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Phytonutrient bioaccessibility and metabolism *in vitro* and *in vivo*

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Abstract

Mangoes and bananas, two of Queensland's major tropical crops are sources of bioactive phytonutrients linked to dietary health based on antioxidant, cardio-protective and vasodilatory properties. Bioaccessibility of macro-, micro- and phytonutrients from whole foods is first determined by mastication followed by gastrointestinal liberation from food matrices (cell wall and membrane). Intact plant cell walls effectively encapsulate cellular components e.g. starch, lipid, protein, carotenoids and polyphenols, physically preventing entry of the mammalian digestive system, which is unable to enzymatically break down plant cell walls. Once bioaccessible, 'free' nutrients are available for absorption, while the unreleased fraction travels to the colon for fermentation. Nutritional recommendations are usually based on extracted contents of raw plant material; therefore true dietary concentrations have not been well established. This thesis focuses on studying the effects of sequential digestive processing of mango (*Kensington Pride*) and banana (*Cavendish*) flesh in the mouth, stomach, small intestine and colon using *in vitro* and *in vivo* approaches.

In vitro nutrient bioaccessibility studies often favour mechanical processing over mastication. Investigating the effect of mastication (Chapter 3) in mango demonstrated microstructural changes, conferring a range of chewed particle sizes (large particle clusters to cell fragments). Actions not replicable with a cutting blade i.e. compression, squashing and formation of bolus were observed in these masticated particles, collectively enhancing encapsulation of carotenoids. Teeth cutting or slicing also occurred simultaneously, rupturing cell walls and releasing cellular contents. Whilst there was a (small) particle size effect on bioaccessibility, this may be secondary for soft (mango) tissues, in contrast to previous reports that a single robust carrot cell wall appeared to be sufficient to prevent bioaccessibility. There was incomplete carotenoid bioaccessibility from solid chewed mango particles (20-50%) and puree (65-75%) after simulated gastrointestinal conditions. Presence of intact mango cells and vascular fibres after *in vitro* digestion indicated acidic hydrolysis or other *in vitro* digestion conditions did not have a major role in breaking down cell walls, and established that unreleased and/or unabsorbed nutrients are expected to survive to the colon because they were shown not to be liberated from the food matrix.

Mango and banana cell structures which survived *in vivo* mastication and *in vitro* gastrointestinal digestion (Chapter 4 part A), were fermented *in vitro* with porcine faecal

inoculum for 48 h. This involved fermentation of non-fibrous cell walls, thereby releasing the effectively encapsulated cell contents for possible metabolism by microbiota. Cumulative gas, short chain fatty acids and ammonia production were greater in mango than banana fermentation. Microscopic and spectroscopic analysis showed that this was due to a major fermentation-resistant starch fraction in banana, which was absent in mango. This study demonstrated distinctive differences in fermentation kinetics between banana and mango, due to preferential degradation of (parenchyma) fleshy cells over resistant starch in banana, and over thick cellulosic vascular fibres in mango. Upon disintegration of fruit matrices, phenolic compounds were consequently exposed to intensive faecal-microbiota metabolism beyond those of human endogenous enzymes. UPLC-PDA and UHPLC-Q-ToF-MS profiles (Chapter 4 Part B) revealed degradation of intact polyphenols within 8-24 h and concomitant formation of intermediate catabolites within 4-8 h.

The time available for nutrient digestion and fermentation in the gastrointestinal tract is determined by the passage rate of intestinal contents, which has often been overlooked in *in vivo* digestibility studies. The influence of dried mango puree (containing pectin as a component of edible pulp) and purified apple pectin on passage time, was examined using pigs as a human model (Chapter 5 Part A). Mango puree and purified apple pectin delayed gastric fractional outflow to the small intestine, reflecting an increased water holding capacity (and corresponding lower dry matter content) of these diets. This increased water holding capacity however reduced overall retention time, leading to a faster passage rate in the small intestine and colon. These results provided insights to the dynamic movement of digesta, which were found on average, to be of 3 h-stomach, 0.3 h-duodenum, 0.5-1.6 h-jejunum, 0.8 h-ileum, 2.3 h-caecum, 7 h-proximal colon, 4.8 h-mid colon and 3.7 h-distal colon. This is important information for comparing the results of *in vitro* bioaccessibility/metabolism studies (where time is a variable) to *in vivo* situations where residence time/passage rate is determined by interactions of food with animals and/or humans. Using samples from the same experiment, the possibility of monitoring mango polyphenol metabolism and uptake along the digestive tract and into the blood stream was assessed. Although it was possible to identify classes of compounds undergoing distinctive digestive processing, the complexity of chromatographic profiles and corresponding mass spectra precluded detailed molecular identification.

This study has evaluated aspects of digestive processing of two archetypal fruits to illustrate the importance of combining *in vitro* and *in vivo* studies to achieve a more complete perspective, and has thereby contributed to the process of defining mechanisms of fruits and vegetables contribution to health and well-being as suggested by many epidemiological studies.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

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Low, D., D'Arcy, B., & Gidley, M. (2015). Mastication effects on carotenoid bioaccessibility from mango fruit tissue. *Food Research International, 67*, 238-246.

Conference abstracts

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Dorrain Low (PhD Candidate)	Designed experiment: Selection of sieve sizes and extraction methodology (100%) Performed experiments (100%) Statistical analysis of data (100%) Analysed data (100%) Wrote paper (100%)
Bruce D'Arcy	Designed experiment: Selection of coarse and fine chewers (50%) Consultation for data analysis (30%) Edited paper (30%)
Michael Gidley	Designed experiment: Selection of coarse and fine chewers (50%) Consultation for data analysis (70%) Edited paper (70%)

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Chapter 4 Part A. Fermentation kinetics of masticated and 'digested' mango and banana during colonic fermentation *in vitro.*

Contributor	Statement of contribution
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Chapter 5 Part A. Passage of gastrointestinal contents in grower pigs as affected by pectin.

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Mark Hodson	Performed experiment: UHPLC-Q-ToF-MS analysis and data extraction (80%) Consultation for data analysis (5%)
Bruce D'Arcy	Consultation for data analysis (25%) Edited chapter (50%)
Gabi Netzel	Consultation for extraction methodologies of different sample matrices (100%) Consultation for data analysis (10%)
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Keywords

Carotenoids, polyphenols, phenolic acids, resistant starch, pectin, cellulose, plant cell wall, bioaccessibility, colonic fermentation, microbial metabolism.

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List of Abbreviations

ca	circa
cv	cultivar
KP	Kensington Pride
QLD	Queensland
NSW	New South Wales
α	alpha
β	beta
%	percent
°C	degree Celsius
cm	centimeters
mm	millimeters
μm	micrometers
nm	nanometers
kg	kilograms
g	grams
mg	milligrams
μg	micrograms
h	hours
min	minutes
s	seconds
mM	milimolar
μM	micromolar
L	liter
mL	milliliter
μL	microliter
ppm	parts per million
rpm	rounds per minute
g	gravity force
i.d.	internal diameter
MHz	megahertz
kHz	kilohertz
Hz	hertz
kV	kilovolt
eV	electron volt
GAE	gallic acid equivalents
RAE	retinol activity equivalent
FW	fresh weight
DM	dry matter
BW	body weight
DMCV	dry matter cumulative volume
SCFA	short chain fatty acids
BCR	branched chain ratio
AAE	acetic acid equivalents
NH_3	ammonia
O_2	oxygen
CO_2	carbon dioxide
JV	jugular vein
HPV	hepatic portal vein
DE	degree of esterification
Cr	chromium
AIA	acid insoluble ash

Yb	ytterbium
Ce	cerium
La	lanthanum
Co	cobalt
SI	small intestine
PC	proximal colon
MC	mid colon
DC	distal colon
WHC	water holding capacity
RO	reverse osmosis
LPH	lactase phloridzine hydrolyase
SGLT1	sodium-dependent glucose transporter
TCR	T cell receptor
UWL	unstirred water layer
LDL	low-density lipoprotein
HDL	high-density lipoprotein
TAG	triacylglycerol
NEPP	non-extractable polyphenols
PPO	polyphenol oxidases
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoulene
EDTA	ethylenediamine tetracetic acid
TFA	trifluoroacetic acid
SPE	solid phase extraction
HPLC-PDA	high performance liquid chromatography-photodiode array detector
HPLC-MS	high performance liquid chromatography-mass spectrometry
UPLC	ultra performance liquid chromatography
ESI	electrospray ionisation
UV	ultraviolet
Vis	visble
TIC	total ion chromatogram
TCC	total compound chromatogram
GC	gas chromatography
TMS	trimethylsilyl
FC	folin-ciocalteu
m/z	mass-to-charge ratio
PBS	phosphate buffer saline
PTFE	polytetrafluroethylene
CLSM	confocal laser scanning microscopy
CP/MAS	cross polarization magic angle spinning
NMR	nuclear magnetic resonance
APTS	3-aminopropyl-trimethoxysilane
H	proton
C	carbon
Eq	equation

Chapter 1. Introduction

1.1. Project Significance

Diets rich in fruits and vegetables have been reported to exert positive health benefits based on biological, nutritional and epidemiological studies of diet and health outcomes, which may be linked to a range of antioxidant, cardio-protective and vasodilatory properties, as well as their vitamin and mineral contents. Although fruits are known to be an essential part of a healthy diet, evidence is primarily based on epidemiological studies, which do not permit underlying mechanisms to be determined. An aspect of consuming whole fruit (pieces) as obtained from e.g. mango and banana is that phytonutrients are likely to be released slowly through the digestive process and may survive to the colon. Fruits such as banana and mango contain a wide range of phytonutrients encapsulated in a cellular tissue structure. Consumption of fresh fruit (pieces) may lead to a more prolonged release of phytonutrients compared with, for example, juices. This project examined the behaviour of a range of phytonutrients such as carotenoids, flavonoids, phenolic acids, and macronutrients such as plant cell walls and starch from banana and mango fruits using models for the small intestine and colon, in order to understand those factors that may promote or undermine the nutritional benefit of these tropical crops. Using a combination of *in vitro* data and the most relevant animal model (pigs) is expected to provide novel scientific information and technical evidence to explain the benefits of eating banana and mango- two key products of the Queensland tropical fruit industry.

Bioaccessibility of phytonutrients from whole foods is first determined by mastication and then gastrointestinal liberation from the encapsulating food matrix (cell walls and membranes). Once released and bioaccessible, the 'free' phytonutrients are available either for small intestinal absorption and/or further colonic-microbial breakdown of unabsorbed phytonutrients. However, truly bioaccessible and bioavailable phytonutrient concentrations in fruits and vegetables have not been well established, and nutritional recommendations are usually based on chemically extracted contents of raw plant material, rather than the body's ability to access these phytonutrients. In particular, the chewing of intact fruits and vegetables is expected to lead to a range of particle sizes in the swallowed food bolus. The consequences of this for the rate and extent of phytonutrient release in the stomach and small intestine, as well as the rate and extent of fermentation in the large intestine remain unexplored. Along with increasing evidence of the importance of the close interaction between the host and its microbiota in health and disease (Van den Abbeele, Van de Wiele, Verstraete, & Possemiers, 2011), specific

changes in the colonic microbiota composition and/or activity are thought to contribute to observed health effects upon polyphenol intake (Bialonska et al., 2010; Queipo-Ortuno et al., 2012; Williamson & Clifford, 2010). *In vitro* gut models are the preferred choice to study polyphenol-induced modulation of colonic microbiota and the persistence of change (Bolca, Van de Wiele, & Possemiers, 2013).

Presently, the chemistry of phytonutrients in mango and banana are not known with certainty, and no *in vitro* release studies of phytonutrients after mastication have been reported. Consequently, digestion in the gastric and small intestine, and fermentation in the colon were evaluated using a combination of laboratory models, human chewing studies and a pig feeding trial. The possible mechanisms limiting bioaccessibility that were considered are: (1) trapping within intact cell walls that prevent passage or release (particularly of carotenoids), (2) binding to plant tissue components, for example, cells walls (particularly of polyphenols), or (3) slow but essentially complete release.

1.2. Project Aims
To investigate the effects of sequential digestive processing of mango (*Kensington Pride*) and banana (*Cavendish*) flesh in the mouth, stomach, small intestine and colon, using a combination of *in vitro* and *in vivo* models.

1.3. Project Objectives
1.3.1. To assess the mechanisms restricting the bioaccessibility of the phytonutrients, and to monitor the roles of macronutrients such as plant cell walls and starch during the digestive process *in vitro* (Chapter 3 and Chapter 4 Part A).

1.3.2. To examine metabolic biotransformations of mango and banana phytonutrients *in vitro* (Chapter 4 Part B).

1.3.3. To examine the passage of a mango ingredient and the main soluble dietary fibre in both banana and mango (pectin) through the digestive tract *in vivo* using a pig model (Chapter 5 Part A).

1.3.4. To assess the practicality of examining metabolic biotransformations of mango phytonutrients within complete diets *in vivo* (Chapter 5 Part B).

Chapter 2. Literature Review

2.1. Tropical fruit

Mango and banana constitute the major tropical fruits of Australia's horticulture industry, with the production of these two fruits mainly concentrated in Queensland.

2.1.1. Mango

Mango, a large fleshy drupe, sometimes fibrous, belongs to the species *Mangifera indica* (Ian, 2006). It is a climacteric fruit where ripeness coincides with the respiratory peak. Its eating quality is closely associated with the onset of the climacteric peak, when fruits change in colour, texture and aroma, and starch is converted into sugars. The ripening rate changes easily with storage temperature and the quality of mango is affected by temperature and relative humidity during ripening (Lillian, 2006).

In Queensland, dry areas with annual rainfall of ~1000 mm (occurs mostly during January to March) and good sun exposure from August to December, are favoured for mango growing. The growing season in Australia stretches from mid-spring to autumn (June to August), with peak production during December and January depending on the regions of growing areas (AAG, 2006). Queensland is the major producing state in Australia, accounting for 70% of the volume of domestic production, with the Northern Territory having 20% of production. The mango season epitomises a Queensland summer and large trees can be found planted on street footpaths in almost every town throughout the state (Australian Mango Industry Association, 2009). Other production areas include New South Wales (NSW) and Western Australia. Mangoes are usually picked green. Quality is improved by controlled temperatures between 15 and 21°C, with the best ripening temperatures from 21-24°C. Ethylene treatment allows green mangoes to develop full colour in seven to ten days depending on the degree of maturity, whereas untreated mangoes require ten to fifteen days (see Fig 2.1 for colour guide).
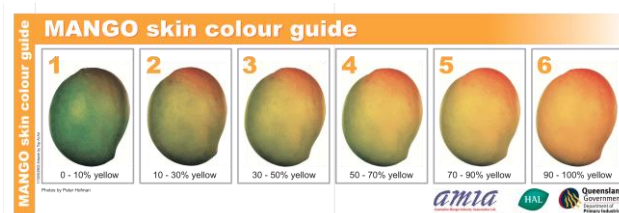
Figure 2.1. Mango skin colour guide at different stages of ripening (Primary Industries & Fisheries, 2012).

Table 2.1. Growth characteristics of some commercial mango cultivars.

Cultivar	Origin	Skin colour	Fibre	Characteristics
Ataulfo/ Manila	Mexico, Philippines	Bright yellow	Low	Small oval fruit, smooth, firm flesh with a creamy flavour, vibrant yellow flesh. Skin turns to a deep golden colour and small wrinkles appear when fully ripe.
Calypso	Australia	Deep orange-red blush on yellow	Low	Firm flesh with little fibre, full sweet flavour, small seed.
Haden	Mulgoba, India, Mexico	Red blush on yellow or green with small white dots	Abundant	Medium to large fruit with an oval shape, firm yellow flesh with fine fibres, rich and aromatic overtones.
Honey Gold	Australia	Golden apricot-yellow	Low	Medium size fruit, firm and fibreless flesh with a honey-yellow colour, rich sweet and aromatic flavour, small seed.
Keitt	Mulgoba, Australia	Small pink blush on green	Little	Large fruit with an oval shape, firm juicy yellow flesh with a very sweet flavour.
Kent	Mexico, Ecuador, Peru, Australia	Small red blush on dark green	Low	Large oval fruit, juicy, tender flesh with a rich flavour.
Tommy Atkins	Mexico, Eucador, Brazil, Peru	Dark red blush on green	Fibrous	Medium to large fruit with an oval shape, firm flesh due to fibres throughout, mild flavour.
Kensington Pride	Australia	Yellow with pink blush	Low	Small to medium size fruit, oval to round shape, golden and fibreless flesh, soft, sweet and tangy flavour.
R2E2	Australia	Orange-red blush on green	Low	Large round fruit, firm flesh and milder flavour, lemon-yellow flesh. Has a long shelf-life.

Images and information adapted from (Australian Mango Industry Association, 2009; Exotic Fruit Market, 2012; Horticulture Australia Limited, 2008; National Mango Board, 2009).

Currently around 70% of the trees grown commercially in Australia are *Kensington Pride*, also known as *Bowen* mangoes, with the other established cultivars including *Irwin, Nam Dok Mai, R2E2, Kent, Tommy Atkins* and *Calypso* (Australian Mango Industry Association, 2009). Extensive plant breeding has generated hundreds of cultivars, the fruits of which show a pronounced diversity in size, shape, colour, flavour, seed size and composition

(Berardini et al., 2005b). Table 2.1 details the varieties of commercial mango grown around the world. The typical yellow-orange colour of ripe mango fruit is due to the presence of carotenoids (Ornelas-Paz, Failla, Yahia, & Gardea, 2008) with β-carotene being the most abundant (see section 2.2.1.).

2.1.2. Banana

Banana is a general term that refers to all wild species, landraces and cultivars belonging to the family *Musaceae*, genus *Musa* (Robert & Odilo, 2011). The banana is a tropical and subtropical herbaceous plant consisting of an underground corm, and a trunk (pseudostem) comprised of concentric layers of leaf sheaths. Bananas do not always attain best eating quality on the plant. The fruit is picked green and then matures (colour stage 1; Fig 2.2). Controlled ripening permits sufficient time for transport and marketing before they ripen (Mercadante & Rodriguez-Amaya, 1998). The peel colour index, which ranges from 1 to 7 (1 = green; 2 = with trace of yellow; 3 = slightly more yellow than green; 4 = more yellow than green; 5 = green tips at end; 6 = completely yellow; 7 = yellow peel with brown spots of sugar), is taken into consideration to estimate the degree of ripening (Isopan, 2011). Peel colour change are largely due to degradation of chlorophyll or the unmasking of carotenoids rather than carotenoid synthesis (Mohapatra, Mishra, & Sutar, 2010). The state of ripeness is indicated by peel colour; best eating quality is considered when solid yellow colour is specked with brown.
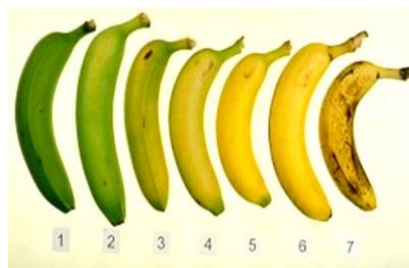
Figure 2.2. Banana colour chart at different stages of ripening (Isopan, 2011).

In terms of world production, banana (genus *Musa*) is one of the top three tropical fruits, along with citrus fruits and pineapple. It is estimated that 97% of bananas traded internationally are of the *Cavendish* group of varieties (Banana Link, 2011). In Australia, bananas are considered the most popular fruit and are consumed steadily throughout the year. The banana industry is one of the largest fruit growing industries, and hence an important contributor to the economies of rural communities in banana growing areas. The banana fruit is amongst Australia's top ten supermarket lines; approximately 28 million bananas are consumed every week, which represents 13 kg per person per year, an

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equivalent of 60-70 bananas per person (Australian Bananas, 2011). They are principally grown in the tropics of North Queensland, now centered around Innisfail and the Tully region. Other smaller scale growing areas include sub-tropical Northern NSW, Southern Queensland, and Carnarvon in Western Australia.

Cavendish bananas account for about 85% of Australian banana production (Australian Bananas, 2011). In recent times, leading supermarket chains in the Australian marketplace have suggested that overall banana sales could be significantly increased by broadening the range of banana cultivars sold (Horticulture Australia Limited, 2011). *Ladyfinger, Ducasse, Sucrier* and *Red Dacca* are some of the other cultivars regularly sold in local markets. Table 2.2 summarises the characteristics of some commercial banana cultivars.

Table 2.2. Growth and characteristics of some commercial banana varieties.

Group	Subgroup	Examples of common cultivar names[a]	Characteristics
AA	*Sucrier*	*Sucrier, Lady's Finger*	Small fruit (8-12cm), thin golden skin, light orange firm flesh, very sweet, 5-9 bands per bunch, 12-18 fingers per hand.
	Lakatan	-	Medium to large straight fruit (12-18cm), golden yellow, light orange flesh, firm, dry, sweet and aromatic, 6-12 hands per bunch, 12-20 fingers per hand.
AAA	*Gros Michel*	*Gros Michel*	Medium to large fruit, thick skin, creamy white flesh, fine textured, sweet and aromatic, 8-12 hands per bunch.
	Cavendish	*Giant Cavendish* (e.g. *Williams*), *Grande Naine,*	Medium to large fruit, yellow skin, white to creamy flesh, melting, sweet, aromatic, 14-20 hands per bunch, 16-20 fingers per hand.
AAB	*Silk*	*Sugar*	Small to medium (10-15cm), thin yellow orange skin, white flesh, soft, slightly subacid, 5-9 hands per bunch, 12-16 fingers per hand, skin frequently has blemishes.
	Plantain	*French, Pisang Ceylan*	Yellow skin, creamy orange firm flesh, few hands per bunch.
ABB	*Bluggoe*	*Bluggoe, Mondolphin*	Medium to large cooking banana, thick coarse skin turns brownish-red when ripe, orange creamy flesh, starchy, 7 hands per bunch.
BBB	*Saba*	-	Stout, angular, medium to large cooking banana (10-15cm), thick yellow skin, creamy white flesh, fine textured, 8-16 hands per bunch, 12-20 fingers per hand.

[a]*Cultivar names varying depending on country (Robert & Odilo, 2011).*

A distinction can be made between the 'dessert' or sweet bananas (*M. acuminata*), which are ripened and best eaten fresh, and 'cooking' or starchy bananas and plantains (*M.*

balbisiana), which are usually cooked or processed. The yellow dessert banana cultivars are the focus of this thesis. Dessert bananas, in general, show great diversity in terms of plant stature, fruit size and colour. During ripening of the banana fruit, the flesh colour changes from a typical opaque white product with high starch content to a soft yellow or orange product (Fig 2.3) as yellowing of the peel intensifies (Englberger, Darnton-Hill, Fitzgerald, & Marks, 2003d; Salvador, Sanz, & Fiszman, 2007). Colour change in the pulp is explained by varying levels of β-carotene in different varieties, ranging from 23-4960 µg/100g (Section 2.2.1). There are many banana cultivars, however, few have been analysed for their carotenoid content, as pointed out in a global review (Englberger et al., 2006b) discussing the potential of carotenoid-rich bananas.

Figure 2.3. Differences in flesh colour of different banana cultivars. The cream coloured banana (left) is a seedless hybrid dessert banana, *Silk* (AAB group) while the yellow flesh banana (right) is a Fe'I banana, *Karat* (Scott, Randy, & Angela, 2006).

2.2. Phytonutrients in mango and banana

Epidemiological studies suggest that diets high in consumption of fruits and vegetables offer possible health benefits such as the prevention of cardiovascular diseases, certain cancers and neurodegenerative disorders. Accumulating evidence has revealed that both mango and banana flesh and the peels contain major bioactive compounds including carotenoids and polyphenols. Proposed mechanisms for their biological activities, vary from being estrogen-like (An, Tzagarakis-Foster, Scharschmidt, Lomri, & Leitman, 2001), inhibiting tyrosine kinases (Takahama, Oniki, & Hirota, 2002), exhibiting antioxidant (Merken & Beecher, 2000; Tsao & Deng, 2004), anti-carcinogenic (Hertog et al., 1995; Yamanaka, Oda, & Nagao, 1997), anti-mutagenic (Agullo et al., 1997; Yoshikawa, Ninomiya, Shimoda, Nishida, & Matsuda, 2002), anti-allergic (Fraga & Oteiza, 2011), anti-arthrogenic (Daud et al., 2010; Erdman et al., 2007), anti-inflammatory (Peri et al., 2005; Suzuki, Isobe, Morishita, & Nagai, 2010), antimicrobial (Zheng & Lu, 1990; Zheng & Wang, 2001), and anti-thrombotic effects and angiogenesis inhibitory activities (Meyer, Heinonen, & Frankel, 1998). In addition, some carotenoids are precursors of vitamin A. The per capita consumption of mangoes and bananas in Queensland are high, therefore

these fruits are an important source of several nutritional and health components. There have been only a few studies that have investigated the carotenoid and polyphenol (the two major phytonutrient families) content of mangoes and bananaa, and even fewer studies that have concentrated on the qualitative identification of these compounds. The following review highlights the main categories of carotenoids and polyphenols present in different cultivars of mango and banana.

2.2.1. Carotenoids in mango and banana flesh

Carotenoids are usually C_{40} tetraterpenoids with a basic structure of symmetrical tetraterpenoid skeletons formed by the conjugation of two C_{20} units, which serves as the light-absorbing chromophore responsible for the yellow, orange or red colour of fruits and vegetables. β-carotene occurs as an orange pigment, while α-carotene is a yellow pigment (Takyi, 2001). Carotenoids are localised in the subcellular plastids, chloroplasts and chromoplasts. In chloroplasts, the carotenoids are associated with proteins and serve as accessory pigments in photosynthesis, photoprotective pigments and membrane stabilisers (Schieber & Carle, 2005). In chromoplasts, they are deposited in crystalline form or as oily droplets in fruits, and may be esterified with fatty acids in ripe fruits. The globulous mango chromoplasts (Fig 2.4), which are the most common type of chromoplasts and widely found in orchard fruits, contain carotenoid-carrying lipid droplets (Pott, Marx, Neidhart, Muhlbauer, & Carle, 2003b; Schieber & Carle, 2005; West & Castenmiller, 1998). Mango chromoplasts contain numerous carotenoid-containing plastoglobuli, which support partial solubilisation of these pigments in lipid droplets. Mango chromoplasts are assigned to globular and reticulotubular types (Vasquez-Caicedo et al., 2006). This underlying chromoplast structure containing different carotenoids, and their physical properties, may be one reason for bioavailability variation.
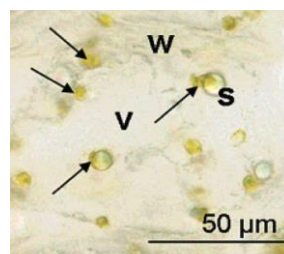
Figure 2.4. Light micrograph of a ripe mango mesophyll cell (showing the typical color of mango chromoplasts (arrows), 'soft cell wall' (w) and enlarged vacuole (v) (Vasquez-Caicedo et al., 2006).

Tropical fruits typically contain more carotenoids than temperate fruits, which usually contain more anthocyanins. Mango is one tropical fruit rich in carotenoids and total

carotenoid (sum of free and esterified carotenoids) concentrations range from 900-9200 µg/100g fresh weight of the edible parts of mango flesh (Corral-Aguayo, Yahia, Carrillo-Lopez, & Gonzalez-Aguilar, 2008; Ornelas-Paz et al., 2008; Pott et al., 2003b; Yahia, Ornelas-Paz, & Gardea, 2006). The content of carotenoids in banana may be low but the high consumption of banana can make it an important nutritional source. *Musa* fruit pulp tissue generally has relatively small amounts of carotenoid species, primarily β-carotene, α-carotene, β-crypoxanthin and/or lutein (Arora, Choudhary, Agarwal, & Singh, 2008; Davey, Keulemans, & Swennen, 2006; Englberger et al., 2006b) with the total carotenoid concentration ranging from 60-5370 µg/100g. Khoo, Prasad, Kong, Jiang, and Ismail (2011) further stated that yellow coloured fruits contain lower amounts of carotenoids including the genera *Musa*. This statement correlates well with carotenoid levels detected in banana and mango mesocarp where it has been observed that most commercial banana cultivars have white to creamy-yellow flesh and contain lower amounts of carotenoids compared to mango, which have deep orangey-yellow flesh and significantly higher carotenoids levels. Colouration of the edible portion of the flesh appears to be a good indicator of its carotenoid content; bright yellow-orange Thai mango cultivars have a higher total carotenoid content (6544-11,249 µg/100g) compared to the pale-coloured cultivars (1019-2195 µg/100g) (Vasquez-Caicedo, Sruamsiri, Carle, & Neidhart, 2005). This was also true for bananas where the flesh colour of most commercial banana cultivars range from creamy-white to pale-yellow. Interestingly, other studies (Arora et al., 2008; Davey et al., 2007; Englberger et al., 2003d; Englberger, Marks, & Fitzgerald, 2003b; Englberger et al., 2006a; Englberger, Schierle, Marks, & Fitzgerald, 2003a) have consistently verified that colour reflects the carotenoid levels in banana cultivars with orange-fleshed plantain varieties (AAB) having substantially higher levels of fruit carotenoids than do dessert bananas (AAA).

During ripening, most varieties of mangoes show a steady increase in carotenoid content (John, Subbaray, & Cama, 1970; Mercadante & Rodriguez-Amaya, 1998; Vasquez-Caicedo et al., 2005; Yahia, Ornelas-Paz, & Gardea, 2006). Ornelas-Paz, Failla, Yahia and Gardea (2008a) recorded exponential changes of the main carotenoids and xanthophyll esters content. Several studies demonstrated that heat treatment apparently enhanced carotenoid synthesis during ripening and accelerated mango ripening (Gonzalez-Montelongo, Lobo, & Gonzalez, 2010b; Talcott, Moore, Lounds-Singleton, & Percival, 2005).

Carotenoids occur predominantly in the all-*trans* configuration, which are more thermodynamically stable than *cis*-isomers. Additionally, carotenoids exhibit pronounced photo- and thermal sensitivity. As mentioned earlier, carotenoids are incorporated in lipoproteins or membranes in fruits and are relatively well protected. During peeling, destoning or cutting of the fruits, extraction or other processing steps, carotenoids readily undergo hydrolysis, oxidation or *trans-cis* isomerisation that is catalysed by UV light, acids and bases, oxygen, heat or traces of metal ions (Davey, Keulemans, & Swennen, 2006; Davey, Mellidou, & Keulemans, 2009; de Rigal, Gauillard, & Richard-Forget, 2000; Gonzalez & Gonzalez, 2010c; Kim & Lee, 2002; Mercadante & Rodriguez-Amaya, 1998; Mordi et al., 1993; Nielsen, 2003b; Ornelas-Paz, Yahia, & Gardea, 2008; Tsao & Deng, 2004). The known susceptibility of carotenoids to oxidation and degradation indicates that it is important to observe the stability of tissue samples and carotenoid extracts during storage.

Carotenoids can be divided into two groups based on their composition: carotenes and xanthophylls. Hydrocarbon carotenoids containing only carbon and hydrogen are collectively called carotenes; those with oxygen containing functional groups such as keto, hydroxy or epoxy groups are termed xanthophylls or oxycarotenoids, and are reported to be the enzymatically formed oxidation products of α- and β-carotene (Van den Berg et al., 2000).

2.2.1.1. Carotenes

Carotenes include several compounds having the general formula $C_{40}H_{56}$. β-Carotene is mostly found as the all-*trans* isomer and less as the *cis*-isomer (Fig 2.5). A study on the effects of drying mango slices revealed that fresh mango fruits contain up to 27% *cis*-isomers of β-carotene. When mango slices are dried in the dark, an increase in 13-*cis*-β-carotene was attributed to the elevated temperature, while solar drying of mangoes results in significantly higher amounts of the 9-*cis*-isomer. This finding correlates with dessert banana and plantain varieties, where exposure of their extracts to light results primarily in the formation of 9-*cis*-β-carotene (Davey, Keulemans, & Swennen, 2006).

Bicyclic β-carotene is the most widespread of all carotenoids in food and occurs as the major constituent, while α-carotene occurs at lower concentrations. The concentration of β-carotene in banana fruit pulp ranges from 21-4940 µg/100g edible portion with average values of 23 µg/100g for *Cavendish*, the primary cultivar marketed globally (NUTTAB,

2011a), and 25 µg/100g α-carotene (USDA, 2011). The β-carotene contents of other banana cultivars from different countries are shown in Table 2.3. β-carotene levels are higher in those bananas with deeper flesh colouration. This trend is supported by Englberger et al. (2006b) who studied carotenoid content in relation to the flesh colour of selected cultivars and showed that all the tested yellow-fleshed cultivars had higher levels of β-carotene than the two cream-fleshed commercially available cultivars- *Williams Cavendish* and *Lady Finger*.
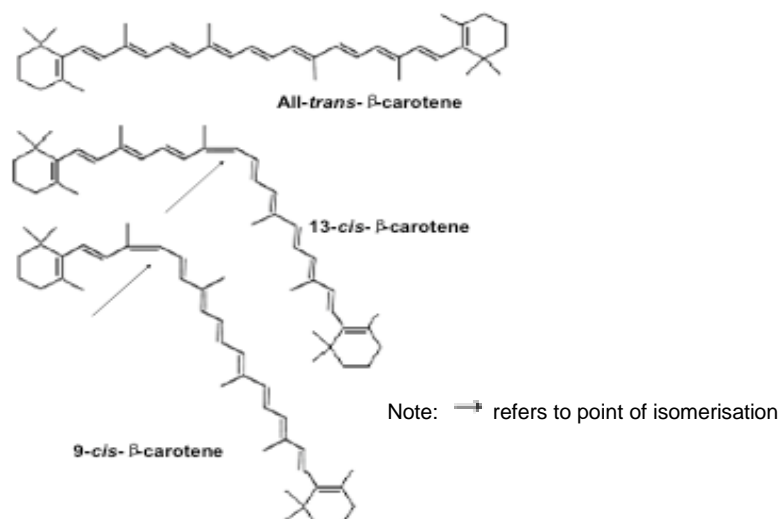
Figure 2.5. Structure of all-*trans*-β-carotene and its two geometric isomers (Boon et al., 2010).

Similarly in mangoes, all-*trans*-β-carotene is the principal carotene comprising 48-84% of the total carotenoid concentration depending on cultivar, fruit maturity and physiological stage (Godoy & Rodriguez-Amaya, 1989). β-Carotene content ranges from 324-4720 µg/100g fresh weight (Table 2.4). The other carotene, α-carotene is present in smaller amounts (Robles-Sanchez et al., 2009a) or not at all. Holden et al. (1999) quantified 17 µg/100g α-carotene, 445 µg/100g β-carotene and 11 µg/100g β-cryptoxanthin in several mango cultivars grown in USA, which was similar to the general values (9 µg/100g α-carotene, 640 µg/100g β-carotene, 10 µg/100g β-cryptoxanthin) reported in the USDA database (2011) (varieties were not indicated). This pattern is comparable to results from other studies (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010) that showed β-carotene was detected in the largest proportion (300-4200 µg/100g β-carotene, 100 µg/100g lutein, 0-1640 µg/100g β-cryptoxanthin). From NUTTAB, α-carotene was quantified at a similar level (9 µg/100g), whereas β-carotene and β-cryptoxanthin were found at significantly higher levels of 1433 µg/100g and 1516 µg/100g respectively (NUTTAB, 2011b). β-Carotene or individual contents of carotenoids have only been

reported in a study focused on the intra- and inter-fruit variability between dried *Kensington Pride* mango powder (Hewavitharana, Tan, Shimada, Shaw, & Flanagan, 2013b).

Table 2.3. β-carotene content based on edible portions of ripe banana flesh.

Banana cultivars/varieties	Country of origin	β-carotene[a] (µg/100g FW)	Reference
Asupina	Australia	1434	(Englberger et al., 2006b)
Kirkirnan		729	
Pisang Raja Udang (Red Dacca)		722	
Horn Plantain		639	
Kluai Khai Bonng		437	
Wain		501	
Pacific Plantain		413	
Lakantan		289	
Red Dacca		214	
Sucrier		227	
Lady Finger		112	
Williams (Cavendish)		65	
Uht en Yap	Federated States of Micronesia	2780	(Englberger et al., 2003a)
Uht Karat		520	
Usr Kulasr		660	
Usr Taiwang		270	
Usr in Yeir		340	
Usr Lakatan		330	
Uht en Ruk		90	
NR	Malaysia	151[b], 66	(Siong & Lim, 1991)
NR (AAA)	NR	56 ± 14	(Mohapatra, Mishra, & Sutar, 2010)
NR (AAB)		97 ± 31	
Red Banana	India	117 ± 19[b]	(Arora et al., 2008)
Karpooravalli		28 ± 7[b]	
Rasthali		30 ± 7[b]	
Hill banana		29 ± 5[b]	
NR	USA	21	(Holden et al., 1999)

[a]*Values obtained via HPLC analyses.* [b]*Values obtained via direct spectrophotometry method (450 nm).*

The proportion of carotenoids varies with the genotype of the fruits analysed. All-*trans*-β-carotene (54-90% of the total carotenoids) have been found to be higher than all-*trans*-α-carotene levels in seventeen banana varieties (Englberger et al., 2003d). Results of Englberger et al. (2006b) also showed that β-carotene levels are greater than α-carotene in most cultivars with exceptions for *Red Dacca* and *Lady Finger*. In five varieties of Belgium banana and plantain, all-*trans*-β-carotene never represented more than 50% of the total carotenoids, and in *Yangambi-5*, it was as low as 30% (Davey, Keulemans, & Swennen, 2006) presumably related to the genetic origins of these varieties.

Table 2.4. Relative amounts of carotenoids of different cultivars of fully ripe mango flesh.

Carotenoids	Mango cultivars/varieties (and their country of origin)						
	Keitt	*Tommy Atkins*	*Keitt*	*Ataulfo*	NR	*Bad-ami*	*Alfon-so*
	Sao Paulo, Brazil[a]		Bahia, Brazil[b]	Mexico[c]	Tainan, Taiwan[d]	India[e]	India[f]
Carotenes (Hydrocarbons)							
All-*trans*-β-carotene	670 ± 160	580 ± 250	1340-1620	3940 ± 260	3654	5064	5950
9-*Cis*-β-carotene	-	-	-	290 ± 14	118		
13-*Cis*-β-carotene	-	-	-	-	148		
γ-Carotene	-	-	-	-	-	21	1
Phytoene	-	-	-	-	-	632	370
Phytofluene	-	-	-	-	-	1170	689
Xanthophylls (Oxycarotenoids)							
Cis-β-cryptoxanthin	tr-100	10 ± 10	tr-10	0.3	-	40	66
All-*trans*-β-cryptoxanthin	20	30 ± 10	30	-			
All-*trans*-β-zeaxanthin	80 ± 30	40 ± 20	60-90	-	116	29	1
Luteoxanthin isomers	270 ± 20	200 ± 60	310-410	-	360	93	1125
All-*trans*-β-violaxanthin	1800 ± 400	2240 ± 910	1820-2390	2420 ± 19	460	-	-
9-*Cis*-violaxanthin	720 ± 140	1450 ± 470	990-1030	800 ± 6	180	708	902
13-*Cis*-violaxanthin	-	tr	130-150	-			
Cis-neoxanthin	30 ± 20	100 ± 100	tr-0.2	-	46	-	-
All-*trans*-neoxanthin	190 ± 90	490 ± 450	100-360	-	142	-	-
Neochrome	-	-	-	-	-	-	-
Cryptoflavin	-	-	-	-	-	6	-
Antheraxanthin	-	-	-	-	-	331	151
Mutatoxanthin	-	-	-	-	-	373	076
Auroxanthin	-	-	-	-	-	1040	271
5,6-Monoepoxy-β-carotene	-	-	-	-	-	75	085
Mutatochrome	-	-	-	-	-	110	152

-Values not detected or not reported. tr: trace amounts. [a]Adapted from (Mercadante & Rodriguez-Amaya, 1998), [b]adapted from (Mercadante, Rodriguez-Amaya, & Britton, 1997), [c]adapted from (Ornelas-Paz et al., 2008), [d]adapted from (Chen, Tai, & Chen, 2004), [e]adapted from (John, Subbaray, & Cama, 1970), [f]adapted from (Jungalwala & Cama, 1963). All values were obtained via HPLC analyses.

Variability in banana carotenoid contents was studied using a selection of Central and West African *Musa* banana varieties cultivated under standardised field conditions (Davey et al., 2007). Within-fruit, within-hand and within-plant analyses, as well as between-plant analyses demonstrated that carotenoid content differed across all sample groups. Even within a single finger, there were statistically different concentrations both longitudinally and laterally with an overall variation of ±20% across all eight sample points in a finger. Thus, to compensate for within- and between-fruit differences, it is important to obtain as

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much of the flesh as practically possible in the preparation of bulk samples for analysis, especially during the removal of the peel and seed of the banana. Variations in carotenoids content appear to be at least partly related to differences in the fruit developmental status that result from the time differences when the flowers of inflorescence emerge and exposure to sunlight or maturity. However, the observed trends were genotype specific (Davey et al., 2007). Within-plant variability of the banana hand position in the bunch appears to be greater than the variability atrributed to finger position in the hand (Davey et al., 2007). A common trend was observed for within-fingers, within-hand and within-plant variations where the proportions of individual carotenoid species (all-*trans*-β-carotene and all-*trans*-α-carotene) remained constant for each genotype across all samples analysed. However, mean carotenoid levels per genotype showed considerable genetic variation in the concentrations between individual plants of a single variety, and between *Musa* cultivars. In addition, disparities in sample preparation, extraction methods and choice of solvents affect the amount of carotenoids quantified. A wide variety of solvents and mixtures that are dependent on the tissue matrix and carotenoid species of interest have been used for carotenoid extraction and analyses (Section 2.4.1 contains a detailed discussion of factors that influence carotenoid extraction from fruit samples).

2.2.1.2. Xanthophylls

Xanthophylls are oxidised derivatives of carotenes. They have the general structure $C_{40}H_{56}O_2$, contain hydroxyl groups and are more polar than the carotenes (Matsuno et al., 1986). Most xanthophylls are yellow-orange pigments, especially lutein and zeaxanthin (Fig 2.6). Lutein appears to undergo limited epoxidation, while zeaxanthin is easily transformed to antheraxanthin and violaxanthin.
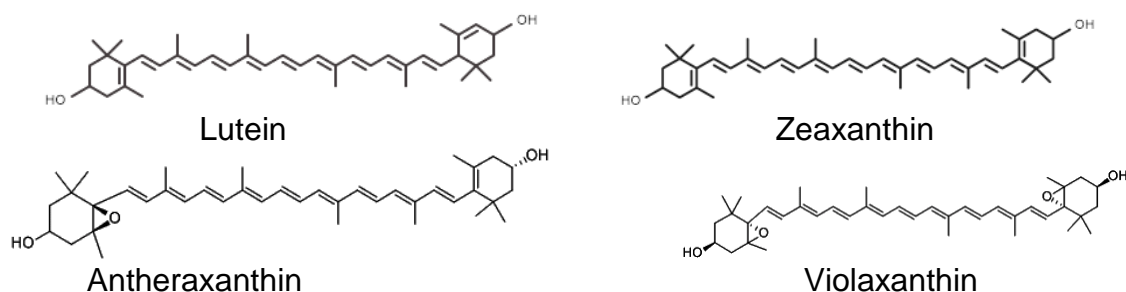
Figure 2.6. Structures of lutein, zeaxanthin, antheraxanthin and violaxanthin.

Lutein (113 µg/100g FW basis) has been reported in *Musa spp.* and β-cryptoxanthin (3 µg/100g FW) in *Musa paradisiacal L.* (Khoo et al., 2011). Here, lutein has been consistently detected in lower amounts than α- and β-carotene in banana cultivars. Lutein

was quantified in selected banana cultivars grown in Australia, ranging from 7-80 µg/100g edible portion (Englberger et al., 2006b) and a mean value of 32 µg/ in Malaysian cultivars (Siong & Lim, 1991).

All-*trans*-violaxanthin and 9-*cis*-violaxanthin are the two other major carotenoids identified in the mango cultivars - *Kent* (Manthey & Perkins-Veazie, 2009; Ornelas-Paz, Yahia, & Gardea, 2008), *Tommy Atkins*, *Keitt* (Mercadante & Rodriguez-Amaya, 1998; Mercadante, Rodriguez-Amaya, & Britton, 1997), *Haden*, *Criollo*, *Paraiso* (Ornelas-Paz, Yahia, & Gardea, 2007), *Ataulfo*, *Manila* (Robles-Sanchez et al., 2011; Robles-Sanchez et al., 2009a) and *Badami* (John, Subbaray, & Cama, 1970). In addition, all-*trans*- and *cis*-β-cryptoxanthin, all-*trans*-zeaxanthin, lutein, luteoxanthin isomers, and all-*trans*- and *cis*-neoxanthin have been identified at lower levels. Because of its facile degradation, violaxanthin levels may be underestimated in fruits, as was shown for mango (Mercadante & Rodriguez-Amaya, 1998). Table 2.4 lists the common carotenoids and their distribution in various mango varieties.

Carotenoid composition differs among mango varieties. Manthey and Perkins-Veasie (2009) found β-carotene to be the predominant carotenoid with all-*trans*-violaxanthin and 9-*cis*-violaxanthin being the second and third most abundant. These results are in agreement with findings generated by other studies (Godoy & Rodriguez-Amaya, 1989; Holden et al., 1999; Jungalwala & Cama, 1963; Ornelas-Paz, Yahia, & Gardea, 2007, 2008; Pott, Breithaupt, & Carle, 2003), which found β-carotene to be the main carotenoid, representing more than 50% of the total carotenoid content. However, Pott, Marx, Neidhart, Muhlbauer and Carle (2003b), and Ornelas-Paz, Yahia and Gardea (2008) showed that all-*trans*-violaxanthin and 9-*cis*-violaxanthin dibutyrates were the main carotenoid esters in *Kent* mangoes (from Brazil and Mexico respectively), and the concentration of 9-*cis*-violaxanthin was slightly lower than that of all-*trans*-violaxanthin. In addition, Mercadante and Rodrigues-Amaya (1997) reported higher concentrations of all-*trans*-violaxanthin in *Keitt* mango, which accounted for 38% of the total carotenoid content, while all-*trans*-β-carotene accounted for 27% of the total carotenoid concentration.

The effects of harvest date, location and varietal differences on the carotenoid pattern were further investigated by Mercadante and Rodrigues-Amaya (1998), and Manthey and Perkins-Veasie (2009). Their findings proved there was modulation of carotenoid values for different cultivars sourced from assorted locations and countries. Similar patterns of all-

trans-violaxanthin, 9-*cis*-violaxanthin and all-*trans*-β-carotene as the major carotenoids were observed in ripe *Keitt* and *Tommy Atkins*; however, *Tommy Atkins* had higher all-*trans*-violaxanthin content (24 µg/g vs 18 µg/g) and 9-*cis*-violaxanthin (15 µg/g vs 7 µg/g) content (Mercadante & Rodriguez-Amaya, 1998). For the carotenoid composition of *Keitt* from Sao Paulo (moderate climate of Brazil) and *Keitt* from Bahia (hot climate of Brazil), the latter had more than twice as much β-carotene (7 µg/g vs 15 µg/g); all-*trans*-violaxanthin and 9-*cis*-violaxanthin levels were also higher in Bahia *Keitt*. A comparison of *Tommy Atkins* from Sao Paulo to the same cultivar from Mato Grosso showed that the β-carotene concentration of the latter was twice the concentration as from Sao Paulo (Godoy & Rodriguez-Amaya, 1989). These results indicate that geographic and/or climate effects could have the same or greater influence on carotenoid composition than cultivar differences, with fruits from hotter regions having generally higher carotenoid contents. This similar geographic tendency was illustrated by results of the study by Manthey and Perkins-Veasie (2009), where for the specific cultivars- *Haden* and *Kent*, generally higher β-carotene values occur at certain harvest dates for the fruit grown in Mexico in comparison to other locations. These findings seem to be supported by the same trend observed in Table 2.6 of compiled β-carotene values.

Inherent variations in carotenoid contents should be expected due to: 1) varying stages of maturity at which the mangoes were collected or left to ripen before analysis, 2) cultivar/varietal differences, 3) geographic or climate effects, and 4) processing and storage conditions. Obviously, a part of this discrepancy is attributed to the extraction and analytical procedures employed by different authors such as inclusion of a saponification step, or choice of solvents and extraction conditions. In some studies, authors employ saponification to hydrolyse xanthophyll esters or remove chlorophylls that, when present in high amounts, can interfere with extraction; this is usually carried out at high temperatures for 10-120 min. However, carotenoid degradation and losses, and artefact formation have been also reported with the use of saponification. Provitamin A carotenoids such as α-carotene, β-carotene, γ-carotene and β-cryptoxanthin can resist saponification conditions, while xanthophylls such as lutein, violaxanthin and other dihydroxy and trihydroxy carotenoids can suffer considerable losses during the saponification process (Kimura & Rodriguez-Amaya, 1999; Kimura, Rodriguezamaya, & Godoy, 1990). In a study that investigated the chromatographic patterns of saponified and unsaponified *Ataulfo* mango extracts (Ornelas-Paz, Yahia, & Gardea, 2007), the two most abundant compounds (all-*trans*-violaxanthin and 9-*cis*-violaxanthin dibuytrates) present in unsaponified extracts were

replaced with all-*trans*-violaxanthin, 9-*cis*-violaxanthin and an unidentified compound. In addition, all-*trans*-violaxanthin ester and 9-*cis*-violaxanthin ester disappeared when crude mango extracts were saponified, and were replaced by the un-esterified compounds (Ornelas-Paz, Yahia, & Gardea, 2008).

2.2.2. Polyphenols in mango and banana flesh

Polyphenols are plant secondary metabolites and contain a wide range of compounds composed of an aromatic benzene ring with one or more hydroxyl moieties, including their functional derivatives such as glycosides, esters or methyl esters. Phenolics, despite only being present in small concentrations are major contributors to colour, sensory characteristics and antioxidant activity. Phenolic compounds present in fruits are found in both free and bound forms (mainly as β-glycosides); however, the free forms as intact conjugates are often excluded from analyses and for this reason, the total phenolic content of fruits are often underestimated (Arranz, Silvan, & Saura-Calixto, 2010). Studies on phenolic composition and concentrations have been carried out for many temperate fruits, to a lesser extent for tropical fruits, and for only a few types of mango and banana.

Existing studies show that there are significant levels of total free polyphenols in different banana cultivars ranging from 5-36 mg GAE/100g edible portion (Table 2.5). Evaluation of banana pulp as a source of cloud components for the juice industry also revealed that banana pulp has a relatively high phenolic content (14 ± 0.2 mg GAE/100g edible portion) when compared to orange (10 ± 0.8 mg GAE/100g edible portion) (Koffi, Phillips, & Wicker, 2007). Mango phenolic contents of 23-160 mg GAE/100g FW (Table 2.8) are significantly higher than those for all banana cultivars on a fresh weight basis. However, significant variations in phenolic levels are observed cross banana cultivars from different origins. Phenolics in banana fruit pulp exist mainly in soluble free forms, where the concentration of free phenolics is 56 ± 2.8 mg GAE/100g edible portion (62.1%). In addition, banana fruit pulp contains significant levels of cell wall bound phenolics at 5 ± 0.04 mg GAE/100g cell wall (ethyl acetate fraction) and 30 ± 0.4 mg GAE/100g cell wall (water-soluble fraction) (Sun, Chu, Wu, & Liu, 2002).

Phenolic concentration and composition vary amongst mango cultivars. The phenolic contents of *Ataulfo*, *Tommy* Atkins, *Haden* and *Kent* cultivars are similar in several countries for each cultivar (Table 2.6) except for *Kent* from Spain where a significantly higher phenolic content has been detected (91 mg/100g FW) - almost twice the

concentration (33-49 mg/100g FW) reported for *Kent* mangoes from other countries. Manthey and Perkins-Veasie (2009) further investigated the influence of harvest dates and location on phenolic levels, and found that the *Haden* cultivar harvested in different months had a two-fold difference in phenolic content. In addition, significantly higher gallotannin contents occurred for fruits collected at later harvest dates in a number of locations. Furthermore, mangiferin concentrations in the *Ataulfo* cultivar harvested at later dates were much higher than those harvested at earlier dates. However, there is a large standard deviation for fruit-to-fruit variations within each set (Manthey & Perkins-Veazie, 2009). Overall, no significant influence of harvest location was observed; in contrast, phenolic compound differences between cultivars were significantly larger than were the differences attributed to different harvest dates and locations, and countries of origin. Generally, mango contains substantially higher phenolic concentrations than bananas. However, bananas are more frequently consumed by the public (all year round) and also in larger quantities. Hence with an increased serving portion, the phenolic intake increases and may exceed that of the mango fruit, especially since mango is a seasonal fruit. The absolute quantity of phenolics present also varies with size of the fruit and the form in which it is eaten- freshly consumed or processed products. Apparently, whole mangoes have higher levels of phenolics when compared to fresh cut mangoes (Robles-Sanchez et al., 2011) or freeze dried mangoes (Shofian et al., 2011), suggesting losses on processing.

Very few studies have investigated the specific composition of phenolics in bananas or mango flesh. Most studies focused on antioxidant activity or on the effects of harvest, location, storage or processing on the total polyphenol content of the fruit peel, seed, bark, leaf and to a lesser extent fruit pulp, rather than on the analysis of individual compounds. There are only four mango studies that have identified specific phenolic compounds and classes in mango pulp and peel, but there have been no reported studies on mango phenolics bioavailability. One study investigated the phenolic profiles of mangiferin, ellagic acid and gallotannin in five mango cultivars from the USA (Manthey & Perkins-Veazie, 2009), while another identified the different classes of phenolic compounds in Australian *Kent* mangoes (Epriliati, 2008). Further, two other studies involved chromatographic separation of flavonols, xanthones and anthocyanins from nine international mango cultivars by Berardini et al. (2005b), and the isolation of mangiferin in Australian *Kensington Pride* mango (Hewavitharana et al., 2013b). So far, only mangiferin has been quantified in *Kensington Pride* and the full range of phenolic classes still remains to be explored.

Table 2.5. Total phenolic content (mg gallic acid equivalents/100g fresh weight), total flavonoid content (mg quercetin equivalents/100g fresh weight), total procyanidin content (µg procyanidin oligomers/g dry weight), gallocatechin and catechins (µg/100g dry weight) based on edible portions of ripe banana flesh.

Banana cultivars/ varieties	Country of origin	Total phenolic content (mg GAE/100g FW)	Total flavonoid content (mg QE/100g FW)	Total procyanidin content (µg procyanidin oligomers/g DW)	Specific phenolics (µg/100g DW)			Reference
					Gallocatechin	(+)-Catechin	(-)-Epicatechin	
Pisang Mas (Sucrier)	Malaysia	14[b]	-	-	-	-	-	(Sulaiman et al., 2011 82)
Kapas		5[b]	-	-	-	-	-	
Berangan (Lakantan)		13[b]	-	-	-	-	-	
Rastali (Silk)		13[b]	-	-	-	-	-	
Pisang Raja		20[b]	-	-	-	-	-	
Pisang Nangka		34[b]	-	-	-	-	-	
Pisang Awak		36[b]	-	-	-	-	-	
Nipah (Saba)		36[b]	-	-	-	-	-	
Gingeli	Mauritius	12 ± 0.1[b]	0.6[b]	-	-	-	-	(Luximon-Ramma, Bahorun, & Crozier, 2003)
NR	Spain	-	-	-	100[a] (FW)	10[a] (FW)	10[a] (FW)	(de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000)
Flhorban920	Martinique	25-41[b]	-	-	-	-	-	(Bugaud, Alter, Daribo, & Brillouet, 2009)
Grande Naine (Cavendish)		10-17[b]	-	-	-	-	-	
NR	USA	90.4[b]	-	-	-	-	-	(Sun et al., 2002)
NR	Malaysia	24-72[b]	4.7-23[b] (CEQ/100g)	-	-	-	-	(Alothman, Bhat, & Karim, 2009)
Pequena Enana	Tenerife	0.87 ± 0.7[a]	-	-	-	6330 ± 3650[a]	-	(Mendez, Forster, Rodriguez-Delgado, Rodriguez-Rodriguez, & Romero, 2003)
Gran Enana		1.08 ± 0.8[a]	-	-	-	6230 ± 3360[a]	-	
NR	Equador	1.06 ± 0.5[a]	-	-	-	10290 ± 3570[a]	-	
Figo	Brazil	8 ± 0.1[b] (DW)	41[b]	2 ± 0.2[b]	37 ± 1[a]	76 ± 11.2[a]	56 ± 3[a]	(Bennett et al., 2010)
Nanicao		10 ± 0.2[b] (DW)	44[b]	4 ± 0.9[b]	542 ± 17[a]	59 ± 2.6[a]	460 ± 16[a]	
Terra		7 ± 0.1[b] (DW)	30[b]	20 ± 0.1[b]	418 ± 23[a]	39 ± 2.2[a]	92 ± 5[a]	
Mysore		10 ± 0.2[b] (DW)	120[b]	86 ±0 .6[b]	255 ± 11[a]	143 ± 7.4[a]	214 ± 10[a]	
Pacovan		7 ± 0.2[b] (DW)	83[b]	2 ± 0.2[b]	295 ± 22[a]	69 ± 9.4[a]	34 ± 6[a]	

-: Values not detected or not reported, GAE: gallic acid equivalents, QE: quercetin equivalents, FW: fresh weight, DW: dry weight. [a]Values obtained via HPC analyses. [b]Values obtained via Folin-Ciocalteu assay or other spectrophotometric assays.

Table 2.6. Total phenolic content (mg gallic acid equivalents/100g fresh weight), total flavonoid content (mg RE/100g dry weight) and β-carotene content (μg/100g fresh weight) based on edible portions of ripe mango flesh.

Mango cultivars/ varieties	Country of origin	Total phenolic content (mg GAE/100g FW)	Total flavonoid content (mg RE/100g DW)	β-carotene content (μg/100g FW)	Reference
Irwin	Korea	2690 ± 376[b] (DW)	330 ± 79	-	(Kim et al., 2010)
R2E2	Australia	1347[b] (DW)	-	-	(Daud et al., 2010)
		-	430 ± 60	-	(Berardini et al., 2005b)
Tommy Atkins	Mexico	-	-	580[a]	(Mercadante & Rodriguez-Amaya, 1998)
		24[b]	-	581[c]	(Manthey & Perkins-Veazie, 2009)
	Germany	-	-	3440[a], 4590[a] (DW)	(Pott et al., 2003b)
		-	-	324-383[a]	(Vasquez-Caicedo, Schilling, Carle, & Neidhart, 2007)
	Brazil	-	460 ± 10	-	(Berardini et al., 2005b)
		-	270 ± 10	-	(Ribeiro, Barbosa, Queiroz, Knodler, & Schieber, 2008)
		24[b]	-	490[a]	(Manthey & Perkins-Veazie, 2009)
	Ecuador	36[b]	-	445[a]	(Manthey & Perkins-Veazie, 2009)
	Peru	30.6[b]	-	507[a]	
Kent	Germany	-	-	5700[a], 5740[a] (DW)	(Pott et al., 2003b)
	Mexico	33[b]	-	2178[a]	(Manthey & Perkins-Veazie, 2009)
	Ecuador	34[b]	-	1230[a]	
	Peru	49[b]	-	838[a]	
	Spain	91[b]	-	1600[c]	(Robles-Sanchez, Rojas-Grau, Odriozola-Serrano, Gonzalez-Aguilar, & Martin-Belloso, 2009b)
Keitt	Mexico	-	-	1340-1620[a]	(Mercadante, Rodriguez-Amaya, & Britton, 1997)
		-	-	670[a]	(Mercadante & Rodriguez-Amaya, 1998)
		30[b]	-	1038[a]	(Manthey & Perkins-Veazie, 2009)
José	La Réunion	-	4780 ± 270	-	(Berardini et al., 2005b)
Mini-mango	Colombia	-	300 ± 50	-	
Haden	Mexico	-	-	400-2800[a]	(Ornelas-Paz, Yahia, & Gardea, 2007)
		31[b]	-	811[a]	(Manthey &

					Perkins-Veazie, 2009)
	Peru	-	1620 ± 270	-	(Berardini et al., 2005b)
		54[b]	-	494[a]	(Manthey & Perkins-Veazie, 2009)
	Brazil	48[b]	-	191[a]	(Ribeiro et al., 2007)
		-	350 ± 10	-	(Ribeiro et al., 2008)
Ubá		-	3390 ± 130	-	
		209[b]	-	2220[a]	(Ribeiro et al., 2007)
Palmer		-	-	661.27[a]	
Ataulfo	Mexico	-	-	3160[a], 7480[a]	(Ornelas-Paz, Yahia, & Gardea, 2007)
		109[b]	-	2610[a]	(Manthey & Perkins-Veazie, 2009)
		125[b], 160[b]	-	4530[a], 4720[a]	(Robles-Sanchez et al., 2009a)
		111 ± 1[b], 116 ± 1[b]	17 ± 0.9, 11 ± 0.2 (mg QE per 100g FW)	303[a], 447[a]	(Robles-Sanchez et al., 2011)
		125[b], 160[b]	17 (mg QE per 100g FW)	-	(Gonzalez-Aguilar, Wang, Buta, & Krizek, 2001)
Kensington Pride	Australia	-	-	6240[a]	(Amitha, 2011)
Kaew	Thailand	-	-	5117[a]	(Vasquez-Caicedo et al., 2005)
		-	-	10300[a], 13910[a] (DW)	(Pott et al., 2003b)
Chok Anan		-	-	4044[a]	(Vasquez-Caicedo et al., 2005)
Maha Chanok		-	-	6107[a]	
Kiew Sawoei		-	-	1349[a]	
Rad		-	-	1438[a]	
Mon Duen Gao		-	-	1413[a]	
Okrang Thong		-	-	1284[a]	
Okrang Kiew		-	-	610[a]	
Nam Dokmai		-	-	6140[a]	
		-	-	3940[a], 4640[a] (DW)	(Pott et al., 2003b)
NR	Malaysia	100 ± 9[b]	-	660 ± 61[a]	(Shofian et al., 2011)
Black Gold		-	-	615[c]	(Siong & Lim, 1991)
	USA	-	-	553[a]	(Khoo et al., 2011)
NR	USA	-	-	445[a]	(Holden et al., 1999)
NR	Indonesia	-	-	1710 ± 950[a]	(Khoo et al., 2011)
Gedong		-	-	3267 ± 2075[a]	
Manalagi		-	-	190 ± 123[a]	
Indramayn		-	-	1606 ± 166[a]	
Harum Manis		-	-	1080 ± 264[a]	
Golek		-	-	1237 ± 626[a]	

-: Values not detected or not reported, GAE: gallic acid equivalents, QE: quercetin equivalents, FW: fresh weight, DW: dry weight. [a]Values obtained via HPC analyses. [b]Values obtained via Folin-Ciocalteu assay. [c]Values obtained via direct spectrophotometry method (450 nm).

