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**BERRY FRUIT ANTHOCYANINS
IN HUMAN NUTRITION – BIOAVAILABILITY AND
ANTIOXIDANT EFFECTS**

A thesis presented in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy
in
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New Zealand.

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ABSTRACT

Anthocyanins (ACNs), which are responsible for the red and blue colours displayed by many vegetables and fruits (particularly berries), belong to secondary plant metabolites, and are a component of our daily diet. There is an increasing interest on their biological activities as they are claimed to enhance health by protecting against some chronic diseases. However, before ACNs can perform health-promoting effects *in vivo*, they must first be sufficiently absorbed, distributed within the human body, and reach target tissues in adequate concentrations. To date, all studies investigating ACN absorption and metabolism came to the conclusion that their bioavailability is extremely low. To benefit from the proposed health effects of ACNs, their bioavailability, including absorption, metabolism, and excretion must first be understood. The main objective of this thesis was to provide further knowledge on ACN absorption, including the absorption site and mechanism, and the influence of food and other flavonoids on ACN absorption, as well as the investigation of their antioxidant effects *in vivo*. *In vitro* experiments using Ussing chambers showed that a strong absorption of ACNs occurred from the jejunum in mice. This was supported with a further *in vivo* study, where the major absorption site for ACNs may be the jejunum in rats. The limitation of ACN absorption to mainly one part of the intestine suggested the participation of a particular transport mechanism. In a further Ussing chamber study it was shown that flavonols, another common flavonoid group present in many fruits and vegetables, strongly inhibit ACN absorption, indicating a specific transport mechanism, with preference for other flavonoid compounds. Further *in vivo* studies have shown that the simultaneous ingestion of food components, such as breakfast cereals, resulted in a delayed absorption profile in two animal species. However, the additional food did not influence the antioxidant effect of ACNs. During a human intervention study, several measures of oxidative stress improved, but this improvement occurred equally in the treatments and placebo control, and may have resulted from changes in lifestyle. The results of these studies aid to understand details of ACN absorption and help to formulate future recommendations for ACN intake with increased bioavailability in humans.

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GENERAL INTRODUCTION

Anthocyanins (ACNs) are widely distributed in the human diet through crops, beans, fruits, vegetables and red wines, suggesting that we ingest considerable amounts from our daily diets. Berry fruits in particular are rich dietary sources, and some can contribute 100-300 mg ACNs in a single serving.

As a potential major component of our daily diet, more and more research has concentrated on their biological activities and possible health benefits in protecting against some chronic diseases, including cancer, atherosclerosis, and diabetes. Recently, some research has also shown that an increasing dietary intake of fruit and vegetables rich in antioxidants like ACNs may retard age-related declines in brain function as well as improve cognitive and motor performance in rats.

Nevertheless, to perform their multiple biological effects, the bioavailability of ACNs present in different fruits and vegetables is an important, but still not well-understood issue. So far, there is only a small number of data available on their ability, in intact or metabolised form, to reach the systemic circulation in humans. Despite the relatively high amounts in food and potential intake in humans, the physiological impact of the ACNs is not well studied and investigations regarding their bioavailability in humans have been conducted only within the last few years. To act as systemic antioxidants and perform health effects for humans, ACNs first need to be ingested and distributed within the body successfully. Therefore, the bioavailability including absorption, metabolism, and excretion must be known.

The main objectives of the present thesis were to provide further information on the absorption site, and mechanisms involved in ACN absorption, with the aim to generate future recommendations on ACN intake with an increased bioavailability. As ACNs are mainly ingested in combination with other food sources, the effect of food matrixes on ACN absorption was also taken into account. Furthermore, the antioxidant capacity of ACNs was investigated, as well as the effect of other food intake on this capacity.

CHAPTER 1

LITERATURE REVIEW: BERRY FRUIT ANTHOCYANINS – BIOAVAILABILITY AND ANTIOXIDANT EFFECTS

ANTHOCYANINS (ACNs)

General. ACNs (Greek anthos = flower and kyanos = blue), are responsible for the red, purple and blue colours displayed in many vegetables and fruits (particularly in berries), and are part of a very large and widespread group of water-soluble plant constituents, known collectively as flavonoids (1). Flavonoids themselves are a subgroup of polyphenols, which belong to secondary plant metabolites.

The most significant function of ACNs is their ability to impart colour to the plant or plant product in which they occur (2). These compounds are the most important group of water-soluble plant pigments visible to the human eye (3), and have been the subject of much investigation of the past 10 years in relation to the natural expression of the colour of parts of plants and the utilization of ACNs as colouring additives in foodstuffs. The use of ACNs as food colorants has attracted considerable interest because of their presumed safety as well as potential nutritional and therapeutic effects (4).

Chemistry. ACNs are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (**Figure 1**) (1), and belong to the phenolic class of flavonoids with the typical A-ring benzoyl and B-ring hydroxycinnamoyl systems (5). The non-sugar components of the glycosides that result from hydrolysis of the molecules are called anthocyanidins, or aglycones. Differences between individual ACNs are the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugars attached to the molecule and the position of the attachment, as well as the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule (1).

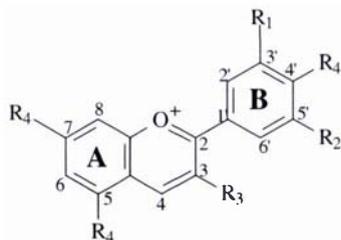


Figure 1. The flavylium cation. $R_1, R_2 = H, OH$ or OCH_3 ; $R_3 =$ glycosyl or H ; $R_4 = OH$ or glycosyl.

ACNs occur as 3-monosides, 3-biosides and 3-triosides as well as 3,5-diglycosides and more rarely 3,7-diglycosides associated with the sugars glucose, galactose, rhamnose,

arabinose, and xylose (5). Additional variations can occur through acylation of the sugar substituents with organic acids. Over 500 different anthocyanin pigments have been identified in plants (6).

Because of their highly reactive nature, ACNs readily degrade, or react with other constituents to form colourless or brown compounds. Loss of ACN pigmentation also occurs in the presence of oxygen and various enzymes, and as a result of high temperature processing. Besides oxygen, temperature, light, and enzymes, pH has a marked effect on ACN stability (7), and on the colour of media containing these pigments (8). Their structure and thus their colour and colour stability vary with pH. ACNs may exist in a variety of protonated, deprotonated, hydrated, and isomeric forms, and the relative proportion of these molecules is strongly dependent on pH. The red flavylium cation is dominant at very acidic pH (pH 1-3). In aqueous media, as the pH is raised to 4-5, hydration reactions generate the colourless carbinol pseudo-base, which can further undergo ring opening to the light yellow chalcones (pH 7-8). The flavylium cation can alternatively be transformed to quinoidal bases through proton transfer reactions and at pH values between 6 and 7 be further converted to the blue-purple quinonoid anions (**Figure 2**) (9, 10).

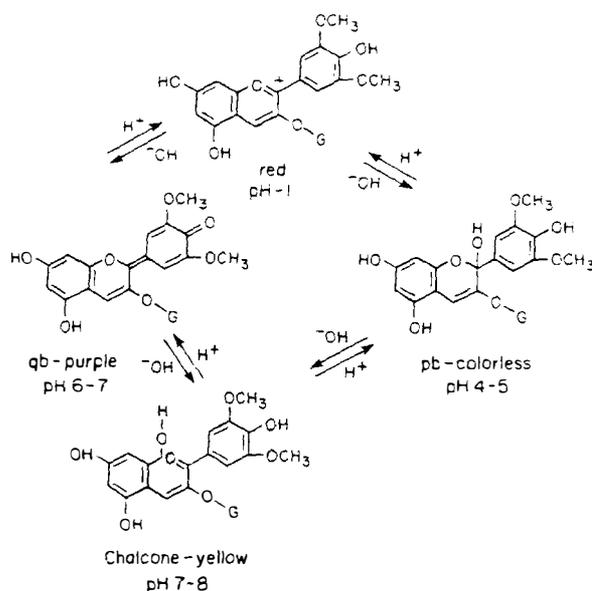


Figure 2. ACN transformation by pH. qb = quinoidal base; pb = pseudo-base (adapted from Belitz and Grosh 1987(11)).

This is an important fact, as ACNs are exposed to several pH values during their passage through the gastrointestinal tract (GIT) and therefore appear as one or more of the transformations mentioned above. Research has shown that in very acidic media (pH 0.5), the red flavylum cation (**Figure 1**) is the only ACN species present. With an increase in solution pH, both the concentration of this species and the pigmentation of the solution decrease, as the cation hydrates to the colourless carbinol base (8).

