations: $Asc^{-} + DPPH^{\bullet} \rightarrow SAsc^{\bullet} + DPPH - H$ $SAsc^{\bullet} + DPPH^{\bullet} \rightarrow DHA + DPPH - H$ $FOH + DHA \rightarrow FO^{\bullet} + SAsc^{\bullet}$	González and Nazareno (2011)
Ascorbate and Flavanones				
ASC + NAR	2.9 μM + 606 μM	DPPH		González and Nazareno (2011)

Table 2. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
Combination of lipophilic antioxidants				
β-CAR + LYC	1:1, 50 μM 1:1, 3 μM	Liposome oxidation is initiated by AMVN and assessed by the formation of TBARS	Regeneration activity Impact on signal transduction pathway Differences in physiochemical properties and spatial compartmentation of the carotenoids in the membrane	Shi et al. (2004) Stahl et al. (1998)
LUT + LYC	1:1, 50 μM 1:1, 3 μM			Shi et al. (2004) Stahl et al. (1998)
β-CRYP + LYC	1:2, 3 μM			Stahl et al. (1998)
ZEA + LYC	1:2, 3 μM			
LUT + β-CAR	2:1, 3 μM			
β-CAR – α-CAR	2:1, 3 μM			
α-CAR + LYC	1:2, 3 μM			
LYC + α-CAR + β-CAR + β-CRYP + ZEA + LUT	2:1:2:1:1:2, 3 μM 1:2:1:1:2, 3 μM 2:1:2:1:1, 3 μM			
α-CAR + β-CAR + β-CRYP + ZEA + LUT	1:2:1:1, 3 μM 2:1:1:1, 3 μM			
LYC + α-CAR + β-CAR + β-CRYP + ZEA	2:1:2:1:1:2:90, 3 μM			
α-CAR + β-CAR + β-CRYP + ZEA LYC + α-CAR + β-CRYP + ZEA LYC + α-CAR + β-CAR + β-CRYP + ZEA + LUT + α-TOC LUT + α-TOC	1:1, 50 μM			Shi et al. (2004)
ASTA + LYC ASTA + β-CAR	1:1, 9 μM 1:1, 9 μM	Liposome oxidation is initiated by AMVN and assessed by the formation of conjugated dienes at 43°C. Lag phase (minutes) of liposome oxidation was calculated	Different spatial distribution in liposome: ASTA anchored to the interface while LYC and β-CAR stayed in the centre. Differences in E_0: LYC and β-CAR are more reducing, so able to transfer electron to regenerate ASTA.	Liang et al. (2009b)

Table 2. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
LYC + α-TOC	5:1, 6 μM 2:1, 7.5 μM 1:1, 10 μM 1:1, 10 μM	Human LDL oxidation is initiated by coper ions ($CuSO_4$) at 37°C and assessed by the formations of TBARS AAPH-induced LDL oxidation	Different spatial distribution in LDL: α-TOC at the surface while LYC at the core, so acts at different sites of LDL and synergistically prevents LDL oxidation α-TOC scavenges unstable lycopene free radicals which are formed when lycopene quenches oxygen molecules	Fuhrman et al. (2000)
Combination of lipophilic and hydrophilic antioxidants ***Carotenoid or α-tocopherol and Flavonols***				
β-CAR + PUE β -CAR + 4'-PROP β -CAR + QUE β -CAR + RUT	 1:1, 3 μM 1:1, 3 μM	Liposome oxidation is initiated by AMVN and assessed by the formation of conjugated dienes at 43°C	Spatial distribution of the compounds facilitates the regeneration of β-CAR by the flavonols	Han et al. (2007) Liang et al. (2010)
α-TOC + QUE	1:4, 0.15 mol% 1:1, 40 μM	Liposome oxidation is initiated by AAPH and assessed by the formation of conjugated dienes AAPH-induced oxidation of linoleic acid in tBuOH/water (3:2) or micelles	High concentration of QUE at interfaces scavenges radicals initiated in aqueous phase and protects α-TOC from oxidation Synergy is mostly because QUE traps initiating radicals, and partly because QUE regenerates α-TOC	Becker et al. (2007) (Zhou et al., 2005b)
α -TOC + MYR α-TOC + RUT α-TOC + KAEM α-TOC + MOR α-TOC + QGP α-TOC + QRP α-TOC + KGP	1:1, 40 μM	AAPH-induced oxidation of linoleic acid in tBuOH/water (3:2) or micelles	Synergy is mostly because the flavonols trap initiating radicals, and partly because the flavonols regenerate α-TOC	(Zhou et al., 2005b)

Table 2. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
α-tocopherol and Flavan-3-ols				
α-TOC + CAT α-TOC + ECAT	1:1, 1:2, 2:1 0.05 or 0.1 mmol/g 0.5% or 0.1% mol of lipid for each antioxidant	Rancidity of sunflower oil AAPH-induced liposome oxidation	Different phase partition of antioxidants protects lipid from oxidation by radicals initiated in both phases	Yin et al. (2012)
α-TOC + ECG α-TOC + EGCG	1:1, 2 μM	Liposome oxidation catalyzed by iron metals	α-TOC located on membrane surface enables synergy with the aqueous polyphenols	Murakami et al. (2003)
Combination of lipophilic and hydrophilic antioxidants				
α-tocopherol and ascorbic acid and flavan-3-ols				
α-TOC + ASC + EGCG α-TOC + ASC + ECG α-TOC + ASC + EGC α-TOC + ASC + EC	7.5:10:10, 27.5 μM	AAPH-induced oxidation of linoleic acid micelles	ASC recycles flavan-3-ols and the latter recycles α-TOC	Dai et al. (2008)
α-TOC + QUE + ASC	1:1, 2 μM	Liposome oxidation catalyzed by iron metals		Murakami et al. (2003)
Carotenoid – Isoflavans				
LYC + GLB β-CAR + GLB	5:1, 6 μM 5:1, 6 μM	AAPH-induced human LDL oxidation Cu^{2+}-induced human LDL oxidation	Spatial distribution of the compounds in LDL facilitates the cooperation of radical scavenging	Fuhrman et al. (2000)
Carotenoid / α-tocopherol – phenolic acids				
LYC + RMA LYC + CNA β-CAR + RMA β-CAR + CNA	1:5, 30 μM 1:5, 30 μM 1:5, 30 μM 1:5, 30 μM	AAPH-induced human LDL oxidation Cu^{2+}-induced human LDL oxidation	Spatial distribution of the compounds in LDL facilitates the cooperation of radical scavenging	Fuhrman et al. (2000)

Table 2. *(continued)*

Mixtures of antioxidants	Ratio, total concentration	Type of assay	Proposed mechanisms	References
β-CAR + CGA	1-10x10^5 (mol/dm^3) + 2 x 10^{-5} (mol/dm^3)	Irradiation using *t*-BuOH/water (4:1) in quantum yield reactor	CGA regenerates β-CAR due to its lower redox potential and bond dissociation energy	Vijayalakshmi et al. (2014)
α-TOC + CFA α-TOC + FRA	1:1, 5 μM or 10 μM 1:1, 5 μM or 10 μM	AAPH-induced liposome oxidation, assessed by the changes in the	The ability of the phenolic acids to penetrate into membrane enables them to regenerate or protect α-TOC from radicals	Neunert et al. (2015)
α-TOC + CGA	1:1, 10 μM	fluorescence intensity of C11-BODIPY 581/591	Chlorogenic acid has low affinity to membrane lipids. High concentration in the membrane is crucial for synergy	

CG: Cyanidin-3-glucoside; MG: Malvidin-3-glucoside, PNG: Peonidin-3-glucoside, PLG: Pelargonidin-3-glucoside; MYR: myricetin; QUE: quercetin; Q3G: quercetin-3-glucoside; KAEM: kaempferol; RUT: rutin; CAT: catechin; GCG: gallocatechin gallate; CTG: catechin gallate; ASC: ascorbic acid; NAR: naringin; β-CAR: β-carotene; LYC: lycopene; LUT: lutein; α-TOC: α-tocopherol; β-CRYP: β-cryptoxanthin; ZEA: zeaxanthin; α-CAR: α-carotene; ASTA: astaxanthin; PUE: puerarin; 4'-PROP: 4'-propylpuerarin; MOR: morin; QGP: quercetin galactopyranoside; QRP: quercetin rutinpyranoside; KGP: kaempferol glycopyranoside; ECAT: epicatechin; ECG: (−)-epicatechin gallate; EGCG: (−)-epigallocatechin gallate; GLB: glabridin; RMA: rosmarinic acid; CNA: carnosic acid; CFA: caffeic acid; FRA: ferulic acid; CGA: chlorogenic acid; AAPH: 2,2'-azobis(2-amidinopropane) dihydrochloride; LDL: low-density lipoprotein; ABIP: 2,2'-azobis[2-(2imidazolin-2-yl)propane] dihydrochloride; DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; FRAP: ferric ion reducing antioxidant power; AMVN: 2,2'-azobis(2,4-dimethylvaleronitrile; TBARS: thiobarbituric acid reactive substances.

Limitations of in vitro antioxidant assays

Conceptual and technical limitations of *in vitro* antioxidant assays have been critically evaluated and reported by Schaich et al. (2015). The authors have pointed out the three major issues of most *in vitro* antioxidant assays, including:

- The radical scavenging activity observed in test tubes does not occur *in vivo* because most bioactive compounds reach circulation at trace levels which are not sufficient to mediate physiological actions merely by scavenging radicals. The microbial or physiological metabolites, instead, may actually be more active *in vivo*. Thus, it may be well erroneous to conclude that radical scavenging is the mode of action of antioxidants *in vivo*.
- The chemistry and molecular targets of most *in vitro* assays are not relevant to *in vivo* conditions.
- Most *in vitro* antioxidant assays do not address radical reactions in lipids, and how natural antioxidants partition into and interact with lipids.

Due to such limitations, the antioxidant capacity of foods measured by most of the test-tube methods cannot be extrapolated to *in vivo* effects in humans (Schaich et al., 2015). Nevertheless, although *in vitro* assays have limitations and there are divergent opinions in the scientific community regarding their use, *in vitro* screening methods should not be ignored since they are low-cost and high-throughput tools to discover potential antioxidant sources (Granato et al., 2018). In addition, data generated by colorimetric antioxidant assays such as ORAC and FRAP are possibly used to anticipate the inhibition of NF-κB activation in cell models (de Camargo et al., 2019). Future studies, however, are encouraged to use at least some *in vitro* biological tests such as cell lines or preferably *in vivo* assessment (Granato et al., 2018).

2.3.3.2. Antioxidant interactions in simple and complex mixtures

Changes of antioxidant capacities when combining pure antioxidants

Combining different pure or isolated phytochemicals could produce non-additive anti-oxidative effects. Lists of antioxidant mixtures that exert antagonistic and synergistic effects on anti-oxidation are shown in **Table 1** and **Table 2** respectively. Interactions can occur between hydrophilic antioxidants. Polyphenolic compounds in green tea extracts have mutual interactions on antioxidant capacity when tested for DPPH$^{\bullet}$ radical scavenging activity. Combinations containing gallocatechin gallate (GCG), epigallocatechin gallate (EGCG), catechin gallate (CTG) and epicatechin gallate (ECG) show synergy (Colon and Nerín, 2016). Hyperoside, one of the major phenolic compounds in *Potentilla fruticosa* L. leaves (a traditional Chinese tea), acts in synergy with ECG in green tea on ABTS$^{\bullet}$ and DPPH$^{\bullet}$ radical scavenging activities (Liu et al., 2016). The combination of the anthocyanins malvidin-3-glucoside or peonidin-3-glucoside with the flavan-3-ol catechin increases the inhibition of linoleic acid oxidation initiated by free radicals (Rossetto et al., 2002). Hidalgo et al. (2010) observed potential synergistic interactions on DPPH$^{\bullet}$ radical scavenging between cyanidin-3-glucoside and kaempferol, delphinidin-3-glucoside and kaempferol, and cyanidin-3-glucoside and myricetin although the interpretation of this study may not be valid because the authors did not use appropriate methods (isobologram or combination index) to determine the interactive effects.

Lipophilic antioxidants also interact. Several mixtures of carotenoids are more efficient than the sum activity of single compounds in oxidative inhibition (Han et al., 2012, Shi et al., 2004, Shixian et al., 2005). Binary mixtures of carotenoids: lycopene-β-carotene, lycopene-lutein and β-carotene-lutein (Zanfini et al., 2010), or the combination of α-

tocopherol and lycopene (Stinco et al., 2016, Zanfini et al., 2010) show stronger ABTS radical scavenging activity than the sum of individual compounds, indicating potential synergy. Lycopene combined with α-carotene, β-carotene or lutein increases the inhibition of *in vitro* lipid peroxidation (Shi et al., 2004, Stahl et al., 1998), among which lycopene-lutein interaction is the strongest (Stahl et al., 1998). A mixture of lycopene and astaxanthin enhances the inhibition of *in vitro* liposome oxidation initiated by 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). Such cooperative antioxidant effect, however, does not occur in the combination of the xanthophylls, astaxanthin and zeaxanthin. Proper spatial orientation of the antioxidants in the lipid phase that could facilitate electron / hydrogen transfer for antioxidant regeneration is crucial for synergy in these systems (Han et al., 2012).

Synergy also occurs with combinations of lipophilic and hydrophilic bioactive compounds: carotenoids and flavonoids; carotenoids and phenolic acids; or carotenoids / tocopherols and water-soluble vitamins. These combinations synergistically improve the antioxidant capacity. For instance, when β-carotene is paired with the flavonoids puerarin, quercetin or rutin, the antioxidant capacity increases by up to 50% (Han et al., 2010, Han et al., 2011). When lycopene is mixed with the polyphenols glabridin, rosmarinic acid or carnosic acid, oxidation of low-density lipoprotein is retarded (Fuhrman et al., 2000). The antioxidant capacity of lycopene together with glabridin is nearly twice as strong as that attained from the sum of the individual activities. Rosmarinic acid or carnosic acid combined with lycopene increases the effect by 32% and 15% respectively (Shi et al., 2004, Fuhrman et al., 2000). α-tocopherol paired with quercetin, caffeic acid or ferulic acid provides stronger inhibitory effects against *in vitro* lipid peroxidation (soybean phosphatidylcholine liposome model) than the single compounds (Becker et al., 2007, Neunert et al., 2015). Regeneration of α-tocopherol by

the antioxidant partner maintains the radical scavenging activity in these systems (Becker et al., 2004).

Some combinations of phytochemicals reduce antioxidant capacity. Although α-tocopherol with caffeic acid or ferulic acid shows synergy against lipid oxidation, its combination with chlorogenic acid (2.5 μM) shows antagonism, possibly, because the steric structure of chlorogenic acid makes it unable to interact with α-tocopherols (Neunert et al., 2015). When β-carotene is combined with the flavonoids daidzein, baicalein or with green tea polyphenols: (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epigallocatechin gallate (EGCG), the antioxidant activity is significantly decreased (Song et al., 2011).

A number of factors are essential for the combination of lipophilic and hydrophilic antioxidants to show synergy in lipid anti-oxidation. These factors include: (i) the ability of the compounds to interact at water/lipid interface, (ii) the differences in the standard redox potential between the two antioxidants, and (iii) the ability to rapidly transfer electrons from one compound to another (Liang et al., 2010, Liang et al., 2009b). Among these factors, the structure and the ability of the hydrophilic compound to orientate and position appropriately at the water/lipid interface is the most important for high anti-oxidative synergy (Han et al., 2012).

Changes of antioxidant capacities in complex phytochemical mixtures

The addition of isolated bioactive compounds to fruit and/or vegetable extracts can improve the antioxidant efficiency. The *in vitro* lipid inhibitory effect of black chokeberry juice significantly increases when added with α-tocopherol (Graversen et al., 2007). The addition of α-tocopherol to the extracts of açaí seed and grape rachis improves the protective efficiency against *in vitro* lipid peroxidation (Melo et al., 2016).

α-tocopherol or quercetin added to lettuce extract improves the delaying of the onset of L-α–phosphatidylcholine liposome oxidation induced by either hydrophilic or lipophilic radicals (Altunkaya et al., 2009, Altunkaya et al., 2016).

Combinations of various fruits, vegetables and/or legumes bring significant changes in the antioxidant efficiency. Combining foods within and across categories results in different degrees of synergy, addition, and antagonism. Wang et al. (2011) examined the *in vitro* antioxidant capacity and the combined effects of a number of mixed extracts made from fruits, vegetables and legumes. Within the same food category, only 13% of the tested combinations show synergy; 68% addition and 21% antagonism. In mixtures across food categories, 21% show synergy; 54% addition and 25% antagonism (Wang et al., 2011).

Table 3 presents a list of fruit and/or vegetable mixtures that show synergy on *in vitro* anti-oxidation. Several vegetable binary mixtures: tomato-lettuce, tomato-onion (Gawlik-Dziki, 2012), eggplant-tomato, and purple potato-tomato (Jiang et al., 2015) produce relatively high synergistic effects in an $ABTS^{\bullet}$ radical scavenging assay. Other vegetable combinations: carrot-eggplant, carrot-purple potato, and eggplant-purple potato (Jiang et al., 2015) show synergy in $DPPH^{\bullet}$ radical scavenging activity. Some pairs of fruit-vegetable mixtures: apple-purple cauliflower, raspberry-mushroom; fruit-legume mixtures: raspberry-adzuki beans, apples-black beans; or vegetable-legume mixtures: tomatoes-soybean, broccoli-adzuki bean show synergy in anti-oxidation (Wang et al., 2011). Lettuce extract mixed with green tea or grape seed extracts demonstrate additive or slightly synergistic effects in the inhibition of liposome peroxidation (Altunkaya et al., 2016).

Table 3. Combination of fruit and/or vegetable extracts showing synergy in antioxidant activities.

Extract combinations (1:1, v/v ratio)	**Antioxidant assay used**	**Synergistic rate* (%)**	**References**
Tomato + onion	ABTS	32	Gawlik-Dziki (2012)
	Xanthine oxidase inhibitory	11	
Tomato + Garlic	ABTS	50	
	Xanthine oxidase inhibitory	35	
Tomato + Lettuce	ABTS	5	
	Xanthine oxidase inhibitory	15	
	Lipoxygenase inhibitory	23	
Tomato + Garlic + Lettuce	ABTS	30	
	Xanthine oxidase inhibitory	22	
Lettuce + Green tea	Liposome oxidation	16[a]	Altunkaya et al. (2016)
Lettuce + Grape seed extract	Liposome oxidation	17[a]	
Eggplant + Purple potato	DPPH	46.6	Jiang et al. (2015)
	ABTS	47.9	
Carrot + Purple potato	DPPH	73.4	
	ABTS	82.1	
Carrot + Eggplant	DPPH	87.4	
	ABTS	81.8	
Tomato + Purple potato	DPPH	35.5	
	ABTS	45.5	
Tomato + Eggplant	DPPH	71.4	
	ABTS	80.6	
Tomato + Purple cauliflower	ORAC	20.3	Wang et al. (2011)
Soybean + Adzuki bean	ORAC	14	
Apple + Purple cauliflower	DPPH	15	
Raspberry + Mushroom	ORAC	12.2	
Apple + Tomato	ORAC	7.5	
Raspberry + Adzuki bean	FRAP	19.5	
	DPPH	31.5	
	ORAC	8.2	
Raspberry + Soybean	ORAC	10.1	

Table 3. *(continued)*

Extract combinations (1:1, v/v ratio)	Antioxidant assay used	Synergistic rate* (%)	References
Apple + Black bean	FRAP	7.6	Wang et al. (2011)
Raspberry + Black bean	ORAC	14.1	
Apple + Adzuki bean	ORAC	9.3	
Apple + Black bean	ORAC	8.5	
Tomato + Soybean	DPPH	5.2	
Broccoli + Adzuki bean	ORAC	13.7	
Tomato + Adzuki bean	ORAC	8.3	
Purple cauliflower + Soybean	ORAC	7.2	
Purple cauliflower + Black bean	ORAC	8.4	
Sumac + Raspberry	ORAC	10	Wang et al. (2015)
	FRAP	15	
	DPPH	45	

DPPH: 2,2-Diphenyl-1-picrylhydrazyl assay; FRAP: ferric ion reducing antioxidant power; ORAC: Oxygen radical absorbance capacity; ABTS/Trolox equivalence antioxidant capacity.
* Synergistic rate (%) = (difference between theoretical effect and experimental effect / theoretical effect)*100 (Jiang et al., 2015). Some of the percentage values might not be directly presented in the original papers, but were calculated using the reported results for comparison purpose in this review.
[a] Reported value of synergy at pH 6.

Wang et al. (2011) observed the percentage of synergy produced by different patterns of food combinations to evaluate which food category combinations are more likely to cause anti-oxidative synergy. The combinations of fruits and legumes are most likely to provide synergistic anti-oxidation (28%), followed by fruit and vegetable mixtures, 22%. Legume combinations are the least effective mixtures in providing anti-oxidative synergy. Among the extract mixtures of fruits, vegetables and legumes analysed in the study, the combination of raspberry and adzuki bean is the only one that exhibits multiple synergistic interactions in all antioxidant assays (DPPH, FRAP and ORAC) (Wang et al., 2011). In that study, all of the food mixtures used were the hydrophilic extracts of fruits, vegetables and legumes, therefore only the combined anti-oxidative effects resulting from the interactions among water-soluble phytochemicals were investigated. Similarly, in the study of Gawlik-Dziki (2012), the author used only the water-soluble extracts of tomato, onion, garlic and lettuce for the evaluation of anti-oxidative interactions. Some food materials, such as tomato and carrot, used in these

studies, however, contain mainly lipophilic phytochemicals: lycopene, α-carotene and β-carotene which were excluded by the interaction tests. Thus, the combined effects on anti-oxidation of the food mixtures containing high content of lipid-soluble antioxidants may be incorrect because these compounds are excluded from possible interactions with water-soluble phytochemicals. When the combinations of food extracts containing hydrophilic antioxidants and those containing lipophilic antioxidants are tested, they show very high synergistic effects on free radical scavenging activities. For instance, the combinations of the lipophilic extracts of tomato or carrot with the hydrophilic extracts of eggplant or purple potato provide high percentage of anti-oxidative synergy (50 – 80%), and the synergistic rate is enhanced with the increase of lipophilic extract ratios (Jiang et al., 2015). In comparison, when the tomato hydrophilic extract is mixed with the water-soluble extract of purple cauliflower, the synergy is as low as 20% (Wang et al., 2011).

2.3.3.3. Anti-oxidative synergy: mechanism and influencing factors

Mechanism of synergistic anti-oxidation

Different mechanisms of antioxidant synergy have been proposed:

- *Regeneration:* one antioxidant is oxidised and becomes a free radical which can receive electrons or hydrogen atoms donated by the other antioxidant to regenerate itself. Generally, in a binary mixture of antioxidants, the compound whose antioxidant capacity is weaker regenerates the stronger one (Becker et al., 2004, Becker et al., 2007, Dai et al., 2008, Shi et al., 2004, Vijayalakshmi et al., 2014).
- *Spatial distribution:* the two antioxidants have different orientation or position at the water/lipid interface or within the membrane that facilitates synergistic

interactions (Becker et al., 2004, Fuhrman et al., 2000, Han et al., 2007, Liang et al., 2009b, Murakami et al., 2003).

- *Sacrificial oxidation:* one antioxidant reacts with free radicals or singlet oxygen to protect the partner from oxidation (Neunert et al., 2015).
- *Metal chelation:* one component chelates metal ions to allow the partner to remain active (Becker et al., 2004).
- *Mutual protection:* the combined antioxidants act in different ways of anti-oxidation that enable them to protect each other from oxidative agents (e.g. a chain breaking antioxidant scavenges free radicals to protect a singlet oxygen quencher from oxidation and that enables the latter to stay active longer to protect the former against singlet oxygen oxidation) (Becker et al., 2004, Becker et al., 2007).

Among the proposed mechanisms, regeneration of antioxidants has been mostly used to explain synergistic interactions between antioxidants, such as the synergy between α-carotene and lycopene; α-tocopherol and lycopene; lycopene and glabridin; lycopene and rosmarinic acid. In these binary mixtures of antioxidants, the less active antioxidant regenerates the more active compound. The regeneration activity is determined by the differences in the standard reduction potential (E^0), which demonstrates the ability of the antioxidants to donate hydrogen atoms or electrons, under standard conditions. For example, the regeneration of α-tocopherol by ascorbic acid in membranes and low-density lipoprotein systems is consistent with E^0 of the half-equation of the reduction of ascorbic acid (0.28 V) being lower than that of α-tocopherol (E^0 = 0.5 V) (Buettner and Jurkiewicz, 1996). Similarly, in the ternary mixture of α-tocopherol, quercetin and ascorbic acid (listed in descending order of the reduction potential), α-tocopherol is regenerated by quercetin, which is subsequently regenerated by ascorbic acid (**Figure 4**)

(Murakami et al., 2003). E^0 can be no more than a guide to antioxidant regeneration activity when temperature, solvent type, phase separation and concentrations are not standard (Becker et al., 2004).

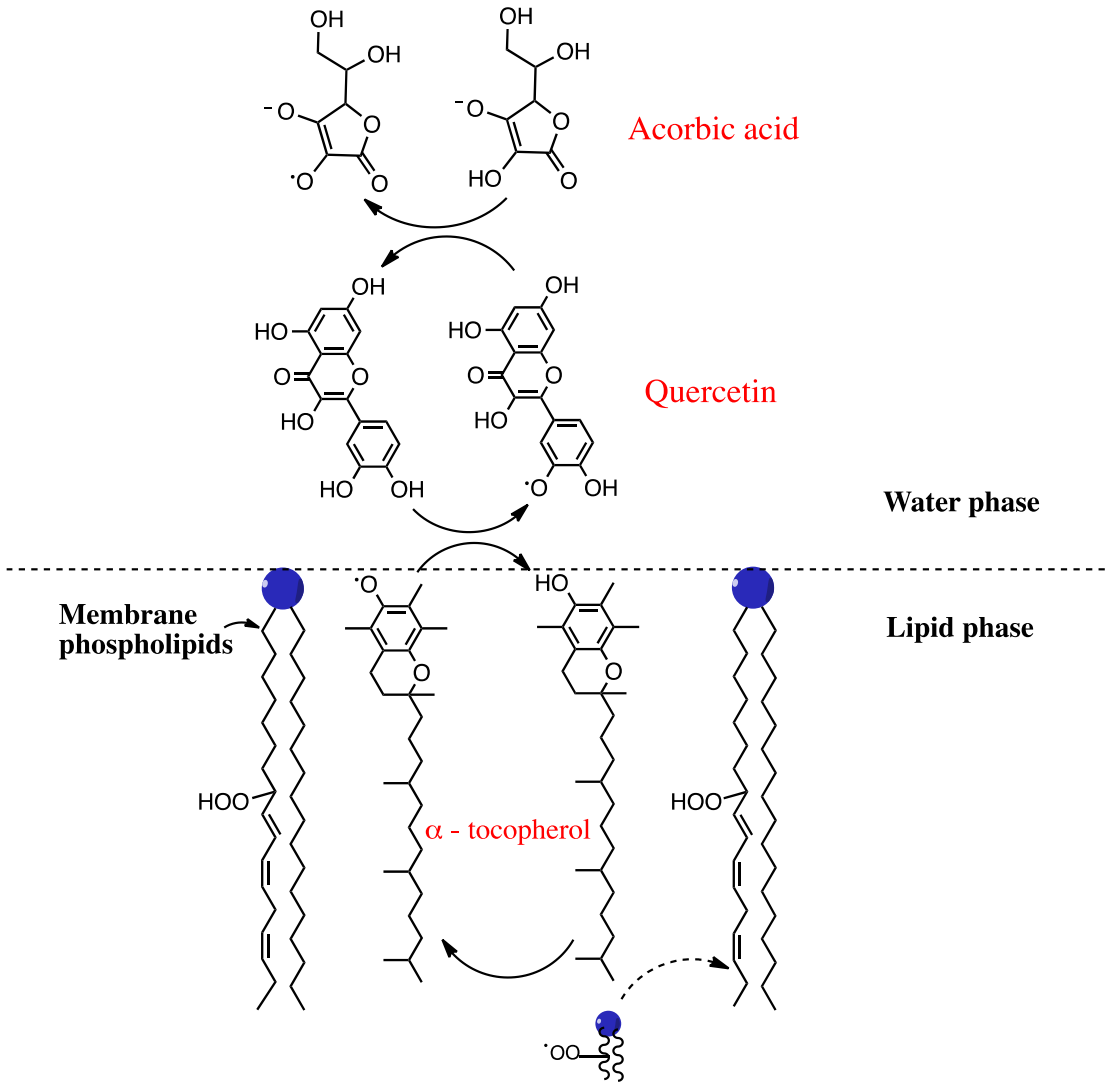


Figure 4. The mechanism of synergy in anti-oxidation of the mixture of α-tocopherol, quercetin and ascorbic acid at the water-lipid interface in a membrane model. In this system, α-tocopherol locates near the water-lipid interface enabling it to interact with quercetin in the aqueous phase. Quercetin regenerates α-tocopherol and is subsequently regenerated by ascorbic acid.

The mixture of lycopene and astaxanthin is a typical example of the importance of proper phase distribution for the exertion of antioxidant synergy (Han et al., 2012). The lipophilic centre of astaxanthin (and other xanthophyll) molecules remains in a membrane with the two polar end groups attached to the lipid/water interface (El-

Agamey et al., 2006, Polyakov et al., 2010). Because of this orientation, astaxanthin is initially oxidised by free radicals to become a radical cation. In contrast, lycopene molecules, with no hydrophilic sites, located within the inner membrane acts as electron donors to the astaxanthin radical cations resulting in the regeneration of the partner (El-Agamey et al., 2006, Polyakov et al., 2010).

One antioxidant in a binary mixture can also protect another from oxidative degradation or isomerization by sacrificial oxidation**.** Lycopene protects all-*trans* β-carotene against isomerization induced by singlet oxygen and free radicals. The isomerization of β-carotene is retarded and is only triggered once lycopene has been completely depleted (Heymann et al., 2015). The ability of lycopene to protect β-carotene against singlet oxygen- and free radical-induced isomerization results from its higher anti-oxidative scavenging activities (Clinton, 1998, Wang, 2012b, Di Mascio et al., 1989). Lycopene acts as a quenching shield that is initially isomerized and degraded in the reaction preventing the accompanying carotenoid from isomerization (Namitha and Negi, 2010).

Factors influencing antioxidant synergy

The types of models used to study the antioxidant interaction: *in vitro* (chemical-based or cell-based) and *in vivo* (animal- or human-based) affect the observation of synergy. Synergistic effects on bioactivities seen in chemical systems (e.g. FRAP, DPPH, ORAC) might not be shown in biological systems (e.g. cancer-cell lines) and *vice versa*, as these two approaches are totally different in assay conditions and ways to evaluate the effects (Wang and Zhu, 2017). On the other hand, the interactive effects of antioxidant mixtures may also be seen differently in different chemical assays. For instance, positive interaction between some anthocyanins (cyanidin-3-glucoside, malvidin-3-glucoside or pelargonidin-3-glucoside) and quercetin appear in FRAP assay but not in DPPH assay (Hidalgo et al., 2010). Although the mixture of malvidin-3-

glucoside and catechin lowers the DPPH scavenging activity (Hidalgo et al., 2010), it increases the inhibition of the oxidation of linoleic acid micelles initiated by ABIP (2,2'-azobis[2-(2imidazolin-2-yl)propane] dihydrochloride) (Rossetto et al., 2002). As chemical methods are widely used in studying phytochemical interactions they are worthy of further discussion on factors that may influence the interactive effects observed in these systems.

A number of factors affect the interactions between antioxidants in chemical systems (**Table 4**). A recent review by Wang and Zhu (2017) has described in detail the reaction medium and solvent effects. The following section summarizes important aspects of these and others to provide a comprehensive overview.

Antioxidant interactions can show differently in different reaction media. The mixture of α-tocopherol and quercetin in methyl linoleate emulsion or liposome shows synergy in lipid oxidation inhibition, but in bulk sunflower oil shows addition (Becker et al., 2007). Antioxidants may perform differently in homogeneous- and heterogeneous-reaction-media (Becker et al., 2007, Zhou et al., 2005b). For instance, α-tocopherol combined with the flavonols quercetin or myricetin inhibits AAPH-induced linoleic acid peroxidation more in single-phase (*t*BuOH/H_2O 3:2) than in multi-phase systems (sodium dodecyl sulfate and acetyl trimethylammonium bromide micelles) (Zhou et al., 2005b). In the single-phase system, the combined antioxidants scavenge the initiating AAPH radicals. In the multi-phase system, they trap the propagating lipid peroxyl radicals on micelle surface and reduce α-tocopheroxyl radicals to regenerate α-tocopherol (Zhou et al., 2005b).

Table 4. Factors that affect antioxidant interactions in chemical assays.

Factors		Influences on	References
Reaction medium	Homogeneous solutions (single-phase systems) Heterogeneous solution (multi-phase systems)	Hydrogen / electron transfer activities Solvation and interfacial phenomena	Becker et al. (2007), (Zhou et al., 2005b)
Solvent	Compositions Polarity	Compatibility between sample preparation solvent and reaction medium	Celik et al. (2010), Serrano et al. (2007) Hidalgo et al. (2010)
Interacting antioxidants	Structural features Concentration Ratio	Orientation of AOXs at interface Interaction of AOXs with membrane lipids Regeneration of AOXs	Hidalgo et al. (2010), Liang et al. (2009a), Jiang et al. (2015)
Nature of radical initiators	Hydrosoluble, e.g. AAPH, AIPH Liposoluble, e.g. AMVN, MeO-AMVN	Participation of AOXs in radical scavenging	Beretta et al. (2006), Yeum et al. (2009), Frankel and Meyer (2000)
Interfering substances	Food matrix components, e.g. proteins, amino acids	Performance of AOXs	Pérez-Jiménez and Saura-Calixto (2006)
	Pro-oxidant agents, e.g. metal ions, free fatty acids		Aubourg (2001), Çelik et al. (2015)

AOXs: antioxidants; AAPH: 2,2'-azobis(2-amidinopropane) dihydrochloride; AIPH: 2,2'-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride; AMVN: 2,2'-azobis(2,4-dimethylvaleronitrile); MeO-AMVN: 2,2'-azobis(4-methoxy-2,4- dimethylvaleronitrile).

Solvent compositions can affect phytochemical interactions (Celik et al., 2010, Hidalgo et al., 2010, Pérez-Jiménez and Saura-Calixto, 2006, Serrano et al., 2007). The impacts of solvents are highest in ORAC assay, less in ABTS and DPPH assays, and least in FRAP assay (Pérez-Jiménez and Saura-Calixto, 2006). When any solvent components used for sample preparation interfere with assay reagents, the interactions between phytochemicals may not appear (Serrano et al., 2007). The mixture of BHA and BHT prepared in dichloromethane (DCM) / ethanol (EtOH) 9:1 shows synergy in CUPRAC and ABTS assays. This mixture, however, does not show synergy in FRAP assay because of the difference in solvent polarity: the sample preparation solvents (DCM) is less polar, whereas the FRAP reagent solution ($Fe(TPTZ)_2^{2+}$ chromophore solution) is high polar (Celik et al., 2010).

The combined effects of a phytochemical mixture depend upon the antioxidant potency of each component, which is determined by the structural characteristics of the compound: the presence of glycosidic moieties, and the number and position of hydroxyl and methoxy groups (Azevedo et al., 2010, Cao et al., 1997, Zhao et al., 2014). When two phytochemical compounds interact and form hydrogen bonds at their active hydroxyl groups, the free-radical-scavenging capacities of the compounds are decreased and antagonism appears (Hidalgo et al., 2010). In multi-phase reaction systems, interaction between a phytochemical in water-phase with the one in lipid-phase depends on the polarity of the hydrophilic compound. The less polar the compound, the higher affinity the compound has to the lipid membrane interior, and the stronger the synergy is. For example, synergy in inhibiting AAPH-induced liposome oxidation appears stronger when α-tocopherol combines with ferulic acid than when it combines with caffeic acid. Ferulic acid is less polar than caffeic acid resulting in stronger interaction with α-tocopherol (Neunert et al., 2015). Specific functional groups on the structure of the hydrophilic compound also determines the occurrence of synergy (Han et al., 2011). When β-carotene combines with puerarin or its derivatives in a liposome model, synergy in anti-lipooxidation shows only in combinations where puerarin component has a free 7-phenolate group on the A ring (puerarin and 4'-propylpuerarin). The A-ring phenolate group functions to regenerate β-carotene.

Antioxidant concentration and ratio in the mixtures affect the interactive effect. In a synergy system with two components, one acts as protector or regenerator of the other by donating its electrons or hydrogen atoms. The alterations of concentration or ratio of each component affect these actions leading to changes in the interactive effect. Tocopherols combined with carotenoids show synergy only when tocopherols are present at higher ratios than carotenoids (Mortensen and Skibsted, 1997, Palozza and

Krinsky, 1992, Wrona et al., 2003). In these mixtures, tocopherols regenerate carotenoids.

The type of free radical impacts the observation of interactions between antioxidants. In heterogeneous systems, the contribution of each phytochemical component in radical scavenging activity depends on which phase the initiating radicals are generated in (Beretta et al., 2006). When radicals are induced in aqueous phase by hydrophilic radical initiators (e.g. AAPH), water-soluble antioxidants mainly contribute to scavenge the free radicals. Fat-soluble antioxidants, especially the ones located far from the interface, cannot approach the initiating radicals (Beretta et al., 2006, Han et al., 2007). Antioxidant interactions, therefore, may not appear in this system. In contrast, when lipophilic azo compounds: 2,2'-azobis(2,4-dimethylvaleronitrile (AMVN) or 2,2'-azobis(4-methoxy-2,4- dimethylvaleronitrile) (MeO-AMVN) are used for radical initiation in lipid phase, lipophilic antioxidants can scavenge the radicals and interact with hydrophilic antioxidants at the interface (Beretta et al., 2006, Han et al., 2007, Liang et al., 2010, Yeum et al., 2009). For instance, the mixtures of α-tocopherol and ascorbic acid (Yeum et al., 2009), or the mixture of β-carotene and puerarin (Han et al., 2007, Liang et al., 2010) provides synergy in inhibition against AMVN-induced liposome oxidation, but does not show in AAPH-induced system. The lipophilic antioxidant (α-tocopherol or β-carotene) scavenges AMVN radicals in the lipid phase, and is subsequently regenerated by the hydrophilic antioxidant (ascorbic acid or puerarin) at the interface.

The presence of non-antioxidant compounds in the reaction can influence phytochemical interactions because these compounds can interfere with the antioxidant activities of phytochemicals. Interfering compounds include food matrix compositions: proteins and amino acids (Pérez-Jiménez and Saura-Calixto, 2006); and pro-oxidant

agents: metal ions (Celik et al., 2010) and free fatty acids (Aubourg, 2001, Kamal-Eldin and Budilarto, 2014). These compounds affect the performance of antioxidants resulting in incorrect estimation of the antioxidant synergy.

Indirect antioxidant effects in vivo

The regular consumption of phytochemicals from fruits and vegetables has been associated with a reduced risk of a number of chronic diseases including cancer, cardiovascular diseases and neurodegenerative disorders (Vauzour et al., 2010). A body of scientific evidence has shown that the mechanisms by which phytochemicals exhibit these beneficial properties do not involve their direct antioxidant effects (Sies, 2010, Vauzour et al., 2010, Stevenson and Hurst, 2007). Instead, these health benefits appear to involve their interaction with endogenous protective enzymes (Sies, 2010, Stevenson and Hurst, 2007), and/or cellular signalling pathways that mediate cellular processes involved in inflammation and the initiation and progression of cancer, cardiovascular diseases and neurodegeneration (Vauzour et al., 2010, Stevenson and Hurst, 2007). On the other hand, the concentrations of phytochemicals required to exert inductive or signalling effects are much lower than that required for effective radical scavenging (Stevenson and Hurst, 2007). For example, several human and animal trials with lycopene or lycopene-containing extracts show that the *in vivo* antioxidant activity for lycopene is not well supported (Erdman et al., 2009). The concentrations of lycopene at tissues appear too low to play a meaningful antioxidant role (Erdman et al., 2009).

2.3.4. Effects of phytochemical interactions on anti-carcinogenic activities

The consumption of whole fruits and vegetables rather than dietary supplements of single bioactive components are recommended (Liu, 2013, Rodriguez-Casado, 2016). Biological effects of a whole food are the combined effects of different bioactive

components presenting in the food (Lila, 2007, Liu, 2013, Singh et al., 2016). Consuming dried tomato powder reduces the mortality from prostate cancer in humans more effectively than diets supplemented with lycopene at equivalent concentrations (Campbell et al., 2004). Lycopene, the major phytochemical component in tomato, and other carotenoids and polyphenolic components contribute to the biological activities of tomato (Boileau et al., 2003, Campbell et al., 2006). Several comprehensive reviews have discussed the role and the mechanisms of phytochemical interactions in the prevention of different cancer diseases (de Kok et al., 2008, DiMarco-Crook and Xiao, 2015, van Breda and de Kok, 2018). The main hypothesis of the underlying mechanism is that the combined phytochemicals affect different molecular targets that activate multiple molecular mechanisms of action, whereas an individual phytochemical activates only one single molecular mechanism (van Breda and de Kok, 2018). A recent overview of phytochemical combinations showing synergistic effects on cancer chemoprevention is provided by van Breda and de Kok (2018). The following section presents some recent findings and highlights some phytochemical combinations showing synergy in inhibiting the development of cancers.