Phenolics can be classified into flavonoids and non-flavonoids. Flavonoids contain a C_6-C_3-C_6 3-ring structure, while the non-flavonoids group of phenolics is classified according to the number of carbon atoms, and includes hydroxycinnamic acids, hydroxybensoic acids, xanthones and hydrolysable tannins. There are other classes of flavonoids such as anthocyanins and non-flavonoids such as flavones, stilbenes, etc, but these will not be discussed here, as they have not been reported to occur in banana or mango flesh.

2.2.2.1. Flavonoids

Flavonoids are low molecular weight compounds consisting of fifteen carbon atoms; two aromatic (A and B) rings enclosing a 3-carbon bridge that is usually a heterocyclic pyran ring (C ring) with oxygen (Fig 2.7). Structural variations are due, in part, to the degree and pattern of hydroxylation, methoxylation, prenylation or glycosylation (Jorge, 2006; Stalikas, 2007). In plants and fruits, flavonoids are usually present as glycosides; sugar substitution on the flavonoid skeleton occurs through the hydroxyl groups of aglycones in the case of O-glycosides or directly to carbon atoms in the ring A of C-glycosides. The more common carbohydrates are rhamnose, glucose, galactose, arabinose or glucuronic acid (de Rijke et al., 2006). Among the classes of flavonoids, of particular interest are flavonols, flavanols, flavanones and proanthocyanidins, which are the major classes in banana flesh and mango flesh across various cultivars (Table 2.7).
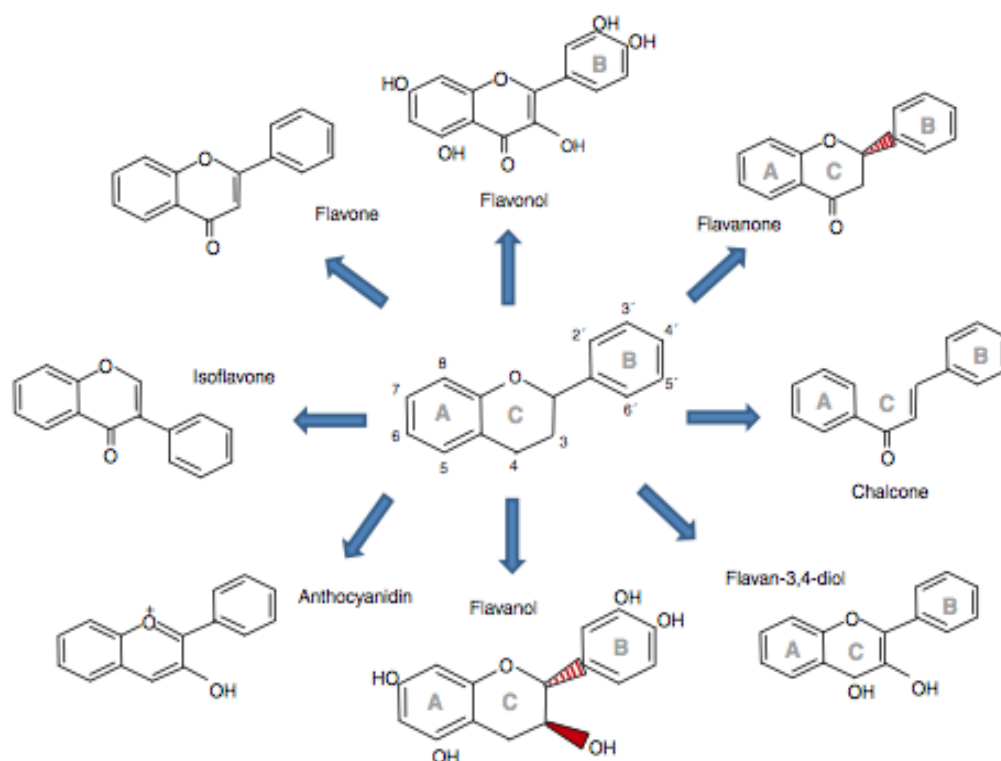
Figure 2.7. Chemical structures of the flavonoid families. The basic generic flavonoid structure showing the A-, B- and C-rings, and the numbers of the various positions in the flavan structure (Fraga & Oteiza, 2011).

Flavonols. The flavonols identified in mango include quercetin, kaempferol, isoquercitrin (quercetin-3-glucoside) and astragalin (kaempferol-3-glucoside) (Fig 2.8). This group is most abundant among the flavonoids in mango fruit. Quercetins are largely responsible for the slight yellow colour of many fruits, flowers and vegetables (Jorge, 2006). Quercetin has been identified in unripe mango, and previously identified in tender fruits, and in mature fruits along with its glycosides, but these disappear on ripening (El Ansari, Reddy, Sastry, & Nayudamma, 1969). Flavonols are mainly accumulated in the outer fruit tissues, since their synthesis is stimulated by sunlight (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004); thus, climate conditions affect flavonol levels in mango. Factors such as fruit maturity and postharvest handling also affect the flavonol content. Quercetin is the main flavonoid present in mango while flavones are less common (Masibo & He, 2008) and occur in foods as O-glycosides with sugars bound at the C_3 position.

Basic flavonol structure

| Astragalin | Quercetin | Isoquercitrin | Kaempferol |
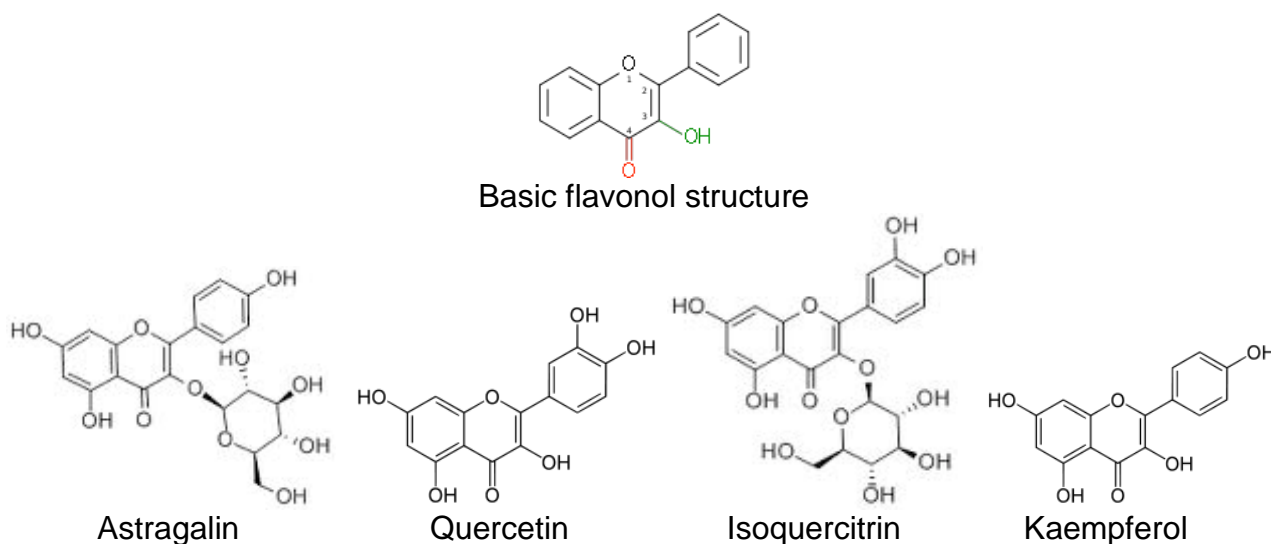
Figure 2.8. Structures of flavonols.

In *Ataulfo* mango (Table 2.7), quercetin and catechin (flavanol) are the major flavonoids (Gonzalez-Aguilar et al., 2001; Shivashankara, Isobe, Al-Haq, Takenaka, & Shiina, 2004). In *Kent* mango, quercetin was also detected as the main flavonoid, followed by kaempferol (Robles-Sanchez et al., 2009a; Robles-Sanchez et al., 2009b). A kaempferol glycoside and five quercetin glycosides are components of mango puree with the predominant glycosides being quercetin-3-galactoside (2.2 mg/100g), quercetin-3-glucoside (1.6 mg/100g) and quercetin-3-arabinosidde (0.5 mg/100g), while the predominant phenolic acid is gallic acid (0.69 mg/100g) followed by mangiferin (0.44 mg/100g) (Schieber, Ullrich, & Carle, 2000). Quercetin aglycon was present at 0.35 mg/100g (Schieber, Ullrich, & Carle, 2000). Quercetin-3-galactoside (0.66 mg/100g) and quercetin-3-glucoside (0.56 mg/100g) are present in *Haden* mango pulp (Berardini et al., 2005b).

Table 2.7. Relative amounts of phenolic compounds in different cultivars of mango flesh.

Mango cultivars/ varieties	Country of origin	Xanthones		Phenolic acid	Flavonoids		Reference
		Mangiferin (µg/g FW)	Iso-mangiferin (µg/g DW)	Ellagic acid (µg/g FW)	Quercetin -3-O-gal (µg/g DW)	Quercetin -3-O-glu (µg/g DW)	
Haden	Mexico	37.3	-	75.4	-	-	(Manthey & Perkins-Veazie, 2009)
	Brazil	16.2 ± 2.7 (DW)	-	-	6.6 ± 0.4	5.6	(Berardini et al., 2005b)
		2.9 ± 0.1	ND	-	ND	0.6	(Ribeiro et al., 2008)
Keitt	Mexico	2.8 ± 5.2	-	92.4	-	-	(Manthey & Perkins-Veazie, 2009)
Kent	Mexico	10.45	-	190	-	-	
	Eucador	tr	-	tr	-	-	
	Peru	110.2 ± 116.3	-	2385	-	-	
Tommy Atkins	Mexico	0-182.7 ± 197.3	-	112.1	-	-	
	Brazil	2.2 ± 0.1	0.5	-	ND	ND	(Ribeiro et al., 2008)
		4.6 ± 0. 1 (DW)	-	-	-	-	(Berardini et al., 2005b)
Ataulfo	Mexico	556.28	-	102.93	-	-	(Manthey & Perkins-Veazie, 2009)
José	La Réunion	19.4 ± 0.2 (DW)	-	-	-	-	(Berardini et al., 2005b)
R2E2	Australia	4.3 ± 0.6 (DW)	-	-	-	-	
Mini-mango	Colombia	3.0 ± 0.5 (DW)	-	-	-	-	
Ubá	Brazil	12.4 ± 0.3, 46.5 ± 4.7	1.1 ± 0.1	-	2.5 ± 0.2	6.3 ± 0.4	(Ribeiro et al., 2008)
Kensington Pride	Australia	0.5 (DW)	-	-	-	-	(Amitha, 2011)

-: Values not reported, ND: not detected, tr: trace, FW: fresh weight, DW: dry weight. All values were obtained via HPLC analyses.

Flavanones. Flavanones contain a saturated 3-carbon chain and an oxygen atom in the C_4 position, and are usually glycosylated at position 7 with a disaccharide, or less frequently with a monosaccharide (e.g. glucose) (Tomas-Barberen & Clifford, 2000). Naringin (naringenin 7-O-rhamnoglucoside) (Fig 2.9) and naringin 7-O-neohesperoside have been previously identified in banana pulp (Aurore, Parfait, & Fahrasmane, 2009; Mendez et al., 2003).

Basic structure of flavanone   Naringin
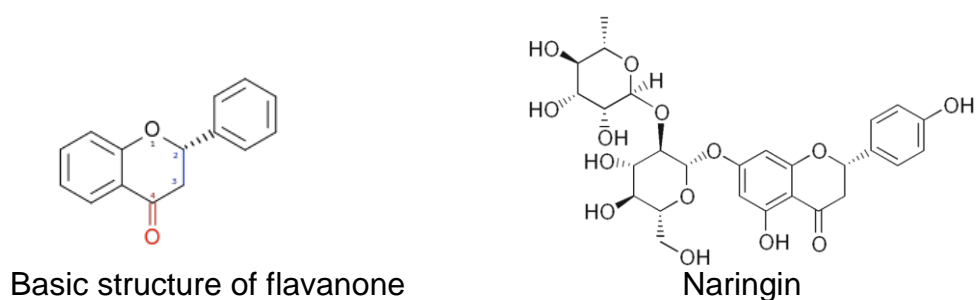
Figure 2.9. Structures of flavanones.

Flavanols. Flavanols or flavan-3-ols have a hydroxyl group on the C_3-ring and occur only as aglycones in fruits. The flavanols identified in banana and mango (Fig 2.10) are present as monomers or as proanthocyanidins. Various soluble flavanols have been identified in banana fruit including (+)-catechin, (-)-epicatechin and (+)-gallocatechin monomeric and oligomeric flavanols (Aurore, Parfait, & Fahrasmane, 2009; Bennett et al., 2010; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Mendez et al., 2003), and they are thought to comprise the bulk of the flavonoids in banana. Collectively, the monomers are referred to as catechins. Catechins are reported to be very reactive when exposed to atmospheric oxygen (Jorge, 2006). Significant increases in (+)-catechin and (-)-epicatechin have been reported in some Brazilian cultivars after harvest (Mendez et al., 2003). The changes in flavanol levels following harvest and ripening are assumed to be due to their incorporation into condensed tannins or catabolic processes (Bennett et al., 2010). Flavanols identified in mango include (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin. Little information on other flavonoids or their concentrations has been reported for mango.

Basic structure of flavanol

(+)-Catechin   (-)-Epicatechin   (+)-Epigallocatechin   (-)-Epigallocatechin gallate
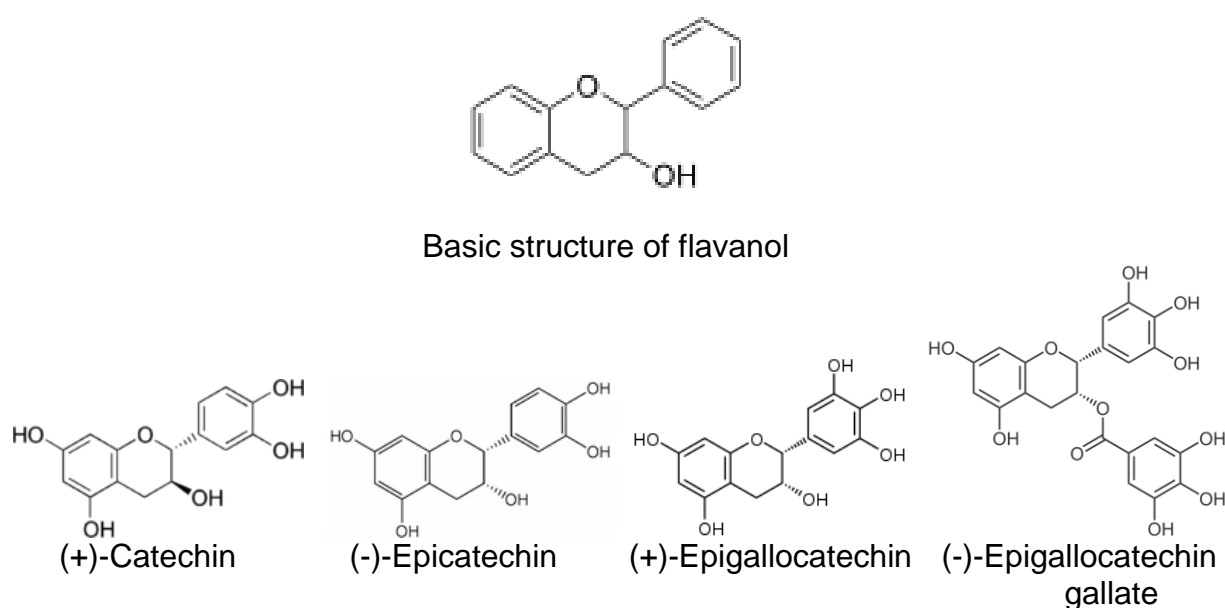
Figure 2.10. Structures of flavanols.

Proanthocyanidins. Proanthocyanidins (PAs), also referred to as condensed tannins, are oligomeric and polymeric flavanols (Fig 2.11) linked through the C_4-C_8 bond. Proanthocyanidins are reported to be colourless when their chain is short, and they turn yellowish to brown with increasing polymerisation (Jorge, 2006). When heated in acidic medium, PAs transform into the corresponding anthocyanidins. These flavanols could be esterified with gallic acid to form 3-O-gallates. PAs can be divided into three groups according to their hydroxylation patterns- prodelphinidins, propelargonidins and procyanidins with the latter being the most common. Soluble tannins (4 mg/100g FW) were quantified using HPLC in banana samples from Virginia, USA (Gu et al., 2003), and were identified as monomers, dimers, trimers and oligomers of the procyanidin type. In addition, homogenous B-type procyanidins (C_4-C_6 linkage) were identified in the banana samples (Gu et al., 2003). Gallocatechin-catechin dimers were measured in Spanish bananas, using a post-column derivatisation HPLC procedure at a level of 0.14 mg/100g FW (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000). Another study identified PAs in soluble extracts of Spanish banana pulp, but no free hydroxycinnamic acid, ferulic acid, soluble anthocyanidins, anthocyanins or naringin were detected (Bennett et al., 2010).
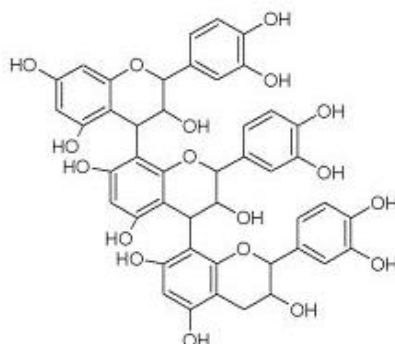
Figure 2.11. Structure of an example oligomeric proanthocyanidin.

2.2.2.2. Non-flavonoids (Other phenolics)

Some non-flavonoid phenolics occur in considerable levels in banana and mango. Xanthones, hydroxycinnamic acids, hydroxybenzoic acids and hydrolysable tannins are the main examples in mango and banana.

Xanthones. Xanthones, generally called C-glucosyl xanthones are heat stable molecules that are widely distributed in higher plants. Mangiferin (C_2-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) (Fig 2.12) was first isolated from mango plant leaves, while homomangiferin was quantified in the mango plant bark (Masibo & He, 2008) at higher levels. In a polyphenol screening of twenty mango cultivars, Saleh and El Ansari (1975)

identified the co-occurrence of mangiferin, isomangiferin and homomangiferin in mango fruit pulp. Schieber et al. (2005) investigated the xanthone glycosides in the flesh of nine different mango cultivars from Brazil, Peru, La Réunion, Colombia, Australia, Thailand and Kenya, and found that mangiferin levels were highest in *Haden* and *José*, while in other cultivars (*Kent, Heidi, Manila* and *Ngowe*), mangiferin was not detected at all. Hewavitharana (2013) reported that the *Kensington Pride* cultivar has 0.5 µg/g DW mangiferin (mean of 2.65 µg/g mangiferin in one mango). Besides this detection of mangiferin, no other phenolic studies on the *Kensington Pride* cultivar have been reported. Factors such as agroclimate, growing conditions, exposure to UV radiation, the ripening stage, and postharvest and storage conditions may all contribute to variations in the phenolic content and composition of mango, and thus inter-fruit variability (Manthey & Perkins-Veazie, 2009; Ornelas-Paz et al., 2008; Ornelas-Paz, Yahia, & Gardea, 2007; Talcott et al., 2005; Veda, Platel, & Srinivasan, 2007; Wilkinson et al., 2011).
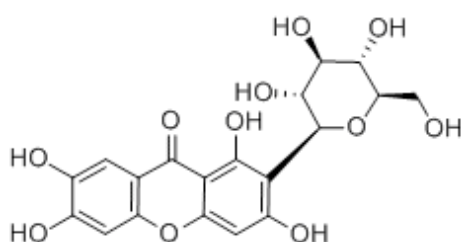
Figure 2.12. Structure of mangiferin.

Hydroxycinnamic acids. Hydroxycinnamic acids are found in all parts of fruits and vegetables even though the highest concentrations are observed in the outer parts of mature fruits, and their concentration decreases during ripening (Manach et al., 2004). These acids are aromatic compounds with a 3-carbon side chain (C_6-C_3). They have been shown to occur in banana cell walls with high levels of *trans*-ferulic acid (5.4 mg/100g FW) and low levels of *p*-coumaric acid (0.46 mg/100g FW) and caffeic acid (0.2 mg/100g FW) being reported (Mattila, Hellstrom, & Torronen, 2006). *m*-Coumaric acid, *p*-coumaric acid and ferulic acid (Fig 2.13) have been also identified in mango (Kim, Brecht, & Talcott, 2007). Flavonol glycosides acylated with these phenolic acids are found in fruits (Shahidi & Naczk, 2004).

p-Coumaric acid   Ferulic acid   Caffeic acid
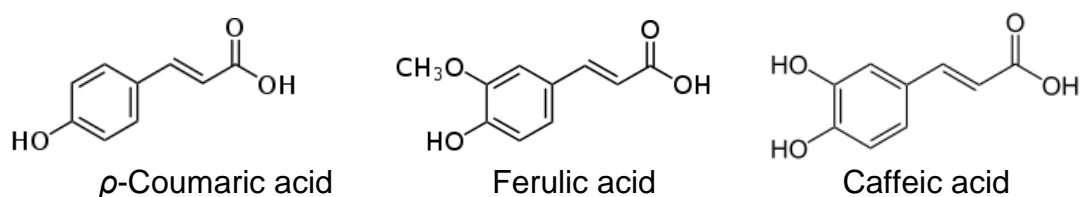
Figure 2.13. Structures of hydroxycinnamic acids.

Hydroxybenzoic acids. Hydroxybenzoic acids have a C_6-C_1 structure with a carboxyl group linked to the benzene ring. Hydroxybenzoic acids are found in banana (e.g. gallic acid) (Aurore, Parfait, & Fahrasmane, 2009; Bennett et al., 2010; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000), and in mango flesh where more studies have been reported. The phenolic acids identified in mango include gallic acid and ellagic acid (Fig 2.14). Gallic acid was identified as the major phenolic acid in mango, followed by six hydrolysable tannins, which together constituted approximately 98% of the total polyphenols identified in *Tommy Atkins* mango (Florida), along with four minor hydroxycinnamic acids (Kim, Brecht, & Talcott, 2007). In another study of *Tommy Atkins* mango, gallic acid was again identified as the predominant phenolic acid in mango flesh, along with four gallic acid precursors in the form of hydrolysable tannins (Talcott et al., 2005). *p*-Hydroxybenzoic acid (20.7 mg/100g DW), *p*-coumaric acid (2.3 mg/100g DW) and two ellagic acid glycosides (<0.5 mg/100g DW) were identified in different stages in fully-ripe mango (day 12-20). Gallic acid and their hydrolysable tannins were found to significantly decrease throughout fruit ripening from mature-green to fully ripe stages, but were unaffected by hot water treatment (46°C for 75 min). Gallic acid concentration decreased by 22% and hydrolysable tannins decreased by 57% during storage and ripening (Kim, Brecht, & Talcott, 2007). In contrast, Talcott et al. (2005) reported a 34% increase in the total hydrolysable tannins concentration in the cultivar *Tommy Atkins* as the fruit ripened, indicating that appreciable differences may occur among fruit cultivars grown under different growing conditions (soil, fertilisation and cultivation practices) or harvest periods. Several studies have shown that climacteric fruits such as mango experience large changes in phenolic content and composition during ripening, especially following respiratory climacteric and shikimic acid pathway activity. Schieber, Ullrich, and Carle (2000) reported there was no evidence of ellagic acid and gallic acid dimers or trimers in mango puree, and suggested that these depsides were hydrolysed as a consequence of heating, acidification or enzymatic treatment. This suggestion was further supported by the detection of aglycones quercetin and kaempferol. Cultivar-dependent differences and changes in phenolic patterns during fruit development have to be considered.

Gallic acid   Ellagic acid
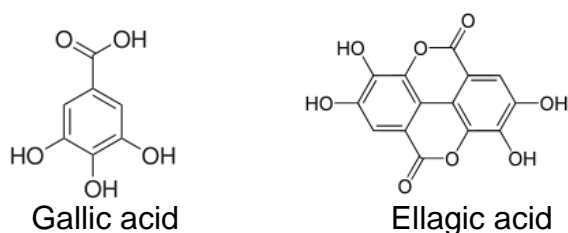
Figure 2.14. Structures of hydroxybenzoic acids.

Hydrolysable tannins. Hydrolysable tannins are mainly gallic acid glucose esters and high molecular weight compounds. Two types are known- gallotannins, which yield only gallic acid upon hydrolysis, and ellagitannins, which produce ellagic acid as the common degradation product. Presently, hydrolysable tannins have not been detected in banana but are found in mango flesh. Tannins occur in ripe mango either as gallotannins (gallic acid component) or other phenolic acids derived from the oxidation of the galloyl residue of ellagitannins (Masibo & He, 2008). Hydrolysable tannins are derivatives of phenolic acids, and their occurrence appears more limited compared to condensed tannins. Some components of the hydrolysable tannins in mango flesh include digallic acid and β-glucogallin (Fig 2.15). A chromatographic study of the phenolic profiles of five mango varieties (*Tommy Atkins*, *Haden*, *Kent*, *Keitt* and *Ataulfo*) was consistent, with the occurrence of hydrolysable gallotannins as the major phenolic along with low concentrations of gallic acid, tetragalloyl glucose, and conjugates of ellagic acid and mangiferin (Manthey & Perkins-Veazie, 2009).

Digallic acid   β-glucogallin
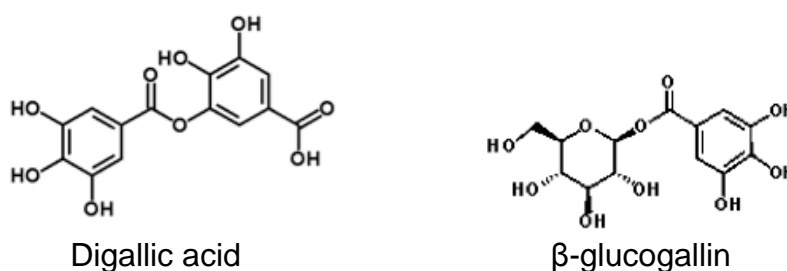
Figure 2.15. Structures of hydroylsable tannins.

2.2.3. Research on nutritional health benefits of carotenoids and polyphenols

Numerous studies have shown an association between the consumption of fruits that are rich in carotenoids, flavonoids and some phenolic acids, and reduced risks of several chronic diseases. Although the health benefits of fruit consumption are strongly indicated from studies of diet and disease, it is not certain what role individual fruit types can play, particularly tropical fruits. Many studies have characterised molecular components and linked these with specific bioactivities as a means of defining the potential health benefits of consuming specific fruits or vegetables. However, cultivar and individual fruit differences have also been reported in some studies that evaluated the bioactivity of fruits (Wilkinson et al., 2011). It is expected that the presence or concentrations of many components would change with maturity level, but there is less information available on the variation of the many secondary metabolites that are likely to contribute to a range of bioactivities. Such differences in bioactive levels or the activity of extracts between individual fruits of the

same cultivar has received less attention in the context of tropical fruits such as mangoes or bananas. Here, the fruit size may contribute to these differences, which has particular significance for bioavailability studies.

2.2.3.1. Carotenoids

Epidemiological studies have shown an inverse correlation between the consumption of carotenoid-rich vegetables and fruits, and the incidence of: cancers including lung, breast, prostate, and those affecting the gastrointestinal tract (gastrointestinal tract) (Bowen, Mobarhan, & Smith, 1993; Gerster, 1993; Kant, Block, Schatzkin, & Nestle, 1992; Kiokias & Gordon, 2004; Mayne, 1996) and the cardiovascular system (Kim et al., 2008; Krinsky, 1998; Murr et al., 2009; Yeum, Beretta, Krinsky, Russell, & Aldini, 2009); diabetes (Yeum & Russell, 2002); some inflammatory diseases (Perera & Yen, 2007); and age-related macular degeneration (Seddon et al., 1994; Snodderly, 1995). In addition, there is also considerable experimental evidence that major dietary carotenoids prevent or delay carcinogenesis at many sites (International Agency for Research on Cancer, 1998). Other biological functions attributed to carotenoids include influencing gene expression and immune function (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010).

In these diseases, free radical damage is thought to play a role in their pathophysiology (Boon et al., 2010; Diplock, 1991; Krinsky, 2001). The most established antioxidant function is singlet oxygen-quenching capacity, which is able to explain the efficacy of β-carotene for skin protection in light-sensitive individuals (Heinen et al., 2007; Van den Berg et al., 2000). The ability of carotenoids to quench singlet oxygen is related to their conjugated double-bond system and maximum protection is given by those carotenoids having nine or more double bonds (Foote, Chang, & Denny, 1970).

The most documented function of some carotenoids is their provitamin A activity. Once converted to vitamin A, derived health benefits include the maintenance of normal eye health, epithelial function, embryonic development and immune system function (Diplock, 1991). Xanthophylls do not possess vitamin A potency, but they are the only carotenoids present in human retinal pigment epithelium, in contrast to other body sites where all carotenoids occur (Bone, Landrum, Hime, Cains, & Zamor, 1993). Xanthophylls probably function as blue light filters and singlet oxygen quenchers (Seddon et al., 1994).

Fruit-derived provitamin A carotenoids have higher bioavailability and bioefficacy for conversion to vitamin A compared to those in green vegetables, presumably due to the chlorophyll-carotenoid or protein-carotenoid complexes in the chloroplasts (West & Castenmiller, 1998). Recent research has shown that β-carotene from some plant sources has a lower bioavailability than once thought, i.e. dark green leafy vegetables may provide only 1 µg of vitamin A from 26 µg of β-carotene (de Pee et al., 1998). Previously, it was accepted that dark green leafy vegetables had a greater bioavailability, providing the same amount of vitamin A from a smaller amount of β-carotene (6µg). However, the bioavailability of β-carotene in orange and yellow fruits and tubers is greater than its bioavailability from dark green leafy vegetables; the ratios have now been set at 12 µg of β-carotene to 1 retinol activity equivalent (RAE) and 24 µg of other provitamin A carotenoids to 1 RAE (de Pee et al., 1998; Institute of Medicine, 2001). It is likely that orange and orange-yellow fruits would have a significant impact on improving vitamin A status.   These differences may result from differences in intracellular location of carotenoids. In leaves, carotenoids are present in chloroplasts whereas in fruits, they are located in chromoplasts. This has led to speculation that chloroplasts may be less efficiently disrupted in the intestinal tract than chromoplasts (de Pee et al., 1998).

As β-carotene accounts for more than half of the total carotenoid content in most mango cultivars, this suggests that mango fruit substantially contributes to the provitamin A supply in tropical and subtropical countries. Therefore, it is important to learn about the variety and levels of carotenoids in mango, especially in *Kensington Pride*, which have not yet been reported. Although ripe mangoes have significantly higher carotenoid concentrations than bananas, it is noteworthy that mangoes are seasonal fruits, being available for only four to five months a year (in Australia), and may not be a year-round better source of provitamin A than bananas.

Among mango carotenoids, β-carotene is considered to be of higher provitamin A relevance since α-carotene, all-*trans*- and *cis*-β-cryptoxanthin are found at lower levels (Mercadante & Rodriguez-Amaya, 1998). As precursors of vitamin A (retinol), the presence of at least one unsubstituted β-ionone ring is a prerequisite for this biological property. All-*trans*-β-carotene has the highest provitamin A capacity, as it possesses two β-rings and may be cleaved theoretically into two molecules of retinal in the intestine by the enzyme β-carotene 15,15' monooxygenase (During, Smith, Piper, & Smith, 2001; Schieber & Carle, 2005). Carotenes in the all-*trans* form have higher bioavailability than

the *cis*-isomers, with β-carotene having the highest theoretical bioconversion rates at 100% on a weight basis (Rodriguez-Amaya, 2001; West & Castenmiller, 1998). Quantifying the proportion of *cis*-β-carotene isomers is essential in provitamin A estimation, since the efficiency of β-carotene conversion into vitamin A is only 53% and 38% for 13-*cis*-and 9-*cis*-β-carotene respectively (Vasquez-Caicedo et al., 2005).

Banana is reported to be a poor source of vitamin A (Helen Keller International, 1993), which may be true for *Cavendish*, the primary banana cultivar marketed globally but is not correct for other cultivars and varieties. There is a huge diversity in the *Musa* family with respect to carotenoids and their provitamin A contents. Some banana cultivars in the Federated States of Micronesia and other Pacific island countries have among the highest carotenoid levels, and these bananas are capable of meeting the estimated daily vitamin A requirements from one to three fruits (Englberger et al., 2003d; Englberger et al., 2003a). This suggests existing provitamin A-rich *Musa* varieties could have significant long term beneficial health impacts for vitamin A deficient populations, particularly in regions where banana fruits are part of a staple diet (Davey, Garming, Ekesa, Roux, & Van den Berg, 2008; Davey, Mellidou, & Keulemans, 2009; O'Neill et al., 2001). Although the carotenoid content of *Cavendish* may be low, the high frequency and quantity of its consumption makes the *Cavendish* banana a potentially important source of provitamin A carotenoids.

2.2.3.2. Polyphenols

The bioavailability of polyphenols differs greatly amongst subclasses, so the most abundant phenolics in the human diet may not necessarily lead to the highest concentrations of active metabolites in target tissues. One of the main objectives of bioavailability studies is to determine which phenolics are better-absorbed and, which of these leads to the formation of active metabolites.

It is essential to determine the nature and distribution of these compounds in the human diet. This will allow evaluation of dietary polyphenol intake and enable more precise epidemiologic analysis that can lead to a better understanding of the relationship between the intake of these compounds and the risk of the development of several diseases. Furthermore, not all carotenoids or phenolic compounds are absorbed with equal efficacy. Phenolics are extensively metabolised by intestinal and hepatic enzymes, and (if not absorbed by the end of the small intestine) by the microbiota of the large intestine. Knowledge of the bioavailability and metabolism of various phenolics is necessary to evaluate their biological activity within target tissues.

Phenolic compounds are receiving much current attention because of their beneficial health effects related to their biological and pharmacological properties: anti-inflammatory, anti-mutagenic, anti-carcinogenic, anti-allergenic, neuro-protective, anti-thrombotic properties, and cardio-protective and vasodilatory effects. Phenolic subclasses are thought to be effective in preventing cardiovascular disease by: acting as antioxidants (Kahkonen et al., 1999; Merken & Beecher, 2000; Shahidi & Naczk, 2004; Takahama, Oniki, & Hirota, 2002; Tsao & Deng, 2004); reducing chronic inflammation; improving endothelial function by up-regulating eNOS expression and increasing production of endothelial cell nitric oxide (Daud et al., 2010; Erdman et al., 2007; Fraga & Oteiza, 2011). Phenolics reduce coronary heart disease mortality (Hertog et al., 1995; Kappus, 1985; Peri et al., 2005; Santos-Buelga, Escribano-Bailon, & Lattanzio, 2011; Steffen, Gruber, Schewe, & Sies, 2008) by suppressing low-density lipoprotein (LDL) oxidation (Meyer, Heinonen, & Frankel, 1998). In addition, phenolics exhibit modulation of cancer-protein functions and agonism or antagonism of carcinogenesis-related receptors e.g. epidermial growth factor (Agullo et al., 1997), atylhydrocarbon receptor (Ashida, Fukuda, Yamashita, & Kanazawa, 2000) and estrogen receptor β (An et al., 2001).

While recent data has shown a strong correlation between serum triacylglyceride levels and coronary atherosclerosis (Hamsten et al., 2005), it has been reported that mangiferin ameliorates hyperlipidemia (Guo et al., 2011) and decreases triacylglyceride levels during animal model studies (Muruganandan, Lal, & Gupta, 2005b; Muruganandan, Srinivasan, Gupta, Gupta, & Lal, 2005a). *In vitro* studies have shown that mangiferin provides antioxidant (Aderibigbe, Emudianughe, & Lawal, 2001; Darvesh, Carroll, Bishayee, Geldenhuys, & Van der Schyf, 2010; Garcia-Rivera, Delgado, Bougarne, Haegeman, & Berghe, 2011; Muruganandan, Gupta, Kataria, Lal, & Gupta, 2002; Viswanadh, Rao, & Rao, 2010; Yoshikawa et al., 2001), anti-diabetic (Masibo & He, 2008), immune-stimulating (Guha, Ghosal, & Chattopadhyay, 1996; Sanchez et al., 2000; Yoshikawa et al., 2002) and anti-viral properties (Zheng & Lu, 1990), while also protecting hepatocytes, lymphocytes, neutrophils and macrophages from oxidative stress (Amazzal, Lapotre, Quignon, & Bagrel, 2007; Jagetia & Baliga, 2005; Pourahmad, Eskandari, Shakibaei, & Kamalinejad, 2010).

Several *In vitro* studies suggest that catechins have beneficial effects owing to the following: (1) their free radical scavenging and antioxidant activities (Augustyniak, Waszkiewicz, & Skrzydlewska, 2005; Rodriguez et al., 2006), (2) their protection against

congestive heart failure (Ishikawa et al., 1997), cancer (Chieli, Romiti, Rodeiro, & Garrido, 2010; Fraga & Oteiza, 2011; Rajendran, Ekambaram, & Sakthisekaran, 2008; Yamanaka, Oda, & Nagao, 1997), and renal failure (Korish & Arafah, 2008), (3) their reduction in the incidence of myocardial ischemia (Modun, Music, Katalinic, Salamunic, & Boban, 2003; Suzuki et al., 2010; van Jaarsveld, Kuyl, Schulenburg, & Wild, 1996), and (4) their support of anti-aging processes (Drouin et al., 2011; El Gharras, 2009). In addition, quercetin has vasodilator and anti-hypertensive effects (Erdman et al., 2007), and reduces the vascular remodeling associated with elevated blood pressure in spontaneously hypertensive rats (Duarte et al., 2001).

In vitro studies of mangiferin, together with catechin and epicatechin, show that these compounds protect human T lymphocytes from T cell receptor (TCR)-induced cell death (AICD) (Hernandez, Rodriguez, Delgado, & Walczak, 2007), and contribute to the protective effects of reducing iron neurotoxicity in cells. However, the effects of these phenolics are not equal, with the order of activity being classified as catechin > epicatechin > mangiferin. The quantities of these compounds may vary among mango cultivars, with their prevalence determining the predominant action. In contrast to mango, there are few studies that detail the effects of banana phenolics on human or animal health. Gorinstein et al. (1999) found that mango is one of the fruits with the highest content of gallic acid among tropical fruits, higher than pineapple, wax apple, guava, rambutan and lichi. More *in vitro* and *in vivo* studies are needed for banana and mango fruits to ascertain the fruit-related effects of phenolic compounds.

2.3. Bioaccessibility and bioavailability of carotenoids and polyphenols
2.3.1. Principles of *in vitro* models
To predict phytonutrient bioavailability, human studies would be most appropriate but there are technical and ethical limitations. For example, high inter-individual variation in plasma concentrations has been reported, suggesting that absorption, distribution and elimination of carotenoids and phenolic compounds is influenced by genetic, metabolic and physiological factors. Large numbers of subjects in human trials are often required to achieve statistical significance, which makes these studies expensive, time-consuming and labour-intensive. There are also possible interactions of these phytonutrients with other components in the diet, leading to further difficulties in studying the target phytonutrient classes.

Interpretation of *in vivo* biological activity of phytonutrients first requires an understanding of their bioavailability based on *in vitro* data. *In vitro* gastrointestinal digestion, colonic fermentation and absorption models have been commonly used to predict the effects of food matrix, processing methods and dietary components on digestive stability, bioaccessibility, microbial degradation and intestinal uptake of dietary carotenoids and polyphenols. Depending on the type of research question and substrates, *in vitro* models can be designed to simulate different phases of digestion. The upper intestinal models are used for measuring the 'removal' of digestible components and changes in non-digestible components, while *in vitro* colonic models elucidate the role of microbial fermentation in the metabolism of the non-digestible parts of the diet. In addition, *in vitro* absorption models using human intestinal cell lines (Hilgers, Conradi, & Burton, 1990; Sambuy et al., 2005), animal brush border membrane vesicles (Moore, Gugger, & Erdman, 1996) or animal intestinal everted sacs (Barthe, Woodley, & Houin, 1999; Wolffram, Block, & Ader, 2002) have been carried out to understand mimic cellular uptake and transport of phytonutrients (During & Harrison, 2001)

2.3.1.1. Mastication

A harmonised static *in vitro* digestion method (Minekus et al., 2014) consisting of an oral, gastric and small intestinal phase was recently proposed by the INFOGEST network to aid in the comparison of *in vitro* digestion studies amongst international labs. The study acknowledged that chewing and the consequent particle size reduction is a major determinant of digestion of solid food, but recommended standardisation of solid food particle size with a commercial meat mincer. This model does not take into consideration the heterogeneity of chewed food particles that are not reflected with 'artificial' mechanical steps.

Ideally, structural properties of a food substrate to be digested *in vitro* should be similar to those of a chewed food bolus. During simulated or real oral chewing, physical barriers to the release of phytonutrients from plant cells may be ruptured and the degree of cellular intactness could be indicative of their potential bioaccessibility. However, oral processing can be difficult to simulate and most *in vitro* studies skip this step or use simplified techniques such as pulverising, sieving, chopping or mincing (Woolnough, Monro, Brennan, & Bird, 2008), and the occasional inclusion of (salivary) α-amylase for starch digestion. As bolus properties strongly influence the subsequent digestion steps, it is important to use a bolus with relevant physicochemical and textural properties during *in*

vitro digestion (Guerra et al., 2012). The effect of mastication is highlighted in Fig 2.16, where a considerable modification of cellular architectures of various fruit and vegetables is apparent.
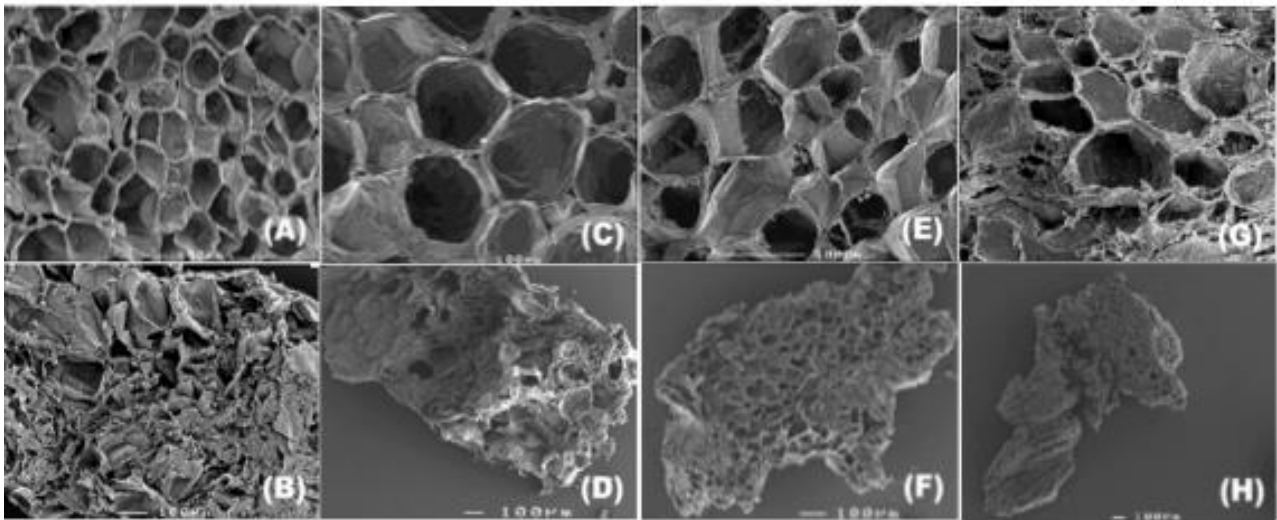
Figure 2.16. Effect of chewing on microstructural changes of fresh apple, rockmelon, carrot and steamed beetroot (A, C, E, G). Chewed structures are shown in the second row (B, D, F, H). Images are adapted from Hoerudin (2012).

Solid fruit samples of mango and banana flesh have been commonly subjected to milling or grinding with hammer mills, blenders, and mortars and pestles (Berardini et al., 2005b; Berardini, Knodler, Schieber, & Carle, 2005a; Davey, Keulemans, & Swennen, 2006; Davey, Mellidou, & Keulemans, 2009; Gonzalez-Montelongo, Lobo, & Gonzalez, 2010a, 2010b; Kim et al., 2010; Wilkinson et al., 2011), and to mechanical/instrumental homogenisation (Ajila, Bhat, & Rao, 2007; Ajila, Rao, & Rao, 2010; Arora et al., 2008; Manthey & Perkins-Veazie, 2009; Mercadante & Rodriguez-Amaya, 1998; Mercadante, Rodriguez-Amaya, & Britton, 1997; Ornelas-Paz et al., 2008; Ornelas-Paz, Yahia, & Gardea, 2007; Sun et al., 2002), often preceded by air-drying (John, Subbaray, & Cama, 1970; Pott et al., 2003b; Robles-Sanchez et al., 2011; Robles-Sanchez et al., 2009a) or lyophilisation (Barreto et al., 2008; Bennett et al., 2010; Berardini et al., 2005b; Bouayed, Hoffmann, & Bohn, 2011; Daud et al., 2010; Davey, Keulemans, & Swennen, 2006; Davey, Mellidou, & Keulemans, 2009; Gonzalez-Montelongo, Lobo, & Gonzalez, 2010a, 2010b; Kim et al., 2010; Shofian et al., 2011; Wilkinson et al., 2011). Wet liquid samples such as purees or juices, have been prepared prior to extraction (Manthey & Perkins-Veazie, 2009) or centrifugation (Alothman, Bhat, & Karim, 2009). In context of the present investigation, one of the objectives must be to prepare fresh fruit samples for analysis, in a way that the state and condition of these samples are similar to the microstructure achieved during consumption with minimal 'artificial' processing.

The few identification and bioaccessibility studies of mango and banana in the current literature are mostly based on extraction of puree (Manthey & Perkins-Veazie, 2009; Ornelas-Paz et al., 2008; Ornelas-Paz, Yahia, & Gardea, 2007, 2008) and cubes (2 x 2 cm) (Robles-Sanchez et al., 2011; Shofian et al., 2011; Sulaiman et al., 2011) except for Epriliati, D'Arcy and Gidley (2009a) who investigated the particle size distribution of chewed mango. Those results emphasised the importance of the simultaneous punch and gentle squash action of teeth. In addition to the breakdown of food particles into smaller particle size, human mastication achieves lubrication, softening and dilution with saliva (Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000; Prinz & Lucas, 1995) and leads to the formation of a cohesive bolus (Lucas et al., 2006) and the melting or hardening caused by phase changes (Bourne, 2004). For beverages and juices, the food may be swallowed directly because no change to inflow characteristics is needed.

Chewing is a subjective process that varies between individuals, and between food matrices and the structural properties of the food bolus. However, characterising food products in terms of structural properties may be feasible and will permit a better simulation of food material used in *in vitro* digestion. It is suggested that the current two-phase *in vitro* digestion model can be improved by including a 'real' chewing phase or a phase that more closely mimics actual chewing behaviour and mechanics.

2.3.1.2. Gastric and small intestinal digestion

Physiological conditions implemented across various static *in vitro* studies can differ considerably. *In vitro* studies are usually conducted to simulate the fasting state of digestion, where the gastric medium is adjusted to pH 2, and the intestinal environment ranges from pH 6.5-7.5. While these conditions may be representative of the gastric and intestinal fasting state, pH can be influenced by the presence of food. The mean stomach pH measured after ingestion of vegetable-rich meals is pH 5.4-6.2, decreasing to pH 1.8-2.9 by 3 h later; however, the intestinal medium, which is ~pH 5 in the fasting state, increased to pH 6.1-6.6 after food intake (Tyssandier et al., 2003).

β-Carotene transfer to the micellar phase was significantly increased under simulated conditions of the fed state (47%) compared to fasting conditions (19%) (Wright, Pietrangelo, & MacNaughton, 2007). These results show there is a need to carefully consider and define experimental parameters used for *in vitro* assays in the study of both carotenoids and polyphenols. While the residence time in the human stomach *in vivo*

varies according to the particle size of food (Guyton & Hall, 1996a), gastric and intestinal incubation periods used in different *in vitro* models are fixed, ranging from 30 min to 2 h. Hur, Lim, Decker and McClements (2011) have provided an extensive review of *in vitro* digestion and absorption studies. *In vitro* models have been adapted to estimate the release of carotenoids by quantifying the fraction of phytonutrients transferred from the food matrix to a supernatant or micellar phase of the digesta, after mimicking conditions of the upper gastrointestinal tract. The fractional release of a phytonutrient from the food matrix is referred to as bioaccessibility (Netzel et al., 2011) and represents the potential for their absorption or bioavailability.

2.3.1.3. Colonic-microbial fermentation

Taking into account the current scientific evidence about the numerous beneficial effects induced by polyphenol intake despite the low bioavailability of these dietary molecules, further studies are required to investigate whether polyphenol colonic-microbial metabolites precede the effects of their parent compounds, which are mostly absorbed in the small intestine (Etxeberria et al., 2013). After undergoing ring fission in the colon, these metabolites (i.e. phenolic acids and hydroxycinnamates) are absorbed and subjected to phase II metabolism in the liver before being excreted in urine in substantial quantities that, in most instances, are well in excess of flavonoid metabolites that enter the circulatory system via the small intestine (Crozier, Del Rio, & Clifford, 2010). *In vitro* colonic models have been designed to exhibit similar conditions to the human colonic region and consist of continuous, semi-continuous, and batch culture systems that maintain colonic or faecal microbiota under strictly anaerobic conditions. Studies on the action of gut microbiota on polyphenols leading to the production of metabolites with diverse physiological relevance have been increasing in recent years (Bolca, Van de Wiele, & Possemiers, 2013). A review of various *in vitro* fermentation studies of polyphenols or polyphenol-rich extracts using human and rat faecal slurries, is presented in Table 2.8. When pure phenolic compounds are used as substrates, their molecular weight and solubility vary according to the degree of conjugation; hence, it is important to measure the amount of substrate on a molar rather than mass basis (Aura, 2005).

Models utilising animal faeces are useful for investigating the metabolic processes mediated by intestinal microbiota, since they can be given controlled diets to avoid prior microbial adaption to certain food substrates, which may bias the gut microbiota composition, unlike the challenges faced in dietary control for humans. It must also be

Table 2.8. Compilation of *in vitro* colonic fermentation conditions for various polyphenols.

Compounds	Faecal slurry	Final concentration	Buffer/pH	Temp.	Time intervals (h)	Reference
Thymol, carvacrol, eriodictyiol and rosmarinic acid	5% human faecal slurry	500 µM, 200 µM (rosmarinic acid)	Carbonate-phosphate buffer		0, 2, 6, 12, 24, 48	(Mosele et al., 2014)
Polyphenol mixture	9% substrate with 45.5% human faecal slurry (20%, w/w)	-	15% (v/v) Dulbecco's phosphate buffer saline (pH 5.5)		5, 24	(Dall'Asta et al., 2012)
Anthocyanin, punicalagin and ellagic acid	1 mL polyphenol in 5 mL human faecal slurry (32%)	-	Phosphate buffer (pH 7)		0, 2, 4, 6, 24, 48	(Gonzalez-Barrio, Edwards, & Crozier, 2011)
Rutin and quercetin	28 µM rutin or 55 µM quercetin in 5 mL human faecal slurry (32%, w/v)	-	Phosphate buffer (pH 7)		0, 2, 4, 6, 8, 30, 48	(Jaganath, Mullen, Lean, Edwards, & Crozier, 2009)
Flavan-3-ols	1 µM polyphenol in methanol with 10 mL human faecal slurry (10 %, w/v)	100 µM	Carbonate-phosphate buffer with 10 mL trace elements (pH 5.5)		0, 1, 2, 4, 6, 8, 24	(Bazzocco, Mattila, Guyot, Renard, & Aura, 2008)
Cyanidin-3-glucoside and cyanidin-3-rutinoside	5 mg polyphenol in 8mL rat caecal slurry (25%)	-	Carbonate-phosphate buffer (pH 7.2) with trace elements and resasurin	37°C	0, 1, 2, 4, 6, 8, 10, 24	(Hassimotto, Genovese, & Lajolo, 2008)
Polyphenol mixture	2 mL rat caecal slurry (100g/L)	-	Phosphate buffer (pH 7.5)		24	(Saura-Calixto, Serrano, & Goni, 2007)
Caffeic, chlorogenic and caftaric acids	1 µM polyphenol in methanol with 10 mL human faecal slurry (5%, w/v)	100 µM	Carbonate-phosphate buffer		0, 2, 4, 6, 8, 24	(Gonthier et al., 2006)
Ellagic acid, punicalagin, daidzein	1% human faecal slurry (w/v)	10 µg/mL, 110 µg/mL, 1 µg/mL	Brain heart infusion medium with cysteine and resazurin (pH 7.4)		5, 24, 48, 72	(Cerda, periago, Espin, & Tomas-Barberan, 2005)
Rutin and anthocyanins	1 µM polyphenol in methanol with 10 mL human faecal slurry (1 or 5%, w/v)	100 µM	Carbonate-phosphate buffer (pH 5.5)		0, 2, 4, 6, 8, 24	(Aura et al., 2005)

noted that animal digestive physiologies differ from humans; for example, rodents are coprophagic, while pigs are generally more similar to humans, although their upper digestive tract is more heavily colonised by microbiota and their lower bowel is proportionally larger in size (Macfarlane & Macfarlane, 2007).

Colonic microbiota ferment substrates to various end-products such as carbon dioxide (CO_2), ammonia (NH_4) and short-chain fatty acids (SCFA). The gas produced as a result of fermentation is an index of fermentative activity and contains a mixture of gases where the predominant gas (CO_2) is derived both from primary fermentation and the reaction of acidic fermentation end-products with basic bicarbonate ions (Davies et al., 2000). The amount of gas produced depends on the amount of fermentable substrate, and the amount and molar proportions of SCFA produced (Awati, Williams, Bosch, Li, & Verstegen, 2006; Beuvink & Spoelstra, 1992; Theodorou, Williams, Dhanoa, McAllan, & France, 1994).

2.3.2. Review of published *in vitro* bioaccessibility and fermentation studies of carotenoids and phenolics in mango and banana

Carotenoid and polyphenol concentrations from chemical extractions of several mango and banana cultivars have been reported, and are used as a means of estimating bioaccessibility and bioavailability in human diets. Recommendations are usually based on nutritional intake or concentrations in the extracts of raw plant material, not taking into account the changes occurring during gastrointestinal digestion, which could result in an overestimation. There is little information as to the health effects of consumption of phytonutrient-rich mango and banana, and presently no bioaccessibility and/or bioavailability studies have been reported for banana.

There have been three reported mango studies, but none has involved the major Australian mango cultivar, *Kensington Pride*. The first study focused on Indian mango (and papaya) varietal differences in β-carotene content, and the effect of milk on β-carotene bioaccessibility (Veda, Platel, & Srinivasan, 2007). Another study examined the impact of ripening stage and ingestion of dietary fat on β-carotene bioaccessibility in Mexican *Ataulfo* mango (Ornelas-Paz et al., 2008). The third study examined processing effects (dried, fresh and juice) on carotenoid release during various *in vitro* digestion steps (Epriliati, D'Arcy, & Gidley, 2009a).

The only *in vitro* study of bacterial fermentation of mango and banana (Vong & Stewart, 2013) looked at the fruit fermentability using freeze-dried residues. The study measured typical end-products e.g. gas volume, pH and SCFA but did not examine the influence of different dietary fibre composition or phytonutrient changes on microbial activity. Other than the above-mentioned studies, information on bioaccessibility and microbial fermentation of β-carotene, other carotenoids and/or polyphenols through the consumption of these fruits is lacking. Therefore, the present study is the first attempt to understand *in vitro* bioaccessibility and biotransformation of carotenoids and polyphenols from *Kensington Pride* mango and *Cavendish* banana, and the first study to attempt *in vivo* validation using an animal (pig) model.

2.3.3. Digestion and absorption of dietary carotenoids

The liberation of carotenoids from the food matrix is necessary before absorption by intestinal cells (Faulks & Southon, 2005). Liberation occurs via the mechanical disruption of food by mastication, ingestion and mixing, and during enzymatic and acid-mediated hydrolysis of carbohydrates, lipids and proteins. Once released, carotenoids must be dissolved in oil droplets, which are emulsified with aqueous components of the chyme (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010). When these oil droplets are mixed with bile in the small intestine, their size is reduced, facilitating hydrolysis of lipids by pancreatic enzymes (Faulks & Southon, 2005; Pasquier et al., 1996). Pancreatic lipase activity specifically accounts for most of the lipid hydrolysis during digestion (Pafumi et al., 2002).