In addition to pH, intensity and stability of colour of ACNs are influenced by several other factors such as temperature, light, oxygen, acetaldehyde, ascorbic acid, and sugars (8, 12).

Berry fruit ACNs. In recent years, the developed world has become very aware of the health-promoting properties of the 'berry fruit' group, which has created a strong world market within this fruit sector. Berries constitute a rich dietary source of phenolic antioxidants (13, 14). Blueberries (*Vaccinium L.* species), blackberries (*Rubus L.* hybrids), and blackcurrants (*Ribes nigrum L.*) are especially rich sources of dietary ACNs and antioxidants (13, 15, 16). The berry fruits used in the studies of this thesis included Boysenberries and blackcurrants.

- **Boysenberries** (*Rubus loganbaccus x baileyanus* Britt) are a hybrid made from raspberries, blackberries and loganberries, which were created by horticulturist Rudolph Boysen. They resemble large raspberries with a purple-red hue. The fruits are large, deep purple in colour and have an acid taste. Boysenberries are grown extensively in the states of Oregon and Washington as well as in New Zealand. According to Luh *et al.* (17), the ACN pigments of Boysenberries are cyanidin-3-glucoside, cyanidin-3-diglucoside, cyanidin-3-rhamno-glucoside and cyanidin-3-rhamnoside-5-diglucoside, whereas Torre and Barritt (18) characterized ACNs of Boysenberries as cyanidin-3-sophoroside, cyanidin-3-glucoside, cyanidin-3-glycosylrutinoside, and cyanidin-3-rutinoside (**Table 1**). A recent study by Cooney *et al.* (19) confirmed the presence of the four ACNs as reported by the latter authors, and described the disaccharide cyanidin-3-sophoroside and the monosaccharide cyanidin-3-glucoside as the two major components, whereas the two others were found less abundant. The total ACN content of Boysenberries has been reported to be over 160 mg/100 g fresh fruit (18).

Table 1. ACN composition of Boysenberries.

Luh <i>et al.</i> (17)	Torre and Barritt (18), Cooney <i>et al.</i> (19)
cyanidin-3-glucoside	cyanidin-3-glucoside
cyanidin-3-diglucoside	cyanidin-3-sophoroside
cyanidin-3-rhamno-glucoside	cyanidin-3-glycosylrutinoside
cyanidin-3-rhamnoside-5-diglucoside	cyanidin-3-rutinoside

- **Blackcurrants** (*Ribes nigrum L.*) belong to the saxifrage family, Saxifragaceae, genus *Ribes*. The berries grow wild in Europe and in cool, moist regions of North America, and are primarily used in the juice processing industry. Blackcurrants contain four major ACNs, which were first reported by Chandler and Harper (20): delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside. Later, Lelous *et al.* (21) identified also mono- and disaccharides of delphinidin and pelargonidin. A more recent and advanced analysis of blackcurrants reported the detection of up to 15 different ACNs (22) (**Table 2**). At maturity, blackcurrants contain about 250 mg ACNs/100 g of fresh fruit (23).

Table 2. Individual ACNs of blackcurrants^a.

	blackcurrants
Major compounds	delphinidin-3-glucoside delphinidin-3-rutinoside cyanidin-3-glucoside cyanidin-3-rutinoside
Minor compounds	petunidin-3-glucoside petunidin-3-rutinoside cyanidin-3-arabinoside pelargonidin-3-glucoside pelargonidin-3-rutinoside peonidin-3-glucoside peonidin-3-rutinoside malvidin-3-glucoside malvidin-3-rutinoside delphinidin-3-(6"-coumaroyl)glucoside cyanidin-3-(6"-coumaroyl)glucoside

^asource: Slimestad and Solheim (22).

Consumption of ACNs. So far, there is only very little data regarding ACN intake. Most papers refer to an evaluation that was made 30 years ago, where the daily intake of ACNs

in humans has been estimated to be as much as 180-215 mg/d in the United States (24) due to their widespread distribution and occurrence in fruits and vegetables. However, according to Timberlake and Henry (25), regular consumers of red wine are likely to have significantly higher intakes since concentrations in red wines of 200 mg/L are not exceptional. Clifford (26) mentioned an estimation of a worldwide annual consumption of 10,000 tonnes ACNs from black grapes alone. A recent estimate on ACN consumption has been made in Finland, where high amounts of berries are eaten. The average daily intake was found to be 82 mg, although some intakes exceeded 200 mg/d (27). A very recent estimation of ACN consumption in the United States reported a daily intake of 12.5 mg (28). However, reliable quantitative data on the intake of ACNs are not yet available (29), and no estimations regarding the daily intake of ACNs in New Zealand have been performed so far.

ABSORPTION & BIOAVAILABILITY

Bioavailability is defined in various ways. With nutrients, for which metabolism is usual and appropriate and the route of administration is nearly always oral, the notion of bioavailability generally designates simply the quantity or fraction of the ingested dose that is absorbed (30). The commonly accepted definition of bioavailability is the proportion of the nutrient that is digested, absorbed and metabolised through normal pathways. Consequently, it is not enough to know how much of a nutrient is present in a dietary supplement; the more important issue is how much of that present is bioavailable (31). Some define bioavailability simply as the proportion of a nutrient or bioactive ingredient that was absorbed from the gastrointestinal tract (GIT), while others include metabolism, excretion, utilisation and a measure of efficacy in their definitions. However, bioavailability is often just characterised as plasma concentration (32).

The nutritional role of flavonoids in humans depends upon the understanding of their behaviour in the GIT and the mechanism of their absorption. Initially it was not possible to identify flavonoids in human blood and tissues, thus it was considered that food flavonoids were not absorbed from the gut or were destroyed by microorganisms before absorption. Early experiments in animals to study flavonoid absorption from the gut were of doubtful value, because most of them used excessively large flavonoid doses, which were not

comparable to those ingested with food. In addition, the use of different species of animals resulted in inconsistent data, that could not be extrapolated to humans (24). Little is known about the bioavailability, absorption and metabolism of polyphenols in humans and it is likely that different groups of polyphenols have different pharmacokinetic properties. A recent review on the bioavailability of polyphenols in humans has clearly shown a wide variability in the bioavailability between the different polyphenols (33).

Polyphenols exist in foods and beverages in various chemical forms, which partly determine their rate and extent of intestinal absorption and the nature of the metabolites circulating in the plasma. After hydrolysis of a polyphenol derivative to the free aglycone, polyphenols are conjugated by methylation, sulfation, glucuronidation or a combination of these. The steps are controlled by the specificity and distribution of the enzymes that catalyse the reactions. The formation of conjugates can dramatically alter the biological properties of the circulating metabolites. Polyphenols that are not absorbed in the stomach or small bowel will be carried to the colon. In addition, polyphenols that are absorbed, metabolised in the liver and excreted in the bile or directly from the enterocyte back into the small intestine will also reach the colon, but in a different chemical form, such as a glucuronide. A major part of the ingested polyphenols (75-99%) is not found in urine. This implies they have either not been absorbed through the gut barrier, absorbed and excreted in the bile or have been metabolised by the colonic microflora or our own tissues (34). Bioavailability studies on flavonoids and anthocyanins, including absorption, metabolism and excretion, have been conducted *in vitro* as well as in animal and human studies.

Intestinal studies (in vitro). Intestinal *in vitro* studies to investigate polyphenol absorption have been mainly carried out using flavonoid classes, such as flavones, isoflavones, and flavonols. A number of studies have used cell culture (Caco-2 cells) monolayers to study flavonoid absorption, and some have suggested that passive diffusion rather than active transport is involved (35, 36). However, Walgren *et al.* (37) have shown the involvement of the sodium-dependent glucose transporter (SGLT1), when quercetin-4'- β -glucoside (Q4'G) was transported across the apical membrane of a Caco-2 cell monolayer. However, they emphasized that the flavonoid was not absorbed all the way across the Caco-2 cells to the serosal side, but accumulated within the cells, which was confirmed by indirect fluorescent microscopy. Furthermore, cellular uptake was sodium-dependent and was inhibited by glucose, a substrate of SGLT1, or phloridzin, a competitive

inhibitor of SGLT1. In a further study, they demonstrated that a multidrug resistance-associated protein (MRP2) was localized to the apical membrane of Caco-2 cells and that this protein both limits the absorption of, and mediates the efflux of Q4'G across Caco-2 cell monolayers (38).