Curcumin is the major active component of turmeric and is known for its high anti-proliferative activities (Basnet and Skalko-Basnet, 2011, Metzler et al., 2013). The mixtures of curcumin with other bioactive compounds show high synergistic effects on cancer chemoprevention *in vitro* (Altenburg et al., 2011, Iwuchukwu et al., 2011, Montgomery et al., 2016, Wang et al., 2016). Turmeric curcumin (12.5 μM) combined with milk thistle silymarin at various concentrations: 3.125, 6.25, 12.5, 25, 50 and 100 μM synergistically inhibits the growth of three colon cancer cell lines (DLD-1, LoVo, HCT116) (Montgomery et al., 2016). The combination of curcumin (12.5 μM) and sylimarin (12.5 μM) shows synergy in inducing apoptosis of DLD-1 colorectal cancer

cells by suppressing nuclear factor-kappa B (NF-κB) activity (Montgomery et al., 2016). Curcumin mixed with berberine provides synergy in inhibiting the growth of MCF-7 and MDA-MB-231 breast cancer cell lines by elevating the induction of apoptosis and autophagic cell death, and by modulating different pathways (JNK, Beclin1, and Bcl-2) (He et al., 2016). Curcumin combined with epigallocatechin gallate (ECGC) provides chemopreventive synergy *in vitro* (Khafif et al., 1998, Balasubramanian and Eckert, 2004, Eckert et al., 2006). This treatment synergistically inhibits the growth of carcinoma cells by blocking the cell cycle at G_1 and S/G_2M phase (Khafif et al., 1998). When curcumin and ECGC are used in combination, the required efficacious dose is diminished (to 12.5-25% for EGCG, and to 33-50% for curcumin (Balasubramanian and Eckert, 2004). Different mixtures of ECGC with the other tea polyphenols epicatechin (EC), epigallocatechin (EGC) or epicatechin gallate (ECG), or the combination of all four compounds provide synergy in suppression of lung tumour and gastric carcinoma cell growths *in vitro* (Horie et al., 2005, Suganuma et al., 1999, Williams et al., 2003, Williams et al., 2000). The mechanisms behind the synergy of tea polyphenol mixtures could be: increase of EGCG cellular uptake, enhancement of apoptosis induction, and/or modulation of transcription of human CYP1A1 (Williams et al., 2003, Williams et al., 2000, Horie et al., 2005, Suganuma et al., 1999).

In addition, synergy in *in vitro* anti-proliferation of cancer cells occurs when EGCG is combined with drugs: 4-methylumbelliferone (4-MU) for suppressing the growth of human breast cancer cells (MDA-MB-231 line) and human microvascular endothelial cells (HMECs); and Paclitaxel (a common prescribed drug in cancer treatment) for inhibiting the proliferation of HMECs (García-Vilas et al., 2016). The combinations of green tea or black tea extract with soy phytochemicals provide synergy in suppressing the development of human prostate and breast tumours in mice (Zhou et al., 2003, Zhou

et al., 2004). Decaffeinated green tea extracts combined with grape skin extracts synergistically inhibit the growth of human cervical carcinoma cells *in vitro*, and combined with grape pomace powder at 25:1 ratio show synergy in inhibiting the growth of 4T1 mammary tumours in mice (Morré and Morré, 2006). Other combinations of dietary phytochemicals providing synergy in *in vitro* anti-proliferation of cancer cell lines (presented in brackets) are: resveratrol-chrysin-curcumin (Caco-2 colon carcinogenic cells) (Iwuchukwu et al., 2011); daidzein-genistein (LNCaP and C4-2B prostate cells) (Dong et al., 2013); genistein-quercetin-biochanin A (PC-3, LNCaP and DU-145 prostate cancer cells) (Kumar et al., 2011); quercetin-kaempferol and/or naringenin (Hepa-1c1c7 mouse liver cancer cells and the LNCaP human prostate cancer cells) (Campbell et al., 2006); quercetin-resveratrol, and quercetin-resveratrol-ellagic acid (human leukemia cells) (Mertens-Talcott et al., 2003); quercetin-EGCG (PC-3 and LNCaP prostate cells); and docosahexanenoic acid-curcumin (SK-BR-3 breast cancer cells) (Altenburg et al., 2011).

Synergy in inhibition of cancer cell growth *in vitro* occurs when isolated bioactive compounds are added with food extracts. For example, the blends of quercetin-3-glucoside and apple extract (Yang and Liu, 2009), or genistein and pomegranate extract (Jeune et al., 2005) synergistically inhibit MCF-7 human breast cancer cells. Grapeseed extracts added with resveratrol enhance the suppression of HCT116 colon carcinogenic cells (Radhakrishnan et al., 2011). The anti-carcinogenic synergy of these combinations possibly results from phytochemical interactions in different biological activities: anti-oxidation, apoptosis induction, cell cycle arrest, enzyme modification, or gene-transcription modulation (Campbell et al., 2006, de Kok et al., 2008, Knowles et al., 2000, Mertens-Talcott et al., 2003). Various mixtures of plant-based extracts: tomato and broccoli (Canene-Adams et al., 2007), or tomato and garlic (Sengupta et al., 2004)

fed to rats, or peppermint and rosemary (Yi and Wetzstein, 2011) treated to SW-480 colon cancer cells *in vitro*, show greater anti-tumour and anti-proliferative effects than each single extract alone (no synergy analysis).

2.3.5. Effects of phytochemical interactions on anti-inflammatory activities

Inflammation is a response of the immune system to protect the cells or tissues from foreign agents. Prolonged inflammation contributes to develop several chronic disorders: diabetes, atherosclerosis and cancers (Libby et al., 2010). Diet-based strategies with regular consumption of fruits and vegetables reduce the risk of inflammation because these foods contain a wide variety of bioactive compounds with antioxidant and anti-inflammatory properties (Vainio and Weiderpass, 2006).

The efficiency of inflammatory treatments can be enhanced by combination approaches: drug-phytochemical or mixed phytochemicals. The combinations of anti-inflammatory drugs with naturally occurring phytochemicals allow the drug's use at lower doses for stronger effects. For instance, atorvastatin (0.1 μM), a common drug for atherosclerosis treatment, in conjunction with cyanidin-3-glucoside (2 μM) provides synergistic suppression of the proliferation of human aortic smooth muscle cells (HASMCs) induced by angiotensin II *in vitro*. When combining with cyanidin-3-glucoside, atorvastatin can be used at a dose lower than when it is used alone which reduces its adverse effects (Pantan et al., 2016). Several combinations of dietary phytochemicals provide synergy to inhibit inflammation. When luteolin is combined with tangeretin, synergy occurs in protecting RAW 264.7 cells against inflammation stimulated by lipopolysaccharide (LPS) *in vitro* (Funaro et al., 2016). Different combinations of polyphenolic compounds: (1) 4'-hydroxymandelic acid, 4-hydroxyphenylacetic acid, 5-(3'-hydroxyphenyl) propionic acid and 3-(4'-hydroxyphenyl) lactic acid; (2) (–)-

epigallocatechin-3-O-gallate, pelargonidin-3-O-glucoside, cyanidin-3-O-glucoside and punicalagin; (3) dihydroferulic acid, feruloylglycine, quercetin and 3-O-methylquercetin; (4) caffeic acid, ferulic acid, isoferulic acid and isoferuloylglycine; (5) hippuric acid, tyrosol, 4'-hydroxyhippuric acid and chlorogenic acid, show synergy in modulating the release *in vitro* of pro-inflammatory cytokines by Jurkat T-lymphocytes (Ford et al., 2016). The combinations of coffee extract with the extracts of cinnamon (Durak et al., 2014); ginger (Durak et al., 2015); or dried coconut meat (Gawlik-Dziki et al., 2016) synergistically inhibit *in vitro* lipoxygenase (LOX-1), which is one of the pro-inflammatory factors. An oral nutraceutical mixture of berberin, red yeast rice, policosanol, astaxanthin, folic acid and coenzyme Q10 enhances anti-inflammatory effects *in vivo*: lowering LDL cholesterol level, and reducing systemic inflammation and endothelial injuries in patients with low-grade systemic inflammation (Pirro et al., 2016).

Inflammation can occur when there is a persistent presence of high concentrations of reactive oxygen species (ROS) (Hsu et al., 2010), which can activate intracellular signaling pathways such as NF-κB and mitogen-activated protein kinase (MAPK) pathways (Fukumitsu et al., 2016, Hsu et al., 2010, Pantan et al., 2016). The activated pathways trigger the expressions of several pro-inflammatory cytokines or chemokines. The potential mechanisms underlying the synergy of phytochemical combinations in anti-inflammation and anti-cancer are related to the synergistic multi-target effects of the combinatory components: single constituents can direct to several targets (e.g. enzymes, or activators) (Imming et al., 2006, Wagner and Ulrich-Merzenich, 2009, Williamson, 2001). The mixture of luteolin and tangeretin provides synergistic effects in inhibiting the formation of nitric oxide free radicals produced by nitric oxide synthase (iNOS), which is one of the pro-inflammatory enzymes mediating inflammatory

processes (Funaro et al., 2016). This mixture also potentiates the suppression of several pro-inflammatory mediators such as PGE_2, IL-1β and IL-6 (Funaro et al., 2016). The synergy between atorvastatin and cyanidin-3-glucoside in the protection of HASMCs against angiotensin II-induced inflammation *in vitro* relies on the ability of the two compounds to synergistically affect different pro-inflammatory pathways: inhibiting NF-κB activity leading to down-regulating iNOS and reducing NO production; and suppressing the expression of NADPH-oxidase resulting in diminishing ROS formation. The two compounds also elevate Nrf2 transcription leading to the activation of several cytoprotective enzymes (Pantan et al., 2016).

2.3.6. Other biological effects of phytochemical combinations

Synergy or potentiation of dietary phytochemical combinations in treatments of other oxidative stress and metabolic disorders is reported. The combinations of resveratrol with quercetin and/or genistein enhance the suppression of adipogenesis. The inhibitory effects of the mixtures of resveratrol (12.5 μM), quercetin (12.5 μM) and genistein (6.5 μM) on lipid accumulation in both human adipocyte and 3T3-L1 mouse cell lines are higher than the calculated additive effects of individual components (Park et al., 2008). Rats fed either resveratrol or quercetin show no significant reduction in adipose tissue weights. When they are fed a diet supplemented with a mixture of resveratrol (15 mg/kg/day) and quercetin (30 mg/kg/day), their body fat accumulation and triacylglycerol metabolism in white adipose tissue are remarkably reduced indicating *in vivo* potentiation in anti-obesity of these two compounds (Arias et al., 2016). The effects of phytochemical combinations on anti-diabetes and neuroprotection are reported. Potentiation in anti-hypoglycemic activities shows in the mixture of loganin and ursolic acid isolated from *Cornus officinalis* fruits (He et al., 2016), or in the blend of 80% apple cider and 20% whole blueberry juice (Agustinah et al., 2016). Fermented

soybeans added with sprouted garlic are more effective in inhibition of oxidation and protection of rat neurons against cognitive dysfunction induced by glutamate (Woo et al., 2016). The mechanisms underlying these combined biological effects of phytochemicals are complicated or unknown in most cases beyond the scope of this review.

2.3.7. Effects of dietary phytochemical interactions on absorption/bioavailability

2.3.7.1. Absorption interferences between hydrophobic phytochemicals

Interactions on intestinal absorption occur when different carotenoids are co-ingested. The carotenoid interactions during absorption and post-absorptive metabolism have been comprehensively reviewed by van den Berg (1999). α-carotene absorption in humans is decreased by 38% when lutein supplementation is increased from 18 mg to 36 mg (Reboul et al., 2007a). Absorption interferences occur both *in vitro* and *in vivo* when any two carotenoids: β-carotene, lycopene, lutein or cathaxanthin are co-supplemented. There are inconsistent findings on the direction and magnitude of the interactions. For instance, an improvement and impairment of lycopene absorption by β-carotene are both reported (Gaziano et al., 1995, Johnson et al., 1997, Prince et al., 1991, Wahlquist et al., 1994, White et al., 1993). β-carotene absorption improved by lycopene is also reported (White et al., 1993). Different findings about carotenoid absorption interferences are due to the differences in study protocols: quantitative methods; dose usage (single-dose, short-term- or long-term supplementation); research models or species used (animals, human or *in vitro* models) (van den Berg, 1999).

Carotenoid interactions may occur at different stages of digestion, metabolism and distribution. Several mechanisms are proposed to explain uptake/bioavailability interactions of carotenoids. Lycopene can scavenge singlet oxygen and free radicals,

which protects β-carotene from isomerization resulting in more active all-*trans* β-carotene remaining for absorption into the plasma (Heymann et al., 2015). An increase in absorption of one carotenoid occurs when the other one can inhibit the activity of carotenoid cleavage enzymes in the intestinal mucosa. For example, the oxycarotenoid lutein or cataxanthin inhibits the cleavage enzyme of β-carotene resulting in an increase of β-carotene in the serum (Kostic et al., 1995). The absorption of a carotenoid is decreased when the co-consumed compound interferes with the carotenoid micellarization in intestinal lumen or with the uptake in intestinal chylomicrons. In systemic circulation, carotenoid compounds may compete to each other for binding with appropriate plasma proteins (Hidalgo et al., 2010, van den Berg, 1999).

Carotenoids can impair the absorption of α-tocopherol. The carotenoids β-carotene, lycopene or lutein, at dietary levels, decrease α-tocopherol absorption in Caco-2 TC7 cell line (Reboul et al., 2007a). Lycopene induces highest impact on α-tocopherol absorption, followed by lutein, and β-carotene. γ-tocopherol, another vitamin E species, can interfere with the absorption of α-tocopherol. Each of these compounds competes with α-tocopherol for uptake at the apical compartment of the Caco-2 cell monolayers and reduces the absorption of α-tocopherol (Reboul et al., 2007a).

2.3.7.2. Absorption interferences between hydrophilic phytochemicals

Hydrophilic compounds can interfere with the absorption of each other. Absorption interferences occur between flavonoids, and between flavonoids and phenolic acids. The mucosal absorption of cyanidin-3-glucoside, in the presence of 50 μmol/L of quercetin-3-glucoside, is significantly decreased by 73.9% ($p < 0.001$) over 2 hours, while the absorption of quercetin-3-glucoside is increased over time (Walton et al., 2006). The competition of these compounds for cellular uptake, or for cellular transportation

contributes to the changes in their absorption. These two compounds can be transported to cells by the same transporter, which has higher affinity to flavonols than to anthocyanins, thus facilitating the absorption of quercetin-3-glucoside. An increase in the absorption of rosmarinic acid in Caco-2 cells occurs when the flavonoids luteolin and apigenin are present (Fale et al., 2013). The absorption rate of rosmarinic acid in Caco-2 cells is significantly boosted in the presence of increasing concentrations of luteolin and apigenin. The simultaneous presence of the flavonoids inhibits the efflux of rosmarinic acid by inhibiting ABC transporters resulting in the increase in the cellular absorption of rosmarinic acid (Fale et al., 2013).

Bioaccessibility/bioavailability of a bioactive compound can increase when its stability and solubility are improved. Phytochemical interactions can result in these improvements. The bioavailability of quercetin is increased in the presence of proanthocyanidins (*Pyracantha fortuneana* fruit) (Zhao et al., 2015). These compounds improve the bioavailability of quercetin by improving its solubility and stability. Proanthocyanidins form hydrogen bonds with quercetin, which possibly contribute to improve quercetin solubility. They improve chemical stability of quercetin by preventing it from oxidation and improving reducing ambient of solvent systems (Zhao et al., 2015).

2.3.7.3. Absorption interferences between hydrophobic and hydrophilic phytochemicals

Hydrophilic compounds can impair the absorption of lipophilic ones. Naringenin can interfere with the absorption of α-tocopherol, lutein or the mixture of β-carotene and β-cryptoxanthin in Caco-2 cells (Reboul et al., 2007a, Reboul et al., 2007b, Claudie et al., 2013). The cellular concentrations of the carotenoid mixture β-carotene-β-cryptoxanthin

increase by 30% when naringenin is present, and by 60% in the presence of hesperidin or hesperetin (aglycone) (Claudie et al., 2013). The cellular uptake of β-carotene or β-cryptoxanthin increases by 150% in the presence of hesperetin (Claudie et al., 2013). Absorption interferences do not occur between ascorbic acid and the carotenoids β-carotene, β-cryptoxanthin or lutein (Reboul et al., 2007a, Reboul et al., 2007b), or between α-tocopherol and vitamin C, caffeic acid, gallic acid or (+)-catechin (Reboul et al., 2007a).

Different explanations for the absorption interferences between hydrophilic and lipophilic phytochemicals have been proposed. Naringenin interferes with the absorption of the lipophilic compounds by interacting with the lipophilic transporter (scavenger receptor class B type I), or with cell membrane lipids, which lead to the invagination of lipid raft domains containing the carotenoids or α-tocopherol receptors (Reboul et al., 2007a, Reboul et al., 2007b). The affinity to cell membrane lipids of water-soluble phytochemicals determines the interfering effects on cellular absorption of fat-soluble compounds. The absorption of the carotenoid mixture β-carotene-β-cryptoxanthin is more enhanced by the flavonone glucoside hesperidin than by the aglycone hesperetin. Hesperidin is poorly bioavailable because of its high affinity to membrane lipids. The sugar moiety of hesperidin interacts with the polar head of the lipids and alters the barrier function of the double-layer membrane facilitating the cellular uptake of the carotenoids (Claudie et al., 2013). The aglycone hesperetin, which is more liposoluble, interacts with the acyl chains of the membrane lipids. Among the three flavonones hesperetin, hesperidin and naringenin, the last has lowest affinity to the membrane lipids and shows the least effect on the carotenoid uptakes (Claudie et al., 2013). Therefore the stronger the affinity of the flavanones to biological membranes, the greater are the enhancing effects on carotenoid absorption. More work is required to

understand the mechanisms of uptake/bioavailability interferences between bioactive compounds.

2.3.8. Research gaps for future studies

The number of studies on uptake/bioavailability interferences between bioactive compounds is very limited. Only few phytochemical mixtures are investigated for their interferences on intestinal uptake. The mechanisms of the absorptive interactions between phytochemicals are not fully understood. In addition, there is limited attention given to studying the impacts of bioaccessibility and bioavailability interferences on bioactivity interactions between phytochemicals. Synergy in biological activities can result only from phytochemical combinations that can promote "solubility, safety, absorption, stability or bioavailability of the principal active compounds" (Kirakosyan et al., 2010). Gawlik-Dziki (2012) measured the antioxidant activities of various vegetable mixtures including raw extracts and their *in vitro* digested and absorbed extracts. If the digested extracts of all vegetable binary mixtures demonstrate higher antiradical activities than their raw combinations, the simulated gastrointestinal digestion must have released more bioaccessible antiradical components from the raw materials. For some vegetable blends such as tomato and onion, or tomato and lettuce, synergy of *in vitro* antiradical activity is seen in the raw and digested extracts, but not in the absorbed extract. Only the combination of tomato and garlic shows synergistic bioactivities in all forms tested (raw, digested and absorbed), probably because tomato and garlic contain highly bioaccessible and bioavailable active constituents.

The mode of interaction and the intensity of the interactive effect in some food combinations can change after the foods undergo gastrointestinal digestion. For instance, a raw water-soluble extract mixture of coffee and cinnamon shows synergy in

the inhibition of *in vitro* lypoxygenase activity but changes to antagonism after digestion (Durak et al., 2014). In contrast, undigested extracts of coffee and ginger act antagonistically but their digested bioaccessible constituents synergistically inhibit lypoxygenase (Durak et al., 2015). The degree of antagonism of the coffee-coconut mixture is higher in its digested extracts than in the combined raw materials (Gawlik-Dziki et al., 2016). These results imply that bioaccessibility and bioavailability of bioactive components should be considered when evaluating the bioactivity interactions. Several factors can contribute to the changes of the interaction between phytochemicals after digestion. They include: the chemical properties of the bioactive components, the ratios of different phytochemicals in the raw extracts and in the digested mixture, and the possible formation of complexes among the compounds and /or with the food matrix during digestion (Gawlik-Dziki et al., 2016). Further studies are required to understand these intricate issues.

Isolated compounds, or fruit / vegetable extracts are used more often than food products in interaction studies. These approaches do not consider the interferences of food matrix components and/or the impacts of food processing on the bioaccessibility and bioactivities of the active compounds. In fact, the bioaccessibility of bioactive compounds can be different in different food matrices. The highest bioaccessibility of vitamin C shows in soy-containing-fruit-juice blends; and of phenolic compounds in water-based fruit-juice blends (fruit juices blended with water) (Rodríguez-Roque et al., 2015). When whole milk is blended with the fruit juices, the bioaccessibility of vitamin C or phenolic compounds is the lowest. Milk proteins can interact with vitamin C and polyphenols; metal ions (Fe, Cu, and Zn) and other vitamins (B_1, B_2, B_{12}) in milk can interact with vitamin C. These interactions result in the degradation of vitamin C and/or

the formation of protein-vitamin and protein-phenol complexes, which cause decreases in bioaccessibility of these compounds.

The bioaccessbility of bioactive compounds in foods can be changed after food processing (Rodríguez-Roque et al., 2015, Swada et al., 2016). Applying non-thermal processes: high-intensity pulsed electric fields or high-pressure processing to fruit juice beverages can increase by up to 38% of the bioaccessibility of several phenolic compounds (e.g. hesperidin, rutin), but can also reduce that of the others (ferulic acid, chlorogenic acid and *p*-hydroxybenzoic acid from water-based beverages) (Rodríguez-Roque et al., 2015). This phenomenon occurs because processing can induce changes in physicochemical properties of phenolic compounds (structure changes: hydroxylation, glycosylation, methylation or dimerization, or formation of phenolic derivatives) resulting in modification (increase or decrease) of the compound bioaccessibility (Dugo et al., 2005). The effects of processing depend on the type of phytochemical substances and of compound-containing food matrices (Rodríguez-Roque et al., 2015); and the duration and intensity of the treatment (Chandrasekara and Shahidi, 2012). Food processing can also affect phytochemical interactions. Different blends of strawberry and papaya nectars (25:75, 50:50 and 75:25) show synergistic effect in anti-oxidation after ultra-high temperature treatments (80 – 135^{o}C) (Swada et al., 2016). Non-heat treated blends show additive effect. Heating at high temperature causes cell rupture that releases more antioxidants trapped in the cell membrane of the fruits to interact with each other. Combining the two fruit nectars followed by heat processing is therefore important for anti-oxidative synergy (Swada et al., 2016).

Another concern in most of the *in vitro* studies on phytochemical interactions is the use of raw food extracts or isolated bioactive compounds at concentrations higher than their

physiological ones. In *in vivo*, however, bioactive compounds are present in plasma or tissues at smaller concentrations. They can be structure-modified or metabolized into other compounds following oral consumption. The active metabolites can interact and produce changes in bioactivities. For example, synergy in radical scavenging activities occurs between the gastric and intestinal metabolites of purple rice anthocyanins (Sun et al., 2015).

Knowing the gaps, future studies, therefore, should be designed to increase more knowledge on:

- The interactive pattern, direction and mechanisms of bioaccessibility/bioavailability interferences;
- The impact of bioaccessibility and bioavailability on bioactivity interactions between phytochemicals: how and why the interactive effects on biological activities of phytochemical mixtures would change after intestinal digestion, absorption and metabolism;
- Phytochemical interactions on bioactivity and bioaccessibility/bioavailability considering the physiological concentrations of phytochemicals and the effects of complex food matrices and food processing;

2.3.9. Conclusion

The combinations of bioactive compounds could result in changes in biological properties and uptake/bioavailability of the compounds. A number of phytochemical mixtures and food combinations provide synergistic effects on inhibiting oxidation, inflammation and cancer cell proliferation. These biological effects of phytochemicals depend on the bioaccessibility and bioavailability of the compounds, which can be impaired with by the co-digestion of other phytochemicals. More research should be

conducted to completely understand the mechanism of bioaccessibility/bioavailability interferences and the inter-relationship between bioaccessibility/bioavailability and bioactivity as the result of interactions between bioactive compounds.

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Pure phytochemicals

All tested phytochemical compounds including cyanidin-3-*O*-glucoside (chloride) (CG), delphinidin-3-*O*-glucoside (chloride) (DG), pelargonidin-3-*O*-glucoside (chloride) (PLG), malvidin-3-*O*-glucoside (chloride) (MG), peonidin-3-*O*-glucoside (chloride) (PNG), petunidin-3-*O*-glucoside (chloride) (PTG), and carotenoids including lycopene, lutein and β-carotene were purchased from Extrasynthese (Lyon, France).

3.1.2. Vegetable materials

Fresh red cabbage (*Brassica oleracea var*. *capitata f*. *rubra*), carrot (*Daucus carota subsp*. *sativus*), baby spinach (*Spinacia oleracea*) and cherry tomato (*Solanum lycopersicum var*. *cerasiforme*) were purchased from local supermarkets (Sydney, NSW, Australia).

3.1.3. Enzymes

Soybean lipoxygenase type I (LOX-1) was purchased from Sigma Aldrich (Sydney, NSW, Australia). Alpha-amylase from porcine pancreas (100,000 U/g) was purchased from Megazyme (Chicago, IL, USA). Pancreatin from porcine pancreas and pepsin from porcine stomach mucosa (> 2000 U/mg) were purchased from MP Biomedicals (Santa Ana, CA, USA). Bile from bovine and ovine sources was purchased from Sigma Aldrich (Sydney, NSW, Australia).

3.1.4. Other chemicals

All chemical reagents were purchased from Sigma Aldrich (Sydney, NSW, Australia). Cell culture medium components were purchased from Gibco^{TM} (Life Technologies, Melbourne, VIC, Australia).

3.2. Evaluation of the resulting biological effects of the combinations of pure anthocyanins and carotenoids

3.2.1. Preparation of stock solutions of anthocyanins and carotenoids

Stock solutions of anthocyanins (1 mg/mL) were prepared in methanol and stored at -80^{o}C. Stock solutions of β-carotene (1 mg/mL), lycopene (1 mg/mL), and lutein (1 mg/mL) were prepared in tetrahydrofuran and were stored under a nitrogen blanket at -80^{o}C no longer than 1 week. The concentration of the carotenoid solution was checked before analysis by reading the UV-VIS absorbance (UV-1800 series spectrometer, Shimadzu, Japan) at 454 nm (extinction coefficient = 134400 L/mol.cm^{-1}) for β-carotene, at 446 nm (extinction coefficient = 144500 L/mol.cm^{-1}) for lutein, and at 474 nm (extinction coefficient = 185000 L/mol.cm^{-1}) for lycopene (Miller et al., 1996).

3.2.2. Liposomal peroxidation inhibition assay

3.2.2.1. Preparation of liposome

Unilamellar liposome was prepared according to Roberts and Gordon (2003) with some modifications. A stock solution (25 mg/mL) of soybean L-α-phosphatidylcholine (PC) was prepared in chloroform. An aliquot of the PC stock was added in an amber vial and diluted to 1.25 mg/mL with chloroform. For samples containing carotenoids, 100 μL of a working solution of each carotenoid was added to the vial to get the final carotenoid concentration of 0.25% mol/mol lipid. The vial was vortexed and kept under a gentle nitrogen stream on a rolling mixer to evaporate chloroform to form a thin lipid layer on

the vial wall. The vial remained under a nitrogen stream for an additional 20 min to remove solvent residue. The lipid film was subsequently rehydrated with phosphate buffered saline (PBS), 10 mM, pH 7.4, to a final PC concentration of 0.5 mg/mL. For samples containing anthocyanins, the lipid film was rehydrated with a working solution of anthocyanins in PBS (final concentration: 0.25% mol/mol lipid). The mixture was mixed for 20 min to yield a homogeneous white suspension of large multilamellar liposomes which was subsequently sonicated for 30-s to completely recover the lipid from the vial wall. The large multilamellar liposomes were passed through a polycarbonate membrane (pore size 100 nm) 11 times in a small extrusion apparatus (Avanti Polar Lipids, Alabaster, AL, USA) to obtain unilamellar liposomes. The final concentration of carotenoid and/or anthocyanins in liposomes was 0.25% mol/mol lipid and the ratio of anthocyanins to carotenoids in liposomes was 1:1. This concentration was selected after preliminary trials. At concentrations higher than 0.25%, loss of carotenoids was seen during the preparation of liposome (i.e., some carotenoids were visibly retained on the polycarbonate membrane when passing the liposome suspension through the membrane to form unilamellar liposomes). In addition, carotenoids at 0.25% mol/mol lipid have been reported to be retained more than 80% in liposomes (Tan et al., 2014). The selected anthocyanin: carotenoid ratio of 1:1 was not necessarily relevant to the typical ratio of these compounds in food materials. The interest was to observe how anthocyanins and carotenoids could interact with each other when they were present at an equal concentration in a chemical reaction (i.e. no dominant compound), and when it came to cell-based activities, the compounds were varied at different concentrations and combinatory ratios to reflect the biological condition.

3.2.2.2. Peroxidation of liposome

Peroxidation of unilamellar liposomes was induced by Fe^{3+}/ascorbate following the method described by Tan et al. (2014) with some modifications. An aliquot of 0.5 mL of unilamellar liposome suspension containing phytochemical(s) was added into a test tube and the lipid peroxidation was initiated by the addition of 10 μL of $FeCl_3$ 15 mM and 10 μL of ascorbic acid 15 mM. A control sample of liposomes without the incorporation of phytochemical(s), a control blank, and a sample blank (no peroxidation induction) were also prepared at the same time. After 60-min incubation at 37°C, 1 mL of the reagent solution comprising of thiobarbituric acid 0.375% w/v, trichloroacetic acid 15% w/v, and HCl 0.25 N (TBA-TCA-HCl solution) was added to each tube. The mixture of liposomes and TBA-TCA-HCl solution was boiled at 100°C for 20 min, then cooled on ice and centrifuged at 3000 rpm for 10 min to remove any flocculants. Absorbance of thiobarbituric acid reagent species (TBARS) was read at 535 nm. The TBARS (%) inhibition was calculated as:

$$\text{TBARS (\%) inhibition} = \frac{\left(A_{control} - A_{control\_blank}\right) - \left(A_{sample} - A_{sample\_blank}\right)}{\left(A_{control} - A_{control\_blank}\right)} \quad (3)$$

Where: $A_{control\_blank}$, A_{sample_blank}: initial absorbance of control liposomes and phytochemical-containing liposomes, respectively, before peroxidation induction; $A_{control}$, A_{sample}: absorbance of control liposomes (containing no phytochemicals) and phytochemical-containing liposomes, respectively, after 60-min Fe^{3+}/ascorbate-induced peroxidation at 37°C.

3.2.3. Lipoxygenase inhibitory assay

The carotenoid working solutions used in this assay were prepared according to the method described by Wu et al. (1999) with some modifications. An aliquot of each carotenoid stock solution was diluted in chloroform containing Tween 80 (0.54%, v/v).

After evaporation of chloroform under nitrogen gas, the carotenoid residues were redissolved in ethylenediaminetetraacetic acid (EDTA) 0.064 mM to get the final carotenoid concentration of 0.024 mM. An aliquot of anthocyanin stock solution was taken into a test tube and evaporated under nitrogen to remove methanol, then redissolved in PBS 50 mM pH 7.4 to get the final concentration of 0.024 mM. Linoleic acid was used as substrate and the solution was prepared as described by Serpen and Gökmen (2006). 2 μL Tween 20 was added into 1 mL of water followed by the addition of 2 μL linoleic acid and the mixture was shaken in an ultrasonic bath for 3 min. 65 μL of 0.1 N NaOH was added to increase optical clarity. The final volume of linoleic acid solution was adjusted to 5.14 ml with PBS 50 mM pH 7.4 to set the final concentration of linoleic acid stock solution to 1.25 mM.

Trials on enzyme reaction kinetics were conducted to determine the optimum enzyme concentration (400 U/mL) for maximal enzyme activity (**Appendix 2** and **Appendix 3**). Lipoxygenase inhibitory activity of anthocyanins and/or carotenoids was assessed following the method described by Durak et al. (2014) with some modifications. A reaction mixture contained: x μL of anthocyanins and/or carotenoids (final concentration of carotenoids: 0.2-2 μM, and of anthocyanins: 2-12 μM), (1100 – x) μL PBS 50 mM pH 7.4, and 50 μL of LOX-1 solution prepared in PBS 50 mM pH 7.4 (final concentration of LOX-1: 400 U/mL). In samples containing both anthocyanins and carotenoids, the ratio of anthocyanins to carotenoids was 1:1. After pre-incubation at 30^{o}C for 10 min, the reaction was initiated by adding 50 μL of 1.25 mM linoleic acid. The absorbance was read at 234 nm (25^{o}C) after 2 min by UV-Vis spectrophotometer (Shimadzu, UV-1800 series, Japan). The lipoxygenase inhibitory activity was calculated as:

$$\text{Inhibition (\%)} = \frac{\left(A_{control} - A_{control\_blank}\right) - \left(A_{sample} - A_{sample\_blank}\right)}{A_{control} - A_{control\_blank}} \quad (4)$$

where:

$A_{control}$: Absorbance of control sample for 100% enzyme activity (-test compounds: anthocyanins or carotenoids, +enzyme)

$A_{control\_blank}$: Absorbance of control blank (to correct for background absorbance of substrate)

A_{sample}: Absorbance of test sample (+test compounds, +enzyme)

A_{sample_blank}: Absorbance of sample blank for 0% enzyme activity (+test compounds, -enzyme, to correct for background absorbance of the test compounds)

3.2.4. General cell culture condition

Human carcinogenic colon Caco-2 cells were routinely maintained on the 75 cm^2 plastic flasks (Corning®, Corning Inc., NY, USA) in a CO_2 incubator (Touch 190S, LEEC, NT, UK) at 37^oC and 5% CO_2. The cells were grown in Dulbecco's modified eagle medium (DMEM, Gibco) supplemented with 10% foetal bovine serum (Invitrogen, Australia), 1% non-essential amino acid (Gibco), 1% GlutaMax^{TM} (Gibco), and 1% of penicillin and streptomycin (Sigma Aldrich, Sydney, NSW, Australia), and subcultured when they reached 80% of confluence.

The following culture condition was applied in all cellular experiments:

Cells at passages 50-55 were used for seeding on plates. They were seeded on microtitre plates at a density of 2.5 x 10^5 cells/mL. The cells were continuously grown for 14 days and the culture medium was changed every day. On the day of the experiment, spent medium was removed and the cells were treated with carotenoids, anthocyanins and/or their mixtures. The concentrations of the phytochemicals when they were used singly or in combinations were 2.5, 5 and 7.5 μM. The ratios of anthocyanins to carotenoids in

combinations were 1:1, 1:3 and 3:1. Carotenoids were delivered into the cells by Tween 40 (20%, w/v, in acetone) at the maximum final concentration of 0.1% in the cell culture medium (O'sullivan et al., 2004).

3.2.5. Cell viability

A protocol of MTT assay described previously (Chiba et al., 1998) was used with some minor modifications. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) stock (5 mg/mL) was prepared in cell culture medium and filtered through 0.22 μm polyethersulfone (PES) membrane (Millex-GP, Merck Millipore, Damdstadt, Germany). A working MTT solution of 0.5 mg/mL was prepared in pre-warmed culture medium. The Caco-2 cells were seeded on 96-well plates and treated with individual carotenoids and anthocyanins at concentrations of 2.5-10 μM for 4 hours at 37^{o}C, 5% CO_2. After the treatment, the medium was discarded and the cells were washed with Dulbecco phosphate buffer saline (DPBS). 100 μL of the working MTT solution was then added into each well and the plate was incubated at 37^{o}C, 5% CO_2 for 4 hours. After that, the MTT media were discarded and the wells were added with 100 μL of 0.04 N HCl-isopropanol to dissolve the formazan crystals. The plates were gently shaken for 5 min at room temperature and the absorbance was read at 560 nm with background subtraction at 620 nm.

3.2.6. Cellular antioxidant assay

A protocol developed by Wolfe and Liu (2007) was used with some modifications. Caco-2 cells were seeded on 96-well plates (CELLSTAR®, micro-clear, Greiner Bio-One GmbH, Frickenhausen, Germany) at 2.5 x 10^5 cells/ml. The cells were cultured and treated with the phytochemicals (100 μL, 2.5-7.5 μM) as described previously. After 3 hours of incubation with the phytochemical(s) at 37^{o}C and 5% CO_2, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (100 μL, 50 μM, prepared in DMEM)

was added and the cells were incubated for 1 hour. The medium was then completely removed and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 100 µL, 0.6 mM, prepared in DPBS) was added. The plate was read at $\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = 520 nm, at 37°C every 5 min for the total of 1 hour (12 cycles). The cellular antioxidant activity (CAA) was calculated as:

$$\text{CAA unit} = 100 - \frac{AUC_{sample}}{AUC_{control}} \times 100 \qquad (5)$$

where AUC_{sample} and $AUC_{control}$ are the integrated area under the sample fluorescence and control fluorescence, respectively, *versus* time curve.

3.2.7. Cell exposure to inflammation induced by human tumour necrosis factor-α (TNF-α)

A protocol described by Peng et al. (2016) was used with some modifications. Caco-2 cells were seeded on 48-well plates (Corning COSTAR®, Corning Inc., NY, USA) at 2.5 x 10^5 cells/mL. The cells were cultured and treated with the phytochemicals (200 µL, 2.5-7.5 µM) as described previously. After 4 hours of incubation with the phytochemical(s) at 37°C and 5% CO_2, 50 µL of human TNF-α (final concentration 100 ng/mL) was added into each well and the plate was incubated for 24 hrs. The supernatants from the culture were then collected and stored at -80°C until further analysis of interleukin-8 (IL-8) and nitric oxide secretion:

- *IL-8 secretion*

The concentration of IL-8 in the medium supernatants was measured by an enzyme immunoassay kit (Human IL-8 ELISA kit, BD OptEIA™, BD Biosciences, San Jose, CA, USA) following the instruction of the manufacturer.

- *Nitric oxide production*

Nitric oxide is not stable and readily oxidized into nitrate and nitrite. The concentration of nitric oxide (as nitrite) in the medium supernatants was measured by the Griess reagent kit (Molecular Probes, Invitrogen, Sydney, NSW, Australia). Nitrate was converted to nitrite by vanadium chloride (VCl_3) (Miranda et al., 2001). Briefly, 100 µL of collected medium supernatants/standards/blank was mixed with 80 µL of VCl_3 (8 mg/mL, prepared in HCl 1 M, stored in the dark at 4^{o}C for less than 2 weeks) on a 96-well-plate, and then 20 µL of Griess reagent mixture (prepared by mixing an equal volume of the two Griess reagents A and B from the kit) was added into each well. The plate was incubated at 37^{o}C for 30 min after which the absorbance was read at 540 nm. A serial of nitrite standards (1-100 µM) was prepared in DMEM.

3.2.8. Cellular uptake of carotenoids

Caco-2 cells were seeded on 6-well plates (Corning COSTAR®, Corning Inc., NY, USA) at 2.5 x 10^5 cells/mL. The cells were cultured and treated with the phytochemicals (2 mL, 2.5-7.5 µM) as described above. After 4 hours of incubation at 37^{o}C and 5% CO_2, the plates were put on ice and the medium was removed. The cells were washed with 2 mL cold DPBS containing 0.1% Tween 40 (20%, w/v, in acetone) to remove carotenoids attached to the cell surface, then washed with 2 mL pure DPBS, and then lysed by osmotic pressure in 3 mL of cold water for 30 min before being collected into 50-ml centrifuge tubes (Biehler et al., 2011).

3.2.9. Carotenoid extraction from cell lysates

The extraction of carotenoids from cell lysates was conducted following the procedure described by Biehler et al. (2011) with some modifications. Briefly, 4 mL of hexane: ethanol: acetone (2:1:1, v/v/v, containing 0.1% butylated hydroxytoluene (BHT) and 20 µL of *trans*-β-apo-carotenal as an internal standard was added into each tube containing the cell lysate. The tubes were vortexed and sonicated for 2 min followed by

centrifugation at 4000 g for 5 min. The supernatant was transferred to another tube and the cell lysate was re-extracted with 2 ml of hexane containing 0.1% BHT, sonicated for 2 min and centrifuged at 4000g for 5 min. All supernatants were combined and dried under nitrogen and stored at -80^{o}C until LC-MS analysis.