Before absorption, carotenoids must be transferred to mixed micelles of lipid digestion products (mainly fatty acids) and bile salts (Pasquier et al., 1996) for delivery to small intestinal epithelial cells (Furr & Clark, 1997; Tyssandier, Lyan, & Borel, 2001) (Fig 2.17). Carotenoids seem to have an absolute requirement for bile salt micelles (Elgorab, Underwood, & Loerch, 1975; Hollander & Ruble, 1978), different from triglycerides, which do not necessarily require micelle formation. In the absence of bile and pancreatin, less than 4% and 8% of β-carotene respectively was transferred to the aqueous phase (Wright, Pietrangelo, & MacNaughton, 2007). Carotenoids intercalate into bile salt micelles, causing them to swell and increasing their solubilisation capacity (Porter & Charman, 2001). However, even with increasing pancreatin or bile concentrations, transfer of carotenoids to the micellar phase reaches a maximum once a certain level of lipolytic activity is achieved (Wright, Pietrangelo, & MacNaughton, 2007). Bile salt micelles must

pass through a ca. 40 µm deep unstirred water layer (UWL) on the surface of the intestinal epithelium, to deliver their contents to the apical portion of the enterocytes across a concentration gradient (Parker, 1996; Perera & Yen, 2007). Recently, it has been shown that the absorption of certain carotenoids is not passive as has been believed for a long time, but is a facilitated process. The cholesterol membrane transporters, scavenger receptor class B type I (SR-B1) and cluster determinant 36 (Cd36) were found to be involved in the intestinal uptake of lutein and β-carotene (Moussa et al., 2008; Reboul et al., 2005; Van Buggenhout et al., 2010).
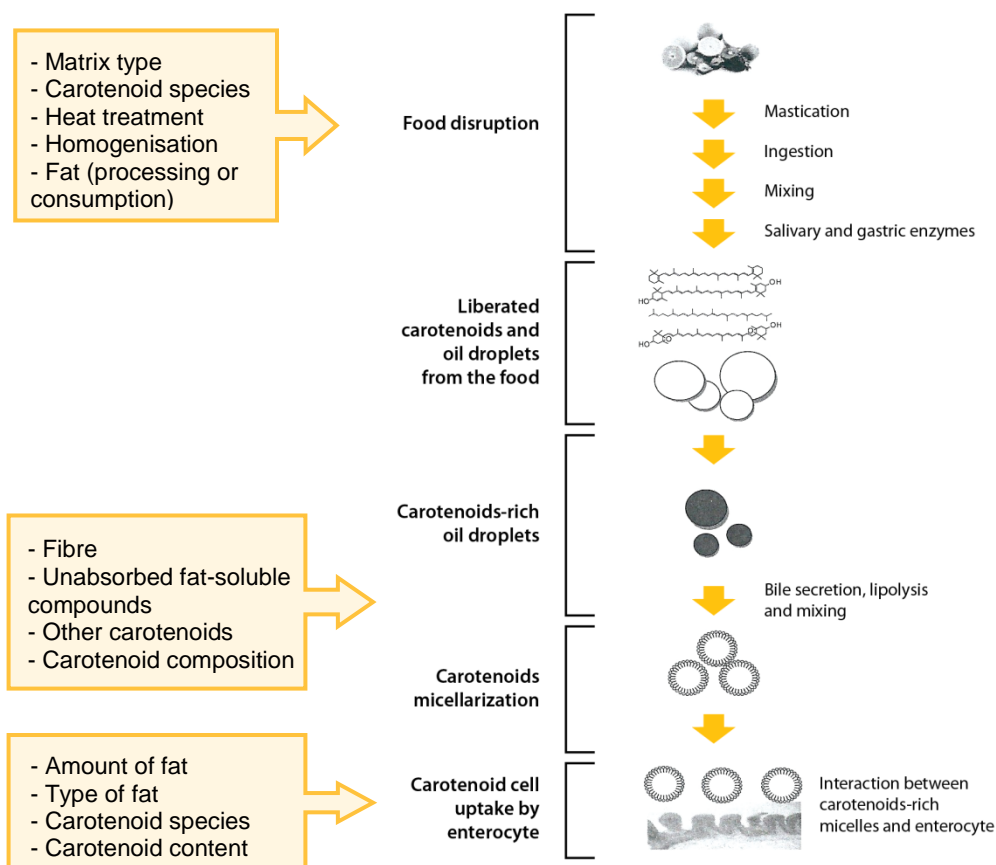
Figure 2.17. Carotenoid absorption process, with dietary factors affecting carotenoid absorption (in left block arrows). Diagram was modified from de la Rosa, Alvarez-Parrilla and Gonzalez-Aguilar (2010).

In the enterocyte, provitamin A carotenoids are converted to vitamin A esters. Carotenoids, vitamin A esters and other lipophilic compounds are packaged into chylomicrons, which are secreted into the lymph and then into the bloodstream. This mechanism means that unlike the water-soluble polyphenols that are directly transported to the liver via the portal vein, lipids and carotenoids enter the general circulation and 'see' the extra hepatic capillary bed first (Faulks & Southon, 2005). Chylomicrons are then attacked by endothelial lipases in the bloodstream, leading to chylomicron remnants, which are then

taken up by the liver (Van den Berg et al., 2000). Carotenoids may be stored there or exported from the liver to various tissues by lipoproteins. Carotenes are predominately transported in the core of low-density lipoprotein (LDL) and very low-density lipoproteins (VLDL), while xanthophylls such as lutein, zeaxanthin and β-cryptoxanthin are distributed approximately equally between high-density lipoproteins (HDL) and LDL in human serum (Erdman, Bierer, & Gugger, 1993; Furr & Clark, 1997; Johnson & Russell, 1992).

It is generally accepted that oxidation of carotenoids begins with epoxidation and cleavage to apocarotenals (e.g. retinal), prior to transformation into other derivatives (Rodriguez & Rodriguez-Amaya, 2007). The precise mechanism of β-carotene cleavage by β-carotene 15,15' monooxygenase (EC 1.14.99.36) has been discovered, as has a second enzyme (β-carotene 9',10' dioxygenase), which cleaves the β-carotene molecule asymmetrically (Bachmann et al., 2002). Wyss et al. (2001) showed that this second enzyme can act on all carotenoids. Other enzymes capable of catalysing the eccentric cleavage of carotenoids probably exist, but under physiological conditions, β-carotene 15,15' monooxygenase is most active, and mainly effective in the small bowel mucosa and liver (Perera & Yen, 2007).

Despite their hydrophobic nature, carotenoids are not very soluble in bulk triglycerides (e.g. vegetable oil, animal and dairy fat). Solubility of apolar carotenoids in bulk triglycerides is estimated at between 112 and 141 mg/100g, while the solubility of polar carotenoids is 22-28 mg/100g (Borel et al., 1996). The amount of lipid normally consumed in a Western-style meal (20-50 g based on 40% of energy from fat) could potentially dissolve up to 70 mg of apolar and 44 mg of polar carotenoids. However, the amount of carotenoid normally ingested in food is only a few mg/kg (Scott, Thurnham, Hart, Bingham, & Day, 1996). The amount of dietary fat required to ensure carotenoid absorption seems to be low (~3-5 g per meal) (Jayarajan, Reddy, & Mohanram, 1980), although it also depends on the physico-chemical characteristics of the ingested carotenoids. However, lipid intake in excess of about 10 g/meal does not increase carotenoid absorption.

Additionally, solubility and location of apolar and polar carotenoids can differ, affecting their micellisation and absorption efficiency. Apolar carotenes are incorporated almost exclusively in the triacylglycerol (TAG) core, which is highly hydrophobic, whereas polar xanthophylls are distributed preferentially on the surface (Borel et al., 1996). The significance of the location in an emulsion is that the surface components can

spontaneously transfer from lipid droplets to micelles, whereas components associated within the core require digestion of the TAG before transfer. Micellarisation of carotenoids can vary by a large extent from 1.7-100% (Ryan, O'Connell, O'Sullivan, Aherne, & O'Brien, 2008), while xanthophyll ester hydrolysis has been found to be incomplete (<40%), suggesting that cholesterol esterase lacks specificity to hydrolyse different esters (Granado-Lorencio et al., 2007).

Several studies have demonstrated that the beneficial effects of carotenoids depend upon the amount that is actually absorbed or metabolised by the body. Small doses appear to be more efficiently absorbed than are large doses (Erdman, Bierer, & Gugger, 1993; Furusho, Kataoka, Yasuhara, Wada, & Masushige, 2000; Tanumihardjo, 2002). The efficient absorption of a relatively large carotenoid dose (10 mg) in a small amount of lipid (10 g) would indicate that most, if not all, of the carotenoid that is transferred to the mixed micelle phase, is absorbed when the micelles dissociate at the enterocytes brush border, unlike bile salts that are not absorbed in the upper gastrointestinal tract (Faulks & Southon, 2005), even assuming that enterocyte absorption and transport mechanisms do not become saturated.

Carotenoids from fruits appear to be, on average, four times more bioavailable than carotenoids from vegetables such as carrots and green vegetables, presumably due to binding to proteins. (de Pee et al., 1998), and involves chemical binding as well as a physical matrix effect, which together reduces bioaccessibility (Castenmiller & West, 1998). Carotenoids in green vegetables have the lowest rate of duodenal transfer when compared to carotenoids in non-green vegetables and fruits, which may be related to their cellular localisation, such as being bound to the thylakoid membranes of chloroplasts (Granado-Lorencio et al., 2007). β-Carotene from carrots and lycopene from tomatoes are reported to be poorly bioavailable, because they exist in the form of large crystals, which may not completely dissolve during their passage through the gastrointestinal tract (de Pee et al., 1998). Carotenoids from mango and papaya were predicted to be readily absorbed, as they are dissolved in oil droplets (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010). Although bioaccessibility may differ between fruits and vegetables, the total amount of carotenoids latent in the tissues, and frequency of consumption by individuals should be taken into consideration. For example, the percentages of bioaccessibility are similar for mango and papaya (24-39%), but it is interesting to note that papaya has to be consumed at nearly three times the amount as for mango, to derive the same beneficial

amount due to lower tissue concentrations in papaya. The bioaccessibility of β-carotene from boiled African bananas is reported to be 10-32% (Ekesa et al., 2012).

Fruit ripening changes such as fruit softening and cleavage of cell-wall polymers (e.g. pectin polysaccharides) could favour bioaccessibility (Ornelas-Paz, Yahia, & Gardea, 2008). This 'biological processing' involves a similar effect to thermal or mechanical processing, resulting in loss of cell integrity. For example, Van het Hof, West, Weststrate and Hautvast (2000) demonstrated that homogenising and thermal processing of tomatoes increased the bioavailability of β-carotene and lycopene. Hedren, Diaz and Svanberg (2012) also reported a 3% increase in β-carotene bioaccessibility after cooking in water for 20min. However, these findings on thermal processing were in contrast to the results of Tydeman et al. (2010a) who found that β-carotene bioaccessibility is greater for raw carrot than for cooked carrot. One explanation for this interesting finding is that heating increased the propensity for intact cell walls to separate, which encapsulated the carotenes, rather than releasing them (Fig 2.18). This suggests cell rupture is an absolute requirement for carotene release, since carotenes remaining within intact cell walls are inaccessible during upper gut digestion. The number of ruptured cells is suggested to be the governing factor in carotene bioaccessibility, rather than surface area; for example, raw carrot released more carotene than cooked carrot tissue despite having similar surface area. Tydeman et al. (2010b) again illustrated the carotenes in intact cells in ileostomists' effluents collected after 10 h, remain largely unaffected by digestion. Therefore, the effect of cell rupture, caused by chewing prior to digestion, on carotene bioaccessibility from fresh fruits needs further investigation.
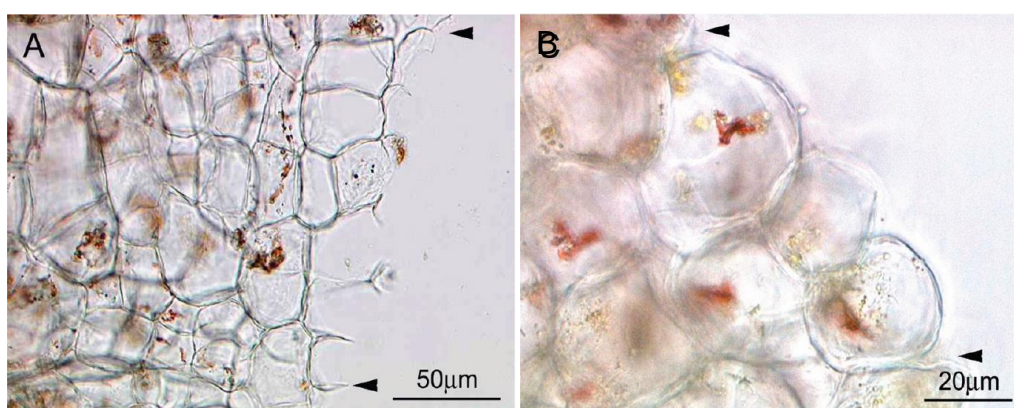
Figure 2.18. Sections of carrot after *in vitro* gastric and duodenal digestion: (A) surface cells of raw carrot cube and (B) surface cells of a steamed carrot cube. Carotenes are present as orange/red bodies. Adapted from Tydeman et al. (2010a).

In vitro digestion findings by Epriliati (2008) showed that carotenoid bioavailability from fruit consumption alone was relatively low, but β-carotene micellarisation efficiency can be increased through the consumption of fat-containing foods; for example, the addition of chicken to mango increased β-carotene micellarisation from moderately and fully ripe mangoes by 114% and 231% respectively as compared to the digestion of mango alone, increasing the absorption potential of carotenoids or their cleavage products (Ornelas-Paz et al., 2008). The addition of milk to mango fruit pulp enhanced β-carotene bioaccessibility from six mango cultivars (Veda, Platel, & Srinivasan, 2007) due to the presence of fat and/or protein. The presence of protein in the small intestine aided in the stabilisation of fat emulsions and enhanced micelle formation (West & Castenmiller, 1998).

Next, the presence of dietary fibre reduced carotene bioaccessibility (van het Hof, West, Weststrate, & Hautvast, 2000) by entrapping carotenoids and interacting with bile acids, resulting in increased excretion (Yeum & Russell, 2002). Dietary fibre components such as pectin, guar gum, cellulose and wheat bran significantly reduced β-carotene bioaccessibility by 33-43% (Riedl, Linseisen, Hoffmann, & Wolfram, 1999). It has been suggested that fibre may exert effects on the activity of pancreatic enzymes (lipase) and micelle formation (Schneeman, 1990), and also influence the morphology of the renewal of the cells of the small intestine (Phillips, 1986). The amount, molecular weight and other physico-chemical properties of pectins have also been shown to affect carotenoid bioavailability from supplements (Ornelas-Paz et al., 2008).

The numerous factors and processing steps mentioned above have demonstrated beneficial or limiting effects on carotenoid bioaccessibility, including: carotenoid speciation, form and quantity; the food matrix; type and intensity of processing; and the presence of dietary components such as fat and fibre. Not all of these have been studied in detail, and there remains more to be explored, particularly related to the underlying properties of fruits and vegetables that are responsible for their different predicted carotenoid bioaccessibilities and bioavailabilities.

2.3.4. Digestion and absorption routes of dietary of flavonoids and phenolic acids

Polyphenol bioaccessibility is affected by differences in cell wall structures and cellular location of glycosides. There is accumulating evidence for the binding of polyphenols to food microstructures, including suggestions as to whether the phenolic contents of food have been underestimated. At the subcellular level, polyphenols accumulate in vacuoles

or cell walls. Yamaki (1984) found that polyphenols are located mainly in vacuoles, some in the periplasmic space and none in the cytoplasm. The seeming homogeneity of this distribution is perhaps confusing, since certain simple flavonoids and ferulic acid esters are located in the cell wall where they can be covalently linked to cell wall polymers, while soluble polyphenols are stored in the vacuoles (Antolovich, Prenzler, Robards, & Ryan, 2000). These intact cells are surrounded by a lipid membrane and enclosed by cellulose-pectin based plant cell walls (Harris & Smith, 2006). The occurrence of polyphenols in a soluble, suspended or colloidal form and in covalent combination with cell wall components (Lichtenthaler & Schweiger, 1998) may likely have a significant impact on their bioaccessibility and hence, bioavailability.

It is generally accepted that polyphenol bioavailability is rather low in comparison with the bioavailability of macro- and micronutrients, with values of relative urinary excretion ranging from 0.3% (anthocyanins) to 38% (gallic acid) (Manach, Williamson, Morand, Scalbert, & Remesy, 2005) as once ingested, polyphenols are recognised as xenobiotics (Crozier, Del Rio, & Clifford, 2010). The key issue of polyphenol absorption is their solubility, which determines their bioaccessibility to the enterocyte. Unlike lipid-soluble carotenoids that are incorporated into mixed micelles, water-soluble polyphenols require solubilisation in the aqueous matrix for efficient absorption. Flavonols may be less water-soluble but they are usually glycosylated, which increases their water solubility (Scholz & Williamson, 2007). Possible metabolism routes for dietary polyphenols in humans are shown in Fig 2.19. After absorption, the polyphenols are subjected to Phase I (oxidation, reduction and hydrolysis) and Phase II biotransformations (conjugation) in the colonic enterocytes and then the hepatocytes, resulting in a series of water-soluble conjugates (methyl, glucuronide and sulfate derivatives) being liberated rapidly into the systemic circulation for distribution to the other organs and for excretion in urine (Cardona, Andres-Lacueva, Tulipani, Tinahones, & Queipo-Ortuno, 2013; Vacek, Ulrichova, Klejdus, & Simanek, 2010). The introduction of methyl ethers decreases the solubility in water; an additional conjugation with glucuronic acid and/or sulfate are necessary for increasing the water solubiliy that favors transport and urinary excretion (Tomas-Barberan, personal communication, March 2015). Enterohepatic circulation may result in some recycling back into the small intestine through bile excretion (Donovan, Manach, Faulks, & Kroon, 2006). Plasma analysis provides useful information on the identity and C_{max} of circulating metabolites with maximum concentrations being roughly correlated with urine excretion (Crozier, Del Rio, & Clifford, 2010). The relatively rapid appearance of polyphenols in

plasma after oral ingestion is not consistent with colonic absorption, and is suggested to be due to a substantial uptake from the small intestine (Gee & Johnson, 2001).

The process of bioavailability of phenolics consists of liberation and digestion in the stomach and gastrointestinal tract, transport across small intestinal epithelial membrane into the bloodstream, tissue distribution, metabolism and elimination. During mastication, it is suggested that flavonoid glycosides may be deglycosylated in the mouth by oral epithelial cells or microbiota (Hirota et al., 2001; Walle, Browning, Steed, Reed, & Walle, 2005). However, this may be unlikely due to the short residence time (in minutes) solid food spends in the mouth during chewing and the inability of the oral epithelial cells and/or microbiota to deglycosylate the glycosides (Ceyman, 2013; Sanz & Luyten, 2006). Spencer et al. (2000) suggested the degradation of flavonol polymers to monomers during residence in the stomach at pH 2. However, Rios et al. (2002) demonstrated that this does not occur *in vivo* in humans, probably due to the food bolus having a buffering effect, making the acidic conditions milder than required for proanthocyanidin hydrolysis. *In vitro* experiments using single layers of Caco-2 cells as an absorption model in the small intestine showed that only flavanol dimers and trimers were able to cross the intestinal epithelial layer (Deprez, Mila, Huneau, Tome, & Scalbert, 2001).
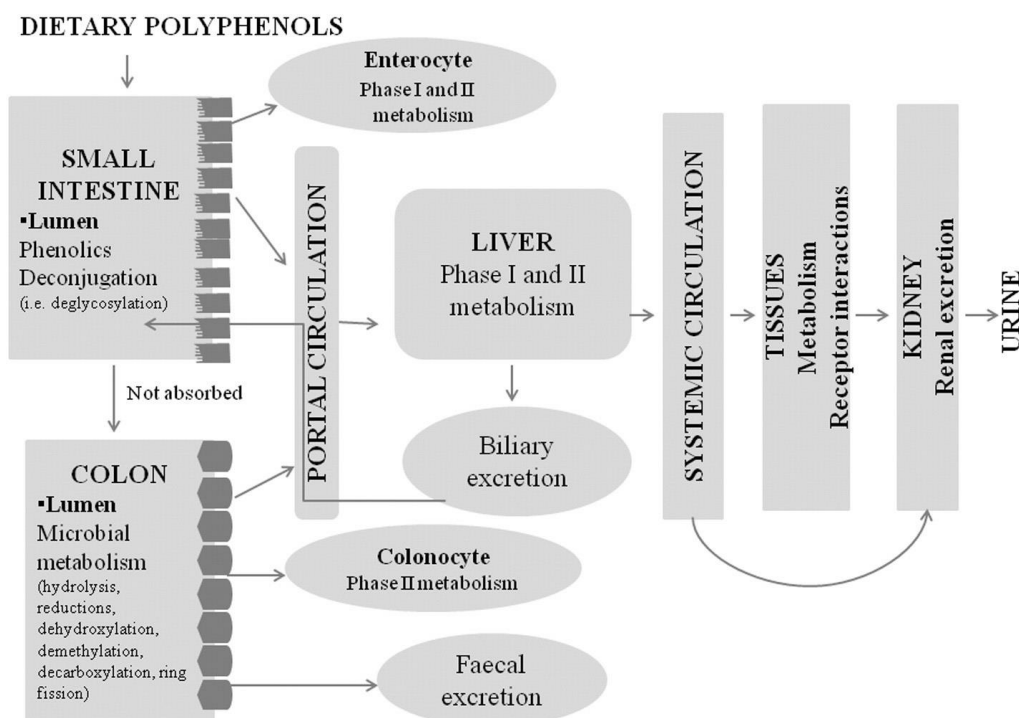
Figure 2.19. Routes for dietary polyphenols and their metabolites in humans. Within the human body, dietary polyphenols and their microbial metabolites successively undergo intestinal and liver Phase I and II metabolism, biliary secretion, absorption in the systemic circulation, interaction with organs and excretion in the urine. Adapted from Cardona, Andres-Lacueva, Tulipani, Tinahones and Queipo-Ortuno (2013).

Gallic acid, quercetin glucosides, catechins and free hydroxycinnamic acids, which are absorbed in the stomach and small intestine reached their maximum concentration (C_{max}) at about 1.5 h, whereas naringin, which is absorbed after release of the aglycone by colonic microbiota, reached C_{max} at 5.5 h (Manach et al., 2005). This demonstrates a great variability in the bioavailability of different phenolic subclasses, and that bioaccessibility is reduced with increasing molecular weight, or by complexing flavonoid conjugates with sugars or acylating them with hydroxycinnamic acids. Gallic acid seems to be most readily absorbed, followed by catechins, flavanones and quercetin glycosides. A characteristic of quercetin bioavailability is that its metabolites are eliminated quite slowly, with half-lives ranging from 11-28 h, which could favour accumulation in plasma with repeated intakes (Manach et al., 2005).
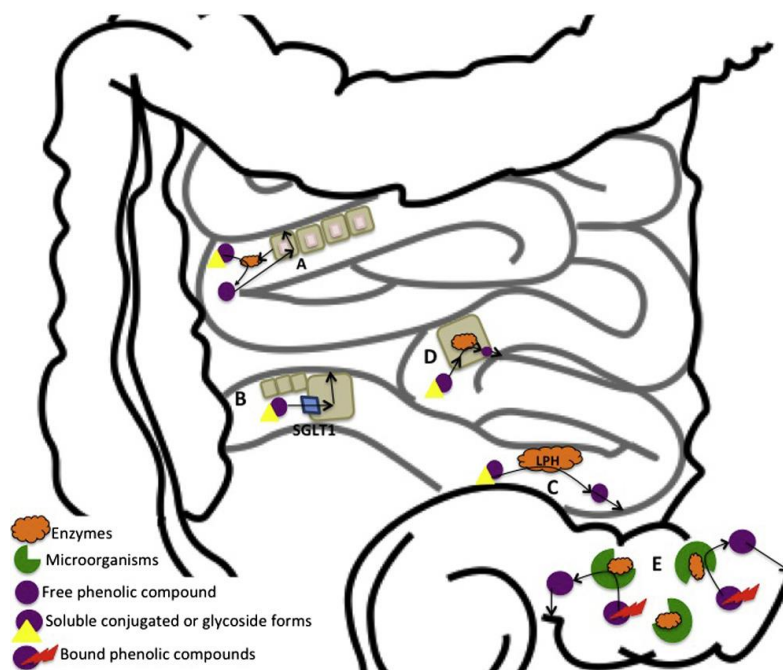
Figure 2.20. Absorption pathways of bound phenolic compounds in the human gastrointestinal tract. (A) Hydrolysis of bound soluble conjugated forms by mucosa cells cinnamoyl esterases. (B) Soluble conjugated forms transport into enterocytes by SGLT1. (C) Brush border LPH (β-glycosidase) hydrolyse soluble conjugated polyphenols. (D) Epithelial cells cytosolic β-glucosidase hydrolyses glycosides, and aglycones are formed after absorption. (E) Esterases and xylanase activities of colon microbes (e.g. *Clostridium spp., and Bifidobacterium adolescentis*) (Acosta-Estrada, Gutierrez-Uribe, & Serna-Saldivar, 2014).

Luminal deglycosylation is catalysed by the action of lactase phloridzine hydrolyase (LPH) in the membrane-bound brush border (Fig 2.20), after which unconjugated phenolics diffuse passively through the UWL into enterocytes (Day et al., 2000; Nemeth et al., 2003; Velderrain-Rodriguez et al., 2014). The LPH enzyme acts outside of the epithelial cells to

deglycosylate phenolic compounds without first having to traverse the enterocyte membrane. In epithelial cells, cytosolic β-glucosidase hydrolyses glycosides, and aglycones are formed after absorption (Aura, 2008). In addition, Selma, Espin and Tomas-Barberan (2009) reported that some glycosides could be transported through the epithelium by the active sodium-dependent glucose transporter (SGLT1). The absorption mechanism of carrier-mediated transport processes, and the possible role of MRP, have also been postulated (Clifford, 2004; Murota & Terao, 2003). Aglycones permeate faster than glycosides on the basis of diffusion and do not require membrane transporters and energy (Tarko, Duda-Chodak, Sroka, Satora, & Michalik, 2009); however, Walle (2004) proved an active influence of SGLT, MRP1 and MRP2 on the transportation of phenolic glycosides though the intestinal epithelium.

2.3.5. Insoluble bound polyphenols

While some polyphenols released from food matrices after mastication are absorbed through the small intestine epithelium, there are unabsorbed, 'insoluble' and entrapped polyphenols that accumulate in the colon, and together with conjugates excreted into the intestinal lumen through bile, are metabolised by colonic microbiota before colonic mucosal absorption and ultimate excretion in the urine (Perez-Vizcaino, Duarte, & Santos-Buelga, 2012; Russell & Duthie, 2011; Selma, Espin, & Tomas-Barberan, 2009). Therefore, the colon is an active metabolism site for polyphenols, rather than just a simple excretion route and thus deserves further attention. In addition to polyphenols and carotenoids, intact plant cell walls will also effectively encapsulate other cellular components such as starch, lipid, and protein, as an intact cell wall provides an effective barrier to the entry of macronutrient-degrading enzymes such as amylase, protease and lipase, and the mammalian digestive system is not able to break down plant cell walls. Here again, the nature of the swallowed food is critical. If cell walls are intact on swallowing, then they are expected to survive to the large intestine where the action of colonic microbiota could be expected to break down cell wall polymers, thereby releasing the effectively encapsulated cell contents for possible metabolism by colonic microbiota.

Human intestinal microbiota has extensive hydrolytic activities and can break down complex polyphenols into simpler phenolic acids and metabolites, which are then available to exert their biological activities systemically within the human body (Booth, Murray, Jones, & Deeds, 1956; Grun et al., 2008; Hertog, Sweetman, Fehily, Elwood, & Kromhout, 1997; Winter, Moore, Dowell, & Bokkenheuser, 1989). Given the diversity of dietary

polyphenols, their possible microbial metabolites, and the fact that they occur as mixtures in whole plants or plant extracts, only a small number of polyphenols have been well studied. Common biological activities recognised for some phenolic metabolites include antibacterial properties especially against Gram-negative species such as *Enterobacteriaceae*, anti-inflammatory activity, anti-AGE formation, stimulation of xenobiotic degradation enzymes and detoxification processes, and phytoestrogenic activity (Hervert-Hernandez & Goni, 2011; Tuohy, Conterno, Gasperotti, & Viola, 2012).

Unabsorbed polyphenols (from the small intestine) and those that are absorbed, circulated enterohepatically and secreted in the bile, enter the colon (Russell & Duthie, 2011; Scalbert & Williamson, 2000). There in the colon, the colonic microbiota carries out hydrolysis, ring-cleavage, reduction, decarboxylation, demethylation, and dehydroxylation reactions (Rechner et al., 2002; Rechner, Spencer, Kuhnle, Hahn, & Rice-Evans, 2001; Rice-Evans, 2001; Tapiero, Tew, Ba, & Mathe, 2002; van Duynhoven et al., 2011; Yeh & Yen, 2006), producing metabolites with different physiological significance. These metabolites are then absorbed from the colon after deconjugation and transformed by human cell enzymes into Phase II conjugates. For example, methyl ether glucuronides and sulfates are metabolised in the liver, resulting in glucuronidated and sulfated derivatives (Aura, 2008; Hervert-Hernandez & Goni, 2011; Rossi et al., 2013; Tapiero et al., 2002; Zhao, Egashira, & Sanada, 2004). Van't Slot and Humpf (2009) investigated caecal bacterial deconjugation and degradation of catechins, procyanidin B2 and gallic acid under anaerobic physiological conditions, and found that these phenolics were almost completely metabolised within 4-8 h.

Glucuronidation and methylation occurs mainly in intestinal cells and liver, while sulfation takes place mainly in the liver and kidney. The primary site of metabolism depends on the dose ingested; smaller doses are metabolised in intestinal mucosa with the liver playing a secondary role, while larger doses are metabolised in the liver (Scalbert & Williamson, 2000). Current evidence suggests that there is no long-term storage of polyphenols in the body (Erdman et al., 2007). However, flavonoids do concentrate in tissues at measurable concentrations (Henning et al., 2006; Hong, Smith, Ho, August, & Yang, 2001). The metabolism of phenolics can depend on: dietary fat intake, the form of polyphenol ingested, dose, gastric and intestinal transit time, fecal degradation rate (Erdman et al., 2007), molecular size of the phenolic compound; degree of phenolic polymerization, type of sugar attached (Tarko et al., 2009), lipophilicity, solubility and pK_a of the phenolic

compound, intestinal membrane permeability, lumen pH; and first-pass metabolism (Shahidi & Naczk, 2004).

Flavonoids that are absorbed in sufficient amounts to exert a possible effect on cardiovascular parameters *in vivo* include flavanones, flavonols and flavanols, where they are predicted to exert biological effects even in conjugated forms (Williamson & Manach, 2005). Mangiferin is reported to undergo phase II liver metabolism, while its aglycone, norathyriol is extensively metabolised by colonic microbiota (Liu et al., 2011). A novel, specific C-glucosyl-cleaving enzyme capable of breaking the C-C linkage of mangiferin to yield norathyriol (and glucose) is produced from *Bacteroides sp.* MANG, and appeared to be different from O-glucosidases (Sanugul et al., 2005a).

It is worth noting that the doses applied *in vivo* in animal experiments, or as part of *in vitro* studies sometimes exceed the doses that human tissues may be exposed to physiologically. The concentration range required for an effect *in vitro* is 0.1-100 µM. However, according to maximum concentrations found in human plasma after nutritional intakes (Manach et al., 2005), the effects observed with doses above 2 µM (or <10 µM) are unlikely to occur *in vivo*. Additionally, it is important that bioavailability studies should use metabolites that are actually found in the human body, since absorption is accompanied by extensive conjugation and metabolism, and the forms appearing in the blood are usually different from those in food. Manach, Masur and Scalbert (2005) suggested that unabsorbed polyphenols such as proanthocyanidins should never be tested in cultured cells or isolated organs since they have no chances of reaching inner tissues.

There is growing evidence from human and animal studies that biologically active polyphenols can have substantial effects on the human gastrointestinal tract microbiota. These compounds appear to modulate bacterial species composition and profile of metabolites absorbed from the gut. However to date, only a small number of studies have focused on the potential biological activities of low-molecular-weight metabolites produced by the colonic-microbial biotransformation of dietary polyphenols. All polyphenol metabolism studies using the pig model (Table 2.9) have used purified compounds rather than complete fruits or vegetables.

Table 2.9. Compilation of *in vivo* phenolic digestion and absorption studies carried out in animal (pig) model.

Animal breed, age and body weight	Phenolic compound(s)	Phenolic or feed daily intake	Diet treatment	Diet composition	Sample/collection intervals	Metabolites	Reference
26 barrows (German LandracexLarge White) (9.9±0.1 kg)	Quercetin	Oral dosage, 10 mg quercetin/kg BW/day and 50 mg/kg fish oil/diet	*ad libitum* twice daily Unlimited water access	56.3% barley, 26% soybean meal, 9% wheat, 5% maize starch or fish oil, 3.7% premixes and minerals	Blood samples from JV weekly prior to feeding and 12 h after fasting Liver, lung, muscle and white adipose tissues collected from neck-shoulder area	Quercetin, isorhamnetin, tamarixetin	(Luehring, Blank, & Wolffram, 2011)
3 6-month old crossbred female pigs (112-119 kg), no medication for 2 weeks before study	Mangiferin, isomangiferin, hesperidin	Oral dosage, 75 g/day of unfermented C genistoides extract dissolved in warm water, mixed with 1.5 kg of total daily diet and 0.2 g ascorbic acid mangiferin 74±2.5 mg/kg BW, isomangiferin 74±2.5 mg/kg BW, hesperidin 1±0 mg/kg BW	11 days, diet was given every morning after overnight starvation before adding rest of daily diet Unlimited water access	79% barley oats, 15% soy groats, 3% mineral diet (Phoskana S 80), 3% soybean oil	Blood samples from JV on days 9 and 11 (5 and 10 h after oral administration), 29, 34, 39, 44 h after last extract intake and on days 1 and 2 after termination (days 12 and 13) Urine via bladder catheter on days 9 and 11 (0-11 and 11-24 h), 24-32, 32-38, 38-48 h after last extract intake (day 12), and 48-56, 56-62, 62-72 h (day 13) Feces excreted on days 9 and 11 (0-5, 5-10, 10-24 h), 24-29, 29-34, 34-38 h	Norathyriol, methyl mangiferin, hesperetin, hesperidin, eriodictyol	(Bock, Waldmann, & Ternes, 2008)

					(day 12) and 48-53, 53-58, 58-72 h (day 13)		
					Feces excreted on days 9 and 11 (0-5, 5-10, 10-24 h), 24-29, 29-34, 34-38 h (day 12) and 48-53, 53-58, 58-72 h (day 13)	3-hydroxyphenylacetic acid (HPA), 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (DHBA), 3,4-dihydroxyphenylacetic acid (DHPA), 2,4,6-trihydroxybenzoic acid (2,4,6-THBA), 3,4,5-trihydroxubenzoic acid, phloroglucinol (PG)	(Bock & Ternes, 2010)
3 female pigs (94-99 kg)	Aspalanthin	Oral administration, 157-167 mg aspalanthin (~96 g rooibos extract containing 16.3% aspalanthin)/kg BW/day or 15.3±1.2 g aspalanthin per animal/day with 500-800 g habitual diet	Fed in the morning after overnight starvation Unlimited water access	70% barley groats, 15% soy groats, 3% mineral diet, 3% soybean oil	Blood samples from JV on days 7 and 10 (6 and 10h), and on day 11 (2, 6, 10 h), 26, 30 h after last ingestion and day 1 after termination Urine via bladder catheter on days 7 and 11 and on days 12 and 13 after termination	Aspalathin, methylated aspalathin, agylcone, methylated eriodictyol Excretion of aspalathin and metabolites finished 36 h after last feed; no detection on day 13, 36-48 h after last ingestion	(Kreuz, Joubert, Waldmann, & Ternes, 2008)

32 barrows (Iberian) (100.1±1.9 kg)	Ellagic acid, ellagitannins	Oral dosage, 4.04 kg acorns/day	Fed for 117 days	Only acorns	Blood samples from JV after deprived for food for 24 h before killing and 3 h postprandial. Terminal jejunum, colon, faeces, urine, bile, brain, kidneys, liver, Longissimus dorsi muscle, subcutaneous fat (rib)	Ellagitannins, ellagic acid, urolithin D, urolithin C, urolithin A, urolithin B	(Espin et al., 2007)
2 castrated crossbred male pigs (122.4 and 138.9 kg)	Quercetin	Oral dosage, 500 mg quercetin/kg BW/day	Thrice daily for 3 consecutive days	Wheat, defatted soybean meal	Deprived for food for 8 h before killing Blood, liver, kidney, spleen, brain, heart tissues	Quercetin, isorhamnetin, tamarixetin	(de Boer et al., 2005)
7 castrated crossbred male pigs (30-35 kg)	Quercetin, quercetin-3-O-glucoside	Oral administration, quercetin or quercetin-3-O-glucoside (10 µral ad BW) directly mixed into meal	Unlimited water access	Barley, wheat, defatted soybean meal (3% fat diet) or enriched with 15 or 30 g lard/100g (17% and 32% fat diet)	Blood samples collected from 0, 4, 8, 12, 20, 24 h	Quercetin, quercetin-3-O-glucoside Peak plasma concentration ~70 min	(Lesser, Cermak, & Wolffram, 2004)
4 castrated crossbred male pigs (30-35 kg)	Quercetin, quercetin-3-O-glucoside, rutin	Oral dosage, single dose of 148 µmol quercetin or Q3G or rutin/kg BW (=50 mg/kg) mixed into 200 g diet, ration was moistened with water	*ad libitum* twice daily Unlimited water access	Ground barley, wheat, defatted soybean meal	Blood samples from JV at 0, 4, 8, 12, 20, 24 h	Quercetin, isorhamnetin, tamarixtein Bioavailability: Q3G > quercetin > rutin	(Cermak, Landgraf, & Wolffram, 2003)

6 castrated crossbred male pigs (30-35 kg)	Quercetin, quercetin-3-O-glucoside	Oral dosage, single dose of 29.6 µral quercetin or Q3G/kg BW (=10 mg/kg) mixed into 200 g diet				Maximal plasma concentration of quercetin: 1.19±0.33 µmol/L (120 min), isorhamnetin: 106.7±40.5 mmol/L (240 min), tamarixetin: 180.9±57.1 nmol/L (210 min)	
3 crossbred female pigs (30-35k g)	Quercetin	Oral dosage, single dose of 29.6 µmol quercetin/kg BW (=10 mg/kg) mixed into 200 g diet, ration was moistened with water. 29.6 µmol Q3G with 15 g grund beef					
			Fed twice daily Unlimited water access	38% barley, 37% wheat, 20% defatted soya bean meal, 2% soya oil, 3% vitamin/mineral premix	Blood samples from jugular catheter over 72 h	Quercetin, kaempferol, isorhamnetin, tamarixtein Bioavailability: 0.5±0.2% free quercetin, 8.6±3.8% free and conjugated quercetin, 17±1% total quercetin and metabolites	(Ader, Wessmann, & Wolffram, 2000)
6 Yorkshire male pigs (40-60 kg)	Quercetin	Oral dosage, 10 mg/kg cyclosporine (Sandimmune® diluted with olive oil) with and without quercetin (50 mg/kg dissolved in glycofurol)	Deprived of food for 12 h before dosing and 4 h after dosing Unlimited water access	(Not reported)	Blood samples from JV at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24 h	Cyclosporin and quercetin Plasma concentration: 50-1600 ng/mL	(Hsiu et al., 2002)

2.3.6. Non-extractable polyphenols

Solid residues from aqueous organic extractions or pomaces are generally discarded, although an appreciable amount of phenolics may remain in these residues and constitute the non-extractable polyphenols (NEPP) (Perez-Jimenez & Torres, 2011). Studies on non-extractable phenolics are quite scarce, mainly focusing on proanthocyanidins, hydrolysable tannins, and some phenolic acids associated with dietary fibres, polysaccharides and proteins (Fig 2.21) by covalent bonds (esters and ether), hydrogen bonding, and hydrophobic and hydrophilic interactions, or those trapped in the core of food matrices that remain in the extract residues (Saura-Calixto, 2014). These NEPP, insoluble or bound phenolics form an interesting group from a nutritional point of view as this 'intact' food fraction may arrive at the colon and upon fermentation, releases the phenolics for absorption through the colonic epithelium or biotransformation by microbial enzymes to smaller molecular weight phenolics and metabolites (Fig 2.22) (Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011).
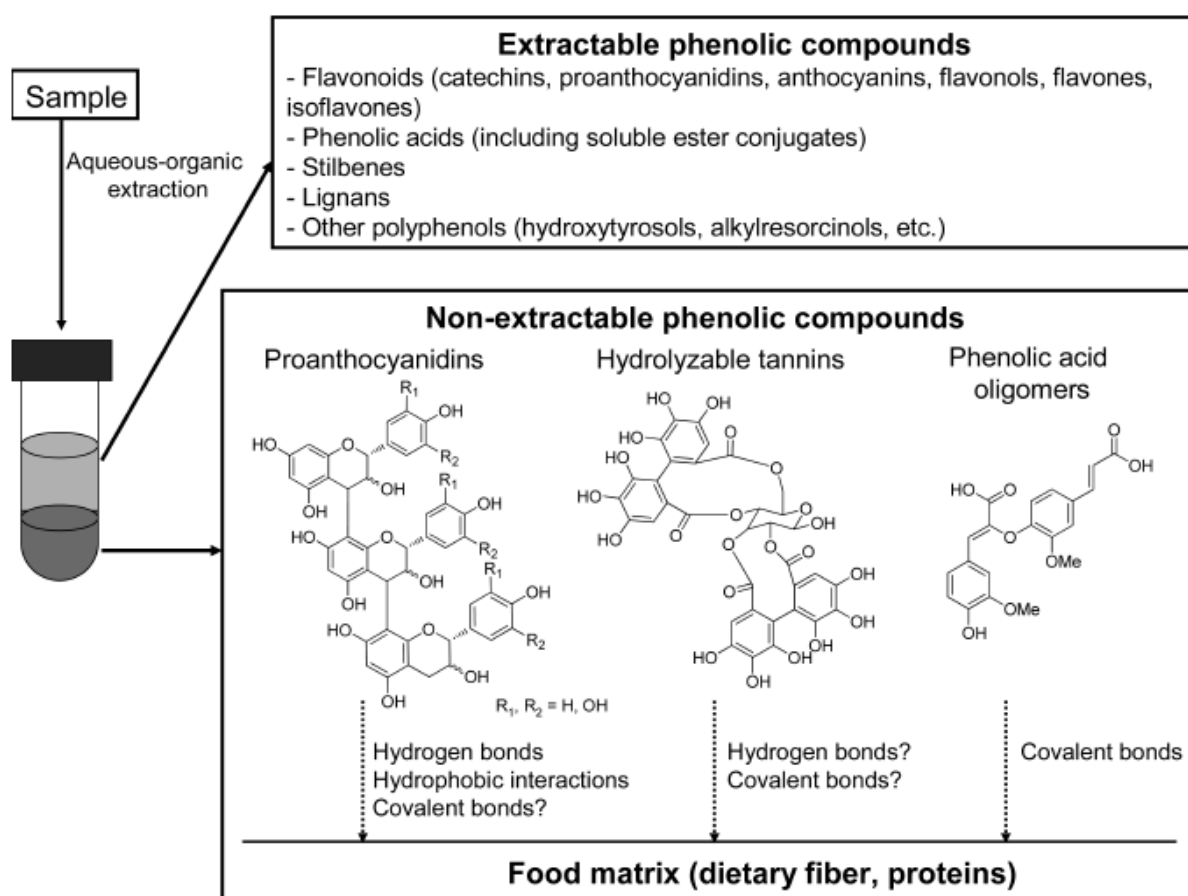
Figure 2.21. Extractable and non-extractable polyphenols in foods (Perez-Jimenez & Torres, 2011).
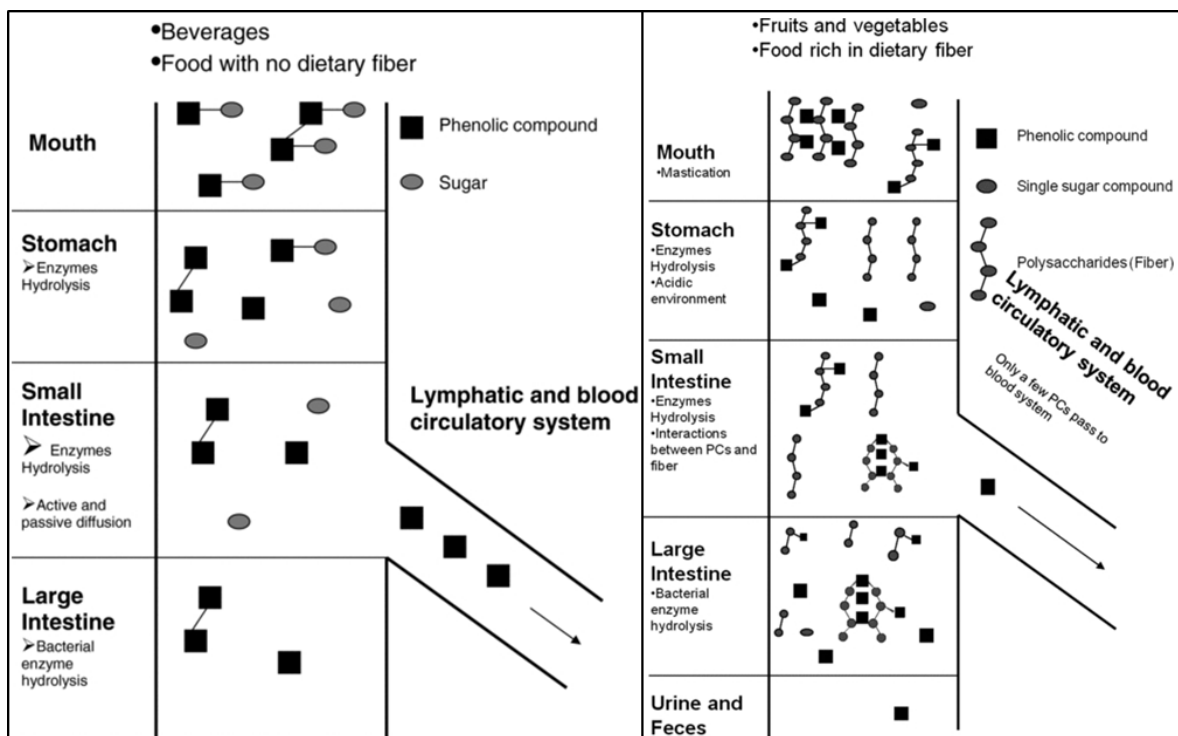
Figure 2.22. Bioavailability of phenolic compounds in beverages or foods poor in soluble fibre (left) and the interference of dietary fibre in the overall process (right). Adapted from Palafox-Carlos, Ayala-Zavala and Gonzalez-Aguilar (2011).

As a result, NEPP are often neglected in bioavailability and metabolism studies. However, these complex conjugated fractions do demonstrate antioxidant activity (Chesson et al., 1999; Perez-Jimenez et al., 2009; Saura-Calixto, Serrano, & Goni, 2007), suggesting potential bioaccessibility and bioactivity after exposure to microbial activity in the colon. The metabolic fate of NEPP has been explored in an *in vitro* colonic fermentation model (Saura-Calixto et al., 2010) and in rats (Mateos-Martin, Perez-Jimenez, Fuguet, & Torres, 2012) confirming that NEPP are a source of absorbable and bioactive metabolites. Generation of an antioxidant environment in the colon may have important effects on gastrointestinal health, including a chemopreventive effect for colorectal cancer (Gao et al., 2006).

In order for such health outcomes to be achieved, dietary polyphenols must be released during mastication or digestion before absorption can occur. If polyphenols are bound to the plant cell wall and/or components, and are not available for small intestinal absorption, these complexes may be transported to the colon, where fermentation of fibrous material occurs. Evidently, apart from regulating the digestive system, plant fibre is important in the controlled release of polyphenols from the gastrointestinal digestive system to the colon. Consequently, the release of bound polyphenols from plant cell material during colonic-microbial fermentation should be further assessed.

2.4. Extraction, identification and quantification of carotenoids and polyphenols

The methods for extraction and quantification of carotenoids and polyphenols are described in this section. Sample preparation procedures are the first important steps, and should be adapted to the nature of the food, the analyte and the analytical method, as well as to the distribution of the analyte in the food. Information such as where and when (sampling times of the crops during the season, or year) the samples were purchased should all be recorded. Extraction and isolation procedures, and solvents are also critical factors for subsequent separation, identification and quantification of individual analytes from various plant-based materials.

2.4.1. Extraction and isolation procedures

Solvent extractions are most often the preferred method for the recovery of phytochemicals from plant or food products. In addition, such extractions are used to separate analytes of interest from materials that may interfere during quantitative analysis, or are used as a 'clean-up' step to achieve concentration of food components prior to analysis (Nielsen, 2003a).

Plant phenols are ionizable with typical pKa values ranging from 8-10 (Gonzalez & Gonzalez, 2010c), and they show considerable diversity in terms of acidity and polarity, from slightly hydrophobic to hydrophilic. In addition, the solubility of the different phenolic classes is governed by their varying degrees of polymerization (Luthria, 2006). Solvents are chosen as a function of the type of flavonoid required, where polarity is an important consideration. Since phenolic acids, flavonoid glycosides and anthocyanins are polar in nature, they are generally at least partially soluble in water, while the presence of attached sugars tends to render the glycosides more water-soluble (Escribano-Bailon & Santos-Buelga, 2003). Extraction and isolation typically involves the use of water, alcohols (methanol or ethanol), acetone, ethyl acetate, or combinations (50-95%) with water under different temperatures and extraction times. Less polar flavonoids (flavanones and flavonols) are extracted with ethyl acetate or diethyl ether, while flavonoid glycosides, higher molecular weight phenolics and their complexes, or more polar aglycones cannot be completely extracted with pure organic solvents; hence, alcohol-water mixtures are recommended (Stalikas, 2007; Tsao & Deng, 2004). Flavan-3-ols (catechins, proanthocyanidins, and condensed tannins) can often be directly extracted with water.

The type and form of the carotenoid, and composition of the food matrix are critical to the amount of sample preparation necessary prior to sample extraction. Carotenoids do not form ester linkages and can be extracted by lipophilic solvents such as hexane, petroleum ether, acetone, ethanol, tetrahydrofuran or mixtures of these solvents. When extracting carotenoids from foods that contain large amounts of water such as fresh plant or food material, a water-miscible organic solvent such as methanol, ethanol, acetone or mixtures thereof should be used to achieve better solvent penetration.

Gonzalez-Montelongo, Lobo and Gonzalez (2010a) recently screened variables that could affect the extraction efficiency of antioxidants from banana peels, and concluded that extracts obtained with methanol had very high antioxidant activity. In addition, Kim and Lee (2002), and Tsao and Deng (2004) commented that methanol had a higher extraction efficiency than ethanol. Other factors such as temperature also contribute to extraction efficiency. For example, high temperatures (between 37°C and 90°C) enhance the diffusion rate and solubility of analytes, although elevated temperatures can cause degradation of the phytochemicals and volatilization losses or thermal decomposition (Davey, Mellidou, & Keulemans, 2009; Escribano-Bailon & Santos-Buelga, 2003; Gonzalez-Montelongo, Lobo, & Gonzalez, 2010b). At higher temperatures, bioactive phytochemicals may also react with other components in the plant material, thus impeding extraction (Gonzalez & Gonzalez, 2010c). Extraction of carotenoids and polyphenols are usually conducted at temperatures ranging from 20-50°C.

The recovery of phytochemicals is also influenced by the extraction time and number of extraction steps. Extraction times ranging from a few minutes to hours, or even days have been used. Longer extraction periods can increase the possibility of the oxidation of phenolic acids, unless reducing agents are used. Usually, a single extraction step is used but sometimes two to eight steps have been used. Gonzalez-Montelongo, Lobo and Gonzalez (2010a) established that to obtain banana peel extracts with a high antioxidant capacity, three sequential extractions are required. Another aspect to take into account is the sample-to-solvent volume ratio. In addition, the pH of water or solvent in the extraction systems determines the solubility of water- or solvent-soluble phytochemicals, and influences the possible solubilisation of hydrolysable fractions (Gonzalez-Montelongo, Lobo, & Gonzalez, 2010a). The optimum pH of the extraction medium depends on the nature of phenolic compounds to be extracted and source of plant material (Ajila et al., 2011; Gonzalez & Gonzalez, 2010c).

Mechanical agitation may be used as part of the extraction steps to maximize the recovery of polyphenols. Gonzalez and Gonzalez (2010c) reported that the mass transfer rate and chemical solubility of phytochemicals could be improved through the use of agitation during the extraction. However, in another study by Davey, Mellidou and Keulemans (2009), vigorous shaking with glass beads did not result in significant differences in carotenoid recoveries between extracts subjected to homogenization and those without homogenization.

Carotenoids and some polyphenols exhibit thermal and photo-sensitivity, resulting in those phytochemicals that are isolated in aqueous extracting mixtures undergoing hydrolysis, oxidation or *trans-cis* isomerization. Additionally, disruption of the cells of a plant matrix as a consequence of peeling and cutting processes, may trigger endogenous enzymatic reactions such as oxidative browning by polyphenol oxidases (PPO). The formation of coloured melanins results in browning of the tissue surface and loss of quality (Robles-Sanchez et al., 2009a). The addition of antioxidants or chelating agents, such as ascorbic acid, butylated hydroxyanisole (BHA), butylated hydroxytoulene (BHT), ethylenediamine tetracetic acid (EDTA), tert-butylhydroquinone and sulfites can limit oxidation. In addition, studies have suggested that laboratory operations should be carried out in dim or yellow light, that evaporation be carried out below 40°C, and that samples should be purged with nitrogen or argon, before being stored in the dark at temperatures of -20°C or below.

Solid-phase extraction (SPE) is becoming increasingly popular for clean-up, isolation, purification and pre-concentration of different classes of phenolics. SPE fractionation is effective in isolating flavonoids and phenolics from mango peel (Schieber, Berardini, & Carle, 2003) and flesh (Berardini et al., 2005b; Epriliati, 2008), fruit juice concentrates (Cilla, Gonzalez-Sarrias, Tomas-Barberan, Espin, & Barbera, 2009) and red wine (Ginjom, D'Arcy, Caffin, & Gidley, 2010). Here, pre-conditioned C18 cartridges are used, where sugars and organic acids are eliminated prior to chromatographic analyses.

2.4.2. Spectrophotometric assays

Most carotenoids exhibit absorption in the visible (vis) region between 400 and 500 nm. As carotenoids obey the Beer-Lambert Law (i.e. absorbance is directly proportional to concentration), absorbance measurements can be used as an estimation of the total carotenoid concentration in food extracts, or in chromatographic fractions where a mixture of carotenoids is expected. In addition, spectrophotometry can be used to quantify the

concentration of a pure (standard) carotenoid using the absorbance values obtained and the extinction coefficient at a specific wavelength (Scott, 2001).

The Folin-Ciocalteu (FC) spectrophotometric assay is most widely used for determination of the total phenolic content in plant and food materials. This method is simple, reproducible and convenient, and has been widely used for studying phenolic antioxidants. The method is an electron transfer based assay and relies on the reduction of the phosphomolybdic-phosphotungstic acid reagent to yield a coloured complex in an alkaline environment in the presence of phenolic compounds (Ajila et al., 2011). The FC reagent is not specific and detects all phenolic groups, including those found in extractable proteins (Shahidi & Naczk, 2004). The total phenolic compounds are assayed colorimetrically as modified by Singleton and Rossi (1965), and Hoff and Singleton (1977). The content of total polyphenols is typically expressed as gallic acid equivalents (GAE).

2.4.3. Chromatographic techniques

Chromatographic techniques have been used for the separation, preparative isolation, purification and identification of carotenoids, flavonoids and phenolic acids. In the past 20 years, high performance liquid chromatography (HPLC) has been the most frequently used analytical technique for separation and quantification of carotenoids and phenolic compounds. The possibility of coupling HPLC to several detection devices such as a photodiode array (PDA) or mass spectrometric (MS) detector has turned HPLC into an even more valuable tool. The introduction of reversed-phase columns has considerably enhanced HPLC separation of carotenoids and the different classes of phenolic compounds. Gas chromatography (GC) has high sensitivity and selectivity, and has been used in studies involving Trimethylsilyl (TMS) derivatives. However, GC is often not practical due to the low volatility of many flavonoids and the necessity of preparing chemical derivatives.

There are a few published reviews of comprehensive summary of chromatographic procedures applied for analyses of carotenoids and various classes of phenolics in banana and mango flesh and peel. Isocratic elution is used for simple phenolic separations; however, most separations rely on gradient elution owing to the diversity of phenolic classes in fruit extracts. Gradient elution systems are usually binary, with one of the solvents being an acidified aqueous solution (Solvent A) and the other being a less polar organic solvent acidified with the same acid (Solvent B). Flavonoid glycosides are eluted

before the aglycones, while the flavonoids that possess more hydroxyl groups are eluted before the less substituted analogs. It is interesting to note that separation systems for phenolics in foods have been oriented towards the measurement of all (usually several sub-classes) of the prominent flavonoids in a single food.

The analysis of carotenoids is complicated due to their diversity and the presence of *trans-cis* isomeric forms. The characteristic conjugated double bond system of carotenoids causes the most problems associated with analysis of carotenoids due to its particular instability towards light, oxygen, heat and acids. For this reason, several precautions are necessary when handling carotenoids. The detection of carotenoids and phenolic compounds is based on the measurement of radiation absorption in the ultraviolet (UV) region for flavonoids and phenolic acids, or the visible spectrum (400-500 nm) for carotenoids (Table 2.11). Carotenoids generally have three maxima, or two maxima and a shoulder with the middle peak (λ_{max}) having the highest intensity (Oneil & Schwartz, 1992), with the main determinant being the number of conjugated double bonds.

Table 2.10 UV-Vis absorption pattern of phenolic subclasses.

Class of phenolic compounds	λ_{max} (nm)
Hydroxybenzoic acids	270-280
Hydroxycinnamic acids	290-300 (shoulder), 320
Anthocyanins	502 or 520
Catechins	210 or 280
Flavonols	270, 280 or 360
Flavan-3-ols	270-280, 300-330 (shoulder)
Flavanones, flavanonols	280 or 290
Flavones	240-280, 300-350

Table 2.11. UV-Vis absorption pattern of carotenoids.

Carotenoid	λ_{max} (nm)
All-*trans*-β-carotene	450 or 452
All-*trans*-α-carotene	444
Lutein	445
β-Cryptoxanthin	450
Neoxanthin	439
Violaxanthin	440
Zeaxanthin	450

Two absorption bands are characteristic of flavonoids. Band II (240-280 nm) is believed to arise from the A-ring and band I (300-550 nm) is attributable to the substitution pattern and conjugation of the C-ring (Stalikas, 2007). Various flavonoids and phenolic acids can be recognized by their UV-spectral characteristics, including the glycosidic substitution pattern, nature of the aromatic acyl group, and the number of hydroxyl groups. There is little or no conjugation between the A- and B-rings of flavanones, so their UV spectra usually have an intense band II and just a small band I. In addition, this lack of conjugation results in small band I peaks for the catechins. Typical wavelengths for analysis and quantification of various classes of phenolic compounds are shown in Table 2.10. To evaluate the overall complexity in a single chromatographic run, the most commonly used wavelength is 280 nm, which represents a suitable compromise.

HPLC coupled with MS is widely used for the efficient identification and characterization of carotenoids and phenolic compounds extracted from food. MS is one of the most sensitive methods of molecular analysis; it has the potential to yield information concerning the exact molecular mass and the structure of compounds through their fragmentation patterns. Electrospray ionisation (ESI) is one of the most versatile ionisation techniques; it is a soft ionisation method that typically generates deprotonated molecules of the compounds analysed in the negative ion mode (Ajila et al., 2011). This is useful in quantitative analysis or molecular mass determination. Negative ionisation mode is said to provide better sensitivity for the identification of many phenolic subclasses (de Rijke et al., 2006). More recently, ultra performance liquid chromatography (UPLC) has been gaining popularity. UPLC is a relatively new technique but has proved more effective than standard HPLC for some analyses; for example, it offers greater sensitivity, enhanced separation efficiency, shorter run times, and lower solvent consumption.

2.5. Conclusions

Nutritional recommendations concerning phytonutrients are usually based on phytonutrient intake or extracted concentrations of raw plant material, not taking into account the changes during gastrointestinal digestion, which could result in overestimation. Moreover, studies have shown that phytonutrient bioaccessibility varies, depending on the consumption of individual fruits and vegetables or meals, with a tendency to be more bioavailable in fruits. However, it is not fully understood why this is so. Could the type of plant tissue (soft or hard) play a role as a limiting factor? Even though evidence is emerging to demonstrate that the food matrix is a key contributor to the release of phytonutrients from plant-based foods, detailed research focusing on the nature of interactions between phytonutrients, and how this affects their bioaccessibility and bioavailability is lacking.

In vitro bioavailability studies through the application of Caco-2 cells are relatively well established for most phytonutrients found in fruits. However, there is a lack of research in the area of bioaccessibility and metabolism, especially in the colon. In addition, studies usually focus on the degree that phytonutrients are bioaccessible, focusing on phytonutrients as a single compounds or a class of related compounds, without directly comparing across the different types of phytonutrients, e.g. carotenoids vs phenolic compounds. A further area in which there is limited information is the fate of any carotenoids or phenolic and other compounds that are not absorbed in the small intestine,

and therefore enter the colon, bound to or trapped within plant cell walls. As fruit cell walls are likely to be fully fermented in the colon, bound or trapped carotenoids or phenolic compounds will be released, and could then either be absorbed through the colon epithelia or be metabolised by colonic bacteria, before being absorbed or excreted. *In vitro* fermentation of mango and banana fractions that survive *in vitro* gastric and small intestinal processing can be used to assess potential bacterial metabolism. Information gained from *in vitro* experiments can be tested *in vivo* through the use of the pig as a model for the human digestive tract. An important aspect of *in vivo* studies that has previously been overlooked, is the passage rate, which determines how long food digesta is present at different sites within the gastrointestinal tract. This is critical information for comparing the results of *in vitro* bioaccessibility/metabolism studies (where time is a variable) to the *in vivo* conditions where residence time/passage rate is determined by the interactions of the food with the animal.