The involvement of the SGLT1 in flavonoid absorption was also confirmed by a study of Wolfram *et al.* (39). Using sections of rat jejunum and proximal colon mounted in Ussing-type chambers, they demonstrated, that SGLT1 is indeed involved in the uptake of quercetin-3-glucoside (Q3G) across the small intestinal brush border membrane, as shown by the inhibitory effect of D-glucose, phloridzin and sodium-free medium on the disappearance of Q3G from the mucosal solution. On the contrary, a study by Murota *et al.* (40) has shown that the quercetin aglycone was more efficiently taken up from Caco-2 cells than its glucosides. Furthermore, it was subsequently methylated and conjugated within the cell, and released into the apical and basolateral solutions (40). In a further study the authors investigated the cellular uptake and metabolism of isoflavones and their glucosides and showed again, that the aglycones were taken up into enterocytes more efficiently than their glycosides (41). It has been shown that the permeabilities of aglycones in the Caco-2 cells were at least 5 times higher than their corresponding glycosides (42). Day *et al.* (43) have shown that lactase phlorizin hydrolase (LPH), a mammalian β -glucosidase present in the brush border of the small intestine, was capable of hydrolyzing various flavonol and isoflavone glucosides. Therefore, aglycones may be released in the small intestine and subsequently absorbed by passive diffusion, as suggested by Kuo *et al.* (35) and Walgren *et al.* (36).

A study with everted sac preparations from rats has demonstrated that quercetin-monoglucosides (Q3G, Q4'G) but not -diglucosides (Q3,4'G) were transported into everted sacs significantly faster than the transport of the aglycone, suggesting, that the glucoside interacts with the SGLT1 (44). They did not detect intact Q3G in the mucosal tissue extract or the serosal solution, but the aglycone and its metabolites (mainly as glucuronides), which indicates its metabolism within the cells as shown by Murota *et al.* (40). Gee *et al.* suggest two possible mechanisms for the transport of quercetin glycosides by enterocytes, namely, transport of the intact quercetin glucosides by SGLT1, and extracellular hydrolysis by lactase phloridzin hydrolase (LPH), followed by passive diffusion of the aglycone (44). Once the flavonoid-glycoside is in the enterocyte it is either secreted back into the lumen

via MRP2, or undergoes hydrolysis and subsequent metabolism within the cell. Therefore, a number of metabolites are expected to appear in serosal solution and possibly in the blood circulation.

Spencer *et al.* (45) studied the perfusion of the jejunum and ileum in an isolated rat intestine model with flavonoids and the influence of glycosylation on the subsequent metabolism. Flavone and flavonol glucosides as well as their aglycones were glucuronidated during transfer across the rat jejunum and ileum without the need for gut flora, which suggests, that glycosidases and UDP-glucuronyl transferase are present in the small intestine.

There is still a controversy as to whether aglycones or glucosides are the main forms absorbed, although the majority of the studies suggest it to be the glycosides. A scheme of possible pathways of flavonoids on a cellular level is shown in **Figure 3**. However, regarding *in vitro* studies on ACN absorption, there is no information available in the literature so far.

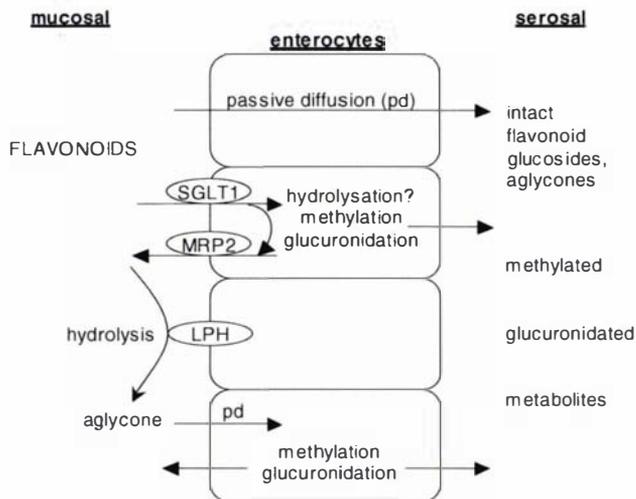


Figure 3. Possible pathways of flavonoid absorption on cellular level. Pd (passive diffusion), SGLT1 (sodium-dependent glucose transporter), MRP2 (multidrug resistance-associated protein), LPH (lactase phloridzin hydrolase).

A common method for *in vitro* investigations into gastrointestinal permeability are the so-called "Ussing chambers", which were developed 1951 by Hans Ussing (46).

Historically, these chambers have provided valuable information on permeability and ion transport when applied to the gastric mucosa of amphibians (47) and fish (48). In recent years however, the Ussing chambers have been applied to the gastrointestinal tissues of higher species. In fact, nowadays virtually all types of epithelial tissues, as well as cell monolayers have been studied using the Ussing chamber.

For the use of gastrointestinal tissues of small animals, the respective intestinal area is dissected out of the body, and immersed in a physiological solution. Small sections of the gastrointestinal tract (GIT) are opened longitudinally along the mesenteric border (**Figure 4 A**), and impaled onto mounting pins of one of two half-cells (**Figure 4 B, C**). The closed tissue clamp (**Figure 4 D**) is vertically mounted into Ussing chambers (**Figure 5**), to represent a mucosal (luminal) and serosal (basolateral) surface of the tissue.

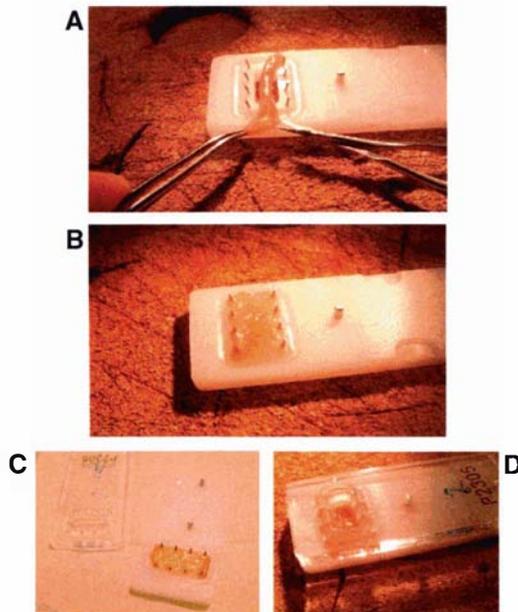


Figure 4. Tissue preparation for Ussing chambers.

The tissue clamp is connected to two compartments (mucosal and serosal) containing physiological solution, which provides the necessary nutrients and electrolytes to maintain tissue viability. The temperature of the chambers is kept at 37°C, and carbogen gas (95%

oxygen, 5% carbon dioxide) is used to oxygenate the tissues, maintain the pH and circulate the physiological solution. Agar-KCl bridges are connected between the intestinal tissue and the physiological solution to measure the potential difference, as well as to pass a current across the tissue in order to maintain the potential difference across the membrane at 0 mV, using automatic clamp units (**Figure 6**).

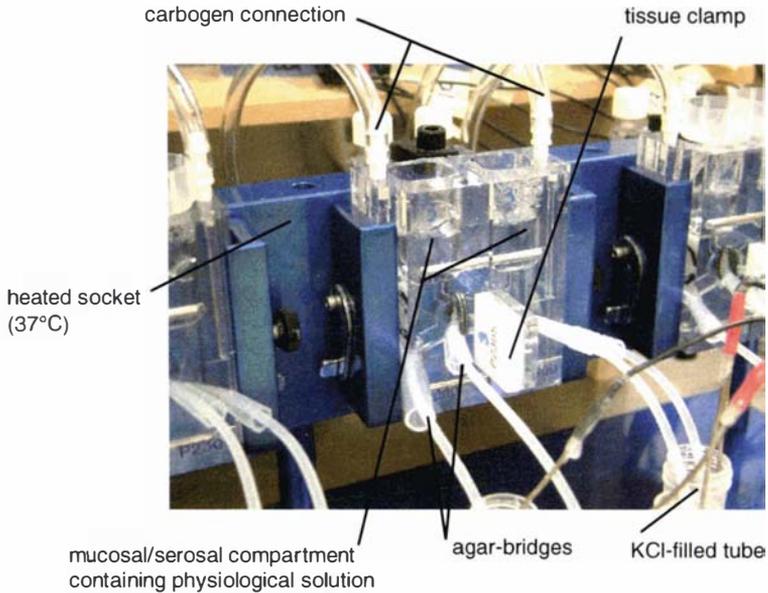


Figure 5. Ussing chamber.

Once the chambers are set up for an experiment (**Figure 6**), the compound to be tested is added to the mucosal compartment of the Ussing chamber and the concentration of that compound subsequently monitored in the mucosal solution over time. The disappearance of the test compound indicates absorption or metabolism of the respective compound, due to the physiological action of the tissue. Compared to animal or human studies, *in vitro* studies, such as the Ussing chambers represent several advantages (inexpensive, controllable, reproducible, fast results) to investigate absorption and mechanisms thereof at a cellular level.