3.2.10. Analysis of carotenoids from cell lysates

The dried carotenoid extract from cell lysates was reconstituted in methanol: acetone (60:40, v/v) and filtered through 0.22 μm PTFE membrane (Kinesis, Redland Bay, QLD, Australia). A series of the standard solutions of each carotenoid added with the internal standard were prepared. 20 μL of samples or standards was injected into a reversed phase liquid chromatography system (Accela LC, Thermo Fisher Scientific Inc., Waltham, MA, USA) interfaced to a Hybrid Ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XL ^{TM}, Thermo Fisher Scientific Inc., Waltham, MA, USA). HPLC separation was carried out using an Acclaim^{TM} C30 column (2.1 x 250 mm, 3 μm particle size, Thermo Fisher Scientific Inc., USA) with a flow rate of 0.5 mL/min at 40^{o}C following a protocol of Van Meulebroek et al. (2014) with some modifications. The mobile phases were (A) acetonitrile: methanol: water (80:19.5:0.5) containing ammonium acetate 0.3 g/L adjusted to pH 6.8 with acetic acid, and (B) methanol: ethyl acetate (50:50, v/v). The gradient was: 0-4 min, 2% B; 4-25 min, 2-35% B; 25-30 min, 35% B; 30-35 min, 35-100% B; 35-37 min, 100% B; 37-38 min, 100-2% B; 38-48 min, 2% B. The Orbitrap mass spectrometer was calibrated on each day prior to the analysis by directly infusing negative calibration solution into the heated electrospray ionization (HESI) source. The mass spectrometer was equipped with an atmospheric pressure chemical ionization probe (APCI) and the detection of carotenoids was operated in negative ionization mode. APCI source working parameters were optimized and involved vaporizer temperature of 320^{o}C, sheath gas flow rate of 80 arbitrary units (au),

auxiliary gas flow rate of 5 au, sweep gas flow rate of 0 au, discharge current of 20 μA, capillary temperature of 275°C, capillary voltage of -2 V, and tube lens voltage of -68 V. A scan range of *m*/*z* 411-575 was selected. Carotenoids were identified according to their relative retention time and accurate mass (*m*/*z* 568.43 for lutein, and *m*/*z* 536.44 for lycopene, α-carotene and β-carotene). Instrument control and data processing were performed using XCalibur™ 2.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Extracted Ion Current chromatograms of *m*/*z* 536.0-536.5 were plotted for the identification of lycopene, α-carotene and β-carotene; and *m*/*z* of 568.0-568.5 for lutein. Calibration curves of pure carotenoid standards (Extrasynthese, Lion, France) were constructed for carotenoid quantification.

3.2.11. Determination of the interactive effects of the pure phytochemical mixtures

The interaction mode between the phytochemicals in every bioactivity assay was determined by comparing the experimental activity to the expected additive activity. The latter was calculated using the equation described by (Fuhrman et al., 2000). The mode of phytochemical interaction is defined as:

- Synergy: the experimental inhibitory effect is greater than the expected effect;
- Antagonism: the experimental inhibitory effect is lesser than the expected effect;
- Addition: the experimental inhibitory effect is equal to the expected effect.

3.3. Evaluation of the resulting biological effects of the co-digestion of different anthocyanin- and carotenoid-containing vegetables

3.3.1. Vegetable sample preparation

Carrots were peeled, sliced and crushed using a kitchen blender (NutriBullet 900 series, Sydney, NSW, Australia). The edible parts of red cabbage without the core and some outer leaves were crushed into smaller pieces. Baby spinach and cherry tomato were

also crushed into smaller pieces using a blender. 10 g of each processed vegetable were weighed into a plastic test tube. Different combinations (1:1 ratio) containing anthocyanin-rich red cabbage and a carotenoid-rich vegetable: carrot, baby spinach or cherry tomato were prepared by mixing approximately 5 g of red cabbage and 5 g of the other vegetable. A mixture of all vegetables containing 2.5 g of each vegetable was also prepared. All processed vegetable samples were stored under -80^{o}C until further analysis.

3.3.2. Simulated gastro-intestinal digestion

3.3.2.1. Preparation of materials

Prior to the digestion experiment, vegetable samples were thawed to room temperature. The simulated digestion was conducted using an artificial gut digester (Nutriscan GI20 Analyser model, Next Instruments, Condell Park, NSW, Australia) at a controlled temperature of 37^{o}C. A simulated salivary solution was made by dissolving 0.238 g Na_2HPO_4, 0.019 g KH_2PO_4, 0.8 g NaCl and 0.5 g pyrogallol in 100 mL milliQ water and the pH of the solution was adjusted to 6.5 by $NaHCO_3$ 1 M (Gawlik-Dziki, 2012). A simulated gastric solution was prepared by mixing 10 mL of NaCl 0.15 M pH 2.0 containing 5 mg/mL pyrogallol with 2 mL of porcine pepsin in 0.1 M HCl (final concentration = 300 U/mL) (Gawlik-Dziki, 2012). A simulated duodenal solution was prepared by dissolving 0.2 g porcine pancreatin and 1.2 g bile extract in 50 mL $NaHCO_3$ 0.1 M (Kaulmann et al., 2016). The preparation of an oil-based salad dressing was based on a standardised high-fat recipe, which is reported to improve carotenoid bioavailability in humans, consisting of: 59 g white vinegar, 44 g water and 112 g canola oil (Brown et al., 2004).

3.3.2.2. Digestion procedure

The digestion of vegetables was according to the protocol of Kaulmann et al. (2016) with some modifications. Briefly, to each vegetable sample (10 g) was added 4.5 mL of the salivary juice and mixed for 30 s at 37^{o}C, then 0.5 mL of α-amylase (2000 U/ml) was added and the mixture was shaken at 100 rpm for 10 min at 37^{o}C. For vegetable samples with added the oil-based dressing, 10 g of vegetables was firstly mixed with 2.5 mL of the prepared dressings prior to oral digestion. A blank sample containing 10 mL of milliQ water instead of 10 g of vegetable was also subjected to the simulated digestion.

After the oral digestion, the samples were adjusted to pH 2 using 4 M HCl, and 12 mL of the gastric solution was then added into each sample to initiate gastric digestion. After shaking at 100 rpm for 60 min at 37^{o}C, the samples were adjusted to pH 5.5 using 1 M $NaHCO_3$ prior to the addition of 6 mL of the duodenal solution to start intestinal digestion. pH of the samples was then adjusted to 7 using 1 M NaOH, and the sample volume was made up to 50 mL with 0.15 M NaCl. After 2 hrs of shaking (100 rpm) at 37^{o}C, the digestion process was completed and samples were cooled on ice, and then centrifuged at 5000 g for 5 min to remove undigested solids and collect the supernatant of the digesta.

The micellar fraction containing bioaccessible carotenoids was separated from the aqueous supernatant by a combination of centrifugation and filtration described by Veda et al. (2006). An aliquot of 20 mL of the supernatant was centrifuged at 5000 g for 20 min at 4 ^{o}C, which is reported as the more practical centrifugation procedure that results in high carotenoid recovery (Granado-Lorencio et al., 2007). The supernatant was subsequently vacuum filtered through a 0.65 μm mixed cellulose ester membrane (47 mm diameter, MF-Millipore^{TM} Membrane filter, Merck, Damdstadt, Germany) for

separation of the micellar fraction (Veda et al., 2006). An aliquot of the filtered micellar fraction was subjected to the extraction of carotenoids as described in the below section.

3.3.3. Total phenolic content (TPC) and total anthocyanin content (TAC) by spectrophotometry

The determination of TPC was according to a method described by Rodriguez-Roque et al. (2013), which was originally developed by Singleton et al. (1998). Briefly, 0.1 mL of the phenolic extracts of fresh vegetables or 0.1 mL of the digesta was added with 0.5 mL milliQ water followed by 0.1 mL of Folin-Ciocalteu's phenol reagent (Sigma Aldrich, Sydney, NSW, Australia). The mixture was left at room temperature for 5 min, and then 2 mL of Na_2CO_3 20% was added. The sample volume was made up to 5 mL with milliQ water, and the samples were left at room temperature in the darkness for 1 hr. After the reaction, any hazy samples were filtered through 0.2 μm nylon membrane before absorbance reading. The absorbance was measured at 725 nm by a UV-VIS spectrophotometer (UV-VIS 1800 series, Shimadzu, Tokyo, Japan). A series of gallic acid standard solutions (40-600 mg/L) was prepared to construct a standard curve. The TPC results were expressed as mg gallic acid equivalent/100 g fresh matter.

TPC determination by Folin-Ciocalteu's phenol reagent is simple, reproducible and robust (Shahidi and Zhong, 2015). However, this method also has several drawbacks such as its sensitivity to pH, temperature and reaction time (Shahidi and Zhong, 2015). In addition, TPC can be overestimated compared to those obtained by HPLC methods because non-phenolic reducing agents such as reducing sugars and certain amino acids possibly contribute to the reduction of the Folin-Ciocalteu's phenol reagent (Blasco et al., 2005). The main focus of our study was anthocyanins and carotenoids, thus we decided to use TPC as a simple method for quantifying total phenolic compounds to provide a glimpse of the changes in TPC after vegetable co-digestion.

The total anthocyanin content of the fresh vegetable extracts and digesta supernatant extracts was measured by a pH differential method (AOAC 2005.02) (Lee et al., 2005). An aliquot of the extract was diluted at an appropriate ratio with potassium chloride buffer (0.025 M, pH 1), and a second aliquot of the extract was diluted with sodium acetate buffer (0.4 M, pH 4.5). The mixtures were left for equilibration at room temperature for 15 min prior to measurements of absorbance at 510 nm and 700 nm (UV-VIS 1800 series, Shimadzu, Japan). The TAC results were expressed as mg cyanidin-3-*O*-glucoside equivalent/100 g fresh matter and calculated as:

$$\text{TAC (mg/L)} = \frac{A \times MW \times Df \times 1000}{\varepsilon \times l} \qquad (6)$$

where:

A: total corrected absorbance, which is calculated as: $A = (A_{510} - A_{700})_{pH\ 1} - (A_{510} - A_{700})_{pH\ 4.5}$, in which $(A_{510}, A_{700})_{pH\ 1}$ is the absorbance of the sample diluted in potassium chloride buffer pH 1 at 510 nm and 700 nm respectively; and $(A_{510}, A_{700})_{pH\ 4.5}$ is the absorbance of the sample diluted in sodium acetate buffer pH 4.5 at 510 nm and 700 nm respectively.

MW: molecular weight of cyanidin-3-*O*-glucoside = 449.2 g/mol

Df: dilution factor of the sample with the buffers

ε: molar extinction coefficient of cyanidin-3-*O*-glucoside = 26900 ($L.cm^{-1}.mol^{-1}$)

l: cuvette pathlength (cm)

3.3.4. Extraction of anthocyanins from fresh vegetables and supernatant of digesta

2 g of the vegetable was mixed with 10 mL of 90% methanol containing 0.5% formic acid, and the sample was sonicated for 10 min, and then centrifuged at 5000 rpm for 10 min. The supernatant was collected and the sample residues were re-extracted 3 times. All supernatants were pooled and evaporated under nitrogen gas to remove methanol. The remaining aqueous extract (0.5-1 mL) was purified by solid phase extraction using

C18 Sep-Pak cartridge (Sep-Pak Vac 3cc 500 mg, Waters, Milford, MA, USA) following a method described by Tian et al. (2005). In brief, the cartridge was activated using 5 mL of acidified methanol (0.1% formic acid) followed by 5 mL of acidified water (0.1% formic acid). The sample extract was then loaded onto the activated cartridge. The cartridge was washed with 10 mL of acidified water and the anthocyanins were eluted using 3 mL of acidified methanol. The methanolic anthocyanin extract was dried under nitrogen gas to remove methanol, and the residue was stored at -80^{o}C until further analysis.

2 mL of each supernatant from the digesta was subjected to solid phase extraction using C18 Sep-Pak cartridge. After activating the cartridge as described above, 2 mL of the digesta supernatant was loaded onto the cartridge followed by 10 mL of acidified water and 3 mL of hexane. 2-3 mL of acidified methanol was then added to elute the anthocyanins. The extract was dried under nitrogen and stored at -80^{o}C. Prior to HPLC analysis, the residue was re-dissolved in acidified methanol, filtered through 0.22 μm nylon membrane (Grace Discovery Sciences, Epping, VIC, Australia) and injected onto the HPLC column.

3.3.5. Analysis of anthocyanins from fresh vegetables and supernatant of digesta

3.3.5.1. Identification of anthocyanins by UPLC-ESI/MS/MS

10 μL of the 0.22 μm-filtered extract was injected onto a UPLC C18 column (Acquity BEH C18, 2.1 x 150 mm, 1.7 μm particle size, Waters, Milford, MA, USA) on a reversed phase liquid chromatography system (Accela UHPLC, Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled with a triple quadrupole mass spectrometer (TSQ Vantage^{TM} EMR, Thermo Fisher Scientific Inc., Waltham, MA, USA). The mobile phases were (A) milliQ water containing 10% formic acid, and (B) acetonitrile containing 10% formic acid. The gradient was: 0-1.5 min, 0.5% B; 1.5-4

min, 0.5-11% B; 4-23 min, 11-15% B; 23-28 min, 15-100% B; 28-38 min, 100% B; 38-39 min, 100-0.5% B; 39-49 min, 0.5% B. Flow rate was set at 0.5 mL/min and column temperature was set at 50^{o}C. Anthocyanins detection was performed in positive ionization mode by the triple quadrupole mass spectrometer interfaced with electrospray ionization (ESI) source, of which the working parameters were optimized as follows: spray voltage, 3500 V; vaporizer temperature, 380 ^{o}C; sheath gas pressure, 50 arbitrary units (au); ion sweep gas pressure, 2 au; auxiliary gas pressure, 55 au; capillary temperature, 270 ^{o}C; and collision energy, 33 V. A scan range of *m*/*z* 600-1400 was selected. A full scan in precursor ion mode followed by parent-product ion scans on selected reaction monitoring (SRM) mode was performed. A scan time of 0.18 s was set for all SRM analysis. Instrument control and data processing were performed using XCalibur^{TM} 2.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.3.5.2. Quantification of anthocyanins by HPLC-PDA

The quantification of anthocyanins was carried out on a reversed phase HPLC (LC-20AD, Shimadzu, Tokyo, Japan) coupled with a photodiode array (PDA) detector (SPD-M20A, Shimadzu, Tokyo, Japan) using the Acquity UPLC BEH C18 column (2.1 x 150 mm, 1.7 µm particle size, Waters, Milford, MA, USA) following a method described by Tian et al. (2005). The mobile phase components were identical as described in the section 3.3.5.1 on page 90-91. The column temperature was set at 50^{o}C and the flow rate was set at 0.15 ml/min to reduce backpressure. The gradient program was therefore modified accordingly as follows: 0-4 min, 0.5-11% B; 4-60 min, 11-15% B; 60-65 min, 15-100% B; 65-95 min, 100% B; 95-96 min, 100-0.5% B; 96-130 min, 0.5% B. Due to the unavailability of anthocyanin standard compounds in red cabbage, cyanidin-3-*O*-glucoside was used as a standard for quantification purpose. Six-point calibration curves of cyanidin-3-*O*-glucoside standard were constructed for anthocyanin

quantification. 2 μL of sample extracts or standards was injected. Anthocyanin compounds were detected at λ = 520 nm. The results were expressed as mg cyanidin-3-*O*-glucoside equivalent/100 g fresh matter.

3.3.6. Extraction of carotenoids from fresh vegetables and micellar fraction of the digesta

A protocol of carotenoid extraction described by Taungbodhitham et al. (1998) was used with some modifications. Briefly, 2 g of each vegetable was mixed with 10 mL of hexane: acetone: ethanol (2:1:1, v/v/v) containing 0.1% butylated hydroxytoluene (BHT). 0.02 g of $MgCO_3$ was added into each sample to neutralise acids if any in the sample. The sample was sonicated for 5 min and centrifuged for 5 min at 5000 g. The hexane phase was collected and the residue was re-extracted 3 times with 10 mL of hexane containing 0.1% BHT, sonicated for 5 min and centrifuged at 5000 g for 5 min. All hexane phases were pooled and dried under nitrogen gas, and stored at -80^{o}C for no longer than 3 days until further analysis.

4 mL of the carotenoid micellar fraction was mixed with 4 mL of hexane: acetone: ethanol (2:1:1, v/v/v) containing 0.1% BHT. The mixture was vortexed for 1 min, then centrifuged at 4000 g for 2 min at 4 ^{o}C to hasten phase separation. The supernatant was collected and the residue was re-extracted with 2 mL of hexane containing 0.1% BHT, vortexed for 1 min, and centrifuged (4000 g, 2 min). All supernatants were combined and dried under nitrogen gas, and the residue was stored at -80^{o}C for no longer than 3 days until further analysis. Prior to HPLC analysis, the residue was re-dissolved in methanol: acetone (60:40, v/v), filtered through 0.45 μm PTFE membrane (Grace Discovery Sciences, Epping, VIC, Australia) and injected onto the HPLC column.

3.3.7. Analysis of carotenoids from fresh vegetables and micellar fraction of the digesta

3.3.7.1. Identification of carotenoids by LC-APCI/MS Orbitrap

Identification of major carotenoids including lutein, lycopene, α-carotene and β-carotene from fresh vegetable and micellar fraction extracts were conducted on a Accela HPLC system (Thermo Fisher Scientific Inc., Waltham, MA, USA) connected to a LTQ Orbitrap XL^{TM} mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with an atmospheric pressure chemical ionization probe (APCI) (Van Meulebroek et al., 2014). Carotenoid separation was carried out at 40 ^{o}C on an Acclaim^{TM} C30 column (2.1 x 250 mm, 3 μm particle size, Thermo Fisher Scientific Inc., San Jose, USA) at a flow rate of 0.5 mL/min. The mobile phase components, the mobile phase gradient and the LC-MS method were identical to what was described in section 3.2.10 on pages 84-85. An *m*/*z* scan range of 411-575 was set. Carotenoids were identified according to their relative retention time and accurate mass (*m*/*z* 568.43 for lutein, and *m*/*z* 536.44 for lycopene, α-carotene and β-carotene). Instrument control and data processing were performed using XCalibur^{TM} 2.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). Extracted Ion Current chromatograms of *m*/*z* 536.0-536.5 were plotted for the identification of lycopene, α-carotene and β-carotene; and *m*/*z* of 568.0-568.5 for lutein.

3.3.7.2. Quantification of carotenoids by HPLC-PDA

Quantifications of the carotenoids from fresh vegetables and the micellar fraction of the digesta were carried out on a reversed phase HPLC (LC-20AD, Shimadzu, Tokyo, Japan) coupled with a PDA detector (SPD-M20A, Shimadzu, Tokyo, Japan) following a protocol of Van Meulebroek et al. (2014) with some modifications. An Acclaim^{TM} C30 column (2.1 x 250 mm, 3 μm particle size, Thermo Fisher Scientific Inc., Waltham,

MA, USA) was used for carotenoid separation at 40 ^{o}C. Carotenoids were eluted by a mobile phase system comprising of (A) acetonitrile: methanol: ammonium acetate 0.3 g/L (80:19.5:0.5, v/v) adjusted to pH 6.8 with acetic acid, and (B) methanol: ethyl acetate (50:50, v/v) at a flow rate of 0.5 mL/min. The gradient was: 0-4 min, 2% B; 4-25 min, 2-35% B; 25-30 min, 35% B; 30-32 min, 35-100%; 32-37 min, 100%; 37-38 min, 100-1.5%; 38-55 min, 1.5% B. Injection volume was 10 μL. Carotenoids were detected at λ = 450 nm. Six-point calibration curves of the carotenoid standards were constructed for carotenoid quantification.

3.3.8. Calculation of digestive bioaccessibility of anthocyanins and carotenoids

Digestive bioaccessibility (dBAC) refers to the fraction of phytochemical compounds that are bioaccessible from undigested food materials at the end of the *in vitro* digestion and available for intestinal absorption. The % digestive bioaccessibility of a phytochemical refers to the percentage content of the compound in the bioaccessible fraction compared to the initial content of the compound in the undigested vegetables (Cilla et al., 2012), and was calculated as follows:

$$\text{Digestive bioaccessibility (\%)} = \frac{\text{Bioaccessible fraction content}}{\text{Total initial content}} \times 100 \qquad (7)$$

where bioaccessible fraction of an anthocyanin was determined from the supernatant of the digesta, whereas bioaccessible fraction of a carotenoid contains the carotenoids that were incorporated into micelles and was determined from the micellar fraction. Total initial content was determined from the undigested materials.

3.3.9. Cellular biological activity assays

3.3.9.1. General cell culture condition

Human carcinogenic Caco-2 cells (passages 50-55) were cultured and maintained following the conditions described previously in section 3.2.4 on page 80-81.

3.3.9.2. Cell viability

A protocol of MTT assay described previously (Chiba et al., 1998) was used with some minor modifications. The Caco-2 cells seeded on 96-well plates were treated with the supernatant from the digesta of each individual vegetable and the digesta of the vegetable mixtures diluted with the growth medium at different ratios (1:4-1:12) for 4 hours at 37^{o}C, 5% CO_2. The cells were then washed with Dulbecco phosphate buffer saline (DPBS) and were subjected to MTT assay following the protocol described in section 3.2.5 on page 81.

3.3.9.3. Cellular antioxidant assay

Differentiated Caco-2 cells grown on 96-well plates (CELLSTAR®, micro-clear, Greiner Bio-One GmbH, Frickenhausen, Germany) for 14 days were treated for 4 h at 37^{o}C, 5% CO_2 with the supernatant from different digesta samples of the fresh vegetables and the ones added the dressing diluted at a supernatant: growth medium ratio of 1:8 and 1:10 respectively at which no significant difference in cell viability in the samples was observed. The cellular antioxidant activity was tested using the protocol of Wolfe and Liu (2007) with some modifications as described previously in section 3.2.6 on page 81-82. Briefly, Caco-2 cells at passages 50-55 were seeded at 2.5 x 10^5 cells/mL on 96-well plates (CELLSTAR®, micro-clear, Greiner Bio-One GmbH, Frickenhausen, Germany) for 14 days with medium changed every day after 100% confluence. The cells were then washed with DPBS and added with the diluted supernatant of different digesta samples (100 µL). The plates were incubated at 37^{o}C and 5% CO_2 for 3 hours and 100 µL of 50 µM DCFH-DA was added followed by incubation for another one hour. The medium was removed and to each well was added 100 µL of 0.6 mM AAPH (prepared in DPBS). The excitation and emission of fluorescence at 37^{o}C was measured at 485 nm and 520 nm respectively (5 min interval

for 2 hours). Cellular antioxidant activity (CAA) was calculated following the equation (5) shown on page 73.

3.3.9.4. Cell exposure to inflammation induced by human TNF-α

Differentiated Caco-2 cells grown on 48-well plates (Corning COSTAR®, Corning Inc., NY, USA) for 14 days were treated for 4 h with the supernatant from different digesta samples of the fresh vegetables diluted at a supernatant: growth medium ratio of 1:8, and from the digesta of the vegetables with added the dressing at a supernatant: growth medium ratio of 1:10 ratio followed by the inflammatory mediation by human TNF-α (100 ng/mL) for 24 h at 37^{o}C, 5% CO_2 Peng et al. (2016). The supernatants from the culture were analysed for the pro-inflammatory cytokine secretion by a human IL-8 ELISA kit (BD OptEIA™, BD Biosciences, San Jose, CA, USA) and nitric oxide secretion by a Griess reagent kit (Molecular Probes, Invitrogen, Sydney, NSW, Australia).

3.3.10. Cellular uptake of carotenoids following vegetable digestion

Differentiated Caco-2 cells grown on 6-well plates (Corning COSTAR®, Corning Inc., Germany) for 14 days were incubated with the supernatant from different digesta samples of the fresh vegetables diluted at a supernatant: growth medium ratio of 1:8, and from the digesta of the vegetable samples with added the dressing at a supernatant: growth medium ratio of 1:10 ratio for 4 h at 37^{o}C, 5% CO_2. The cells were then washed with 2 mL cold bile salt solution (2 g/L) to remove adsorbed carotenoids on the cell surface followed by a wash with 2 mL cold DPBS. The cells from 12 wells were pooled and collected into test tubes after being lysed by osmotic pressure in 3 mL of cold water for 30 min (Biehler et al., 2011). Carotenoids from cell lysates were extracted following the protocol described in section 3.2.9 on page 83, and analysed by LC-APCI/MS Orbitrap as described in section 3.2.10 on pages 84-85.

3.3.11. Calculation of intestinal cellular uptake and intestinal cellular bioaccessibility of carotenoids

Intestinal cellular uptake (%) of a carotenoid refers to the percentage content of the compound uptake by Caco-2 cells compared to the bioaccessible content of the compound in the micellar fraction, and was calculated as follows:

$$\text{Cellular uptake (\%)} = \frac{\text{Cellular uptake content}}{\text{Micellar fraction content}} \times 100 \qquad (8)$$

whereas cellular uptake content is the amount of the carotenoids absorbed by the cells; micellar fraction content is the amount of the carotenoids that were incorporated into micelles.

Intestinal cellular bioaccessibility (cBAC) refers to the fraction of carotenoid compounds from undigested food materials that are absorbable by the intestinal Caco-2 cells. The % intestinal cellular bioaccessibility of a carotenoid refers to the percentage content of the compound uptake by Caco-2 cells compared to the initial content of the compound in the undigested vegetables, and was calculated as follows:

$$\text{Intestinal cellular bioaccessibility (\%)} = \frac{\text{Cellular uptake content}}{\text{Total initial content}} \times 100 \qquad (9)$$

whereas cellular uptake content is the amount of the carotenoids absorbed by the cells and was determined as described in section 3.3.10 on page 96. Total initial content was determined from the undigested materials.

3.3.12. Determination of the mode of combined bioactivities of the co-digested vegetables

The mode of the combined biological effect of the co-digested vegetables was determined by comparing the experimental effect (EPE) to the expected additive effect (EAE). The latter was calculated according to the equation described by Fuhrman et al. (2000) as below:

$$EAE_{A-B} = EPE_A + EPE_B - EPE_A \times EPE_B/100 \quad (10)$$

where EAE_{A-B} is the expected additive effect resulting from the exposure of the Caco-2 cells to the diluted digesta of a mixture comprising of vegetables A and B; and EPE_A, EPE_B is the experimental effect resulting from the exposure of the Caco-2 cells to the diluted digesta of the vegetable component A and B respectively.

The experimental and expected bioactivity of each mixed vegetable was based on the same total weight of the vegetables that were originally subjected to the digestion procedure. For example, the experimental activity of 1 g of a vegetable mixture consisting of 2 vegetable components (1:1 ratio) subjected to digestion was compared to the expected additive activity resulting from 0.5 g of each vegetable component. Similarly, the experimental activity of 1 g of a vegetable mixture consisting of 4 vegetable components (1:1:1:1 ratio) was compared to the expected additive activity resulting from 0.25 g of each vegetable component.

The biological effect of a vegetable mixture is considered to be synergistic when the experimental effect is higher than the expected additive effect, antagonistic when the experimental effect is lesser than the expected additive effect, and additive when the experimental effect is equal to the expected additive effect (Fuhrman et al., 2000).

3.3.13. Statistical analysis

All experiments were done at least in triplicate. Data were expressed as means ± standard deviation. The means were tested for significant difference by performing one-way analysis of variance (ANOVA) and Tukey's test, and significance was considered at $p < 0.05$. All statistical analyses were executed by Minitab (version 9.0, Minitab Inc., State College, PA, USA).

INTRODUCTION TO CHAPTERS 4-6

The literature review shows that among major groups of food phytochemicals, the interaction between anthocyanins and carotenoids have yet to be understood. Therefore, the first major objective of this thesis was to investigate the interactive effects on bioactivities and cellular uptake of different combinations of six common anthocyanins and three major dietary carotenoids (β-carotene, lutein and lycopene) at three different combinatory ratios: 1:1, 1:3 and 3:1. The selection of these ratios was not based on dietary significance or intake of the phytochemicals. These ratios were selected aiming to see how the interactive effects between anthocyanins and carotenoids would exhibit when they were present at the same molar ratio (1:1) and when carotenoids were at a higher concentration than anthocyanins (3:1) and *vice versa* (1:3). They are also commonly used ratios in several other studies (Hidalgo et al., 2010, Jiang et al., 2015). The selected anthocyanins are the monoglucosides of six anthocyanidins whose structural features are different from each other (He and Giusti, 2010). Likewise, the selected carotenoids are different in the chemical structure as well as the cell membrane orientation (Saini et al., 2015, Han et al., 2012). The use of the different anthocyanin and carotenoid compounds enables us to gain insights into the interactive effects between the two groups of pigmented phytochemicals.

To study the combined effects of anthocyanins and carotenoids, chemical and Caco-2 cell models were used for testing the antioxidant and anti-inflammatory effects of the individual compounds in comparison to that of the compound mixtures. Cellular uptake of carotenoids was evaluated using the Caco-2 cell model. Each of the following chapters presents the interactive effects on cellular uptake, antioxidant and anti-inflammatory activities between the anthocyanins and each of the selected carotenoids:

- Chapter 3: interaction between anthocyanins and β-carotene
- Chapter 4: interaction between anthocyanins and lutein
- Chapter 5: interaction between anthocyanins and lycopene

Each of these chapters is presented in the original format of the accepted manuscript. The journal-formatted version is also provided in the Appendices. There are certain inevitable repetitions in some sections including introduction and discussions in Chapters 4-6 due to the fact that these chapters are the published papers sharing similar and related objectives.

CHAPTER 4

Interactive effects of β-carotene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity *in vitro* and *ex vivo*

(This is an Accepted Manuscript of an article published by Elsevier in Journal of Functional Foods volume 45, pages 129-137 on 14 March 2018, available online: https://doi.org/10.1016/j.jff.2018.03.021*)*

Abstract

This study investigated the bioactivity interactions *in vitro* and *ex vivo*, and cellular uptake interaction between β-carotene and some common anthocyanins. The combined antioxidant and anti-inflammatory activities of β-carotene and different anthocyanins were assessed in both chemical and biological systems. Bioactivity synergy was seen in none of the combinations in no system studied. Some mixtures even showed antagonistic effects. All of the tested anthocyanins except for delphinidin-3-glucoside, tested at 7.5 μM, significantly improved the cellular uptake of β-carotene (2.5 μM) by 68-200% ($p < 0.05$) although such increases of β-carotene intracellular content did not lead to an enhancement of the combined bioactivities. The increase in β-carotene absorption at a particular concentration facilitated the pro-oxidant activity of β-carotene. This effect could be partly responsible for the bioactivity antagonism seen in some of the combinations.

4.1. Introduction

Anthocyanins are water-soluble food pigments abundant in several red- or purple-coloured fruits and vegetables. They are powerful antioxidants which can deactivate reactive oxygen species (free radicals, singlet oxygen and peroxides) and chelate metal

ions (He and Giusti, 2010). β-carotene is one of the lipophilic plant pigments possessing several biological properties: anti-oxidation, anti-inflammation, and anti-cancer (Saini et al., 2015).

Anthocyanins and β-carotene are found together in vegetables such as purple carrots and purple tomatoes. They can also be co-digested from different food sources in diets. The co-presence of different active phytochemicals can result in addition, synergy (higher effects than addition), or antagonism (lower effects than addition) on biological activities (Phan et al., 2018c). Some phytochemical mixtures containing either anthocyanins or carotenoids produce anti-oxidative effects other than additive effect in chemical-based systems. For instance, the mixtures of cyanidin-3-glucoside and quercetin (Hidalgo et al., 2010), malvidin-3-glucoside or peonidin-3-glucoside and catechin show synergy (Rossetto et al., 2002). The mixtures of delphinidin-3-glucoside and quercetin, malvidin-3-glucoside and kaempferol (Hidalgo et al., 2010), β-carotene and tea polyphenols (Song et al., 2011), or β-carotene and baicalein (Liang et al., 2010) produce antagonism on *in vitro* antioxidant activity. The combined biological activities of anthocyanins and carotenoids, however, are not yet understood. The interaction between phytochemicals is mostly investigated using chemical-based models because these methods are cheaper and less complex (Phan et al., 2018c). Cell-based models, although more costly and more complicated, can evaluate bioactivity interaction between phytochemicals by considering the cell membrane-phytochemical interaction, the cellular uptake and the metabolism of the phytochemicals. Cell-based models are therefore an improvement over the chemical models (Wolfe and Liu, 2008).

There are very limited studies about absorption interferences between bioactive compounds (Phan et al., 2018c). In addition, bioactivity interaction and interference in cellular uptake between phytochemicals are usually reported separately. There is also no

study investigating the impact of cellular uptake interaction on the bioactivity interaction between phytochemicals. This chapter, therefore, reports the interactions between some common anthocyanins and β-carotene on both bioactivities and cellular uptake. Antioxidant and anti-inflammatory activities of individual compounds and their mixtures were assessed by both *in vitro* chemical assays and *ex vivo* biological models using human carcinogenic colon Caco-2 cells. The cellular uptake of β-carotene in the absence and presence of different anthocyanins was measured to evaluate the absorption interference and its impact on the bioactivity interaction between the compounds.

4.2. Materials and methods

As described in Chapter 3, section 3.1.1, 3.1.4, and 3.2.1-3.2.11 on pages 75-85.

4.3. Results

4.3.1. β-carotene-anthocyanin interaction on antioxidant activities

4.3.1.1. Liposome peroxidation inhibition

The combined effect of various β-carotene-anthocyanin mixtures at 1:1 molar ratio on lipid peroxidation inhibition in a membrane model (liposome) was examined in order to understand the water-lipid interfacial interaction between these compounds. The experimental and the expected additive TBARS inhibition percentages of each mixture are presented in **Figure 5**. The inhibitory capacity against liposome peroxidation of the β-carotene-anthocyanin mixtures was only 50-80% of the expected additive effect. There was therefore antagonism in all of the tested mixtures.

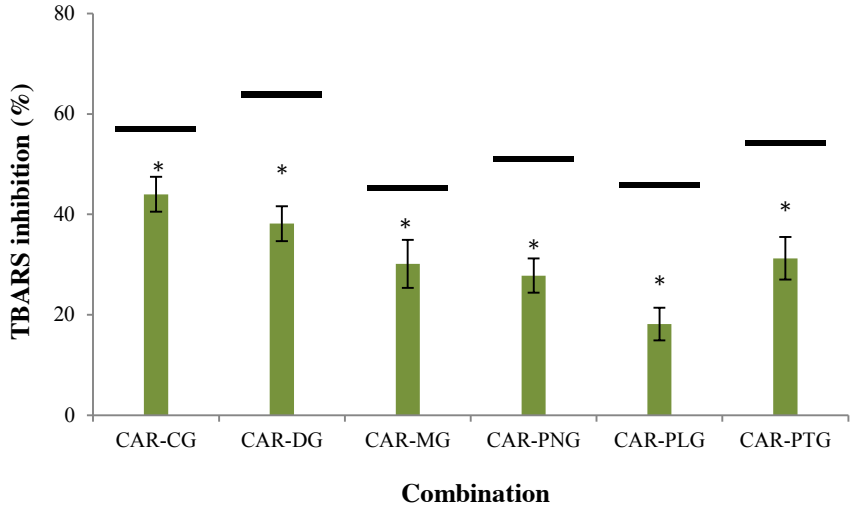


Figure 5. Liposome peroxidation inhibitory activity of different combinations of β-carotene and anthocyanins at 1:1 ratio (n = 3). The horizontal lines showed the expected additive activity of the mixtures, which was calculated based on an equation of Fuhrman et al. (2000): $TBARS_A$ + $TBARS_B$ - TBARSA x $TBARS_B$/100 in which $TBARS_A$, $TBARS_B$ is the TBARS (%) inhibition of a single anthocyanin or β-carotene respectively, which was calculated following the equation (3) on page 78. Columns marked with an asterisk indicate that the experimental activity of the mixture was significantly different ($p < 0.05$) from the calculated additive value. TBARS: thiobarbituric acid reagent species, CAR: β-carotene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

4.3.1.2. Cell viability

Prior to the biological assays, the cell cytotoxicity of each tested compound was checked. No phytochemical at 2.5-10 μM significantly reduced the viability of the Caco-2 cells (**Appendix 1**).

4.3.1.3. Cellular antioxidant activity (CAA)

The combined CAA of β-carotene and anthocyanins at different ratios was evaluated. At the β-carotene: anthocyanin ratio of 1:3, the effect was additive when β-carotene was mixed with cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside or malvidin-3-*O*-glucoside, but was decreased by 26-57% when mixed with the other anthocyanins (**Table 5**). An additive effect was shown in most of the mixtures when β-carotene was mixed with each anthocyanin at the 1:1 and 3:1 ratios.

Table 5. Cellular antioxidant activity (CAA) of different mixtures of β-carotene and anthocyanins at different ratios.

Mixture	β-carotene: anthocyanin ratio					
	1:3		1:1		3:1	
	Experimental effect[1]	Expected additive effect[2]	Experimental effect	Expected additive effect	Experimental effect	Expected additive effect
CAR-CG	76.6 ± 3.8[a]	73.4	63.8 ± 3.6[a]	70.1	42.8 ± 10.9[a]	44.6
CAR-DG	70.6 ± 4.0[a]	67.2	62.6 ± 3.9[a]	63.6	49.8 ± 8.4[a]	40.7
CAR-MG	67.3 ± 5.1[a]	65.2	61.1 ± 5.3[a]	64.5	50.6 ± 9.7[a]	46.0
CAR-PNG	42.8 ± 8.7[b]	57.8	52.8 ± 9.3[ab]	64.1	40.8 ± 4.8[a]	40.7
CAR-PLG	25.1 ± 9.0*[, b]	58.1	55.7 ± 6.7[ab]	65.1	40.0 ± 3.0[a]	43.2
CAR-PTG	42.6 ± 14.0[b]	58.1	46.3 ± 4.9*[, b]	65.6	32.0 ± 9.6[a]	44.0

Values marked with an asterisk indicate that the experimental activity of the mixture was significantly different from the calculated additive value ($p < 0.05$).
At a specific ratio, rows that share the same letter are not significantly different from each other ($p < 0.05$).
CAR: β-carotene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.
[1] The experimental effect is expressed as mean of CAA unit ± SD of four individual replicates ($n = 4$).
[2] The expected additive activity of the mixtures was calculated as $CAA_A + CAA_B - CAA_A \times CAA_B/100$ in which CAA_A, CAA_B is the CAA unit of a single anthocyanin or β-carotene respectively, which was calculated following the equation (5) on page 82.

4.3.2. β-carotene-anthocyanin interaction on anti-inflammatory activities

4.3.2.1. Lipoxygenase inhibitory activity

At physiological concentrations, β-carotene (0.2-2μM) inhibited lipoxygenase in a dose-dependent manner, whereas anthocyanins (2-12 μM) were not active inhibitors. The LOX-1 IC_{50} of β-carotene recorded in our study was 0.9 μM. The LOX-1 IC_{50} values of anthocyanins previously reported are in the range of mM, for example: LOX-1 IC_{50} of CG: 0.5 mM, LOX-1 IC_{50} of PNG: 38 mM (Knaup et al., 2009). When one component in the mixture is inactive and the other is active, the modes of interaction (synergy/addition/antagonism) cannot be determined (Phan et al., 2018c). The effect of anthocyanins on the LOX-1 inhibitory activity of β-carotene, therefore, was evaluated instead by comparing the IC_{50} of each β-carotene-anthocyanin mixture with that of β-carotene. All of the tested combinations showed higher IC_{50} than β-carotene (**Figure 6**), which indicates that, the lipoxygenase inhibitory activity of β-carotene was decreased in the presence of the anthocyanins.

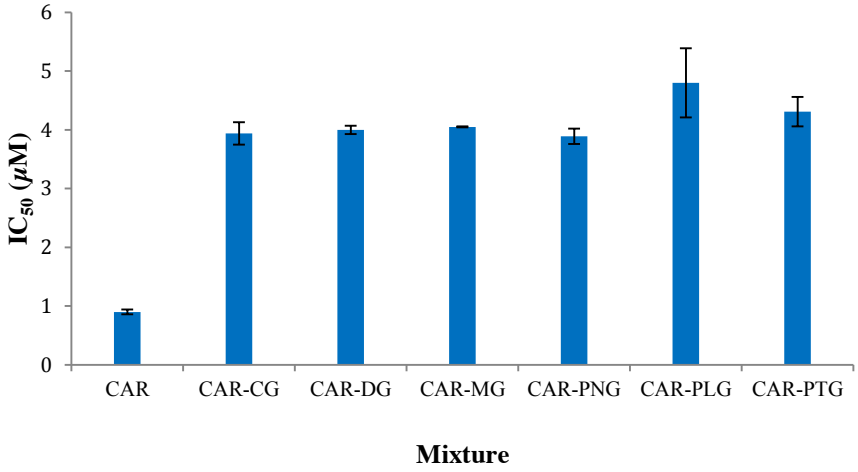


Figure 6. Lipoxygenase inhibitory activity of β-carotene and its mixtures with different anthocyanins (n = 3), expressed as IC_{50}: concentration at which 50% of the enzyme activity is inhibited. CAR: β-carotene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

4.3.2.2. Pro-inflammatory cytokine interleukin-8(IL-8) secretion

All combinations of β-carotene and anthocyanins (total concentration of 10 μM) at different ratios significantly reduced the secretion of pro-inflammatory cytokine IL-8 induced by human TNF-α (100 ng/ml). The combined effect, however, was additive or antagonistic. No synergy was detected in any tested mixtures. In particular, β-carotene showed antagonistic interaction with CG ($p < 0.05$) and PLG ($p < 0.01$) at all three ratios studied (**Figure 7**).

4.3.2.3. Nitric oxide (NO) production

β-carotene (2.5-7.5 μM), anthocyanin (2.5-7.5 μM) and any combination of β-carotene and anthocyanins (total concentration of 10 μM) were not effective on reducing the production of nitric oxide induced by human TNF-α (100 ng/ml). The combined activity of all mixtures showed an additive effect (**Figure 7**).