The subsequent chapters of the thesis will describe the work that has been undertaken to study the effects of sequential digestive processing in the mouth, stomach, small intestine and colon using mango (*Kensington Pride*) and banana (*Cavendish*) flesh as a model fruit system. Mechanisms restricting the bioaccessibility of carotenoids during *in vivo* human mastication and *in vitro* gastrointestinal digestion (Chapter 3), and the differential *in vitro* colonic fermentation kinetics of macronutrients (plant cell walls and starch), and phenolic compounds (Chapter 4 Part A and B) will be critically examined. As *in vitro* digestion studies are generally carried out in shorter time intervals (~2-4 h), an *in vivo* pig model was carried out to study the 'real' digesta passage time through the digestive tract with a mango ingredient and pectin (as the main soluble dietary fibre in banana and mango) (Chapter 5 Part A and B).

Chapter 3. Mastication effects on carotenoid bioaccessibility *in vitro* from mango fruit tissue

3.1. Introduction

Epidemiological studies have shown an inverse correlation between consumption of carotenoid-rich fruits and vegetables, and the incidence of cancers of the gastrointestinal tract (Kant et al., 1992; Kiokias & Gordon, 2004; Mayne, 1996; Rock & Swendseid, 1992), cardiovascular diseases (Krinsky, 1998; Murr et al., 2009), diabetes (Yeum & Russell, 2002), some inflammatory diseases (Perera & Yen, 2007), as well as age-related macular degeneration (Snodderly, 1995). The most documented function of β-carotene is its provitamin A activity, with consequent health benefits, such as maintenance of epithelial function, embryonic development, and immune system function (Diplock, 1991). Xanthophylls e.g. zeaxanthin and lutein, are only present in human retinal pigment epithelia, in contrast to other body sites where all other carotenoids occur (Bone et al., 1993), and probably function as blue light filters and singlet oxygen quenchers (Seddon et al., 1994).

Human studies are most appropriate to predict nutrient bioavailability, but these studies have technical and ethical limitations (Netzel et al., 2011). Metabolic and physiological factors have been reported to influence the absorption, distribution and elimination of carotenoids (Bowen, Mobarhan, & Smith, 1993; Johnson, Qin, Krinsky, & Russell, 1997; Kostic, White, & Olson, 1995), resulting in inter-individual variability in plasma concentrations. In addition, host-related factors such as gut health, nutritional status or discrepancies, and genotype are typically encountered in most laboratory rodent models (Van Buggenhout et al., 2010). However, these factors can be avoided through the use of *in vitro* models. *In vitro* models are relatively easy to apply to large sample numbers, and are suitable for studying the effects of various digestion conditions or other factors linked to nutrient bioaccessibility (Fernandez-Garcia et al., 2012). *In vitro* digestion models can be used to simulate the physiological conditions of gastric and intestinal digestion. In addition, nutritional recommendations are often based on intakes or concentrations present in extracts of raw plant material, not taking into account bioaccessibility and any changes during gastrointestinal digestion. This could result in nutrient overestimation, and emphasises the importance of estimating bioaccessibility.

Current *in vitro* digestion procedures have proven useful for the analysis of carotenoid release and/or bioaccessibility (Castenmiller & West, 1998; Tydeman et al., 2010a).

However, the reliability of the two-phase (stomach and small intestine) *in vitro* digestion model would be expected to be improved by including a 'real' chewing phase, or a phase that more closely mimics actual chewing behaviour and mechanics, which has been excluded in most digestion studies. Mastication is often the first step of food digestion, where the process of breaking down solid foods into smaller particle sizes and mixing with saliva takes place. During simulated or real oral chewing, the physical barriers to the release of nutrients from plant cells may be ruptured. Therefore, the degree of cellular intactness could be indicative of their potential bioaccessibility, particularly as cell breakage is likely to be a major requirement for carotenoid bioaccessibility (Lemmens, Van Buggenhout, Van Loey, & Hendrickx, 2010; Tydeman et al., 2010b). Ideally, the structural properties of a food product digested *in vitro* should be similar to that of a chewed food bolus, since mastication varies subjectively between individuals, which impacts on food matrices and the structural properties of food boluses. Currently, simulated oral chewing has been mimicked using techniques such as pulverising, sieving, chopping or mincing (Woolnough et al., 2008), and the occasional inclusion of (salivary) α-amylase for starch digestion (Bornhorst, Hivert, & Singh, 2014; Miao et al., 2014). However, such mechanical steps do not adequately reflect the heterogeneous nature of chewed food. Epriliati, D'Arcy and Gidley (2009a) demonstrated the importance of the simultaneous punch and gentle squash action of teeth, while Hoerudin (2012) found that mastication has a considerable effect on the cellular architectures of vegetables. In addition, mastication involves lubrication, softening and dilution with saliva (Lucas et al., 2006; Prinz & Lucas, 1995), and the formation of a cohesive bolus (Barry et al., 1995).

Mangoes are the second most important tropical fruit in terms of production and consumption, and have high carotenoid contents, particularly of β-carotene (Chen, Tai, & Chen, 2004; Yahia, Soto-Zamora, Brecht, & Gardea, 2007), which is responsible for the yellow-orange colour of ripe mango flesh (Pott, Breithaupt, & Carle, 2003). Current carotenoid studies have focused on the compositional profile or content (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010; Manthey & Perkins-Veazie, 2009; Mercadante & Rodriguez-Amaya, 1998; Robles-Sanchez et al., 2009a), the impacts of ripening stages (Ornelas-Paz, Yahia, & Gardea, 2008), the presence of fat (Veda, Platel, & Srinivasan, 2007), and effects of processing (dried, fresh, juice) (Epriliati, D'Arcy, & Gidley, 2009a). However, mastication effects on carotenoid gastrointestinal release from mango fruit have not been reported. Comparisons of the carotenoid content before and after *in vitro* digestion can provide information on their stability during gastrointestinal digestion. *In vitro*

digestion models can be adapted to estimate the bioaccessibility of carotenoids by quantifying the fractions of phytonutrients transferred from the food matrix into the aqueous digesta or micellar phase, which then represents their potential for absorption or bioavailability. Studies have shown that the bioaccessibility of carotenoids can be as inefficient as 1.7% or as high as 100% (Tydeman et al., 2010a) depending on the type of carotenoids, as well as raw versus cooked conditions. The different solubility of polar xanthophylls and apolar carotenes can also affect their ability to be incorporated into micelles and thus, affect both release and absorption efficiency.

It is hypothesised that the mechanism limiting carotenoid release involves intact cell walls (Tydeman et al., 2010a), which prevent the free passage of carotenoids into lipid-soluble components or micelles, thus affecting bioaccessibility. The objectives of this study were to investigate how the degree of mastication results in varying size distributions of ready to swallow bolus particles, and how this affects subsequent simulated gastrointestinal release of carotenoids from masticated mango tissue.

3.2. Materials and Methods

3.2.1. Plant material

Fully ripe mangoes (*cv. Kensington Pride*) were purchased from local stores in St. Lucia, Brisbane (Australia) two to three days before each of three chewing sessions, in the month of November 2012. Mango ripeness was selected based on typical eating maturity at stage 6 when the peel is yellow with pink-red blush and the flesh is slightly firm, according to the Department of Agriculture, Fisheries and Forestry (Queensland Government) mango-ripening guide (Primary Industries & Fisheries, 2012). Mangoes were stored between 4-6°C prior to the chewing sessions.

3.2.2. Chewing and bolus collection

Chewing experiments were approved by the Medical Research Ethics Committee at The University of Queensland (Ethical clearance No. 2012000683) (Appendix 1). Twenty healthy participants (aged 18-55) were recruited on the basis of frequent mango consumption and all gave informed consent to the study for mastication of fresh fruit. From the twenty participants, their expectorated boluses were collected, size fractionated and each sieve fractions were weighed to obtain a % distribution of particle sizes ranging from >5.6 mm to 0.075 mm. This generated an individual mastication profile, allowing the participants to be categorised into fine or coarse chewers. For example, the fine chewer

has a higher proportion of smaller particle size fractions while the coarse chewer has a higher proportion of particle size fractions. This profile was then used for the selection of a fine and a coarse chewer, in addition to another criteria: their consistency in producing a similar particle size distribution in each chewing experiment. Two participants representing a fine and a coarse chewer each were then selected from the twenty participants and re-invited for three subsequent chewing sessions for bolus collection.

The three independent chewing sessions were carried out on three different days of each consecutive week to account for inter-day variation. The chewing sessions were held between 0900 and 1100, after the chewers had consumed a light breakfast meal. Five to six mangoes (300-600 g each) were cut into cubes and 300 g of cubes were randomly selected from the sample pile, and given to each of the fine and coarse chewer. The remaining cubes were combined and blended (Rocket blender DJL-1017, Cafe™ Essentials, China) for 1 min to a puree to determine the carotenoid composition of the fresh mango. The chewers were instructed to chew the mango as per their habitual chewing behaviour, and to expectorate when they 'desired' to swallow. The expectorated boluses were collected, washed with 70% ethanol to prevent further biochemical changes, and fractionated via a wet sieving method, where water was flushed through a stack of sieves of apertures 5.6, 2.8, 1, 0.5 and 0.075 mm (Fig 3.1). The sieved particles were drained and collected for *in vitro* digestion. Chewing, fractionation, *in vitro* digestion and blending processes were carried out in a single day. Particles collected on the 5.6 mm sieve were excluded as the largest bolus particle or longest fibre collected on this sieve, varied to a great extent in each chewing session. Unlike the subseuqnt sieve size of 2.8 mm, there was no capped maximum size or length for boluses collected on the 5.6 mm sieve, which would have to be physically measured each time and as the boluses have to be processed as soon as possible to avoid contamination, oxidation and degradation, it was decided not to use the particles collected on the 5.6 mm sieve.
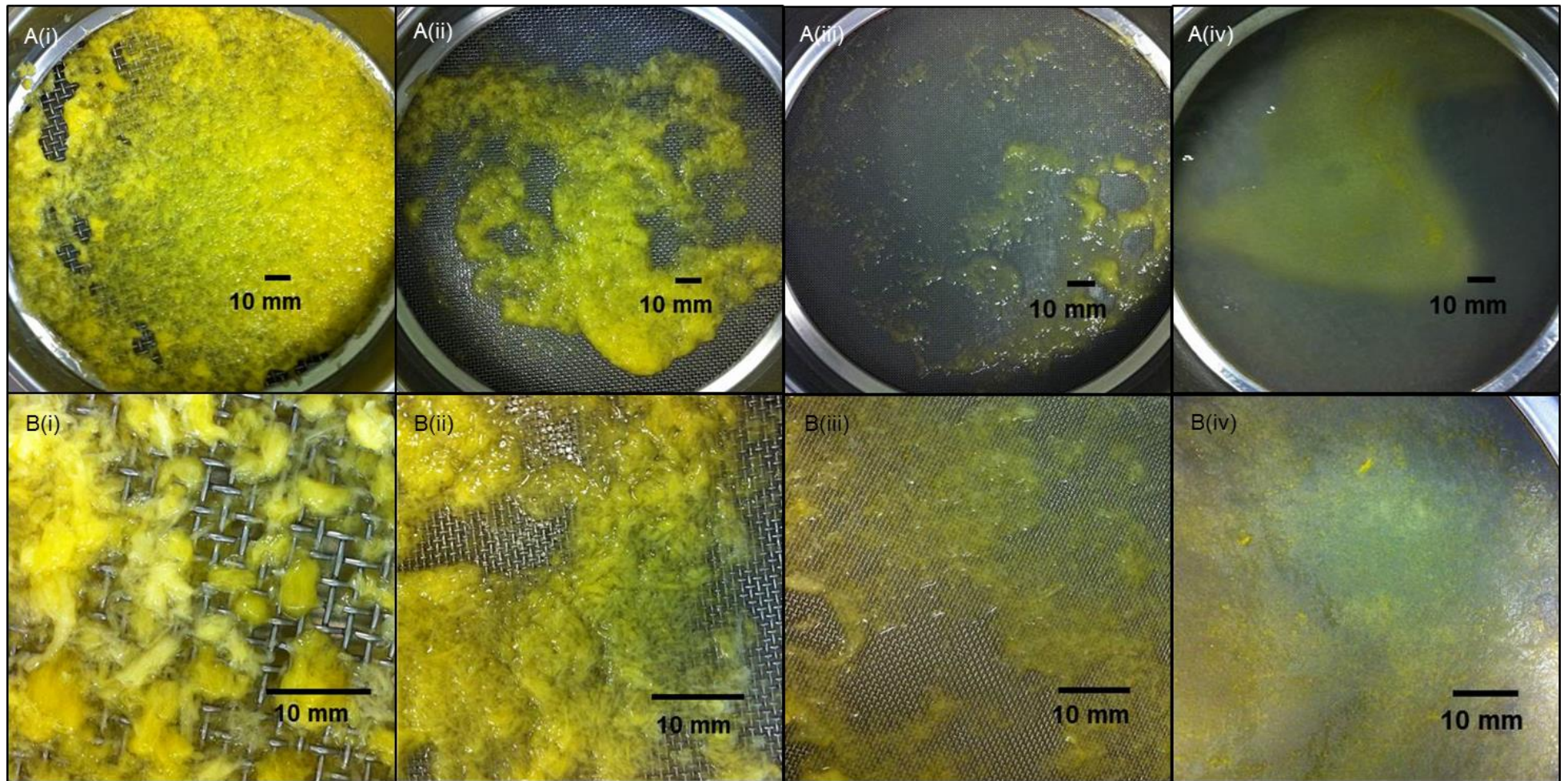
Figure 3.1. Images of A(i-iv) fractionated and chewed mango boluses particles of 2.8, 1, 0.5 and 0.075 mm respectively, and B(i-v) magnified view of each fraction (collected from the coarse chewer). Larger particle clumps and vascular fibres are observed in 2.8 mm B(i) fractions. A finer texture is observed with each decreasing sieve size.

3.2.3. *In vitro* gastrointestinal digestion

Gastrointestinal conditions were modified from Hoerudin (2012). Gastric digestion (1 h) of puree and bolus samples (2 ± 0.05 g) was initiated with 10 mL of emptying gastric secretion (130 mM NaCl, 5 mM KCl, 5 mM PIPES), followed by addition of 1 M HCl to reduce the pH to 2, and 1 mL porcine pepsin (1:2500 U/mg protein, Sigma-Aldrich, NSW, Australia) solution. Subsequently, transition from gastric to small intestinal phase was reflected by raising the pH to 6 with 1 M NaHCO$_3$. Small intestinal digestion (1 h) was mimicked by adding 5 mL pancreatin (lipase activity ≥8 USP U/mg, protease and amylase ≥4 USP U/mg, Chem Supply, Adelaide, Australia)-bile (Sigma-Alrich, NSW Australia) extract, adjusting the overall pH to 7, and diluting with 5 mL intestinal salt secretion (120 mM NaCl, 5 mM KCl). To simulate physiological movement, the mixtures were incubated in a shaking water bath at 37°C, 55 rpm. Digesta samples were then centrifuged at 3000 g, 10 min (Centrifuge 5702R, Eppendorf, USA) to separate the bioaccessible fraction from residual pellet, flushed with nitrogen and stored at -80°C.

3.2.4. Carotenoid extraction

Carotenoid extractions of the puree, digesta and residual pellets were carried out the very next day after chewing and digestion, as modified from Ornelas-Paz et al., (2008). Puree (0.8 g) and digested pellets were vortex mixed with 2.5 mL and 1.5 mL PBS respectively. Digesta supernatants were homogenized three times with an Ultra-Turrax® at 4200 rpm with 20 mL petroleum ether:acetone (2:1) containing 0.1% BHT, or until the digesta pellets turned white. In between each homogenization step, samples were centrifuged at 3000 g for 5 min. Organic fractions were collected, combined, evaporated under nitrogen, dissolved in methanol:tetrahydrofuran (1:1) with 0.1% BHT and filtered through 0.22 µm PTFE membrane. The extracts were flushed with nitrogen and stored at -80°C prior to HPLC analysis. Care was taken to evaporate just to dryness, to prevent degradation and preferential adhesion of carotenoids to vial walls (Emenhiser, Englert, Sander, Ludwig, & Schwartz, 1996). Sample preparation and extraction procedures were performed under reduced light, and all glassware and tubes were wrapped in aluminum foil to avoid contact with light. Extracts were analysed within three days of extraction, or after one freeze-thaw-cycle (frozen storage at -20 °C).

3.2.5. HPLC-PDA analysis

Separation and quantification of carotenoids were carried out on a Waters Acquity™ UPLC-PDA system using an existing method developed by the Analytical Services unit,

School of Agriculture and Food Sciences, The University of Queensland (Waanders, personal communication, March 2012). Isocratic elution was performed at 2 mL/min on a Hypersil® OBS C18 (250 x 4.6 mm, 5 µm i.d.) RP column (ThermoQuest) using a mobile phase of methanol:tetrahydrofuran:water (67:27:6). The column temperature was maintained at 25°C. An injection volume of 5 µL was used, and UV-Vis spectra of column eluent were recorded from 210-498 nm. β-carotene was identified by comparing the retention time and UV-Vis absorption spectrum with all-*trans*-β-carotene reference standard (>98% purity, Sigma-Aldrich, NSW, Australia) and xanthophylls were tentatively identified by comparing to those of published literature. Xanthophylls concentrations were calculated as β-carotene equivalents. β-Carotene calibration curve for quantification was constructed by plotting peak area against concentration (µg) (r^2=0.999). β-Carotene working standards (0.2, 0.5, 1, 5, 10, 25, 50 µg/mL) were prepared fresh on a daily basis from a β-carotene stock (50 µg/mL) in methanol:tetrahydofuran (1:1) with 0.1% BHT, and injected daily. β-Carotene standard concentration was calculated at 453 nm spectrophotometric absorbance at 453 nm and a molar absorption coefficient of 2592. β-Carotene stock solution was found to be stable for two months at -20°C (<5% loss).

3.2.6. Moisture analysis

Moisture contents of the pureed mango and chewed particles (2-5 g) were determined by vacuum oven drying (65°C for 24 h).

3.2.7. Confocal laser scanning microscopy (CLSM)

Autofluorescence of carotenoids was detected using CLSM (LSM700, Carl Zeiss, Germany), differential interference contrast (DIC) and Zen (Black) 2011 software. Observations of carotenoid chromoplasts were carried out under 10x, 20x and 63x objective lens, at an excitation λ of 488 nm, emission λ above 488 nm, and laser power intensity of 2%. Fluorescence of cell walls was observed at excitation λ of 355 nm and emission λ from 400-440 nm, after staining with Calcofluor.

3.2.8. Statistical analysis

Significant differences between mean values of carotenoid quantification were tested using one-way ANOVA, while differences between chewers and particle sizes were determined using Tukey's HSD multiple rank test ($P<0.05$) (Minitab v.16, USA).

3.3. Results and Discussion

3.3.1. Carotenoid composition and content

A typical HPLC carotenoid profile of *Kensington Pride (KP)* mango contains twelve peaks (Fig 3.2). Peaks 1-4, 6, 8-10, and 5, 7, 11 were tentatively identified as all-*trans*-violaxanthin and 9-*cis*-violaxanthin, and/or their derivatives respectively. The absorption spectra of all-*trans*-violaxanthin (λ_{max} 416, 441, 472 nm) and 9-*cis*-violaxanthin (λ_{max} 413, 436, 465 nm) are similar to those reported by Ornelas-Paz, Failia, Yahia and Gardea (2008). The 9-*cis*-violaxanthin isomer was distinguished from the all-*trans* isomer based on a characteristic 3nm hypochromic shift (Ornelas-Paz, Yahia, & Gardea, 2007). The identification of *trans* and *cis*-violaxanthins is based on comparison to published absorption spectra, and has not been characterized as free xanthophylls or xanthophyll esters so in this study, they are collectively termed as xanthophylls. Peak 12 was identified as β-carotene by comparing elution time and spectral maximum (λ_{max} 453, 481 nm) to an authentic standard (λ_{max} 453, 481 nm).
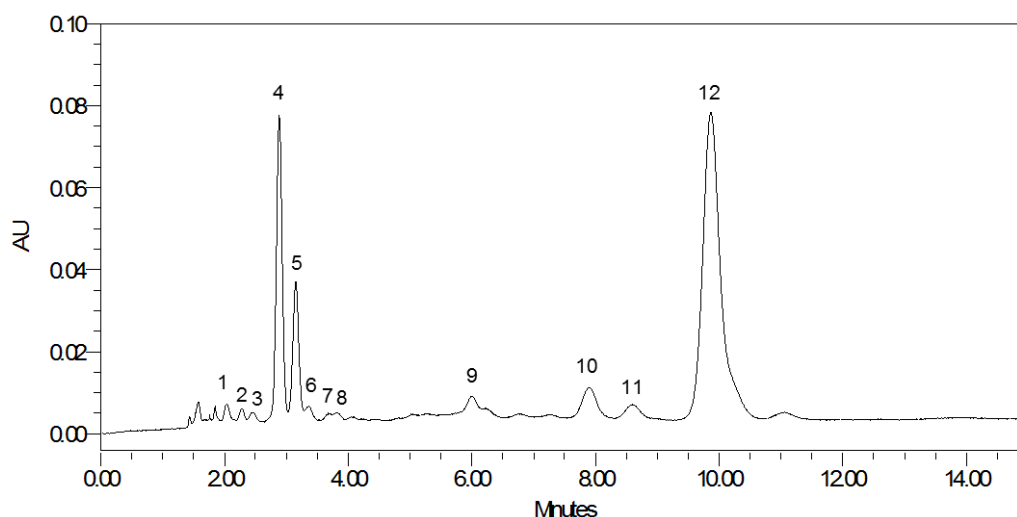
Figure 3.2. Representative HPLC-PDA chromatogram of ripe *KP* mango extract at 453 nm. Peaks 1-4, 6, 8-10 were assigned to all-*trans*-violaxanthin (and derivatives), and peaks 5, 7, 11 were assigned to 9-*cis*-violaxanthin (and derivatives). Peak 12 was identified as all-*trans*-β-carotene.

KP mango carotenoid composition is reported for the first time, and is comprised principally of all-*trans*-β-carotene (54%), followed by all-*trans*-violaxanthin (34%) and some 9-*cis*-violaxanthin (12%). Similar compositions have been recorded for *Tommy Atkins, Manila, Ataulfo, Haden* and Taiwanese cultivars (Chen, Tai, & Chen, 2004; Manthey & Perkins-Veazie, 2009; Ornelas-Paz, Yahia, & Gardea, 2007), with β-carotene being the predominant carotenoid in mangoes, although β-carotene as a percentage of total carotenoids can vary from 48 to 84% (Godoy & Rodriguez-Amaya, 1989) depending on

the cultivar or fruit physiological maturity stage. The β-carotene content in *KP* mango (1282-2081 µg/100g FW) is higher than in most other cultivars (de la Rosa, Alvarez-Parrilla, & Gonzalez-Aguilar, 2010), where it ranges from 191-1340 µg/100g, with the exception of *Ataulfo* cultivars.

In contrast, all-*trans*-violaxanthin (930-1150 µg/100g FW) and 9-*cis*-violaxanthin (318-425 µg/100g FW) was present in lower concentrations to those reported for other cultivars (Mercadante & Rodriguez-Amaya, 1998; Mercadante, Rodriguez-Amaya, & Britton, 1997; Ornelas-Paz et al., 2008). β-Carotene shows ~40% variability between individual mangoes, reflecting fruit to fruit variation (Hewavitharana, Tan, Shimada, Shaw, & Flanagan, 2013a), which is large even for fruits from the same source. *Keitt* mangoes grown in different regions of Brazil also had a two-fold difference in β-carotene content (Mercadante & Rodriguez-Amaya, 1998), indicating that environmental effects may have a similar influence on carotenoid content as cultivar-related differences.

3.3.2. Mastication, blending, particle size and carotenoid locations

Carotenoids are observed within globules in the cells of mango flesh (Fig 3.3A), supporting similar observations from other chromoplast morphology studies (Rock & Swendseid, 1992; Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012; Vasquez-Caicedo et al., 2006). During blending to a puree, the high shear rate breaks up both cell walls and globules to an almost homogenous mixture (Fig 3.3B). The puree consists of 5-10 µm cell components, with some containing carotenoids as evidenced by their color (Fig 3.3B(i)) and autofluorescence (Fig 3.3B(ii)); however, most structural cell walls are no longer present. Mastication confers actions that are not replicable with a cutting blade such as compression, compaction, squashing, and lubrication of food material with saliva to form a cohesive bolus. While these actions collectively encapsulate carotenoids, teeth cutting or slicing can be considered a prerequisite for releasing cell contents, where the physical barriers of plant cell walls are ruptured. In larger chewed fractions (captured on 5.6 and 2.8 mm sieves), clusters of intact and stacked cells encapsulating carotenoids are held tightly together by vascular fibre strands (Fig 3.4A-B). The 1 mm tissue fraction comprises single cells and cell fragments, while a reduction in cell size is observed (Fig 3.4C). In the 0.075 mm fraction, the cells are more sparsely dispersed with empty pockets of ruptured cells (Fig 3.4D); additionally, 'free' carotenoids were detected, indicating release from the broken mango cells (Fig 3.4D(ii)).

Hutchings and Lillford (1986) proposed that boluses should reach a degree of structure and lubrication before swallowing, and so information on the bolus water content before and after chewing could give a measure of saliva incorporated into the masticated matrix. The moisture content of masticated fractions (96 ± 1%, 98 ± 0.2%, 99 ± 0.3% in 2.8, 1, and 0.075 mm fractions respectively) was always higher than in fresh mango (84 ± 1%), showing a considerable portion of saliva is retained by the boluses during chewing.
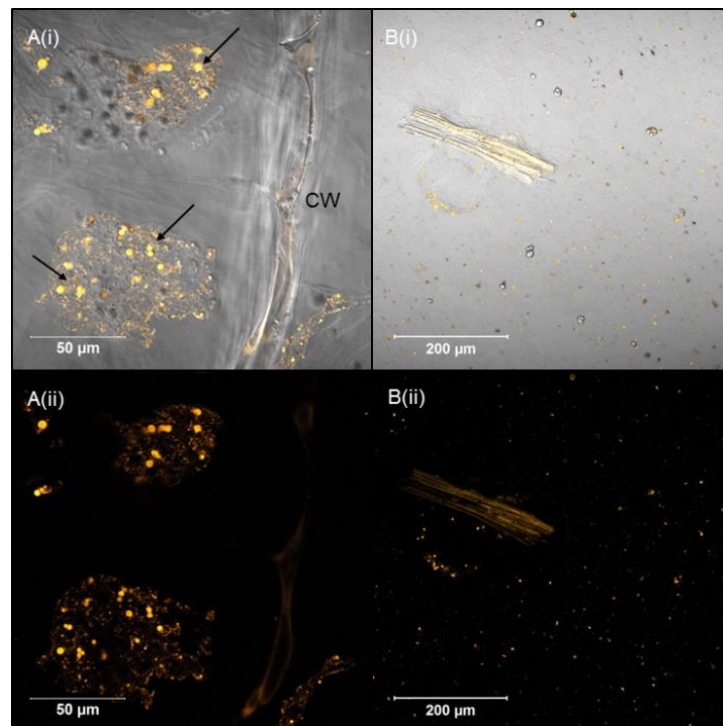
Figure 3.3. Differential interference contrast images of mango flesh A(i) and puree B(i); A(ii) and B(i) showing autofluorescence of carotenoids (orange globules). Arrows in A(i) represent carotenoid-containing chromoplasts, and 'CW' represents cell walls.

3.3.3. Release of carotenoids from solid particles is dominated by small intestinal digestion

Most *in vitro* digestion studies ignore oral mastication or replace it with mechanical processing, and only a few studies have investigated the effects of mastication on nutrient bioaccessibility (Bornhorst, Hivert, & Singh, 2014; Epriliati, D'Arcy, & Gidley, 2009a; Hoerudin, 2012; Ranawana, Monro, Mishra, & Henry, 2010). Thus, carotenoid release from solid chewed fractions as a starting material was of particular interest in this study. Relating the release of carotenoids in the fresh mango tissue to that of the expectorated boluses posed an initial challenge due to the constant production and dilution of saliva during chewing. Therefore, the bioaccessibility of each particle size fraction was determined as the bioaccessible fraction in the supernatant relative to the sum of combined contents in the digesta supernatant and bolus pellet following digestion, rather

than to the absolute content of fresh mango. As such, the fraction of carotenoids readily lost to the aqueous environment, i.e. the liquid phase in the mouth during chewing and washing during the wet sieving process, is not taken into account, in contrast to the puree, which still contains the liquid phase.

After simulated gastric digestion (1 h), mango cells and vascular fibres were still intact (Fig 3.5A, 3.5C), and apparently encapsulating the carotenoids, indicating that acidic hydrolysis did not have a major role in breaking down the cell walls or releasing the carotenoids. This is consistent with a relatively low level of release from chewed bolus particles under these conditions (Fig 3.6). In contrast, the effect of the emulsifying activity of bile salts on carotenoid aggregation was evident after subsequent small intestinal digestion *in vitro*. Cellular-trapped and 'free' carotenoids are shown as assorted clusters of globular aggregates (Fig 3.5B, 3.5D), which are consistent with a significant increase in release of β-carotene from 8% to 33% (Fig 3.6A), and xanthophylls from 20% to 57% (Fig 3.6B) in the chewed solid fractions following *in vitro* small intestinal digestion. This illustrates the critical importance of bile salts for carotenoid release into aqueous digesta. Being lipophilic, carotenoid dissolution in micelles is essential, unlike water-soluble polyphenols that are readily dissolved in aqueous digesta. Other studies of carotenoid release, notably from tomato, have shown that the presence of triglyceride oils increases bioaccessibility (Colle, Van Buggenhout, Lemmens, Van Loey, & Hendrickx, 2012; Huo, Ferruzzi, Schwartz, & Failla, 2007), presumably by facilitating the transfer of carotenoids to the emulsion phase. However, unlike tomatoes, mango fruit is not typically consumed with oil so this was not investigated in the current study.

The release of β-carotene from mango puree (67%) following small intestinal digestion is substantially greater than from chewed particles (<33%), and likewise for xanthophylls (puree: 75% and chewed particles: <57%). Hedren, Diaz and Svanberg (2002) confirmed that mechanical homogenization leading to carrot cell rupture, increased β-carotene release from 3% to 21% with an expansion of surface area. In addition, Reboul et al. (2006) reported that for carrots, juicing increased bioaccessibility levels from 3% to 14%. However, the absolute values of released carotenoids cannot be directly compared between puree and chewed particle forms as the latter is expected to have lost readily released carotenoids during the chewing and sieving/washing stages. Due to the relatively low % release following *in vitro* gastric treatment, particularly for β-carotene, it is proposed that the difference in carotenoid release between puree and chewed particles, after *in vitro*
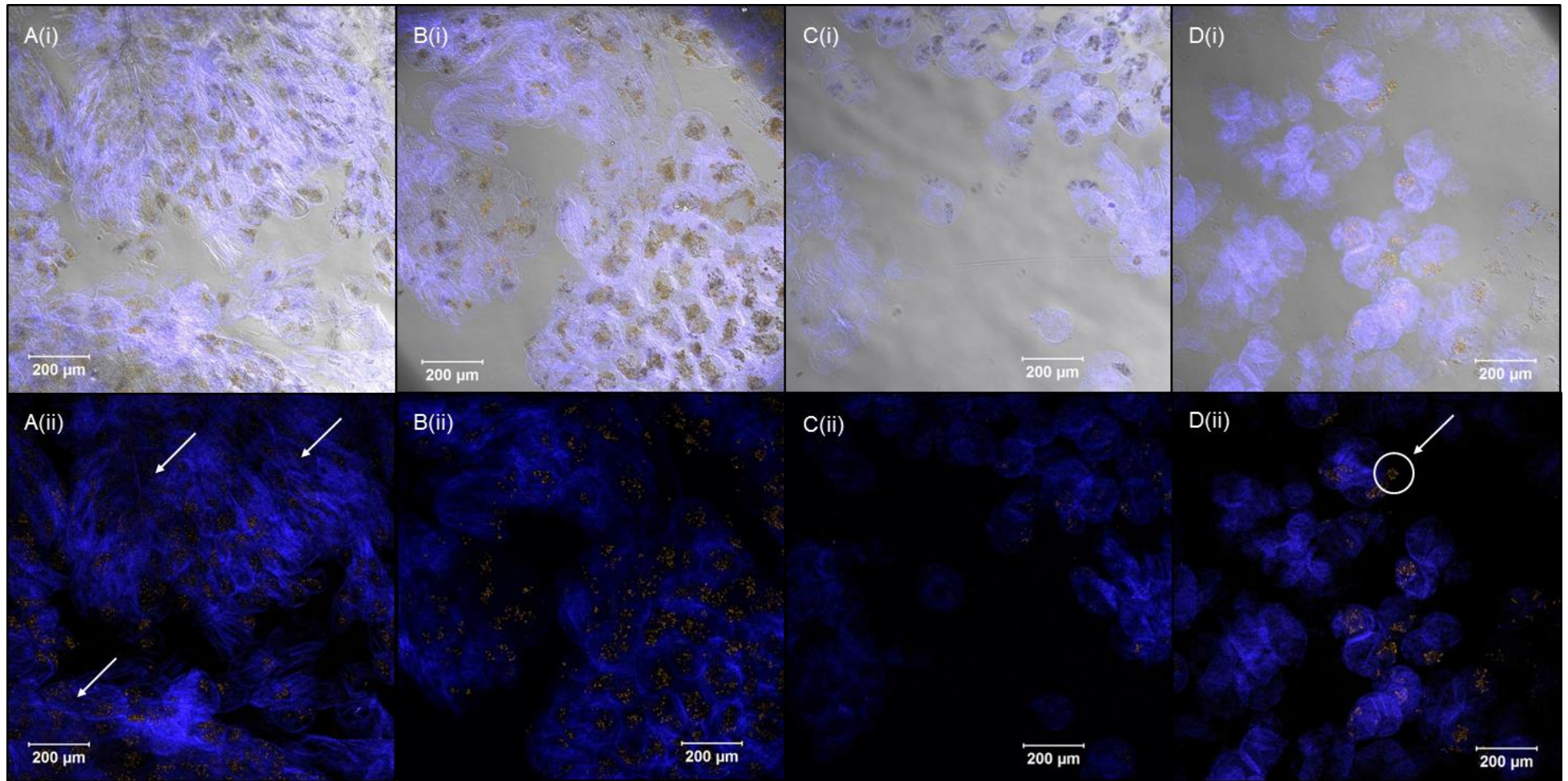
Figure 3.4. Effect of *in vivo* chewing on mango cellular microstructure for particles captured on sieves of screen size: (A) 5.6 mm, (B) 2.8 mm, (C), 1 mm, and (D) 0.075 mm. A(i)-D(i) differential interference contrast images showing carotenoids (orange) located within residual cellular structures; A(ii)-D(ii) the same fields of view showing fluorescence of carotenoids (orange globules) and cell walls (purple-blue). Arrows in A(ii) show connective vascular fibres and in D(ii) shows released carotenoids.

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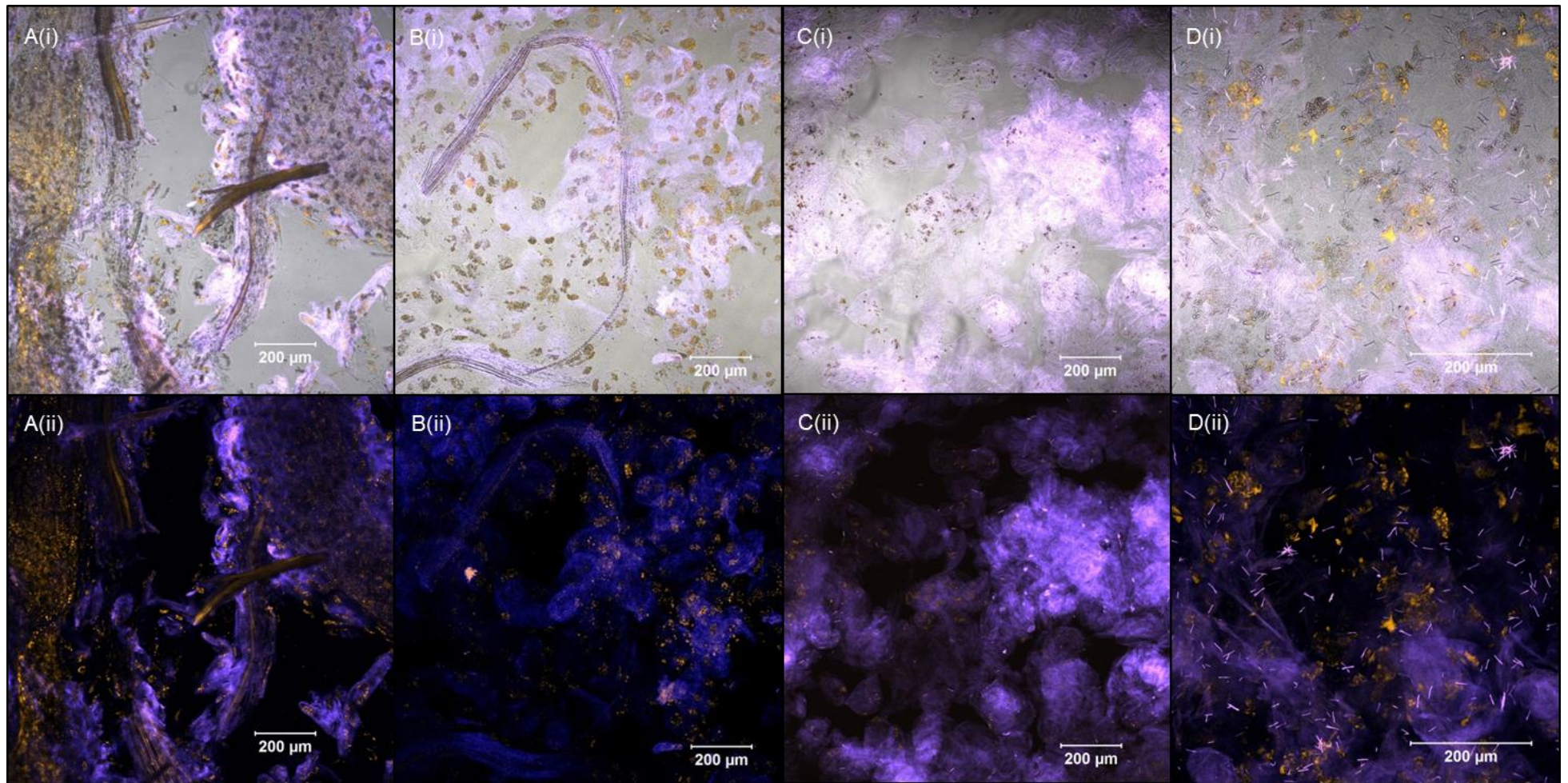
Figure 3.5. Effect of *in vitro* digestion on two sieve size fractions of chewed mango following *in vitro* gastric digestion: (A) 5.6 mm, (C) 0.075 mm, and following small intestinal digestion: (B) 5.6 mm, (D) 0.075 mm; A(i)-D(i) differential interference contrast; A(ii)-D(ii) fluorescence of carotenoids (orange globules) and cell walls (purple-blue).

gastric digestion, provides an estimate of the fraction of carotenoids lost during the oral processing and isolation of solid chewed particles, as indicated in Fig 3.6A.

A greater relative % release is observed for the xanthophylls in comparison to β-carotene, presumably due to xanthophylls being less hydrophobic. Transfer efficiency seems to be influenced by solubility as reflected in the micellarised localisation of different carotenoid types. Carotenes are embedded in the triacylglycerol-rich core of micelles, while xanthophylls with more hydroxyl or other functional groups are more polar (Matsuno et al., 1986; Tyssandier et al., 2003), and are expected to reside closer to the surface monolayer, together with proteins, phospholipids, and partially ionised fatty acids (Canene-Adams & Erdman, 2009). This suggests that xanthophylls are more readily incorporated into lipid-bile micelles (Garrett, Failla, & Sarama, 1999; Van Buggenhout et al., 2010), although this may vary amongst green vegetables containing membrane or protein-bound chloroplasts (Failla, Huo, & Thakkar, 2008).
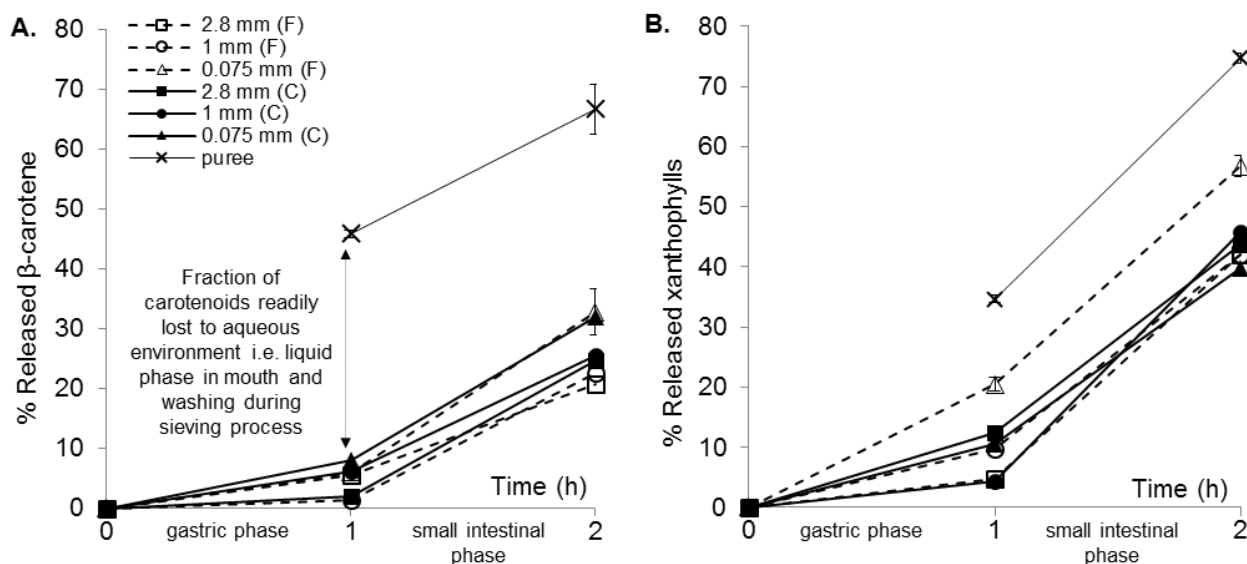
Figure 3.6. Percentage release of (A) β-carotene and (B) sum of total xanthophylls and derivatives, from masticated mango fractions and puree following simulated gastric (1 h) and small intestinal (2 h) digestion, into the aqueous digesta phase. Solid particles captured on three sieves (2.8, 1, 0.075 mm) from a fine (F) and a coarse (C) chewer were studied. For clarity, error bars are only shown for puree and 0.075 mm samples from the fine chewer.

Further to this, there is a fraction of carotenoids that is not released following small intestinal digestion (Fig 3.6). There is still a limited extent of bioaccessibility from the solid chewed particles (20-30% for β-carotene and 40-50% for xanthophylls) or incomplete bioaccessibility after pureeing (65-75%). We propose that the incomplete bioaccessibility

of purees is due to the crystallinity of mango carotenoids or residual chromoplast structure, since there is no evidence for the presence of intact cell wall material. For the chewed particles, additional restrictions on bioaccessibility are proposed to arise from residual embedding in cell wall residues (Fig 3.5D). Pectin from fruit matrices has been suggested to interfere with micelle formation by partitioning bile salts and fat in the pectin gel phase (Palafox-Carlos, Ayala-Zavala, & Gonzalez-Aguilar, 2011; Parada & Aguilera, 2007; Rock & Swendseid, 1992) that is necessary for the absorption of lipophilic carotenoids.

3.3.4. Higher carotenoid concentration in particles from coarse chewing and in larger particle size fractions

Mango pieces chewed to the same particle sizes (of 2.8, 1, 0.075 mm) from the fine and coarse chewers were examined to show variability in chewing behaviour. The carotenoid bioacessibility from the mango tissue of the same particle size produced from the fine and coarse chewers was similar (Fig 3.6). However, larger absolute concentrations of β-carotene and xanthophylls were found in equivalent sieve fractions from a coarse chewer compared with a fine chewer ($P<0.05$) (Table 3.1) for both the bioaccessible carotenoids and those trapped in the residual plant material following *in vitro* small intestinal digestion. This relatively large difference (coarse chewed particles typically have about 30% higher carotenoid concentration than fine chewed particles - Table 3.1) suggests that fine chewing causes more carotenoids to be released into the solution phase, which in this experiment either remained in the mouth or were removed during sieve capture of particles. In contrast, the percentage release of either β-carotene or xanthophylls from all particle sizes was very similar for both coarse and fine chewers (Fig 3.6), apart from xanthophylls from the smallest fraction. This illustrates, in this trial, the type of inter-individual habitual chewing (coarse or fine) as influenced by the number of chews, preferential use of tongue to molars or a longer chewing period, have a larger impact than the chewed particle size in determining the total release of carotenoids from mango fruit.

In addition, total carotenoids were present in higher concentrations in larger particles, which suggest a distribution effect due to a greater mass of plant cell walls, cell clusters and insoluble fibre network in larger particles that entraps more carotenoids. The highest concentration per 100 g (fresh weight) was found in the 2.8 mm fraction, consisting of bulky cell clusters, followed by the 1 mm fraction containing single cells and some cell fragments, while the lowest concentration was found in the 0.075 mm fraction, which consists mostly of fragmented cells.

Table 3.1. Carotenoid concentrations (μg/100g fresh weight) in each mango particle size fraction following gastrointestinal digestion *in vitro* (2 h)

Chewed mango fractions	Bioaccessible fraction			Trapped in plant matrix	
	Gastric digestion	Small intestinal digestion	β-carotene/ xanthophylls[1]	Small intestinal digestion	β-carotene / xanthophylls
Coarse					
2.8 mm	48 ± 27[a]	320 ± 96[a]	211 ± 90[a]/127 ± 9[x]	788 ± 220[a]	649 ± 198[a]/159 ± 28[x]
1 mm	47 ± 12[a]	249 ± 28[ab]	154 ± 13[ab]/115 ± 16[xy]	561 ± 171[abc]	447 ± 139[abc]/132 ± 35[xy]
0.075 mm	50 ± 23[a]	199 ± 42[ab]	152 ± 42[ab]/93 ± 20[yz]	397 ± 81[bc]	326 ± 58[bc]/ 90 ± 24[yz]
Fine					
2.8 mm	44 ± 13[a]	211 ± 14[ab]	131 ± 14[ab]/99 ± 5[xyz]	610 ± 108[ab]	499 ± 79[ab]/129 ± 29[xy]
1 mm	24 ± 19[a]	192 ± 18[b]	118 ± 22[ab]/93 ± 3[yz]	506 ± 66[abc]	404 ± 67[abc]/121 ± 2[xy]
0.075 mm	34 ± 17[a]	136 ± 18[b]	87 ± 20[b]/68 ± 4[z]	216 ± 20[c]	179 ± 15[bc]/56 ± 10[z]

Data is expressed as means±standard error of three independent chewing and digestion experiments. Values with different letters in each column denote a significant difference (P<0.05) in carotenoid concentration. [1]Xanthophylls consist of violaxanthins and derivatives.

The smallest particle size (75 μm) fraction resembles a 'mash' (Fig 1C(v)), for which the increased surface area explains the higher % release in the smaller particles (Fig 3.6), in agreement with Lemmens et al. (2010) and Hedren et al. (2002), who reported that smaller particles had a higher release or digestibility. Netzel et al. (2011) also showed that disruption of the cell wall matrix led to improved release rates, with bioaccessibility of carotenoids in single carrot cells (70-80 μm) increasing two-fold compared to 230 μm cell clusters. There is a general trend for smaller particles to be associated with a greater % release of carotenoids, but the differences between particle sizes or fine vs coarse chewing (Fig 3.6) are unexpectedly small. Whilst there is indeed a (small) particle size effect on bioaccessibility, this may be secondary to the extent of chewing for soft tissues such as mango, which contrasts with carrot tissues where a single cell wall appears to be enough to retain carotenoids (Tydeman et al., 2010a). This contrast with carrots is likely to be a consequence of the robustness of the cell wall matrix. In carrots and many other vegetables, cell walls are relatively robust and can survive cooking processes. In ripe mango and possibly other ripe fruits, chewing and *in vitro* digestion results in a gel-like solid structure (Fig 3.5) without a discrete wall. Therefore, phytonutrient bioaccessibility may vary amongst fruits, vegetables, grains and legumes due to differences in cell wall thickness and structure, which determine how intact cellular structures remain after chewing and digestion.

3.4. Concluding Remarks

This study of mastication effects on carotenoid release emphasises the importance of including chewing in *in vitro* digestion studies. Mastication confers a combination of size reduction and compaction processes as part of the digestive process, with plant cell wall structures being one of the limiting factors for carotenoid release, and chromoplast location of carotenoids being another. Some carotenoids (25-33%) may be present as crystallites or may remain trapped in the residual plant matrix, and could potentially be fermented in the large intestine, releasing more carotenoids during degradation of cell walls there. This warrants further investigation of carotenoid release following colonic fermentation of *in vitro* digestion residues. Factors influencing individual chewing behaviour or habit could also be investigated in future work. Particle size and the type of chewing resulted in differences in the relative amounts of carotenoids in the solid fraction after chewing, but had a surprisingly small impact on carotenoid bioaccessibility from the solid particles, suggesting that cell wall factors are not necessarily the most important in determining carotenoid bioaccessibility in soft tissues in contrast to tissues with more robust cell walls such as carrot.

Chapter 4. Fermentation kinetics and microbial biotransformation of polyphenols during colonic fermentation of masticated mango and banana *in vitro*

4.1. Introduction

In vivo Investigations of dietary polysaccharide fermentation in the human colon can be challenging due to inaccessibility, inconsistency, and the limitations of dietary control for human volunteers (Edwards et al., 1996). There is continued interest in the development of relevant *in vitro* models for the digestive process, but uncertainty over how best to represent the unit processes involved. As an adjunct to *in vivo* studies, non-invasive *in vitro* colonic fermentation models can be used to monitor changes in substrate fermentability before and after digestion, and to elucidate the potential role of microbiota in the metabolism of partially and/or non-digestible components of the diet e.g. dietary fibre. *In vitro* colonic models involving rats or pig caecal microbiota (Shahidi & Naczk, 2004) have proved useful for investigating the metabolic processes mediated by intestinal microbiota, since pigs can be fed controlled diets, and there is direct access to the intestinal contents. Even so, it is important to remember that animal digestive physiologies do differ from humans e.g., rodents are coprophagic, while pigs are generally more similar to humans in terms of digestive anatomy and physiology (Labib, Erb, Kraus, Wickert, & Richling, 2004), although their upper digestive tract is more heavily colonised by bacteria (Macfarlane & Macfarlane, 2007). For comparative purposes, it is important that the microbiota used as an inoculum is from a well-defined source.

Amongst a range of fermentable substrates, fruit and vegetable samples have been examined *in vitro* using colonic fermentation techniques (Bazzocco et al., 2008; Day, Gomez, Oiseth, Gidley, & Williams, 2012; Molan, Lila, Mawson, & De, 2009; Piquer et al., 2009; Vong & Stewart, 2013). Previous authors have used a variety of pre-treatments on fruits and vegetables in *in vitro* digestion studies preceding fermentation step such as milling or grinding with hammer mills, blenders, and mortars and pestles (Davey, Keulemans, & Swennen, 2006; Kim et al., 2010; Kim, Brecht, & Talcott, 2007), and homogenisation (Ajila, Rao, & Rao, 2010; Manthey & Perkins-Veazie, 2009; Mercadante, Rodriguez-Amaya, & Britton, 1997; Ornelas-Paz, Yahia, & Gardea, 2007), often preceded by air-drying (Robles-Sanchez et al., 2011) or lyophilisation (Bouayed, Hoffmann, & Bohn, 2011; Shofian et al., 2011). In addition, wet liquid samples such as purees or juices have been prepared (Alothman, Bhat, & Karim, 2009; Manthey & Perkins-Veazie, 2009). However, these high-shear techniques result in the disintegration and collapse of cellular structures, which does not necessarily simulate the human mastication process,

consequently leading to significant overestimates in phytonutrient bioaccessibility. For example, in Chapter 3, the carotenoid bioaccessibility of pureed mango was significantly higher and twice that of *in vivo* masticated mango fractions of varying particle sizes. In addition, findings from Chapter 3 also showed that there is still a fraction of carotenoids not completely released from the puree or chewed particles after small intestinal digestion *in vitro,* which is therefore hypothesised to be transported to the colon.

The aims of this study were to investigate the fermentation kinetics and end-products of fresh mango and banana flesh (Chapter 4 Part A), with an additional focus on the release and metabolism of their polyphenols (Chapter 4 Part B) using a standardised batch fermentation model after *in vivo* human mastication and *in vitro* digestion processing. In this context, the fruits were prepared in such a way such the state and condition of these samples are comparable to the microstructures achieved at the beginning of the colon, i.e. using minimal 'artificial' processing. Following mastication, samples were size-fractionated to allow comparison of the effect of particle size on subsequent fermentation behaviour. Mango and banana, two widely consumed tropical fruits were used as model systems.

Part A. Fermentation kinetics of masticated mango and banana tissue during colonic fermentation *in vitro*

4.2. Introduction to Part A

Williams et al. (2005) developed a cumulative gas production technique for use with monogastric inocula, which involves a batch culture to measure gas production kinetics (Theodorou et al., 1994) and generated end-products such as short chain fatty acids (SCFA) and ammonia (NH_3) (Jacobs, Gaudier, van Duynhoven, & Vaughan, 2009). The volume of gas produced as a result of fermentation acts as an index of fermentation activity, and contains a mixture where the predominant gas (CO_2) is derived from primary fermentation and the reaction of acidic fermentation end-products with basic bicarbonate ions (Davies et al., 2000). SCFA such as acetic, propionic and butyric acids are major products of carbohydrate fermentation and are known to be beneficial in terms of energy contribution and health (Chiu & Stewart, 2012), whereas NH_3, one of the end-products of protein and peptide fermentation (Hendriks, van Baal, & Bosch, 2012), has potentially negative effects on the long-term health of colonic epithelial cells (Mosenthin, 1998).

4.3. Materials and methods

4.3.1. Preparation of fruit substrates

Ripe *Kensington Pride* mangoes and *Cavendish* bananas were procured from local stores in Brisbane, Queensland, Australia and selected based on typical eating maturity. The fruits were first subjected to *in vivo* human mastication and wet-sieve fractionation (Fig 4.1) as described in Chapter 3 (ethical clearance No. 2012000683) (Low, D'Arcy, & Gidley, 2015) followed by *in vitro* gastrointestinal digestion (Day et al., 2012) and centrifugation at 3000 g for 10 min (Avant®JE centrifuge, JA14 rotor). The pellets were washed three times with water (1:3) to remove salivary components such as enzymes, soluble sugars and amino acids. The samples were stored at 4°C prior to fermentation *in vitro*.
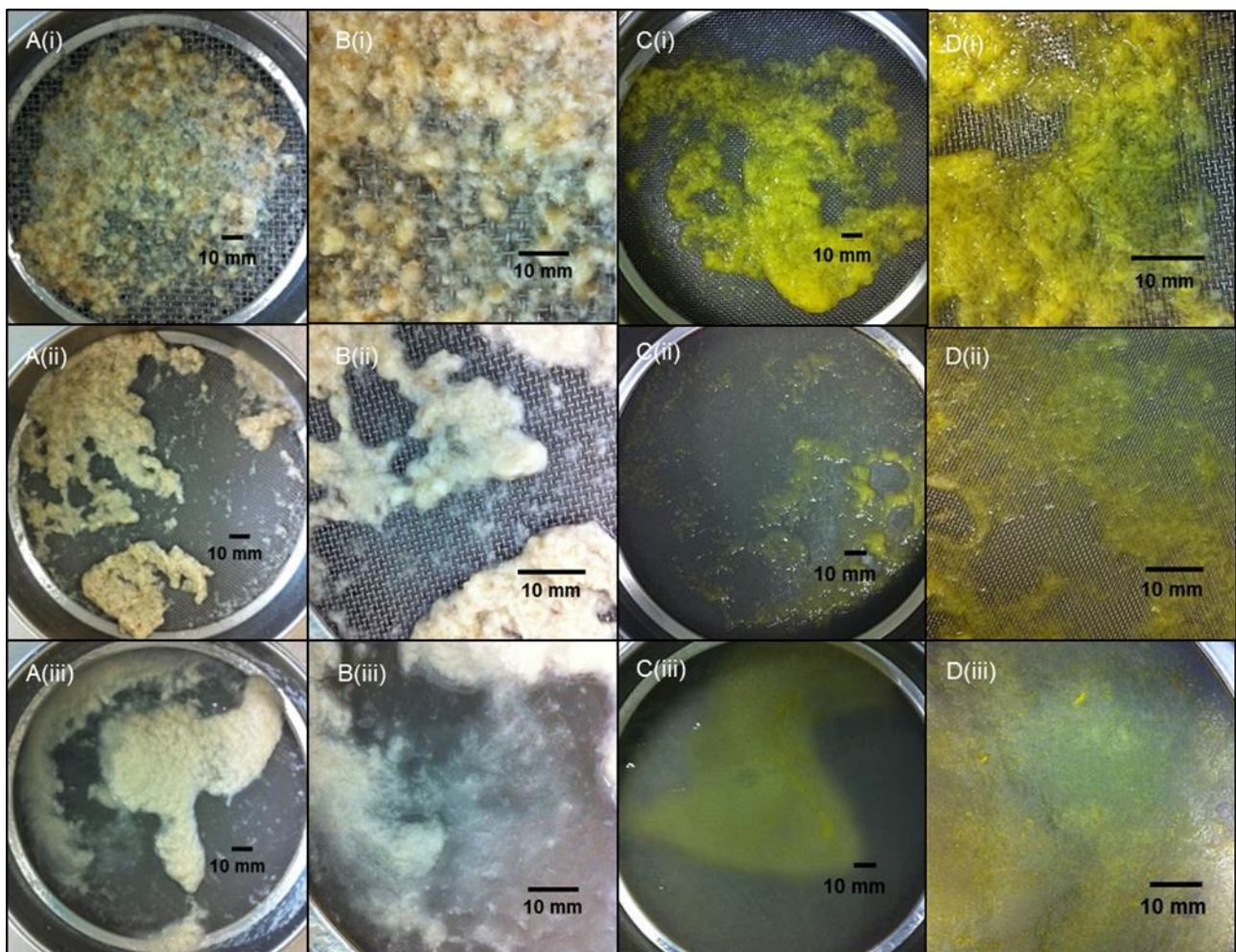
Figure 4.1. Images of chewed and fractionated (A) banana and (C) mango bolus particles of (i) 2.8 mm, (ii) 1 mm and (iii) 0.075 mm apertures. B(i-iii) and D(i-iii) show a magnified view of each fraction respectively.

4.3.2. Collection and preparation of fresh faecal inoculum

Faecal inoculum was prepared as described previously (Williams, Bosch, Boer, Verstegen, & Tamminga, 2005b). Faeces were collected directly from five pigs (animal weight ~35 kg) under ethics approval of the University of Queensland Animal Ethics Committee (SAFS/111/13/ARC). Prior to faecal collection, the pigs were strictly fed a semi-purified diet based on highly digestible maize starch and fishmeal for ten days to avoid adaption of the gut microbiota to any of the substrates being used (Wang, Williams, Ferruzzi, & D'Arcy, 2013). Faeces were kept in pre-warmed CO_2-filled vacuum flasks during transport to the laboratory. To avoid (as far as possible) any effect of genetic variation of the pigs, the faeces from all five pigs were combined to make an inoculum representative of pigs as a whole. The faeces were then mixed (1:5) with pre-warmed saline (9 g/L NaCl), homogenised for 1 min under CO_2 and strained through four layers of muslin cloth.

4.3.3. Cumulative gas production

The cumulative gas production technique was slightly modified from Williams et al. (2005b). Fresh unfractionated and fractionated mango (4.8 ± 0.4 g) and banana (3.1 ± 1.4 g) particles (each particle size, n=4) were weighed into 120 mL serum bottles containing 82 mL fermentation medium (76 mL basal solution, 1 mL reducing agent, 1 mL vitamin/phosphate buffer solution, 4 mL bicarbonate buffer). Unfractionated mango and banana refers to expectorated mango and banana boluses that were not sieved, containing mixed size particles. Faecal inoculum (4 mL) was added to each serum bottle within 2 h of faecal collection and incubated at 39°C (the body temperature of pigs). A steady stream of O_2-free CO_2 flowed into the fermentation bottles at all times prior to sealing with butyl rubber stoppers and aluminium crimp seals. Experimental blanks containing only inoculum and medium were also included. Cumulative gas readings were measured via a pressure transducer (Type 453A, Bailey and Mackay Ltd., Birmingham, UK) and a LED digitial readout voltmeter (Tracker 200) after insertion of a hypodermic syringe needle through the butyl rubber stopper above the fermentation solution. The head-space pressure and volume of gas were measured in each fermentation bottle (178 bottles) at 0, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 43, 46 and 48 h of the fermentation period according to the method of Theodorou et al. (1994). Then, the pressure and volume of gas recorded for each bottle was regressed to provide a corrected volume at each time per bottle. After cumulative gas readings were carried out for the bottles at their respective time intervals, they were placed immediately in iced-water to inhibit further microbial activity.