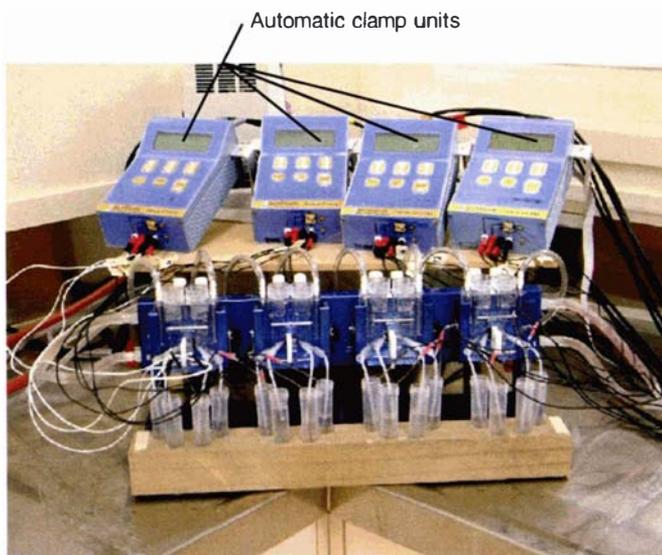


Figure 6. Four Ussing chambers set up for an experiment.

Animal studies. Besides *in vitro* studies, there have been an increasing number of animal studies investigating ACN absorption and metabolism during the last few years. Most studies have found that ACNs are absorbed mainly in their intact glycosidic form, and rapidly reach the circulatory system within 0.25-2 h. After a single oral administration (400 mg/kg body weight (BW)) of *Vaccinium myrtillus* ACNs, the plasma concentrations reached peak level (2-3 $\mu\text{g}/\text{mL}$) after only 15 min and then rapidly declined within 2 h (49). A quick appearance of intact ACN in plasma (C_{max} : 3.8 μmol [1.8 $\mu\text{g}/\text{mL}$] at 15 min) after oral administration of red fruit ACN (320 mg cyanidin-3-glucoside (C3G)/kg BW) via stomach intubation into rats was confirmed (50). However, the authors did not detect the aglycones or conjugates of ACN in rat plasma, suggesting that the flavylum cation structure is much more stable against bacterial hydrolysis than other flavonoids, and impart resistance against enzymatic conversion into conjugates. A further study by Tsuda *et al.* (51) found that after oral administration of C3G to rats (400 mg/kg BW), the intact form appeared rapidly in the plasma (C_{max} : 0.3 μmol [0.14 $\mu\text{g}/\text{mL}$] at 30 min), but the aglycone cyanidin was not detected, although it was present in the jejunum. In addition, the authors found protocatechuic (PC) acid in the plasma, which they suggest to be produced by degradation of cyanidin. The concentration of PC was 8-times higher than that of C3G.

Since a maximum C3G-concentration after only 15 min in stomach tissue was found, ACNs may already be absorbed from this organ. This assumption was recently confirmed by studies, which suggest ACN-absorption from the stomach to explain the rapid appearance of ACNs in plasma of rats and humans (52, 53). After *in situ* gastric administration of purified ACNs, or a mix of grape ACNs respectively, ACNs appeared only after 6 min in blood samples, with ~25% ACN-monoglucosides being absorbed from the stomach. None of the studies detected aglycones or metabolized ACN in plasma samples. It was suggested that the ability of ACNs to permeate gastric mucosa was due to a specific transport mechanism, bilitranslocase, an organic anion membrane carrier localized in the liver (54) and in the gastric mucosa (55). It has been shown, that ACNs are a substrate of this transport mechanism and that it could play a role in ACN-bioavailability (56).

Recently there has been a first study using rabbits to investigate ACN absorption and excretion. After a single dose of ACNs from blackcurrant juice (182 mg ACN/animal) an excretion of 0.035% of the ingested amount was found in rabbit urine within the first 4 h (57). Besides plasma samples, urine samples are often investigated for the presence of ACN. Blackcurrant ACNs were found to be directly absorbed, rapidly distributed to the blood (t_{max} at 0.5-2 h), and excreted into urine as the glycosylated forms in rats after oral administration of three purified ACNs (delphinidin 3-*O*- β -rutinoside, cyanidin 3-*O*- β -rutinoside, and cyanidin 3-*O*- β -glucoside) prepared from blackcurrant juice. No other peaks were detected after administration (58).

Felgines *et al.* (59) have shown that blackberry ACN are excreted in urine as intact as well as methylated forms, but no aglycones or conjugated forms. Furthermore, they detected low amounts of ACNs as well as aglycones in caecal contents, suggesting an adaptation of microflora to ACN degradation. In addition, ACNs and their metabolites have been reported in bile for the first time, already after 20 min, which suggests quick absorption and metabolism (53).

More recent ACN absorption studies in rats reported the occurrence of methylated ACNs in plasma, indicating their production during absorption from the GIT (60). After an oral administration of 100 mg delphinidin-3-glucoside (D3G)/kg BW, a maximum concentration (0.4 μ mol) appeared in plasma within 15 min. The methylated form of D3G showed a maximum plasma concentration after 1 h. Further studies detected the

glucuronides of ACNs in rat plasma, and it was suggested, that these metabolites are mainly produced in the liver, rather than by intestinal flora (61, 62). Both studies applied a 100 mg C3G/kg BW dose to rats, and reported maximum concentrations for the original compound (0.18 μmol ; t_{max} : 15 min), methylated metabolites (< 0.021 μmol ; t_{max} : 15-120 min), and glucuronidated compounds (< 0.07 μmol ; t_{max} : 15-60 min). The glucuronidated and methylated conjugates of ACNs have also been shown to be the two major types of metabolites that appear in urine in pigs (63). The original ACNs showed a maximum plasma concentration of 0.103 μmol after 1 h. The urinary recovery of the original ACNs and their related metabolites was 0.088%. Wu *et al.* (64) administered three kinds of berry types with different ACN profile to pigs and suggested that the aglycone and the sugar moieties alter the absorption and metabolism of ACNs. Talavera *et al.* (65) were the first to report the original as well as methylated and glucuronidated metabolites of ACNs in the jejunum, liver and kidneys of rats. A summary of ACN absorption studies in animals is shown in **Table 3**.

Table 3. Animal ACN absorption studies.

Species	Material	ACN dose ^a (per kg BW)	C _{max} ^b	t _{max} ^c (h)	Urinary excretion ^d (%)	Ref.
Rat	Bilberry	400 mg	2-3 $\mu\text{g}/\text{mL}$	0.25		(49)
Rat	Elderberry	360 mg	3.80 $\mu\text{mol}/\text{L}$	0.25		(50)
	Blackcurrant					
Rat	Purple corn	400 mg	0.31 $\mu\text{mol}/\text{L}$	0.5		(51)
Rat	Blackcurrant	359 mg C3G ^e	0.84 $\mu\text{mol}/\text{L}$	0.5		(58)
		476 mg C3R ^f	0.85 $\mu\text{mol}/\text{L}$	0.5		
		489 mg D3R ^g	0.58 $\mu\text{mol}/\text{L}$	2.0		
Rabbit	Blackcurrant	117 mg	780 ng/mL	0.5	0.035 (4 h)	(57)
		164 mg	100 ng/mL	0.25	0.009 (4 h)	
		53 mg	450 ng/mL	0.5	0.023 (4 h)	
Rat	Blackcurrant	100 mg D3G ^h	0.4 $\mu\text{mol}/\text{L}$	0.25		(60)
Pig	Marion berry	74 mg	0.103 $\mu\text{mol}/\text{L}$	1	0.088 (24 h)	(63)
Rat	Purple black rice	100 mg C3G ^e			0.005 (4 h)	(61)
Rat	Blackcurrant	100 mg C3G ^e	0.18 $\mu\text{mol}/\text{L}$	0.25		(62)
Rat	Blackcurrant		0.36 $\mu\text{mol}/\text{L}$	3	0.190 (24 h)	(65)
	Chokeberry	229 μmol			0.096 (24 h)	(64)
Pig	Blackcurrant	140 μmol			0.067 (24 h)	
	Elderberry	228 μmol			0.131 (24 h)	
Pig	Raspberry	50 mg			0.073 (4 h)	(66)

^atotal ACNs, if not stated otherwise.

^bmaximal plasma concentration.

^ctime to reach C_{max}.

^d% of intake.