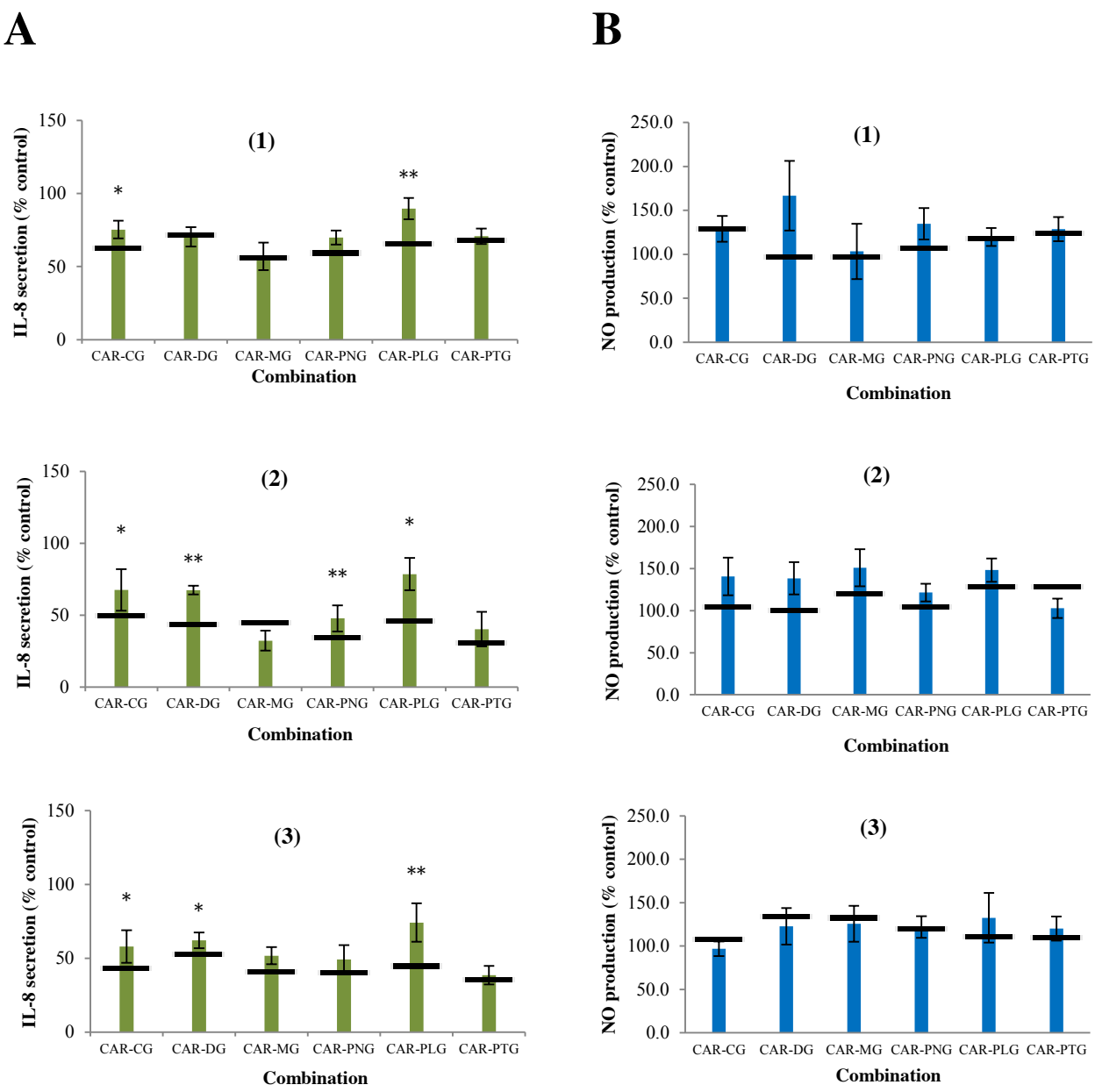


Figure 7. (A) IL-8 cytokine secretion (% control) and (B) nitric oxide (NO) production (% control) induced by pro-inflammatory TNF-α (100 ng/ml) in the presence of different mixtures of β-carotene and anthocyanins at different ratios: (1) 1:3, (2) 1:1, and (3) 3:1. Controls were not treated with phytochemicals, and were induced inflammation with TNF-α (100 ng/ml). The horizontal lines show the expected additive activity of the mixtures, which was calculated as 100 - (A + B - A x B/100) in which A, B is the percentage reduction of the IL-8 or NO secretion of the cells to the control when treated the cells with an anthocyanin or β-carotene respectively. Columns marked with asterisk(s) indicate that the experimental activity of the mixture was significantly different from the calculated additive value (*, $p < 0.05$; **, $p < 0.01$). Experimental values are mean ± SD of three independent replicates ($n = 3$). CAR: β-carotene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

4.3.3. Effect of anthocyanins on the cellular uptake of β-carotene

The effects of anthocyanins (5-7.5 μM) on the absorption of β-carotene (2.5-5 μM) at 1:1 and 1:3 ratios are shown in **Figure 8**. β-carotene uptake was not altered in the presence of anthocyanins at the 1:1 ratio (5μM: 5μM). At the 1:3 ratio (2.5μM: 7.5μM), the presence of anthocyanins, except for DG, significantly increased the cellular uptake of β-carotene ($p < 0.05$). Particularly, β-carotene absorption was enhanced by ~70% when β-carotene was in combination with CG or PTG, and by 130-200% with PNG, MG or PLG. The highest increase of β-carotene absorption was attained in the presence of PLG.

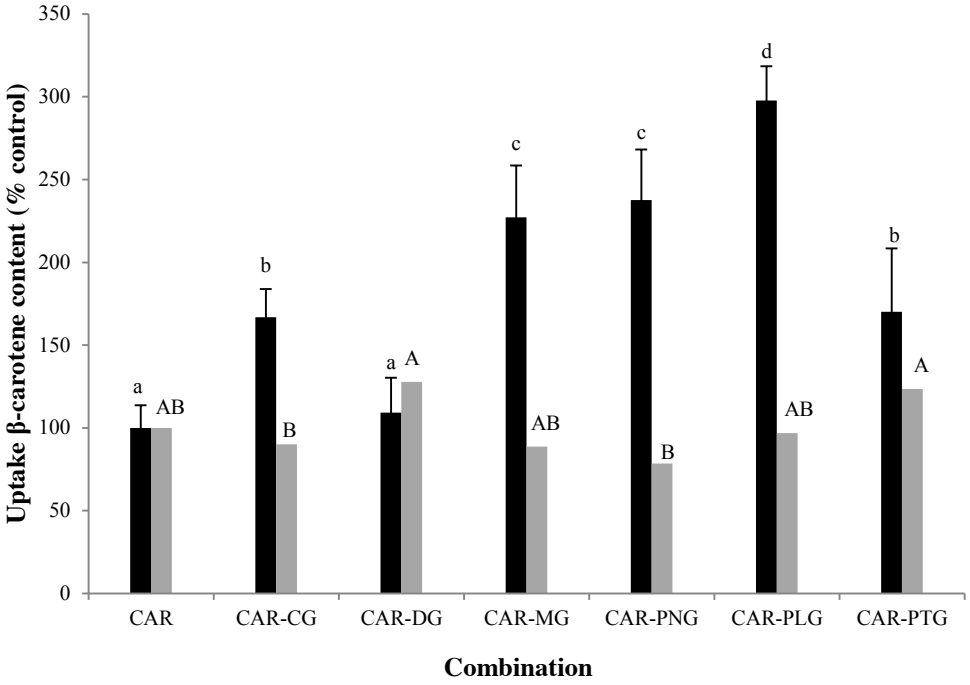


Figure 8. β-carotene uptake (% control) by Caco-2 cells in the absence (control) and presence of different anthocyanins at β-carotene: anthocyanin ratios of 1:3 (2.5:7.5 μM, represented in black bars) and 1:1 (5:5 μM, represented in grey bars). Columns of the same colour marked with different letters indicate a significant difference from each other ($p < 0.05$). Values are mean ± SD of three independent replicates ($n = 3$). CAR: β-carotene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

4.4. Discussion

The interactive effects between β-carotene and anthocyanins on antioxidant and anti-inflammatory activities were assessed in chemical-based and cell-based systems. Liposome membrane is often used to study the interactions between hydrophilic and lipophilic phytochemicals over a water-lipid interface (Liang et al., 2010, Liang et al., 2009b, Stahl et al., 1998). Each of the tested anthocyanins showed antagonistic interaction with β-carotene over the water-lipid interface in the liposome model. Interaction between hydrophilic and lipophilic bioactive compounds in a multi-phase system depends upon the spatial distribution of the compounds (Phan et al., 2018c), and the membrane partition of the hydrophilic component which depends on its polarity and affinity to the lipid membrane interior (Han et al., 2012).

The mechanism of antagonism between hydrophilic and lipophilic phytochemicals on lipid peroxidation inhibition has been proposed. β-carotene shows antagonistic interaction with green tea polyphenols on the inhibition of liposome peroxidation, which is explained by (i) the incorrect penetration of the tea polyphenols (either shallow or too deep penetration) into the lipid bilayer; and (ii) the possible formation of β-carotene/catechin adducts (Song et al., 2011). The formation of adducts is also proposed for the antagonism between β-carotene and daidzein (Liang et al., 2010). The antagonism on liposome peroxidation seen between β-carotene and the anthocyanins in our study could possibly be incurred by the same mechanism although further studies may be required to confirm. Anthocyanins are located mainly in the hydrophilic compartment of the outer monolayer of the membrane. They do not penetrate deeply into the hydrophobic region (Bonarska-Kujawa et al., 2012). β-carotene has two β ionone rings, so can have flexible location and orientation in a lipid membrane system (Van de Ven et al., 1984). The radical form of β-carotene, which is produced after β-

carotene scavenges lipid peroxyl radicals, can rotate and move towards the more polar interface owing to its positive charge and higher polarity than the parent molecule (Han et al., 2012). The β-carotene radical cation and the anthocyanin compound could probably react to form a β-carotene-anthocyanin adduct leading to a decrease in the active antioxidant concentration in the liposome system, and an increase in the rate of oxidation, resulting in the observed antagonistic effect.

Chemical assays are more commonly used to study phytochemical interaction than biological assays because of their simplicity and the lower cost but they do not mimic *in vivo* conditions as effectively as the tissue cultures do (Wang and Zhu, 2017, Phan et al., 2018c). Our study used a biological antioxidant assay, using a human colorectal Caco-2 cell model, to understand the interactive patterns between β-carotene and anthocyanins in a more biologically relevant condition. Most combinations of β-carotene and the anthocyanins showed additive effect on the cellular antioxidant activity when they were mixed at an equal molar ratio (1:1) or at a high ratio of the carotenoid (3:1) (**Table 5**). At a high ratio of the anthocyanins (1:3), each pair of β-carotene with PNG, PLG or PTG showed an experimental CAA lower than the expected effect, and significant CAA antagonism was seen in the β-carotene-PLG mixture.

The combined effects of β-carotene and anthocyanins on anti-inflammatory activities were also evaluated. β-carotene was a very effective lipoxygenase inhibitor at physiological concentrations (IC_{50} = 0.9 μM). In contrast, each tested anthocyanin showed very low or no anti-inflammatory activity and interfered with the activity of β-carotene. The carotenoid, when in combination with the anthocyanins, was required at concentrations of 1.2-1.7 times higher than when it was alone to bring about the same 50% of LOX-1 inhibition. Anthocyanins and carotenoids are non-competitive lipoxygenase inhibitors which bind to the enzyme-substrate complex (Knaup et al.,

2009, Serpen and Gökmen, 2006). Although the anthocyanins in the present study were not as active LOX-1 inhibitors as β-carotene at low concentrations (2-12 μM), they could still possibly interfere with the binding of β-carotene to the enzyme-substrate, and thus reduced the LOX-1 inhibitory activity of the carotenoid.

On the other hand, the combinations of β-carotene and anthocyanins did not result in a synergy on modulating pro-inflammatory mediators in Caco-2 cells. Some combinations showed antagonism. The interactive effects of β-carotene and other polyphenolic compounds on anti-inflammation have been reported. For instance, the mixture of β-carotene and carnosic acid (a phenolic acid) also showed only additive or antagonistic effect on inhibiting the production of selective pro-inflammatory cytokines and nitric oxide (Hadad and Levy, 2012). The ultimate cellular antioxidant and anti-inflammatory effects of a phytochemical combination rely on the combined activities of the phytochemicals on modulating the following biomarkers: reactive oxygen species (ROS) or reactive nitrogen species (RNS); pro-inflammatory cytokines or chemokines (e.g. IL-8, IL-6, IL-1β, PGE_2); oxidative/pro-inflammatory enzymes involved in the formation of ROS/RNS; defensive enzymes (catalase, superoxide dismutase); intracellular signalling pathways (e.g. NF-κB, mitogen-activated protein kinase); and expression of genes associated with redox and/or inflammatory processes (Wang and Zhu, 2017, Phan et al., 2018c). Synergy of mixed phytochemicals on biological activities in cell-based models can be derived from the synergistic multi target effects of the phytochemical components which can target to different biomarkers (Imming et al., 2006, Wagner and Ulrich-Merzenich, 2009, Williamson, 2001). The molecular pathways responsible for the anti-inflammatory activity of individual phytochemicals are reported, but the cellular mechanisms of bioactivity antagonism between phytochemicals are not yet understood. There is no method to be able to assess the

difference between the actual expressions of a molecular pathway resulted from the effect of a phytochemical combination and the expected expression resulted from the sum effect of each phytochemical component.

The interaction between β-carotene and anthocyanins over the water-lipid interface of the cell membrane could determine their combined effect. β-carotene and each of the anthocyanins showed antagonistic interaction in the liposomal membrane system, but some of the mixtures did not show antioxidant antagonism in the Caco-2 cell model. There might be therefore other factor(s) responsible for the observed combined effects of the compounds in the cell system.

The combined biological effects of phytochemicals in a cellular system result from the interaction between the absorbed portions of the components. Phytochemicals interfere with absorption (Phan et al., 2018c). Hydrophilic phytochemicals may interfere with the absorption of lipophilic compounds, and may consequently affect the ultimate bioactivities of phytochemical mixtures (Phan et al., 2018c). The effects of phytochemical combinations on bioactivities and cellular uptake are often studied separately, thus the interplay between absorption interaction and bioactivity interaction of phytochemicals is not well displayed. We conducted an absorption study firstly to investigate the effect of anthocyanins on the cellular uptake of β-carotene and secondly to see the relation of the absorption interaction to the bioactivity interaction between the compounds. The uptake study showed that all of the anthocyanins tested (7.5 μM), except for delphinidin-3-glucoside significantly increased the cellular absorption of β-carotene (2.5 μM) by 70-200% (**Figure 8**).

The structural properties of the anthocyanins seem to affect the β-carotene uptake. β-carotene absorption was improved more by the anthocyanin compounds that contain only one hydroxyl group on the B ring (PLG, PNG and MG) than by those containing 2

or 3 hydroxyl groups (CG, PTG and DG). PLG containing only one OH group on the B ring induced the highest enhancing effect on β-carotene absorption, whereas DG which has 3 OH groups induced insignificant effect. Methoxyl substitutes on the B ring of the anthocyanins seem not to favour the uptake of β-carotene. When we compared anthocyanins with the same number of OH groups on the B ring, the methoxylated derivatives showed similar or lower enhancement of β-carotene uptake than did the non-methoxylated ones. For example, PNG and MG (containing one B-ring OH, and methoxylated) were less effective than PLG (containing one B-ring OH, and non-methoxylated) on the enhancement of β-carotene uptake. MG has two methoxyl groups but showed an effect similar to PNG. These results apparently show that the number of hydroxyl groups on the B ring of anthocyanins determines the enhancing effect of anthocyanins on the cellular absorption of β-carotene: the less the number, the stronger the enhancement.

The incorporation of anthocyanins into the erythrocyte membrane of red blood cells decreases the general polarization of the membrane, which indicates that the packing order in the hydrophilic region of the membrane is decreased (Bonarska-Kujawa et al., 2012). The increasing disorder of the polar heads of the membrane lipids induced by the anthocyanins possibly altered the barrier function of the cell membrane enabling more β-carotene transported through the membrane leading to an increase in β-carotene absorption. Pelargonidin-3-glucoside disorders the hydrophilic compartment of the erythrocyte membrane to a greater extent than cyanidin-3-galactoside (Bonarska-Kujawa et al., 2012). This may explain the higher enhancing effect of PLG on β-carotene absorption than that of the other anthocyanins observed in our study. The effect of some flavanones, including hesperetin, hesperidin and naringenin, on the cellular uptake of β-carotene and β-cryptoxanthin has been previously published (Claudie et al.,

2013). Only hesperetin and hesperidin, which could interact with the polar heads and the acyl chains of the membrane lipids respectively, could effectively improve the absorption of the carotenoids. Anthocyanins that have strong affinity to the hydrophilic region of the membrane (Bonarska-Kujawa et al., 2012, Yi et al., 2006), enhanced the uptake of β-carotene in our study. The ability of a flavonoid to interact with the membrane lipids is determined by the structural features of the flavonoid (glycoside/aglycone, the degree of hydroxylation/methoxylation) (Abram et al., 2013, Arora et al., 1998). Comprehensive research, therefore, is needed to study the effect of different classes of flavonoids on the cellular absorption of carotenoids in order to thoroughly understand the structure-effect relationship.

The absorption interaction study showed that the anthocyanins enhanced the cellular uptake of β-carotene. The interaction studies on antioxidant and anti-inflammatory activities between the compounds, however, showed that the combinations of β-carotene and anthocyanins provided no enhancing effects, and some mixtures even showed antagonistic effects. The enhancement of β-carotene absorption in the Caco-2 cells by the concomitant anthocyanins did not result in higher cellular antioxidant or anti-inflammatory activities than the expected additive activities of the individual compounds. The intracellular concentration of β-carotene most significantly increased in the presence of 7.5 μM PLG but the corresponding measured biological activities of the β-carotene-PLG mixture (1:3 ratio) were lower than that of the other β-carotene-anthocyanin mixtures at the same ratio (**Table 5**). We also measured the cellular antioxidant activity of β-carotene alone at various concentrations ranging from 0.01-10 μM to verify the effect of increasing β-carotene absorption on the antioxidant activity. The absorption of β-carotene by the Caco-2 cells increases linearly with increasing initial β-carotene concentrations of up to 6-8 μM and plateaus at initial concentrations higher

than 10 μM (During, 2002, Liu et al., 2004), which means that β-carotene is absorbed at the same rate in the linear range of concentrations. We calculated the intracellular β-carotene content absorbed from the initial β-carotene concentrations of 0.01-10 μM based on the absorption rate previously measured at 2.5 μM. The corresponding CAA of β-carotene was 26-30.7% at the initial concentrations of 0.01-1 μM (corresponding to the β-carotene absorbed amount of < 11 ng), and increased to 61.5-62.7% at 2.5-5 μM (corresponding to the β-carotene absorbed amount of 27-54 ng), but then decreased to 40.7% at 7.5 μM (80.5 ng β-carotene absorbed) and to 37.2% at 10 μM (107.4 ng β-carotene absorbed) (**Table 6**).

Table 6. Cellular antioxidant activity (CAA) of single β-carotene at different concentrations.

Initial β-carotene concentration in cell culture medium (μM)	Absorbed β-carotene content[1] (ng)	CAA unit[2]
0.01	0.1	26.0 ± 11.2[a]
0.1	1.1	30.8 ± 9.0[a]
1	10.7	30.7 ± 12.6[a]
2.5	26.8	62.7 ± 3.9[b]
5	53.7	61.5 ± 7.4[b]
7.5	80.5	40.7 ± 10.5[a]
10	107.4	37.2 ± 7.7[a]

[1]Calculated from a constant absorption rate of 1% in the linear range of concentrations (0.01-10 μM). The β-carotene absorption rate of 1% was measured at an initial β-carotene concentration of 2.5 μM. See texts for more explanation.
[2]Values are mean ± SD of four replicates (n = 4). Different letters indicate a significant difference (p < 0.05).

These results again showed that the increase in β-carotene uptake to a certain level resulted in a decrease in the cellular antioxidant activity, which is consistent with the previous findings in the interaction studies. In the presence of PLG (7.5 μM), the absorption of β-carotene increased to 78.7 ng at which a reduction of CAA was observed in the CAA study of β-carotene alone. This may, to some extent, explain for the lowest CAA of the β-carotene-PLG (2.5 μM: 7.5 μM) mixture and the CAA antagonism seen in this combination. In the presence of the other anthocyanins (CG, MG, PNG or PTG), β-carotene absorption was increased to levels at which the CAA of the β-carotene

component was similar to that of β-carotene when it was alone (61.5-62.7%), which resulted in the observed additive cellular antioxidant effect.

β-carotene is known to have antioxidant activity at low concentrations but exhibits pro-oxidant activity at high concentrations (Young and Lowe, 2001). For example, β-carotene at 1-3 μM in the culture medium could protect HT29 cells from DNA oxidative damage induced by xanthine/xanthine oxidase, but the DNA protective ability of β-carotene rapidly decreased at higher concentrations of 4-10 μM (Lowe et al., 2009). In our study, β-carotene showed increasing CAA at 0.01-5 μM, but showed decreasing CAA at 5-10 μM. The decreased biological activities and/or the antagonism seen between β-carotene and some anthocyanins, therefore, could be related to the increase in β-carotene absorption induced by the presence of anthocyanins reaching concentrations at which β-carotene started to exhibit pro-oxidant activity. This result shows that a possible mechanism of bioactivity antagonism between phytochemicals in a cell-based system involves absorption interference that can facilitate the pro-oxidant activity of the relevant compound.

4.5. Conclusion

The co-presence of each of the tested anthocyanins, except for delphinidin-3-*O*-glucoside, significantly increased the cellular absorption of β-carotene in the Caco-2 cells. The enhancement of intracellular β-carotene content by the anthocyanins, however, did not result in any synergy on the cellular antioxidant or anti-inflammatory activities of the combinations. The antagonistic bioactivities seen in some mixtures of β-carotene and the anthocyanins could be related to the exhibition of pro-oxidant activity of β-carotene when its cellular concentration increased to a certain level by the presence of the anthocyanin compounds.

CHAPTER 5

Interactive effects of lutein and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity *in vitro* and *ex vivo*

(This is an Accepted Manuscript of an article published by MDPI in Molecules volume 23, pages 2035-2046 on 14 August 2018, available online: https://doi.org/10.3390/molecules23082035)

Abstract

The interactive effects on anti-oxidation and anti-inflammation of lutein combined with each of the six common anthocyanins were studied in both chemical and cellular systems. The combined phytochemicals showed an antagonism in the inhibition of lipid oxidation in a liposomal membrane, but showed an additive effect on cellular antioxidant activity in Caco-2 cells. Lutein was an active lipoxygenase inhibitor at 2-12 μM while anthocyanins were inactive. The concentration of lutein when it was used in combination with anthocyanins was 25-54% higher than when lutein was used alone (i.e., IC_{50} = 1.2 μM) to induce 50% of lipoxygenase inhibition. Only the combination of lutein with malvidin-3-*O*-glucoside showed anti-inflammatory synergy in the suppression of interleukin-8, and the synergy was seen at all three ratios tested. Some mixtures, however, showed anti-inflammatory antagonism. The presence of anthocyanins (5-7.5 μM) did not affect lutein uptake (2.5-5 μM) by Caco-2 cells.

5.1. Introduction

Lutein is a xanthophyll carotenoid mainly present in dark green leafy vegetables (Reboul et al., 2007b). Lutein shows antioxidant and anti-inflammatory activities by targeting reactive oxygen species, and downregulating inflammatory proteins and pro-

inflammatory cytokines (Qiao et al., 2018). Lutein is one of the three xanthophyll carotenoids that can cross the blood-brain barrier and selectively accumulate in the retina and brain tissues (Reboul et al., 2007b, Thurnham, 2007, Wallace, 2018). The xanthophyll lutein is often co-ingested with other plant phytochemicals such as carotenoids and/or flavonoids in a normal human diet containing plant-based foods. Anthocyanins are one of the largest classes of flavonoids and are present abundantly in many fruits and vegetables (Bueno et al., 2012a). Thus, there are chances for lutein and anthocyanins to be concurrently consumed in a meal, after which they can interact with each other during digestion and absorption to effect biological activities. Water-soluble phytochemicals may interfere with the uptake of lipid-soluble bioactive compounds (Phan et al., 2018c). For instance, lutein uptake by Caco-2 cells is impaired by the flavonoid naringenin, but is not affected by (+)-catechin, a phenolic acid, or vitamin C (Reboul et al., 2007b). Absorption interference between phytochemicals may result in changes on combined biological effects of the compounds (Phan et al., 2018c). We previously reported that anthocyanins increased β-carotene uptake by Caco-2 cells to levels that triggered β-carotene's pro-oxidant activity, which resulted in an antagonistic cellular antioxidant effect seen in some combinations (Phan et al., 2018b). Phytochemical interactions on cellular uptake and biological activities are often studied separately, so the mutual influences between these aspects are not well addressed. This study aimed to investigate the effect of different common anthocyanidin glucosides on lutein uptake by Caco-2 cells, and the combined effects of anthocyanins and lutein on oxidative inhibition and anti-inflammation in both chemical and cellular models.

5.2. Materials and methods

As described in Chapter 3, section 3.1.1, 3.1.4, and 3.2.1-3.2.11 on pages 75-85.

5.3. Results and discussion

5.3.1. Effects of lutein-anthocyanin combinations on oxidative inhibition in chemical and cellular models

5.3.1.1. Liposome peroxidation inhibition

The percentage of thiobarbituric acid reagent species (%TBARS) inhibition when lutein was present alone was 39%, and when anthocyanins were present alone, %TBARS inhibition was 14–43%. Lutein combined with each of the tested anthocyanins did not enhance the inhibitory effect on lipid peroxidation in the liposomal membrane. The expected additive effects of TBARS inhibition of lutein-anthocyanin mixtures were 48–66%, but the actual effects of the mixtures were less than 35% (**Figure 9**). This indicates that lutein and anthocyanins showed an antagonistic interaction at the interface of the liposomal membrane. Lutein is a xanthophyll carotenoid characterized with polar groups at the two ends of its molecule. Lutein can position itself in parallel closely to the polar heads of the membrane, or it can span the molecule across the membrane with the polar ends anchoring to the polar lipid heads (Han et al., 2012). Anthocyanin compounds are normally positioned in the aqueous region of the membrane outer monolayer (Bonarska-Kujawa et al., 2012). Such orientations of the compounds in the lipid bilayer membrane may enable them to interact and form lutein-anthocyanin adducts, which result in the reduced capability of lipid peroxidation inhibition. The formation of adducts between other carotenoids and flavonoids, for example: β-carotene and green tea polyphenolic compounds (Song et al., 2011) or β-carotene and daidzein (Liang et al., 2010), has been previously reported to impart antioxidant antagonism in liposomes.

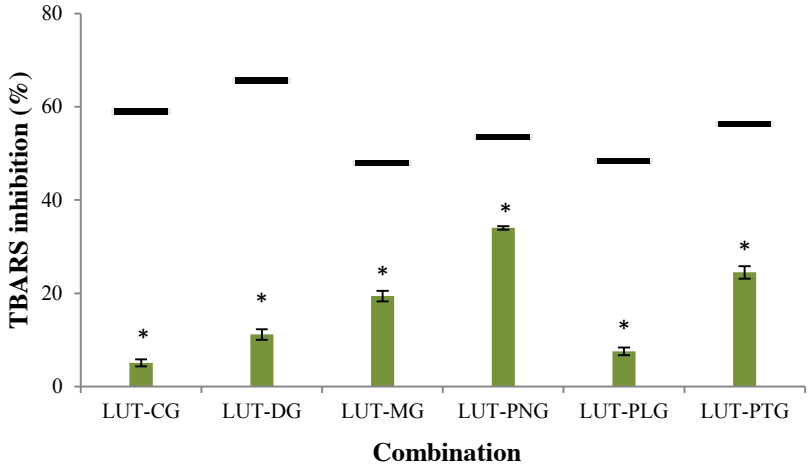


Figure 9. Liposome peroxidation inhibitory activity of different lutein-anthocyanin combinations (1:1 ratio). The horizontal lines illustrate the expected additive effect of lutein-anthocyanin combinations. Asterisk-marked columns indicate a significant difference ($p < 0.05$) between the observed effect of the mixture with its calculated additive effect. Calculation of the expected additive effect was based on an equation of Fuhrman et al. (2000): $TBARS_A + TBARS_L - TBARS_A \times TBARS_L/100$ ($TBARS_A$ and $TBARS_L$ are %TBARS inhibition of anthocyanin alone and lutein alone respectively, which was calculated following the equation (3) on page 78. TBARS: thiobarbituric acid reagent species, LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

5.3.1.2. Cellular antioxidant activity (CAA)

The interactive effects on anti-oxidation of lutein-anthocyanin combinations at 1:1, 1:3 and 3:1 ratios were assessed in a Caco-2 cell model. There was no synergistic or antagonistic effect seen in any of the mixtures at the tested ratios. All combinations showed additive CAA in Caco-2 cells (**Table 7**). Lutein and the anthocyanidin glucosides showed antagonistic interaction in the phosphatidylcholine (PC) liposome membrane, but did not show the same interaction in the cell membrane. The different interactions between phytochemicals can be seen in different assay models (Phan et al., 2018c, Wang and Zhu, 2017). A combination of phytochemicals may show synergy/antagonism in chemical models, but may not show the same in cellular models, and *vice versa*. For example, the combination of raspberry and adzuki bean extracts

shows antioxidant synergy in chemical assays but does not show the same effect in MCF-7 cancerous cells (Wang et al., 2013).

Table 7. Cellular antioxidant activity (CAA) of lutein-anthocyanin mixtures.

Mixture	Lutein: anthocyanin ratio					
	1:3		**1:1**		**3:1**	
	Experimental effect[1]	Expected additive effect[2]	Experimental effect	Expected additive effect	Experimental effect	Expected additive effect
LUT-CG	38.1 ± 12.5	52.1	36.9 ± 12.8	52.1	46.3 ± 7.5	45.2
LUT-DG	48.2 ± 12.3	49.4	45.5 ± 5.9	36.4	44.1 ± 5.3	44.0
LUT-MG	50.8 ± 7.1	48.1	44.0 ± 3.1	42.5	50.3 ± 6.8	50.4
LUT-PNG	42.9 ± 6.9	43.9	33.6 ± 8.8	38.1	48.7 ± 2.4	46.8
LUT-PLG	38.6 ± 4.8	44.3	32.5 ± 8.6	38.9	49.2 ± 4.0	45.8
LUT-PTG	38.2 ± 6.5	42.6	34.6 ± 11.5	39.2	47.6 ± 5.9	45.7

LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.
[1]Each value of the experimental effect was mean of CAA unit ± SD of four individual replicates ($n = 4$).
[2] The expected additive effect was calculated as $CAA_A + CAA_L - CAA_A \times CAA_L/100$ (CAA_A and CAA_L is the CAA unit of an anthocyanin alone and lutein alone respectively, which was calculated following the equation (5) on page 82).

On the other hand, membrane lipid composition has a pronounced effect on the localization of phytochemicals and the interaction of the phytochemicals with the membrane, which may lead to changes in biological activities (Selvaraj et al., 2015). The interactive effect of lutein and anthocyanins in the PC liposome membrane being different from that in the Caco-2 cell membrane might be partly due to the differences in the composition of the two membrane models.

5.3.2. Effects of lutein-anthocyanin combinations on anti-inflammation in chemical and cellular models

5.3.2.1. Lipoxygenase inhibitory activity

Lutein showed strong inhibition of LOX-1 (IC_{50} = 1.2 μM). None of the anthocyanins showed potent LOX-1 inhibitory activity at 2-12 μM (% LOX-1 inhibition of 0.5-12.3%). They have been reported to have high LOX-1 IC_{50}, for example: peonidin-3-glucoside (PNG): 38 mM, or cyanidin-3-glucoside (CG): 0.5 mM (Knaup et al., 2009). The mode of the interactive effect upon LOX-1 inhibition between lutein and anthocyanins could not be determined because lutein was an active LOX-1 inhibitor at

low concentrations while anthocyanins were not. The lipoxygenase inhibitory effects of all lutein-anthocyanin mixtures were still measured to evaluate whether the presence of anthocyanins affected the LOX-1 inhibitory activity of lutein. IC_{50} values of lutein-anthocyanin mixtures ranged from 3.1-3.8 μM, which were higher than that of lutein (IC_{50} = 1.2 μM) (**Figure 10**).

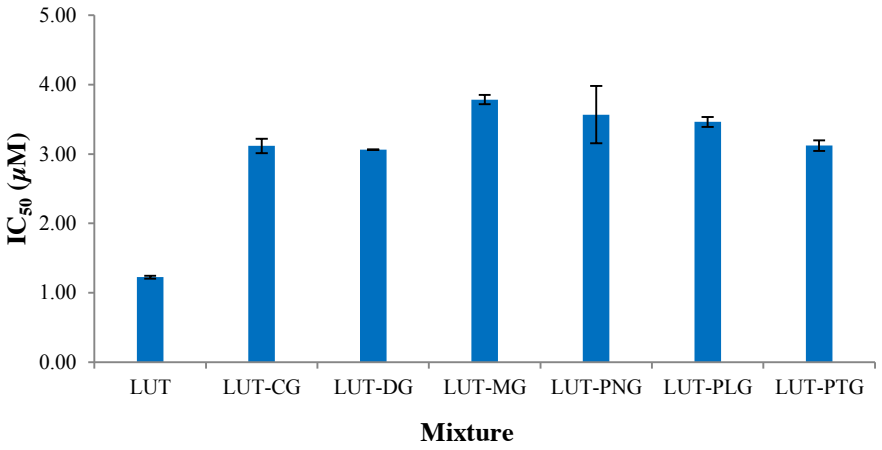


Figure 10. Lipoxygenase IC_{50} of lutein alone and different lutein-anthocyanin combinations. IC_{50} (n = 3): inhibitory concentration that exerts 50% enzyme inhibition. LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

This indicates that lutein combined with anthocyanins inhibited LOX-1 less effectively than lutein alone. The concentrations of lutein required for the mixtures to exhibit 50% of LOX-1 inhibition increased by 25-54% of the IC_{50} of lutein when it was applied alone. These results show that the presence of anthocyanins affected the LOX-1 inhibitory activity of lutein. Anthocyanins and carotenoids inhibit LOX-1 non-competitively (Knaup et al., 2009, Serpen and Gökmen, 2006) by binding to the lipoxygenase-substrate complex. The reduced LOX-1 inhibitory effect of lutein when it was present with anthocyanins might be due to the interference of the anthocyanins with the binding of lutein to the lipoxygenase-substrate complex.

5.3.2.2. Secretion of IL-8

The % IL-8 secretion compared to the control when lutein (2.5, 5, 7.5 μM) was applied alone was 70%, 78% and 57%, respectively. Most of the lutein-anthocyanin mixtures effectively reduced the amount of IL-8 secreted by Caco-2 cells after TNF-α-induced inflammation. The effectiveness of suppressing IL-8 secretion when lutein was combined with CG or DG was lower than when it was combined with the other anthocyanins. The mixtures of lutein with MG, PNG, PLG or PTG increasingly reduced IL-8 secretion when the ratio of lutein to anthocyanins was increased (**Figure 11**). The lutein-malvidin-3-*O*-glucoside combination (LUT-MG) was the only combination that showed a synergistic effect on interleukin-8 suppression, and the synergy was seen at all three ratios tested. The LUT-PNG mixture showed an additive effect at all three tested ratios, and some mixtures showed an antagonistic effect, including: LUT-CG and LUT-DG at all three ratios tested; LUT-PLG at the lutein: anthocyanin ratios of 1:3 and 1:1; and LUT-PTG at the 1:1 and 3:1 ratios.

5.3.2.3. Nitric oxide production

The % NO production compared to the control when lutein (2.5, 5, 7.5 μM) was applied alone was 95%, 81% and 76%, respectively. Most of the combinations of lutein with anthocyanins did not effectively inhibit the production of nitric oxide (**Figure 11**). Synergy was not seen in any of the mixtures. An antagonistic effect was observed in most of the combinations at the 1:1 and 3:1 ratios of lutein to anthocyanins. All mixtures showed an additive effect at the lutein: anthocyanin ratio of 1:3.

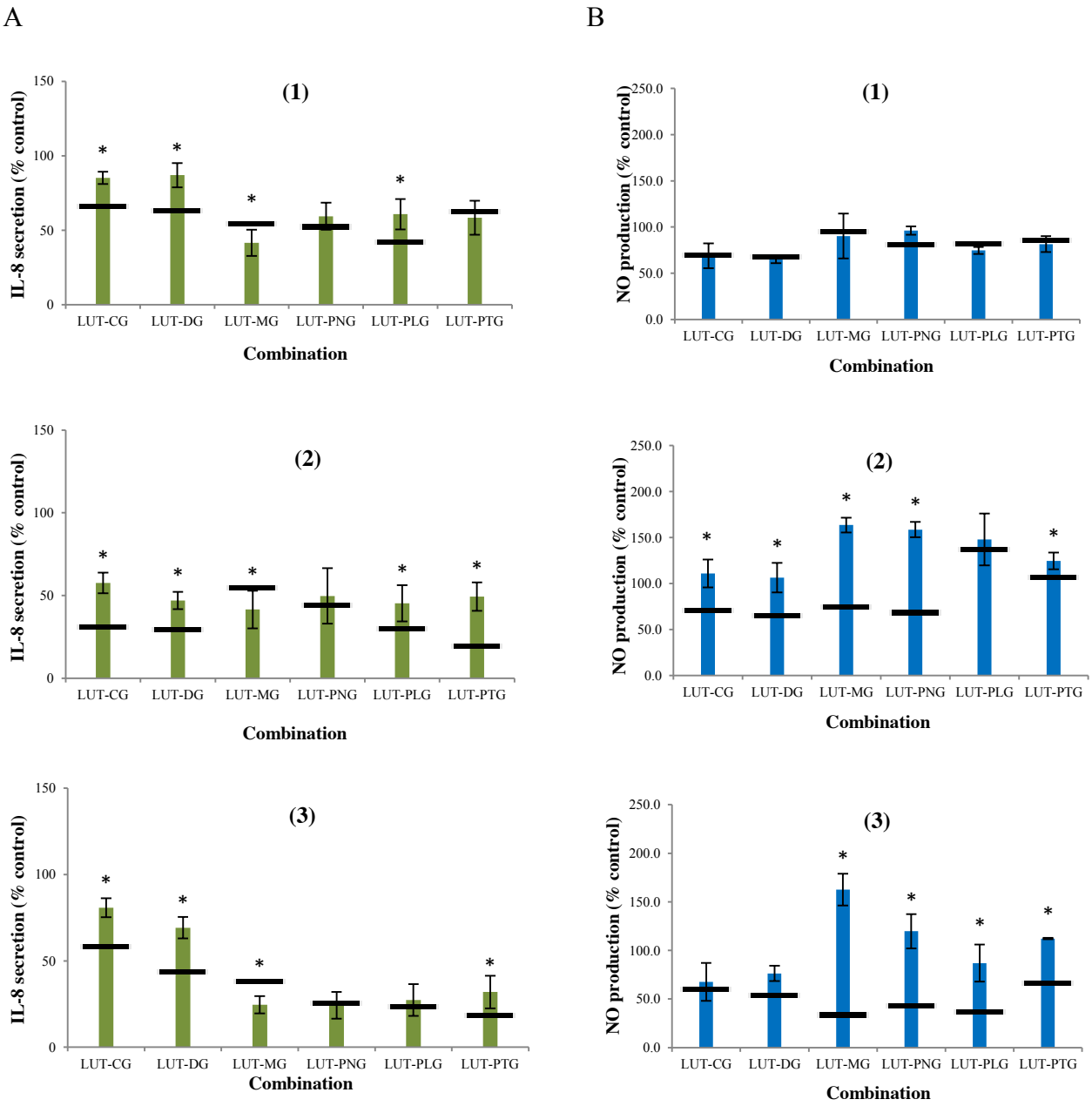


Figure 11. (A) Secretion of (A) IL-8 (% control) and (B) nitric oxide (NO) (% control) by Caco-2 cells after being treated with different lutein-anthocyanin mixtures followed by TNF-α-induced inflammation (100 ng/mL). Total concentration of the bioactive compounds in cell culture was 10 μM and the ratio of lutein to anthocyanin was varied at (1) 1:3, (2) 1:1, and (3) 3:1. Controls are samples collected from the cells that underwent TNF-α-induced inflammation without pre-treatment with phytochemicals. The horizontal lines illustrate the expected additive effect, which was calculated as 100 − (A + L − A × L/100) (A and L are % reduction of IL-8 or NO secreted by Caco-2 cells compared to the control when treating the cells with anthocyanin alone and lutein alone respectively). Asterisk-marked columns indicate a significant difference ($p < 0.05$) between the observed effect of the combination with its calculated additive effect. Experimental values show as mean ± SD of three independent replicates ($n = 3$). LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

5.3.3. Interferences of anthocyanins on lutein uptake by Caco-2 cells

The cellular uptake of lutein (5 μM) in the presence of each of the tested anthocyanins (5 μM) was not significantly different ($p > 0.05$) from the lutein uptake when it was present alone (**Figure 12**). The same trend was observed when the ratio of anthocyanin to lutein was increased to 7.5 μM: 2.5 μM (**Figure 12**). These results indicate that anthocyanins did not affect the uptake of lutein by Caco-2 cells. The effects of some polyphenols on lutein uptake by Caco-2 cells have been previously reported. (+)-catechin, gallic acid and caffeic acid do not affect the cellular absorption of lutein, whereas naringenin causes an impairment of lutein uptake (Reboul et al., 2007b). The latter has been suggested to be the consequence of the interaction of naringenin with the membrane lipids, which influences the invagination of the lipid raft domains containing lutein receptors (Reboul et al., 2007b).