4.3.4. Post fermentation analyses

pH of fermentation solutions were recorded and aliquots taken for SCFA, NH_3 and polyphenol analyses for all the fermentation bottles after removal at their respective time intervals. The remaining bottle contents were centrifuged at 4000 g for 10 min at 4°C and then washed twice with water. Dry matter (DM) of fermented samples (and substrates before fermentation) was determined by drying to a constant weight at 103°C (ISO 6496, 1999) and then ashing by combustion at 550°C (ISO 5984, 1978).

Analysis of the SCFA concentration of fermented samples was modified from Vreman, Dowling, Raubach, and Weiner (1978) with modifications to the microvacuum distaillation apparatus, which has been expanded to distaill 12 samples at one time. Sample aliquots (0.9 mL) and 1 M sulphuric acid (0.1 mL) containing 500 mM formic acid were added to the Thunberg tube, frozen with liquid N_2 and vacuum distillated. SCFA concentrations of the extracted aliquots were analysed by an Agilent GC-FID (HP6890) (Agilent Technologies, Mulgrave, VIC, Australia) and DB-FFAP capillary column (30 m x 0.5 mm) at a flow rate of 5.3 mL/min with helium as the carrier gas. Injector and detector temperatures were 250°C, and oven temperature was programmed from 90°C (1 min) to 190°C (1 min) at 10°C/min. The injection volume was 0.5 µL. Iso-caproic acid was used as an internal standard. The SCFA mixed reference comprised of acetic acid (52.51 mM), propionic acid (13.4 mM), iso-butyric acid (1.07 mM), n-butyric acid (5.45 mM), iso-valeric acid (0.91 mM), n-valeric acid (0.92 mM), n-caproic acid (0.16 mM) and heptanoic acid (0.15 mM) (Sigma-Aldrich, Castle Hill, NSW, Australia). The branched-chain ratio (BCR) was calculated as the ratio of the concentrations of branched chain acids (isobutyric, isovaleric and valeric acids) to straight chain acids (acetic, propionic acid and butyric acids) that had all been corrected to mg of acetic acid equivalents (AAE) using their respective molar masses.

The analysis of NH_3 in the fermented samples was modified from (Baethgen & Alley, 1989). Sample aliquots were mixed with 0.2 N HCl (1:1) with the concentrations of ammonium and nitrogen being determined using the reduction of ammonium ions by sodium salicylate and nitroprusside in a weakly alkaline buffer (free chlorine). The resulting coloured complex was measured with a UV-Vis spectrophotometer (OlympusAU400, Tokyo, Japan) at 650 nm.

4.3.5. Nuclear magnetic resonance (NMR) spectroscopy

Solid-state ^{13}C CP/MAS NMR

Mango and banana particles, before and after fermentation (0 h and 48 h respectively), were freeze-dried and analysed by solid-state ^{13}C CP/MAS NMR spectroscopy using a Bruker MSL-300 spectrometer (Bruker, Karlsruhe, Germany) at a frequency of 75.46 MHz, to detect for the presence of cellulose and starch. Samples were lightly ground and stirred to ensure homogeneity, from which 200 mg was packed into a 4 mm diameter, cylindrical, PSZ (partially stabilized zirconium oxide) rotor with a KelF end cap. The rotor was spun at 5-6 kHz at the magic angle (54.7°). The 90° pulse width was 5 µs and a contact time of 1 ms was used for all starches with a recycle delay of 3 s. The spectral width was 38 kHz, acquisition time 50 ms, time domain points 2 k, transform size 4 k, and line broadening 50 Hz. At least 2400 scans were accumulated for each spectrum.

Solution state ^{1}H NMR

Similarly, mango and banana samples, before and after fermentation (0 h and 48 h respectively) were analysed for the presence of galacturonic acid, pectic acid and various sugars. The freeze-dried samples (5 mg) were dissolved at 80°C overnight in 650 µL of d_6-DMSO containing 0.5 wt % LiBr. After the samples were cooled to room temperature, sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) in D_2O was added as an internal standard. The addition of 50 µL of deuterated trifluoroacetic acid (d_1-TFA) directly before each measurement moved the HOD peak (Tizzotti, Sweedman, Tang, Schaefer, & Gilbert, 2011). ^{1}H NMR spectra were measured on a Bruker Avance 500MHz spectrometer operating at 298K equipped with a 5 mm PABBO probe using 12 µs 90° pulse, 3.91 s acquisition time, 1 s relaxation delay and 64 scans.

4.3.6. Confocal laser scanning microscopy (CLSM)

Microscopy of mango and banana particles before and after fermentation (0 h and 48 h respectively) was carried out under 10x and 40x objective lenses using CLSM (LSM700, Carl Zeiss, Germany), differential interference contrast (DIC) and Zen (Black) 2011 software. Fluorescence of cell walls was observed at an excitation λ of 355 nm, emission λ from 400-440 nm, and laser power intensity of 2% after staining with Calcofluor. Fluorescence of starch granules was observed at an excitation λ of 488 nm after staining with 3-aminopropyl-trimethoxysilane (APTS) followed by washing samples with 70% ethanol, incubating in APTS solution (10 mM APTS in 15% acetic acid) at 30°C overnight

(Chen et al., 2011; Chung & lai, 2006; Wei et al., 2010), washing five times with Milli-Q water and centrifuging at 3000 g for 10 min.

4.3.7. Curve fitting and statistical analysis

The cumulative gas production measured over time was corrected to the mL of gas produced per g of substrate DM, and the dry matter cumulative volume ($DMCV_{48}$) was fitted to the monophasic Michaelis-Menten model (Groot, Cone, Williams, Debersaques, & Lantinga, 1996) and calculated from Eq. (1):

$$DMCV_{48} = A/(1 + (C/t)^B) \tag{1}$$

where A is the asymptotic gas production (mL), B is the switching characteristic of the curve, C is the time at which half of the asymptotic value is reached ($T_{1/2}$) and t is the fermentation time (h). The maximal rate of gas production, R_{max} (mL/h) and the time at which it occurs, TR_{max} (h) were calculated from Eq. (2) and (3):

$$R_{max} = (A(C^B)B(TR_{max}^{(-B-1)}))/(1+(C^B)TR_{max}^{(-B)})^2 \tag{2}$$

$$TR_{max} = C(((B-1)/(B+1))^{1/B}) \tag{3}$$

All parameters were tested for significant differences (effects of fruit, particle size and interaction between fruit and particle size) using the Tukey-Kramer multiple comparison procedure as defined in Eq. (4):

$$Y = \mu + F_i + P_i + (F_i \times P_i) + \varepsilon_i \tag{4}$$

where Y is the dependent variable, μ is the mean, F_i is the effect of fruit, P_i is the effect of particle size, $(F_i \times P_i)$ is the interaction between fruit and particle size, and ε_i is the error term. Effect of replicate bottles was also examined separately but did not contribute significantly to any of the parameters (P>0.05), and was therefore excluded from the main model effects. Statistical analyses were performed using SAS (9.3) NLIN (curve fitting) and GLM (significant difference) procedures.

4.4. Results

4.4.1. Fermentation kinetics of mango and banana

The cumulative gas profiles for mango and banana are shown in Fig 4.2. Measured data points fitted well to the mathematical predictions of Groot's model (Groot et al., 1996) for all substrate types except for the unfractionated mango, where the measured gas values were higher than the predicted curve. A comparison of replicates (n=4 per particle size

per fruit) indicated that the sample bottles 1, 2, 7, 11, 15, 17 behaved as outliers (Fig A2.1, Appendix 2) and were therefore excluded from Proc GLM analysis to avoid false means because the high variation in raw data contributed to exaggerated estimates for $T_{1/2}$, TR_{max} and R_{max} values based on the curve fitting results. Updated $DMCV_{48}$ means are shown in Fig 4.3. Interestingly, there was an opposite trend for mango and banana as a function of particle size, although the absolute differences between particle sizes were small (P=0.43). An inverse relationship between particle size and gas production was observed for mango, in contrast to banana, where larger particles showed faster and more extensive gas production. All substrates started with an initial lag phase of 2-6 h, suggesting an adaption time for physical adhesion of cellulolytic microbial species to the fibrous plant cell wall components (Flint, Scott, Duncan, Louis, & Forano, 2014; Van Soest, 1994).
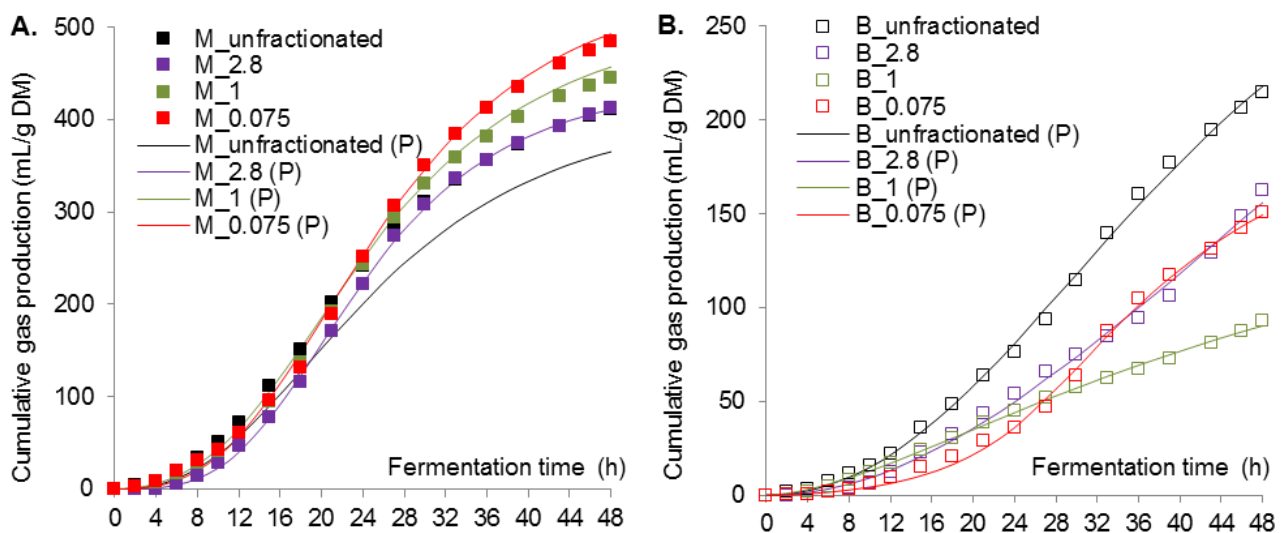
Figure 4.2. Cumulative dry matter cumulative volume profiles of (A) mango (■) and (B) banana (□) particle size fractions and fitted monophasic curve predictions (−) according to Groot's mathematical model during 48 h microbial fermentation *in vitro*. Note the different y axis scales for the two profiles. M: mango, B: banana, P: predicted, DM: dry matter.

Mango was more readily fermentable (Fig 4.3), and to a greater extent, as the total gas production for mango (440 mL/g DM) notably greater than for banana (113 mL/g DM). Mango reached its maximum rate of gas production (R_{max}=17 mL/h) at 19 h while banana only reached its maximum rate (R_{max}=3 mL/h) after 31 h, reflecting a significant fruit effect (P<0.0001). The half-time to reaching asymptotic gas production also differed significantly between mango and banana (25 h and 54 h respectively, P<0.0001). Neither the end-point gas production (48 h) nor R_{max} were significantly different between particle sizes (P>0.05), but TR_{max} occurred significantly later for the 0.075 mm particles (P=0.0002). The effects of fruit type and particle size, and any interactions of the fermentation kinetic parameters ($DMCV_{48}$, $T_{1/2}$, TR_{max}, R_{max}), are shown in Table 4.1. However, fermentation of the

substrates apparently would have extended beyond 48 h, so gas production asymptotes were extrapolated rather than observed (Figs 4.2 and 4.3), particularly for banana.
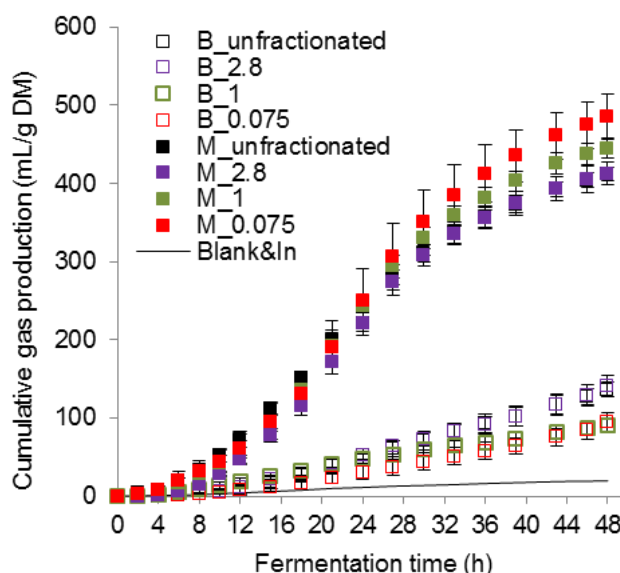
Figure 4.3. Combined dry matter cumulative volume profiles of mango (■) and banana (□) particle size fractions, and experimental blank (−) during 48 h microbial fermentation *in vitro*. Data is expressed as means±standard error. M: mango, B: banana, Blank&In: blank containing only inoculum and medium, DM: dry matter.

Table 4.1. Fruit and particle size effects on fermentation kinetics of masticated mango and banana during 48 h microbial fermentation *in vitro*.

Treatment	n	$DMCV_{48}$ (mL/g DM)	$T_{1/2}$ (h)	TR_{max} (h)	R_{max} (mL/h)
Effect of fruit					
mango	15	440.39[a]	24.90[b]	19.41[b]	16.96[a]
banana	11	113.65[b]	53.52[a]	30.51[a]	3.18[b]
MSD		26.23	5.87	2.85	1.08
Effect of Psize					
whole	5	301.53[a]	31.11[b]	22.00[bc]	10.53[a]
2.8 mm	7	296.03[a]	37.75[ab]	25.46[ab]	11.39[a]
1 mm	7	293.40[a]	33.04[b]	18.95[c]	10.70[a]
0.075 mm	7	317.48[a]	44.45[a]	29.42[a]	11.73[a]
MSD		49.83	11.15	5.41	2.04
Probability					
fruit		<.0001	<.0001	<.0001	<.0001
Psize		0.43	0.020	0.0002	0.180
fruit x Psize		0.0065	0.016	0.0002	0.041

Data is expressed as means of replicates. [a,b,c]*Different letters within each treatment effect denote significance differences (P<0.05). $DMCV_{48}$: cumulative gas production during 48 h fermentation, $T_{1/2}$: half time of asymptote gas production, TR_{max}: time of maximum rate of gas production, R_{max}: maximum rate of gas production. MSD: minimum significant difference, Psize: particle size.*

4.4.2. pH, and short chain fatty acids and ammonia production in mango and banana

At the end of fermentation, pH values formango and banana ranged from 6.15-6.55 (Table 4.2), while blanks were in the pH range of 6.34-6.57 for all time points up to 48 h. There

was no significant difference in pH considering the effects of fruit or particle size, or any interaction amongst them (P=0.52), indicating there were similar pH environments during the fermentation of both fruits and that the buffering capacity of the medium was sufficient for the fermentations taking place.

Table 4.2. Fruit and particle size effects on pH, short chain fatty acids (mmol/g dry matter), ammonia (mmol/g dry matter) and branched chain ratio of masticated mango and banana during 48 h microbial fermentation *in vitro.*

Treatment	n	Acetic acid	Propionic acid	Butyric acid	Total SCFAs	NH_3	BCR[1]	pH
Effect of fruit								
mango	15	9.93[a]	2.14[a]	1.41[a]	15.42[a]	11.42[a]	0.26[b]	6.36[a]
banana	11	2.91[b]	0.86[b]	0.60[b]	4.99[b]	4.15[b]	0.28[a]	6.50[a]
MSD		0.25	0.076	0.047	0.41	0.95	0.008	0.20
Effect of Psize								
whole	5	7.47[a]	2.15[a]	1.24[a]	12.44[a]	9.78[a]	0.27[b]	6.31[a]
2.8 mm	7	6.69[b]	1.44[c]	0.99[b]	10.39[b]	7.26[b]	0.24[c]	6.51[a]
1 mm	7	6.31[b]	1.29[c]	0.89[c]	9.75[b]	8.12[ab]	0.29[a]	6.44[a]
0.075 mm	7	7.51[a]	1.67[b]	1.18[a]	11.88[a]	9.20[a]	0.28[ab]	6.37[a]
MSD		0.48	0.14	0.089	0.79	1.78	0.015	0.37
Probability								
fruit		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.16
Psize		<.0001	<.0001	<.0001	<.0001	0.0026	<.0001	0.49
fruit x Psize		<.0001	<.0001	<.0001	<.0001	0.048	<.0001	0.52

Data is expressed as means of replicates. [a,b,c]Different letters within each treatment effect denote significance differences (P<0.05). [1]All individual SCFA production values are also converted using acetic acid equivalents, i.e. AAC=acetic x 60; APR=propionic x 74 x 1.21; ABU=butyric x 88 x 1.36; AIBU=isobutyric x 88 x 1.36; AIVAL=isovaleric x 102 x 1.46; AVAL=valeric x 102 x 1.46. BCR=(AIBU+AIVAL+AVAL)/(AAC+APR+ABU). MSD: minimum significant difference, Psize: particle size, SCFA: short chain fatty acids, NH_3: ammonia, BCR: branched-chain ratio.

Changes in total SCFA and NH_3 concentrations with time are shown in Fig 4.4A-B. All mango fractions consistently produced significantly larger amounts of total SCFA in comparison with banana (P<0.0001), which is in agreement with the retarded fermentability of banana as evidenced in the lower volume and rate of gas production. The total SCFA concentration initially started from 0.7 mmol/g and showed a gradual increase over 48h for both fruit types. Small but significant particle size effects were observed for total SCFA (P<0.0001). The unfractionated particles and smallest particles (0.075 mm) produced significantly higher total SCFA (12.4 and 11.9 mmol/g DM) than the 2.8 mm and 1 mm particles (10.4 and 9.6 mmol/g DM). This trend was also observed for the major individual SCFA- acetic, butyric and propionic acids (Table 4.2.). The higher values for total SCFA contents of unfractionated fruit particles compared with all subsequent fractions (Table 4.2) suggests that there was a small loss of fermentable material during the fractionation process, particularly for banana (Fig 4.4).

NH$_3$ concentrations were at least two-fold higher in all mango particle sizes compared to banana (P=0.0026). Similarly to SCFA production, the unfractionated and 0.075 mm mango particles had significantly higher NH$_3$ concentrations than in banana. On the other hand, there was no significant difference between particle sizes for banana. The concentration of NH$_3$ peaked between 8 and 18 h, and then declined. From 0 h, NH$_3$ concentrations of >2 mmol/g DM for both fruits suggest that some bacterial species within the porcine faecal microbiota have actively fermenting some peptide/amino acid source present in the inoculum and/or medium.
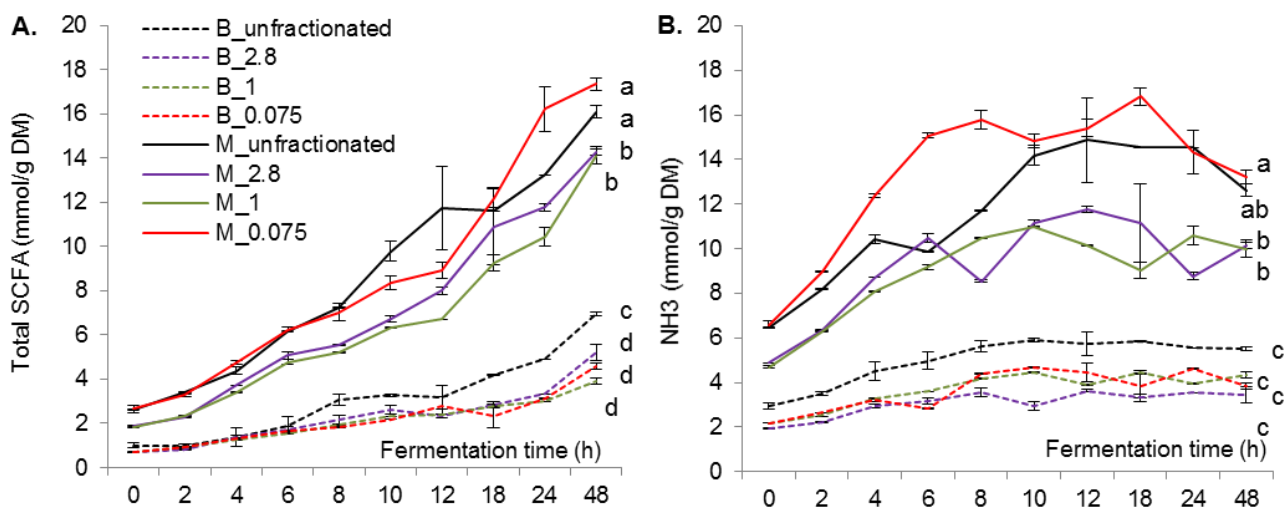
Figure 4.4. (A) Total short chain fatty acids (SCFA) and (B) ammonia (NH$_3$) production profiles of mango (–) and banana (··) particle size fractions (n=4) during 48 h microbial fermentation *in vitro*. SCFA and NH$_3$ concentrations are reported as mmol/g dry matter of starting substrates weighed into fermentation bottles. Data is expressed as means±standard error. [a,b,c,d]Different letters within substrates denote significance differences for the end-point values (48 h) at P<0.05.
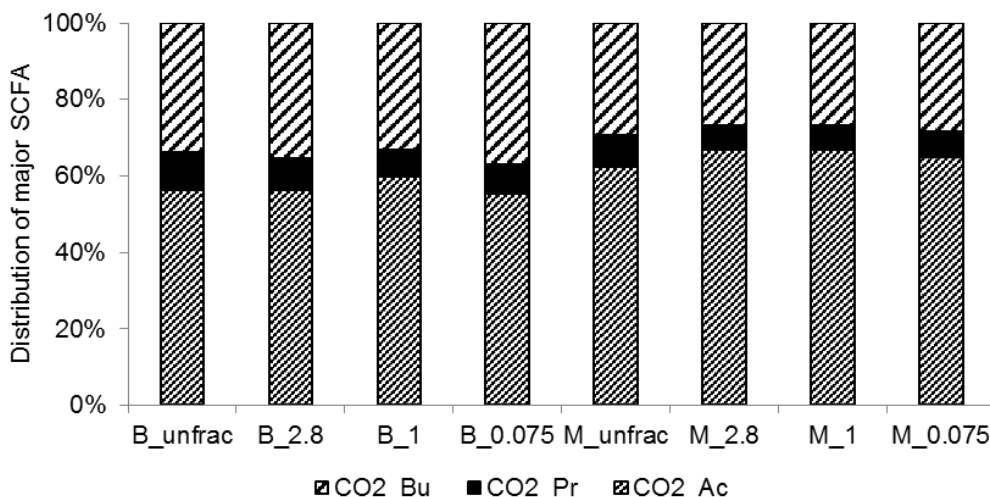
Figure 4.5. % Distribution of individual major short chain fatty acids (SCFA): acetic acid (Ac), propionic acid (Pr) and butyric acid (Bu) in mango and banana particle size fractions (n=4). M: mango, B: banana.

Following the usual pattern for gut fermentation, acetic acid was the major SCFA produced (54-66%) in both fruits, followed by propionic acid (13-19%) and butyric acid (9-17%) (Fig 4.5), whereas valeric, isovaleric and isobutyric acids were minor SCFA, collectively accounting for <10%. All these SCFA concentrations (mmol/g DM) were subsequently converted into acetic acid equivalents (Table 4.2) using their respective molar mass to obtain a branched-chain ratio (BCR) to allow a comparison of individual SCFA according to their carbon content. The BCR gives an indication of the proportion of SCFA more likely to be related to protein fermentation (Williams et al., 2005b). Banana fermentation was associated with a higher proportion of branched-chain SCFA (isobutyric, isovaleric and valeric acids) to the straight chain acids (acetic, propionic and butyric acids), leading to a significantly higher BCR (P<0.0001) for banana than for mango.

4.4.3. Physical structures and major polysaccharide composition affecting fermentation

From confocal microscopy, it could be seen that chewed mango particles subjected to *in vitro* gastrointestinal digestion contained soft parenchyma (fleshy) tissue, which disappeared after microbial fermentation, leaving mostly cellulosic vascular fibres (Fig 4.6A). This was confirmed by the ^{13}CP/MAS NMR spectra of fermented mango (Fig 4.7) where the dominant signal at 105 ppm was assigned to cellulose C-1 (Dick-Perez et al., 2011; Ng et al., 2014). These vascular fibres are structured but loosely detached from the rest of the sample material. Vascular fibres were also present in banana after fermentation, but these were less pronounced than in mango, apparently thinner (Fig 4.6Cii), and were not resolved from more major peaks by NMR.

Banana after chewing and *in vitro* gastrointestinal digestion comprised mostly of starch, before and after fermentation (Fig 4.7). Before fermentation, starch granules were generally observed to be encapsulated by intact cell walls (Fig 4.6Bi). After fermentation, cell walls surrounding the starch were no longer detected, but liberated starch granules were still clearly visible (Fig 4.6Bii). This was supported by the solid state ^{13}C NMR spectra showing a characteristic starch spectrum (Tan, Flanagan, Halley, Whittaker, & Gidley, 2007), which overlapped with lower intensity cellulose signals (95-105 ppm), for samples before and after fermentation. Microscopic evidence of partial fermentation of banana starch was observed by the apparently roughened or scratched granule surfaces, a typical morphology of early stages of amylolytic starch breakdown as seen in banana (Zhang, Whistler, BeMiller, & Hamaker, 2005) and potato (Dhital, Shrestha, & Gidley, 2010).
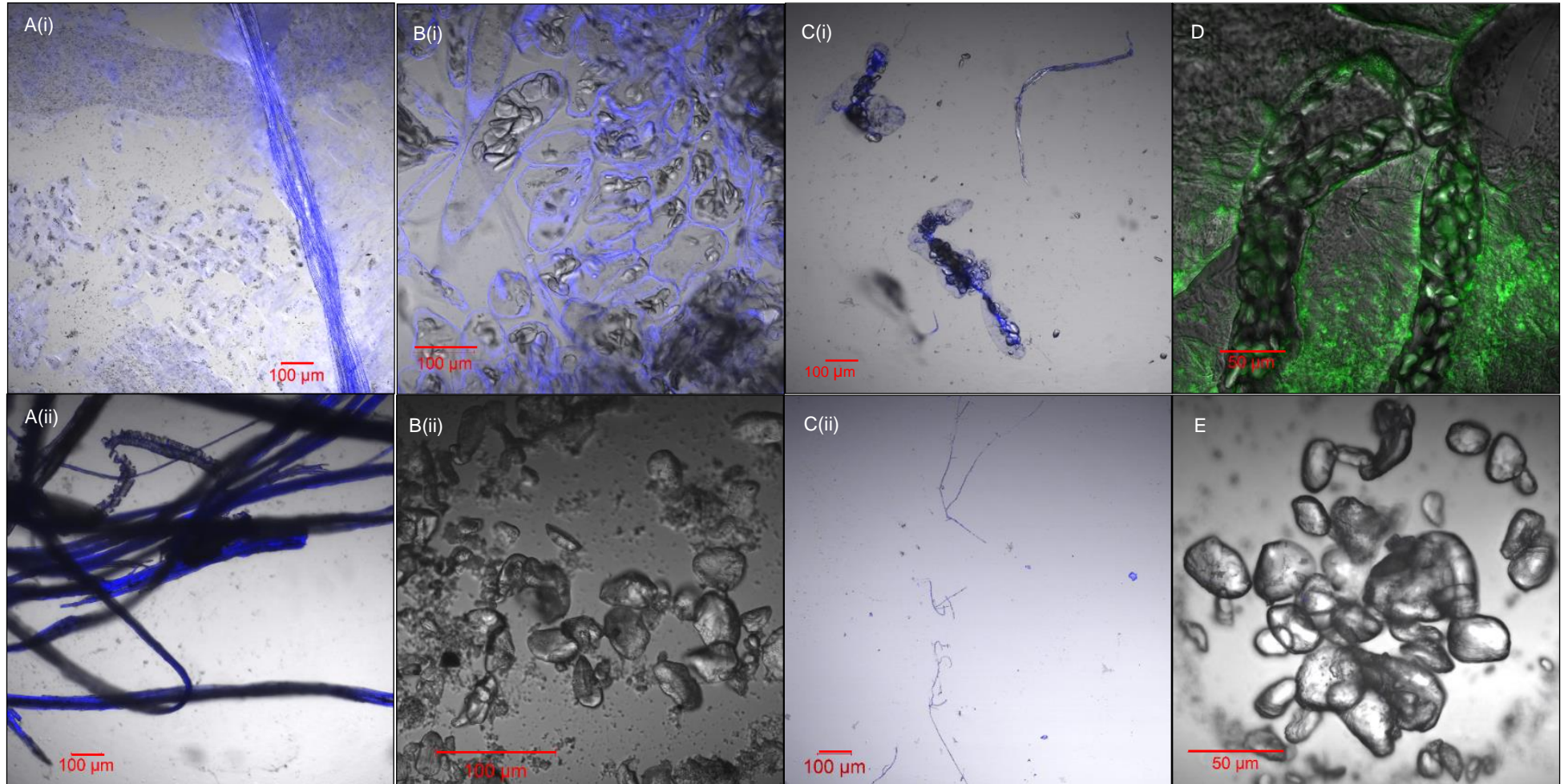
Figure 4.6. Differential interference contrast images of A(i) mango (2.8 mm), B(i) banana (2.8 mm) and C(i) banana (0.075 mm) cellular structures in blue fluorescence before fermentation, and (D) banana starch granules (10-30 μm in length) in green fluorescence before fermentation. Thick cellulose vascular fibres remained in A(ii) mango after fermentation, whereas fermented banana comprised mostly of B(ii) starch and C(ii) some vascular fibres. The original image presented as (E) shows the rough and/or scratched surfaces of banana starch granules after 48 h fermentation (63x magnification).
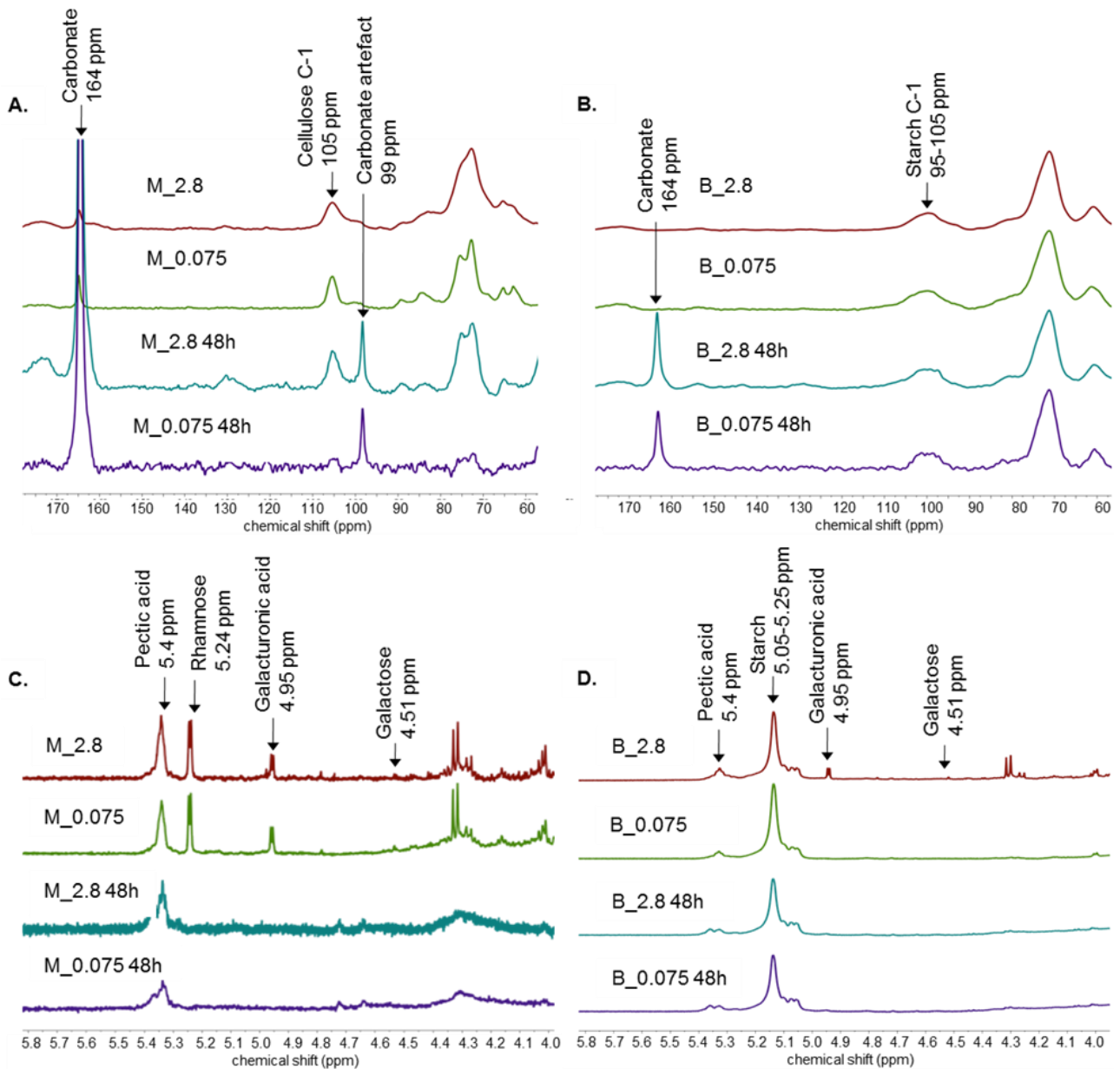
Figure 4.7. ^{13}CP/MAS and ^{1}H NMR spectra of masticated (A, C) mango and (B, D) banana particles of 2.8 mm and 0.075 mm respectively, before and after 48 h microbial fermentation *in vitro*. In (A) mango samples after fermentation, the chemical shift at 99 ppm is an artefactual spinning side band from the intense carbonate residue (164 ppm) and at 105 ppm is the C-1 of cellulose. In (B), the shifts between 95 and 105 ppm is the C-1 of starch. In (C), the sharp peaks before fermentation are anomeric protons of pectic acid residue (5.4 ppm) (Dinola et al., 1994), rhamnose residue (5.24 ppm) (de Bruyn, de Gussem, & Dutton, 1976), galacturonic acid and galactose residues (Marcon et al., 2005). The broad shift from 5.05-5.25 ppm in (D) banana is from anomeric protons of starch. M: mango, B: banana, 2.8: 2.8 mm chewed fraction, 0.075: 0.075 mm chewed fraction.

Pectin was present in both mango and banana as observed in the proton (^{1}H) NMR spectra (Fig 4.7C-D) but this was not obvious in the ^{13}CP/MAS NMR spectra because of the overlap of the spinning side band (ca 99 ppm) from the carbonate peak (164 ppm) with galacturonan C-1, which would appear from 98-101 ppm (Dick-Perez et al., 2011). Rhamnose residue was present in mango as the major neutral sugar (Fig 4.7C), which has

been similarly reported in *Ataulfo* and *Tommy Atkins* cultivars (Garcia-Magana, Garcia, Bello-Perez, Sayago-Ayerdi, & de Oca, 2013), but was not observed in banana. Rhamnose, along with galactose and galacturonic acid residues were apparently utilised by the faecal microbiota, as they were not observed for either fruit after fermentation. Bacterial species capable of degrading pectin and/or cellulose in porcine fecal microbiota have been reported in numerous studies (Rink, Bauer, Eklund, & Mosenthin, 2011; Sappok et al., 2012; Williams et al., 2005a).

4.5. Discussion
4.5.1. Effects of fruit and particle size on fermentation kinetics
Gas kinetics profiles showed significant differences between between mango and banana both in terms of kinetics and end-points. Disintegration of the plant cell wall network and cell structures during *in vivo* mastication led to particles of varying sizes (Chapter 3). The largest chewed fraction (2.8 mm) consisted of more fermentation-resistant cellulosic vascular tissues, whereas the 1 mm and 0.075 mm fractions comprised mostly single cells and ruptured cell fragments, and less or no vascular fibres (shown in Chapter 3, Fig 3.4). There was no significant particle size effect (P=0.43) on cumulative gas production, however, there was a likely trend where smaller mango particles of 0.075 mm were fermented more rapidly and extensively, and produced more gas (485 mL/g DM) than the larger (>1 mm) and unfractionated particles (411-445 mL/g DM). While decreasing particle size confers an expansion of surface area available for microbial accessibility and/or attachment (Guillon, Auffret, Robertson, Thibault, & Barry, 1998; Parada & Aguilera, 2007), the relative amount of vascular fibres is also a potential factor influencing particle size effect. Fig 4.3 shows that the significant difference in surface area due to particle size was associated with kinetic rate (i.e. active fermentation) rather than lag (i.e. colonisation).

In contrast, the larger banana cell-cluster particles (2.8 mm) produced more gas (136 mL/g DM) than the smallest (0.075 mm) fraction (93 mL/g DM). Larger banana particles may have contained a higher proportion of more fermentable cell wall structures. Similarly, in a previous study, multi-cellular (137-298 µm) carrot particles were fermented faster (23 mL/h) compared to 50-75 µm single carrot cells and fragments (8 mL/h) (Day et al., 2012). The plant cellular composition or architecture appears to have a more significant impact than particle size or available surface area (to faecal microbiota) during fermentation. Fruit and vegetable matrices of varying physical and structural characteristics, i.e. taproot or fruit, appeared to have a strong influence on substrate fermentability, as did the cell

contents. In this study, differences in substrate fermentability were due to a fruit effect rather than a particle size effect, presumably due to the soft tissue structures of mango and banana.

4.5.2. Degradation of cell walls is more extensive than that of resistant starch

Chewed pieces of mango fruit were readily fermented based on the 48 h DMCV and SCFA values, leaving mostly long cellulose vascular fibres after fermentation, which would be expected to be difficult to ferment by intestinal microbiota (Ismailbeigi, Reinhold, Faraji, & Abadi, 1977; Otles & Ozgoz, 2014; Yu, Liu, Shen, Jiang, & Huang, 2005) depending on the chemical structure, colonic microbiota and residence time in the colon (Anderson & Chen, 1979). The strands of cellulose in mango appear not strongly connected to the parenchyma (fleshy) tissue, and were sometimes observed as separate strands before microbial fermentation, but clearly separated after fermentation. There appears to be a hierarchy in substrate utilisation as evidenced by the preferential degradation of (thinner) primary parenchyma cell walls over cellulosic vascular fibres.

Banana was far less efficiently fermented than mango, likely due to clear differences in polysaccharide compositions. Cellulose was the major polysaccharide component of mango before and after fermentation while starch was the major component of banana before and after fermentation. Starch granules encapsulated within intact banana cell walls survived mastication, as well as gastrointestinal digestion *in vitro*. The thin banana cell walls present before fermentation were apparently all fermented, releasing the starch granules, which the cell walls had encapsulated previously. Most starch granules appeared smooth after mastication and digestion, but exhibited a parallel-striated surface after fermentation (Fig 4.6E). The fact that numerous and relatively intact starch granules were observed at the end of fermentation shows that they were not rapidly fermented as soon as their encapsulating cell wall had been degraded. Indeed, banana starch in the granular form is relatively resistant to digestion by pancreatic enzymes (Soares et al., 2011), similarly to other B-type starches such as potato. When treated with pancreatic amylases and amyloglucosidases *in vitro*, potato starch granules showed the same type of 'scratching' or exo-corrosion (Dhital, Shrestha, & Gidley, 2010) as found in the present study for banana starch granules after microbial fermentation.

The smooth dense surface of banana starch granules could also partially account for the intrinsic resistance of such granules to enzyme-catalysed hydrolysis by faecal microbiota.

Additionally, banana starch has been previously reported to be highly resistant to *in vivo* human small intestinal digestion (Cummings, Beatty, Kingman, Bingham, & Englyst, 1996; Faisant et al., 1995a). The thick external layer (several µm) of larger blockets (Faisant, Gallant, Bouchet, & Champ, 1995b) composed of a hard and well organised material (Soares et al., 2011), surrounding the banana starch granules has been proposed to impede enzyme action and thus reduce the hydrolysis rate. Colonic bacteria reportedly utilise a Starch Utilisation System to get at these starch structures to extract glucose for energy (Martens, Koropatkin, Smith, & Gordon, 2009), but the evidence from this study suggests that colonic microbiota may not be any more effective than pancreatic amylases in overcoming the hard surface layer of banana starch granules.

Striations on the starch granule surface indicate the presence of microbial amylolytic activity in the fermentation medium, leading to erosion, but with limited hydrolytic effect. Some areas of the starch granule are more likely to be difficult to hydrolyse than other areas (crystalline regions appearing after partial hydrolysis/digestion) (Zhang et al., 2005), and banana starch was described as a B-type crystalline entity (Hamaker & Tuncil, 2014). Additionally, during weighing of the masticated fractions into the fermentation bottles, it was noted that the banana fractions had a more physically compacted structure, which made it more difficult to obtain seemingly homogenous sub-samples. Accessibility, as influenced by the entrapping matrix of banana cells and/or cell clusters appeared to restrict access of the microbiota and/or their enzymes into the fruit substrates.

4.5.3. Higher short chain fatty acids and ammonia concentrations in mango but higher branched-chain ratio in banana

Differencesin SCFA and NH_3 between fruits were more pronounced than differences in particle sizes, where 68% and 64% significantly ($P<0.0001$) greater concentrations of SCFA and NH_3 respectively, were observed in mango compared with banana ($P<0.0001$). This, correlates well with the 74% greater $DMCV_{48}$ value for mango and is consistent with the expectation that more rapid and extensive fermentation is generally associated with higher SCFA production *in vitro* (Topping & Clifton, 2001). However, the higher NH_3 production in mango was not accompanied by a higher BCR. Particle size played a small role in SCFA production and had no significant effect on NH_3 production ($P>0.05$). Another fermentation study of fine wheat bran also found that finer wheat particles (50 µm) produced higher SCFA concentrations than did the larger clusters (758 µm) (Jenkins et al., 1999).

A lower total SCFA production typically corresponds to a proportionate increase in NH_3 level. SCFA production in banana showed a lower total production, but there was no concomitant increase in NH_3, which would have been expected. However, banana fermentation was associated with a slightly higher BCR ($P<0.0001$), further validating the differences between these two fruits. Branched-chain SCFA are usually formed as a result of bacteria metabolising undigested and endogenous proteins, peptides and amino acids, particularly when carbohydrates are in short supply as an energy source (Hendriks, van Baal, & Bosch, 2012) or difficult to utilise as in this study. Here, NH_3 production was reduced for banana, which reflected the differential availability of fermentable carbohydrate (Cone, van Gelder, & Driehuis, 1997; Sappok et al., 2012), thus increasing the BCR.

The composition of dietary polysaccharides available for fermentation also influenced the proportions of SCFA produced. Resistant starch in banana appeared to favour an increased production of butyric acid (Fig 4.5), agreeing with previous reports that *in vitro* colonic fermentation of resistant starch is associated with elevated butyrate levels (Casterline, Oles, & Ku, 1997; Rose et al., 2009). Conversely, the higher % acetic acid observed in mango can be ascribed to the presence of higher levels of cellulose and pectin, which is consistent with reported studies (Annison & Topping, 1994; Low, 1993) showing that acetate production predominates over propionate and butyrate for diets containing higher levels of non-starch polysaccharides.

4.6. Concluding Remarks

The investigation of fermentation kinetics of masticated particles of mango and banana has demonstrated distinctive differences between the two fruits in terms of cellular architecture and starch content, which seemed to outweigh any effects of particle size on colonic fermentability. A decrease in particle size and the concomitant increase in available surface area would have been expected to increase the total gas production by enhancing microbial accessibility. However, colonic fermentation differences between larger particle clusters (2.8-1 mm) and single cells or cell fragments (0.075 mm) were not as significant in the soft tissues of mango and banana studied here, as compared to a previous study on carrot with a more robust cellular structure (Day et al., 2012). The fruit (parenchyma) fleshy cells were fully or mostly fermented during fermentation, preferentially over resistant starch in banana, and over the thick cellulosic vascular fibres in mango. The slow fermentability of banana starch conferred by its intrinsic resistance and cell-wall encapsulation may have implications on calorific content, satiety, glucose metabolism,

transit rates along the colon, and deserves further study. The higher absolute levels of butyrate production from mango could actually be more important in terms of contributing to anti-inflammatory and anti-carcinogenic properties (Tedelind, Westberg, Kjerrulf, & Vidal, 2007; Vinolo, Rodrigues, Nachbar, & Curi, 2011; Williams, Coxhead, & Mathers, 2003) rather than the higher % ratio of butyrate to acetate/propionate in banana. Further studies investigating the extended fermentation of both fruits over 72 h and longer, and the microscopic degradation of banana cell walls with time, preferably 3-hourly should also be explored in future work.

Chapter 4. Fermentation kinetics and microbial biotransformation of polyphenols during colonic fermentation of masticated mango and banana *in vitro*

Part B. Polyphenol-microbial biotransformation during colonic fermentation of masticated mango and banana *in vitro*

4.7. Introduction to Part B

In vitro bioavailability studies, such as through the application of Caco-2 cells are relatively well established for many fruit polyphenols (Boyer, Brown, & Liu, 2004; Cilla et al., 2009; Teng et al., 2012; Vaidyanathan & Walle, 2001; Van Buggenhout et al., 2010; Walgren, Lin, Kinne, & Walle, 2000; Walle, Galijatovic, & Walle, 1999). However, little work has been reported concerning bioaccessibility of polyphenols from fresh fruit, especially in the colon. A further area with limited information is the fate of polyphenols that are not released or absorbed in the small intestine, and therefore enter the colon, bound to or trapped within plant cell walls. As plant cell walls are likely to be fully fermented in the colon, these trapped/bound polyphenols will be released and available for absorption directly through the colon epithelium, or metabolised by colonic bacteria before being absorbed, or excreted without any further metabolism. These metabolites and/or catabolites may be responsible for part of the health effects attributed to dietary polyphenols (Crozier, Del Rio, & Clifford, 2010; Dall'Asta et al., 2012; Gonzalez-Barrio, Edwards, & Crozier, 2011; Scalbert, Manach, Morand, Remesy, & Jimenez, 2005; Wilkinson et al., 2008).

Solid residues from organic solvent extractions or pomaces from fruits and vegetables have previously tended to be disregarded, although a significant amount of polyphenols may remain in these residues and constitute the non-extractable polyphenols (NEPP). Extractable polyphenols may only be the tip of the iceberg (Saura-Calixto, 2012) while NEPP or polyphenols associated with the indigestible fraction represent the majority of dietary polyphenols. Studies on NEPP (proanthocyanidins, hydrolysable tannins and some phenolic acids) are quite scarce, indicating NEPP is an interesting group from a nutritional point of view as some may be hydrolysed by intestinal/colonic microbiota and/or secreted enzymes, and become potentially bioavailable in the human gut.

In a Spanish diet consisting of solid fruits, vegetables, legumes and cereals, 42% of the total polyphenols were estimated as bioaccessible in the colon, while 10% were inaccessible and remained in the food matrices after the whole digestion process and were

excreted (Saura-Calixto, Serrano, & Goni, 2007). Proanthocyanidins in the water-insoluble polymer fraction of Spanish banana cell walls were found to persist after 16 h of acid hydrolysis (Bennett et al., 2010), implying that C-C (or carbon-carbon) bond linkages between some proanthocyanidins and cell walls are not hydrolysed by acid, compared to the acid-labile C-O (or carbon-oxygen) linkages between other proanthocyanidins and polyphenols and/or polysaccharides. Most known fermentation or metabolism studies have focused on the degradation products of polyphenols from berries or single compounds, but none has been reported for either mango or banana.

Polyphenols must be released from the food matrix during chewing or digestion in the upper gastrointestinal tract for direct absorption to occur. If they are bound to plant cell walls and become unavailable for small intestinal absorption, these complexes may be transported to the colon where fermentation of fibrous material ensues (Padayachee et al., 2013). Therefore, the objectives of this study are to investigate the colonic release and metabolism of dietary polyphenols in a standardised *in vitro* model mimicking colonic fermentation of the solid residues remaining after *in vivo* human mastication and *in vitro* gastrointestinal digestion of fresh mango and banana fruits.

4.8. Materials and methods
4.8.1. Fruit substrates, *in vivo* mastication and *in vitro* digestion
Unfractionated (mixed sized particles) particles that were produced from *in vivo* mastication of mango and banana flesh were then subjected to *in vitro* digestion using the methods described in Chapter 3 and Chapter 4 Part A. The solid residues of both fruits left after these two processes were the samples subjected to colonic fermentation detailed in section 4.8.2.

4.8.2. Fresh faecal inoculum and cumulative gas production
Preparation of faecal inoculum, and the cumulative gas production technique are described in Chapter 4 Part A. Additionally, experimental blanks containing only the faecal inoculum and basal medium were included for comparison of matrix background absorption during UPLC analysis. Residues of digested fruit particles (Section 4.8.1) were simultaneously fermented in individual fermentation bottles to monitor polyphenol metabolism at time intervals of 0, 2, 4, 6, 8, 10, 12, 18, 24 and 48 h.

4.8.3. Polyphenol analyses

Fermented solution (1 mL) was added to formic acid (200 µL), centrifuged at 4°C for 5 min at 10,000 g, filtered through 0.2 µm GHP Acrodisc filters (Pall, Surry Hills, NSW, Australia), flushed with CO_2, and stored at -80°C prior to UPLC analysis. A Waters Acquity UPLC-PDA system (Waters, Rydalmere, NSW, Australia) with a VisionHT C18 Basic RP column (100 x 2 mm, 1.5 µm) fitted to a guard column (5 x 2 mm) was used. Mobile phases A and B were 0.1% formic acid (aq) and 0.1% formic acid in acetonitrile respectively. The gradient elution was developed and optimised to maximise separation, and was as follows: 98% A (1 min), 98-94% A (1.5 min), 94-70% A (7.5 min), 70% A (9.5 min), 70-40% A (3 min), 40% A (1 min), 40-2% A (6.5 min) 2% A (2 min), 2-98%A (0.1 min), 98% A (5.9 min) at 0.3 mL/min, 30°C and with an injection volume of 5 µL. UV-Vis spectra were recorded from 210-498 nm. Data acquisition was carried out using Empower Pro v.2 software. A calibration curve, that was constructed from ferulic acid (0.25, 0.5, 1, 5, 10, 20 µg/mL) by plotting peak areas against concentration, showed good linearity (r^2=0.999). Final phenolic concentrations were calculated as ferulic acid equivalents (at 280 nm), corrected to per g DM, and were the average of two or four replicates. Compounds occurring exclusively in the samples were assigned numbers common to mango and banana. Compounds detected in both experimental blanks and samples were ignored.

Tandem mass spectrometry analysis was carried out on a UHPLC-Q-ToF-MS system equipped with a Dual AJS ESI interface (Agilent Technologies, Mulgrave, VIC, Australia) in negative ion mode and connected to a PDA (UV-Vis spectra from 190-500 nm). Chromatographic separation was achieved using a Zorbax C18 Bonus RP column (100 x 2.1 mm, 1.8 µm) fitted with a compatible guard column (5 x 2.1 mm). A different gradient elution from the previous analysis was used to optimise separation of compounds: 99% A (2 min), 99-0% A (30 min), 0% A (1 min), 0-99% A (1 min), 99% A (6 min) at 0.3 mL/min, 30°C and an injection volume of 5 µL. The MS parameters were as follows: 50-1700 m/z range, 0 eV collision energy, 4 kV capillary voltage, 200°C source temperature, and 14 L/min gas flow rate. Data acquisition was carried out using MassHunter Workstation v.6 software.

Commercial standards of the following analytes were purchased from Sigma-Aldrich (NSW, Australia): chlorogenic acid, coumaric acid, caffeic acid, cinnamic acid, quinic acid, syringic acid, benzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, 4-hydroxyphenylacetic acid, protocatechuic acid, neochlorogenic acid, 3-(4-

hydroxyphenyl)propanoic acid, hippuric acid, 3-(3-4-dihydroxyphenyl)propanoic acid, mangiferin, epicatechin, quercetin, pyrogallol, kaempferol and ethyl ferulate (>98% purity). Their retention times, chemical formulae, molecular masses, m/z values and UV-Vis absorbance wavelengths are shown in Table A3.1 (Appendix 3). All solvents used for UPLC/UHPLC analyses were HPLC grade (>99%).

4.8.4. Statistical analysis

The 48 h endpoint samples (n=4 for each fruit) and time interval samples from 2-48 h (n=2 for each fruit) were analysed for the statistical effects on the polyphenol concentrations of substrate, time and the interaction, substrate*time, using SAS (9.3) (SAS Institute, Inc., Cary, NC, USA). Group means were analysed using Proc GLM procedure (Tukey-Kramer multiple comparison) for individual compounds and the significance of treatment differences was set at $P<0.05$.

4.9. Results and Discussion

4.9.1. Phenolic compounds in fermented mango and banana

For analysis and identification of the intact polyphenols and metabolic products of colonic-microbial degradation, masticated and 'digested' mango and banana pulp were incubated with fermentation suspensions as a function of time. The supernatants were analysed by UPLC with a PDA detector to characterise and quantify the compounds to per g DM of starting substrate. Where applicable, further confirmation through the application of UHPLC-Q-ToF-MS (via molecular feature extraction, chemical formula and/or extraction of specific mass) was used. In addition, compounds detected in the samples that were also present in the experimental blanks (basal solution and faecal inoculum) were ignored, were ignored, permitting only those compounds specific to the fermentation of the fruit residues to be selected for study.

Ten and twelve phenolic compounds (intact compounds and metabolites) were detected in banana and mango respectively at 0 h fermentation after the *in vivo* mastication and *in vitro* 'digestion' processes. A representative UPLC-PDA (280 nm) chromatogram of mango at 0 h fermentation time is displayed in Fig 4.8, and the extracted chromatograms from 0-48 h of colonic fermentation for both fruits are illustrated in Fig A3.2 (Appendix 3). The identity and characteristics, including retention times, UV-Vis and mass spectral data, of these twelve compounds are shown in Table 4.3. In addition, Fig A3.3 details the full UV-Vis spectra for these twelve compounds.
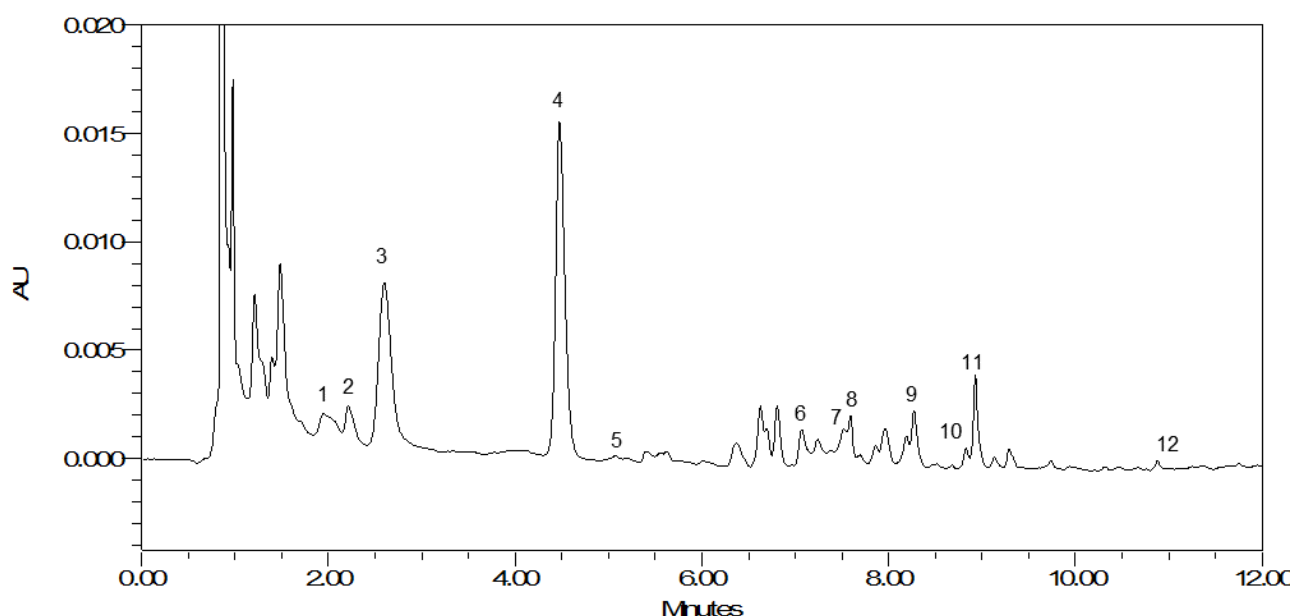
Figure 4.8. UPLC-PDA chromatograms (280 nm) of mango at 0 h of colonic fermentation. The chromatograms from 2-48 h are shown in Fig A3.1 (Appendix 3). Information for each detected peak is presented in Table 4.3 with common peak numbers used for mango and banana.

Table 4.3. Compounds detected in masticated mango and banana samples after 48 h microbial fermentation *in vitro,* and their characterisation based on UV-Vis and mass spectral analysis.

Peak no	Compound	Retention time (min)[1]	Present in mango	Present in banana	Chemical formula	Molecular mass (Da)[3]	λ_{max} (nm)[4]
1	Quinic acid	1.945	✓	✗	C7H12O6	192.167	215/258
2	Unidentified	2.211	✓	✓	-	-	286/344
3	Unidentified	2.599	✓	✓	-	-	224/288
4	Epigallocatechin/ gallocatechin (1)[4]	4.474	✓	✓	C15H14O7	306.267	219/279
5	4-hydroxyphenylacetic acid	5.074	✓	✓	C8H8O3	152.147	222/271
6	3-(4-hydroxyphenyl)propanoic acid	7.068	✓	✓	C9H10O3	166.174	210/302/361
7	Unidentified	7.557	✓	✗	-	-	211/258/290
8	Chlorogenic acid	7.591	✓	✓	C16H18O9	354.309	222/298/382
9	Epicatechin	8.271	✓	✓	C15H14O6	290.268	211/280
10	Unidentified	8.928	✓	✓	-	-	222/268/368
11	Epigallocatechin/ gallocatechin (2)[5]	9.148	✓	✓	C15H14O7	306.267	219/279
12	Unidentified	11.222	✓	✓	-	-	218/262/351
13	Hippuric acid[2]	8.999	✓	✓	C9H9NO3	179.0586	-

[1,4]*Retention time and UV-Vis absorbance of peaks were obtained from UPLC-PDA chromatograms except* [2]*hippuric acid, which was extracted from the UHPLC-Q-ToF-MS chromatogram.* [3]*Molecular masses of compounds (epicatchin, chlorogenic acid, quinic acid, 3-(4-hydroxyphenyl)propanoic acid and hippuric acid) were extracted from the UHPLC-Q-ToF-MS data, while the molecular masses of 4-hydroxyphenylacetic acid and epigallocatechin/epicatechin are theoretical masses as reported in Chemspider database.* [5]*Two compounds suggestive of epigallocatechin/gallocatechin were detected and are named as epigallocatechin/gallocatechin (1) and (2) respectively.*

4-Hydroxyphenylacetic acid and epicatechin were detected in both fruits and their identities were confirmed by matching against their elution time and UV-Vis spectrum of the respective standards (Table 4.3; Table A3.1 in Appendix 3; Fig A3.3). The UV-Vis

spectrum of 4-hydroxyphenylacetic acid (peak 5) showed absorbance at 222/275nm and eluted at 5.074 min, which matched with the authentic standard (222/274 nm, 5.094 min). However, a molecular ion of m/z 151.0462 [M-H]⁻, which is indicative of 4-hydroxyphenylacetic acid was not found in the total or extracted ion chromatograms despite reducing the collision energy to 0 eV.