^eC3G = cyanidin-3-glucoside; ^fC3R = cyanidin-3-rutinoside; ^gD3R = delphinidin-3-rutinoside, ^hD3G = delphinidin-3-glucoside.

Human studies. The investigation of ACN absorption in humans has increased over the last decade. Lapidot *et al.* (67) investigated the bioavailability of red wine ACNs (218 mg ACNs/300 mL) as detected in human urine (max. 5% of the oral dose within 12 h). Their study demonstrates the absorption, in part, of ACNs in humans, after ingestion of a normal amount of red wine (two glasses). However, none of the more recent studies were able to find urinary recoveries nearly as high as those reported by these authors. Cao and Prior (68) report direct evidence of the absorption of ACNs (intake total ACNs: 1.5 g) in their glycosidic form in humans. The plasma ACN concentration was reported to be at least 100 $\mu\text{g/L}$, 30 min after consumption of the elderberry extract.

Matsumoto *et al.* (58) found blackcurrant ACNs to be directly absorbed, distributed to the blood and excreted into urine as the glycosylated forms in rats and humans. In their human study, they detected the blackcurrant ACNs in both the plasma (0.120 $\mu\text{mol/L}$) and the urine (0.06-0.11% of the dose ingested) also as the intact form, after subjects ingested a single dose of blackcurrant concentrate (3.57 mg C3G/kg BW). Their results indicate that ACN-glycosides can be absorbed rapidly within 2 h of ingestion and are excreted in urine as the intact forms. The extremely low bioavailability of ACN has also been shown in a human study by Murkovic *et al.* (4). After ingestion of 180 mg ACN (spray-dried elderberry juice as gelatinous capsules), the maximum plasma concentration was found to be 35 ng/mL, as a result of quick degradation or excretion of the compounds. Another study, using 200 ml blackcurrant juice (153 mg of ACN), found that only 0.02-0.05% of the oral dose was excreted in urine (69). It was suggested that the poor bioavailability of these unstable compounds, like incomplete absorption, decomposition in the lumen, elimination with the faeces, or substantial first-pass elimination may contribute to their low urinary excretion rate.

Malvidin-3-glucoside (M3G), an ACN occurring in red wine and red grape juice was studied by Bub *et al.* (70). After ingestion of red wine (68 mg M3G/500 mL) or red grape juice (117 mg M3G/500 mL), M3G was found in plasma (C_{max} : 1.4 nmol at 20 min, red wine; C_{max} : 2.8 nmol at 180 min, red grape juice) and urine (< 0.03% of the ingested amount of red wine, and red grape juice respectively) of human volunteers. Neither aglycones nor glucuronate or sulfate conjugates were found in plasma and urine samples, indicating that M3G is absorbed in its glucosylated form (70).

More recent studies still confirm low bioavailability of ACNs. After ingestion of 11 g elderberry (containing 1.9 g ACN), very low recoveries of ACN were found in urine (0.003-0.012% of the oral dose) (71). McGhie *et al.* (72) also found a low ACN excretion in urine (0.01-0.06%) over a 7 h period after ingestion of Boysenberry concentrate (344.5 mg ACNs), blackcurrant concentrate (188.5 mg ACNs), and blueberry extract (439.1 mg ACNs). Frank *et al.* (73) found urinary excretions of ACNs between 0.18 and 0.23%, after the ingestion of a single oral dose of either 400 mL red grape juice (283.5 mg total ACNs), or 400 mL red wine (279.6 mg total ACNs). Felgines *et al.* (74) on the other hand reported a urinary excretion of 1.80%, after consuming 200 g strawberries providing 179 μ mol pelargonidin-3-glucoside, which is to date the highest reported recovery. In a more recent study, investigating blackberry ACNs (mainly C3G), the same authors showed a urinary excretion of only 0.16% (75). The considerable difference in urinary excretion indicates a possible difference in the bioavailability of individual ACNs, as had been shown in pigs by Wu *et al.* (64). A recent study which compared the absorption and excretion of blackcurrant ACNs in humans and rabbits found no differences between the two species in the percentage of the ingested dose excreted in urine at 4 h after ingestion (57). A review of human studies on ACN absorption is shown in **Table 4**.

More recent studies reported methylated and glucuronidated conjugates of ACNs in human urine (19, 74-79), as well as sulfoconjugates and aglycones (74, 75). A few studies also showed the methylated and glucuronidated metabolites in human serum (76, 77).

Overall, these studies indicate that ACNs are mainly absorbed in their intact forms as glycosides. However, the absorption mechanism involved has not been identified yet. Within the intestinal cells, ACNs are partly metabolized via methylation and/or glucuronidation. In the liver and kidneys further metabolism takes place, and ACNs are excreted in the urine either as the intact glycosides, or as methylated or glucuronidated forms. **Figure 7** represents possible pathways of ACNs during their absorption, metabolism, and excretion. All animal and human studies on ACN absorption agree on the extremely low bioavailability of these compounds. In addition, the review of the literature on ACN bioavailability shows, that there is a large variability in the reported dose-plasma concentration ratios, which is most likely due to the different applied methods for measuring ACN concentration in plasma. To date there is no general validated assay for the extraction of ACNs in plasma or urine samples. It is therefore questionable if the reported

low plasma concentrations are sufficient enough for ACNs to exert health-related effects *in vivo*. If it is possible to maximize ACN absorption into the human body, the resulting ACN concentrations in the blood circulation may be sufficient enough to provide target tissues with adequate amounts to perform health-related effects.

Table 4. Human ACN absorption studies.

Material	ACN dose ^a (total intake)	C _{max} ^b	t _{max} ^c (h)	Urinary excretion ^d (%)	Ref.
Red wine (300 mL)	218 mg			5.10 (12 h)	(67)
Elderberry extract (25 g)	1.5 g	100 ng/mL	0.5		(68)
Blackcurrant	236 mg	0.120 μ mol/L	1.25-1.75	0.06-0.11 (8 h)	(58)
Elderberry juice (spray dried capsules)	180 mg	35 ng/mL	1		(4)
Blackcurrant juice (200 mL)	153 mg			0.02-0.05 (5 h)	(69)
Red wine (500 mL)	68 mg M3G ^e	0.0014 μ mol/L	0.8	0.02 (6 h)	
Dealcoholized red wine	56 mg M3G ^e	0.0017 μ mol/L	1.5	0.02 (6 h)	(70)
Red grape juice (500 mL)	117 mg M3G ^e	0.0028 μ mol/L	2.0	0.02 (6 h)	
Elderberry (11 g)	1.9 g			0.003- 0.012 (6 h)	(71)
Blueberry powder (100g)	1.2 g	0.029 μ mol/L	4		(80)
Elderberry extract (12 g)	720 mg	0.097 μ mol/L	1.2	0.06 (24 h)	(81)
Elderberry extract (12 g)	720 mg			0.08 (4 h)	(78)
Blueberry (189 g)	690 mg			0.004 (6 h)	(78)
Red wine (400 mL)	180 mg	43 ng/mL	1.5	0.23 (7 h)	(73)
Red grape juice (400 mL)	284 mg	100 ng/mL	0.5	0.18 (7 h)	(73)
Blackcurrant juice	1.24 g	53 ng/mL	0.75	0.07 (4 h)	
	0.72 g	16 ng/mL	0.75	0.05 (4 h)	(57)
	0.75 g	32 ng/mL	1.5	0.05 (4 h)	
Blackcurrant concentrate (300 mL)	189 mg			0.06 (7 h)	(72)
Boysenberry concentrate (300 mL)	345 mg			0.03 (7 h)	(72)
Blueberry extract (300 mL)	439 mg			0.02 (7 h)	(72)
Strawberries (200 g)	76 mg			1.80 (24 h)	(74)
Chokeberry extract (7.1 g)	721 mg	0.096 μ mol/L	2.8	0.15 (24 h)	(77)
Blackberries (200 g)	431 mg			0.16 (24 h)	(75)

^atotal ACNs, if not stated otherwise.

^bmaximal plasma concentration.

^ctime to reach C_{max}

^d% of intake.

^eM3G – malvidin-3-glucoside.