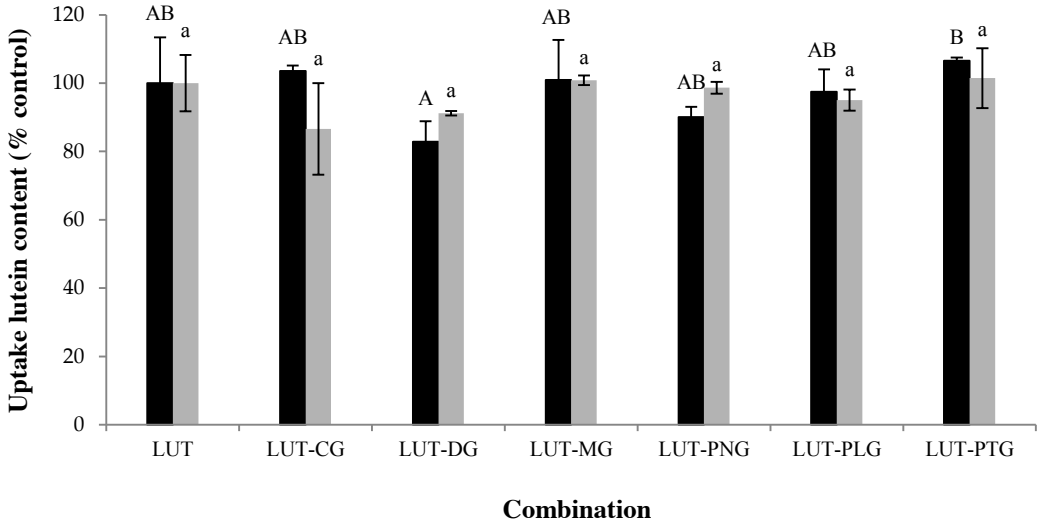


Figure 12. Lutein uptake (% control) by Caco-2 cells in the absence (control) and presence of different anthocyanins at lutein: anthocyanin ratios of 1:3 (2.5:7.5 μM, represented in black bars) and 1:1 (5:5 μM, represented in grey bars). Columns of the same colour marked with different letters indicate a significant difference from each other ($p < 0.05$). Values are mean ± SD of three independent replicates ($n = 3$). LUT: lutein, CG: cynidin-3-*O*-glucoside, DG: delphnidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

Anthocyanins can incorporate into the polar interface of the membrane outer monolayer (Bonarska-Kujawa et al., 2012) leading to an increase in the polarization area, which may result in a mismatch between the area of the polar heads and the area of the hydrophobic tails (Tarahovsky et al., 2008). Consequently, the interspace between the two lipid layers may increase, giving additional freedom to the hydrocarbon chains. This effect is called membrane fluidization, which may influence the appearance and development of lipid rafts (the so-called raft-breaking effect (Tarahovsky et al., 2008)), leading to a reduced diffusion of some lipid molecules. Membrane fluidization, on the other hand, decreases lipid-melting temperatures which possibly results in an increase in lipid diffusion (Tarahovsky et al., 2008). These contradictory effects of polar flavonoids upon the diffusion of lipophilic molecules were seen in anthocyanins affecting the uptake of carotenoids. We previously reported in chapter 4 that some anthocyanins (7.5 μM) increased β-carotene uptake (2.5 μM) (Phan et al., 2018b). These anthocyanin compounds, however, decreased lycopene absorption (present in Chapter 6) (Phan et al., 2019) and did not influence lutein uptake. It seems that the interaction of anthocyanins with the cellular lipid membrane did not affect the lipid raft domains that contain lutein receptors.

The combinations of lutein with anthocyanins showed neither synergy nor antagonism in cellular antioxidant activity (CAA) in Caco-2 cells. Lutein uptake by Caco-2 cells was not significantly altered by the presence of anthocyanins. The maintained intracellular lutein content may partly explain the additive CAA seen in all of the lutein-anthocyanin combinations. It seems that the interaction between anthocyanins and carotenoids on cellular antioxidant activity is partly relevant to the interference of anthocyanins with the cellular uptake of carotenoids. In the previous study presenting in chapter 4, we found that some anthocyanins increased the intracellular content of β-

carotene to certain levels where it exerted pro-oxidant activity, which partly explains the observed antagonism of CAA in some of the mixtures (Phan et al., 2018b). In this study, we found that the cellular uptake of lutein was not affected by the presence of anthocyanins, and the interactive cellular antioxidant effects in all tested lutein-anthocyanin mixtures were additive. The effect of anthocyanins on lutein uptake, however, did not show relevance to the interactive anti-inflammatory effects. The intracellular content of lutein was not significantly changed by the presence of anthocyanins, but some of the lutein-anthocyanin mixtures showed non-additive anti-inflammatory effects on the suppression of interleukin-8 secretion and NO production. This indicates that the combined anti-inflammatory effects between lutein and anthocyanins might not be a consequence of the uptake interaction between the compounds.

The synergistic effect of a phytochemical mixture on cellular bioactivities can be the result of the multi-target effects of its phytochemical components on different biomarkers (e.g., oxidative and/or defensive enzymes, inflammatory mediators, gene expression) (Phan et al., 2018c, Wang and Zhu, 2017). Molecular mechanisms of anti-inflammatory antagonism between phytochemicals, however, have not been uncovered. There is a limitation of method availability for the prediction of expected gene expressions of inflammatory markers resulting from the combined activity of phytochemicals.

5.4. Conclusion

The combinations of lutein and anthocyanins did not show synergistic antioxidant effects in the tested chemical and cellular models. Lutein and anthocyanins (1:1, 2 μM) showed an antagonistic interaction on lipid peroxidation in a phosphatidylcholine liposome membrane. All of the combinations at the tested ratios (1:1, 1:3 and 3:1, total

concentration of 10 μM), however, showed additive effects on cellular antioxidant activity in a Caco-2 cell model. The cellular uptake of lutein (2.5–5 μM) was not affected by the presence of anthocyanins (5–7.5 μM), which could partly explain the observed additive cellular antioxidant activity. Only the mixture of LUT with MG showed anti-inflammatory synergy in the suppression of interleukin-8 at all tested ratios. Some lutein-anthocyanin combinations showed antagonism in the suppression of pro-inflammatory mediators (IL-8, NO) despite the fact that at the concentrations tested, lutein uptake was not affected by the presence of anthocyanins. Future studies should be designed to unravel the molecular mechanisms of anti-inflammatory antagonism of mixed phytochemicals. An understanding of phytochemical combinations and the appropriate concentrations can lead to designing foods or supplements with better targeted functions and absorption.

CHAPTER 6

Interactive effects of lycopene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity *in vitro* and *ex vivo*

(This is an Accepted Manuscript of an article published by Elsevier in Food Chemistry volume 276, pages 402-409 on 15 March 2019, available online: https://doi.org/10.1016/j.foodchem.2018.10.012)

Abstract

Lycopene was combined with the glucosides of each of the six common anthocyanidins at 3 different ratios to investigate their interactions on antioxidant and anti-inflammatory activity, and cellular uptake. The bioactivity interaction between lycopene and anthocyanins was studied in both chemical and cellular models. Anti-oxidative synergy was not seen in any of the tested lycopene-anthocyanin mixtures, nor in the models studied. When lycopene was paired with the methoxylated anthocyanins, the anti-inflammatory effect on the inhibition of the cytokine IL-8, which is a pro-inflammatory biomarker, was increased by 15-69% of the expected additive activity, indicating synergistic interaction between the compounds. The cellular uptake of lycopene was significantly impaired by the presence of the anthocyanins: reduced by 50-80% at the lycopene: anthocyanin combinatory ratios of 2.5:7.5 μM (1:3) or 5:5 μM (1:1). The reduced intracellular lycopene content might be partly responsible for the antagonistic cellular antioxidant property seen in some of the tested mixtures.

6.1. Introduction

Phytochemicals in food materials can interact with each other and produce synergy or antagonism in biological activities. Anti-oxidative synergy is seen in several mixtures of hydrophilic phytochemicals: anthocyanins and flavonols, flavan-3-ol and flavonols, tea polyphenols; or in lipophilic phytochemical mixtures: mixed carotenoids, carotenoids and α-tocopherol; and in flavonoid-carotenoid mixtures: carotenoids and flavonols, carotenoid and isoflavans (Phan et al., 2018c). The combination of phytochemicals can also show antagonistic antioxidant effects, for example: the mixtures of different anthocyanins; anthocyanins and other flavonoids (Hidalgo et al., 2010); lycopene and γ-tocopherol (Fuhrman et al., 2000); β-carotene and flavones or flavan-3-ols (Liang et al., 2010, Song et al., 2011).

Synergistic and antagonistic interactions between phytochemicals and their resultant effects on anti-inflammatory activities have also been reported. Luteolin combined with tangeretin produced synergistic protection of RAW 264.7 cells from lipopolysaccharide-induced inflammation (Funaro et al., 2016). β-carotene combined with cyanidin-3-*O*-glucoside or pelargonidin-3-*O*-glucoside showed antagonism on the suppression of cytokine secretion (Phan et al., 2018b). Interferences in absorption between phytochemicals also occur. For example, β-carotene uptake was increased in the presence of hesperidin or hesperetin (Claudie et al., 2013), or in the presence of some anthocyanidin glucosides (Phan et al., 2018b). Bioactivity and bioavailability interactions between bioactive compounds, however, are often studied separately, so the mutual influence between these aspects of phytochemical interaction is not yet completely understood.

Anthocyanins and carotenoids are natural plant pigments possessing antioxidant and anti-inflammatory activities (Saini et al., 2015, He and Giusti, 2010). They are co-

present in several vegetables (for example: purple tomato, purple carrot) or can be concurrently digested from human diets. The interactive effects between carotenoids and anthocyanins are not well reported. We previously reported in chapters 4 and 5 the combined effects of some anthocyanins and β-carotene or lutein respectively on biological activities and their cellular uptake interference (Phan et al., 2018b). In this chapter, we report the combined effect of six common anthocyanidin glucosides and lycopene on anti-oxidation and anti-inflammation in both chemical and cellular models. The interference of anthocyanins on the cellular uptake of lycopene by Caco-2 cells was also studied at the same time to evaluate the relevant impact of interaction on uptake and the consequential combined bioactivities of the compounds.

6.2. Materials and methods

As described in Chapter 3, section 3.1.1, 3.1.4, and 3.2.1-3.2.11 on pages 75-85.

6.3. Results

6.3.1. Lycopene-anthocyanin interactive effects upon antioxidant activities in chemical and cellular models

6.3.1.1. Liposome peroxidation inhibition

Mixtures of anthocyanins and lycopene at a 1:1 ratio were examined for the combined inhibitory activity against lipid oxidation in a liposomal membrane to understand their interaction over a water-lipid interface. The results showed that the experimental TBARS inhibition percentage of each lycopene-anthocyanin mixture was only 45-76% of the expected additive value (**Figure 13**), which indicated an antagonistic interaction between the compounds.

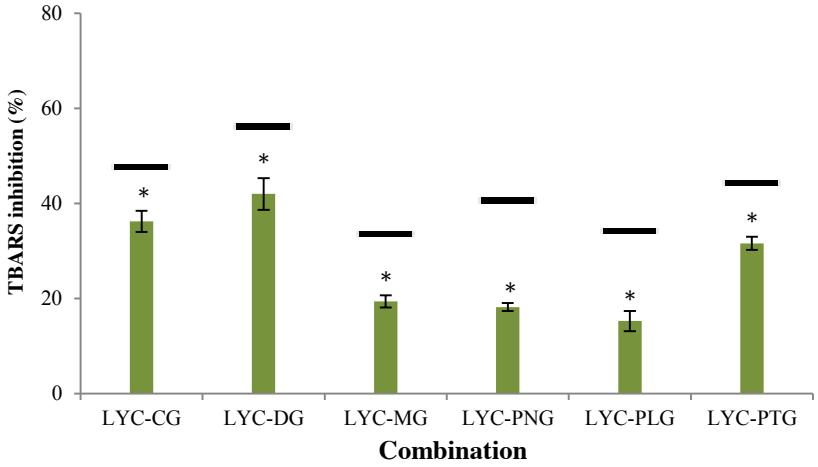


Figure 13. Liposome peroxidation inhibitory activity of different lycopene-anthocyanin combinations (1:1 ratio). The horizontal lines illustrate the expected additive effect of lycopene-anthocyanin combinations. Asterisk-marked columns indicate a significant difference between the observed effect of the mixture with its calculated additive effect ($p < 0.05$). Calculation of the expected additive effect was based on an equation of Fuhrman et al. (2000): $TBARS_A + TBARS_L - TBARS_A \times TBARS_L/100$ ($TBARS_A$ and $TBARS_L$ are % TBARS inhibition of anthocyanin alone and lycopene alone respectively, which was calculated following equation (3) on page 78). TBARS: thiobarbituric acid reagent species, LYC: lycopene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

6.3.1.2. Cellular antioxidant activity (CAA)

The interactive effect on anti-oxidation of anthocyanins and lycopene was determined in the intestinal Caco-2 cells at 3 different lycopene: anthocyanin ratios of 1:1, 1:3 and 3:1 at a total concentration of 10 μM. The results are presented in **Table 8**. All of the mixtures showed an antagonistic effect at the 1:1 and 1:3 ratios, except for the mixture of lycopene and PNG which showed an additive effect at the 1:3 ratio. The antioxidant effect was reduced by 25-30% when lycopene was mixed with CG, MG or PTG, and by 36-43% with DG and PLG. At the 3:1 ratio of lycopene to anthocyanin, an additive antioxidant activity was seen in every combination tested.

Table 8. Cellular antioxidant activity (CAA) of lycopene-anthocyanin combinations.

Mixture	Lycopene: anthocyanin ratio					
	1:3		1:1		3:1	
	Experimental effect[1]	Expected additive effect[2]	Experimental effect	Expected additive effect	Experimental effect	Expected additive effect
LYC-CG	44.7 ± 9.4*	63.1	45.1 ± 10.9*	63.8	49.4 ± 8.9	56.0
LYC-DG	37.4 ± 5.2*	58.8	30.8 ± 4.3*	54.5	48.1 ± 4.2	55.1
LYC-MG	40.3 ± 9.0*	53.5	40.9 ± 3.9*	58.3	60.9 ± 9.3	60.2
LYC-PNG	54.4 ± 4.4	56.3	31.8 ± 11.2*	58.1	51.5 ± 5.6	57.3
LYC-PLG	31.4 ± 4.6*	53.9	33.0 ± 9.8*	56.4	54.3 ± 6.1	56.5
LYC-PTG	40.4 ± 3.1*	53.9	39.0 ± 3.9*	56.6	39.8 ± 7.6	56.4

*A significant difference ($p < 0.05$) was seen between the experimental effect and the calculated additive value.
LYC: lycopene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.
[1] Each value of the experimental effect was mean of CAA unit ± SD of four individual replicates ($n = 4$).
[2] The expected additive effect was calculated as $CAA_A + CAA_L - CAA_A \times CAA_L/100$ (CAA_A and CAA_L is the CAA unit of an anthocyanin alone and lycopene alone respectively, which was calculated following the equation (5) on page 82).

6.3.2. Lycopene-anthocyanin interactive effects upon anti-inflammatory activities in chemical and cellular models

6.3.2.1. Lipoxygenase inhibitory activity

The lipoxygenase inhibitory activity of lycopene at 0.2-2 μM was dose-dependent. Lycopene was an effective LOX-1 inhibitor with the IC_{50} of 0.33 μM. All of the tested anthocyanins at the concentrations of 2-12 μM did not inhibit LOX-1 activity. Anthocyanins have been previously reported to have high LOX-1 IC_{50} values: CG: 0.5 mM, PNG: 38 mM (Knaup et al., 2009). Because lycopene is an active LOX-1 inhibitor but anthocyanins are not, the interactive mode between them was not able to be determined. The LOX-1 inhibitory activity of lycopene-anthocyanin mixtures was measured to compare with that of lycopene alone. All of the lycopene-anthocyanin mixtures (1:1 ratio) showed higher LOX-1 IC_{50} (4-4.5 μM) than that of lycopene (0.33 μM) (**Figure 14**). These results showed that the presence of the anthocyanins reduced the lipoxygenase inhibitory activity of lycopene.

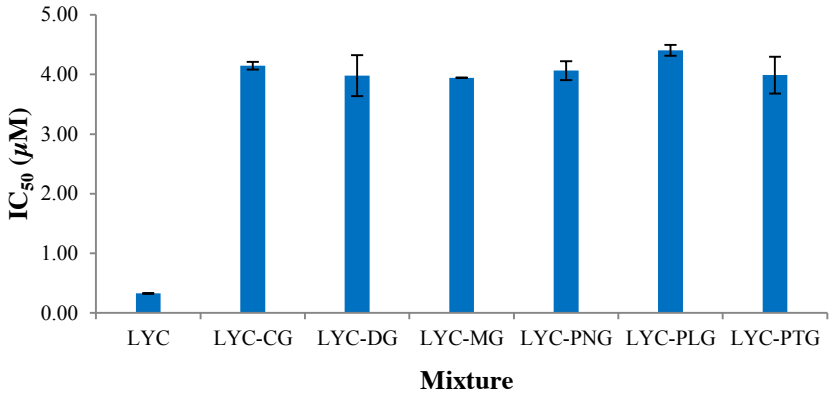


Figure 14. Lipoxygenase IC_{50} of lycopene alone and different lycopene-anthocyanin combinations. IC_{50} (n = 3): inhibitory concentration that exerts 50% enzyme inhibition. LYC: lycopene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

6.3.2.2. Secretion of IL-8

IL-8 secretion by Caco-2 cells was significantly reduced in the presence of lycopene-anthocyanin mixtures (10 μM in total) at all ratios tested. The higher the ratio of lycopene: anthocyanin, the greater the reduction of IL-8 secretion (**Figure 15**). Some mixtures of lycopene and anthocyanins showed synergistic effect on the reduction of IL-8 secretion. At the lycopene: anthocyanin ratios of 1:1 and 3:1, the combinations of lycopene with MG, PNG or PTG synergistically suppressed the secretion of the pro-inflammatory cytokine IL-8. The combined effect of LYC-MG or LYC-PNG mixtures on the IL-8 reduction was increased by 15-16% of their expected additive effect at the 3:1 ratio, and by 49-69% at the 1:1 ratio. LYC mixed with PTG increased the suppression of IL-8 secretion by 53-54% of their expected additive activity. LYC-PNG was the only mixture that showed synergy at all tested ratios. The mixtures of LYC with CG, DG or PLG showed an additive effect at all three ratios tested.

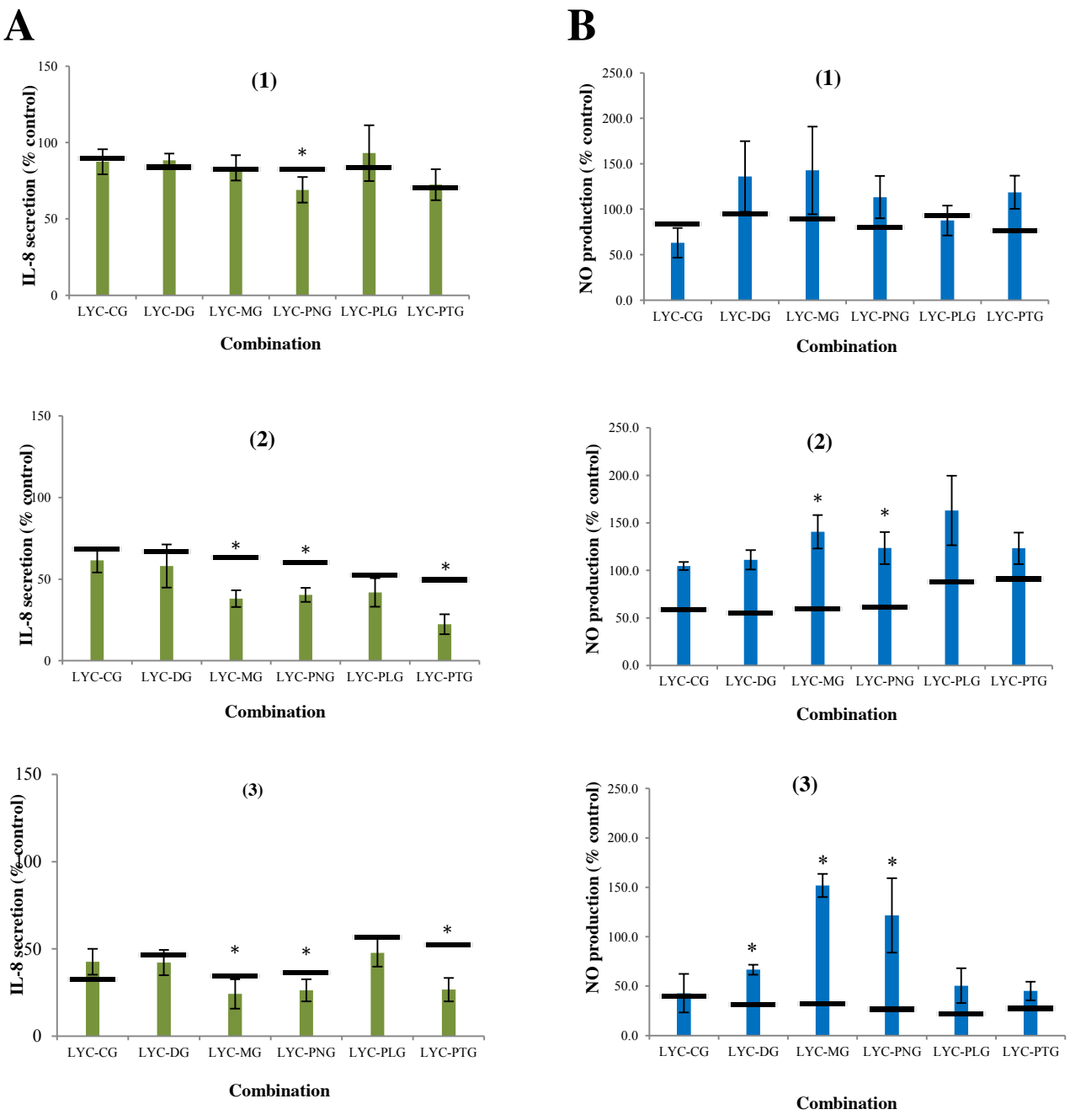


Figure 15. (A) Secretion of (A) IL-8 (% control) and (B) nitric oxide (NO) (% control) by Caco-2 cells after being treated with different lycopene-anthocyanin mixtures followed by TNF-α-induced inflammation (100 ng/ml). Total concentration of the bioactive compounds in cell culture was 10 μM and the ratio of lycopene to anthocyanin was varied at (1) 1:3, (2) 1:1, and (3) 3:1. Controls are samples collected from the cells that underwent TNF-α-induced inflammation without pre-treatment with phytochemicals. The horizontal lines illustrate the expected additive effect, which was calculated as 100 - (A + L - A x L/100) (A and L are % reduction of IL-8 or NO secreted by Caco-2 cells compared to the control when treating the cells with anthocyanin alone and lycopene alone respectively). Asterisk-marked columns indicate a significant difference between the observed effect of the combination with its calculated additive effect ($p < 0.05$). Experimental values show as mean ± SD of three independent replicates ($n = 3$). LYC: lycopene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

6.3.2.3. Nitric oxide production

Lycopene-anthocyanin combinations (1:3, 1:1 and 3:1 ratios, 10 μM in total) did not result in synergy in the suppression of NO production. Most of the mixtures showed an additive effect, except for LYC-MG and LYC-PNG which showed an antagonistic effect at the 1:1 and 3:1 combinatory ratios (**Figure 15**).

6.3.3. Interferences of anthocyanins on lycopene uptake by Caco-2 cells

The impact of anthocyanins on the absorption of lycopene in Caco-2 cells was studied at the two lycopene: anthocyanin ratios of 1:1 and 1:3 in which the anthocyanin concentration was equal or higher than that of lycopene respectively. All of the tested anthocyanins, except for PNG when it was mixed with LYC at the 1:3 ratio, significantly impaired the cellular uptake of LYC (**Figure 16**). At the combination of 1:1 ratio (5 μM: 5 μM), the uptake of LYC was decreased by 75-80% by DG, MG, PLG or PTG, and by 52-63% by CG or PNG. At the combination of 1:3 ratio (2.5 μM: 7.5 μM), all of the anthocyanins, except for PNG, decreased LYC uptake by 50-70%.

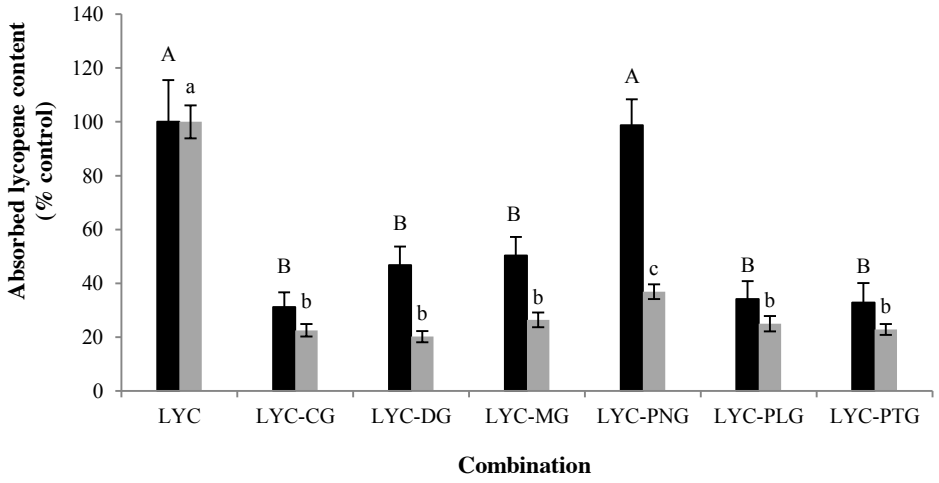


Figure 16. Lycopene uptake (% control) by Caco-2 cells in the absence (control) and presence of different anthocyanins at lycopene: anthocyanin ratios of 1:3 (2.5:7.5 μM, represented in black bars) and 1:1 (5:5 μM, represented in grey bars). Columns of the same colour marked with different letters indicate a significant difference from each other ($p < 0.05$). Values are mean ± SD of three independent replicates ($n = 3$). LYC: lycopene, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

6.4. Discussion

The interaction between lycopene and anthocyanins was studied in both chemical and cellular systems. Some of the combinations resulted in bioactivities other than the expected additive effect. In the liposome model, lycopene and anthocyanins interacted antagonistically resulting in the reduced effect on the inhibition of lipid peroxidation. The antagonistic effect on liposome peroxidation inhibition has been previously reported on other combinations of carotenoids and flavonoids, for example: β-carotene and green tea polyphenols (Song et al., 2011), β-carotene and daidzein (Liang et al., 2010), or β-carotene and anthocyanins (Phan et al., 2018b). The formation of carotenoid-flavonoid adducts was reported to result in the antagonism between these compounds (Liang et al., 2010, Song et al., 2011). Lycopene is located deeply in the centre of the hydrophobic region of the lipid membrane and parallel to the membrane surface (Han et al., 2012). Anthocyanins locate themselves in the hydrophilic region of the membrane outer monolayer and do not orientate deeply into the lipophilic compartment (Bonarska-Kujawa et al., 2012). When two compounds locate themselves in the distant regions of the lipid membrane, it is less likely for them to react to each other to form adducts. Additional experiments may be required to confirm whether or not the lycopene-anthocyanin adducts exist.

The orientation of the hydrophilic phytochemicals in the lipid bilayer may affect the interaction with the hydrophobic compounds. For example, tea polyphenols were seen to penetrate shallowly into the lipid membrane, which resulted in the antagonistic interaction with β-carotene (Song et al., 2011). The observed antagonistic effect upon the inhibition of lipid oxidation between lycopene and anthocyanins could be because of the improper orientation of the two compounds: anthocyanin mainly attaches to the outer hydrophilic layer of the membrane while lycopene is in the hydrophobic core.

The interactive effects between lycopene and anthocyanins upon the inhibitory activity against lipoxygenase were evaluated, because lipoxygenase inhibitors are potential agents for the control of inflammation and allergy as well as cardiovascular-related diseases and cancers (Nguyen et al., 2013). Lycopene was a very potent LOX-1 inhibitor (IC_{50} = 0.33 μM), whereas all of the tested anthocyanins did not exhibit potent inhibition of LOX-1 and demonstrated an interference with the LOX-1 inhibitory activity of lycopene. In the presence of the anthocyanins, the required concentration of lycopene for the mixtures to inhibit 50% of the LOX-1 activity was increased by 5-5.7 times from the IC_{50} of lycopene when it was applied alone. Anthocyanins and carotenoids non-competitively inhibit lipoxygenase (Knaup et al., 2009, Serpen and Gökmen, 2006) by binding to the lipoxygenase-substrate complex. The reduced LOX-1 inhibitory activity of lycopene might be because of the interference of anthocyanin compounds with the binding of lycopene to the enzyme-substrate complex.

The interactions between lycopene and anthocyanins on biological activities were also studied in a cellular model using human colorectal Caco-2 cells. Synergy of cellular antioxidant activity was not seen in any of the lycopene-anthocyanin combinations, as with the liposome model explained earlier. All of the tested mixtures of lycopene and anthocyanins, except for LYC-PNG, showed a CAA antagonism at the 1:3 ratio (**Table 1**). All of the combinations also showed antagonism at a 1:1 ratio of anthocyanins to lycopene, but showed an additive effect at a 3:1 ratio of lycopene to anthocyanins. The anthocyanins when combined with β-carotene or lutein also did not show any synergy in cellular antioxidant activity, as reported in chapters 4 and 5 (Phan et al., 2018b).

The interactive effects upon the cellular anti-inflammatory activities of the lycopene-anthocyanin mixtures were assessed by the measurement of the pro-inflammatory cytokine IL-8 and nitric oxide. Synergy on the suppression of the IL-8 secretion was

seen in the mixtures of lycopene with the methoxylated anthocyanins: MG, PNG and PTG. The mixtures of lycopene with the non-methoxylated anthocyanins: CG, DG and PLG produced additive effects on the IL-8 reduction. PNG was the only tested anthocyanin when combined with lycopene that produced a synergistic effect on the reduction of the IL-8 secretion at all tested ratios. Nevertheless, none of the mixtures of lycopene and the anthocyanins produced synergy on the reduction of the NO production. MG, PNG and PTG contain methoxyl groups on the B-ring which make these anthocyanins less polar than CG, DG and PLG which contain hydroxyl groups. It should be noted that carotenoids remain stable in the cell culture condition (pH 7-7.4, 37 ^{o}C) up to 24 hrs (O'sullivan et al., 2004), but anthocyanins are unstable, which can be chemically broken down into phenolic acids and aldehydes (Kay et al., 2009, Woodward et al., 2009). More than 50% of C3G is lost after 4 hr of incubation in cell cultured DMEM medium and is spontaneously degraded into protocatechuic acid and phloroglucinaldehyde, which are subsequently metabolised to glucuronide and sulphate conjugates (Kay et al., 2009). Methoxylation on the B-ring of flavonoids improves the compound stability during cell culture (Xiao and Högger, 2015). Woodward et al. (2009) also demonstrated that the degradation of anthocyanins is mediated by the hydroxylation on the B-ring of the compound. Therefore, it seems that the methoxylated anthocyanins are more stable than the non-methoxylated compounds during cell culture. This higher stability of the methoxylated anthocyanins might contribute to the synergistic effect on the IL-8 reduction seen in the present study although further experiments are required to reveal the underlying mechanisms.

Due to the spontaneous degradation in cell culture medium, the absorbed portions of anthocyanins may comprise of the parent anthocyanins and/or the conjugated metabolites of their degradation products. The combined biological activities of

anthocyanins and lycopene observed in this study may therefore be a consequence of complex interactions between the absorbed lycopene, the parent anthocyanins and/or the metabolites. Phytochemical synergy on cellular bioactivities can be the result of the multi-target effects of the phytochemical components on different biomarkers (Phan et al., 2018c, Wang and Zhu, 2017). For instance, lycopene (1 μM) combined with carnosic acid (1-2 μM) produced synergistic anti-inflammatory effects on the inhibition of the productions of TNF-α, superoxide, PGE_2 and NO (Hadad and Levy, 2012). The underlying molecular mechanisms of the cellular bioactivities of a single phytochemical can be studied, but it is not easy to understand thoroughly the molecular mechanisms of the interactive effects between phytochemicals. A limiting factor is the unavailability of a methodology to predict the expected gene expression of a molecular pathway resulting from the combined effect of phytochemicals. In some previous reports, the effect of a phytochemical mixture on the expression of an inflammatory molecular pathway is often compared with that of each phytochemical component in the mixture (Hadad and Levy, 2012, Funaro et al., 2016). The determination of the interactive mode between phytochemicals based on such comparisons, however, is not meaningful, because the combined effect of two or more compounds can be apparently higher than that of a single compound, which cannot be claimed as synergy (Chou, 2006).

An advancement of cell-based models over chemical models is that the assessment of the bioactivity interaction between phytochemicals considers the cellular uptake of the compounds. The observed combined effects on biological activities of a phytochemical mixture in a cellular system, in fact, is a consequence of the compound portions absorbed by the cells. The interactive effects of mixed phytochemicals, therefore, can be affected by the uptake interference between the compounds. The bioactivity interaction and the uptake interference between bioactive compounds are often studied separately,

which makes their interplay poorly understood. In our study, both of these two aspects of phytochemical interactions were assessed in each of the lycopene-anthocyanin mixtures tested. Hydrophilic bioactives may affect the cellular uptake of hydrophobic phytochemicals (Phan et al., 2018c). The reverse effect has not been reported in literature. Factors affecting anthocyanin absorption *in vitro* and *in vivo* have been reviewed (Kamiloglu et al., 2015, Yang et al., 2011), and carotenoids are not listed as one of the factors. In addition, the degradation of anthocyanins during cell culture is solely due to the spontaneous chemical breakdown in neutral pH medium (Kay et al., 2009). The presence of lycopene at the tested concentrations would not modify the pH of the cell culture, so it is unlikely to have any effect on the pH-induced degradation of anthocyanins. Thus, in this study, we focused to investigate the impact of anthocyanins on the uptake of lycopene by Caco-2 cells and the relevance of the uptake interference to the combined bioactivities. Lycopene uptake was measured in the presence of every tested anthocyanin at the ratio of lycopene: anthocyanin of 1:1 and 1:3, and that was compared with the absorption of lycopene alone (control) at the respective concentrations. The results showed that lycopene uptake by Caco-2 cells was decreased by 50-80% when anthocyanins were present at the tested concentrations (**Figure 16**). The uptake of lycopene was seen unaffected when it was combined with PNG at the 1:3 ratio.

Anthocyanins locate mainly in the hydrophilic compartment of the lipid bilayer membrane and interact with the polar heads of the lipid molecules (Bonarska-Kujawa et al., 2012). The incorporation of anthocyanins into the polar interface of the membrane decreases the packing order of the membrane hydrophilic region (Bonarska-Kujawa et al., 2012), which means that their incorporation increases the polarization area. The increase in the polarization area may cause a mismatch between the area occupied by

the polar heads and the area of the hydrophobic moieties, which may result in an increase in the internal space between the lipid layers providing additional freedom for the hydrocarbon chains (Tarahovsky et al., 2008). This phenomenon is called membrane fluidization which leads to a decrease in lipid melting temperature and an increase of the lateral diffusion of lipid molecules. The membrane fluidization, however, may induce raft-breaking effects that influence the appearance and development of lipid rafts which are composed of tightly packed lipid domains containing cholesterol, saturated sphingolipids and peculiar proteins (Tarahovsky et al., 2008). The steric mismatch of the polar and hydrophobic areas may also lead to the formation of interdigitated bilayer structures which increase the integrity of the lipid bilayer structure and prevent the diffusion of lipid compounds (Tarahovsky et al., 2008).

Flavonoids that preferably locate in the hydrophilic region of the lipid membrane may favour the development of a fluidizing membrane or an interdigitating membrane. As the consequences of the interaction between polar flavonoids and the lipid bilayer membrane, some lipophilic molecules may increase or decrease their diffusion through the cell membrane. For example, β-carotene uptake was improved when hesperetin or anthocyanins were present (Phan et al., 2018b, Claudie et al., 2013). The diffusion of the lipophilic compounds may be decreased when the interaction of the flavonoids with the membrane causes the disruption of the lipid raft domains. For example, the cellular uptake of lutein was reported to be impaired by naringenin, which was suggested to be the consequence of the naringenin-lipid membrane interaction that affected the invagination of the lipid draft domains containing lutein receptors (Reboul et al., 2007b). Cyanidin-3-*O*-β-glucoside is able to disrupt the formation of lipid rafts in the cell membrane of murine alveolar macrophages from mice by depleting cholesterol from the lipid rafts (Fu et al., 2014). Our research group found that the effect of

anthocyanins on the cellular uptake of different carotenoids in Caco-2 cells is different. The tested anthocyanins were seen to improve β-carotene uptake (present in chapter 4) (Phan et al., 2018b), but were seen to decrease the cellular uptake of lycopene, and did not affect the uptake of lutein (present in chapter 5) (Phan et al., 2018a), which suggests that the interaction of anthocyanins with the cell lipid membrane may induce different effects on the lipid raft domains containing the receptor of each of the carotenoids. The antagonistic effect on the cellular antioxidant activity (CAA) seen between lycopene and the anthocyanins might be a consequence of the decreased uptake of lycopene induced by the anthocyanins. At the lycopene: anthocyanin ratio of 1:3, all tested lycopene-anthocyanin mixtures, except for LYC-PNG, showed antagonism in CAA. On the other hand, the cellular uptake of lycopene at this ratio was significantly decreased when the anthocyanins were present, except for PNG. The mixture of LYC-PNG (1:3) produced additive CAA. Similarly, at the 1:1 combinatory ratio, lycopene uptake was impaired by all of the anthocyanins, and their combined effect on CAA was antagonistic. These results show that the reduction in lycopene uptake caused by the presence of the anthocyanins might be partly responsible for the reduced CAA in the lycopene-anthocyanin mixtures.

The reduced uptake of lycopene, however, did not affect the anti-inflammatory activity of the mixtures. The IL-8 secretion into the growth medium was reduced at increasing initial loaded concentrations of lycopene (2.5-7.5 μM) when lycopene was applied alone or in combination with the anthocyanins (**Figure 15**). It might be expected that the decreased lycopene uptake would result in lower anti-inflammatory effect, but none of the lycopene-anthocyanin mixtures showed antagonism, and some of them even produced synergistic inhibitory activity against the production of IL-8. The percentage uptake of lycopene in all samples at the 1:1 ratio (containing 5 μM lycopene) was lower

than that at the 1:3 ratio (containing 2.5 μM lycopene). Because the previous measurements of the lycopene cellular uptake at different ratios as part of this study were conducted on different days, it may not be very meaningful to compare the cellular uptake of lycopene between samples of these two ratios. Therefore, the cellular uptake of lycopene was tested again at the initial loaded concentrations of 2.5 and 5 μM in the absence of anthocyanins and in the presence of MG, PNG, or PTG that together with lycopene produced synergistic effect on the suppression of IL-8. All of the tested samples again showed that the uptake amount of lycopene by the Caco-2 cells when initially incubated with 5 μM was significantly lower than that with 2.5 μM of lycopene (**Table 9**).

Table 9. % cellular uptake[1] of lycopene at different initial lycopene concentrations.

	Initial lycopene concentration loaded into the cell culture medium	
Compound/Mixture	**2.5 μM[a]**	**5 μM[b]**
LYC (single)	0.43 ± 0.039	0.13 ± 0.008
LYC-MG	0.33 ± 0.023	0.03 ± 0.005
LYC-PNG	0.47 ± 0.056	0.05 ± 0.005
LYC-PTG	0.16 ± 0.038	0.03 ± 0.003

[a, b] Different letter indicates a significant difference ($p < 0.05$) on % uptake of lycopene seen in every sample at the two initial lycopene concentrations.
[1] % cellular uptake = absorbed content of lycopene (ng)/initial content of lycopene loaded (ng)
Values are mean ± SD of three replicates ($n = 3$).
LYC: lycopene, , MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

Similar findings have been reported previously in the literature. For example, the absorption of pure lycopene by Caco-2 cells at the concentration of 1.4 μg/mL was significantly lower than that at 0.4 μg/mL (Déat et al., 2009). The absorption percentage of lycopene in humans after a dose of 10 mg lycopene in a tomato beverage was significantly greater than at the higher doses (30-120 mg) (Diwadkar-Navsariwala et al., 2003). These findings and our finding show that the higher the initial dose of lycopene, the lower the lycopene absorption percentage. Although the anti-inflammatory activity

of lycopene and its mixtures with the anthocyanins on the inhibition of IL-8 secretion seemed to follow a lycopene dose-dependent trend, it was in fact inversely related to the intracellular contents of lycopene. This could explain why the reduced uptake of lycopene, due to the presence of the anthocyanins, did not reduce but increased the anti-inflammatory effect of some lycopene-anthocyanin mixtures. Further experiments should be carried out to investigate how lycopene at lower intracellular contents could result in higher anti-inflammatory effects. Our research shows that the study of biological activities of phytochemicals and/or their interactive effects should be conducted together with determination of phytochemical absorption in order to have a better understanding about the relationship between the bioactivities of the compounds and their corresponding absorbed concentrations.