The identity of epicatechin (peak 9) with UV-Vis absorbance at 211/280 nm (maximum absorbance at 280 nm) and retention time of 8.271 min was confirmed by its respective standard (219/279 nm, 8.263 min). Catechins, a class of flavonoids, also known as flavanols include monomers such as catechin, epicatechin, gallocatechin and epigallocatechins, generally show an intense band II (240-280 nm) and just a small band I (300-550 nm) due to little or no conjugation between the A- and B-rings (Stalikas, 2007). Again, a deprotonated ion of m/z 289.0783, which is characteristic of epicatechin molecular ion (290.268) analysed in negative electrospray ionisation MS, was not found in the samples. Peaks 4 (219/279 nm) and 11 (217/279 nm) are tentatively identified as gallocatechin or epigallocatechin, as standards were not available. The UV-Vis spectra of these two compounds in the samples are similar to that of epicatechin and catechins, but do not have similar retention times to the commercial authentic epicatechin standard. Gallocatechin and/or epigallocatechin have been previously reported to comprise the bulk of flavonoids in banana pulp extracts (Aurore, Parfait, & Fahrasmane, 2009; Bennett et al., 2010; de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Mendez et al., 2003; Someya, Yoshiki, & Okubo, 2002). These flavanols have been also identified in mango but have not been quantified.

Chlorogenic acid (peak 8 in Fig 4.8) was identified based on matching the UV-Vis spectrum (222/298/382 nm) and m/z of 353.0888 to those of the standard UV-Vis: 218/241/326 nm and m/z: 353.0882 (Fig 4.9). Quinic acid (peak 1 in Fig 4.8, UV-Vis: 215/258 nm) was identified via comparison of its UV spectrum to that of the commercial authentic standard (215/287 nm), and using the [M-H]⁻ ion at 191.0567 in the mass spectrum (ESI negative) of quinic acid (peak 1) (Fig 4.9), which corresponds to the molecular mass of 192.0639. Quinic acid has been reported as a hydrolysis product of chlorogenic acid (Ludwig et al., 2013). The presence of 3-(4-hydroxyphenyl)propanoic acid was confirmed in both fruits by comparing the UV-Vis spectrum of compound 6 in Fig 4.8 (210/302 nm, 7.068 min) to the that of the authentic standard (222/276 nm, 6.958 min) (Fig A4.2), and using the deprotonated ion of m/z 165.0559 in the mass spectrum of both

compound 6 and authentic 3-(4-hydroxyphenyl)propanoic acid standard, which corresponds to a molecular mass of 166.0629. Compound 13 was detected in mango and banana samples only at the 0 h fermentation time. Identification of compound 13 as hippuric acid was based on the mass spectrum (from the Q-ToF-MS instrument) containing a deprotonated [M-H]- ion (m/z 178.0509) (Fig 4.9) that was also in the mass spectrum of the commercial authentic hippuric acid standard (molecular mass: 179.0586).
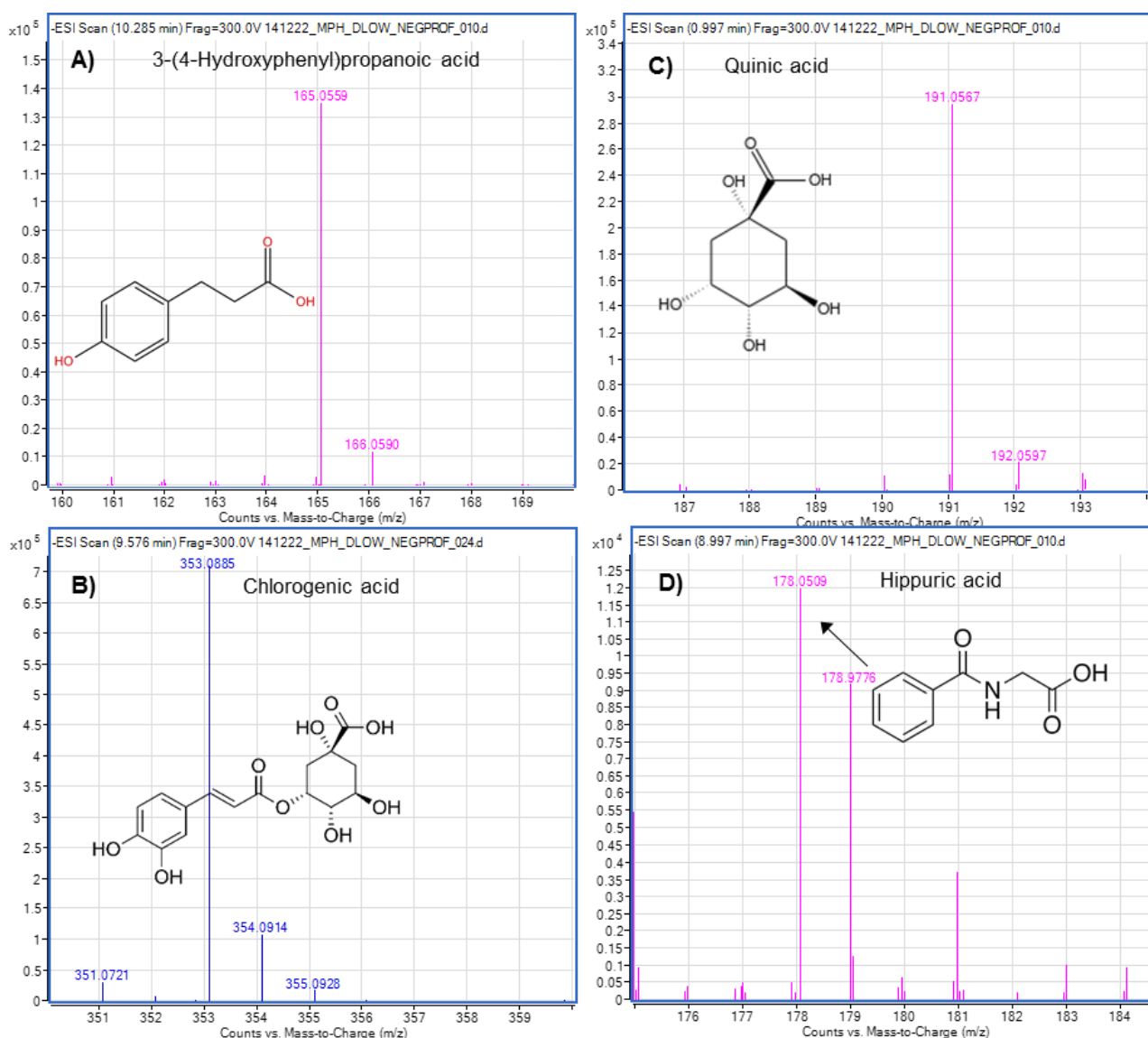
Figure 4.9. ESI-MS spectral identification of the deprotonated ions of (A) 3-(4-hydroxyphenyl)propanoic acid, (B) quinic acid, (C) chlorogenic acid and (D) hippuric acid. The identities of these compounds were confirmed by comparing their mass spectra and retention times to respective authentic standards.

4.9.2. Methodological challenges

The identities of seven compounds remained unelucidated, with their UV-Vis spectral characteristics detailed in Table 4.3, and the fermentation kinetics of five of these seven compounds displayed in Fig 4.10. These data for compounds 2, 3 and 10 are indicative of

the degradation of parent phenolic compounds in the food matrix, which were metabolised after 6-10 h of *in vitro* colonic fermentation, while such data for compounds 7 and 12 suggest these compounds are metabolites that were simultaneously produced and accumulated. The concentration of compound 7, present only in mango, reached a maximum (301 µg/g DM) after 4 h of *in vitro* colonic fermentation, and was followed subsequently by a swift decline. Compound 12 reached its maximum concentration of 292 µg/g DM in mango and 800 µg/g DM in banana after 18 h of *in vitro* colonic fermentation, and then decreased in concentration to <10 µg/g DM after 48 h.
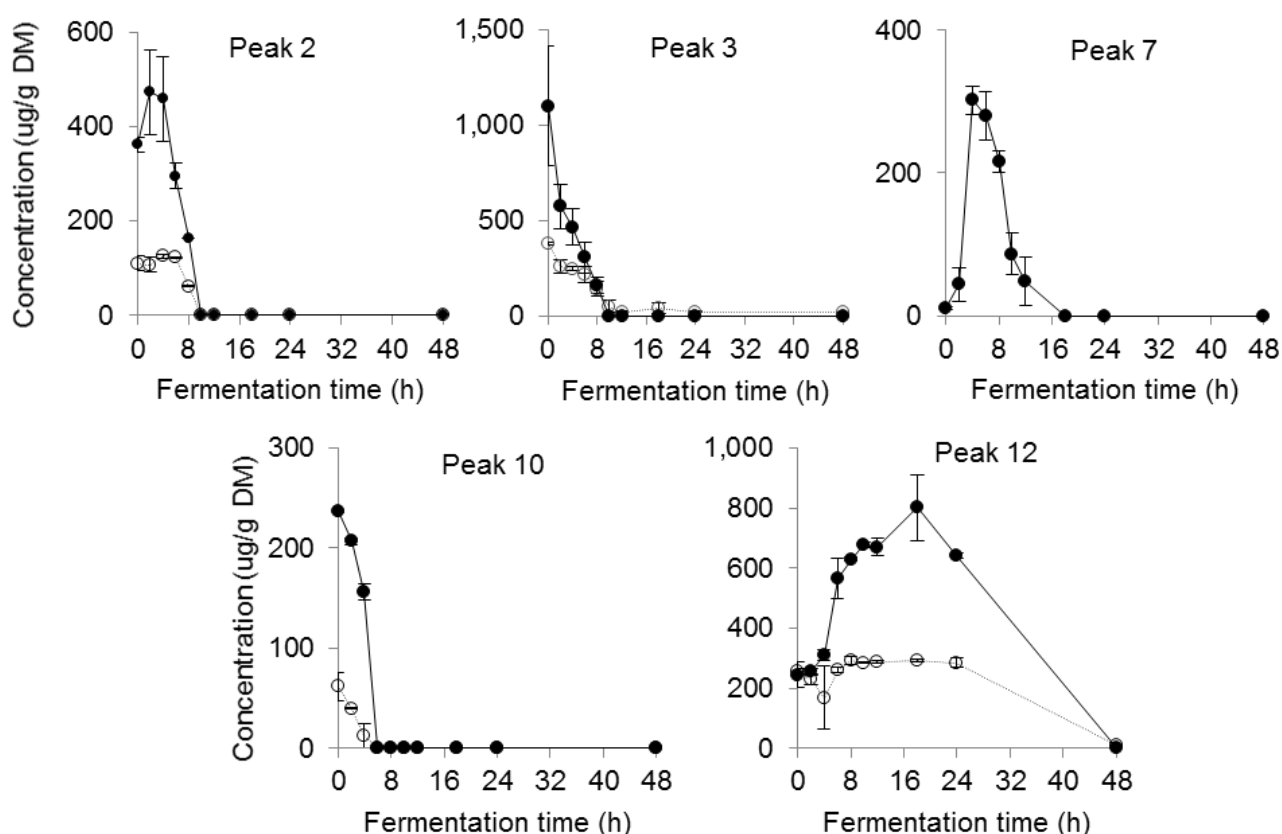
Figure 4.10. Time course profiles illustrating the degradation of unidentified individual compounds and production of their metabolites (0-48 h) as detected in fermented mango (-●-) and banana (··o··). Data is expressed as mean±standard error of µg/g dry matter of ferulic acid equivalents. Quantification was calculated at 280 nm for these compounds. Note the different y axis scales for each compound.

UPLC-PDA (280 nm) identification and quantification was chosen for the search for phenolic compounds; however, proteins and peptides with aromatic amino acids such as tryptophan, tyrosine or phenylalanine absorb at 280, 274 and 257 nm respectively (Desbois, Seabrook, & Newman, 2013; Held, 2003; Knapik, Fernandes, de Azevedo, & Porto, 2014), while DNA absorbs at 240-310 nm (Stapleton & Walbot, 1994). This made the identification of phenolic compounds difficult to achieve.

Compounds 6 (210/302/362 nm) and 12 (213/261/351 nm) absorbed more strongly at 320 nm (Table 4.3), which is a more selective wavelength for the presence of polyphenol aromatic rings, however, unexpectedly, these two compounds did not match in retention times and UV-Vis absorption with any of the available authentic standards. These two compounds appear to be hydroxycinnamic acids and/or flavones, which tend to have stronger UV absorbance in the band I spectrum (320 nm), attributed to the substitution pattern and conjugation of the C-ring (Manach et al., 2004; Stalikas, 2007). Ferulic acid, caffeic acid, coumaric acid and hydroxybenzoic acid were specifically searched for in this set of microbially-fermented fruit substrates using both techniques of UPLC-PDA and UHPLC-Q-ToF and via comparison to the standards elution time, UV spectrum and mass spectrum, however, these compounds were not found despite having been previously reported in ripe banana and mango flesh (Kim, Brecht, & Talcott, 2007; Mattila, Hellstrom, & Torronen, 2006; Shahidi & Naczk, 2004). This indicates these compounds are likely to have been released during the *in vivo* mastication and *in vitro* gastrointestinal digestion processes or there may be an increased susceptibility of these compounds to degradation or oxidation, and therefore, not present in these fermented fruit residues.
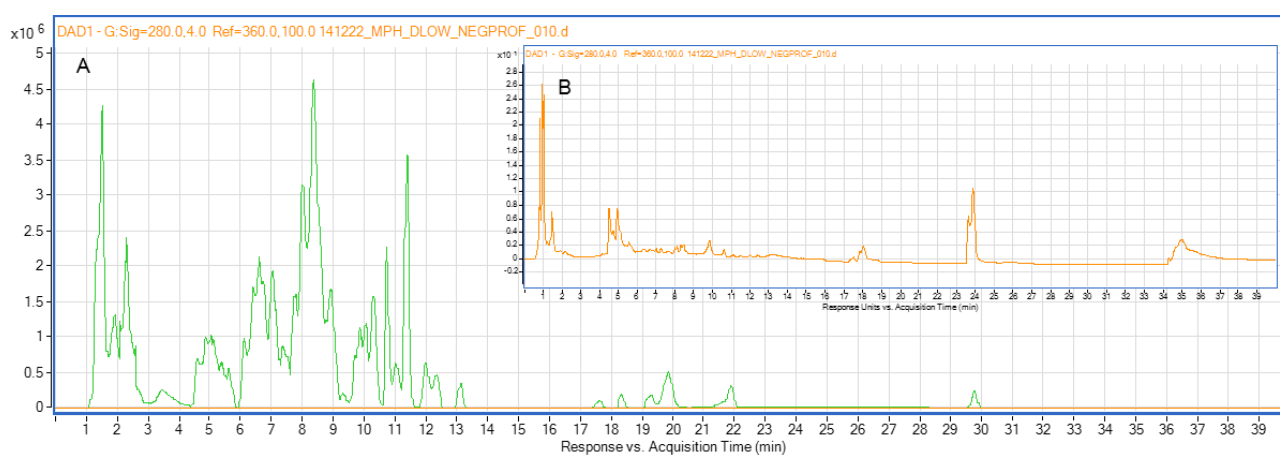
Figure 4.11. (A) Total compound chromatogram and (B) UV-Vis chromatogram of mango at 0 h microbial fermentation *in vitro* (1-30 min) extracted from UHPLC-Q-ToF-MS. Note the different y axis intensities in both chromatograms.

Ideally, compound identification using the total ion current chromatogram (TIC) from UPLC-MS and UPLC-MS/MS analysis is desired, but this proved complicated and challenging in the present study. In a typical TIC chromatogram of mango residue (Fig 4.11), there are a very large number of peaks (note the abundance intensity), many more than present in the UV chromatogram from the same instrument (Fig 4.11) (the mass spectra of compounds are not shown as there are >2000 features). Q-ToF-MS is a highly sensitive technique, so there is little doubt that the instrument is also detecting and/or

ionising the trace minerals in solution, vitamin/phosphates in solution, microbial metabolites, or other components not explicitly from the target class of phenolic compounds or catabolites. There may be more compounds of interest from the TIC chromatogram, in addition to the seven identified phenolic compounds, but this requires a targeted search for specific compounds, which is time and resource intensive. Currently, besides a small class of known phenolic compounds reported in the literature, available databases usually favour pharmaceutical drugs, proteins/peptides, nutraceuticals, human intestinal transporters and enzymes.

Additionally, thirteen phenolic compounds (pyrogallol, caffeic acid, benzoic acid, cinnamic acid, neochlorogenic acid, chlorogenic acid, 4-hydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propanoic acid, 3-(3-4-dihydroxyphenyl)propanoic acid, syringic acid, ethyl ferulate, epicatechin and kaempferol) were selectively detected using UV absorbance at 280 nm, but each compound produced a mass spectrum with weak ion intensities in the mixed standard UPLC runs (Fig A4.4), suggesting that these phenolics do not ionise well even in the soft negative ionisation mode, although polyphenol analysis and characterisation studies are generally performed in the negative mode (Ajila et al., 2011; de Rijke, Zappey, Ariese, Gooijer, & Brinkman, 2003; Mosele et al., 2014; Rechner et al., 2004), except for anthocyanins, which are carried out in the positive ionisation mode (Gonzalez-Barrio, Edwards, & Crozier, 2011).

4.9.3. Biotransformation of intact phenolic compounds to respective metabolites

The intact parent mango flavonoids and phenolic acids studied were rapidly catabolised (78%) *in vitro* within the first 10 h, while the produced catabolites gradually disappeared after 24-48 h (Fig 4.12), except for 3-(4-hydroxyphenyl)propanoic acid, chlorogenic acid and epigallocatechin/gallocatechin (2), which were still detected at 48 h (15 µg/g, 32 µg/g and 76 µg/g DM). A similar degradation behaviour was observed for banana phenolics with a concentration decrease of 72% after 10 h (with similar phenolic compounds to those in mango being detected), apart from quinic acid and an unidentified compound (peak 7) that were unique to mango. Concomitant with such degradations were the formation and accumulation of lower-weight metabolites at 4 h into the colonic-microbial fermentation (Fig 4.12), which were followed by a continuing degradation and/or further biotransformation of these metabolites to other as yet unidentified catabolites.
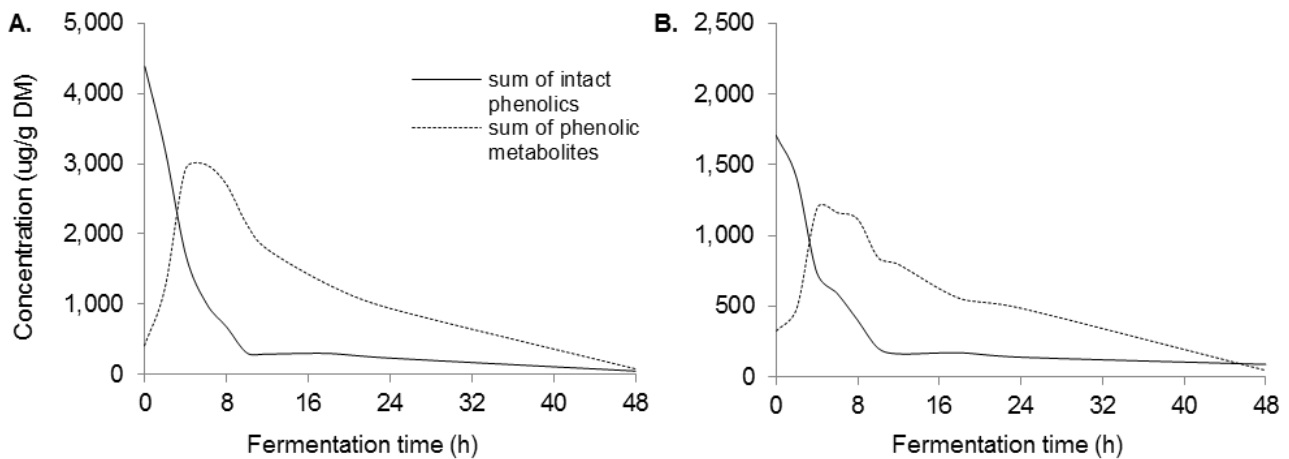
Figure 4.12. Time course plots of intact phenolic compounds (−) and derived metabolites (- - -) of (A) mango and (B) banana during 48 h microbial fermentation *in vitro*. Phenolic concentrations are expressed as µg/g dry matter of ferulic acid equivalents (quantification at 280nm). The sum of intact phenolics include compounds 1-4, 6-10, 12, and the sum of phenolic metabolites include compounds 5, 7, 11. Note the different y axis scales for both profiles.

The kinetics of chlorogenic acid fermentation showed significant ($P<0.0001$) degradation after 24 h (Fig 4.13), with the first intermediate microbial metabolite derived from the hydrolysis of chlorogenic acid was quinic acid, which reached its maximum concentration (100 µg/g DM) at 2 h of *in vitro* colonic fermentation, before degrading completely in the next 4 h. The proposed colonic microbial metabolic pathway of chlorogenic acid reported by Ludwig, de Pena, Cid and Crozier (2013) (Fig 4.14) involves the cleavage of the ester bond between the caffeic acid and quinic acid components of chlorogenic acid, with the colonic microbiota being the exclusive site for human metabolism (Plumb et al., 1999). *Escherichia coli, Bifidobacterium lactis and Lactobacillus gasseri* have been shown to be capable of this cleavage by expressing cinnamoyl esterase activity (Couteau, McCartney, Gibson, Williamson, & Faulds, 2001). The same authors revealed the rapid degradation of chlorogenic acid within 3-4 h of human faecal incubation with coffee, and 2 h using standard compounds (Gonthier et al., 2006). Caffeic acid, one of the main metabolites after colonic fermentation of chlorogenic acid (Ludwig et al., 2013; Parkar, trower, & Stevenson, 2013; Rechner et al., 2004; Tomas-Barberan et al., 2014) was not found despite a targeted search, which may be due to an increased tendency to degradation or oxidation. Free quinic acid was also absent in the Gonthier et al. (2006) study, indicating immediate metabolism in a soluble fermentation medium. The slow degradation rate of chlorogenic acid in this study demonstrates that intact plant cell walls/structure may be a controlling factor to microbial susceptibility, and that future studies of polyphenol catabolism should take this into account.
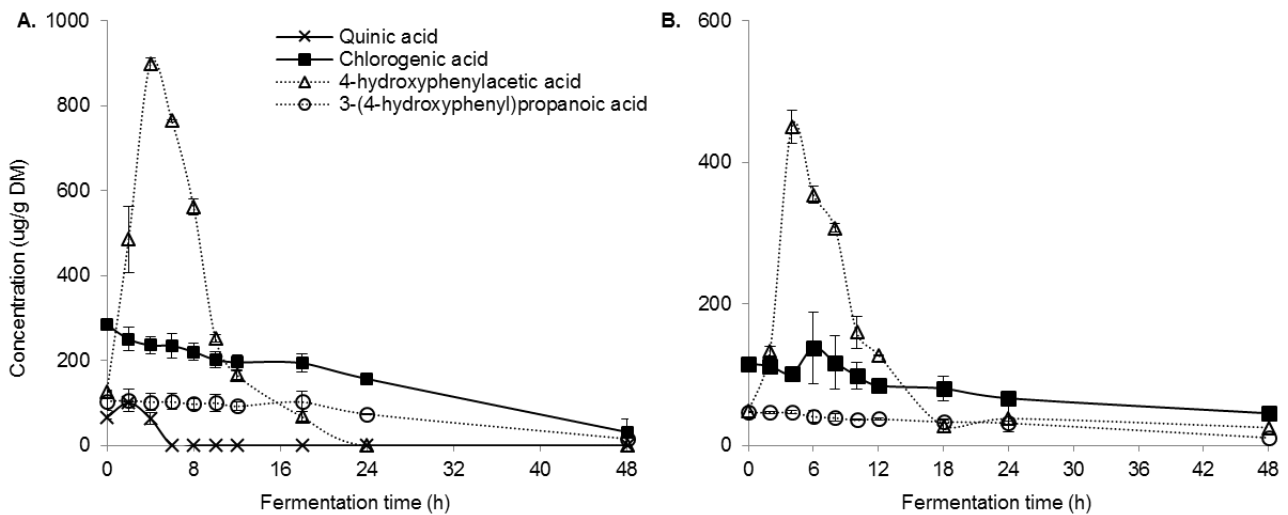
Figure 4.13. Fermentation-time profiles of chlorogenic acid (■) and its metabolites in (A) mango and (B) banana during 48 h microbial fermentation *in vitro*, demonstrating the similar degradation patterns through the proposed sequence of chlorogenic acid and hydrolysis to quinic acid (x) and dehydroxylation to 3-(4-hydroxyphenyl)propanoic acid (o), or decarboxylation and further dehydroxylation to 4-hydroxyphenylacetic acid (Δ). Data is expressed as mean±standard error. Note the different y axis scales.

The subsequent metabolites detected in the substrate faecal medium are 3-(4-hydroxyphenyl)propanoic acid and 4-hydroxyphenylacetic acid. 3-(4-Hydroxyphenyl)propanoic acid is formed from reduction of a double bond in chlorogenic acid to caffeic acid or ferulic acid and then dehydroxylation at the C4 position (Fig 4.14). 3-(4-Hydroxyphenyl)propanoic acid and 4-hydroxyphenylacetic acid have been proposed to originate from various precursors such as quercetin, quercetin-3-O-glycosides, kaempferol-rutinoside, myricetin and naringenin through ring fission by rat faecal microbiota (Serra, Macia, Romero, Ortega, & Motilva, 2012), and by human faecal microbiota (Aura et al., 2002; Crozier, Del Rio, & Clifford, 2010; Rechner et al., 2004), and from precursors such as flavanols (Aura, 2008; Chen & Sang, 2014; Henning et al., 2013) and mangiferin (Bock & Ternes, 2010). Mangiferin and the xanthone glycosides were identified in the mango pulp of various cultivars (Berardini et al., 2005b; Daud et al., 2010; Saleh & EL Ansari, 1975; Wilkinson et al., 2011), but were not detected in the present study, presumably due to very low initial concentrations or losses during the preceding mastication and 'digestion' processes. Another mango study also found that the concentration of mangiferin in the flesh of *Kensington Pride* mango was below the detection limit, and between 0.34% and 0.95% in the peel (Daud et al., 2010; Pierson, Monteith, Roberts-Thomson, Dietzgen, & Gidley, 2014). Further, environment factors such as growing conditions, ripening stage, and postharvest and storage conditions may contribute to phenolic composition variation and thus, inter-fruit variability (Hewavitharana et al., 2013a; Manthey & Perkins-Veazie, 2009; Talcott et al., 2005).
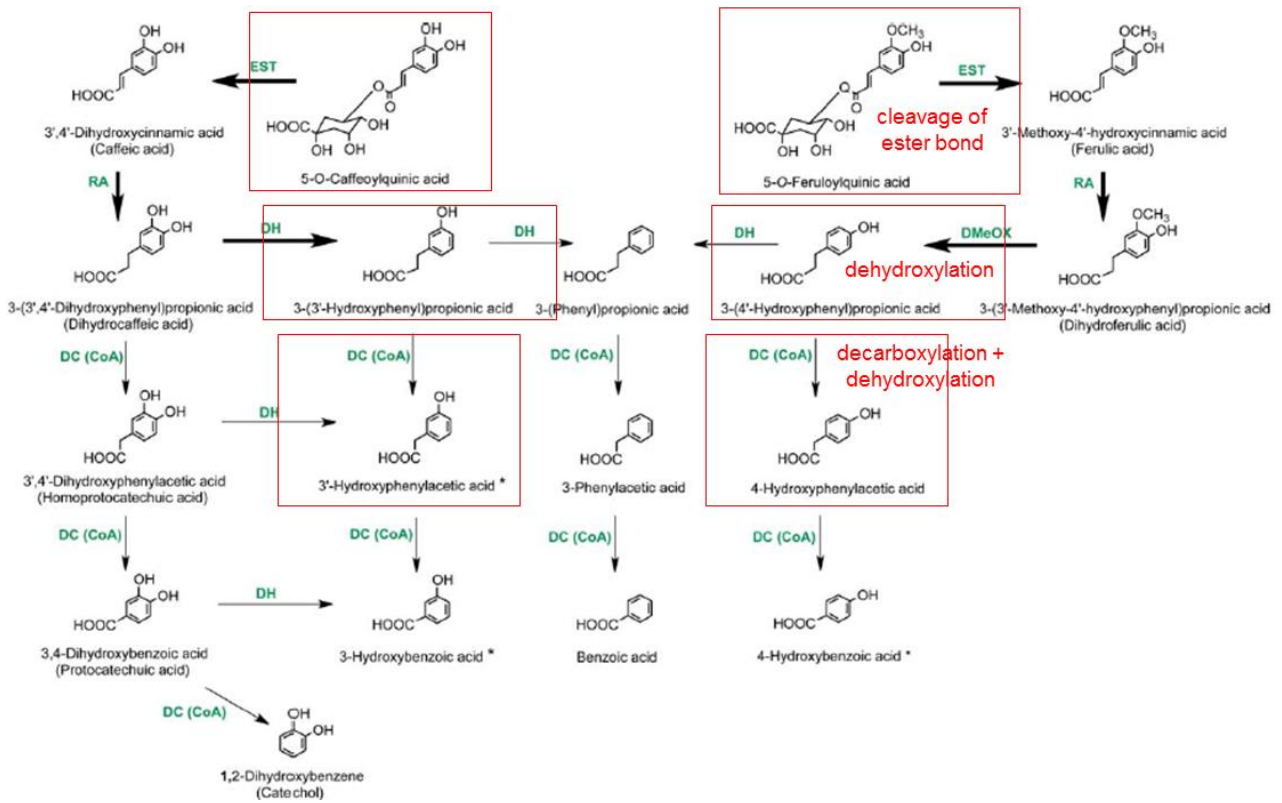
Figure 4.14. Catabolic pathways for chlorogenic acid microbial degradation in the colon (Ludwig et al., 2013). The red boxes represent transformations to identified metabolites observed in this study.

3-(4-Hydroxyphenyl)propanoic acid steadily decreased for 24 h, and became significantly (P=0.004) very low by 48 h (15 μg/g DM and 11 μg/g DM in mango and banana respectively) This finding is similar to that of Rechner et al. (2004), where the 3-(3-hydroxyphenyl)propanoic acid concentration decreased by only a small amount during 24 h fermentation. 4-Hydroxyphenylacetic acid was the major *in vitro* colonic fermentation end-product of chlorogenic acid, with a maximum concentration of 898 μg/g DM and 450 μg/g DM for mango and banana respectively, after 4 h; however, it disappeared after 24 h (Fig 4.13). Next, the decline in 3-(4-hydroxyphenyl)propanoic acid was concomitant with the swift formation and accumulation of 4-hydroxyphenylacetic acid, which may be generated through the decarboxylation and further dehydroxylation of 3-(4-hydroxyphenyl)propanoic acid (Rechner et al., 2002; Rechner et al., 2001).

Tomas-Barberan et al. (2014) found 3-(3-hydroxyphenyl)propanoic acid as the bioconversion end-product in six of nine subjects in an *in vitro* fermentation study utilising human faeces, which did not proceed towards the formation of phenylpropanoic acid, unlike in this study, possibly suggesting higher enzymatic activities of decarboxylation and dehydroxylation in porcine faeces. Caffeoyl-glycerol metabolites such as caffeoyl-glycerol

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and 3-(3,4-dihydroxyphenyl)propanoyl-glycerol were described in the same study, for the first time as microbial metabolites of chlorogenic acid. The presence of these metabolites disclosed a novel degradation pathway where the cyclohexane ring was broken, leaving a glycerol residue esterified with caffeic acid, and further degradation of the quinic acid residue before the ester link, resulted in the caffeoyl-glycerol residues. Metabolites from this pathway, however, were not present in the current set of fermented samples. No formation of benzoic acid from the quinic acid component of chlorogenic acid, as previously suggested (Indahl & Scheline, 1973), was observed during the time scale of the present *in vitro* colonic fermentation experiment. The presence of quinic acid, 3-(4-hydroxyphenyl)propanoic acid and 4-hydroxyphenylacetic acid in the fermentation medium at 0 h incubation suggests some degree of biotransformation of these metabolites may have been initiated during addition of the faecal inoculum at 0 h, which relies on efficient inactivation of microbial activity.

Hydroxyphenylacetic acids have also been characterised as specific metabolites during the colonic degradation of quercetin and its glycosides via ring fission of the C-ring to 3-(3-4-dihydroxyphenyl)propanoic acid, subsequent degradation to 3-4-dihydroxyphenylacetic acid, and finally dehydroxylation to hydroxyphenylacetic acid and phenylacetic acid (Selma, Espin, & Tomas-Barberan, 2009; Serra et al., 2012). Quercetin has commonly been reported as the main flavonol in mango (Gonzalez-Aguilar et al., 2001; Masibo & He, 2008; Robles-Sanchez et al., 2009a; Robles-Sanchez et al., 2009b; Schieber, Ullrich, & Carle, 2000; Shivashankara et al., 2004); however, quercetin and dihydroxyphenylacetic acids were not found in the substrate fermentation medium based on either UV or MS detection, presumably due to active degradation or losses occurring in the earlier digestive steps, similarly to chlorogenic acid and its intermediate catabolites. Quercetin aglycone and glycosides are easily hydrolysed within 20 min and 4 h respectively, and are described as being highly bioavailable in the small intestine (Boyer, Brown, & Liu, 2004; Crespy et al., 2002; Hollman et al., 1997c; Hollman, vanTrijp, Mengelers, deVries, & Katan, 1997b; Manach et al., 2004). 4-Hydroxyphenylacetic acid was discussed previously as being a metabolite of chlorogenic acid, thus, the degradation metabolites of hydroxyphenylpropanoic acids and hydroxyphenylacetic acids can be classified as being non-specific metabolites formed from flavanols and hydroxycinnamic acids, due to their structural properties.
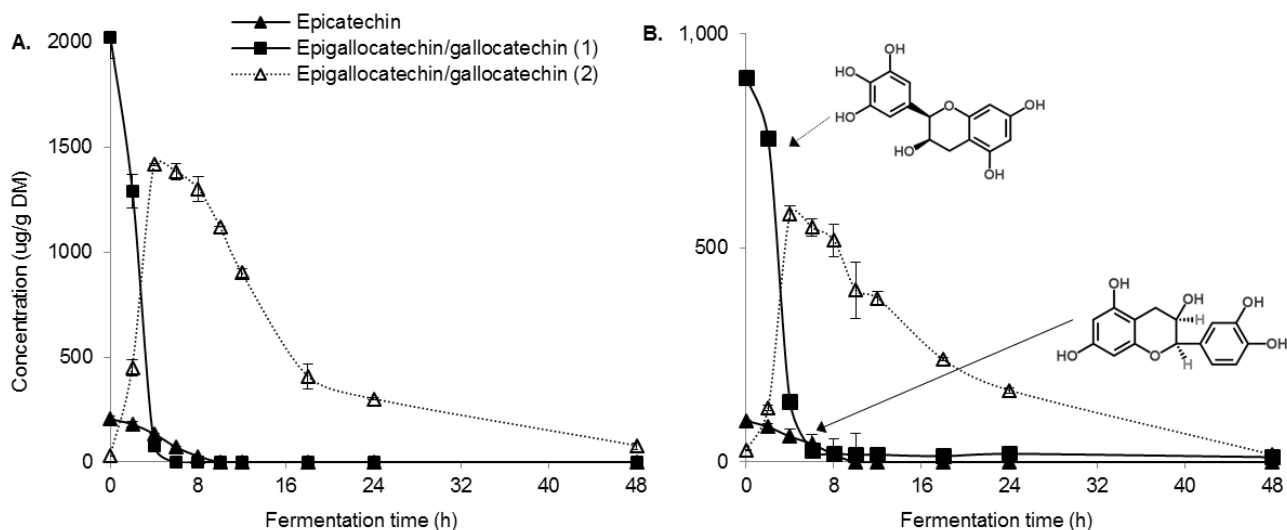
Figure 4.15. Fermentation-time profiles of epicatechin (▲) in (A) mango and (B) banana during 48 h microbial fermentation *in vitro*, and derivatives of epigallocatechin/gallocatechin (1) (■) and (2) (Δ), demonstrating the similar patterns through the degradation of epicatechin and epigallocatechin/gallocatechin (1), and formation and accumulation of epigallocatechin/gallocatechin (2). Data is expressed as mean±standard error. Note the different y axis scales.

With regards to the colonic metabolism of epicatechin, the kinetic response is shown in Fig 4.15. Degradation of epicatechin was complete within 8 h of fermentation, indicating high microbial activity for this flavanol. As peaks 4 and 11 were tentatively assigned as epigallocatechin, gallocatechin or catechin monomers, they are broadly termed as catechin derivatives (1) and (2) in this discussion. The main intact phenolic compound in mango and banana was the catechin derivative (1), with the highest starting concentrations occurring at time 0 h of *in vitro* colonic fermentation (2023 μg/g DM and 898 μg/g DM respectively); this compound was then rapidly metabolised within 8 h. Previous studies of the incubation of flavanols with pig caecal microbiota similarly showed metabolism within 4-8 h (Hein, Rose, Van't Slot, Friedrich, & Humpf, 2008; Van't Slot & Humpf, 2009). Degradation of epicatechin and the catechin derivative (1) occurred simultaneously, in parallel with a progressive 4 h increase in the concentration of the catechin derivative (2), which then gradually declined in concentration until the end of the 48 h colonic fermentation. Hippuric acid, as detected by the Q-ToF-MS, was found to be present in mango and banana at low levels only at 0 h, and was proposed to originate from 4-hydroxybenzoic acid (Fig 4.16) (Gao et al., 2006; Roowi et al., 2010). In addition, there are pathways to hippuric acid from benzoic acid, quinic acid (Clifford, 2000), tryptophan, tyrosine and phenylalanine (Bridges, French, Smith, & Williams, 1970; Grumer, 1961; Self, Brown, & Price, 1960). Hippuric acid is well recognised as a urinary excretion metabolite

(Gonthier, Verny, Besson, Remesy, & Scalbert, 2003; Mulder, Rietveld, & van Amelsvoort, 2005; Rechner et al., 2002; Van Dorsten, Daykin, Mulder, & Van Duynhoven, 2006).
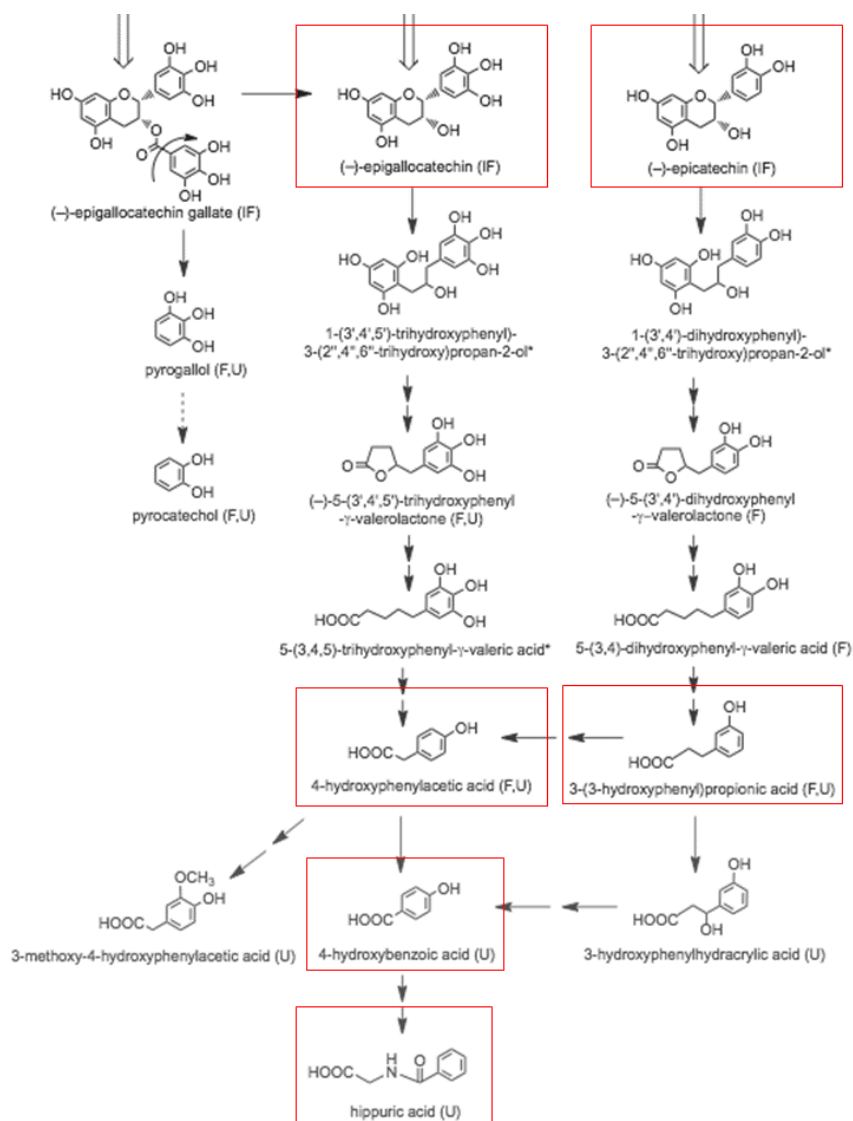
Figure 4.16. Proposed metabolic pathways for epicatechin and catechin (flavanols). Single arrows indicate known conversions, double arrows indicate unknown conversions, the dotted arrow between pyrogallol and pyrocatechol indicates this is a minor conversion (Roowi et al., 2010). The red boxes represent transformations to identified metabolites observed in this study.

Studies of microbial metabolism of dietary catechins and procyanidins showed that benzoic, phenylacetic, phenylpropanoic, phenylvaleric and phenyllactic acid derivatives and phloroglucinol were produced with different hydroxylation patterns (Groenewoud & Hundt, 1986; Rios et al., 2002; Tzounis et al., 2008). However, phenylvaleric acids or valerolactones were not detected in the present study. Interestingly, the formation of these metabolites from (+)-catechin required an initial conversion to (+)-epicatechin, which has been linked to the dramatic change in the growth of distinct microbiota populations in the presence (+)-epicatechin (Tzounis et al., 2008).

4.10. Concluding Remarks

This study of colonic-microbial biotransformation has demonstrated an intensive metabolic activity of the faecal microbiota, including the disintegration of banana and mango fruit cell wall structures (as shown in Chapter 4 Part A), and the subsequent release and metabolism of phenolic compounds during the *in vitro* fermentation process. UPLC-PDA and UHPLC-MS analyses revealed degradation of intact polyphenols (chlorogenic acid and epicatechin), and the formation and accumulation of the intermediate catabolites, quinic acid, 3-(4-hydroxyphenyl)acetic acid, 4-hydroxyphenylacetic acid, hippuric acid and two catechin derivatives. Q-ToF-MS may be effective in identifying known compounds, given sufficient time to elucidate the mass, formula, and structure, and the availability of a broad range of authentic standard compounds but this technique has also proved challenging with cost and time constraints. Continuation of this study is warranted with a further investigation of studying the unidentified metabolites, including non-phenolic-associated compounds, through the use of MS/MS fragmentation or selected reaction monitoring mode (SRM) of individual compounds, as approached in some reported studies (de Oliveira et al., 2013; Jenner, Rafter, & Halliwell, 2005; Mosele et al., 2014). Future work could also focus on preserving or increasing the longevity of the compounds of interest; there may be degradation of the phenolic compounds in the samples, given that the MS-based analysis was a latecomer to proceedings and some expected signals were not observed. Considering that some of the polyphenols present in mango and banana may be non-extractable, characterisation and identification of these compounds may be improved via the application of chemical depolymerisation techniques, i.e. phloroglucinolysis and butanolysis. The present study has shown that unabsorbed polyphenols are likely to be transported to the colon, because they were not released from the food matrix during the use of the *in vitro* digestion model for the small intestine digestion. Exposure (upon release from cell walls) to faecal microbiota and/or their microbial enzymes capable of metabolic activities (e.g. ring fission, dehydroxylation, decarboxylation and hydrolysis) beyond those of human endogenous enzymes resulted in the production of lower molecular-weight metabolites. This will contribute to a better understanding of the fruit matrix-phenolic-microbiota interactions, and subsequently the physiological impact on gut health. The site of maximum fermentation activity *in vivo* may be influenced by passage kinetics of the digesta with changes to the proportions of dietary components and this is investigated in Chapter 5 Part A.

Chapter 5. Passage of mango and pectin, and polyphenol metabolism in the gastrointestinal tract of pigs – a human model

5.1. Introduction

The total nutritive value of a meal is influenced by its components and the processing steps that the components have undergone before they are consumed (Schwizer et al., 1997; Siddons, Paradine, Beever, & Cornell, 1985), physiological processes including mastication (Bach Knudsen, 2001), acid hydrolysis in the stomach (Näslund, Gutniak, Skogar, Rössner, & Hellström, 1998), enzymatic hydrolysis in the small intestinal lumen (Souza da Silva, van den Borne, Gerrits, Kemp, & Bolhuis, 2012), and microbial fermentation along the large intestine (Jian, Vigneron, Najean, & Bernier, 1982). These physiological processes are heavily dependent on the duration that nutrients spend in each compartment of the gastrointestinal tract.

There have been human feeding studies where human volunteers were fed radioactive tracers and the passage of these tracers was recorded as they progressed along the gastrointestinal tract (Brunner et al., 2003; Feldman, Smith, & Simon, 1984; Weaver et al., 2014). More recently, magnetic resonance imaging has been used to investigate the passage of gastrointestinal tract contents (Evans et al., 1993; Gamarra et al., 2010; Martin, Danrad, Herrmann, Semelka, & Hussain, 2005). Despite these current techniques, they remain costly and are difficult to apply to large-scale trials. Although there are inherent differences between humans and pigs, including feeding quantity, length and size of the large intestine, and gastric luminal environment, pigs have frequently been used as a human model to determine the movement of gastrointestinal tract contents (Guilloteau, Zabielski, Hammon, & Metges, 2010).

The aims of the current study were to quantify the passage kinetics along the digestive tract (from the stomach to the distal colon) *in vivo*, and the fractional outflow rates from the stomach and small intestine. Since insoluble dietary fibres have previously been shown to influence passage dynamics, diets containing a mango ingredient, and the main soluble dietary fibre (pectin) in both banana and mango were used to investigate the influence on these parameters (Chapter 5 Part A). The next aim was to study the release of polyphenols from the mango pulp ingredient and their biotransformation to respective catabolites in defined sites of the entire gastrointestinal tract in 'real time' (Chapter 5 Part B).

Part A. Passage of gastrointestinal contents in grower pigs as affected by dietary components - mango and pectin

5.2. Introduction to Part A

It has been shown previously that dietary fibres have the ability to alter the retention time of nutrients in each gastrointestinal tract compartment. Generally, there has been an association between a reduced rate of gastric emptying and delayed nutrient absorption. This, in turn, then attenuates post-prandial glucose and insulin responses (Miao et al., 2014), and prolongs feelings of satiety (Pond, Ellis, James, & Deswysen, 1985). Several studies have suggested that soluble dietary fibres would increase the viscosity of the liquid phase of gastric contents, which will lead to a significantly longer stomach retention time (Bach Knudsen, 2001; Hartnell & Satter, 1979; McIntyre, Gibson, & Young, 1993; Rainbird & Low, 1986). In the small intestine, soluble dietary fibre is thought to primarily interfere with digestion and absorption of nutrients, yet few studies have referred to changes in passage rates.

Due to practical difficulties in collecting small amounts of intestinal digesta from numerous sites between the duodenum and ileum, only a limited number of studies have investigated the rate of passage along the length of the small intestine. These studies have relied on the insertion of one or two cannulas into specific regions, usually the duodenum or ileum (Gidenne, 1992). The effects of insoluble dietary fibres in the large intestine have been extensively studied (Ehle, Jeraci, Robertson, & Van Soest, 1982; Hendriks, van Baal, & Bosch, 2012; Owusu-Asiedu et al., 2006), with the conclusion that insoluble dietary fibre promoted large intestinal passage due to increased bulk, and the retention of water by digesta.However, the effects of soluble dietary fibre either isolated or as a component of food, on small intestinal rate of passage remain unclear.

The feeding of dietary markers to investigate gastrointestinal tract passage is a useful approach. From reviews of a variety of markers, a summary of ideal digesta marker criteria is as follows: 1) the marker must be strictly non-absorbable, (2) the marker must be physically similar to or intimately associated with the material it is to mark, and 3) its method of estimation in digesta samples must be specific and sensitive, and the marker must not interfere with other analyses. The most commonly used method for measuring digesta flow involves administration of markers at a constant rate, either in the diet or by infusion at a point proximal to the points at which flow is to be measured, followed by sampling at those points, once equilibrium (steady-state) conditions have been achieved.

Steady-state conditions exist when the marker pools and flows that are proximal to the sampling points are constant, and are reflected as constant marker concentrations in the samples when the animal is fed continuously (Faichney, 1975), or at regular short intervals, or in a repeating pattern of concentrations related to feeding and/or marker dosing patterns (Faichney, 1980). The form of the markers (e.g. as oxides or chlorides, inert metals) makes them indigestible to mammals and often tightly bound to plant material, which are therefore expected to flow through the gastrointestinal tract in close association with digesta. Such an association could be desirable in reducing variation in faecal marker concentration attributable to differential flow of feed residue relative to flow of the marker, from various gastrointestinal tract compartments (Kotb & Luckey, 1972).

5.3. Materials and Methods

5.3.1. Pigs, diets, experimental design, housing and feeding

The experiment was approved by The University of Queensland Animal Ethics Committee (Ethical Clearance: CNAFS/179/11/CSIRO). Thirty Large White male pigs, obtained from The University of Queensland Piggery, Gatton, QLD, Australia, were randomly distributed into three groups of ten pigs each. Each group was assigned to one of three experimental diets shown in Table 5.1. The control diet contained wheat starch as the major source of carbohydrate, while in the other two diets, dried mango powder or purified apple pectin was included at the expense of wheat starch. The mango diet contained 15% dried mango puree powder, a component of which is pectin (10.7%); the addition of 15% dried mango powder in the mango diet is qpproximately equivalent to 0.81 kg of fresh mango puree per kg of dried material consumed. This mango powder was sourced from Nutradry (Hendra, QLD, Australia). The pectin diet contained 10% purified apple pectin sourced from Hawkins Watts Pty Ltd. (Mulgrave, VIC, Australia). Edible portions (pulp) of mango were used in the production of the Nutradry mango powder and as a commercial source of mango pectin was not available, the common commercial source of purified pectin extracted from apple pomace was selected over citrus peels. The selected apple pectin is a high methoxyl pectin with a degree of esterification (DE) of 59-64%, which is similar to published DE values of apple pomace of 58-62% (Constenla, Ponce, & Lozano, 2002; Garna et al., 2007) and mango pulp of 58-70% (Saeed, El Tinay, & Khattab, 1975). Pectin comprises the major fraction of soluble dietary fibre in both mango and apple pulp (Fernandez, 2001; Goni, Torre, & Saura-Calixto, 1989; Gullon, Falque, Alonso, & Parajo, 2007; Thompson, 2010). Protein, fat, fibre, vitamins, minerals and energy were kept constant and isocaloric between diets (Table 5.2). The pigs were housed individually on

Table 5.1. Chemical composition of the experiments diets (g/100 g).

Ingredients	Control	Pectin	Mango
Whey protein concentrate	3.000	3.000	3.000
Calcium caseinate	12.985	12.985	12.895
Nutradry mango powder	0.000	0.000	15.000
Pectin Classic AF 401 85%	0.000	11.765	0.000
Arbocell[1]	6.000	6.000	6.000
Celite[2]	26.46	24.88	24.44
Dried whole egg powder	2.000	2.000	2.000
Palm oil	3.145	3.191	3.007
Sunflower oil	0.791	0.798	0.798
Wheat starch	64.907	54.867	52.478
Limestone- 1mm + 250 um dust free	2.096	1.321	0.793
Dicalphos[3]	1.443	1.443	1.440
Salt	0.000	0.001	0.199
Sodium bicarbonate	0.712	0.308	0.243
Potassium chloride	0.156	0.448	0.195
Potassium carbonate	0.724	0.000	0.000
Causmag (Mgo) [4]	0.830	0.661	0.649
AN GSH-PX selenium yeast 0.2% Se	0.011	0.011	0.011
Choline chloride 60%	0.045	0.045	0.046
DL methionine	0.210	0.210	0.210
Lysine HCl	0.137	0.137	0.137
L-Threonine	0.725	0.725	0.725
L-Tryptophan	0.043	0.043	0.043
Pig Mineral 1 (Applied Nutrition Pty Ltd*)	0.080	0.080	0.080
Pig Vitamin 1 (Applied Nutrition Pty Ltd*)	0.050	0.050	0.050

[1]Arbocel was used as a source of cellulose (fibres). [2]Celite (or diatomaceous earth) was measured as acid insoluble ash. [3]Dicalphos was used as a calcium supplement. [4]Causmag (magnesium oxide) was used as a magnesium supplement. *Applied Nutrition Pty Ltd, Alexandra Hills, QLD, Australia. All ingredients were sourced locally in Queensland.

Table 5.2. Calculated nutritional composition of the experimental diets (%).

Ingredients	Control	Pectin	Mango
Volume	100.00	100.00	100.00
Dry matter	91.723	91.404	92.063
Protein	16.019	15.978	16.382
Crude fibre[1]	4.411	10.800	5.558
Pectin	0.510	6.929	1.658
Ash	7.440	6.660	6.120
Digestible energy (mega joules)[2]	15.150	15.150	15.150
Arginine	0.499	0.499	0.506
Histidine	0.414	0.424	0.417
Isoleucine	0.667	0.667	0.673
Lysine	1.095	1.095	1.103
Methione	0.571	0.571	0.574
Threonine	0.764	0.764	0.770
Tryptophan	0.219	0.219	0.222
Valine	0.841	0.841	0.848
Ailyspig	1.061	1.061	1.061
Calcium	1.300	1.076	0.833
Phosphorus	0.361	0.361	0.362
Av_phosphorous	0.300	0.300	0.300
Sodium	0.210	0.160	0.160
Potassium	0.300	0.280	0.280
Chloride	0.112	0.250	0.250
Fat	5.500	5.500	5.500
Saturated fat	2.030	2.053	2.010
Monounsaturated fatty acids (MUFA)	0.009	0.010	0.009
Polyunsaturated fatty acids (PUFA)	0.812	0.821	0.804

[1]Crude fibre is comprised primarily of cellulose (from Arbocell) and pectin. [2]Digestible energy was measured as mega joules.

raised floors in 1.8 m^2 pens. Individual housing was realised by placing stainless steel pens side-by-side with a feeding trough at one end. Pigs could see and hear each other but were restricted from touching by the pens. Lights were on from 0600 to 1800. The shed was mechanically ventilated. Post-weaning, the pigs were fed a commercial diet before adaption. The pigs were adapted to their allocated experimental diets by gradually changing the ratio of commercial diet to experimental diet over a one-week period, i.e. the pigs were fed 100% commercial diet on day 1 of the changeover week, 75% commercial diet to 25% experimental diet on days 2 and 3, then 50% commercial diet to 50% experimental diet on days 4 and 5, 25% commercial diet to 75% experimental diet on days 6 and 7, and finally 100% of the experimental diet on day 8 and thereafter for 21 days prior to slaughter. Pigs were weighed each week and fed individually according to their weight at 2.5 times the metabolisable energy requirements for maintenance. The average weight of the pigs at sampling was 54.6 ± 3.2 kg. Blood was collected from the pigs prior to the diet changeover week, and during sample collection at the end of three weeks on the experimental diets, but no pigs showed any signs of disease. The diets were fed as a mash twice daily at 0800 and 1600, and allowed *ad libitum* access to water.

5.3.2. Dietary markers

Gastrointestinal contents have been widely reported to consist of a fluid phase and heterogeneous (particulate) solid phase with differential flow rates (Johansen, Bach Knudsen, Sandstrom, & Skjoth, 1996; Lentle & Janssen, 2011). In the current study, the fluid phase was a result of ingested water (*ad libitum)* and gastrointestinal secretions, while the heterogeneous solid phase was primarily made up of varying particles sizes of feed ingredients and boluses formed on hydration within the digestive tract. Therefore, indigestible solid and liquid phase markers that associate and distribute exclusively throughout each phase were incorporated into the experimental diets to measure the flow rate of digesta as two independent phases in various gastrointestinal sites. Solid phase markers adsorb onto particles, which may be hydrolysed by digestive enzymes and when completely hydrolysed, attach onto adjacent undigested particles (Hartnell & Satter, 1979). The dietary markers were categorised as constant and pulse dose markers.

Constant feed markers

Chromium chloride ($CrCl_3$) (198 mg/kg DM) and Celite (measured as acid-insoluble ash (AIA)) (25-26 g/kg DM) were constant (solid) markers mixed with the dry diet ingredients and fed daily to the pigs. This feeding regime was designed to mimic a steady state

infusion, which allowed the recovery of chromium (Cr) and AIA to calculate retention time, transit time and rate of passage of digesta.

Pulse dose markers

Cerium chloride ($CeCl_3$), ytterbium chloride ($YbCl_3$) and lanthanum chloride ($LaCl_3$) were selected as pulse dose (solid) markers, while cobalt-EDTA (Co-EDTA) was selected as a pulse dose (liquid) marker. $CeCl_3$, $YbCl_3$ and $LaCl_3$ have a strong affinity for small particulates and when fed at known time intervals, can provide insight into the passage of undigested solid digesta (Cuddleford, Pearson, Archibald, & Muirhead, 1995; Miyaji et al., 2008; Pagan, Harris, Brewster-Barnes, Duren, & Jackson, 1998; Pearson, Archibald, & Muirhead, 2001; Pond et al., 1985; Rosenfeld, Austbo, & Volden, 2006; Uden, Colucci, & Van Soest, 1980). At 6 h prior to euthanasia, the pigs were fed a portion of the diet (20%) dosed with $CeCl_3$ (5.3 mg/kg DM) and at 4 h prior to euthanasia, the pigs were fed another 20% of the diet dosed with $YbCl_3$ (6.6 mg/kg DM). Lastly, At 2 h prior to euthanasia, the pigs were 20% of the diet dosed with $LaCl_3$ (5 mg/kg DM) and Co-EDTA (7.6 mg/kg DM).

5.3.3. Sampling

Pigs were anesthetised 2 h postprandially (Zhang et al., submitted 2014). Pigs were laid dorsally on an operating table, intubated and their blood levels were monitored for CO_2 and O_2. The abdominal cavity was opened by midline laparotomy and the digestive tract ligated to prevent digesta flow between gastrointestinal tract sections. Digesta was collected from nine sites along the gastrointestinal tract- stomach, small intestine, caecum and colon by gentle squeezing (Fig 5.1).
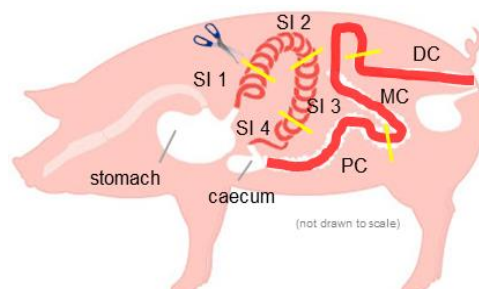
Figure 5.1. Sampling sites of the pig gastrointestinal tract consisting of the stomach, small intestine (split into four sections), caecum and colon (split into three sections). SI: small intestine, PC: proximal colon, MC: mid colon, DC: distal colon.

The small intestine was split into four sections (SI1-4): SI1 corresponded to the duodenum and the first part of the jejunum, comprising the first 2 m of tract after the pyloric sphincter, SI4 corresponded roughly to the terminal ileum and consisted of the last 1 m, while the remaining small intestinal tract (corresponding to the rest of the jejunum) was split into

equal lengths labelled SI2 and SI3 (~4 m each). The colon was divided into three sections based on equal lengths into the proximal colon (PC), mid colon (MC) and distal colon (DC) (~0.88 m each). Each section was weighed before and after careful removal of the contents, the lengths were recorded and the pH measured, immediately before digesta samples were frozen at -20°C.

5.3.4. Chemical analyses

5.3.4.1. Dry matter

Dry matter (DM) was determined by drying to constant weight at 105°C (ISO standard 6496:1999) (International Organisation for Standardisation, 1978).

5.3.4.2. Water holding capacity (WHC)

Water holding capacity (WHC) of the diets was measured by centrifugation method (Stephen & Cummings, 1979). Samples (10 g) was weighed, added to 35 mL distilled water and mixed in a water bath at 250 rpm at 37°C for 24 h, followed by centrifugation at 4000 g (Avant®JE centrifuge, JA14 rotor) for 30 min. The supernatant was removed and the tubes were kept upside down for 3 min to remove all the liquid. The swollen sample weights were measured, and water binding capacity described in g of water absorbed per g of dried diet weight.

5.3.4.3. Acid insoluble ash (AIA)

Analysis of AIA content in the experimental diets and digesta was adapted from Van Keulen and Young (1977). Samples (~100 mg AIA) were weighed into a pre-weighed sintered-glass crucible (Pyrex, porosity 4), dried at 105°C for 24 h and re-weighed. The samples were then ashed at 500°C for 6 h, heated to 100°C with 4 M hydrochloric acid for 30 min and thoroughly washed with reverse-osmosis (RO) water. The processes of drying, ashing, boiling and washing were repeated until the ash appeared white.