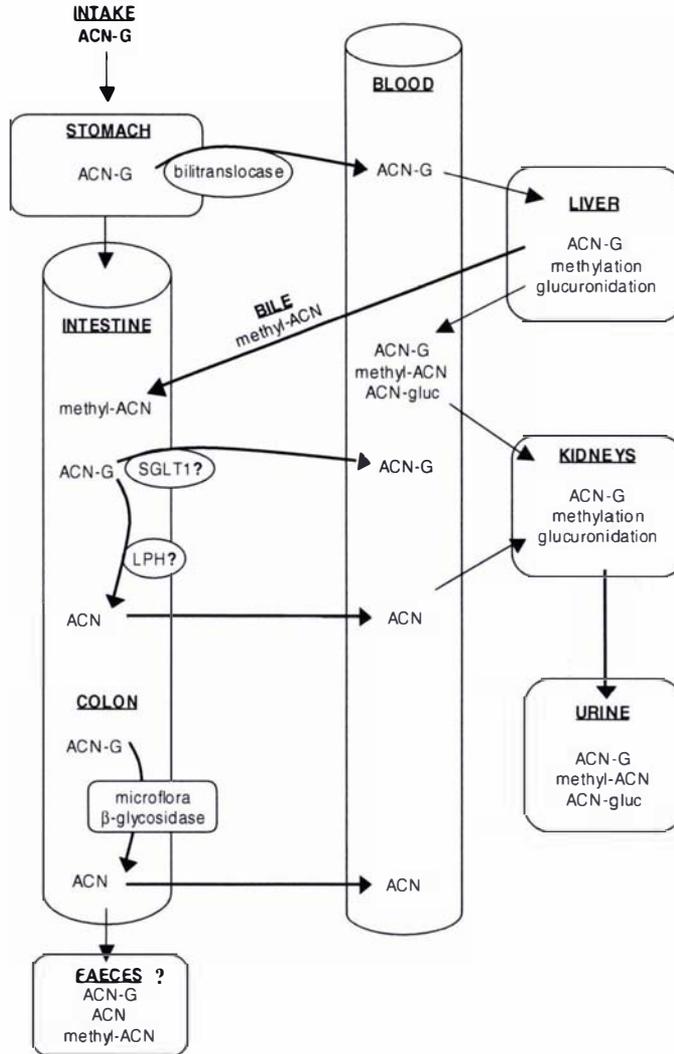


Figure 7. Possible pathways of ACN absorption, metabolism, and excretion in the animal/human body. ACN (ACN aglycone), ACN-G (ACN-glucoside), methyl-ACN (methylated ACNs), ACN-g luc (glucuronidated ACNs), SGLT1 (sodium-dependent glucose transporter), LPH (lactase phloridzin hydrolase).

ANTIOXIDANT EFFECTS

General. Numerous epidemiologic and clinical trials have shown that consumption of fruits and vegetables, many of which are rich in ACNs, are related to the decreased incidence of many chronic and degenerative diseases, including heart disease, cancer, and aging (82-84). The preventive effect of plant products have been largely ascribed to their high content of antioxidants, such as vitamin C, tocopherols, carotenoids, and polyphenols (85). In fact, antioxidant mechanisms have been suggested as potential means of disease prevention (86, 87). However, the major components of fruits that act as antioxidants are phytochemicals such as phenolic compounds, flavonoids, and ACNs. Among all fruits and vegetables in the diet, dark blue and red coloured berries were especially reported to have the highest antioxidant capacities (88). Indeed, fruits with a high ACN content have a high antioxidant capacity (1, 15, 89), which has been shown *in vitro* (89-98) and *in vivo* (80, 99-103). Thus the beneficial effects of ACNs may mainly be related to their potent antioxidant activity. The phenolic structure of ACNs (**Figure 1**) conveys marked antioxidant activity in model systems via donation of electrons or transfer of hydrogen atoms from hydroxyl moieties to free radicals (104).

Reactive oxygen species (ROS), including superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{\bullet}), and singlet oxygen (1O_2), are generated as by-products of normal metabolism (105). Increased levels of these ROS or free radicals create oxidative stress, which may impair metabolism, cause oxidative damage to essential cellular components, and eventually result in cell death (94, 106). Oxidative stress occurs when exposure to oxidants overcomes the native antioxidant defences, which include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, as well as macromolecules, such as albumin, ceruloplasmin, and ferritin, resulting in oxidative damage to biomolecules like lipids, proteins, and DNA (**Figure 8**) (15). The consequences are lipid peroxidation and protein degeneration, which subsequently damage cell components such as lipoprotein, and result in degenerative diseases like atherosclerosis. The oxidative damage to DNA leads to mutation and consequently results in cancer. In fact, oxidative stress has been associated with the development of many chronic and degenerative diseases, including cancer (107), heart disease (108), neuronal degeneration

such as Alzheimer's (109) and Parkinson's diseases (110), as well as being involved in the progress of aging (108).

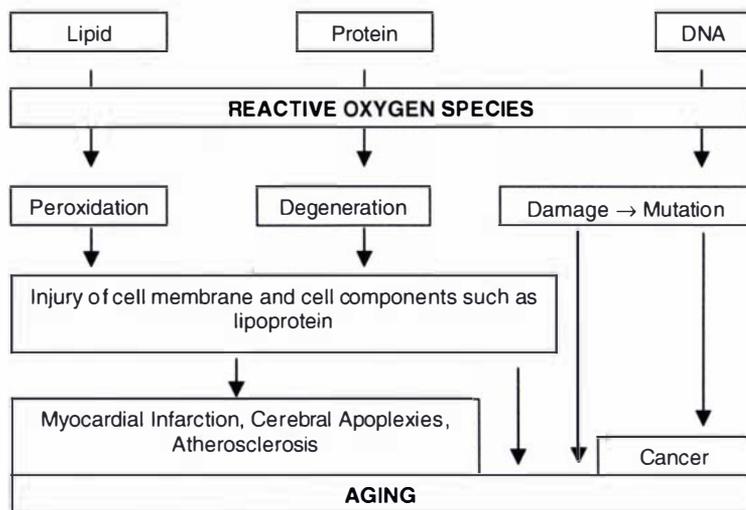


Figure 8. Consequences of reactive oxygen species attack on lipids, proteins and DNA.

Antioxidants can reduce oxidative molecular and cellular damage by preventing the initial attack of biomolecules by free radicals, or by interrupting the perpetuation of free radical species (111). Thus, antioxidants, such as ACNs may be of importance in the prevention of oxidative stress related diseases. The oxygen radical absorbance capacity (ORAC), which measures antioxidant scavenging activity against peroxy radical induced by 2,2'-azobis(2-amidinopropane)dihydrochloride, has been shown to be high in several berry fruits, including strawberries, blackberries, blueberries, blackcurrants and red raspberries (15, 112-115), as well as for purified ACNs (89). It has further been demonstrated that several berry fruits also possess antioxidant activities against superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (105). However, the antioxidant activity of berry fruits is expected to vary, due to seasonal reasons, and growing conditions (116). In addition, interactions with other phenolic compounds present in berries, or with nutrient compounds present in the diet may alter the original antioxidant capacity of berry fruit ACNs (106).

ACNs exhibit a variety of biological effects based on their antioxidant activity. Oxidative modification of low-density lipoprotein (LDL) in the arterial wall plays a key role in the pathogenesis of atherosclerosis. Ghiselli *et al.* (117) reported a free radical scavenging activity, as well as the inhibition of LDL oxidative modification and platelet aggregation, two events in the pathogenesis of atherosclerosis, by an ACN fraction obtained from an Italian red wine. Purified extracts of a number of berries, including lingonberry, bilberry, blackcurrants and raspberry, and ACNs from tart cherries were also reported to provide protection toward lipid and protein oxidation (118, 119). The most abundant ACN found in fruits, C3G, has been shown to reduce serum thiobarbituric reactive substances and decreased the sensitivity toward *ex vivo* lipid peroxidation in normal rats, and rats subjected to oxidative stress by hepatic ischemia-reperfusion injury (99, 100). The latter studies indicate that ingestion of ACNs or ACN-rich food can protect against oxidative stress in animals. However, *in vivo* studies have shown, that the supplementation with cranberry juice in humans, or blackcurrant material in rabbits had no, or rather an increasing effect of biomarkers such as LDL and cholesterol (120, 121). These results indicate the importance to differentiate between *in vitro* findings and actual effects of dietary components on human health *in vivo*.

A further biological activity of ACNs has been reported to be the protection against age-related declines in cognitive behaviour and neuronal dysfunction in the central nervous system (122). A short-term supplementation (8 weeks) of rats with either a strawberry or spinach extract retarded age-related decrements in cognitive and neuronal function, and a long-term dietary supplementation (9 months) with a blueberry extract was effective in reversing age-related deficits in neuronal and motor function (122-124). Strawberries have additionally been suggested to protect oxidative stress-induced neuronal damage, and the protection has been shown to be due to the antioxidant properties against neurodegeneration (125). These studies suggest that an optimum intake of coloured food, such as berries, may play an important role in preventing or perhaps reversing the effects of oxidative stress in the progress of aging and neurodegenerative disease by preserving normal neuronal functions.