6.5. Conclusion

The combination of 2.5 μM of lycopene with 7.5 μM of the anthocyanins (except for PNG), or 5 μM of lycopene with 5 μM of the anthocyanins resulted in the reduction of lycopene absorption in Caco-2 cells. The decreased cellular uptake of lycopene seemed to affect the cellular antioxidant activity of the mixtures because antagonism was seen in the lycopene-anthocyanin mixtures that showed reduced lycopene uptake. The impairment of lycopene uptake did not result in antagonism on the inhibition of the cytokine IL-8 production of the mixtures. The higher the initial dose of lycopene (single or in mixtures with the anthocyanins) loaded onto the cells, the lower the intracellular lycopene content, but the stronger the IL-8 inhibitory activity. This requires further investigation to understand the underlying molecular mechanisms.

INTRODUCTION TO CHAPTERS 7-8

The interactive effects between some anthocyanins and carotenoids combined in pairs on antioxidant and anti-inflammatory activities and on carotenoid uptake were reported in Chapters 4-6. The results presented in those chapters provide an understanding of the combined biological effects that the two compound molecules in a carotenoid-anthocyanin mixture, without the interference of the food matrix, might produce. This reveals the potential molecular interaction between these compounds when none of other food components is present.

Phytochemicals including anthocyanins and carotenoids are in fact present with other nutrients/chemical compounds in fruits and vegetables. They are released from their food matrix during digestion for absorption by the intestinal cells to enter the blood stream. The second major objective of this thesis was to study the interactive effects on bioaccessibility and bioactivities of anthocyanins and carotenoids from different vegetables that were co-digested. The specific aims were to see how the bioaccessibility of anthocyanins and carotenoids from different vegetables could change as a result of vegetable co-digestion, and the consequences of the interaction between phytochemicals from the co-digested vegetables on cellular bioactivities.

- Chapter 7 presents the changes in the digestive bioaccessibility of anthocyanins from red cabbage and carotenoids from carrot, cherry tomato and/or baby spinach when individual vegetables and different vegetable mixtures were digested.
- Chapter 8 presents the consequences of phytochemical interaction on cellular antioxidant and anti-inflammatory effects resulting from the co-digestion of vegetables, as well as the changes in the intestinal cellular uptake and intestinal cellular bioaccessibility of carotenoids from the mixed vegetables.

CHAPTER 7

Digestive bioaccessibility of anthocyanins and carotenoids after simulated *in vitro* gastro-intestinal digestion of mixed vegetables

(This is the original manuscript submitted to Food Chemistry on 10/01/2019, which includes additional data that were not presented in the submitted manuscript)

Abstract

The effects of co-digestion of anthocyanin-rich red cabbage with different carotenoid-rich vegetables including carrot, baby spinach and cherry tomato on the digestive bioaccessibility of anthocyanins and carotenoids were examined using a simulated *in vitro* gastro-intestinal digestion model. The individual vegetables and their mixtures were digested in a fresh raw form (without added the oil-based dressing) and in a salad form in which a standardised salad dressing was added. Digestive bioaccessibility of total anthocyanins was enhanced by 10-15% ($p < 0.05$) when red cabbage was co-digested with the carotenoid-rich vegetables, except with carrot. In contrast, the co-digestion of red cabbage with carrot decreased the digestive bioaccessibility of total carotenoids by 21-33% ($p < 0.05$) and with cherry tomato by 42-56% ($p < 0.05$). The digestive bioaccessibility of a given carotenoid was varied depending on the vegetable matrix. Lutein was the most bioaccessible of all carotenoids studied from the tested vegetable matrices, and its digestive bioaccessibility was not altered in the presence of red cabbage during the digestion. Among the tested vegetable mixtures, red cabbage and baby spinach when co-digested with and withthout added the oil-based dressing demonstrated that both anthocyanins and carotenoids were equally bioaccessible (total anthocyanin bioaccessibility of 62-66% and total carotenoid bioaccessibility of 66%).

7.1. Introduction

Anthocyanins and carotenoids are the two major groups of phytochemical pigments present in several purple/red and/or yellow/orange coloured fruits and vegetables respectively (He and Giusti, 2010, Saini et al., 2015). Anthocyanins locate mainly in the vacuoles of plant cells and are majorly released from the plant matrix during gastric digestion (Bohn, 2014). Carotenoids are lipophilic compounds which, after being released from the food matrix, are incorporated into micelles in order to be absorbed by intestinal cells (van het Hof et al., 2000).

A number of factors can influence the bioaccessibility of phytochemicals from plant-based sources including: the chemical nature of the phytochemicals; food material microstructure which affects the release of phytochemicals from food matrix; and interactions with other food components (Parada and Aguilera, 2007). For instance, cellular structures (e.g. cell wall or chromoplast substructures) are natural barriers of carotenoid liberation from food matrix (Lemmens et al., 2014); the physical state of food (liquid or solid) affects the bioaccessibility of polyphenolic phytochemicals (Bohn, 2014). There are some recent studies reporting the impact of food matrix during digestion on the bioaccessibility of phytochemicals. For instance, the digestion of a blended fruit juice mixed with soymilk led to a decreased bioaccessibility of several carotenoid compounds as compared to each food component digested alone (Rodríguez-Roque et al., 2014). The bioaccessibility of α-tocopherol and ascorbic acid was highest when a blended fruit juice was mixed with whole milk but lowest when the fruit juice was blended with soymilk (Cilla et al., 2012). The actual bioaccessibility of lutein, α-carotene and β-carotene from a mixture of carrot, baby spinach and grape tomato was lower than the expected bioaccessibility of these compounds from the individual vegetables estimated with the assumption of no food matrix interference during

digestion (Rodrigues et al., 2017). These studies suggest that there are interactions among constituents of different food sources during digestion leading to changes in carotenoid bioaccessibility (Rodrigues et al., 2017).

Food macromolecules such as sugar, starch and cellulose in diets influence the bioaccessibility of anthocyanins from pomegranate (Sengul et al., 2014), whereas fats and oils (Nagao et al., 2013, Huo et al., 2007), and dietary fibre (Palafox-Carlos et al., 2011) can influence the bioaccessibility of carotenoids. In most past studies, the bioaccessibility of a specific group of phytochemicals is often assessed from singly-administered fruit or vegetable although in fact different fruits and/or vegetables are usually co-ingested in a meal. Changes in the bioaccessibility of different distinct groups of phytochemicals (e.g. hydrophilic anthocyanins and hydrophobic carotenoids) as a result of co-ingestion of fruits and/or vegetables are still poorly understood. This study, therefore, reports on how the bioaccessibility of anthocyanins and carotenoids could be altered after the co-ingestion of different vegetables with and without added the oil-based dressing using an *in vitro* simulated gastro-intestinal digestion model. Red cabbage, carrot, cherry tomato and baby spinach were singly ingested and co-ingested in pairs or altogether. These vegetables were selected because they are often used in salads in human diets. Among anthocyanin-containing vegetables that can often go in salads with spinach, carrot and tomato, red cabbage contains a substantial amount of anthocyanins with the total anthocyanin content of 322 mg/100 g fresh weight, whereas other vegetables such as red leaf lettuce and red onion contain much lesser anthocyanin content: 2.2 and 48.5 mg/100 g fresh weight respectively (Wu et al., 2006). The majority of anthocyanins in these purple-pigmented vegetables are cyanidin glycosides (Li et al., 2012, Wu et al., 2006). Most of vegetables containing anthocyanins have their anthocyanin profile containing mostly the glycosides of one of the following

anthocyanidins including cyanidin, delphinidin, petunidin and pelargonidin, among which cyanidin glycosides are the most common (Li et al., 2012). Because of its significantly high content of anthocyanins, red cabbage was selected as the source of anthocyanins for the vegetable co-digestion experiments. Anthocyanins and major dietary carotenoids including α-carotene, β-carotene, lycopene and lutein that were contained in the digesta as a result of co-digestion of the vegetables chosen were measured for comparison of bioaccessibility.

7.2. Materials and methods

As described in Chapter 3, section 3.1.1-3.1.4 on pages 75-76, and 3.3.1-3.3.8 on pages 85-94.

7.3. Results and discussion

7.3.1. Identification of anthocyanins and carotenoids

Seven anthocyanin compounds were detected in the Sep-Pak C18-purified extract of red cabbage using UPLC-ESI/MS/MS. The anthocyanin profile of red cabbage before and after digestion analysed by HPLC-PDA at 520 nm is shown in **Figure 17,** and by LC-ESI/MS/MS using selected reaction monitoring (SRM) analysis is shown in **Figure 18**. The MS data of the anthocyanins are presented in **Table 10**. There were three major anthocyanin compounds in the red cabbage extract used in this study, including cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside (peak 5, $[M]^+$ m/z = 919), cyanidin-3-(feruloyl)-diglucoside-5-glucoside (peak 6, $[M]^+$ m/z = 949) and cyanidin-3-(sinapoyl)-diglucoside-5-glucoside (peak 7, $[M]^+$ m/z = 979). All of the anthocyanin compounds detected in our red cabbage extract are monoacylated cyanidin derivatives structured with a core unit of cyanidin-3-diglucoside-5-glucoside bound with different hydroxycinnamic acids: *p*-coumaric, ferulic, sinapic or caffeic acids, which are similar

to what has been reported in previous studies (McDougall et al., 2007, Wiczkowski et al., 2014).

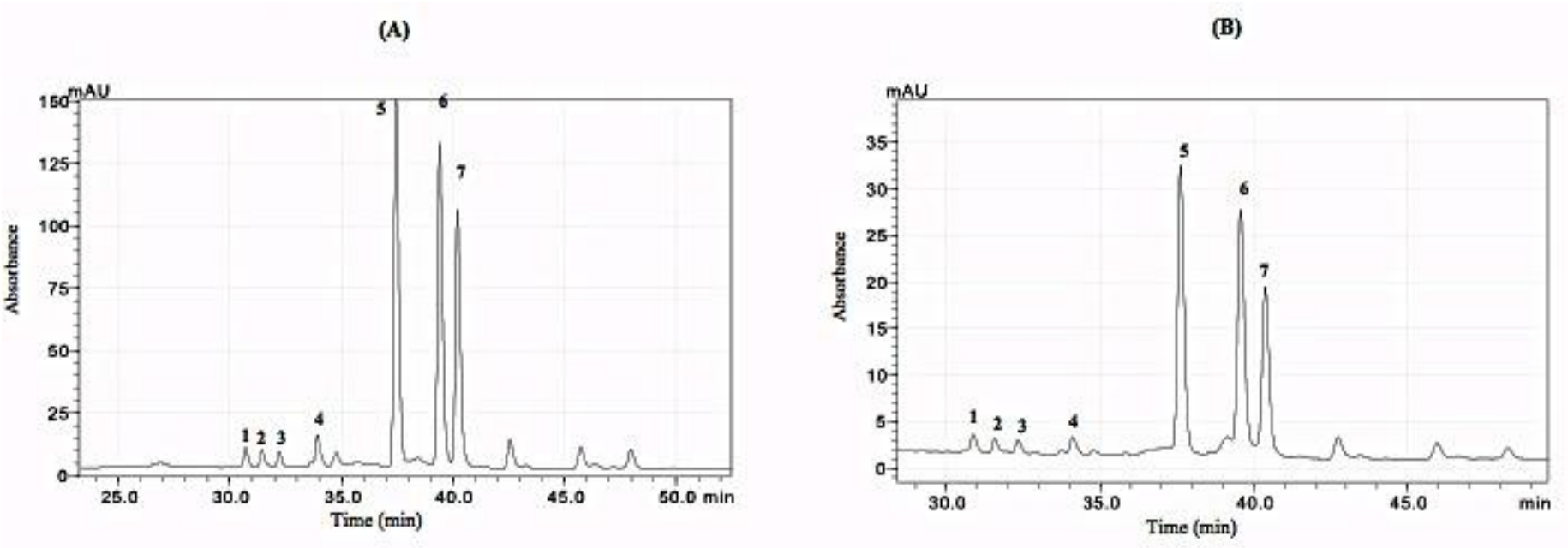


Figure 17. HPLC-PDA chromatogram of anthocyanins in red cabbage at 520 nm (A) before and (B) after digestion. Identity of anthocyanin compounds corresponding to a number is provided in Table 10. Only peaks whose MS/MS spectrum was identified on full scan and SRM scan modes (Figure 2) following the method described in section 3.3.5.1 on pages 90-91 are labelled.

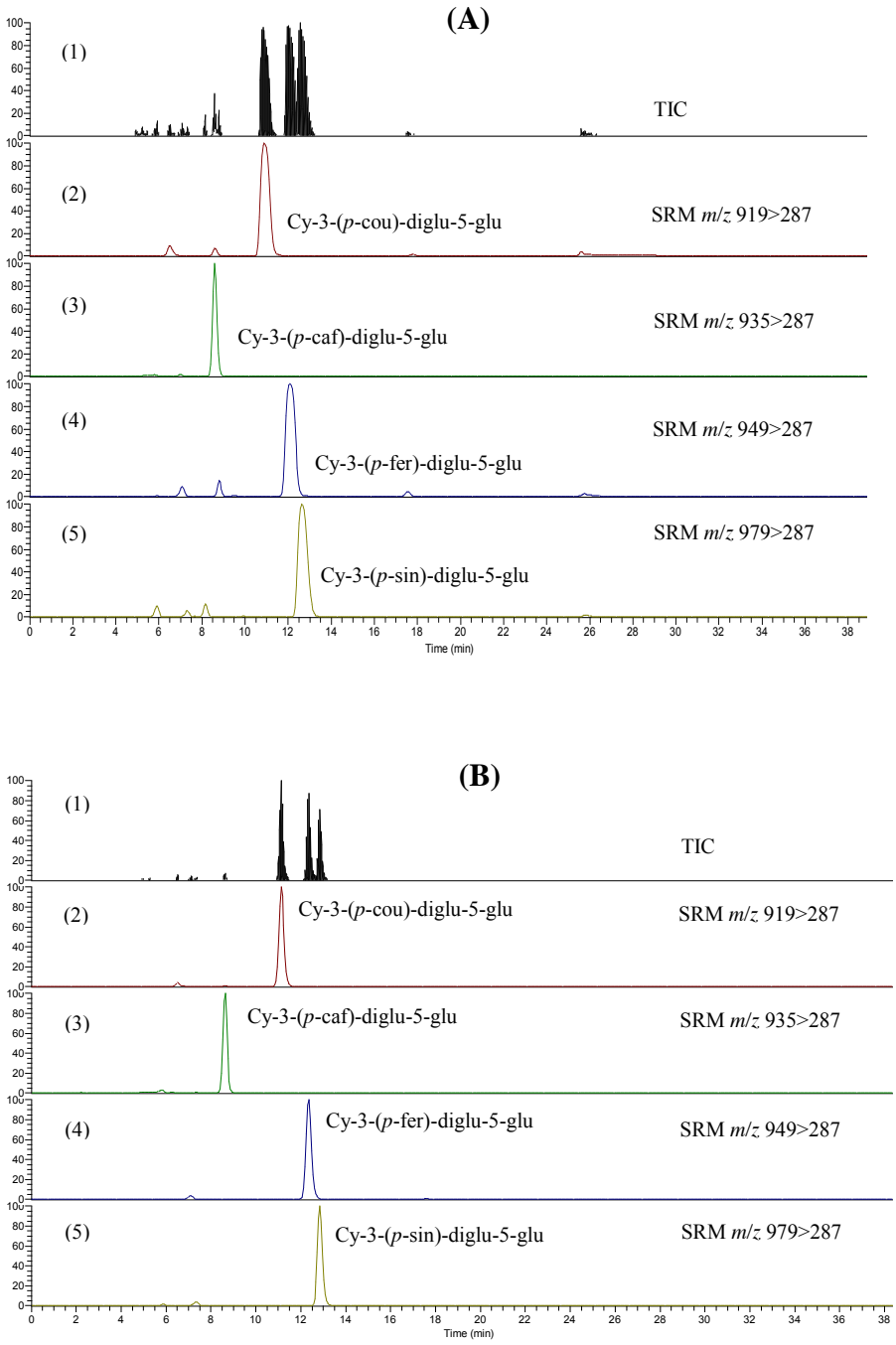


Figure 18. LC-ESI/MS/MS of anthocyanins in red cabbage (A) before and (B) after *in vitro* digestion using selected reaction monitoring (SRM) analysis. (1) Total ion current (TIC) chromatogram; SRM of (2) *m/z* 919>287 for cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside; (3) *m/z* 935>287 for cyanidin-3-(caffeoyl)-diglucoside-5-glucoside; (4) *m/z* 949>287 for cyanidin-3-(feruloyl)-diglucoside-5-5glucoside; and (5) *m/z* 979>287 for cyanidin-3-(sinapoyl)-diglucoside-5-glucoside. The retention time of the peaks differs from that shown in the HPLC-PDA chromatogram because of the use of different flow rates on these systems to ensure backpressure stay within limits of each LC system.

Table 10. LC-ESI/MS/MS data for red cabbage anthocyanins.

Peak	Retention time on HPLC-PDA (min)	$[M]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	Putative identification*
1	30.71	919	757 449 287	Cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside
2	31.43	949	787 449 287	Cyanidin-3-(feruloyl)-diglucoside-5-glucoside
3	32.54	979	817 449 287	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside
4	33.92	935	773 449 287	Cyanidin-3-(caffeoyl)-diglucoside-5-glucoside
5	37.45	919	757 449 287	Cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside
6	39.41	949	787 449 287	Cyanidin-3-(feruloyl)-diglucoside-5-5glucoside
7	40.20	979	817 449 287	Cyanidin-3-(sinapoyl)-diglucoside-5-glucoside

* Referenced from McDougall et al. (2007) and Wiczkowski et al. (2014)

The chromatograms of carotenoids from carrot, cherry tomato and baby spinach before and after digestion analysed using HPLC-PDA at 450 nm and LC-APCI/MS are shown in **Figure 19** and **Figure 20** respectively. The identification of major carotenoids including lutein, lycopene, α-carotene and β-carotene was based on the relative retention time to the standards, UV-VIS spectrum and the accurate mass (*m*/*z*), which are shown in **Table 11**. The chromatograms show that there was a tiny little peak eluted closely after lutein which was suspected to be the isomer zeaxanthin (**Figure 19**). These two peaks however were successfully separated by the developed HPLC method. Thus, the quantitation of lutein reported in subsequent sections was solely based on the lutein peak in the chromatograms. The extracts of fresh carrot and the micellar fraction of the digested carrot contained mainly β-carotene, α-carotene and lutein. Lutein and β-carotene were also the major carotenoids detected in the micellar fraction of the

digested baby spinach, whereas lycopene, β-carotene and lutein were the main carotenoids found in the micellar fraction of the digested cherry tomato.

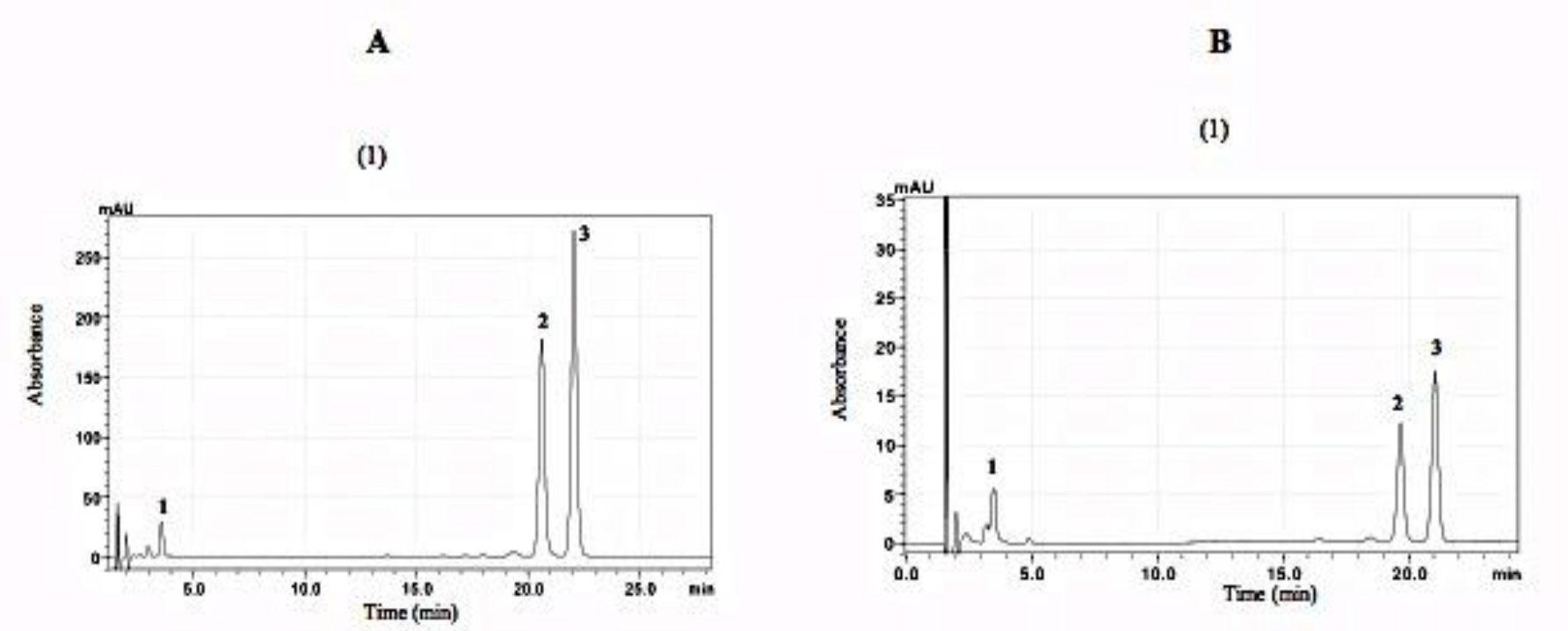


Figure 19. HPLC-PDA chromatogram of carotenoids in (1) carrot, (2) baby spinach, and (3) cherry tomato at 450 nm (A) before and (B) after digestion. Only peaks of the major dietary carotenoids selected in the study are labelled. (1) Carrot: peak 1: lutein, peak 2: α-carotene, peak 3: β-carotene; (2) Baby spinach: peak 1: lutein, peak 2: β-carotene; (3) Cherry tomato: peak 1: lutein, peak 2: lycopene, peak 3: β-carotene. Peaks marked with an asterisk on the chromatogram of the fresh baby spinach (A2) represent chlorophyll compounds (identified according to their UV-VIS spectrum) which disappeared after digestion.

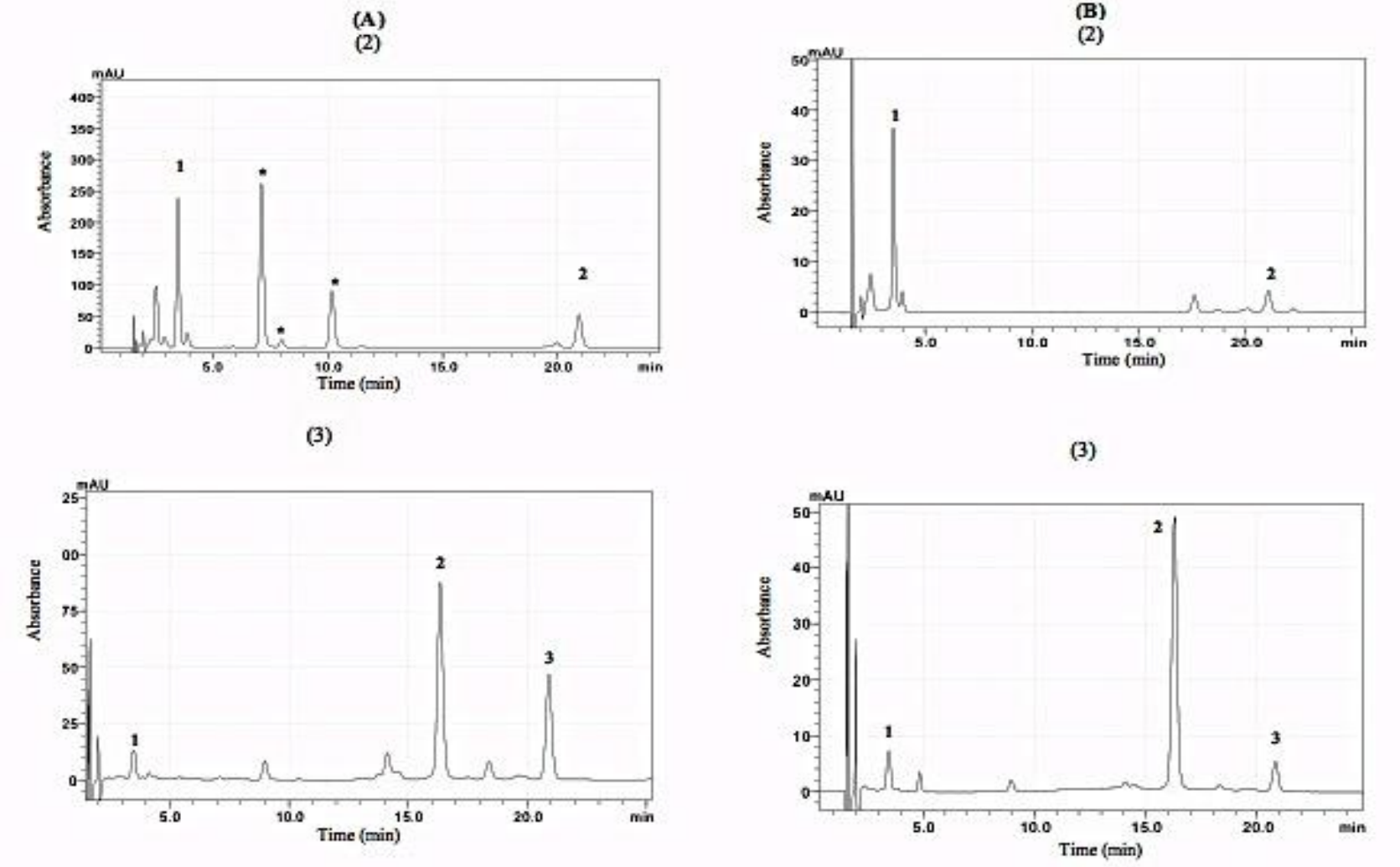


Figure 19. *(continued)*.

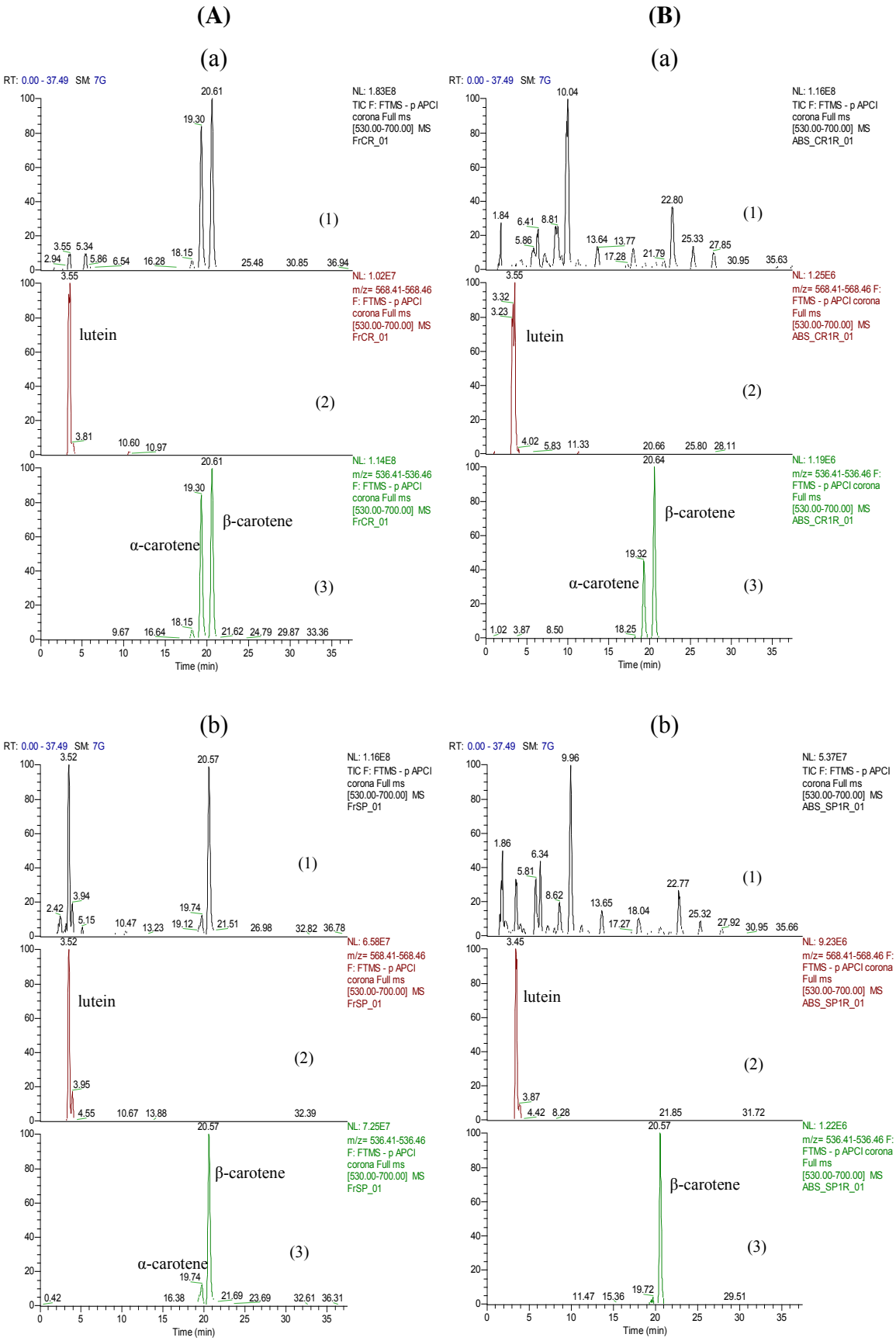


Figure 20. LC-APCI/MS of carotenoids from (a) carrot; (b) baby spinach; (c) cherry tomato (A) before and (B) after simulated *in vitro* digestion. (1) Total ion current (TIC) chromatogram; APCI/MS of (2) *m/z* 568.44 for lutein; (3) *m/z* 536.44 for lycopene, α-carotene and β-carotene.

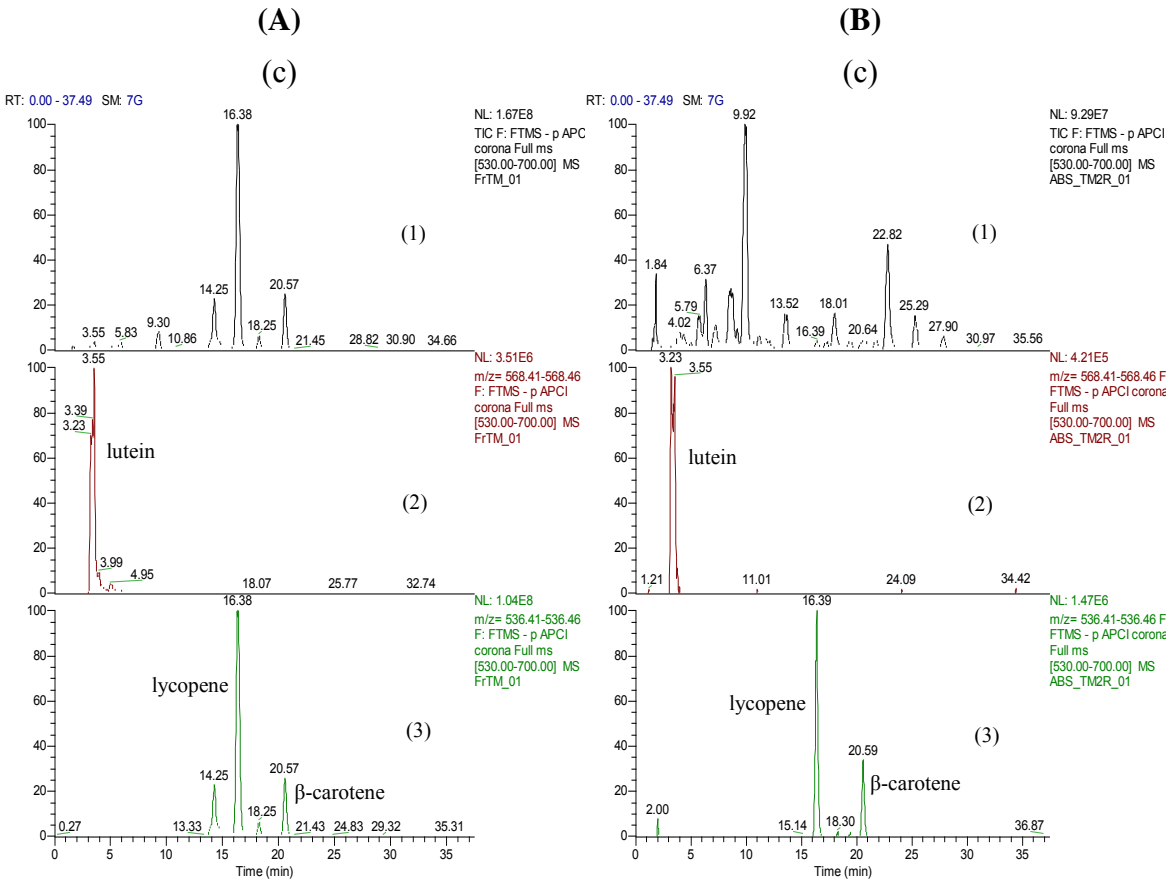


Figure 20 (*continued*).

Table 11. Retention time, UV-VIS λ_{max} and MS *m/z* data of carotenoids from carrot, cherry tomato and baby spinach.

Vegetable	Peak	Retention time (min)	*m/z*	λ_{max}	Identification
Carrot	1	3.48	568.34	445	Lutein
	2	19.41	536.44	446	α-carotene
	3	20.78	536.44	453	β-carotene
Baby spinach	1	3.50	568.34	445	Lutein
	2	20.79	536.44	453	β-carotene
Cherry tomato	1	3.50	568.34	445	Lutein
	2	16.32	536.44	472	Lycopene
	3	20.83	536.44	453	β-carotene

7.3.2. Digestive bioaccessibility (dBAC) of anthocyanins and polyphenols from singly-digested red cabbage

The definition and calculation of the digestive bioaccessibility are presented in chapter 3, section 3.3.8 on page 94. The percentage digestive bioaccessibility of polyphenols from all vegetable samples (with and without added the oil-based dressing) was higher than 45% (**Table 12**). The content of the three major anthocyanin compounds in fresh

red cabbage and the supernatant of the digesta quantified by HPLC-PDA and the total anthocyanin content (TAC) analysed by HPLC-PDA and by spectrophotometry are presented (**Table 13**). We used HPLC-PDA to quantify the three major anthocyanins in red cabbage and then calculated the TAC based on the total amount of these major anthocyanins. We also used a spectrophotometric assay to determine TAC, which took into account the content of both major and minor anthocyanins in the extract. Data are reported in fresh weight of vegetables whose moisture contents are given in the legend of **Table 12**. The % digestive bioaccessibility of total anthocyanins after the simulated gastro-intestinal digestion is calculated and presented in **Table 13**. The results showed that more than 50% of the anthocyanins from fresh red cabbage were accessible after the simulated digestion of vegetables with or without added the oil-based dressing.

Table 12. Mean total phenolic content (TPC) in undigested fresh vegetables, bioaccessible TPC, and % dBAC of TPC after the simulated *in vitro* digestion.

Sample		**TPC (mg gallic acid equivalent/100 g FW)**	**Digestive bioaccessibility (%)**
Fresh red cabbage		123.1 ± 11.3	-
Fresh carrot		9.5 ± 1.0	-
Fresh spinach		64.8 ± 5.4	-
Fresh tomato		38.3 ± 5.7	-
Digestion without added salad dressing	R	63.0 ± 3.2	51.2 ± 2.6
	C	5.4 ± 1.6	56.5 ± 16.6
	S	32.8 ± 1.8	50.6 ± 2.8
	T	19.3 ± 5.9	50.5 ± 15.4
	R-C	42.2 ± 4.5	63.6 ± 6.8
	R-S	44.8 ± 1.4	47.7 ± 1.2
	R-T	41.0 ± 6.8	50.8 ± 8.4
	R-C-T-S	35.9 ± 1.0	60.9 ± 1.7
Digestion with added salad dressing	R	68.8 ± 6.4	55.9 ± 5.2
	C	5.3 ± 0.5	55.8 ± 5.7
	S	37.1 ± 4.1	57.3 ± 6.3
	T	17.3 ± 5.6	45.1 ± 14.5
	R-C	42.2 ± 3.1	63.7 ± 4.7
	R-S	49.4 ± 0.6	52.6 ± 0.6
	R-T	40.4 ± 1.7	50.0 ± 2.2
	R-C-T-S	35.2 ± 1.2	59.7 ± 2.0

R: red cabbage (90.6 ± 0.1 % moisture); C: carrot (86.0 ± 0.2 % moisture); S: baby spinach (91.3 ± 0.1 % moisture); T: cherry tomato (91.2 ± 0.2 % moisture); R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach; FW: fresh weight; TPC: total phenolic content.
Results are expressed as mean ± standard deviation (n = 3).

Table 13. Mean anthocyanin content in fresh vegetables, digestive bioaccessible anthocyanin content, and % digestive bioaccessibility after the simulated *in vitro* digestion.

Sample		Major anthocyanin compounds (mg CG equivalent/100 g FW)			Total ACN[1] (mg CG equivalent/100 g FW)	Total ACN[2] (mg CG equivalent/100 g FW)	Digestive bioaccessibility of total ACN[3] (%)
		Cy-3-(*p*C)-diGlc-5-Glc	Cy-3-(fer)-diGlc-5-Glc	Cy-3-(sin)-diGlc-5-Glc			
Fresh vegetable	R	30.4 ± 2.1	28.0 ± 1.8	22.6 ± 1.1	80.9 ± 5.0	93.2 ± 8.9	-
	C	n.d	n.d	n.d	n.d	n.d	-
	S	n.d	n.d	n.d	n.d	n.d	-
	T	n.d	n.d	n.d	n.d	n.d	-
Digestion without added salad dressing	R	15.9 ± 1.3	15.0 ± 1.3	10.1 ± 0.8	41.1 ± 3.3	46.0 ± 6.8	50.8 ± 4.1^{a}
	R-C	16.2 ± 3.2	15.2 ± 2.7	11.3 ± 2.0	42.6 ± 4.9	52.8 ± 2.7	52.7 ± 6.1^{ab}
	R-S	20.0 ± 0.1	18.5 ± 0.4	11.5 ± 0.3	50.0 ± 0.8	62.6 ± 5.6	61.8 ± 0.9^{b}
	R-T	21.0 ± 0.5	19.3 ± 0.6	12.8 ± 1.0	53.1 ± 1.2	61.4 ± 6.0	65.7 ± 1.5^{b}
	R-C-T-S	21.1 ± 3.6	19.4 ± 3.2	11.5 ± 1.1	52.1 ± 7.7	64.7 ± 11.7	64.4 ± 9.6^{b}
Digestion with added salad dressing	R	16.3 ± 1.6	16.3 ± 2.0	11.0 ± 1.4	43.5 ± 4.9	42.0 ± 4.7	53.8 ± 6.1^{a}
	R-C	18.3 ± 1.9	16.8 ± 2.1	12.3 ± 1.6	47.4 ± 5.9	52.3 ± 11.9	58.6 ± 7.3^{ab}
	R-S	21.6 ± 2.4	19.9 ± 2.3	11.9 ± 1.9	53.5 ± 6.3	55.9 ± 11.2	66.1 ± 7.7^{b}
	R-T	23.6 ± 1.8	21.7 ± 1.4	11.8 ± 2.4	57.1 ± 2.9	52.2 ± 7.5	70.6 ± 3.6^{b}
	R-C-T-S	21.5 ± 2.7	19.7 ± 2.7	11.9 ± 1.9	53.1 ± 7.2	53.1 ± 13.7	65.7 ± 8.9^{ab}

[1] Equal to the sum of the content of the three major anthocyanin compounds analysed by HPLC-PDA using the standard curve of cyanidin-3-*O*-glucoside.
[2] Analysed by spectrophotometry (pH differential method).
[3] Calculated using the anthocyanin content analysed by HPLC-PDA.
R: red cabbage; C: carrot; T: cherry tomato; S: baby spinach; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach; Cy: cyanidin; *p*C: *p*-coumaroyl; fer: feruloyl; sin: sinapoyl; Glc glucoside; CG: cyanidin-3-glucoside; FW: fresh weight; ACN: anthocyanins.
Results are expressed as mean ± standard deviation ($n = 3$).
Values marked with different letters within the same sample treatment (i.e. with or without added salad dressing) indicate a significant difference ($p < 0.05$).