5.3.4.4. Mineral (markers) content

The content of markers- $CrCl_3$, $CeCl_3$, $YbCl_3$, $LaCl_3$ and Co-EDTA in the experimental diets and digesta was measured as individual mineral- Cr, Ce, Yb, La and Co content. This was determined by digesting 300 mg of dried sample in 6 mL nitric acid and 2 mL perchloric acid followed by the addition of RO water to 20 mL total volume. The digested samples were analysed using an inductively coupled plasma atomic emission spectrometer (Optima7300 DV, Perkin Elmer; Wellesley, MA, USA) (Isherwood, 2014).

5.3.5. Terminology and calculations

Terminology used in this study is defined here to avoid confusion from common terms used by other authors, for example, (mean) retention time or passage rate are often defined or interpreted differently. In this study, retention time is defined as the average time (in hours) digesta components are retained in a mixing pool compartment- i.e. stomach and caecum, which slowly releases its contents; mixing pool refers to the mass, weight or volume of contents (Mertens, 2005). Transit time is defined as the time (in hours) the digesta takes to travel through a particular tubular section of the small intestine (SI1-4) and colon (PC-DC) (Faichney, 2005), where migrating myoelectrical complexes occur due to segmentation motility from the generation of electrical activity from the gastro-duodenal junction, which was first discovered by Szurszewski (1969). Calculation of retention and transit times were applied to all markers (Cr, AIA, Ce, Yb, La and Co). Digesta flow from the ileum to caecum and proximal colon is intermittent, and can be followed by periods of quiescence due to both peristaltic and anti-peristaltic contractions that result in the digesta being mixed and moved towards the DC (Szurszewski, 1969). Retention time (RT) and transit time (RT), in hours, are calculated from Eq. (1) (Lascano & Quiroz, 1992):

$$RT \text{ or } TT = FD \ (M_s / M_D) \tag{1}$$

where FD is feed hours, which is the time period from when the pigs were fed at 0800 the day before, to time of anesthesia or slaughter. M_s is the concentration of marker (mg or g) determined in each gastrointestinal tract section and M_D is the total amount of marker (mg or g) added to the diets. The marker concentration was corrected for total % recovered in the entire digestive tract, rather than the amount of marker offered to the pigs (Fig 5.2). Assuming steady-state conditions, the passage rate (in m/h) can be calculated using the constant feed markers, Cr and AIA. Passage rate (PR) is a measure of digesta flow through a known length of a gastrointestinal tract section per unit time, and was calculated from Eq. (2) as the inverse of transit time (Letourneau-Montminy et al., 2011):

$$PR = L \times (1 / TT) \tag{2}$$

where L is the length (m) measured for each site (*see section 5.2.3. Sampling*).

Fractional outflow rates were calculated for the stomach and (combined) small intestinal compartments using the pulse dose (solid) markers (Ce, Yb and La) (Table A4.1, Appendix 4). The post marker dose at 0 h refers to the averaged feeding dose (mg) of Ce, Yb and La fed to the pigs. The post marker feeding dose (mg) for Ce, Yb and La has been

set to 6 h, 4 h and 2 h respectively, and the pool size at these time intervals refers to the (normalised) quantity of Ce, Yb and La recovered in the digesta of the stomach and small intestine. Influx into the stomach has been set to 0 mg, assuming the pulse dose markers instantaneously end up in the stomach after oral ingestion. The stomach outflow rate for each 2 h time window was calculated as the difference in the pool size at 0 h and at 2 h, and similarly for 4 h and 6 h, for example in Eq. (3):

$$\text{Stomach outflow rate} = \text{pool size at 0 h} - \text{2 h} \tag{3}$$

where the stomach outflow rate (mg/2 h) equals influx into the small intestine. The small intestinal outflow rate into the colon was subsequently calculated as Eq. (4):

$$\text{Small intestine outflow rate} = \text{influx} - (\text{pool size at 2 h} - \text{0 h}) \tag{4}$$

In addition, Eq. (4) was also applied to the flow rates at 4 h and 6 h. A graphical representative of these flow rates in mg/h is shown in Fig 5.7. Fractional outflow rates were not calculated for the colon compartment as these pulse dose markers have not reached the colon within 2 h after the last marker feeding dose.

5.3.6. Statistical Analysis

The experimental unit for all parameters was a group of thirty pigs. DM, pH, % recovery, retention time, transit time and passage rate were analysed by mixed model analysis using Proc Mixed in SAS 9.3 (SAS Institute, Inc., Cary, NC, USA). The effects of Diet, Gastrointestinal tract site and the interaction of Diet*Gastrointestinal tract site were determined by the slice statement using the same model. Group means were analysed using Proc GLM and the significance of treatment differences was set at $P<0.05$.

5.4. Results

5.4.1. Digesta dry matter is lower in mango- and pectin-fed pigs

The control diet contained wheat starch as the major source of carbohydrate while in the other diets, dried mango powder and purified apple pectin were included at the expense of some of the wheat starch. Digesta DM was influenced by ingestion of soluble fibre (pectin) and mango in the diet, where the DM digesta (%) in these diets were significantly lower ($P<0.0001$) than in the control diet (mango < pectin < control). Digesta DM significantly decreased ($P<0.05$) during transit from the stomach to small intestine, and increased again in the colon (Fig 5.2A, Table 5.3). The water holding capacities of the dry diets were determined to be 0.49 ± 0.01, 0.40 ± 0.01 and 1.24 ± 0.05 (g of water/ g of dried diet) for

the control, mango and pectin diets respectively, suggesting that the pectin diet was able to retain significantly (P<0.05) more water than the control and mango diets.

5.4.2. Digesta pH along the gastrointestinal tract

Digesta pH was lowest (~5) in the stomach and gradually increased to 7.4 in the terminal ileum (SI4) (Fig 5.2B). The sharp increase in duodenal (SI1) pH is explained by secretion of bicarbonate (Grendell, 2014). In the caecum, the pH dropped to 6.7 and increased again to pH 8 in the DC, similarly as observed by Fallingborg (1999) due to an excess of rapid fermentation of carbohydrates supplied from the caecum, overwhelming the buffering capacity (Van Soest, 1994).

Table 5.3. Digesta dry matter (%) in various gastrointestinal tract sites of pigs fed the control, 15% mango or 10% pectin diets.

GIT site	n*	Diets			Probability of mixed effects		
		Control	Mango	Pectin	Diet	GIT	Diet x GIT
STO	10	31.4±1.4	23.8±1.4	24.4±1.4			0.0008
SI1	10	8.6±1.7	8.1±1.7	12.1±1.7			0.208
SI2	10	12.6±0.7	11.9±0.7	10.9±0.7			0.226
SI3	10	21.1±0.6	17.9±0.6	13.5±0.6			<0.0001
SI4	10	21.3±0.9	15.9±1.0	15.7±0.9	<0.0001	<0.0001	0.0003
CAE	10	32.4±3.3	16.6±3.3	23.5±3.3			0.009
PC	10	26.4±0.7	18.9±0.7	21.4±0.7			<0.0001
MC	10	30.3±0.9	20.8±0.9	21.3±0.9			<0.0001
DC	10	42.3±1.2	23.5±1.2	24.0±1.2			<0.0001

*GIT: gastrointestinal tract, STO: stomach, SI: small intestine, CAE: caecum, PC: proximal colon, MC: mid colon, DC: distal colon. Data is expressed as mean±standard error. *10 pigs per diet per GIT site.*
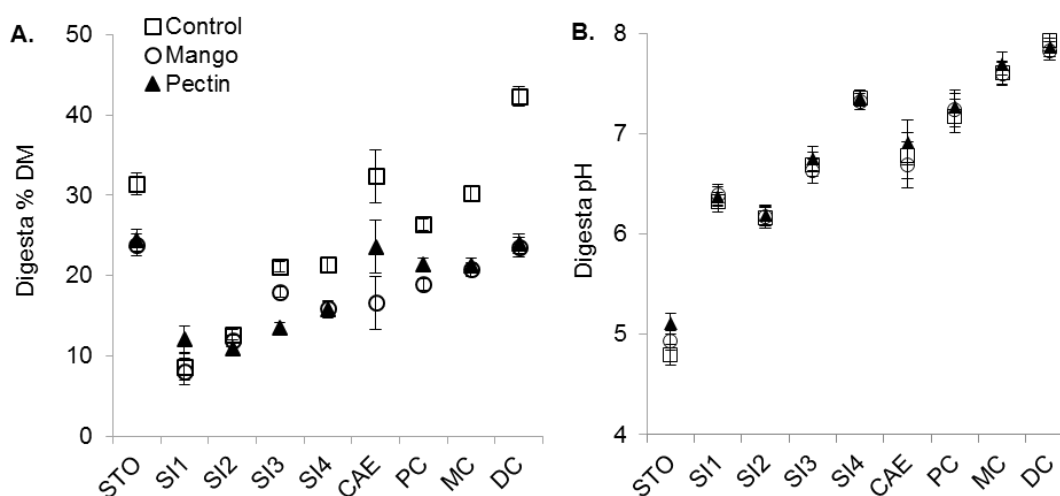
Figure 5.2. Digesta (A) dry matter (%) and (B) pH along the gastrointestinal tract of pigs fed the control (□), 15% mango (O) or 10% pectin (▲) diets. Data is expressed as mean±standard error. STO: stomach, SI: small intestine, CAE: caecum, PC: proximal colon, MC: mid colon, DC: distal colon.
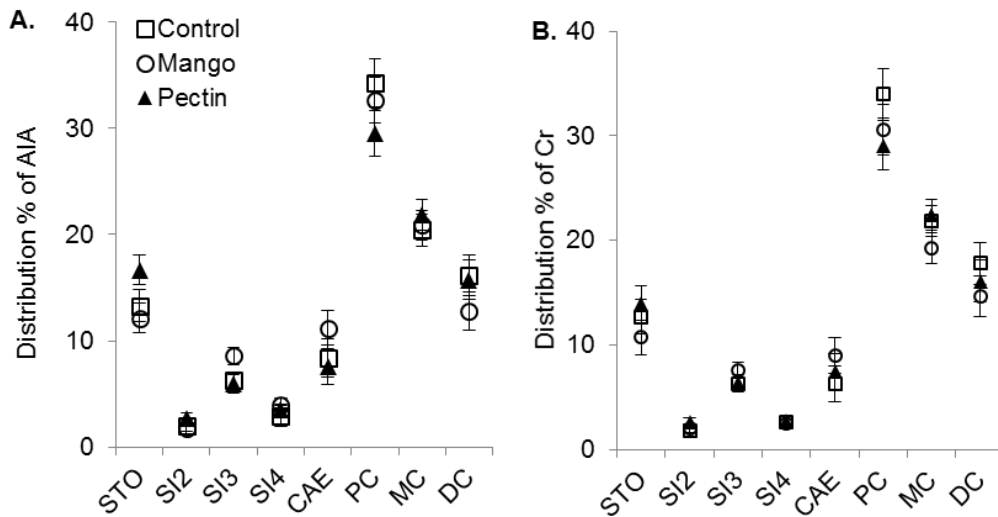
Figure 5.3. Distribution of constant feed markers (A) acid insoluble ash (AIA) and (B) chromium (Cr) recovered in the gastrointestinal tract of pigs fed the control, 15% mango (O) or 10% pectin (▲) diets. Data is expressed as mean±standard error. STO: stomach, SI: small intestine, CAE: caecum, PC: proximal colon, MC: mid colon, DC: distal colon.
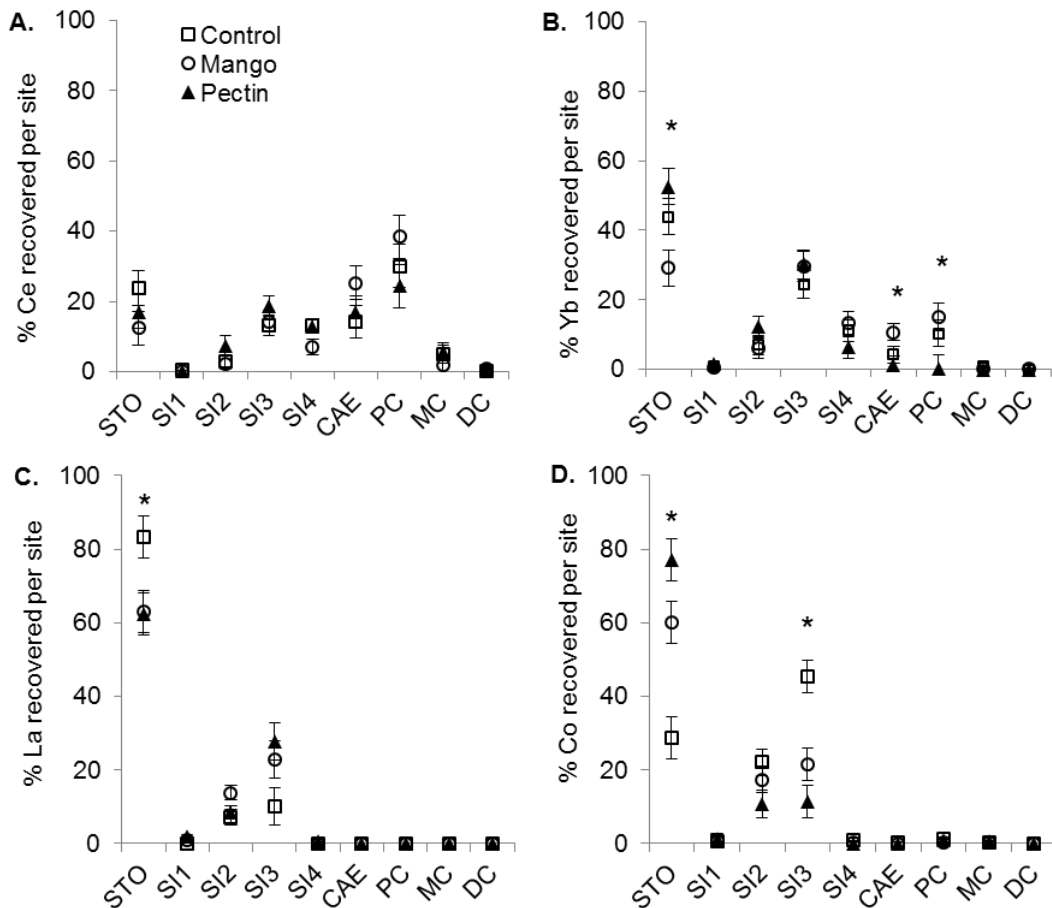
Figure 5.4. Distribution of pulse dose markers (A) cerium (Ce), (B), ytterbium (Yb), (C) lanthanum (La) and (D) cobalt (Co) recovered in the gastrointestinal tract of pigs fed the control (□), 15% mango (O) or 10% pectin (▲) diets. Pulse dose (solid) markers were added to the diets at various time intervals before anaesthesia (0 h): Ce: -6 h; Yb: -4 h; La: -2 h, and (liquid) marker: Co: -2 h. Any significant (P<0.05) significant interactions between diet and gastrointestinal tract site (P<0.05) are marked by an asterisk (*). Data is expressed as mean±standard error. STO: stomach, SI: small intestine, CAE: caecum, PC: proximal colon, MC: mid colon, DC: distal colon.

5.4.3. % Marker recovery in each gastrointestinal tract section

The distribution of constant feed markers, AIA and Cr along the gastrointestinal tract is 86 ± 4% and 77 ± 3% shown in Fig 5.3. Pulse dose marker recovery for Ce, Yb, La and Co averaged 72 ± 4%, 71 ± 4%, 69 ± 6% and 59 ± 5% respectively (Fig 5.4). The markers fed at earlier intervals to the pigs had a higher recovery across all diets, for example, 40% of Ce (fed 6 h prior to euthanasia) had reached the PC, 20% of Yb (4 h) was recovered in PC and after 2 h, La and Co had only reached SI3. La was recovered mostly in the stomach (60-80%), whereas a higher proportion of Co was recovered in SI3.

5.4.4. Retention and transit times of constant feed markers

The retention and transit times relative to each gastrointestinal tract section show a similar trend for both constant feed markers (AIA and Cr), where transit time was longest in the colon, followed by small intestine and stomach (Fig 5.5). The retention time of AIA and Cr averaged 3 h in the stomach, and 2.3 h in the caecum, while transit time averaged 0.5 h in SI2, 1.6 h in SI3, 0.8 h in SI4, 7 h in PC, 4.8 h in MC and 3.7 h in DC. Interestingly, even though SI2 and SI3 were split into equal lengths, transit time in SI3 is significantly longer (P=0.05). A longer transit time was also observed in the PC compared to MC and DC (P=0.03). The total retention and transit times for the pectin diet was 21 h, mango diet was 24 h and control diet was 25 h, suggesting that a higher pectin content in the digesta reduced the transit time, consistently with a faster passage rate along the gastrointestinal tract (Fig 5.6). The passage of digesta was faster along the small intestine relative to the colon.
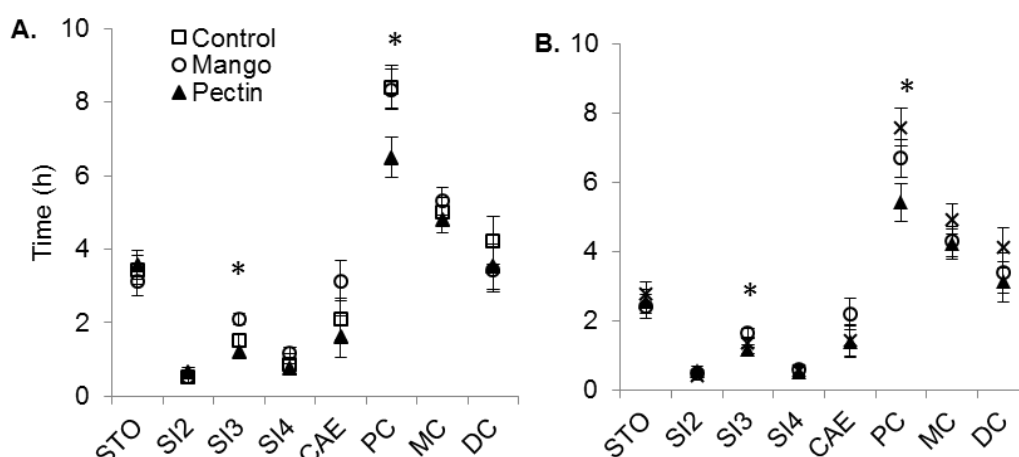
Figure 5.5. Time (hours) that (A) acid insoluble ash and (B) chromium spent in the gastrointestinal tract of pigs fed the control (□), 15% mango (O) or 10% pectin (▲) diets. Time spent in the stomach (STO) and caecum (CAE) is represented as retention time, while time spent in the small intestine (SI1-4) and colon (PC-DC) is represented as transit time. Any significant (P<0.05) interactions between diet and gastrointestinal tract site (P>0.05) are marked by an asterisk (*). Data is expressed as mean±standard error. SI: small intestine, PC: proximal colon, MC: mid colon, DC: distal colon.

There were no AIA measurements for SI1 due to insufficient digesta collected for AIA anlysis. This was a common problem for all thirty pigs where SI1 always contained the least digesta, ranging between 3-43 g as compared to other sites, which ranged between 800 and 1600 g. The transit time of SI1 using Cr was obtained from only three pigs and was very short (<0.5 h), but does not reflect a statistical representation, therefore this value was not used to calculate the passage rate for SI1 (Fig 5.6).
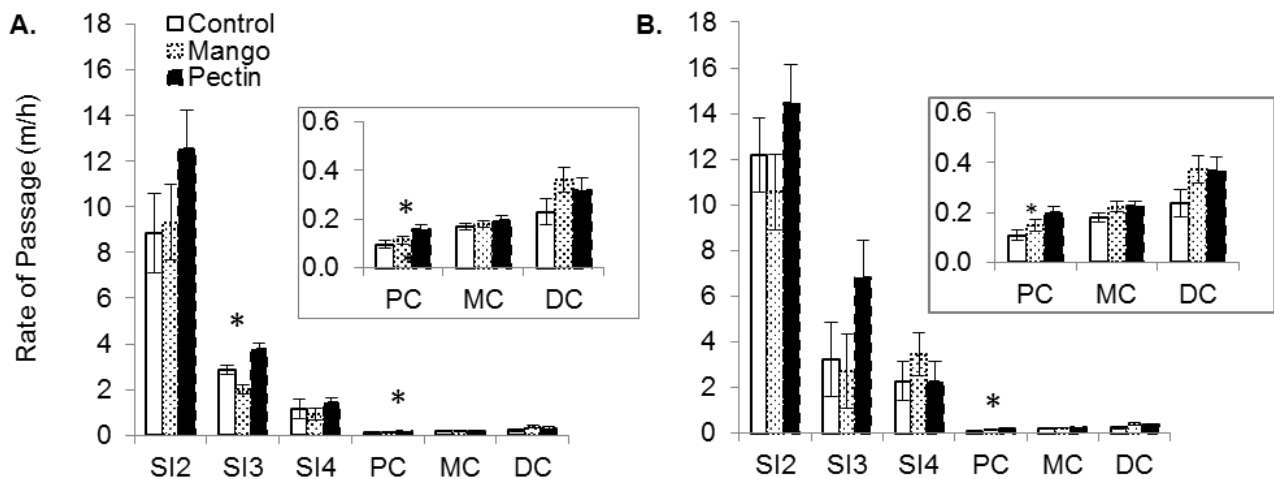
Figure 5.6. Rate of passage (RoP) at which (A) acid insoluble ash and (B) chromium pass through the gastrointestinal tract of pigs fed the control, 15% mango or 10% pectin diets. The rate of passage for the proximal to distal colon has been expanded for (A) and (B). Stomach and caecum are mixing pools and therefore, excluded in this calculation and figure. Any significant (P<0.05) interactions between diet and gastrointestinal tract site (P>0.05) are marked by an asterisk (*). Data is expressed as mean±standard error. SI: small intestine, PC: proximal colon, MC: mid colon, DC: distal colon.

5.4.5. Retention and transit times of pulse dose markers

The passage of pulse dose solid and liquid phase markers at different time intervals along the gastrointestinal tract is shown in Fig 5.7 (and Fig A4.1, Appendix 4). Ce was administered at an earlier time interval (2 h before the other markers) and had travelled furthest along the tract to the DC, mostly being retained in the caecum and PC. Yb fed 4 h pre-euthanasia, travelled to the MC with a longer transit time in SI3 whereas La and Co which were fed 2 h pre-euthanasia, only reached the ileum (SI4) and mostly remained in the stomach. Transit time in the duodenum (SI1) was <0.06 h for all markers, although this was only determined from pigs who had sufficient digesta in SI1. An significant effect of diet (P<0.05) was observed for the pulse dose markers (Fig 5.7), where retention and transit times were highest in the mango digesta for Ce and Yb, particularly in the caecum and PC. Between the 2 h markers, La, the solid marker had a sigificantly longer transit time (P=0.02) in the jejunum (SI2-3) for the mango digesta, while Co, the liquid marker, in the control diet had the shortest retention time (0.3 h) in the stomach and fastest transit

time in SI3, suggesting that Co travelled more rapidly through the tract in the liquid phase of digesta in the absence of the pectin. Increased concentration of pectin or mango in the diet led to a significantly (P=0.002) increased retention time in the stomach and transit time in the small intestine.
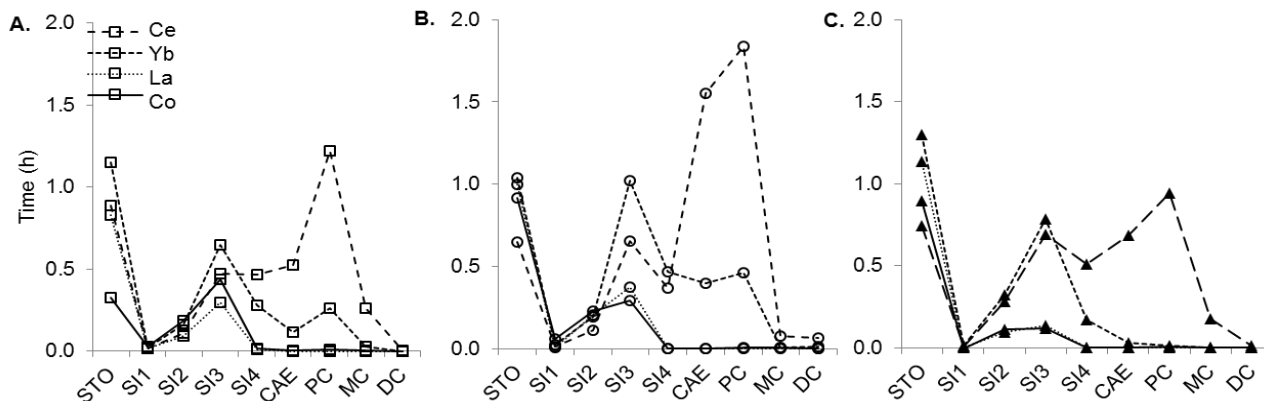
Figure 5.7. Retention and transit times of pulse dose markers: cerium (- - -), ytterbium (− −), lanthanum (· ·) and cobalt (·····) along the gastrointestinal tract of pigs fed the (A) control (□), (B) 15% mango (O) or (C) 10% pectin (▲) diets. Time spent in the stomach (STO) and caecum (CAE) is represented as retention time, while time spent in the small intestine (SI1-4) and colon (PC-DC) is represented as transit time. SI: small intestine, PC: proximal colon, MC: mid colon, DC: distal colon.

5.4.6. Fractional outflow rates of stomach and small intestinal contents

Fractional outflow rates of digesta leaving the stomach, and influx and outflow rates into the small intestine are shown graphically in Fig 5.8. Table A4.1 (Appendix 4) shows the numerical fractional outflow and influx rates. At 6 h pre-euthanasia, the stomach fractional outflow rate of the pectin-fed pigs was more rapid than the stomach fractional outflow from control-fed pigs, but became similar after 2 h pre-euthanasia.
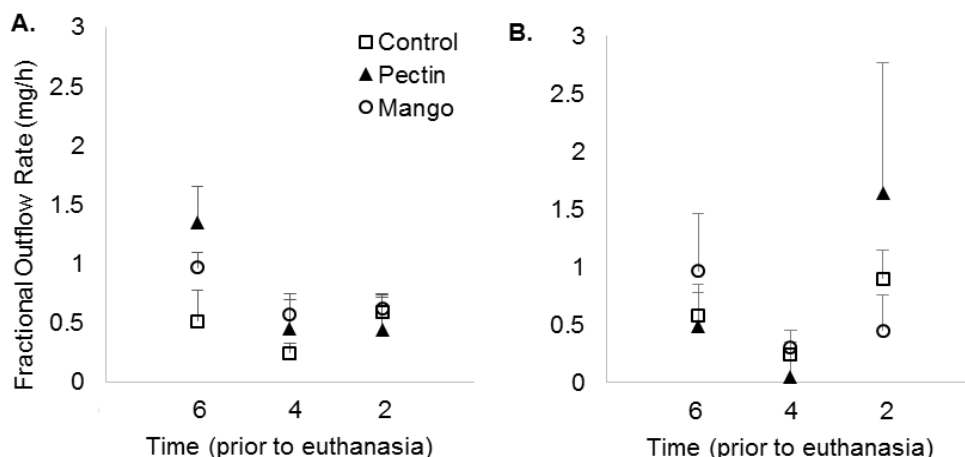
Figure 5.8. Fractional outflow rate of (A) stomach and (B) small intestinal digesta at 6 h, 4 h and 2 h post-euthanasia from pigs fed the control (□), 15% mango (O) or 10% pectin (▲) diets. Data is expressed as mean±standard error. Fractional outflow rate (mg/h) was calculated as outflow divided by pool size, per hour.

5.5. Discussion

5.5.1. Methodological considerations

Unlike other reported studies, gastrointestinal tract contents were not recovered using cannulae, rather the contents were collected at one time-point post-euthanasia. Additionally, the length of each gastrointestinal tract site was determined, allowing for the passage rate to be expressed in terms of meters per hour as opposed to a quantified marker concentration passing a specific site with unknown length or volume approximates, as did Wilfart et al. (2007a).

During formulation of the experimental diets, Cr was one of the last feed components to be added to the feed mixing vessel. Cr was added in a lesser quantity than AIA, and equal dispersion of this marker may not have been entirely achieved despite rigorous mixing. Analysis of Cr in feed samples revealed concentrations varying from 191-222 mg/kg DM. To improve the homogeneity of Cr or any marker to be added in very low quantities, it is suggested that mordanting to a dietary component be done prior to mixing into the diet. AIA was added in considerably larger quantities (24-26 g/kg DM); hence achieving homogeneity was not a concern. Despite the challenge of obtaining Cr homogeneity in the feed, the amounts of Cr and AIA recovered in each gastrointestinal tract site were not significantly different (Fig 5.3). Consequently, the profiles of retention and transit times between the two markers were very similar (Fig 5.5), leading to similar digesta passage rates regardless of homogeneity concerns.

5.5.2. Lower dry matter content in the digesta reduced retention and transit times

The digesta DM had a significant impact ($P<0.05$) on the total tract transit time from the stomach to the DC (Table 5.3 and Fig 5.5). Generally, a higher digesta DM contributed to a longer transit time. The averaged total digesta retention and transit times was 21 h for the pectin diet, 24 h for the mango diet and 25 h for the control diet. The pectin diet had the highest WHC, while the pectin-fed pigs had the shortest transit time and therefore had a faster passage rate along the gastrointestinal tract. An accelerated rate of flow of digesta through the colon by the addition of soluble and insoluble dietary fibres such as supplementary bran, lactulose and pectin to the diet has been reported for pigs (Kass, Van Soest, Pond, Lewis, & Macdowell, 1980; Low, Nelson, & Sporns, 1988; Wilfart, Montagne, Simmins, Noblet, & van Milgen, 2007b; Wilfart, Montague, Simmins, Noblet, & Van Milgen, 2007a).

It should be noted that there is a 2 h period from the last feeding to time of slaughter so the reduction in dry matter content of the diets (91-92%) after ingestion to digestion in the stomach (24-31%) is not immediate. During ingestion of the dry feed, water was added to the feeding trough to improve palability and for ease of swallowing the feed, and the pigs had *ad libtum* access to water. These conditions, along with the addition of saliva and gastric secretions all contributed to increased moisture content of the stomach digesta after passage from oral ingestion. In the stomach, the digesta DM in pigs fed the control diet was higher than in pigs fed the mango and pectin diets due to increased water retention of these two diets, resulting in easier passage of the liquid digesta phase into the small intestine for the control diet. This is validated by Co, the 2 h liquid phase marker, in Fig 5.4D where 30% of Co was recovered in the stomach digesta of control-fed pigs compared to 60-80% recovery in the mango- and pectin-fed pigs. Digesta contents are known to be heterogeneous and in addition to the presence of pectin with water holding capacity (in the mango and pectin diets), led to differences in particle drainage and rate of digesta transit into the small intestine.

In the duodenum (SI1), the digesta from mango and pectin-fed pigs were expected to have a lower DM content due to their swelling/solubility properties as observed from the increased WHC of the pectin diet. From SI1 to SI2, digesta DM decreased in the pectin diet but rose in the other two diets, suggesting that less water was taken up from the pectin (diet) digesta for absorption from this segment compared with the other two. In SI3, irrespective of diet, water was removed from the digesta with residual DM% reflecting again the higher WHC of pectin component in the diet and digesta. In the ileum (SI4), there was a clear expression of WHC of pectin, where pectin digesta held significantly more water than the control (P=0.0003). In the caecum, water was removed from the control digesta due to lack of structuring; however, water was removed from the pectin digesta presumably as a result of rapid fermentation, leaving residual pectin digesta with similar properties to the control residual digesta. The mango digesta maintained a low DM% potentially due to less extensive fermentation of the cell wall material in the mango component compared with fermentation of the soluble pectin diet.

Throughout the colon from PC to DC, the digesta continued to lose water due to the colon's high capacity for water absorption (Sandle, 1998) and depletion of available substrates by fermentation. The increase in digesta DM from the PC to DC suggests there was a cease in the colonic-microbial activity, leading to an accumulation in non-

fermentable components in the DC. Wiggins (1983), and Cummings and Macfarlane (1991) have previously reported fermentation activity being greatest in the left (proximal) colon, and then the digesta was ejected though the transverse (mid) colon to the right (distal) colon for storage and eventual excretion in humans. Digesta from control-fed pigs reached 42% DM, also potentially due to lower numbers of water-holding bacteria (Friedman & Henry, 1938). The digesta DM from mango-fed pigs started to increase and become similar to that of pectin-fed pigs as the mango component fermented. The digesta from pectin and mango-fed pigs had lower DM% than the digesta from the control-fed pigs potentially due to greater levels of water-holding bacteria. As pectin is readily fermentable in the colon (Jorgensen, Zhao, & Eggum, 1996; Sunvold, Hussein, Fahey, Merchen, & Reinhart, 1995), those colonic bacteria utilising pectin as a substrate proliferated rapidly too. Those bacteria having a high water content (Bratbak & Dundas, 1984; Friedman & Henry, 1938) contributed to the higher water content of the digesta from the pectin-fed pigs. In addition, Satchithanandam, Vargofcakapker, Calvert, Leeds and Cassidy (1990) suggested that fibre induced increments in gastrointestinal mucin production may be responsible for the increased transit time found in their rat feeding study.

These results however, are in disagreement with other previous findings (Owusu-Asiedu et al., 2006; Potkins, Lawrence, & Thomlinson, 1991). Potkins et al. (1991) concluded that guar gum and pectin (10 and 50 g/kg) did not significantly affect the total tract transit time, while Owusu-Asiedu et al. (2006) found that guar gum and cellulose decreased digesta passage rate to the ileum by 0.42%/h and 0.3%/h respectively. A number of factors in addition to diet have been described to influence retention time, including animal weight (Le Goff, Van Milgen, & Noblet, 2002), age (Almirall & Esteve-Garcia, 1994) and feeding frequency (Goetsch & Galyean, 1983).

5.5.3. Acid insoluble ash and chromium as passage markers for retention time, transit time and passage rate

Digesta transit through the stomach and small intestine averaged 3 h and 3.2 h respectively, whereas a relatively longer transit time was recorded for the caecum and colon (19 h). Similar retention and/or transit times have been reported for pigs, varying between 2-16 h in the stomach and small intestine (Guerin et al., 2001; Hendriks, van Baal, & Bosch, 2012) and 20-40 h through the colon (Low, 1993). The present study is the first known study to report digesta transit time and passage rate along numerous gastrointestinal tract sections, and provided insights into the dynamic passage of digesta,

particularly in the presence of a soluble fibre both in its isolated form and as a component of food. The small intestinal compartment showed variable transit times from 0.3 h in the duodenum (SI1), 0.5-1.6 h in the jejunum (SI2-3) and 0.8 h in the ileum (SI4). Through mathematical modelling, Letourneau-Montminy et al. (2011) estimated transit time in the proximal segment of the small intestine to be 0.2 h, which is comparatively similar to results from the present study. But their transit time calculations of 3 h in the distal segment (Letourneau-Montminy et al., 2011) may have been grossly overestimated.

After leaving the stomach mixing pool, the digesta travelled rapidly through the first half of the jejunum (SI2) at 10 m/h, then slowed down to 3 m/h in the remaining half (SI3) and 1 m/h in the ileum (SI4) (Fig 5.6). A decrease in passage rate was observed in the small intestine, which agrees with a lower frequency of segmental contractions in the distal ileum in comparsion to the duodenum (Laplace, Aumaitre, & Rerat, 2001; Van Weyenberg, Sales, & Janssens, 2006). Van Weyenberg et al. (2006) also reported a quicker transit rate of 30 cm/min or 18 m/h for most of the digesta in the small intestine (in horses), which may not be entirely true for all the sections of the small intestine in other domestic animals. As transit time and passage rate are inferred from the quantity of markers recovered in the pig digesta post-euthanasia, a time delay resulting from myoelectric migrating complexes and colonic (aboral, propagated and isolated) contractions were not seen in this study. This leads to a probable conclusion that the rate of digesta passage may be more rapid in the small intestine.

Interestingly, across all diets, transit time in the PC (7.2 h) was significantly (P=0.03) higher than that in the MC (4.7 h) and DC (3.6 h) despite being divided equally into three portions based on length (0.88 m each). It has been suggested that peristaltic actions stimulating propulsive colonic motility may be responsible for this differential passage of digesta (Crema, Frigo, & Lecchini, 1970; Edwards, 1990; Laplace, 1981). The total colon transit time, including the caecum averaged 18 h for the solid digesta phase and was much shorter than the 35 h reported by Wilfart et al. (2007a).

5.5.4. Retention time in the stomach is reduced by the presence of pectin and mango in the digesta

In the stomach, the retention of Co from pigs fed the control diet (Fig 5.7) was shorter (0.3 h) than the retention of La (0.8 h), suggesting that the liquid phase of the digesta moved faster than the solid phase except when pectin was present, which is consistent with pectin

being able to hydrodynamically retain water (Liu, Cooke, Coffin, Fishman, & Hicks, 2004; Vandamme, Lenourry, Charrueau, & Chaumeil, 2002). Latymer et al. (1985) also reported that there was a lack of liquid and solid phase separation in the digesta from pigs that were fed pectin and guar gum, and this was attributed to the high viscosity and colloidal nature of the solutions (Latymer, low, & Woodley, 1985). Hypertonic solutions have also demonstrated a slower gastric emptying rate (Paraskevopoulos, Houghton, Eyre-Brooke, Johnson, & Read, 1988).

Retention of digesta in the stomach averaged 3 h in the present study compared to literature retention values in pigs varying greatly from 1-7 h (Guerin et al., 2001; Hendriks, van Baal, & Bosch, 2012), which are strongly influenced by dietary components. Previous studies have reported that retention time in the stomach decreased when diets were supplemented with guar gum and pectin (Potkins, Lawrence, & Thomlinson, 1991) or not influenced at all (Latymer & Low, 1985). Potkins, Lawrence and Thomlinson (1991) determined the rate of gastric emptying via the decrease in total DM content in the stomach at various time intervals (0.5, 1, 2, 4, 7.5 h) post-feeding to the total DM of the given meal. They recovered less DM in the stomach of pigs fed guar gum and pectin 1-4 h postprandially, but did not determine the amount of liquid in the stomach at these time points. Inferring the gastric emptying rate based solely on stomach DM contents neglects the fact that stomach contents comprise both a solid and liquid phase. The results from the present study showed that those pigs that were fed pectin retained significantly more liquid in the stomach (Fig 5.7), and at 6 h postprandially (Fig 5.8). The liquid phase of the stomach contents from the pigs fed the control diet had progressed significantly more to the small intestine. Pectin had a higher affinity for water, based on the WHC results in the present study, and has been described to form a gel in the stomach (Drochner, Kerler, & Zacharias, 2004), which restricts the liquid phase on passing through the pyloric valves. However, pectin did not affect the retention time of the solid digesta phase in the stomach based on the use of any of the other three pulse dose markers.

5.6. Concluding Remarks

The selection of solid and liquid markers to measure the independent flow in defined (solid and liquid) phases improved the reliability of digesta flow estimates in the diets containing wheat starch, mango and pectin. The mango ingredient containing soluble fibre (pectin) and purified pectin delayed gastric fractional outflow to the duodenum, showing pectin being able to hydrodynamically retain water, as reflected in a lower dry matter content and

increased water holding capacity of the pectin diet. However, after leaving the stomach compartment, the rate of digesta transit through the small intestine and colon, for pigs fed these two diets, was significantly quicker as a result of increased digesta viscosity. This is the first study to show differences in transit times within segments of the small intestinal tract (SI1: 0.3 h, SI2: 0.5 h, SI3: 1.6 h, SI4: 0.8 h) and within the colon (PC: 7.2 h, MC: 4.7 h, DC: 3.6 h). The constant feed marker, AIA confirmed its reliability in providing insights into the dynamic passage of digesta along the digestive tract, and the use of pulse dose markers permitted the determination of the fractional outflow rate of digesta, specifically in the stomach and small intestine. Recommendation for future work continuing from this study includes improving the homogeneity of Cr or any markers to be added in very low quantities by mordanting to a dietary component prior to mixing with the other ingredients in the diet.

Chapter 5. Passage of mango and pectin, and polyphenol metabolism in the gastrointestinal tract of pigs – a human model

Part B. Can mango polyphenol metabolism in the gastrointestinal tract and blood of pigs be monitored after feeding mango-containing whole diets?

5.7. Introduction to Part B

Polyphenols are receiving much current attention due to potential beneficiary health effects related to their biological and pharmacological properties e.g. (Drouin et al., 2011), anti-inflammatory (Peri et al., 2005; Suzuki et al., 2010), anti-carcinogenic (Hertog et al., 1995; Yamanaka, Oda, & Nagao, 1997), anti-aging (Drouin et al., 2011; El Gharras, 2009), and cardio-protective and vasodilatory potentials (Erdman et al., 2007; Fraga & Oteiza, 2011), suggesting an association between consumption of polyphenol-rich foods and a reduced risk of several chronic diseases. These studies have characterised molecular components and signalling pathways in cells using a range of *in vitro* and laboratory techniques, and linked these to specific bioactivities as a means of defining potential health benefits of consuming specific fruits or vegetables.

Over recent years, research on the *in vivo* metabolic fate of polyphenols has been actively growing, focusing on detecting original/parent compounds and secondary metabolites in plasma, urine, tissues or faeces. It is crucial that bioavailability studies use metabolites that are actually found in the human body, since absorption is accompanied by extensive conjugation and metabolism, and the forms appearing in the plasma and target organs, and those as a result of digestive or hepatic activity, are different from the native structures in food (Manach et al., 2004).

Absorbed metabolites appear transitorily in plasma, but are treated by the human body as xenobiotics and are quickly removed from the bloodstream. While plasma analysis provides insights into pharmacokinetic profiles of circulating metabolites after short-term supplementation, 'area under the curve' estimates do not provide accurate quantitative assessments of uptake from the gastrointestinal tract (Crozier, Del Rio, & Clifford, 2010). Urinary excretion provides a more realistic estimate, but excludes the possibility of sequestration in body tissues and underestimates the degree of absorption. Vital information such as the specific intestinal site of metabolism, tissue distribution and accumulation is still lacking. Approaches to quantify the intestinal absorption of polyphenols in humans include intake-excretion balance and total plasma 'phenolic

response' (Clifford, van der Hooft, & Crozier, 2013), but are estimates per se. Phenolic metabolism in animal models has been investigated mostly in rats, but pigs would be a preferable animal model as the porcine digestive system is more physiologically and anatomically similar to humans (Almond, 1996; Miller & Ullrey, 1987).

Simple pig feeding studies have been carried out using single purified compounds (Kreuz et al., 2008; Lesser, Cermak, & Wolffram, 2004; Luehring, Blank, & Wolffram, 2011; Wang et al., 2007) or as a mixture (Bock, Waldmann, & Ternes, 2008) but these fall short of capturing the confounding variables present in a 'complex' food system. Only one study has examined a system approaching that of a whole food (ellagitannin metabolism in pigs that were fed acorns) and reported that the degradation of ellagitannins started from the jejunum and the last metabolite was produced in the colon (Espin et al., 2007). However, this study included an acorn-only diet, which is not a diet of normal human consumption. Digesta from the jejunum and colon were collected, but no measurements regarding the two sites were specified. In contrast, the present study aimed to add food ingredients found in human diets into the pig feed, and to examine the fate of dietary polyphenols through the digestive system.

So far, no reported studies have examined the *in vivo* intestinal metabolism of polyphenols using fruits. Therefore, a pig feeding trial using a diet containing 15% dried mango fruit powder was carried out to address this knowledge gap. Here, the aim was to study the degradation of intact original polyphenols in mango fruit, and their biotransformation to respective catabolites, in defined sites of the entire gastrointestinal tract in 'real time' (Chapter 5 Part B), based on measured digesta passage rates (Chapter 5 Part A).

5.8. Materials and Methods
5.8.1. Pigs, diets, experimental design, housing and feeding
All animal and experimental procedures are described in Part A (sections 5.3.1-5.3.4).

5.8.2. Collection of digesta, blood and urine
At two weeks prior to the introduction of the experimental diets (June 2013), baseline blood sample (20-30 mL) from the pig's jugular vein (JV) was withdrawn into EDTA-vacutainers and centrifuged at 4500 g for 10 min at 4°C to obtain (6-12 mL) plasma. Additionally, after two weeks of feeding the experimental diets to these pigs, JV blood samples were similarly collected. The plasma was acidified with 10.44 M TFA (1.2 mL) and

immediately stored at -80°C. During the slaughter week, digesta samples from six gastrointestinal tract sites (stomach, SI1, SI2, SI4, caecum, PC, and DC) (Fig 5.1) were collected and stored (section 5.3.3). Blood from the JV and hepatic portal vein (HPV) were also collected, centrifuged and stored similarly as to the baseline plasma. Any haemolysis in plasma samples was recorded, which may interfere with polyphenol extraction. Urine (30 mL) was collected, acidified with formic acid (10:1), and immediately stored at -20°C.

5.8.3. Sample preparation and extraction

Digesta samples were thawed overnight at 4°C. Next, digesta sample (1 g) was spiked with 10 µL quercetin (50 µg/mL in 80% methanol) as an internal standard, homogenised with 5 mL acidified 80% methanol (180 rpm, 15 min) and then centrifuged at 14,000 g for 10 min. Samples were then processed according to Espin et al. (2007) with modifications. The supernatant was collected and the methanolic phase was vacuum evaporated to <2 mL at 50°C (2 h) using a MiVac Speedvac (Scitek, NSW, Australia). Formic acid (12 mM; 10 mL) was added to the remaining aqueous extract and sonicated for 5 min. Sep-Pak C18 (12 CC, 2 g) cartridges (Waters, NSW, Australia) were conditioned sequentially with 2 x 5 mL methanol, equilibrated with 2 x 5 mL formic acid (12 mM), loaded with previously prepared extracts, washed with 2 x 5 mL formic acid (12 mM) and the phenolic fraction was eluted with 10 mL acidified methanol (12 mM formic acid) using a vacuum manifold. Eluates were vacuum evaporated at 50°C (~10 h) to <2 mL, frozen, and freeze-dried overnight. The extracts were reconstituted with methanol:acetonitrile (50:50) acidified with 0.1% formic acid, filtered through 0.2 µm GHP Acrodisc syringe filters (Pall, NSW, Australia), kept at -20°C, and analysed by UPLC-PDA and UHPLC-Q-ToF-MS.

Plasma samples (3 mL) were thawed at room temperature for 1 h, spiked with 40 µL quercetin (50 µg/mL) and centrifuged at 140,000 g for 10 min. Polyphenol extraction from plasma used a method that was slightly modified from that of Frank, Netzel, Strass Bitsch and Bitsch (2003). C18 cartridges (900 mg, Grace Davison, Victoria, Australia) were activated with methanol (2 x 5 mL) and equilibrated with 1.5 M formic acid (2 x 5 mL), after which plasma (3 mL) was loaded. The cartridges were then washed again with 2 x 5 mL 1.5 M formic acid and 2 x 5 mL dichloromethane to remove dissolved proteins, and lipid soluble hormones and fatty acids. The phenolic fraction was eluted with 10 mL 0.44 M TFA in methanol, vacuum evaporated at 50°C for 3 h, reconstituted with 50% acidified acetonitrile (0.1% formic acid) to 1 mL, filtered through 0.2 µm GHP Acrodisc syringe filters, and finally, kept at -20°C prior to UPLC-PDA and UHPLC-Q-ToF-MS analysis.

5.8.4. UPLC-PDA analysis

UPLC-PDA analysis was carried out on a Waters Acquity^{TM} UPLC-PDA system with a VisionHT C18 Basic column (100 x 2 mm, 1.5 μm) equipped with a guard column (5 x 2 mm), and operating at 30°C. Mobile phases A and B were 0.1% formic acid and 0.1% formic acid in acetonitrile respectively. The best separation was obtained using the following gradient elution: 98% A (3 min), 96-70% A (10 min), 70% A (5 min), 70-40% A (2 min), 40-2% A (0.1 min), 2% A (4.9 min), 2-98% A (0.1 min), 98%A (4.9 min) at 0.3 mL/min. The injection volume was 5 μL. UV-Vis spectra were recorded from 210-498 nm. Data acquisition was carried out using Empower Pro v.2 software. A calibration curve was constructed from ferulic acid (0.25, 0.5, 1, 5, 10, 20 μg/mL) at 320 nm and phenolic concentrations were corrected to per g DM, reported as the average of six (pig) replicates and calculated as ferulic acid equivalents at 320 nm. Chromatographic peaks were gathered and assigned peak numbers common to the diets and digesta.

5.8.5. UPLC-ESI-Q-ToF-MS analysis

UHPLC-Q-ToF-MS conditions were the same are described in Chapter 4 Part B (section 4.8.3), except that 20 eV collision energy was used.

5.8.6. Calculations and statistical analysis

For polyphenol metabolism in the *in vivo* trial, only pigs fed the mango and control diets are of interest so the experimental unit was twelve pigs (six pigs per diet). Compound peaks detected in the chromatograms of digesta, plasma, urine, and diets were assigned numbers common across all UPLC-UV chromatograms. Individual phenolic concentrations in the digesta were corrected to per g of AIA marker recovered at each gastrointestinal tract site and pooled content was calculated from total digesta DM. Plasma phenolic concentrations were calculated as per mL. Polyphenol concentrations were analysed using Proc mixed in SAS 9.3 (SAS Institute, Inc., Cary, NC, USA), with diet as a mixed effect. The effects of Diet, Gastrointestinal tract site, and the Diet*Gastrointestinal tract site interaction were determined by the slice statement. Group means were analysed using Proc GLM and significance differences was set at $P<0.05$.

5.9. Results and Discussion
5.9.1. Methodological considerations

Quantification of peaks in the diets and digesta chromatograms was carried out at 320 nm, rather than 280 nm as described in Part B of Chapter 4, since the intense absorbance of a

large number of compounds at 280 nm resulted in the poor separation and co-elution of many individual peaks, leading to inaccurate quantification despite optimisation of the gradient elution program and serial dilution. An example showing the large disparity in chromatographic absorbance between 280 and 320 nm is shown in Fig 5.9 for the diets and the original mango powder. Because an absorbance wavelength of 320 nm was used for quantification, most of the detected compounds are assumed to be phenolic compounds consisting of extended aromatic ring structures, which have a characteristic absorbance at 320 nm. No other dietary compounds are expected to absorb at 320 nm.

5.9.2. Compounds detected in the original diets and mango powder

There are compounds unique to the mango powder and mango diet that are not present in the control diet, which indicates these compounds originate from the mango powder. These include compounds responsible for the peaks 1-4, 9-14, 17, 22 and 33 (Table 5.4 and Fig 5.10). Compounds 5 and 6 were only present in the mango powder, suggesting that they occur in low initial concentrations in the mango powder and therefore, may not be detected in the diet. Compounds 7, 8, 16, 18, 20, 25-30 were present in all the control diet, mango diet and mango powder, suggesting common compounds.

Table 5.4. Compounds detected in the chromatograms of extracts of the control and mango (15% mango powder) diets, and dried mango powder.

Peak no[1]	Retention time (min)	Control diet	Mango diet	Mango powder
1	2.218	✗	✓	✓
2	2.473	✗	✓	✓
3	2.704	✗	✓	✓
4	2.880	✗	✓	✓
5	3.851	✗	✗	✓
6	4.478	✗	✗	✓
7	6.251	✓	✓	✓
8	6.307	✓	✓	✓
9	6.377	✗	✓	✓
10	6.843	✗	✓	✓
11	7.043	✗	✓	✓
13	7.460	✗	✓	✓
14	7.504	✗	✓	✓
16	8.684	✓	✓	✓
17	8.488	✗	✓	✓
18	8.998	✓	✓	✓
20	9.514	✓	✓	✓
22	9.982	✗	✓	✓
25	12.253	✓	✓	✓
26	11.136	✓	✓	✓
27	11.707	✓	✓	✓
30	15.633	✓	✓	✗
33	12.093	✗	✓	✓

[1]*Peak numbers may not be assigned in order of increasing elution time. ✓refers to compounds that are detected in the diets or powder. ☐ refers to the absence of these compounds in the diets or powder.*
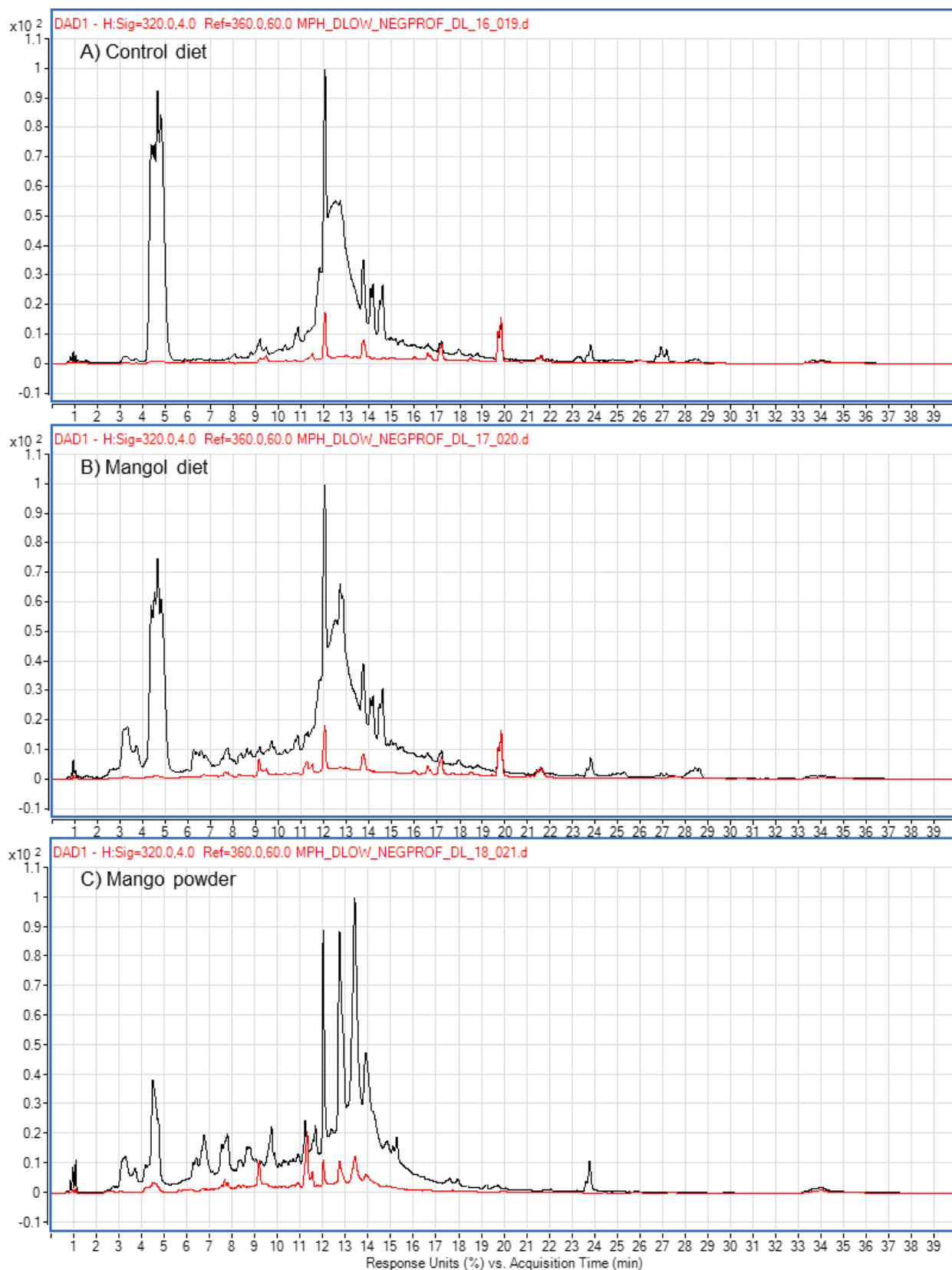
Figure 5.9. Chromatogram overlay showing differences in UV-Vis absorbance intensities between (A) control diet, (B) mango diet and (C) mango powder, at 280 nm (black) and 320 nm (red). Vertical scale reads 1.1 x 10^2 on all chromatograms.
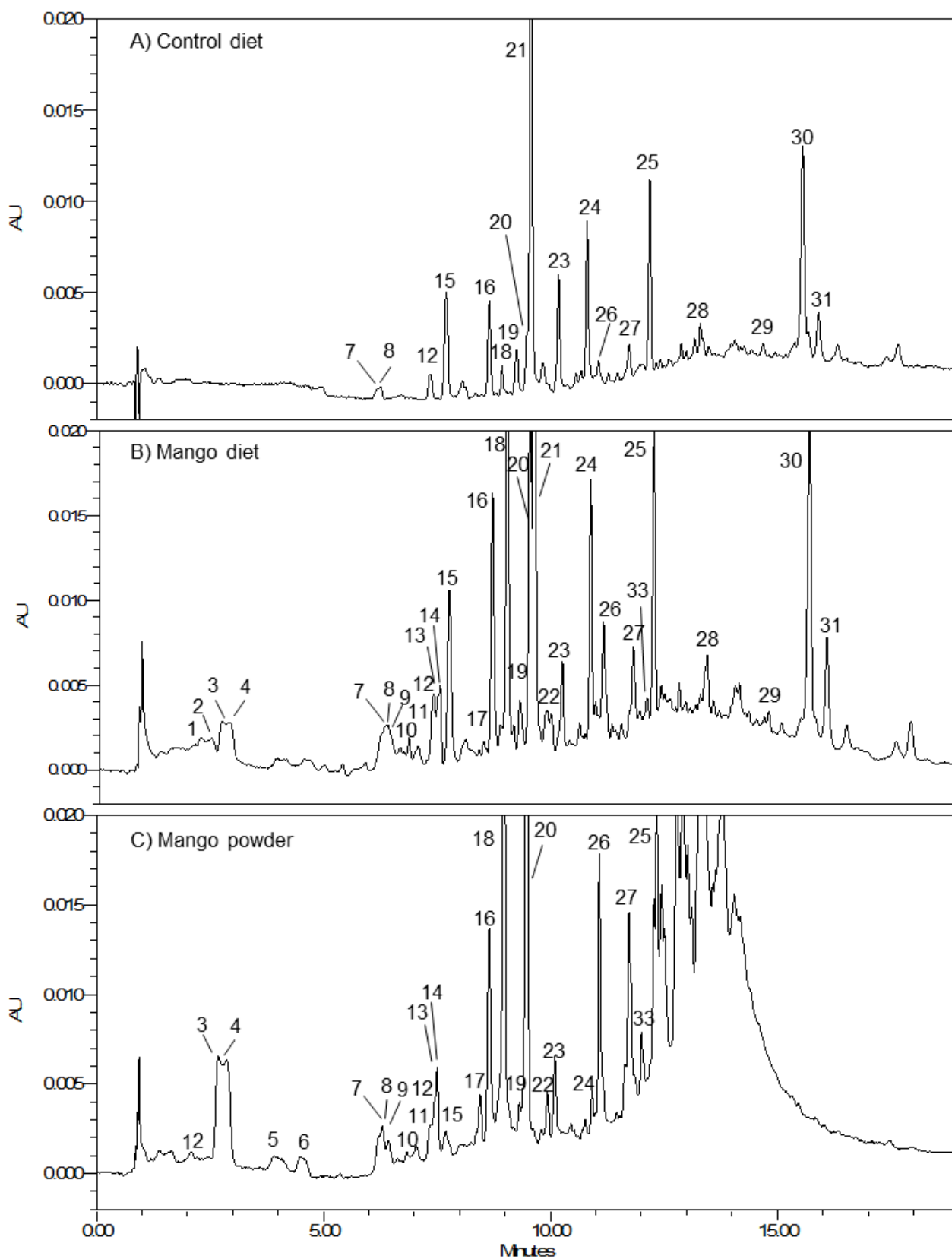
Figure 5.10. UV-Vis (320 nm) chromatograms of extracts of (A) control diet, (B) mango diet, and (C) mango powder. Peak numbers here correlate to those in Table 5.4. The chromatogram of the mango powder extract shows the chromatographic profile after a 2x dilution.

5.9.3. Biotransformation in the digestive tract

The addition of Celite (measured as AIA) as a constant marker in the diets was used to quantify the disappearance of the intact polyphenols and the production of metabolites by correcting their measured concentrations in the digesta to per g of AIA at various sites of the gastrointestinal tract. This removes the variables of total digesta volumes and DM content, which is influenced by simultaneous digestive processes including the absorption of dietary components, such as macronutrients and water, or the endogenous secretions of mucus, bile and enzymes.