Moreover, ACN fractions extracted from a number of different sources, including berry extracts have demonstrated anticancer activity (126). Endogenous oxidative damage, particularly to DNA, has been considered to be a significant factor in the initiation of

human cancer. Several antioxidants in fruits and vegetables have been suggested to contribute to the anticarcinogenic effect, by scavenging free radicals, thus preventing DNA damage and subsequently mutation (127). The antioxidant activity shown by ACNs is suggested to be the most important property that can be exploited for their use as cancer preventive agents (128). Flavonoids have been shown to inhibit cancer cell proliferation (129), and quercetin for example, was reported to inhibit the proliferation of azoxymethanol-induced colonic epithelial tumor cells in mice (130). The inhibition of tumor cell proliferation by natural food colours such as the ACN C3G were also demonstrated to be significant (131). Extracts of several berry fruits strongly inhibited cell proliferation in colon cancer, breast cancer (129), and human liver cancer cells in a dose-dependent manner (127). An *in vitro* study by Lazze *et al.* (132) has demonstrated that ACNs are furthermore effective against cytotoxicity, and DNA single strand break formation induced by tert-butyl-hydroperoxide, and another study indicated that ACNs could inhibit H₂O₂ induced DNA damage (DNA strand breaks) in human colon cells (133). In fact, it has been suggested that the anticancer activity of ACNs may be due to their ability to protect DNA from single strand breaks (128). In Vitamin E deficient rats, the hepatic level of 8-hydroxy-2'-deoxyguanosine (8-OHdG), another marker for oxidative DNA damage, was reduced following a 2-week supplementation with ACN extracts (102). Black raspberries were reported to significantly reduce urinary 8-OHdG levels in rats with azoxymethane-induced colon carcinogenesis (134). However, a recent human intervention study has shown controversial results, where the daily supplementation with an ACN-rich cranberry juice for 2 weeks did not alter blood or cellular antioxidant status, or several biomarkers, including urinary 8-OHdG or endogenous or H₂O₂-induced DNA strand breaks (120). Thus, these results indicate the importance of distinguishing between the *in vitro* and *in vivo* antioxidant activities of dietary ACNs in relation to human health.

Biomarkers of oxidative damage. Several biomarkers have been reported for the measurement of oxidative damage *in vivo*, including oxidative damage to lipids (plasma malondialdehyde), proteins (carbonylated plasma protein), and DNA (urinary excretion of 8-OHdG; strand breaks – comet assay).

Lipids are readily damaged by free radicals, and result in the generation of a number of further products that damage other biomolecules. One of the most frequently used biomarkers providing an indication of the overall lipid peroxidation level is the plasma

concentration of malondialdehyde, one of several byproducts of lipid peroxidation processes (135).

Proteins can also be damaged by free radicals resulting in the oxidation of amino acids forming protein carbonyls (136). The presence of protein carbonyls is one of the most widely used means of detecting oxidative damage to proteins and has been associated with aging, diabetes and neurodegenerative disease (136, 137). Protein carbonyls are measured by reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine followed by spectrophotometric, immunochemical, or radiometric techniques.

Oxidative damage to DNA is, at least in real life, a rare event. However, it is important to be able to assess it accurately, since it may represent one of the earliest stages of carcinogenesis, and it is a valuable biomarker of oxidative stress (138). Oxidative damage to DNA occurs *in vivo* and produces lesions that may be mutagenic and lethal (139). One of the most abundant lesions, 8-OHdG, is widely used as a marker for oxidative damage to DNA and has been used to establish that a wide range of environmental and lifestyle factors are associated with increases in oxidative damage. 8-OHdG can be measured in cells and urine, but its measurement in urine provides an assessment of oxidative damage to DNA in the whole body. The exact origin of 8-OHdG in urine is not known, but nucleotide excision repair provides a plausible mechanism for its excretion in urine (140).

Another approach to assess DNA damage is to measure strand breaks of DNA by the comet assay (also known as single cell gel electrophoresis). This assay represents a rapid, simple, visual and sensitive technique for measuring and analysing DNA breakage within single mammalian cells (141, 142). A flow chart of the method is shown in **Figure 9**. It can be applied to lymphocytes and is especially suitable for use in human biomonitoring studies (138). It has become one of the standard methods for assessing DNA damage. The generally adopted comet assay technique is that of Singh *et al.* (141), in which the procedure of Ostling and Johanson (142) is modified by performing the electrophoresis at high pH instead of under neutral conditions. Briefly, cells are embedded in agarose on a microscope slide and lysed with detergent and high salt. After lysis, histones are removed but the supercoils of DNA persists, so that the DNA remains tightly packed in a nucleus-like structure, the nucleoid. Subsequently, the liberated DNA undergoes electrophoresis under alkaline conditions (pH > 13), where loops containing a strand break lose their supercoiling and become free to extend toward the anode. The migrated DNA is finally

stained with a DNA binding dye (e.g. ethidium bromide) and analysed by a fluorescence microscope. The resulting pictures of damaged DNA resemble “comets” (143), whose size, and the distribution of fluorescence within have been correlated quantitatively with frequency of DNA breaks (144). Quantitation of DNA damage is either assessed using commercial software packages for image analysis, where the comets are analysed by charge-coupled device camera, giving parameters such as tail length or % DNA in tail, or by visual analysis/scoring.

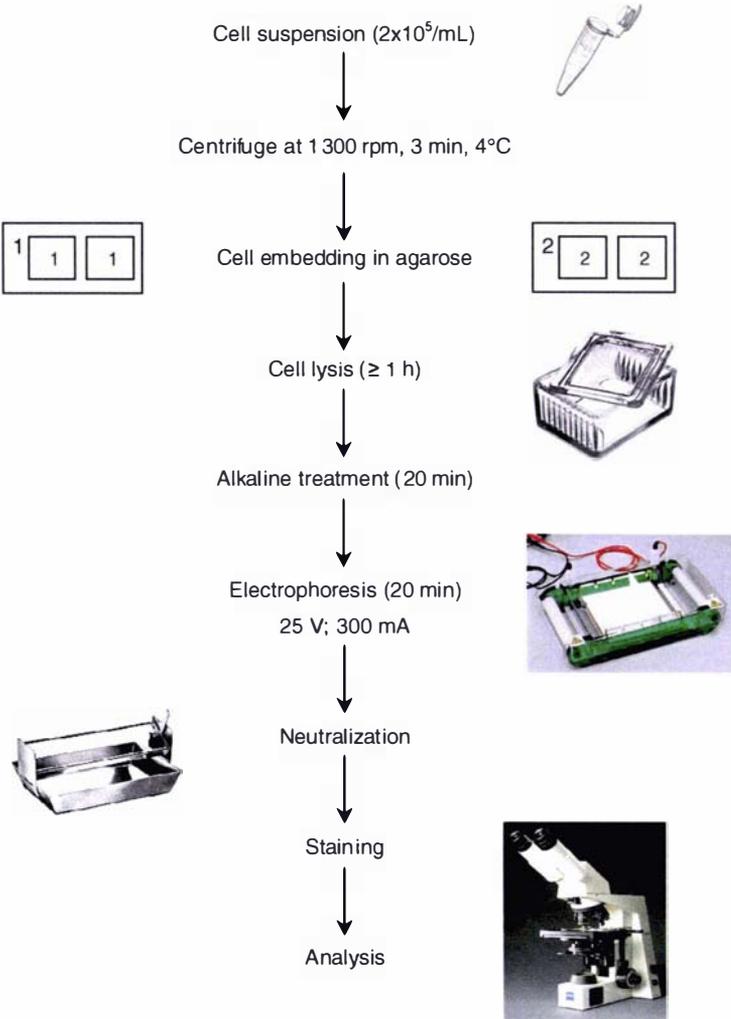


Figure 9. Flow chart of the comet assay.

For analysis by visual scoring, comets are classified into one of 5 classes according to the relative intensity of fluorescence in the 'comet'-tail (0 = no tail; 4 = almost all DNA in tail) with the total score per sample being between 0 and 400 "arbitrary units" (**Figure 10**). The latter is a widely used, simple and less time consuming method, which has been shown to be faster and more sensitive than computerized image analysis (145).