7.3.3. Changes in digestive bioaccessibility of anthocyanins and polyphenols as a consequence of the simulated co-digestion of red cabbage and carotenoid-rich vegetables

The % dBAC of total anthocyanins was significantly enhanced ($p < 0.05$) by 10-15% when red cabbage was co-digested with baby spinach, with cherry tomato, or with a mixture of carrot, baby spinach and cherry tomato. Similar trend in anthocyanin digestive bioaccessibility was also seen in the corresponding co-digested vegetables with added the salad dressing. The co-digestion of red cabbage with carrot only, however, did not affect the anthocyanin dBAC. In particular, changes in the anthocyanin dBAC as a result of the co-digestion were seen only in the two major red cabbage anthocyanin compounds: cyanidin-3-*O*-(*p*-coumaroyl)-diglucoside-5-*O*-glucoside and cyanidin-3-*O*-(feruloyl)-diglucoside-5-*O*-glucoside, which were more bioaccessible by 26-33% and 23-29% respectively when fresh red cabbage was co-digested with the fresh carotenoid-rich vegetables, except for carrot; and by 32-45% and 21-33% respectively in the corresponding co-digested vegetable samples with added the salad dressing. The co-digestion of red cabbage with the carotenoid-containing vegetables, however, did not significantly change the content of the third major red cabbage anthocyanins: cyanidin-3-*O*-(sinapoyl)-diglucoside-5-*O*-glucoside.

The increase in the anthocyanin digestive bioaccessibility could be a consequence of the increased stability of anthocyanins during the simulated co-digestion. The selective vegetables that were co-digested with red cabbage contain carotenoids and other antioxidants. Carotenoids are potent antioxidants whose structure contains extended conjugated double bonds which enable them to quench singlet oxygen and free radicals (Palozza et al., 2011). In addition, other antioxidant compounds in the vegetables such as ascorbic acid could also protect anthocyanins from degradation (Kaack and Austed,

1998, Özkan, 2002). It is, therefore, possible that the antioxidant activity of the released carotenoids and other antioxidants in the digesta contributed to reduce the susceptibility of anthocyanins to oxidative degradation during digestion resulting in the increase in the anthocyanin bioaccessibility. The actual digestive bioaccessibility of total polyphenols from a vegetable mixture was compared with the theoretical value which is the average of the digestive bioaccessibility of total polyphenols from the vegetable components of the mixture. A significant increase ($p < 0.05$) in the digestive bioaccessibility of total polyphenols by 12-18% was observed when red cabbage was co-digested with carrot or the mixture of carrot, baby spinach and cherry tomato with and without added the oil-based dressing.

7.3.4. Digestive bioaccessibility of carotenoids from singly-digested vegetables

The percentage digestive bioaccessibility of each major carotenoid including lutein, lycopene, α-carotene and β-carotene and the % dBAC of total carotenoids after the digestion of single vegetables with and without added the salad dressing are reported in **Table 14**.

Table 14. Mean carotenoid content in fresh vegetables, bioaccessible carotenoid content, and their % digestive bioaccessibility after the simulated *in vitro* digestion.

Sample		Major carotenoid compounds (mg/100 g FW)				Total carotenoids (mg/100 g FW)	% Digestive bioaccessibility of individual carotenoid				% Digestive bioaccessibility of total carotenoids
		Lutein	Lycopene	α-carotene	β-carotene		Lutein	Lycopene	α-carotene	β-carotene	
Fresh vegetable	R	n.d	n.d	n.d	n.d	-					
	C	1.2 ± 0.3	n.d	7.2 ± 0.4	11.0 ± 0.6	19.4 ± 1.1					
	S	9.3 ± 0.5	n.d	0.6 ± 0.1	3.2 ± 0.2	12.4 ± 0.5					
	T	0.5 ± 0.1	6.7 ± 0.8	n.d	3.7 ± 0.2	11.0 ± 0.9					
Digestion without added salad dressing	C	0.3 ± 0.0	n.d	0.5 ± 0.1	0.6 ± 0.1	1.4 ± 0.2	23.3 ± 1.8^{a}		6.2 ± 0.8^{a}	5.8 ± 0.8^{a}	7.0 ± 0.8^{a}
	S	7.9 ± 0.7	n.d	n.d	0.5 ± 0.0	8.5 ± 0.7	85.6 ± 7.9^{b}			16.6 ± 1.3^{b}	68.1 ± 5.6^{b}
	T	0.3 ± 0.1	1.2 ± 0.2	n.d	0.05 ± 0.0	1.5 ± 0.2	56.1 ± 1.0^{c}	17.4 ± 3.5^{a}		1.3 ± 0.2^{c}	13.8 ± 2.2^{c}
	R-C	0.3 ± 0.6	n.d	0.3 ± 0.1	0.5 ± 0.1	1.1 ± 0.2	23.1 ± 4.1^{a}		4.7 ± 1.4^{b}	4.1 ± 1.2^{a}	5.5 ± 1.4^{a}
	R-S	7.8 ± 0.4	n.d	n.d	0.5 ± 0.1	8.2 ± 0.4	83.7 ± 4.3^{b}			14.4 ± 3.7^{b}	66.1 ± 3.1^{b}
	R-T	0.3 ± 0.2	0.4 ± 0.1	n.d	0.03 ± 0.0	0.7 ± 0.1	53.3 ± 16.4^{c}	5.3 ± 0.8^{b}		0.8 ± 0.1^{d}	6.1 ± 1.2^{a}
	R-C-T-S	5.1 ± 0.7	0.4 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	6.2 ± 0.9	46.7 ± 6.7^{c}	6.1 ± 1.7^{b}	3.0 ± 0.9^{b}	2.3 ± 0.4^{e}	16.6 ± 2.4^{c}
Digestion with added salad dressing	C	0.2 ± 0.0	n.d	0.3 ± 0.0	0.5 ± 0.0	1.0 ± 0.1	16.1 ± 2.8*^{a}		4.7 ± 0.2*^{a}	4.3 ± 0.2*^{a}	5.1 ± 0.3*^{a}
	S	7.5 ± 0.4	n.d	n.d	0.6 ± 0.1	8.1 ± 0.3	80.9 ± 3.9^{b}			17.7 ± 0.4^{b}	65.4 ± 2.7^{b}
	T	0.4 ± 0.1	1.2 ± 0.1	n.d	0.07 ± 0.0	1.5 ± 0.1	71.6 ± 5.7*^{c}	17.3 ± 2.1^{a}		2.0 ± 0.0*^{c}	14.8 ± 1.1^{c}
	R-C	0.2 ± 0.1	n.d	0.2 ± 0.0	0.3 ± 0.1	0.7 ± 0.1	14.7 ± 0.9*^{a}		2.8 ± 0.5^{b}	2.6 ± 0.5^{c}	3.4 ± 0.4*^{d}
	R-S	7.9 ± 0.5	n.d	n.d	0.4 ± 0.0	8.2 ± 0.5	85.5 ± 3.5^{b}			11.5 ± 0.1^{d}	66.2 ± 4.4^{b}
	R-T	0.3 ± 0.1	0.6 ± 0.1	n.d	0.04 ± 0.0	0.9 ± 0.0	61.8 ± 8.3^{c}	8.6 ± 0.9*^{b}		1.2 ± 0.0*^{e}	8.6 ± 0.2*^{e}
	R-C-T-S	5.5 ± 0.1	0.4 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	6.7 ± 0.1	49.9 ± 1.3^{d}	6.0 ± 0.5^{c}	4.0 ± 0.8^{a}	2.8 ± 0.7^{c}	18.0 ± 0.3^{c}

R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach; FW: fresh weight; n.d: not detected.

α-carotene was quantified using the standard curve of β-carotene.

Results are expressed as mean ± standard deviation ($n = 3$).

Values marked with different letters within the same sample treatment (i.e. with or without added salad dressing) indicate a significant difference ($p < 0.05$).

Values marked with an asterisk indicate a significant difference to the corresponding vegetable without added salad dressing ($p < 0.05$).

Among the selected carotenoid-rich vegetables, baby spinach appeared to contain highly bioaccessible carotenoids: more than 60% of the total carotenoids were incorporated into micelles after the simulated digestion, followed by cherry tomato: 14-15%, and carrot: 5-7%. Carotenoids in carrots are present in crystalline form in the chromoplasts, which makes them hardly bioaccessible (Schweiggert et al., 2012). The digestive bioaccessibility of a given carotenoid in different vegetables varied widely. Lutein was the most accessible carotenoid in all tested vegetables (with and without added the salad dressing). The digestive bioaccessibility of lutein was decreasing in the following order: baby spinach > cherry tomato > carrot. Likewise, β-carotene from baby spinach (17-18%) appeared to be more accessible than from carrot (4-6%) and cherry tomato (1.3-2%). The results are in line with earlier reports that xanthophylls are more bioaccessible than carotenes because of their higher polarity (Eriksen et al., 2017, Goñi et al., 2006, Granado-Lorencio et al., 2007, Nagao et al., 2013). The % dBAC of lutein (85.6%) and β-carotene (16.6%) in baby spinach measured in this study is similar to what has been reported previously (Goñi et al., 2006). Lutein and β-carotene from vegetables are bioaccessible at 83% and 26% respectively (Goñi et al., 2006). The bioaccessibility of carotenoids from fruits and vegetables vary depending upon natural structural barriers of the plant tissues, including cell wall integrity, cellular network and chromoplast substructures (Lemmens et al., 2014). For instance, the major barriers for β-carotene in carrot to be released during digestion are cell walls and chromoplast substructures, and for lycopene in tomato are chromoplast substructures and organisation (Palmero et al., 2013). In carrot and tomato, these carotenes are deposited in crystalloid plastidal substructures in which the carotenes are present in a solid-crystalline physical state (Schweiggert et al., 2012), which makes the compounds hardly bioaccessible.

7.3.5. Changes in digestive bioaccessibility of carotenoids as a consequence of the simulated co-digestion of red cabbage and carotenoid-rich vegetables

The percentage digestive bioaccessibility of each major carotenoid including lutein, lycopene, α-carotene and β-carotene and the % dBAC of total carotenoids after the digestion of mixed vegetables with or without added the salad dressing are reported in **Table 14**. The % dBAC of lutein was ranging from 15-23% in the carrot-containing samples to 53-70% in the cherry tomato-containing samples, and more than 80% in the baby spinach-containing samples. However, the digestive bioaccessibility of the carotenes were less than 20% in all samples. The co-digestion of the carotenoid-rich vegetables with red cabbage adversely affected the digestive bioaccessibility of carotenoids, except when digested with baby spinach. The digestive bioaccessibility of total carotenoid decreased by 42-56% in the red cabbage-cherry tomato mixture (with and without added the dressing), by 21-33% in the red cabbage-carrot co-digested mixture, and by 16-25% in the red cabbage-carrot-cherry tomato-baby spinach mixture (**Table 14**). No significant loss of carotenoids was observed when red cabbage was co-digested with baby spinach. Among the tested vegetable combinations, red cabbage and baby spinach appeared to be the most suitable mixture that provided 66% of total bioaccessible carotenoids, followed by the mixture of 4 vegetables: 17-18%; red cabbage-cherry tomato: 6-9%; and red cabbage-carrot: 3-6%. The effect of red cabbage on the digestive bioaccessibility of different dietary carotenoids was also varied. Lutein was the only carotenoid of which the digestive bioaccessibility from the co-digested vegetables (with and without added the oil-based dressing) was the same as from the singly digested vegetables. The co-digestion with red cabbage, however, significantly impaired the micellarisation of the carotenes α-carotene, β-carotene and lycopene. The % dBAC of α-carotene from carrot reduced by 24-40% whereas that of lycopene from

cherry tomato dropped by 50-70%. The reduction in β-carotene digestive bioaccessibility from different vegetable combinations varied from 14-45%. Red cabbage contains dietary fibre which is reported to interfere with the micellarization of carotenoids during digestion (Palafox-Carlos et al., 2011). Dietary fibre may entrap lipid molecules and bile salts or partition these compounds into the gel phase of the fibre, which prevents them from forming micelles with carotenoids (Palafox-Carlos et al., 2011) leading to a reduction in carotenoid bioaccessibility. In the present research, the matrix effect of red cabbage showed a pronounced impact on the digestive bioaccessibility of the hydrocarbon carotenes, but not on the xanthophyll lutein. The incorporation of carotenoids into aqueous micelles is inversely related to the compound hydrophobicity (Tyssandier et al., 2001). Lutein is less hydrophobic than the carotenes, so its transfer into the solubilized portion is more efficient and less dependent on lipid molecules as compared to the carotenes (Nagao et al., 2013). The major carotenoid identified in baby spinach was lutein of which the bioaccessibility was not significantly changed ($p > 0.05$) after the co-digestion with red cabbage. This possibly explains why the co-digestion of red cabbage and baby spinach showed no significant loss of carotenoids and provided the highest bioaccessible carotenoids as compared to the other vegetable combinations.

7.3.6. Effects of the addition of salad dressing on the digestive bioaccessibility of anthocyanins and carotenoids

The anthocyanins were slightly more bioaccessible in the vegetables with added the salad dressing than in the fresh vegetables. The impact of the food matrix on anthocyanin bioaccessibility after *in vitro* food co-digestion has been reported previously. For instance, different foodstuffs such as meat, soymilk and cream reduced the bioaccessibility of anthocyanins when they were co-digested with pomegranate

(Sengul et al., 2014). Some food components such as sugars, starch, cellulose, citric acid or salt were seen to improve the total anthocyanin content in the dialyzed fraction obtained after each of these components was co-digested with pomegranate (Sengul et al., 2014). The addition of salad dressing which contains vinegar (acetic acid) in the digestion may explain the slight increase in the digestive bioaccessibility of red cabbage anthocyanins in our study. The effect of salad dressing on carotenoid digestive bioaccessibility was varied depending upon the vegetable matrix. The digestive bioaccessibility of any of the measured carotenoids (e.g. lutein and β-carotene) as well as the digestive bioaccessibility of total carotenoids from carrot decreased; remained unaffected in spinach; and increased from cherry tomato in the presence of salad dressings.

The same trend was also seen in the corresponding mixture with red cabbage that was added the salad dressing. The salad dressing used in the present study contained 52% (w/w) of canola oil, which constituted a fat content of 12.5% (w/w) in the prepared vegetable salads prior to the simulated digestion. The presence of oil in the salad dressing might partly contribute to the changes in the carotenoid digestive bioaccessibility in the vegetable samples with added the dressing. Some of the findings in this research with regards to the effect of fat/oil on the bioaccessibility of carotenoids from spinach are in agreement with earlier reports. For instance, the *in vitro* bioaccessibility of lutein and β-carotene from raw spinach or steamed spinach puree was not enhanced by the addition of fat or oil (3.3%) into the food prior to the digestion (Eriksen et al., 2017). Nagao et al. (2013) also reported that the addition of various dietary fats/oils (1%) did not improve lutein bioaccessibility from spinach, whereas β-carotene bioaccessibility could be improved by several types of fats/oils except safflower oil and sesame oil. In our study, the digestive bioaccessibility of lutein and β-

carotene from baby spinach or baby spinach-red cabbage with added the salad dressing containing 12.5% of canola oil were similar to that from the fresh raw vegetables (no oil). The addition of salad dressing, however, reduced the bioaccessible carotenoids from carrot/carrot-red cabbage mixture, but increased the bioaccessible carotenoids from the cherry tomato-red cabbage mixture. These findings are partly contradictory to what has been reported previously. Carotenoids from both carrot and tomato appeared to be more bioaccessible in the presence of sunflower oil (1% and 2.5%) (Schweiggert et al., 2012). The bioaccessibility of carotenoids from a salad puree containing spinach, tomato, carrot, romaine lettuce and orange pepper was enhanced by the addition of 0.25-1% canola oil, but slightly decreased at higher concentration of canola oil (1-2.5%) (Huo et al., 2007). In these studies, the concentration of fat or oil added into food ranged from 0.25-2.5%, whereas a much higher fat content (12.5%) was used in our study. Some studies have reported that the addition of high amount of fat into vegetables/fruits does not result in an enhancement of the *in vitro* bioaccessibility of carotenoids (Huo et al., 2007, Schweiggert et al., 2012). For example, the *in vitro* bioaccessibility of lycopene from tomato and papaya was not enhanced further when the lipid content was increased from 1-2.5% to 15% (Schweiggert et al., 2012). The increase in dietary fat content, on the contrary, shows an enhancement of carotenoid bioavailability in humans. The use of high-fat (12.5%) salad dressings added into a mixed vegetable comprising of spinach, romaine lettuce, shredded carrot and cherry tomato was seen to improve *in vivo* carotenoid bioavailability in human plasma chylomicrons as compared to the use of fat-free or reduced-fat (2.7%) salad dressings (Brown et al., 2004). In Brown *et*. *al*.'s study, the authors investigated the effect of fat intake on the bioavailability of carotenoids from a vegetable mixture, but not from a single vegetable matrix. In our study, the addition of salad dressing containing 12.5% of

fat slightly increased ($p \geq 0.05$) the *in vitro* digestive bioacessibility of lutein, β-carotene, and total carotenoids from the mixed 4-vegetable sample, but showed a different effect on carotenoid digestive bioaccessbility in different single vegetable matrices. Future studies should be undertaken to find out how high fat containing meals affect the bioaccessibility and bioavailability of carotenoids from different food matrices.

7.4. Conclusion

The co-digestion of red cabbage with different carotenoid-rich vegetables in the form of raw fresh mixture or mixed vegetables with added salad dressing resulted in changes in the digestive bioaccessibility of anthocyanins and carotenoids. The digestive bioaccessibility of total anthocyanins was increased in all digested vegetable mixtures, except for the mixture of red cabbage and carrot, whereas the digestive bioaccessibility of total carotenoids was decreased when red cabbage was co-digested with carrot or cherry tomato. The digestive bioaccessibility of total polyphenols was also increased by the co-digestion of red cabbage with the carotenoid-rich vegetables, except with tomato. Among the vegetable mixtures tested in this study, red cabbage and baby spinach appeared to be effectively co-ingested because the co-digestion of these vegetables (1:1, w:w) with and without added salad dressing provided higher bioaccessible anthocyanins whilst not reducing the carotenoid digestive bioaccessibility. The addition of salad dressing slightly increased the digestive bioaccessibility of anthocyanins, and showed different effects on the digestive bioaccessibility of different carotenoids. Further studies should be carried out to investigate how the changes in the digestive bioaccessibility of anthocyanins and carotenoids as a consequence of the co-digestion of vegetables as well as the presence of fats would affect the ultimate biological activities.

CHAPTER 8

Intestinal cellular bioaccessibility of carotenoids and cellular biological activity as a consequence of vegetable co-ingestion

(This is an Accepted Manuscript of an article published by Elsevier in Food Chemistry volume 286, pages 678-685 on 15/07/2019, available online on 19/02/2019: https://doi.org/10.1016/j.foodchem.2019.02.046, which includes additional data that were not presented in the published article)

Abstract

The effects of co-digestion of a carotenoid-rich vegetable such as carrot, cherry tomato or baby spinach with an anthocyanin-rich vegetable such as red cabbage with and without salad dressing on the intestinal cellular bioaccessibility of carotenoids and the resultant cellular antioxidant and anti-inflammatory activities were investigated. The % intestinal cellular bioaccessibility (% cBAC) of lutein from the tested vegetables was 0.23-1.42%, lycopene 0.07-0.39%, α-carotene 0.01-0.12% and β-carotene 0.03-0.61% respectively. The % cBAC of each of these carotenoids from the co-digested vegetables was significantly higher ($p < 0.05$) than from carrot, cherry tomato or baby spinach digested alone. % cBAC of total carotenoids was significantly increased by 46-191% ($p < 0.05$) as a result of the co-digestion. The vegetable co-digestion did not result in any impairment on the resultant cellular anti-oxidation and anti-inflammation (NO, IL-8 secretion). Among the tested vegetables, baby spinach co-digested with red cabbage showed synergistic bioactivities in all tested assays.

8.1. Introduction

Fruits and vegetables contain a wide array of phytochemicals that have been claimed for several potent biological effects including anti-oxidation, anti-inflammation and anti-cancer (Liu, 2013). The consumption of a high proportion of different variety of fruits and vegetables in diets is therefore recommended to maintain human health and well-being, and to prevent the onset of non-communicable diseases (Graf et al., 2005, Liu, 2013).

The biological effects resulting from different combinations of fruits and/or vegetables have been reported. Wang et al. (2011) tested the antioxidant activities of a number of combinations of fruits and/or vegetables using different *in vitro* chemical assays. These authors found that the combinations of different fruits, vegetables and/or legumes produced 13-21% synergy, 54-68% addition and 21-25% antagonism in *in vitro* antioxidant activities. For example, several mixtures, such as: apple and purple cauliflower, raspberry and mushroom, or apple and tomato were seen to exert synergistic effect in radical scavenging (Wang et al., 2011). However, the raw food materials were extracted with methanol for hydrophilic phytochemicals and then the combined effects of the mixed extracts were measured, which eliminated the digestion and absorption of foods. In another study, the combined anti-oxidative and anti-inflammatory properties of different mixtures of tomato with onion, garlic or lettuce in the form of raw, digested and absorbed fractions were reported (Gawlik-Dziki, 2012). The dialysates (absorbed fraction) of all the tested mixtures showed synergy in the inhibition of xanthine oxidase but show additive or antagonistic effect on antiradical activity and lipoxygenase inhibition. The author of this study used a dialyzed membrane as the model for absorption and chemical assays for bioactivity interaction, which did not reflect biological conditions. Thus, the present study aimed to report for the first

time how vegetable co-digestion could affect phytochemical intestinal cellular bioaccessibility and the resultant bioactivities using a more biologically relevant model: human Caco-2 cells following a simulated *in vitro* digestion. Some flavonoids including anthocyanins and flavanones can impact on the cellular uptake of carotenoids when they are in pairs in the pure form (Claudie et al., 2013, Phan et al., 2018b, Phan et al., 2018a, Phan et al., 2019), but this effect in food materials is not yet understood. In this study, anthocyanin-rich red cabbage was co-digested with carotenoid-rich carrot, cherry tomato and/or baby spinach in pairs and altogether using an *in vitro* digestive model. The filtered supernatant from the digesta of each single vegetable and each vegetable mixture was subjected to cellular uptake of carotenoids by Caco-2 cells. The cellular biological effects on anti-oxidation and anti-inflammation of the digesta from individual and mixed vegetables were examined and compared. It was hypothesized that the cellular uptake of carotenoids from mixed vegetables could be altered because of the ability of anthocyanins from red cabbage to interact with cell membrane lipids, which could consequently result in enhanced cellular biological activity.

8.2. Materials and methods

As described in Chapter 3, section 3.1.1-3.1.4 on pages 75-76, and 3.3.9-3.3.12 on pages 94-98.

8.3. Results

8.3.1. Cell viability

The filtered supernatant from the digesta of fresh vegetables (single and mixed) was diluted with the growth medium to 1:4-1:8 ratios and the filtered supernatant from the digesta of vegetables (single and mixed) with added the salad dressing was diluted to 1:8-1:12 ratios before being used for the Caco-2 cell study. An appropriate dilution ratio

was selected according to the following criteria: (i) providing more than 75% of viable cells; (ii) no significant difference in cell viability among samples; and (iii) as low as possible to increase the testability of cellular biological activities and cellular uptake of carotenoids. Based on these criteria, the dilution ratio of 1:8 was selected for the digesta of fresh vegetables and 1:10 for the digesta of vegetables with added the salad dressing (**Table 15**) to be used in the bioactivity and uptake experiments.

Table 15. Cell viability (%) after exposure to the supernatant of the digesta of different single/combined vegetables diluted with growth medium at different ratios.

Cell viability (%)	**Supernatant of digesta: Growth medium ratio**					
	Digestion without added salad dressing			***Digestion with added salad dressing***		
	1:4	**1:6**	**1:8**	**1:8**	**1:10**	**1:12**
R	75.5 ± 8.3	88.3 ± 12.7	92.9 ± 8.8	92.9 ± 2.1	96.7 ± 0.4	95.7 ± 3.5
C	72.8 ± 4.1	78.1 ± 3.6	80.8 ± 6.4	88.1 ± 6.4	92.1 ± 0.5	87.6 ± 3.1
S	56.7 ± 3.7	68.3 ± 4.2	74.1 ± 4.4	55.7 ± 1.2	89.4 ± 0.4	87.1 ± 0.2
T	64.7 ± 4.0	76.0 ± 1.7	74.9 ± 1.9	55.2 ± 8.9	76.0 ± 9.4	74.6 ± 2.6
R-C	77.1 ± 3.8	83.9 ± 1.2	87.8 ± 8.2	95.8 ± 0.3	101.9 ± 9.8	98.8 ± 5.2
R-S	60.1 ± 3.7	70.1 ± 4.4	73.8 ± 3.6	92.2 ± 3.6	95.1 ± 2.4	92.3 ± 6.3
R-T	69.0 ± 2.7	76.8 ± 1.9	79.1 ± 3.9	56.5 ± 14.5	79.9 ± 6.0	83.0 ± 12.5
R-C-S-T	62.0 ± 2.3	73.1 ± 5.0	74.1 ± 3.6	92.7 ± 2.3	99.2 ± 10.5	95.5 ± 3.1

Values are expressed as mean ± SD of three replicates (n = 3).
R: red cabbage; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach.

8.3.2. Cellular antioxidant activity (CAA) following the exposure to the digesta of different single/combined vegetables

The experimental CAA following the exposure to the digesta of the individual/combined vegetables and AAPH-induced oxidation are presented in **Figure 21**. The results are expressed as CAA unit (% control) per g fresh weight (FW). The experimental CAA following the exposure to the mixed vegetable digesta was compared with the expected additive CAA of the digested vegetable components based on the same total fresh weight (as explained in section 3.3.12 on page 98). The results showed that the digesta of every tested vegetable exerted similar cellular antioxidant activity. However, the experimental CAA/g FW following the exposure to the co-digested vegetables showed a significant increase ($p < 0.05$) by 26-31% as compared to

the expected additive CAA resulting from 0.5 g of each vegetable component, which indicates a synergistic effect.

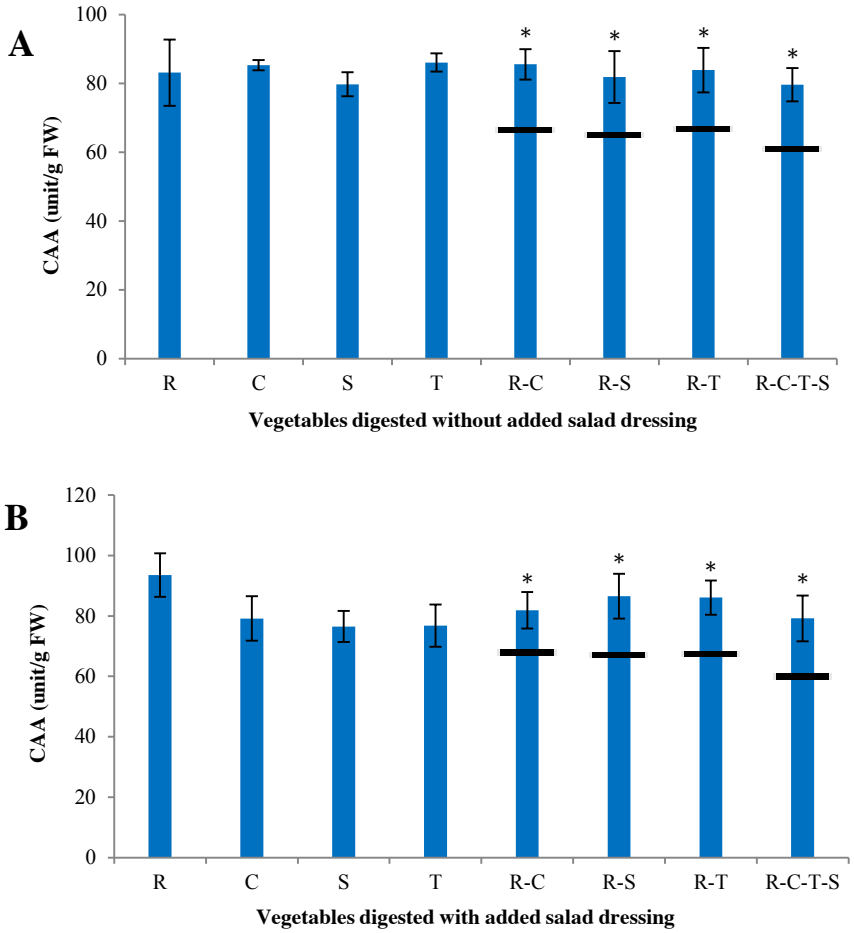


Figure 21. Cellular antioxidant activity (CAA) following the exposure to the supernatant from the digesta of different vegetables (single and combination) (A)without added salad dressing and (B) with added salad dressing after the induction of oxidation by AAPH. Data are expressed as mean of CAA unit/g fresh weight (FW) of the vegetable. The horizontal lines indicate the expected additive CAA following the exposure to the supernatant from the digesta of the vegetable components, which was calculated based on the same total FW according to the equation of Fuhrman et al. (2000): $CAA_{A\text{-}B}$ (unit/g FW) = CAA_A + CAA_B – CAA_A x CAA_B/100, in which $CAA_{A\text{-}B}$ is the expected CAA unit per g FW of the mixture, and CAA_A, CAA_B is the CAA unit per 0.5 g FW of the vegetable component A and B respectively. Columns marked with an asterisk demonstrate a significant difference ($p < 0.05$) between the experimental CAA and the expected CAA. Experimental values are mean ± SD ($n = 6$). R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach.

8.3.3. Anti-inflammatory effects following the exposure to the digesta of different single/combined vegetables

8.3.3.1. Pro-inflammatory cytokine IL-8 secretion

The secretion of interleukin-8 (% control) by Caco-2 cells following TNF-α-induced inflammation (100 ng/mL) in the presence of the digesta of different single or combined vegetables with and without added the salad dressing is presented in **Figure 22**.

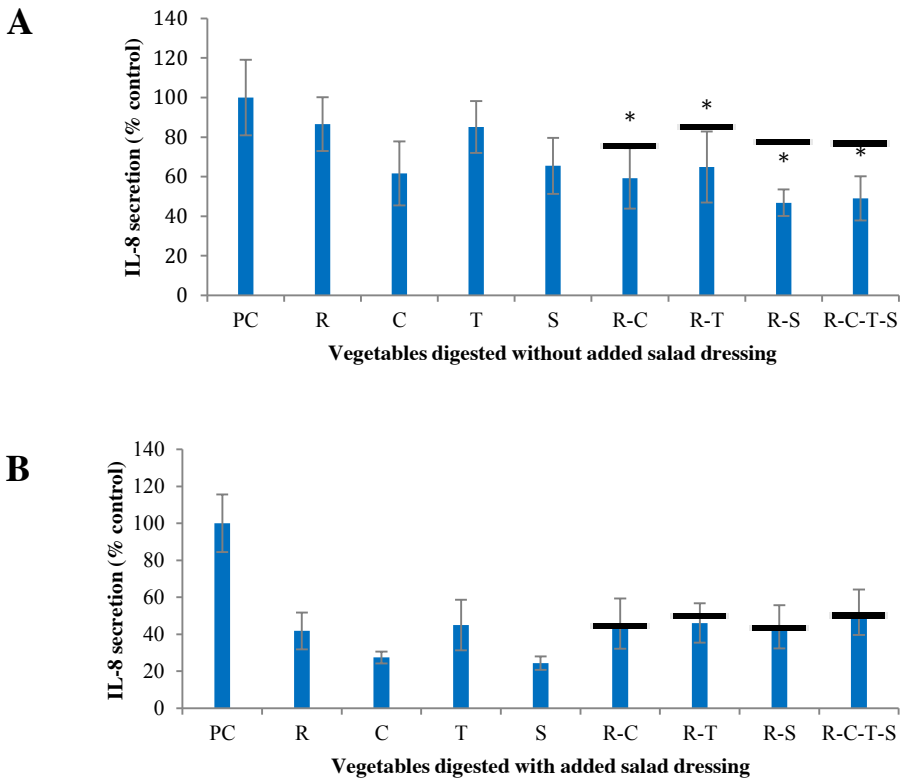


Figure 22. IL-8 secretion (% control) by Caco-2 cells following the exposure to the supernatant from the digesta of different vegetables (single and combination) (A) without added salad dressing and (B) with added salad dressing after TNF-α-induced inflammation (100 ng/mL). The horizontal lines show the expected additive activity of the vegetable mixtures based on the same total fresh weight, which was calculated as 100 - (A + B - A x B/100) in which A, B is the percentage reduction of the IL-8 secretion of the cells to the control when exposed the cells to the digesta supernatant of the componential vegetables. Columns marked with an asterisk indicate that the experimental activity of the mixture was significantly different from the calculated additive value ($p < 0.05$). Experimental values are mean ± SD ($n = 8$). PC: positive control in which cells were exposed to the supernatant of blank digesta (4 h) and TNF-α (24 h) which was set as 100%. Blank digesta contained 10 g of milliQ water instead of vegetables that was subjected to the digestion. In the following samples, the cells were exposed to the digesta of the abbreviated vegetable (4 h) and TNF-α (24 h). R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach.

The results showed that the inhibition of IL-8 secretion was significantly enhanced ($p < 0.05$) by 27-65% in the presence of the digesta of mixed fresh vegetables as compared to the expected additive effect based on the same total FW of vegetables (10 g), which indicates a synergy. The red cabbage-baby spinach mixture showed the highest inhibitory effect on the secretion of IL-8. However, there was no significant enhancement ($p \geq 0.05$) in the inhibition of IL-8 by the mixed vegetable with added the salad dressing, which indicates an additive effect.

8.3.3.2. Nitric oxide production

The production of NO by Caco-2 cells in response to TNF-α-induced inflammation (100 ng/mL) when the cells were incubated with the digesta of different single or combined vegetables with and without added the salad dressing is presented in **Figure 23**. All of the tested vegetables significantly suppressed the NO production by 25-62%. Among the individual vegetables, red cabbage (with and without added the salad dressing) showed lower inhibition of NO production than the other carotenoid-containing vegetables. When fresh red cabbage was co-digested with fresh baby spinach or the mixture of carrot, cherry tomato and baby spinach, the inhibition of NO production was significantly increased ($p < 0.05$) by 14% and 23% respectively as compared to the expected additive effect of the vegetable components based on the same total FW, indicating a synergistic effect. The co-digested mixtures of fresh red cabbage with carrot or with cherry tomato showed an additive effect on NO suppression. In contrast, a synergistic effect on NO suppression was seen in all combinations of vegetables with added the salad dressing which suppressed the NO production by 11-36% higher than the expected additive activity.

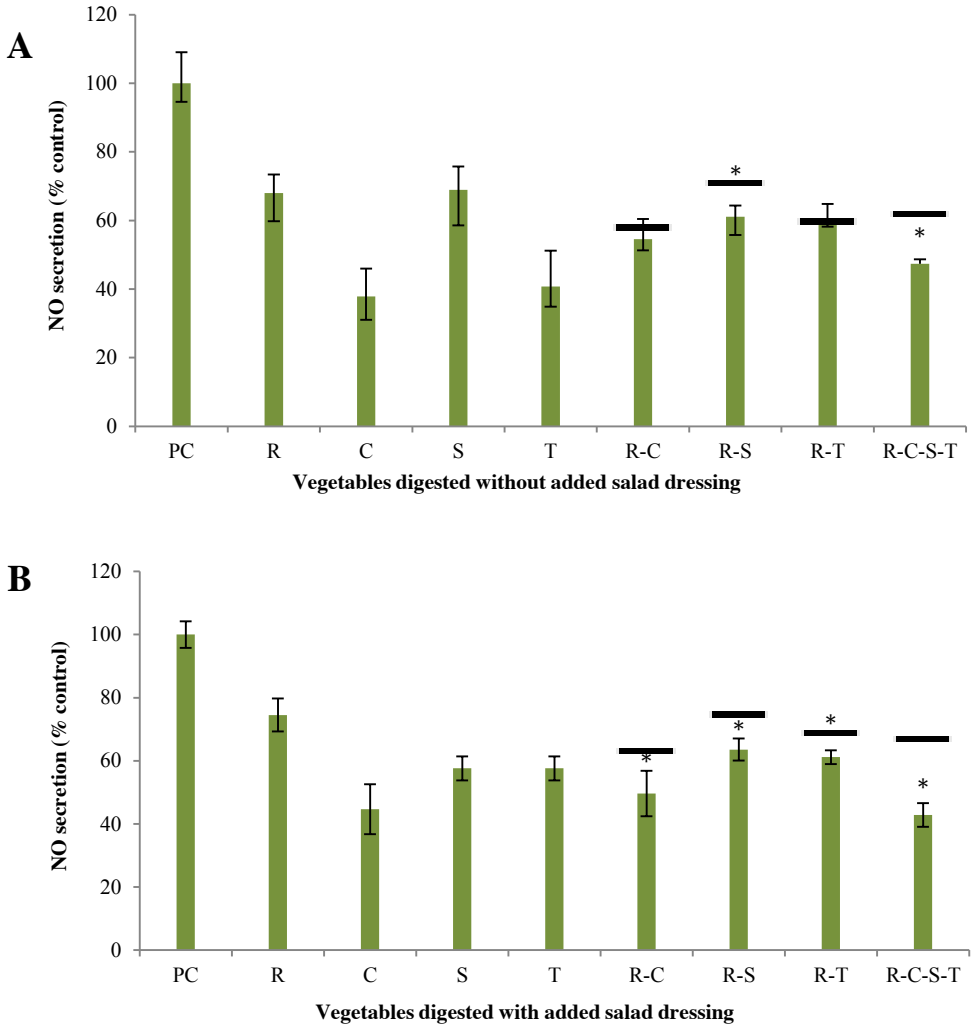


Figure 23. NO secretion (% control) by Caco-2 cells following the exposure to the supernatant from the digesta of different vegetables (single and combination) (A) without added salad dressing and (B) with added salad dressing after TNF-α-induced inflammation (100 ng/mL). The horizontal lines show the expected additive activity of the vegetable mixtures, which was calculated as 100 - (A + B - A x B/100) in which A, B is the percentage reduction of the NO secretion of the cells to the control when exposed the cells to the digesta supernatant of the componential vegetables. Columns marked with an asterisk indicate that the experimental activity of the mixture was significantly different from the calculated additive value ($p < 0.05$). Experimental values are mean ± SD ($n = 8$). PC: positive control in which cells were exposed to the supernatant of the blank digesta (4 h) and TNF-α (24 h) which was set as 100%. Blank digesta contained 10 g of milliQ water instead of vegetables that was subjected to the digestion. In the following samples, the cells were exposed to the digesta supernatant of the abbreviated vegetable (4 h) and TNF-α (24 h). R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach.

8.3.4. Effect of co-digestion with red cabbage on the intestinal cellular bioaccessibility of carotenoids

The uptake of major carotenoids including lutein, lycopene, α-carotene and β-carotene expressed in ng/mL digesta and μg/100 g FW from vegetables with and without added

the salad dressing are presented in **Table 16** and **Table 17** respectively**.** The results show that the uptake of carotenoids (μg/100 g FW) from the co-digested vegetable mixtures, excepting for lutein from the red cabbage-cherry tomato (R-T) mixture, was significantly higher ($p < 0.05$) than that from the singly digested carotenoid-containing vegetables. The % intestinal cellular uptake and % intestinal cellular bioaccessibility (% cBAC) of each carotenoid are also reported (**Table 16**). The % intestinal cellular uptake of each carotenoid was significantly greater ($p < 0.05$) from the digesta of the mixed vegetables than from the digesta of the individual vegetables, except for lutein from red cabbage-cherry tomato and lycopene from the mixture of all 4 tested vegetables ($p \geq 0.05$). The % cBAC of carotenoids from fresh vegetables was significantly enhanced ($p < 0.05$) by 43-147% for lutein, by 40-59% for lycopene, by 177-205% for α-carotene, and by 42-279% for β-carotene (**Table 16**).

Similarly, the % cBAC of carotenoids from mixed vegetables with added the oil-based dressing was also significantly higher than from singly-digested vegetables with added the dressing. The absorbed amount of carotenoids from vegetables with added the salad dressing, however, was seen be lower than that from fresh vegetables, and lycopene was not detected in any of the vegetables that were added the salad dressing. All carotenoid extracts were usually subjected to the LC-MS analysis no longer than 1 week after the extraction but this set of samples had to be stored for much longer because of the unexpected technical issues of the LC-MS Orbitrap that had postponed the carotenoid analysis for a month. The carotenoids from the supernatant of the salad digesta might have degraded during the prolonged storage at -80^{o}C of the carotenoid cellular extracts resulting in a significant reduction of the absorbed carotenoids measured. There were not enough salad digesta to re-conduct the uptake experiment, and time and money did not allow repeating the whole experiments from the beginning.

Table 16. Absorption and % intestinal cellular bioaccessibility of carotenoids from different single and mixed fresh vegetables after simulated *in vitro* digestion and intestinal absorption by Caco-2 cells.