The chromatographic profiles along the digestive tract from the stomach to the end of the colon of pigs fed the mango diet are shown in Fig 5.11. Investigation of the identities of the large number of compounds detected in the digesta was attempted, but due to the complex natures of the UPLC chromatograms produced by UV-Vs and MS detection, no compounds were identified in the present *in vivo* study. However, based on the used solvent gradient elution program, it is expected that those phenolic classes containing more hydroxyl groups and glycosides (being more polar) will elute earlier than the relatively less hydrophilic flavonols. In addition, those phenolic compounds or small intestinal metabolites that are absorbed and recycled back to the small intestinal sites (SI2-4), through enterohepatic circulation and bile excretion (Donovan et al., 2006), may undergo glucuronidation and/or sulfation which would render them more hydrophilic and early eluting (Manach et al., 2004). In the sampling sites of the stomach, SI2 and SI4, a larger number of early eluting and thus more hydrophilic compounds are observed, which suggest that highly hydrophilic compounds may be directly absorbed in the earlier segments of the gastrointestinal tract. However, for extracts of the caecum digesta, there is a shift in the compound elution profile to longer elution times (Fig 5.11), which reflects the passage and fate of less hydrophilic compounds along the digestive tract. Compounds detected by UPLC in extracts of the lower gastrointestinal tract samples, PC and DC, suggest that aglycones, microbial catabolites, and trapped phenolic compounds are likely to be released only in the caecum and colon. These findings are important, since no studies have reported the elution profiles of *in vivo* phenolic metabolites, especially after colon-microbial fermentation or liver conjugation.
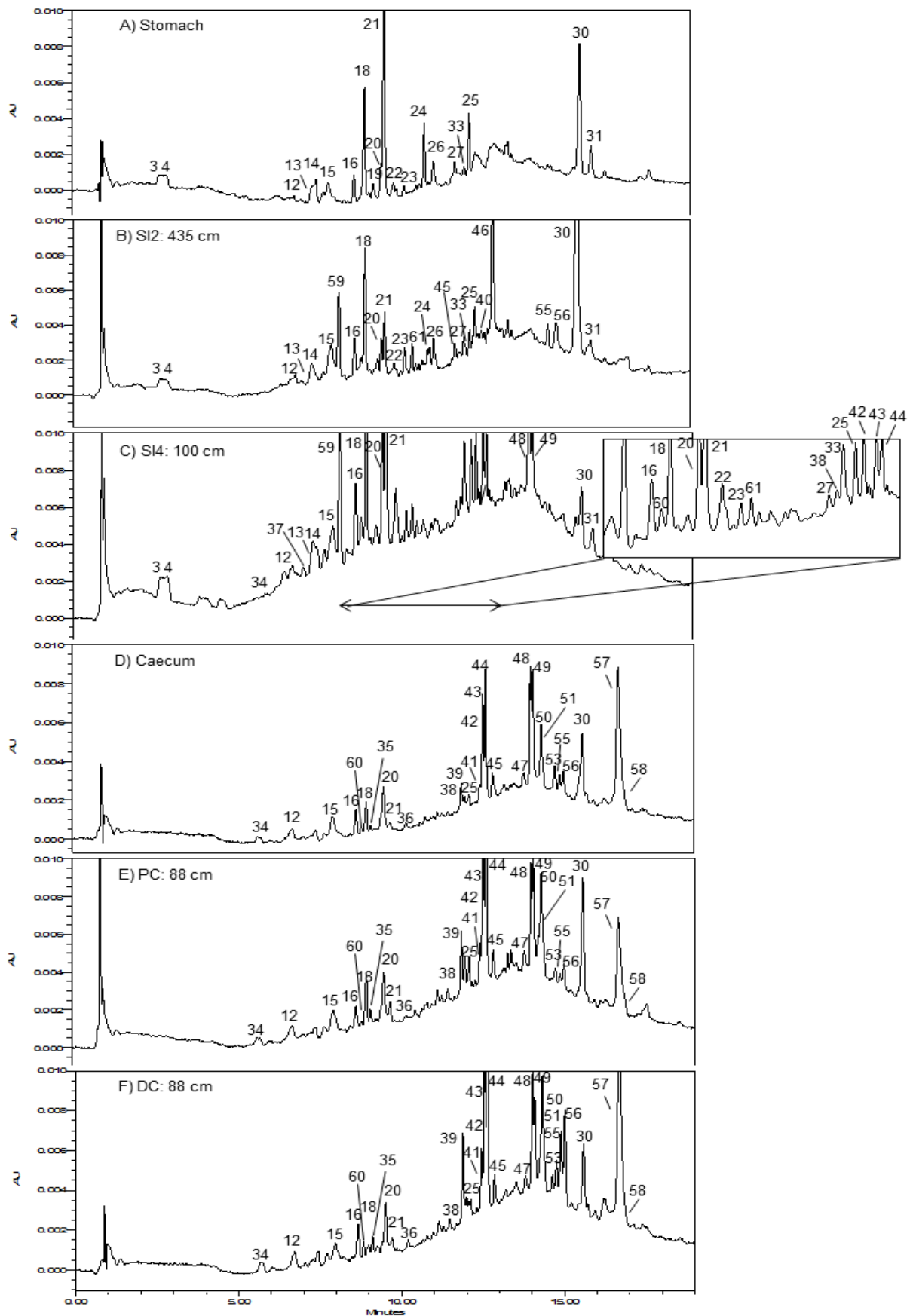
Figure 5.11. UV-Vis chromatographic profiles (320 nm) of digesta extracts from various gastrointestinal sites, A) stomach, B) SI2, C) SI4 (area containing dense number of peaks has been expanded), D) caecum, E) PC and F) DC of pigs fed the mango diet. Peak numbers correlate to those in Fig 5.12. Peak numbers may not be assigned in order of increasing elution time. SI: small intestine, PC: proximal colon, DC: distal colon.

The relative concentrations of compounds associated with peaks detected in the chromatograms from digesta of the control- and mango-fed pigs are shown in Fig 5.12. Overall, most of these compounds are present in higher concentrations in the digesta of the mango-fed pigs. However, compounds 22, 25, 27, 35, 38, 39, 41, 42, 44, 45, 47-50, 56 and 57 are present at similar or higher concentrations in the control-fed pigs digesta relative to the mango-fed pig digesta, and therefore, are not relevant in this discussion. However, all other peaks of interest show an interesting digestive profile. For example, compounds 1, 2, 13 and 14 displayed a distinctive disappearance profile from the stomach to SI2, suggesting these compounds were being degraded by intestinal porcine enzymes or absorbed into the intestinal epithelium within the duodenum and first half of the jejunum.

The concentrations of peaks 4, 10, 16, 18, 20, 26, 30, 33, 34, 40, 46, 54, 55 and 59 increased after leaving the stomach and reached their maximum concentration in SI2 before gradually decreasing during passage to SI4. As the concentration of these peaks have been corrected/adjusted for the indigestible AIA marker, it is unlikely this apparent increase in SI2 represents a greater concentration of slowly absorbing compounds from the uptake of water and rapidly absorbed nutrients from protein and starch. These compounds are more suggestive of intact phenolic compounds being liberated from protein interactions or other diet components during gastric digestion, or due to amplified mechanical interactions between the digesta and the finger-like projections of intestinal villi (Waltona et al., 2012), explaining the significant spike in concentrations in SI2 ($P<0.05$). There is currently no available literature on the gastrointestinal tract sites of, for example, de-glycosylation and/or uptake of polyphenols within the small intestine in pig models, except that reported by Espin et al. (2007) who reported that degradation of one class of polyphenols, the ellagitannins, started from the jejunum. These fourteen compounds are further metabolised, potentially leading to the formation of compounds 36, 51, 52, 53 and 58, which are produced from the end of the small intestine (SI4) and caecum (Fig. 5.12). These compounds accumulate through to the DC, suggesting they are derived from microbial metabolism and would, at least in part, be excreted out of the body through faeces. The complexity of chromatographic profiles may be challenging but classes of compounds were clearly observed to undergo distinctive digestive processing as a function of gastrointestinal tract location in this study, which would not be achievable in *in vitro* bioaccessibility and/or metabolism studies.
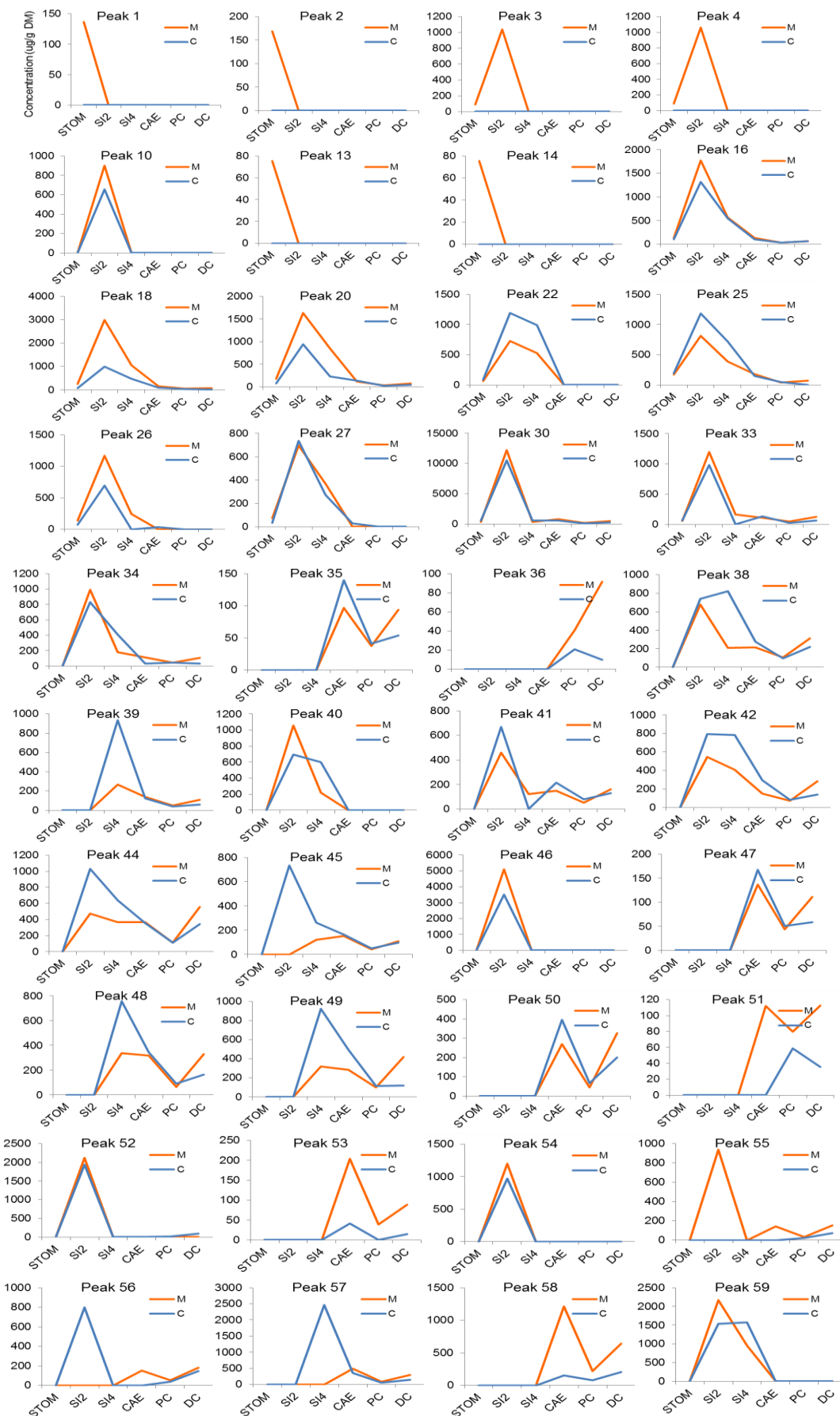
Figure 5.12. Compounds detected in the digesta (stomach, small intestine (SI2, SI4), caecum and colon (PC, DC) of pigs fed the mango (M) and control (C) diets. Peak numbers correspond to those of the diets. Concentrations of individual peaks are a pooled average of six pigs (for each diet).

5.9.4. Distribution of circulating metabolite profiles in plasma pre- and postprandial

There was a relatively smaller number of compounds (four) occurring in the HPV and JV plasma (Fig 5.13) in comparison to the diets or digesta extracts at the UV-Vis absorbance of 320 nm. However, quantification of these four compounds (peaks 3, 5, 8 and 13) was carried out at 280 nm, where these compounds had a stronger absorbance. In addition to peaks 3, 5, 8 and 13, fifteen compounds were detected in the HPV and JV plasma extracts of both sets of pigs fed the mango and control diets (Fig 5.14). Their circulation kinetics and concentrations are shown in Fig 5.15, but their identities remains un-elucidated.

All ninteen compounds are present in all the plasma extracts, i.e. there are no unique compounds in the HPV or circulating JV plasma from the mango diet. Most of the compounds are present in higher concentrations in the plasma of pigs fed the mango diet than the control diet, except for compounds 12, 13, 14 and 17, which suggest they are components from other diet ingredients rather than the mango powder. Compounds 3, 13 and 16 are an interesting group; although they are present in the baseline plasma of pigs fed the control and mango diets, they only appeared in the HPV and JV plasma of the mango-fed pigs after euthanasia.

Compound 10, although present in the plasma of both mango- and control-fed pigs, was not present in the baseline plasma and only appeared in the JV after the pigs were put on the mango diet for two weeks, suggesting this compound may be a phenolic metabolite (whatever was the source of the original polyphenol). Phenolic metabolites have been reported to have a long residence time in plasma (24-48 h after a single dose of administration of the precursor) (Kuijsten, Arts, Vree, & Hollman, 2005) (Vetrani et al., 2014). Compounds 1 and 2 are present in larger amounts in the HPV plasma than in the JV, indicating high absorption levels from the small intestine and/or colon, which are then transported through the tributaries of the super mesenteric vein (connecting the HPV) to the liver for Phase I and II metabolism. It is not confirmed where these two compounds are absorbed in the intestine, but their presence in the HPV plasma indicates that ingestion of these two compounds (or their respective parent compounds), and passage though the digestive tract, absorption through the intestinal epithelial cells and passage into the blood in the HPV took place rapidly within 2 h. Espin et al. (2007) have reported the absorption of free ellagic acids within 30 min to 1 h after intake.
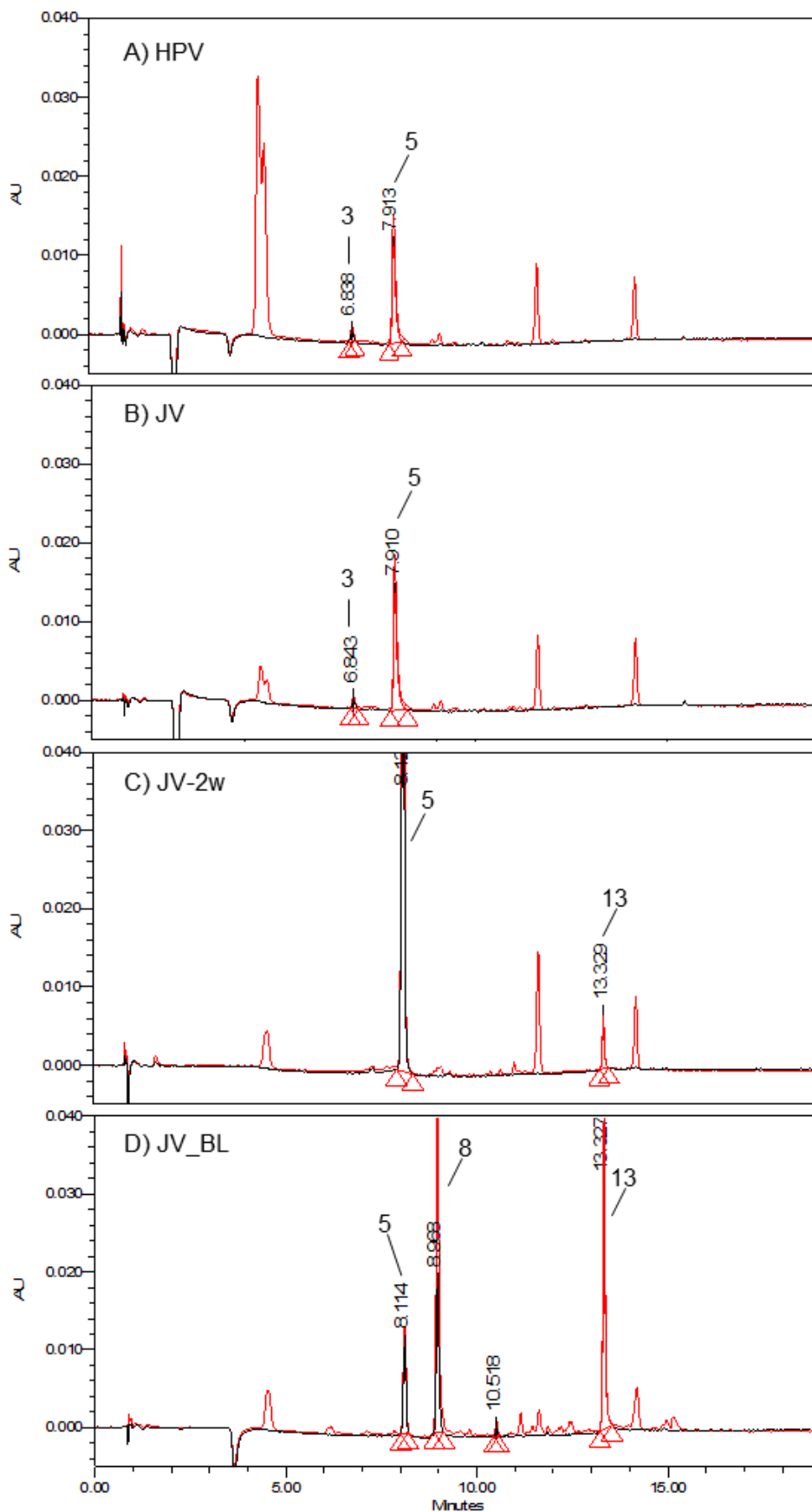
Figure 5.13. UV-Vis chromatograms at 280 nm (red) and 320 nm (black) of plasma from (A) hepatic portal vein (HPV), (B) jugular vein (JV), (C) JV_2w and (D) JV_BL of pigs fed the mango diet. JV_BL: JV baseline plasma (pigs on commercial diet), JV_2w: JV (two weeks after pigs were fed the experimental diets), HPV and JV: five weeks after the pigs were put on the experimental diets. Peak numbers correlate to those in Fig 5.15.
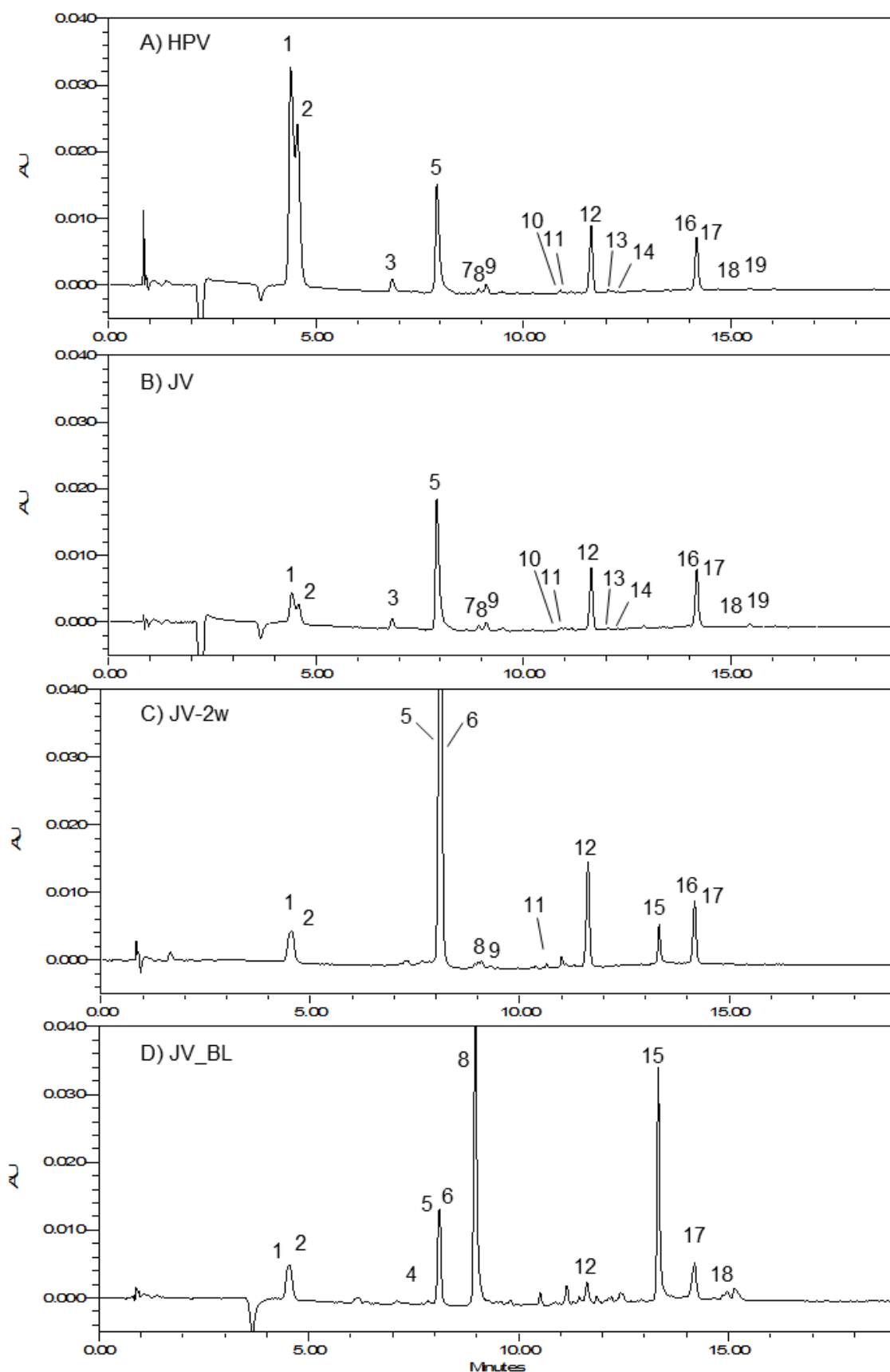
Figure 5.14. UV-Vis chromatograms (280 nm) of plasma from (A) hepatic portal vein (HPV), (B) jugular vein (JV), (C) JV_2w and (D) JV_BL of pigs fed the mango diet. JV_BL: JV baseline plasma (pigs on commercial diet), JV_2w: JV (two weeks after pigs were fed the experimental diets), HPV and JV: five weeks after the pigs were put on the experimental diets. Peak numbers correlate to those in Fig 5.15.
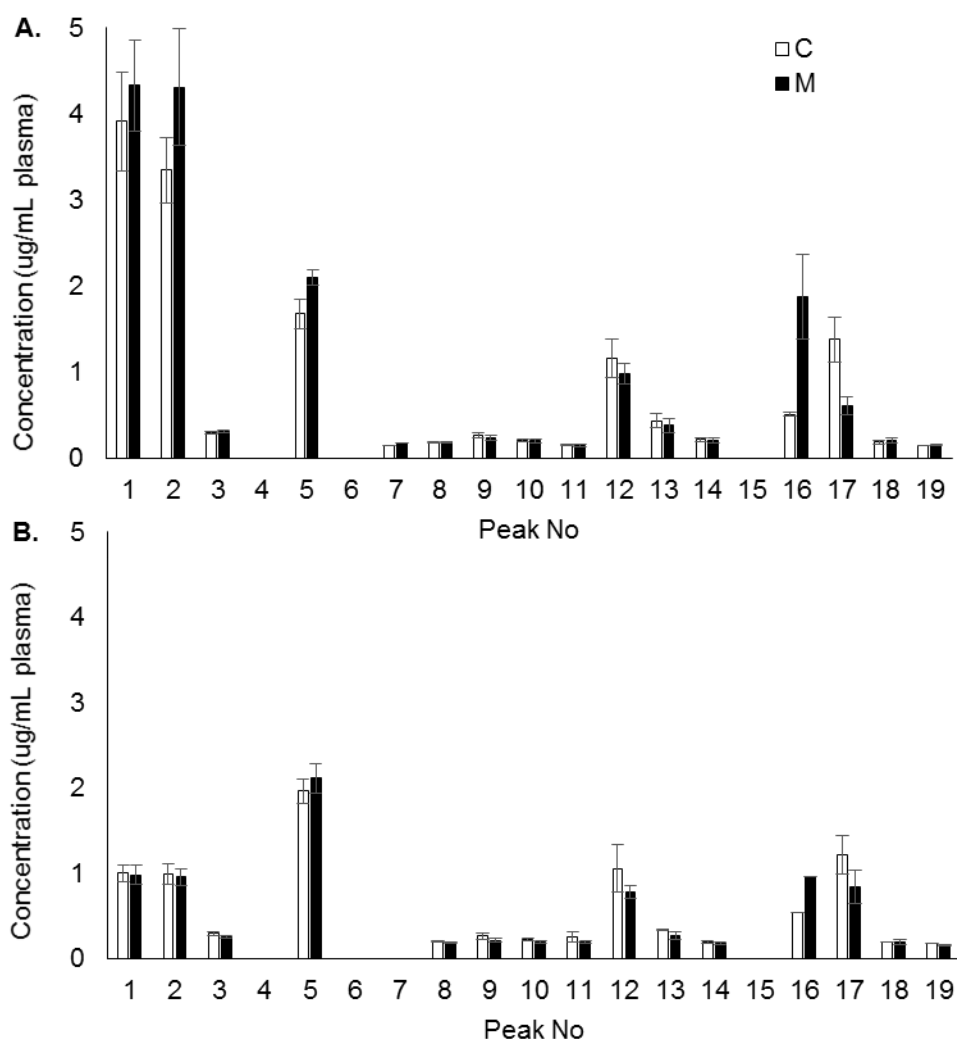
Figure 5.15. Concentrations (µg/mL plasma) of nineteen detected compounds from (A) hepatic portal vein and (B) jugular vein plasma extracts of pigs fed the mango (M) and control (C) diets. Data is expressed as mean±standard error of µg/g dry matter of ferulic acid equivalents. Concentrations of individual peaks are a pooled average of six pigs (for each diet).

5.10. Concluding Remarks

The possibility of monitoring mango polyphenol metabolism and uptake along the digestive tract and into the blood stream (hepatic portal and jugular vein) appears promising. The fate of individual compounds as a function of location in the digestive tract can be studied, but identification of molecular structures within such complex chromatographic profiles is a major challenge. There is evidence for at least three types of polyphenol type behaviour- rapid uptake, slow uptake and/or intermediary metabolism in the small intestine, and microbial fermentation primarily in the colon. Despite mango powder inclusion at 15% of the diet, and all other major components being relatively pure protein, starch and lipid (refer to diet list earlier in thesis), the polyphenolic content of the control diet was apparently comparable to or greater than that from mango. The reasons for this need to be further explored. The next step of this area of study would be to analyse individual diet

ingredients, using UPLC-PDA to assess their absorbance at 320 nm in the UV-Vis chromatograms. This would enable the determination of the components that are absorbing at the same wavelength as those polyphenols that have an extended aromatic ring structure. Feeding standard phenolic compounds to pigs may appear to be a more realistic and simpler approach, but the true residence passage time and site of absorption along the gastrointestinal tract would be different without a real fruit system to provide interactions with other diet cellular components. In order to understand whole diet effects (including passage rates), future work should continue to focus on the whole diet effect, but perhaps with more stringent screening of dietary materials to minimise complications from unintended dietary phenolics.

Chapter 6. General conclusions and recommendations

This thesis has focused on studying the effects on mango (*Kensington Pride*) and banana (*Cavendish*) fruit flesh of sequential digestive processing in the mouth, stomach, small intestine and colon, using a combination of *in vitro* and *in vivo* approaches. This study has shown that bioaccessibility of macro- and phytonutrients from whole food is initially affected by the mastication process, and then by potential gastrointestinal liberation from the encapsulating fruit matrix (cell wall and membrane). *In vivo* mastication conferred a range of particle sizes (large particle clusters from >5.6 mm to cell fragments of <75 μm) that was not achieved by simple blending (pureed cell components are 5-10 μm in size), in addition to the consequent actions of compression, squashing and formation of a bolus. Since previous *in vitro* digestion studies of whole fruits and vegetables have typically not investigated the influence of mastication on the release and/or bioaccessibility of macro- and micro-nutrients, both bioaccessibility and bioavailability values may have been overestimated in some reported studies. Despite some reported digestibility studies favouring mechanical processing or pureeing, there is a lack of emphasis on the critical importance of the mastication process. Results from the present study (Chapter 3) showed that the bioaccessibility of carotenes and xanthophylls is significantly overestimated in pureed mango (65-75%) in comparison to *in vivo* masticated fractions of varying particle sizes (20-50%); however, there was incomplete bioaccessibility after simulated gastrointestinal digestion. Future studies of nutrient release from fruit tissue involving standard two-phase *in vitro* digestion models will be improved by including an *in vivo* human chewing phase to realistically represent oral processing effects on bioaccessibility.

Microscopic observations of intact mango cells and vascular fibres after gastrointestinal digestion *in vitro*, established that some unreleased phytonutrients will survive to the colon. The subsequent *in vitro* fermentation of (mango and banana) cell structures after *in vitro* gastric and small intestinal digestion resulted in degradation of non-fibrous cell walls within 48 h, releasing the effectively encapsulated cellular contents for microbial metabolism. Upon disintegration of the cell walls, phenolic compounds were consequently liberated and exposed to faecal-microbial metabolism involving ring fission, dehydroxylation and decarboxylation that is beyond the capabilities of human endogenous enzymes. UPLC-PDA and UHPLC-Q-ToF-MS profiles revealed degradation of intact polyphenols within 8-24 h with concomitant formation and accumulation of catabolites within 4-8 h, confirming the colon as an active metabolism site for phytonutrients.

The presence of a fermentation-resistant starch fraction in banana, which was absent in mango, led to distinctive differences in fermentation kinetics (cumulative gas and short chain fatty acid and ammonia production) between these two fruits, reflecting a preferential degradation of (parenchyma) fleshy cells over resistant starch and over thick cellulosic vascular fibres (particularly from mango). Unexpectedly, the thin banana cell walls present before *in vitro* microbial fermentation were more readily fermented than the starch granules, which the cell walls had encapsulated previously. As microscopic degradation of banana cell walls was only investigated at 48 h, the time required for degradation of these cell walls is not known; therefore, more time intervals, preferably 3-hourly examinations should also be explored in future work following the present study, as well as an extended fermentation of ≥72 h to investigate the complete degradation of resistant starch and cellulosic fibres. The slow fermentability of banana starch conferred by its intrinsic resistance to pancreatic enzymes and faecal microbiota, and the presence of cell-wall encapsulation, may have implications on calorific content, satiety, glucose metabolism and passage rates (via faecal bulking) along the colon. The rapid butyrate production from mango fermentation, or the slow fermentability of banana starch (favouring butyrate production) is suggestive of a longer colon residence time, which is important in contributing to the anti-inflammatory, anti-carcinogenic and anti-proliferative properties that are so important to colon health. Butyrate impacts on cellular metabolic pathways in colonic cancer cells by inducing cell growth inhibition and differentiation (Blouin et al., 2011; Otles & Ozgoz, 2014).

The (retention) time available for nutrient digestion and fermentation in the gastrointestinal tract is determined by the passage rate of intestinal contents, which is influenced by dietary components. The present *in vivo* pig-feeding study showed that mango fruit pulp cellular components (cellulosic fibres and pectin) and purified pectin increased the passage rate of digesta through the digestive tract. There was a decreased dry matter content in the digesta of mango- and pectin-fed pigs, compared to the control diet containing mostly wheat starch, which demonstrated a differential water-holding capacity of the diets, resulting in an increased digesta viscosity and a reduction in overall passage time. The present study provided insights into the dynamic movement of gastrointestinal contents, which were found on average, to be 3 h in the stomach, 0.3 h in the duodenum, 0.5-1.6 h in the jejunum, 0.8 h in the ileum, 2.3 h in the caecum, 7 h in the proximal colon, 4.8 h in the mid colon and 3.7 h in the distal colon. Such retention and/or transit times for specific locations in the digestive tract have not been reported previously, and this

information is critical for comparing the results of *in vitro* bioaccessibility/metabolism studies (where time is a variable) to the *in vivo* situation where residence time/passage rate is determined by interactions of the food with humans and/or animals (Fig 6.1).
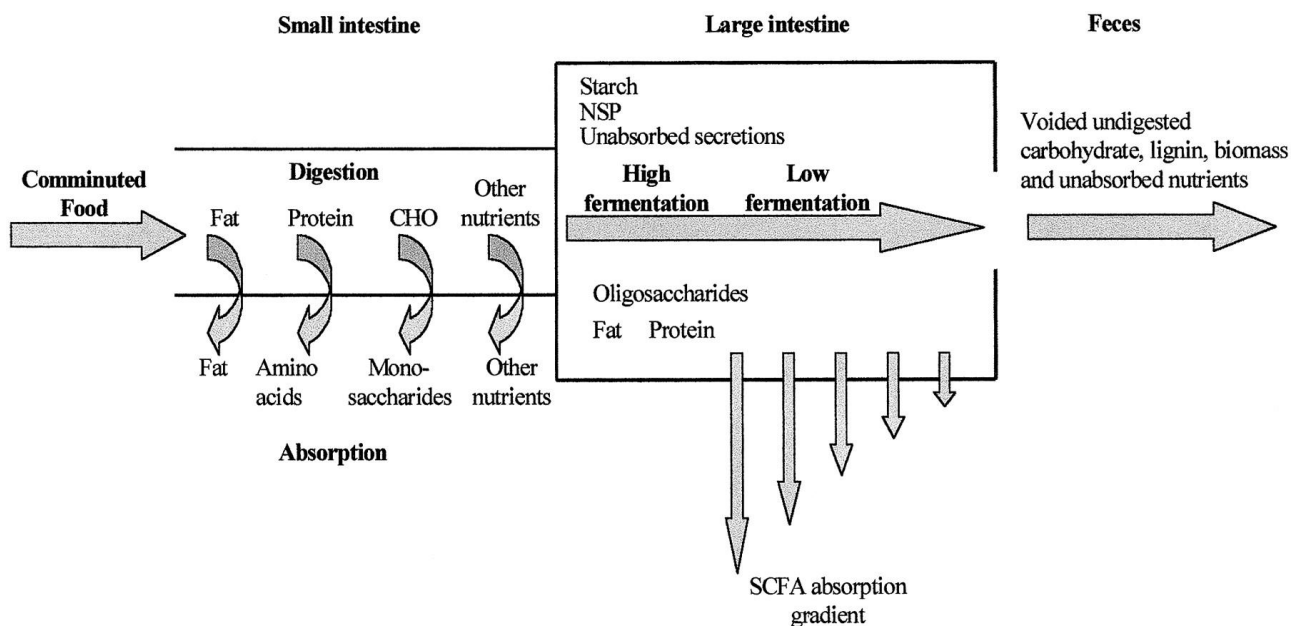
Figure 6.1. Overview of the relationship between the passage of food through the gastrointestinal tract, digestion of nutrients in the small intestine and fermentation in the colon. Adapted from (Topping & Clifton, 2001).

The monitoring of polyphenol (e.g. in mango) metabolism and uptake along the digestive tract and into the blood stream (hepatic portal and jugular vein) appears promising as a means to assess the fate of individual compounds as a function of location in the digestive tract/body site. In the present study, various classes of compounds were found to undergo distinctive digestive processing, with evidence for at least three types of polyphenol behaviour- rapid uptake; slow uptake in the small intestine; and microbial fermentation primarily in the colon. Although the complexity of chromatographic profiles and corresponding mass spectra precluded detailed molecular identification, future *in vivo* pig feeding experiments should continue to focus on the whole diet effect, but perhaps with more stringent screening of dietary materials to minimise complications from unintended dietary phenolics. Future considerations following from this study should also include assessing the usefulness of the MS technique of selected reaction monitoring mode (SRM) of individual compounds, as approached in some reported studies (de Oliveira et al., 2013; Jenner, Rafter, & Halliwell, 2005; Mosele et al., 2014), and the study of other relevant samples reflecting post-absorptive phenolic metabolism, such as the liver and urine.

Although fruits and vegetables are known to be an essential part of a healthy diet, evidence of diet and health outcomes linked to a range of antioxidant, cardio-protective and vasodilatory properties is primarily based on epidemiological studies, which do not permit underlying mechanisms to be determined. In the past, the concept of prebiotics was limited to non-digestible carbohydrates, where fermentable carbohydrates and fibres can alter greatly the microbial ecology, by acting as substrates or supplying short chain fatty acids. This ignores associated nutrients such as polyphenols which may also have an effect on the microflora composition. Much attention has been directed toward the study of specific beneficial lactic acid bacteria such as probiotics (usually *Bifidobacteria* or *Lactobacilli*) rather than a study of intestinal microbiota as a whole (Peng, Li, Luo, Wu, & Liu, 2013; Tuohy et al., 2012). However in recent years, diet-induced changes in microbiota are gaining more attention, where the health effects attributed to dietary polyphenols and their metabolism is suggested to modulate gut microbial composition through the stimulation of beneficial species and inhibition of pathogenic species (Hervert-Hernandez & Goni, 2011; Jacobs et al., 2009; Selma, Espin, & Tomas-Barberan, 2009).

In conclusion, the present study has evaluated aspects of the digestive processing of two archetypal fruits, mango and banana, to illustrate the importance of combining *in vitro* and *in vivo* studies to achieve a more complete perspective of human digestion. Consequently, the project has contributed to the process of defining mechanisms of how fruits (and vegetables) contribute to health and well-being as suggested by many epidemiological studies. In addition, the present study has shown how fruits contribute to other health aspects due to differences in the structural fruit matrix and the proportion of cellular components in different fruits, which lead to varying digestion and fermentation kinetics, and different nutrient metabolism and uptake. These results have contributed to a better understanding of the fruit matrix-phenolic-microbiota interactions, and subsequently their physiological impact on gut health. Therefore, the regular consumption of diets rich in fruits (and vegetables) with high phytonutrient content may beneficially balance the gut microbial ecology, helping to prevent gastrointestinal disorders and thus, enhance the health of the host.

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APPENDICES

Appendix 1.

THE UNIVERSITY OF QUEENSLAND
Institutional Approval Form For Experiments On Humans
Including Behavioural Research

Chief Investigator:	Dorrain Low Yanwen
Project Title:	Analysis Of Fresh Fruit Flesh After Human Mastication
Supervisor:	Dr Bruce D'Arcy, Prof Mike J Gidley
Co-Investigator(s):	None
Department(s):	School of Agriculture and Food Sciences, Centre for Nutrition and Food Sciences
Project Number:	2012000683
Granting Agency/Degree:	PhD
Duration:	31st July 2015

Comments:

Name of responsible Committee:-
Medical Research Ethics Committee
This project complies with the provisions contained in the *National Statement on Ethical Conduct in Human Research* and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative:-
Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Date: 10 July 2012   Signature:

Figure A1.1. Human ethics approval form for approval of human mastication of fresh fruits experiments from 2012-2015 (approved by the Medical Research Ethics Committee).
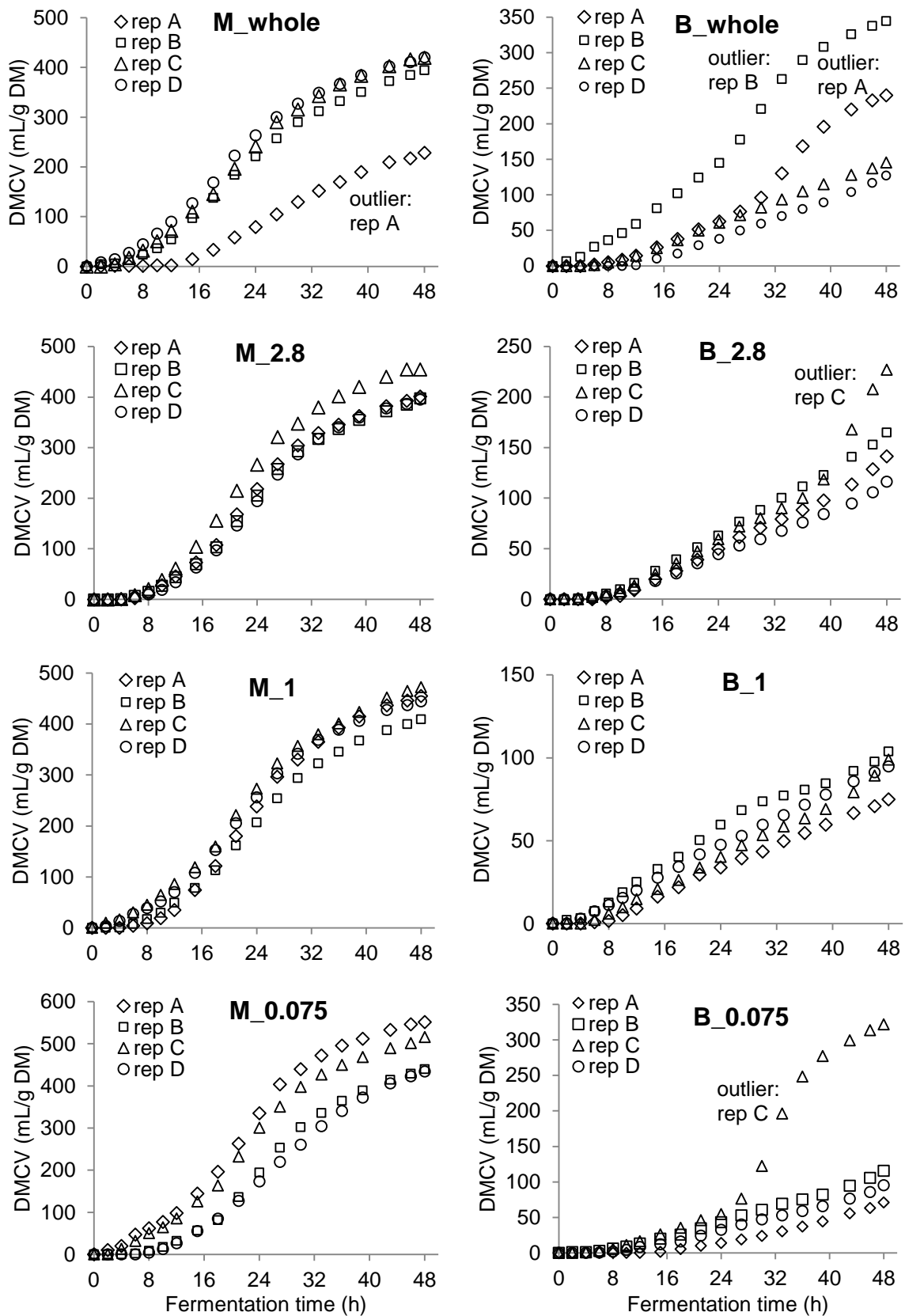
Figure A2.1. Dry matter cumulative volume time course profiles (g/mL dry matter) of replicates of masticated mango and banana substrates (unfractionated, 2.8, 1 and 0.075 mm) during 48 h microbial fermentation *in vitro*.

Appendix 3.
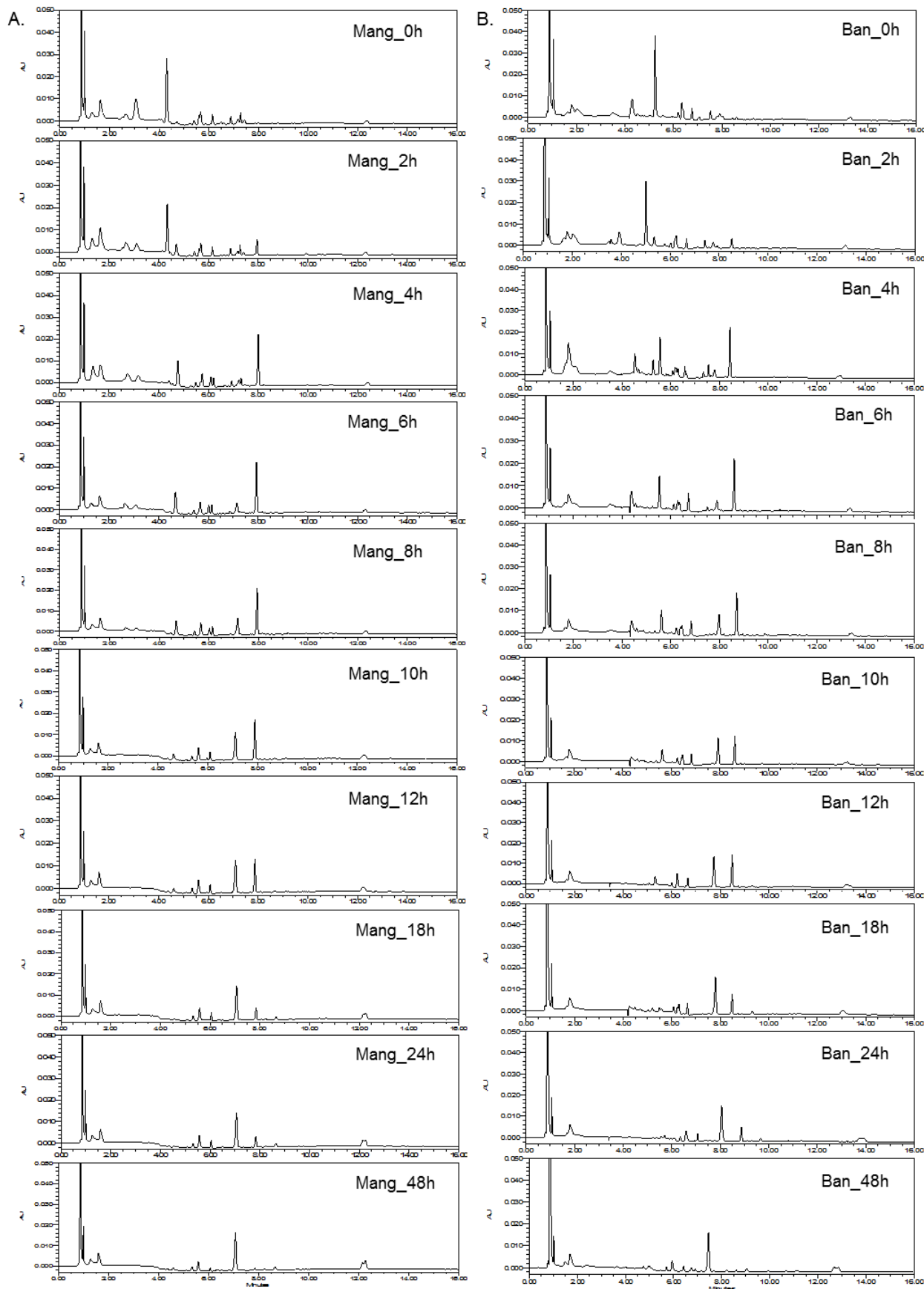

Figure A3.1. UPLC-PDA chromatograms of fermented (A) mango and (B) banana from 0-48 h at 280 nm. Disappearance of peaks was observed during the first 8 h, along with the concomitant increase in 'metabolite' peaks.
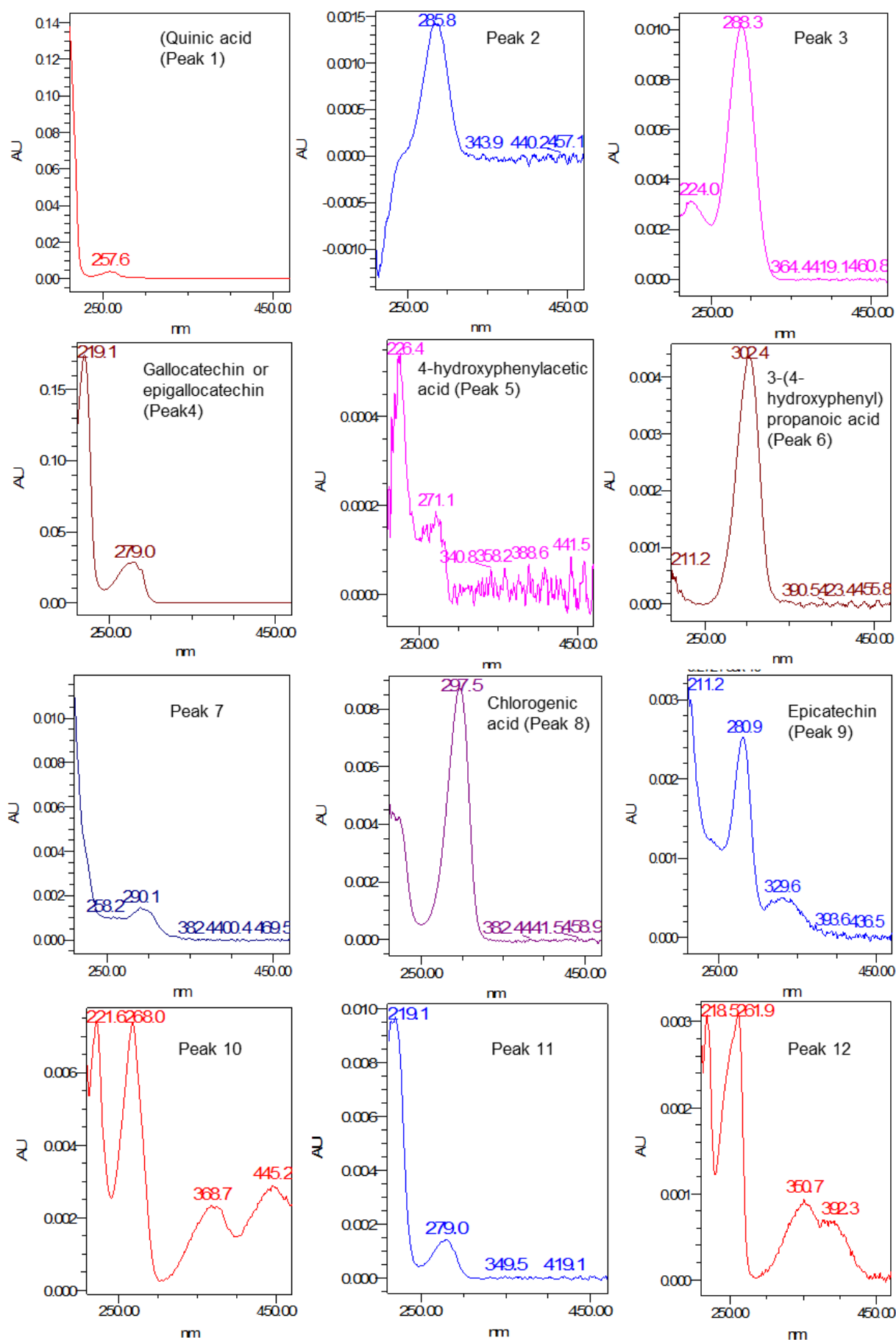
Figure A3.2. UV spectra of twelve compounds detected in fermented mango and banana samples. The identity and characteristics of each peak correlates with those in Table 4.3.

Figure A3.3. TCC and UV chromatograms of polyphenol authentic standards at 280 nm. (A) Standard mix of chlorogenic acid, coumaric acid, mangiferin, epicatechin, ferulic acid, quercetin and caffeic acid, (B) standard mix of benzoic acid, pyrogallol, 3- and 4-hydroxybenzoic acid, cinnamic acid, 4-hydroxyphenylacetic acid, protocatechuic acid, neochlorogenic acid, (C) standard mix of hippuric acid, quinic acid, syringic acid, ethyl ferulate, kaempferol, 3-(4-hydroxyphenyl)propanoic acid, 3-(3,4-dihydroxyphenyl)propanoic acid. Note that while some phenolics appear in both TCC and UV chromatograms, some compounds do not show up on the TCC or if they do, appear in extremely low intensities unless an 'Extracted Ion Chromatogram' feature was performed for a targeted m/z.

Table A3.1. The retention time, chemical formula, mass, [M-H]⁻ m/z and UV-Vis absorbance maxima of commercial authentic standards analysed using UPLC-PDA and UHPLC-Q-ToF-MS instruments.

Compound	Retention time (min)[1]	Retention time (min)[2]	Chemical formula	Molecular mass (Da)	[M-H]⁻ m/z	λ_{max} (nm)
Pyrogallol	11.223	2.979	C6H6O3	126.110	125.0250	301/268
Benzoic acid	7.776	11.603	C7H6O2	122.121	121.0250	230/273
3-Hydroxybenzoic acid (m-hydroxybenzoic acid)	5.683	8.800	C7H6O3	138.121	137.0247	200/236/296
4-hydroxybenzoic acid (p-hydroxybenzoic acid)	5.106	9.299		138.121	137.0249	200/255
4-Hydroxyphenylacetic acid	5.094	7.703	C8H8O3	152.147	151.0462	222/274
3-(4-Hydroxyphenyl)propanoic acid	6.956	9.870	C9H10O3	166.174	165.0556	222/276
3-(3,4-Dihydroxyphenyl)propanoic acid (dihydrocaffeic acid)	5.248	7.766	C9H10O4	182.170	181.0508	210/280/318
Protocatechuic acid	2.935	6.850	C7H6O4	154.120	153.0196	219/259/293
Cinnamic acid	11.510	14.205	C9H8O2	148.159	147.0452	217/278
Hippuric acid	4.741	8.997	C9H9NO3	179.173	178.0515	228/314
Caffeic acid	7.321	9.670	C9H8O4	180.157	179.0360	217/324
Quinic acid	11.364	1.139	C7H12O6	192.167	191.0568	215/287
Ferulic acid	9.445	11.416	C10H10O4	194.184	193.0485	218/235/323
Syringic acid	7.650	9.188	C9H10O5	198.173	197.0456	217/275
Coumaric acid	7.336	11.632	C9H8O3	164.158	163.0392	227/309
4-Caffeoylquinic acid	7.244	9.632	C16H18O9	354.309	353.0882	218/326/366
5-Caffeoylquinic acid		9.632		354.309	353.0882	218/241/326
Ethyl ferulate (ethyl 4-hydroxy-3-methoxycinnamate)	14.468	17.670	C12H14O4	222.237	221.1185	218/236/324
Kaempferol (aglycon)	14.883	18.150	C15H10O6	286.236	285.0400	221/364
Quercetin dihydrate (aglycon)	13.318	16.331	C15H10O7	302.236	301.0390	2000/255/371
Mangiferin/isomangiferin	7.553	9.246	C19H18O11	422.340	421.0823	258/318/366/425
Epicatechin	8.263	9.113	C15H14O6	290.268	289.0753	219/279

[1]Retention time based on gradient elution from UV chromatograms. [2]Retention time based on gradient elution from Q-ToF-MS chromatograms.
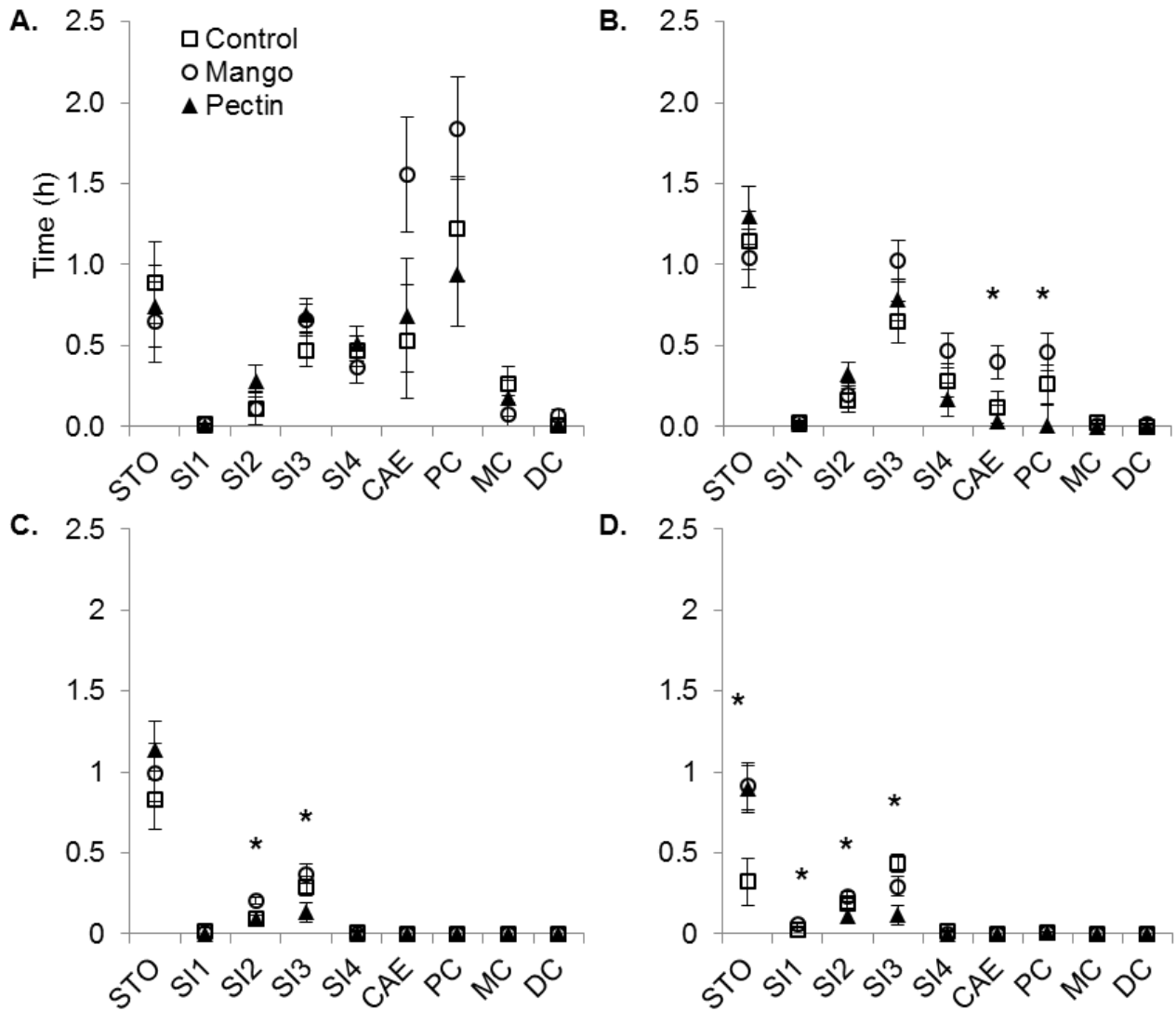
Appendix 4.

Figure A4.1. Retention and transit times of pulse dose markers (A) cerium, (B) ytterbium, (C) lanthanum and (D) cobalt passage along the gastrointestinal tract of pigs fed the control (□) diet, or 15% mango (O) or 10% pectin (▲) at the expense of wheat starch. Time spent in the stomach (STO) and caecum (CAE) is represented as retention time, while time spent in the small intestine (SI1-4) and colon (PC-DC) is represented as transit time. Any significant (P<0.05) interactions between diet and gastrointestinal tract site (P>0.05) are marked by an asterisk (*). Data is expressed as means±standard error. SI: small intestine, PC: proximal colon, MC: mid colon, DC: distal colon.

Table A4.1. Two-hourly outflow and influx rates (mg/2 h) from stomach and small intestine respectively, from pigs fed the control diet, or 15% mango or 10% pectin at the expense of wheat starch.

Control diet (n=9)						
Post marker dose (h)	Stomach			Small intestine[1]		
	Pool size[2] (mg)	Influx (mg/2 h)	Outflow[3] (mg/2 h)	Pool size (mg)	Influx (mg/2 h)	Outflow (mg/2 h)
0	500.00	0	0	0	0	0
2	228.53	0	271.47	97.09	271.47	174.38
4	153.76	0	74.77	115.59	74.77	56.27
6	76.01	0	77.76	89.28	77.76	104.07
Pectin diet (n=7)						
Time after feed (h)	Pool size (mg)	Influx (mg/2 h)	Outflow (mg/2 h)	Pool size (mg)	Influx (mg/2 h)	Outflow (mg/2 h)
0	500.00	0	0	0	0	0
2	269.82	0	230.18	56.41	230.18	173.78
4	133.54	0	117.87	169.46	117.87	4.82
6	38.37	0	109.32	130.82	109.32	147.96
Mango diet (n=9)						
Time after feed (h)	Pool size (mg)	Influx (mg/2 h)	Outflow (mg/2 h)	Pool size (mg)	Influx (mg/2 h)	Outflow (mg/2 h)
0	500.00	0	0	0	0	0
2	222.69	0	277.31	146.18	277.31	131.14
4	104.48	0	118.21	164.21	118.21	100.18
6	35.62	0	68.86	79.49	68.86	153.57

[1]Small intestine here refers to the sum of combined small intestinal (SI1-4) sites. [2]Pool size at 0 h refers to the averaged feeding dose (mg) of cerium, ytterbium and lanthanum to the pigs, and pool size at 6, 4, 2 h refers to cerium, ytterbium and lanthanum respectively, measured in the stomach or combined small intestinal digesta. [3]Outflow rate of digesta from the stomach equals to influx rate into the small intestine, and is specific for each marker, fed 2 h apart.