Absorption and bioaccessibility	**Carotenoids**	**Digesta from fresh vegetables**								
		R	**C**	**R-C**	**S**	**R-S**	**T**	**R-T**	**C-T-S**	**R-C-T-S**
Absorbed content from the digesta (ng/mL)[1]	Lutein	n.d	11.5 ± 2.7	14.2 ± 0.7	184.5 ± 3.5	131.7 ± 4.6*	3.6 ± 0.9	2.6 ± 0.9	49.9 ± 1.3	85.0 ± 5.8*
	Lycopene	n.d	n.d	n.d	n.d	n.d	37.6 ± 7.1	26.6 ± 1.3*	9.4 ± 1.8	11.6 ± 0.6
	α-carotene	n.d	6.5 ± 0.7	9.0 ± 2.1	n.d	n.d	n.d	n.d	1.6 ± 0.2	3.8 ± 0.6*
	β-carotene	n.d	14.3 ± 1.5	23.8 ± 5.9*	23.1 ± 2.5	19.2 ± 4.7	2.9 ± 1.0	5.5 ± 0.2*	10.1 ± 0.5	11.1 ± 1.9
Absorbed content from fresh material (μg/100 g FW)[2]	Lutein	n.d	5.7 ± 1.3	14.2 ± 0.7*	92.4 ± 1.9	132.1 ± 4.9*	1.8 ± 0.4	2.5 ± 0.9	25.0 ± 0.7	54.7 ± 2.0*
	Lycopene	n.d	n.d	n.d	n.d	n.d	18.8 ± 3.6	26.4 ± 1.3*	4.7 ± 0.9	7.5 ± 0.2*
	α-carotene	n.d	3.2 ± 0.3	9.0 ± 2.1*	n.d	n.d	n.d	n.d	0.8 ± 0.1	2.5 ± 0.3*
	β-carotene	n.d	7.1 ± 0.7	23.7 ± 5.9*	11.6 ± 1.2	19.2 ± 4.8*	1.4 ± 0.5	5.4 ± 0.2*	5.0 ± 0.3	7.1 ± 1.0*
% Cellular uptake[3]	Lutein	-	2.11 ± 0.49	5.25 ± 0.27*	1.16 ± 0.02	1.70 ± 0.06*	0.62 ± 0.15	0.92 ± 0.33	0.29 ± 0.01	1.07 ± 0.04*
	Lycopene	-	-	-	-	-	1.61 ± 0.31	7.41 ± 0.38*	1.61 ± 0.31	1.82 ± 0.04
	α-carotene	-	0.72 ± 0.08	2.62 ± 0.62*	-	-	-	-	0.72 ± 0.08	1.15 ± 0.15*
	β-carotene	-	1.13 ± 0.12	5.23 ± 1.30*	2.21 ± 0.24	4.25 ± 1.06*	2.71 ± 0.94	18.90 ± 0.57*	0.42 ± 0.02	1.78 ± 0.24*
% Intestinal cellular bioaccessibility (cBAC)[4]	Lutein	-	0.49 ± 0.12	1.21 ± 0.06*	1.00 ± 0.02	1.42 ± 0.05*	0.35 ± 0.08	0.49 ± 0.18	0.23 ± 0.01	0.50 ± 0.02*
	Lycopene	-	-	-	-	-	0.28 ± 0.05	0.39 ± 0.02*	0.07 ± 0.01	0.11 ± 0.00*
	α-carotene	-	0.04 ± 0.00	0.12 ± 0.03*	-	-	-	-	0.01 ± 0.00	0.03 ± 0.00*
	β-carotene	-	0.06 ± 0.01	0.22 ± 0.05*	0.37 ± 0.04	0.61 ± 0.15*	0.04 ± 0.01	0.15 ± 0.00*	0.03 ± 0.00	0.04 ± 0.01*
	Total	-	0.08 ± 0.01	0.24 ± 0.04*	0.84 ± 0.02	1.22 ± 0.07*	0.20 ± 0.04	0.32 ± 0.02*	0.08 ± 0.00	0.17 ± 0.01*

[1] Carotenoid content (ng) that was absorbable from 1 mL digesta.
[2] Carotenoid content (μg) that was absorbable from 100 g fresh weight (FW) of the raw vegetable(s).
[3] Defined as the ratio of the absorbed carotenoid content to the micellarised carotenoid content of the digesta that was subjected to the intestinal absorption.
[4] Defined as the ratio of the absorbed carotenoid content to the original carotenoid content of the fresh vegetable.
Values in the same row that are marked with an asterisk indicate a significant difference from the value displayed in the instant previous column, which demonstrates that carotenoid uptake/bioaccessibility from the co-digested vegetables was significantly different from the corresponding singly-digested vegetable.
Data are expressed as mean ± standard deviation (n = 4).
n.d: not detected. R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; C-T-S: carrot-cherry tomato-baby spinach; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach.

Table 17. Absorption and % intestinal cellular bioaccessibility of carotenoids from different single and mixed vegetables with added the salad dressing after simulated *in vitro* digestion and intestinal absorption by Caco-2 cells.

Absorption and bioaccessibility	**Carotenoids**	**Digesta with added salad dressing**								
		R	**C**	**R-C**	**S**	**R-S**	**T**	**R-T**	**C-T-S**	**R-C-T-S**
Absorbed content from digesta (ng/mL)[1]	Lutein	n.d	1.5 ± 0.2	0.6 ± 0.0*	67.2 ± 0.2	44.3 ± 2.4*	7.2 ± 1.0	4.4 ± 0.2*	75.7 ± 1.4	76.3 ± 8.8
	Lycopene	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	α-carotene	n.d	3.6 ± 0.1	26.9 ± 2.0*	n.d	n.d	n.d	n.d	0.9 ± 0.0	2.0 ± 0.1*
	β-carotene	n.d	7.9 ± 0.3	37.6 ± 0.1*	9.6 ± 3.2	39.7 ± 2.5*	3.5 ± 1.1	11.5 ± 1.6*	21.1 ± 3.9	10.4 ± 0.2*
Absorbed content from fresh material (μg/100 g FW)[2]	Lutein	n.d	0.6 ± 0.1	0.6 ± 0.0	33.6 ± 0.1	43.8 ± 2.4*	3.6 ± 0.5	4.4 ± 0.2	37.8 ± 0.7	50.2 ± 5.8*
	Lycopene	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	α-carotene	n.d	1.8 ± 0.0	27.1 ± 2.0*	n.d	n.d	n.d	n.d	0.4 ± 0.0	1.3 ± 0.1*
	β-carotene	n.d	4.0 ± 0.2	37.9 ± 0.1*	4.8 ± 1.6	39.3 ± 2.5*	1.8 ± 0.5	11.6 ± 1.6*	10.5 ± 2.0	6.8 ± 0.1*
% Cellular uptake[3]	Lutein	-	0.32 ± 0.06	0.38 ± 0.01	0.45 ± 0.00	0.56 ± 0.03	0.97 ± 0.14	1.37 ± 0.08*	0.47 ± 0.01	0.92 ± 0.11*
	Lycopene	-	-	-	-	-	-	-	-	-
	α-carotene	-	0.53 ± 0.01	13.39 ± 0.98*	-	-	-	-	0.53 ± 0.01	0.38 ± 0.02*
	β-carotene	-	0.85 ± 0.03	13.37 ± 0.05*	0.77 ± 0.25	10.87 ± 0.69*	2.40 ± 0.72	26.99 ± 3.67*	0.90 ± 0.17	1.36 ± 0.03*
% Intestinal cellular bioaccessibility (cBAC)[4]	Lutein	-	0.05 ± 0.01	0.06 ± 0.00*	0.36 ± 0.00	0.47 ± 0.03*	0.69 ± 0.10	0.85 ± 0.05	0.34 ± 0.01	0.46 ± 0.05
	Lycopene	-	-	-	-	-	-	-	-	-
	α-carotene	-	0.02 ± 0.00	0.37 ± 0.03*	-	-	-	-	0.01 ± 0.00	0.02 ± 0.00*
	β-carotene	-	0.04 ± 0.00	0.34 ± 0.00*	0.16 ± 0.05	1.25 ± 0.08*	0.05 ± 0.01	0.32 ± 0.0*4	0.04 ± 0.01	0.06 ± 0.00*
	Total	-	0.03 ± 0.00	0.34 ± 0.01*	0.31 ± 0.01	0.67 ± 0.00*	0.05 ± 0.00	0.15 ± 0.01*	0.11 ± 0.00	0.14 ± 0.01*

[1] Carotenoid content (ng) that was absorbable from 1 mL digesta.
[2] Carotenoid content (μg) that was absorbable from 100 g fresh weight (FW) of the raw vegetable(s).
[3] Defined as the ratio of the absorbed carotenoid content to the micellarised carotenoids content of the digesta that was subjected to the intestinal absorption.
[4] Defined as the ratio of the absorbed carotenoid content to the original carotenoid content of the fresh vegetable.
Values in the same row that are marked with an asterisk indicate a significant difference from the value displayed in the instant previous column, which demonstrates that carotenoid uptake/bioaccessibility from the mixed vegetable with added the salad dressing was significantly different from the corresponding singly-digested vegetable with added the dressing.
Data are expressed as mean ± standard deviation (n = 4).
n.d: not detected. R: red cabbage; C: carrot; S: baby spinach; T: cherry tomato; R-C: red cabbage-carrot; R-S: red cabbage-baby spinach; R-T: red cabbage-cherry tomato; C-T-S: carrot-cherry tomato-baby spinach; R-C-T-S: red cabbage-carrot-cherry tomato-baby spinach..

8.4. Discussion

The co-digestion of red cabbage with carotenoid-rich vegetables showed a positive impact on the resultant cellular bioactivities of anti-oxidation and anti-inflammation. No antagonism in the cellular antioxidant and anti-inflammatory activities was observed. The digestion of the mixed vegetables with added the salad dressing produced a synergistic effect on cellular antioxidant activity (CAA). The CAA enhanced by up to 30% from the expected additive effect of the digested vegetable components. Synergy in anti-inflammatory activities was also seen as a result of the co-digestion of the vegetables. The interleukin-8 secretion by Caco-2 cells was increasingly suppressed following the exposure to the digesta of every tested fresh vegetable mixture, while the NO production more effectively reduced by the mixed vegetables with added the salad dressing as compared to the expected additive effect of the digested vegetable components.

The synergistic effects on cellular bioactivities seen from the co-digested vegetables could be the result of the interaction between phytochemical compounds and/or their metabolites. The major anthocyanin compounds identified in our red cabbage extract and the digesta samples were cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside (30.4 mg cyanidin-3-glucoside (CG) equivalent/100 g FW), cyanidin-3-(feruloyl)-diglucoside-5-glucoside (28.0 mg CG equivalent/100 g FW) and cyanidin-3-(sinapoyl)-diglucoside-5-glucoside (22.6 mg CG equivalent/100 g FW) (**Figure 17**, **Figure 18**, **Table 13**). The major carotenoids identified in carrot, cherry tomato and baby spinach used in our study were lutein, lycopene, α-carotene and β-carotene. We previously reported the interactive effects of different anthocyanidin glucosides with various carotenoids including β-carotene in chapter 4 (Phan et al., 2018b), lutein in chapter 5 (Phan et al., 2018a) and lycopene in chapter 6 (Phan et al., 2019). There was no

synergistic interaction in cellular antioxidant activity seen in any of the tested anthocyanin-carotenoid combinations (Phan et al., 2019, Phan et al., 2018a, Phan et al., 2018b). Some combinations such as lutein and malvidin-3-glucoside (at 1:1, 1:3 and 3:1 ratios), or lycopene and methoxylated anthocyanins: malvidin-3-glucoside, peonidin-3-glucoside or petunidin-3-glucoside (at 1:1 and 3:1 ratios) showed synergistic suppression on IL-8 secretion, whereas the other tested combinations showed additive or antagonistic effect (Phan et al., 2019, Phan et al., 2018a). In these studies, the anthocyanin and carotenoid compounds were combined in pairs in the pure form (no food matrix effect) to investigate their interactive effects at the molecular level. Bioactive compounds that are fractionated or isolated from their food matrix can be less effective than the whole plant extract because the synergistic interaction between these compounds and the other compounds in the extract may vanish (Efferth and Koch, 2011). In the present study, the complex interaction between multiple phytochemical compounds released from their food matrix during digestion resulted in either synergistic or additive effect on cellular anti-oxidation and anti-inflammation. The combined extracts of several vegetables are reported to produce synergistic antioxidant effects in chemical models (Gawlik-Dziki, 2012, Jiang et al., 2015, Wang et al., 2011). For instance, mixtures of carotenoid extract of tomato or carrot with anthocyanin extract of eggplant or purple potato at appropriate ratios synergistically enhanced free radical scavenging activities in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate (ABTS) assays (Jiang et al., 2015). A mixture comprising methanolic extracts of tomato and purple cauliflower (1:1 ratio) exerted synergistic antioxidant effects in total phenolic content (TPC) assay and oxygen radical absorbance capacity (ORAC) assay (Wang et al., 2011). It was suggested that the antioxidant synergy in these combined vegetable systems resulted from the

combinatorial interaction of the existing phytochemicals (Wang et al., 2011). The combined extracts in these studies were from raw vegetables that did not undergo gastrointestinal digestion. Gawlik-Dziki (2012) showed that the membrane dialysate (considered as the absorbed fraction after intestinal digestion) of the mixture of tomato with garlic produced synergistic effects on ABTS antiradical activity and catalase activity, and the dialysates of tomato mixed with onion, garlic and/or lettuce exhibited synergy in xanthine oxidase inhibition. The bioactivities of the dialyzed fractions of the co-digested vegetables/spices in Gawlik-Dziki's study were assessed using chemical models, which might not be biologically relevant. In our study, we used human Caco-2 cells as a model for intestinal absorption and bioactivity assessment to better represent the biological system. The present study is so far the first report on the consequent effects of vegetable co-digestion on cellular bioactivities and phytochemical uptake using a human Caco-2 cell model.

Synergy in cell-based biological activities could be the result of multi-target effects of interactive phytochemical components contained in the food to different biomarkers (Imming et al., 2006, Wagner and Ulrich-Merzenich, 2009, Williamson, 2001): reactive oxygen species (ROS) or reactive nitrogen species (RNS); pro-inflammatory cytokines or chemokines; oxidative/pro-inflammatory enzymes involved in the formation of ROS/RNS; defensive enzymes; intracellular signalling pathways; and expression of genes associated with redox and/or inflammatory processes (Wang and Zhu, 2017, Phan et al., 2018c). Although experiments can be set up to study the underlying molecular mechanisms of the cellular bioactivities of a single phytochemical, it is hard to understand thoroughly the molecular mechanisms of the interactive effects between phytochemicals because of the unavailability of a methodology to predict the expected

gene expression of a molecular pathway resulting from the combined effect of phytochemicals.

Synergistic cellular activities may also result from the improved absorption, stability or bioaccessibility of the bioactive compounds (Kirakosyan et al., 2010, Wagner and Ulrich-Merzenich, 2009). The impact of hydrophilic bioactive compounds on the uptake of hydrophobic phytochemicals has been documented while there is no evidence reported on the reverse effect. Thus, in the present study, changes on cellular uptake of major carotenoid compounds as a consequence of vegetable co-digestion were investigated. The uptake of each major carotenoid including lutein, lycopene, α-carotene and β-carotene from the mixed vegetables was compared to that from the individual vegetables. The outcome of the changes on carotenoid uptake when the uptake efficiency was expressed in absorbed carotenoid content per mL digesta was slightly different from that when the uptake efficiency was expressed in absorbed carotenoid content per 100 g fresh weight of the vegetable. The digesta volume of all vegetable samples was made up to the same value of 50 mL at the end of the digestion to ensure consistency in digestive protocol for comparison. The expression of carotenoid uptake per volume of the digesta demonstrates the carotenoid uptake efficiency from the digestion perspective (liquid medium) where the initial total fresh weight of vegetables and the final digesta volume were kept the same in every sample. On the contrary, the expression of carotenoid uptake per 100 g FW of the vegetable demonstrates the carotenoid uptake efficiency from the raw material perspective (solid medium). Therefore, it is necessary to discuss the results from both perspectives.

The comparison of carotenoid uptake (ng) per mL digesta shows that the co-digestion of red cabbage with baby spinach resulted in significant reduction ($p < 0.05$) in lutein uptake, but this was not evident in the co-digestion with carrot or cherry tomato ($p \geq$

0.05). Lycopene uptake from the cherry tomato-red cabbage mixture was significantly lower ($p < 0.05$) than that from the cherry tomato alone. A significant increase in β-carotene uptake ($p < 0.05$) was observed from the mixture of red cabbage with carrot or with cherry tomato as compared to that from the singly digested vegetables. These results are somehow consistent with what we have found previously regarding the impact of anthocyanins on carotenoid uptake when these compounds are present together in a liquid medium: anthocyanins (7.5 μM) did not impair lutein (2.5 μM) uptake (Chapter 5) (Phan et al., 2018a), but increased the cellular uptake of β-carotene (2.5 μM) (Chapter 4) (Phan et al., 2018b) and decreased lycopene (2.5 μM) uptake in Caco-2 cells (Chapter 6) (Phan et al., 2019).

Red cabbage used in this study majorly contains monoacylated diglucoside anthocyanins of which total anthocyanin content analysed using HPLC is 80.9 mg CG equivalent/100 g FW (**Table 13**). The total anthocyanin content remaining after the gastrointestinal digestion of red cabbage (with/without added the salad dressing) was 41.1-43.5 mg CG equivalent/100 g FW, and 42.6-57.1 mg CG equivalent/100 g FW after the digestion of the mixed vegetables (with/without added the salad dressing) (**Table 13**). This shows that the digestive bioaccesssibility of total anthocyanins from red cabbage and the other vegetable mixtures was higher than 50%. The co-digestion of vegetables in fact resulted in a significant increase ($p < 0.05$) in the digestive bioaccessibility of total anthocyanins from red cabbage by 10-15% as reported in Chapter 7. These anthocyanins and other flavonoids contained in the vegetables might influence the cellular uptake of carotenoids due to their ability to interact with the cell membrane (Bonarska-Kujawa et al., 2012). Anthocyanins can interact with the polar heads of the lipid molecules on the outer membrane layer in the hydrophilic compartment (Bonarska-Kujawa et al., 2012). The incorporation of anthocyanins into

the polar interface of the membrane increases the polarization area, which results in a mismatch between the polar head area and the hydrophobic moiety area leading to the fluidization of the membrane and/or the formation of interdigitated bilayer structures (Tarahovsky et al., 2008). The membrane fluidization may increase the lateral diffusion of lipid molecules, but may also induce raft-breaking effects which affect the appearance and development of lipid rafts (Tarahovsky et al., 2008). On the other hand, the interdigitation of the membrane increases the integrity of the lipid bilayer structure and prevents the diffusion of lipid compounds (Tarahovsky et al., 2008). The flavonoid-membrane interaction may therefore result in an increase or decrease in the diffusion of some lipophilic molecules through the cell membrane. Hesperetin and anthocyanins improved the uptake of β-carotene (Phan et al., 2018b, Claudie et al., 2013), whereas naringenin decreased lutein uptake because of the invagination of the lipid draft domains containing lutein receptors (Reboul et al., 2007b). The various effects of anthocyanins on the intestinal cellular uptake of carotenoids could be because of the interaction of anthocyanins with the cell lipid membrane which may induce different effects on the lipid raft domains that contain the receptor of each carotenoid.

The uptake efficiency based on fresh weight of the vegetable (μg/100 g FW) of each of the carotenoids from every tested vegetable mixture showed a significant increase ($p < 0.05$) as compared to that from the singly digested vegetable, except for lutein uptake from the red cabbage-cherry tomato mixture ($p \geq 0.05$). The co-digestion with red cabbage resulted in a significant enhancement ($p < 0.05$) in the % intestinal cellular bioaccessibility (% cBAC) of lutein, lycopene, α-carotene and β-carotene leading to a significant improvement ($p < 0.05$) in the % cBAC of total carotenoids. Lutein (% cBAC = 0.23-1.42%) was absorbed by the Caco-2 cells more than the carotenes (lycopene: % cBAC = 0.07-0.39%; α-carotene: % cBAC = 0.01-0.12%; β-carotene: %

cBAC = 0.03-0.61%). The % cBAC of lutein and β-carotene was highest from baby spinach, followed by carrot and cherry tomato. It was seen that the % digestive bioaccessibility of total carotenoids from baby spinach was also the highest and was not changed when baby spinach was co-digested with red cabbage (**Table 14**). These results show that baby spinach contains the most bioaccessible and absorbable carotenoids among the tested vegetables. The effect on carotenoid uptake as a result of the co-digestion with red cabbage with the addition of salad dressing was seen similarly: none of the tested carotenoids showed a reduction in the % cBAC. As a result of the co-digestion with red cabbage, the % digestive bioaccessibility of total carotenoids from the tested vegetable mixtures (except with baby spinach) was decreased (**Table 14**), but the % intestinal cellular bioaccessibility of total carotenoids from every tested vegetable mixture was ultimately increased by 46-191% (**Table 16** and **Table 17**). The improvement of the % intestinal cellular bioaccessibility of total carotenoids might have contributed to the synergistic effects on cellular antioxidant and anti-inflammatory activities seen in some of the mixed vegetables.

8.5. Conclusion

The cellular antioxidant and anti-inflammatory effects that resulted from the co-digestion of red cabbage with carrot, cherry tomato and/or baby spinach were either additive or synergistic as compared to that resulting from the digestion of single vegetables. The enhanced biological effects seen in the co-digested vegetables could be the result of complex interactions between multiple phytochemical components and/or their metabolites. The remarkable increase in the % intestinal cellular bioaccessibility of carotenoids from the co-digested vegetables could have also partly contributed to the observed synergistic bioactivities. Among the tested vegetable combinations, red cabbage and baby spinach appeared to be a potential mixture for co-ingestion because

they provide a great source of highly bioaccessible carotenoids and imparted synergistic effects on all tested bioactivities including the cellular anti-oxidation, and the suppression of IL-8 secretion and NO production. Further study should be carried out to verify these effects of the co-digestion of red cabbage and baby spinach *in vivo* because *in vivo* human studies are essential to substantiate/confirm any *in vitro* results. An understanding of the effect of food co-ingestion would help consumers to make better food choices and attain the most benefit out of their diets. This is also important for the food industry in the development of new food products.

CHAPTER 9

Overall Conclusion and Recommendations

This research project was conducted to investigate the interactive effects of different pairs of common dietary anthocyanins and carotenoids in their pure forms on antioxidant and anti-inflammatory activities using both chemical and cellular models. The impact of anthocyanins on the cellular uptake of carotenoids and the relevance of the uptake interference to the combined bioactivities of the tested phytochemicals was also studied. In addition, the project also investigated the interactive effects of anthocyanins and carotenoids within the food matrix after the *in vitro* co-ingestion of vegetables that are rich in these compounds.

Changes in the *in vitro* bioaccessibility of anthocyanins and carotenoids as well as the resultant cellular activities as a result of vegetable co-digestion were reported. A simulated *in vitro* gastrointestinal digestion followed by intestinal cellular uptake using a human carcinogenic Caco-2 cell model was used to study the interactive effect of anthocyanins and carotenoids on their bioaccessibility and bioactivity when red cabbage was co-digested with different carotenoid-containing vegetables such as carrot, cherry tomato and/or baby spinach.

Each of the anthocyanins including cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and petunidin-3-*O*-glucoside was paired with each of the following carotenoids: β-carotene, lutein and lycopene at different ratios (1:1, 1:3 and 3:1, total concentration of 10 μM). The combined activity of each anthocyanin-carotenoid mixture on liposome peroxidative inhibition, lipoxygenase inhibition, cellular antioxidant activity and the inhibition of cellular pro-inflammatory mediators (IL-8 and NO) was evaluated and

compared with the expected additive activity of the two phytochemical components to determine the mode of the interaction. The results show that in chemical systems all tested combinations of anthocyanins and carotenoids (1:1 ratio) provided antagonistic effect on the inhibition of liposome peroxidation and had lower lipoxygenase inhibitory activity than the corresponding carotenoids. On the other hand, none of the tested anthocyanin-carotenoid mixtures showed synergy in cellular antioxidant activity; some of the mixtures showed an antagonistic effect. When β-carotene was paired with pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside or petunidin-3-*O*-glucoside at the 1:3 ratio, the cellular antioxidant activity was reduced by 26-57% of the expected additive activity. All mixtures of lycopene with the anthocyanins at the 1:1 and 1:3 ratios, except with peonidin-3-*O*-glucoside at the 1:3 ratio, also showed a reduced cellular antioxidant activity by 25-43% of the expected additive activity. Neither synergy nor antagonism in cellular antioxidant activity was seen in any tested lutein-anthocyanin mixtures.

With regards to anti-inflammatory effect, synergy in reducing the secretion of pro-inflammatory IL-8 was observed in some of the anthocyanin-carotenoid mixtures, for example: LUT-MG and LYC-PNG mixture (at all tested ratios), and LYC-MG and LYC-PTG mixture (at 1:1 and 3:1 ratios). Some other mixtures, however, showed antagonistic effect, such as β-CAR-CG, β-CAR-PLG, LUT-CG, and LUT-DG. In addition, there was no synergy in the suppression of NO production seen in any combinations of anthocyanins and carotenoids tested in this study.

The interference of anthocyanins with the cellular uptake of carotenoids was also studied and the relevance of the uptake interference to the combined bioactivities of the compounds was observed. The impact of the tested anthocyanins on the uptake of different carotenoids was different. β-carotene uptake was increased significantly by

70% in the presence of CG or PTG, and by 130-200% in the presence of PNG, MG or PLG. The increase of β-carotene intracellular content to a certain level, however, triggered pro-oxidant activity of β-carotene which possibly led to a reduction in cellular antioxidant activity and antagonistic effect seen in some of the β-carotene-anthocyanin combinations. On the contrary, the anthocyanins did not interfere with the uptake of lutein but significantly impaired the uptake of lycopene. These results are relevant to the combined cellular antioxidant activity of the compounds. Lutein uptake was not affected by the presence of anthocyanins and their combined cellular antioxidant activity was additive. Likewise, lycopene uptake was reduced when anthocyanins were present and their combined cellular antioxidant activity was antagonistic.

The second major part of the project aims to understand the interactive effects on bioaccessibility and bioactivity of anthocyanins and carotenoids from co-digested vegetables with and without added salad dressing. Different combinations of anthocyanin-rich red cabbage with carotenoid-rich carrot, cherry tomato and/or baby spinach underwent *in vitro* gastrointestinal digestion and the digestive bioaccessibility of the major anthocyanins and carotenoids was measured and compared with that resulting from the digestion of individual vegetables. Major anthocyanin compounds identified in the red cabbage used in this study are cyanidin-3-(*p*-coumaroyl)-diglucoside-5-glucoside, cyanidin-3-(feruloyl)-diglucoside-5-glucoside and cyanidin-3-(sinapoyl)-diglucoside-5-glucoside, among which the digestive bioaccessibility of the first two compounds increased significantly after the co-digestion of the vegetables. The % digestive bioaccessibility of total anthocyanins was significantly enhanced when red cabbage was co-digested with baby spinach, with cherry tomato, or with a mixture of carrot, baby spinach and cherry tomato when the salad dressing was or was not added in. In contrast, the co-digestion with red cabbage decreased the digestive

bioaccessibility of total carotenoids, except from baby spinach. The digestive bioaccessibility of the carotenes such as α-carotene, β-carotene and lycopene significantly decreased whereas that of the xanthophyll lutein remained unchanged. Although the vegetable co-digestion was seen to reduce the % digestive bioaccessibility of carotenoids after the digestion, it ultimately increased the % intestinal cellular bioaccessibility of carotenoids.

The resultant cellular bioactivity appeared to be enhanced as a result of the increased intestinal cellular bioaccessibility of total carotenoids. Synergy in cellular antioxidant activity was seen in all co-digested vegetables. The co-digestion of vegetables also resulted in additive or synergistic effects on anti-inflammatory activities. The complex interaction between phytochemicals absorbed by the cells and/or their metabolites and the increase in carotenoid cellular uptake would contribute to the enhancement of the cellular bioactivities observed in this study. Among the vegetable mixtures studied, red cabbage-baby spinach was a potential combination that provided highly bioaccessible carotenoids and anthocyanins after the co-digestion.

In conclusion, this study has provided considerable new information about the interactions between phytochemicals, and has shown a great level of insights to several important aspects of the co-digestion of vegetables. However, the study has also had some pitfalls and limitations due to time constraint and cost-related issues. One of the limitations is that the measurement of anti-inflammatory activity was based only on the secretion of two biomarkers including IL-8 and NO because the kits for the analysis of pro-inflammatory markers are very costly. The analysis would be more comprehensive if more pro-inflammatory markers and/or gene expression of molecular pathways involving in inflammation could have been included in the study. The second limitation of our study is that the effects of vegetable co-digestion were evaluated using only one

combined ratio of vegetables (1:1). More combined ratios should be tested in future studies to represent different dietary intakes of vegetables in human diets. Another limitation is that the simulated digestion model used in our study did not include colonic fermentation, so we were unable to assess the involvement of microbial metabolites generated by the colon microbiota.

Recommendations for future studies:

The findings of this research expand the knowledge of the interactive effects of food phytochemicals on their bioaccessibility and bioactivities. This research will ultimately lead understanding how to maximise health benefits by combination of appropriate fruits and/or vegetables in one's diet. In addition, this research provides an understanding of phytochemical combinations and the appropriate concentrations that can lead to designing foods or supplements with better targeted functions and absorption.

- Explore the phytochemical interaction to evaluate phytochemical uptake and bioactivity interaction altogether in order to understand how the uptake interference would affect the bioactivity interaction.
- Further studies on co-digestion of fruits and vegetables should include different combining ratios to better reflect the typical dietary intakes of the population which may then provide greater insights in terms of phytochemical bioaccessibility and bioactivity compared to singly digested fruits/vegetables.
- Studies should also focus on the effects of co-ingestion of other food materials (macro- and micro-nutrients) that are often consumed together in human diets. Foods contain a variety of different phytochemicals and other nutrients, so the interaction between phytochemicals can be influenced by their interaction with other food components. It is more complex to understand the effects and the

mechanisms of phytochemical interaction in this context. Extensive efforts are required in future studies in order to completely unravel the complex interaction between food phytochemicals in human diets *in vitro* and *in vivo*.

- The gut microbiota play a key role in modulating the production, bioavailability and, thus, the biological activities of phytochemical metabolites. Thus, it is desirable for future studies to incorporate colonic fermentation in the digestion model to investigate how the interaction between phytochemical gut metabolites generated by the colonic microbiota would ultimately be beneficial to human health.
- Food processing also plays a role in food digestibility and food component bioaccessibility. Future studies, therefore, should also focus on the impact of food processing such as cooking and fermentation of mixed fruits/vegetables on the bioaccessibility of phytochemicals and the resultant bioactivity.
- Development of sequential intestinal and liver models to study the bioavailability of these compounds will contribute to a better understanding of the absorption of these compounds leading to understanding of their requirements for humans.
- Development of human organoids models (3-D cell models) instead of using 2-D tissue cultures to evaluate phytochemical absorption and metabolism would be more desirable for such studies.
- Animal studies and/or human clinical trials are essential to substantiate and confirm the findings observed in any *in vitro* work.

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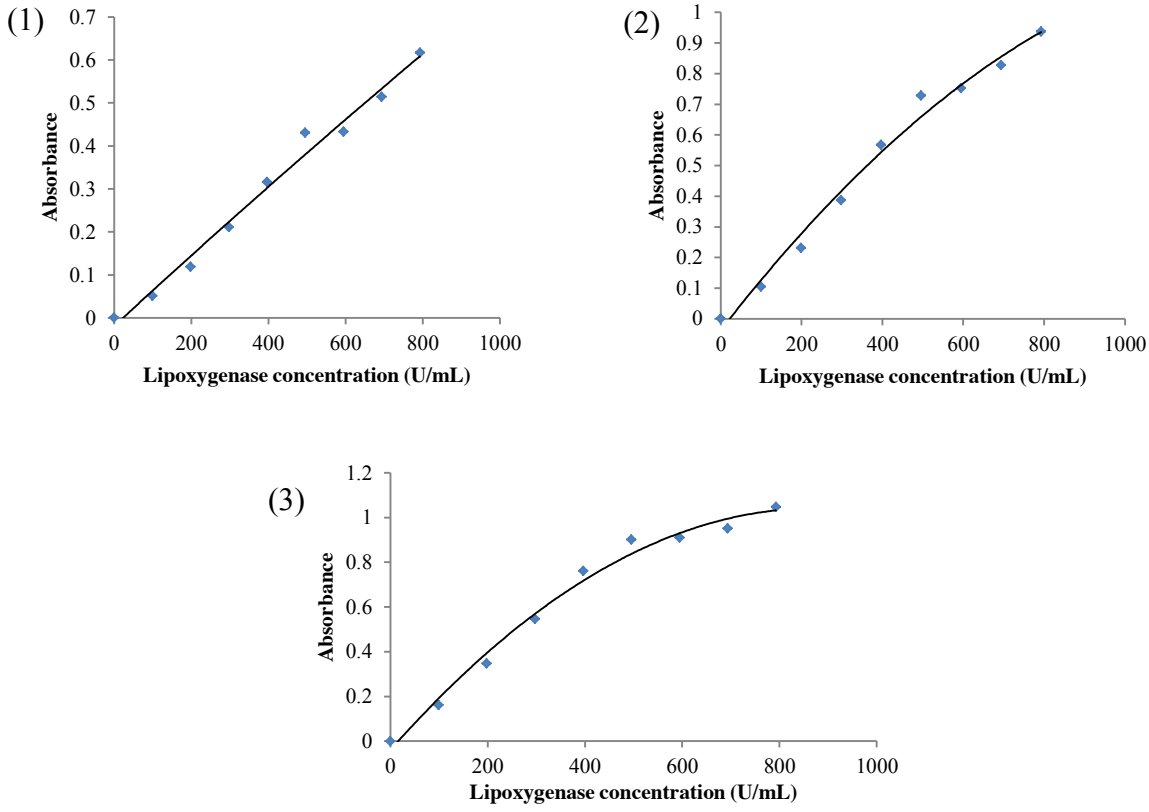
APPENDICES

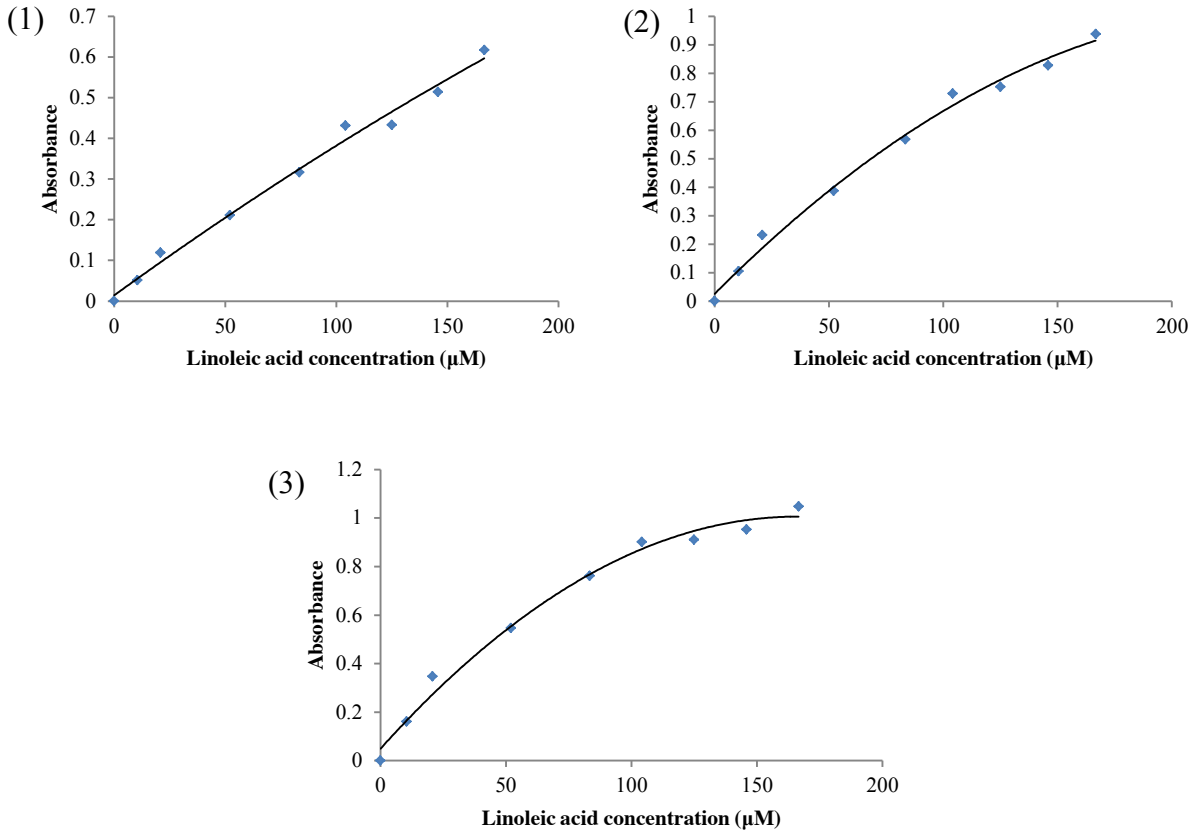
Appendix 1. Caco-2 cell viability in the presence of β-carotene and some anthocyanins compared to the control*.

Compound	**Cell viability (%)**	
	1 µM	**10 µM**
β-carotene	134.3 ± 4.8	97.3 ± 13.6
cyanidin-3-*O*-glucoside	135.3 ± 14.1	129.1 ± 23.9
delphinidin-3-*O*-glucoside	129.8 ± 5.4	133.3 ± 18.8
malvidin-3-*O*-glucoside	145.8 ± 9.6	123.9 ± 19.0
peonidin-3-*O*-glucoside	120.7 ± 11.7	97.0 ± 21.1

*Control included growth medium with none of the compounds.



Appendix 2. Lipoxygenase kinetic reaction at various concentrations of lipoxygenase after (1) 1 min, (2) 2 min and (3) 3 min of reaction time. The concentration of linoleic acid was kept constant at 52 µM.



Appendix 3. Lipoxygenase kinetic reaction at various substrate concentrations of linoleic acid after (1) 1 min, (2) 2 min and (3) 3 min of reaction time. The concentration of lipoxygenase was kept constant at 400 U/mL.

Appendix 4. Cellular antioxidant activity (% control) of individual carotenoids and anthocyanins at different concentrations.

Cellular antioxidant activity (% control)	**Concentration (µM)**		
	2.5	**5**	**7.5**
β-CAR	62.7 ± 3.9	61.5 ± 10.4	40.7 ± 16.0
LYC	47.6 ± 11.9	53.4 ± 8.4	52.9 ± 13.0
LUT	36.8 ± 5.9	34.6 ± 7.7	41.3 ± 10.2
CG	6.5 ± 2.3	22.4 ± 6.8	29.5 ± 7.1
DG	4.6 ± 3.2	2.5 ± 1.2	21.4 ± 4.3
MG	15.5 ± 1.5	10.5 ± 3.7	16.7 ± 5.0
PNG	9.4 ± 6.4	10.1 ± 2.7	11.3 ± 2.5
PLG	7.6 ± 2.9	6.6 ± 2.5	12.0 ± 2.7
PTG	7.4 ± 2.1	7.1 ± 3.2	12.0 ± 6.6

Data are presented as mean ± standard deviation of at least three replicates.
β-CAR: β-carotene, LYC: lycopene, LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

Appendix 5. IL-8 secretion by Caco-2 cells (pg/mL) in the presence of individual carotenoids and anthocyanins at different concentrations.

IL-8 secretion (pg/mL)	**Concentration (μM)**		
	2.5	**5**	**7.5**
β-CAR	703.6 ± 257.4	718.2 ± 178.7	684.5 ± 140.5
LYC	943.0 ± 229.3	1000.3 ± 73.1	751.9 ± 146.1
LUT	786.7 ± 78.7	872.1 ± 87.7	636.1 ± 127.0
CG	750.8 ± 187.7	811.5 ± 185.4	871.0 ± 143.9
DG	919.4 ± 215.8	710.3 ± 170.8	1001.4 ± 125.9
MG	675.5 ± 180.9	728.3 ± 183.2	782.2 ± 105.6
PNG	699.1 ± 173.1	560.8 ± 120.3	828.3 ± 345.0
PLG	778.9 ± 222.5	755.3 ± 264.1	914.9 ± 205.7
PTG	621.5 ± 230.4	768.7 ± 154.0	1091.3 ± 198.9

Data are presented as mean ± standard deviation of at least three replicates.
β-CAR: β-carotene, LYC: lycopene, LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

Appendix 6. NO secretion (μM) by Caco-2 cells in the presence of individual carotenoids and anthocyanins at different concentrations.

NO secretion (μM)	**Concentration (μM)**		
	2.5	**5**	**7.5**
β-CAR	17.2 ± 6.1	12.9 ± 5.4	18.1 ± 2.6
LYC	11.1 ± 1.1	9.9 ± 5.9	20.1 ± 1.6
LUT	11.9 ± 1.2	7.5 ± 1.4	7.5 ± 2.2
CG	16.2 ± 12.0	11.8 ± 3.3	13.1 ± 2.3
DG	15.6 ± 8.2	11.2 ± 3.4	11.8 ± 5.3
MG	14.0 ± 10.0	10.8 ± 2.1	12.8 ± 3.6
PNG	12.5 ± 7.3	11.9 ± 3.6	11.5 ± 3.1
PLG	10.9 ± 6.0	17.6 ± 1.7	13.0 ± 4.0
PTG	12.9 ± 7.0	16.6 ± 3.9	12.3 ± 1.9

Data are presented as mean ± standard deviation of at least three replicates.
β-CAR: β-carotene, LYC: lycopene, LUT: lutein, CG: cyanidin-3-*O*-glucoside, DG: delphinidin-3-*O*-glucoside, MG: malvidin-3-*O*-glucoside, PNG: peonidin-3-*O*-glucoside, PLG: pelargonidin-3-*O*-glucoside, PTG: petunidin-3-*O*-glucoside.

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2. Phan, M. A. T., Bucknall, M., & Arcot, J. (2018). Interactive effects of β-carotene and anthocyanins on cellular uptake, antioxidant activity and anti-inflammatory activity in vitro and ex vivo. *Journal of Functional Foods, 45*, 129-137.
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