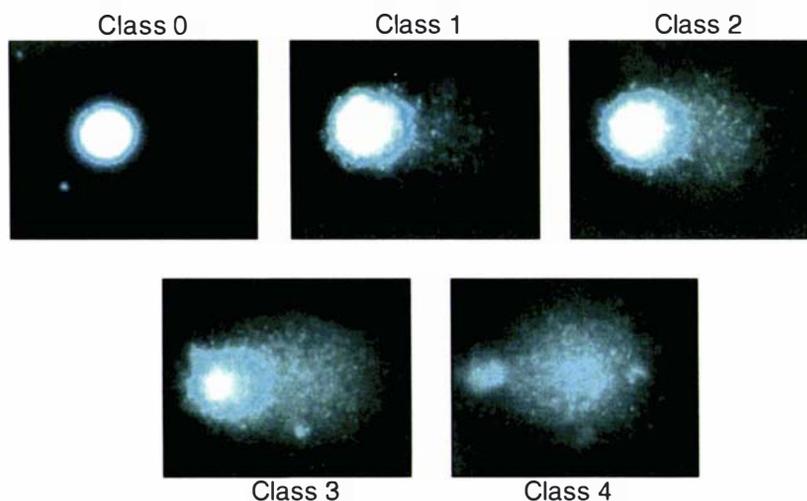


Figure 10. Classification of comets into classes 0-4 for visual analysis.

JUSTIFICATION OF STUDIES

Over the last decade, there has been an increased interest in the health benefits of secondary plant metabolites, such as the polyphenols, including flavonoids and ACNs. Especially their antioxidant properties have received great interest in protecting against degenerative diseases. However, to act as antioxidant *in vivo*, these compounds first have to be efficiently absorbed from the GIT into the blood stream, and circulated within the body in concentrations high enough to exert health-promoting effects. Numerous studies have been conducted to investigate the bioavailability of ACNs, including absorption, metabolism, and excretion. However, all those studies have reported extremely low bioavailabilities for ACNs. Furthermore, the exact absorption mechanisms for ACNs are still not fully understood. From the review of the literature ACNs may initially be absorbed from the stomach via bilitranslocase and reach the blood circulation as the intact glycosidic form. ACN, which reach the small intestine, might be either absorbed as their intact form

via the SGLT1 transporter, or subsequent to hydrolysis by LPH and appear in blood circulation as the aglycone or glycoside. However, to date it has not yet been investigated, where ACNs are absorbed throughout the entire GIT, neither what exact absorption mechanism is involved. Furthermore, almost none of the previous studies have investigated the effect of additional ingested food on ACN absorption, as the majority of the studies administered ACNs in an aqueous form. However, as ACNs are rarely ingested on their own, but rather in combination with other flavonoids present in fruit, or in combination with foodstuff, it is necessary to take possible ACN-food or ACN-flavonoid interactions into account, which could have an impact on ACN bioavailability. Furthermore, the influence of other food components on the antioxidant activity of ACNs has not been shown yet. To benefit from possible health effects of ACNs, their bioavailability, and in particular ways to enhance their absorption need to be further studied. Detailed knowledge regarding ACN absorption and mechanisms thereof is an important first step toward recommendations on ACN intake. A possible improvement of ACN absorption into humans could result in increased ACN plasma levels, and subsequently enhance their proposed health-related benefits.

The major objectives of the present thesis were:

1. To provide further knowledge on ACN absorption, including
 - a. *In vitro* studies (Ussing chambers) to evaluate the main absorption site for ACNs within the GIT (Chapter 2)
 - b. *In vitro* studies (Ussing chambers) to assess the absorption mechanism involved with ACN absorption (Chapter 3)
 - c. Analysis of the influence of food and other flavonoids on ACN absorption *in vitro* and *in vivo* (Chapter 3-5)

2. To investigate antioxidant effects of ACNs, including an
 - a. *In vivo* study to investigate the effect of a food matrix and other flavonoids on the antioxidative capacity of ACNs (Chapter 5)
 - b. *In vivo* study to investigate the antioxidative capacity of ACNs on parameters related to oxidative stress (Chapter 6)

ABBREVIATIONS USED

ACNs, anthocyanins; BW, body weight; C3G, cyanidin-3-glucoside; GIT, gastrointestinal tract; LPH, lactase phlorizin hydrolase; M3G, malvidin-3-glucoside; MRP2, multidrug resistance-associated protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; Q3G, quercetin-3-glucoside; Q4'G, quercetin-4'- β -glucoside; ROS, reactive oxygen species; SGLT1, sodium-dependent glucose transporter.

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CHAPTER 2

THE JEJUNUM IS THE MAIN SITE OF ABSORPTION FOR ANTHOCYANINS IN MICE

Prior to investigating proposed health benefits of anthocyanins, it is first necessary to gain further knowledge in the details and mechanisms of anthocyanin absorption. The aim of the study reported in this chapter was to determine where anthocyanins are absorbed within the gastrointestinal tract.

ABSTRACT

Intestinal absorption of anthocyanins (ACNs) was studied *in vitro* by comparing ACN disappearance from the mucosal solution of Ussing chambers not containing any tissue (controls), and Ussing chambers containing segments of mouse duodenum, jejunum, ileum or colon. The tissues were mounted in the chambers and bathed with Ringer's solution (RS) adjusted to a pH representative of the respective segments *in vivo*. The chambers were kept at 37°C and the RS perfused continuously with carbogen (95% O₂ /5% CO₂). After the addition of an ACN extract to the mucosal solution, samples from both the mucosal side and serosal side were withdrawn at 10, 40, 80 and 120 min and analyzed for ACN concentration using a reversed-phase HPLC with photodiode array detection. The highest absorption of ACNs occurred in chambers mounted with jejunal tissue (max absorption rate, 55.3 ± 7.6%). Minor absorption occurred with duodenal tissue (10.4 ± 7.6%), with no absorption recorded when tissues from the ileum or colon were used. This study demonstrates for the first time that ACN absorption in mice occurs predominantly in the jejunum.

INTRODUCTION

Anthocyanins (ACNs) are responsible for the red, purple and blue colors displayed by many vegetables and fruits (particularly berries) and are part of a large and widespread group of water-soluble plant constituents, known collectively as flavonoids (1). Their intake in humans has been estimated to be as much as 180-215 mg/d in the United States (2) due to their widespread distribution and occurrence in fruits and vegetables. Berry fruits in particular are rich dietary sources, and some can contribute 100-300 mg ACNs in a single serving (3). As a potential major component of our daily diet (2), increasing attention has focused on the biological activities and possible health benefits of ACNs. In particular the antioxidant properties of flavonoids (4) are thought to protect cells against oxidative damage that contributes to various pathologies, including diabetes (5), arteriosclerosis (6), neurodegeneration (7) and some cancers (8-10). However, to evaluate the health effects of ACNs in detail, their bioavailability, including absorption, metabolism and excretion must be known (11). At present, these aspects of ACNs are still not fully understood. Most studies have found that the glycosidic forms of ACNs are absorbed intact and appear in

plasma and urine samples (3, 12-17), whereas other studies have found metabolites of ACNs in urine, as well as in tissues such as liver and kidneys (11, 12, 17, 18). Nevertheless the fate of ACNs within the gastrointestinal tract (GIT) is largely unknown.

The pH has a marked influence on the color and stability of ACNs in aqueous media (19, 20), where ACNs exist as a mixture of at least four different molecular species: the colored basic flavylum cation and three secondary structures, the quinoidal base, the carbinol pseudobase, and the chalcone pseudobase (21). The concentration with which each compound is present in a solution is pH dependent. During the passage of ACNs through the GIT, they are exposed to different pH environments and therefore might exist as different forms. The ACN forms present in the different regions and tissues of the GIT and eventually during absorption are not known with certainty. It is likely that the flavylum cation will exist only in the lumen of the stomach due to low pH, and that the other forms will predominate lower down the GIT and in the epithelium if absorbed. This assumption was recently supported by a study of McGhie *et al.* (3). Sixty minutes after dosing rats with a Boysenberry extract, an intense red color in the stomach indicated the presence of the red flavylum cation, whereas no ACNs as the cation were observed in the small intestine. However, after acidifying the intestinal tissue, a red color appeared, indicating the conversion of colorless forms of ACNs into the red cation. It is possible that ACNs are converted to the carbinol pseudobase, quinoidal base, or chalcone pseudobase during passage through the small intestine, and that these compounds are the potential forms that are absorbed from the gut into the blood system (22). Whether or not these forms are the ones responsible for the observed biological activity is not known yet.

The aim of the present study was to evaluate ACN absorption at different locations in the intestine, and in particular, to identify the main absorption site within the GIT. For this purpose, Ussing chambers were used to compare the absorption (as disappearance of ACNs from the mucosal solution) of a Boysenberry ACN in four intestinal segments of mice, the duodenum, jejunum, ileum and colon.

MATERIALS AND METHODS

Animals. The study was approved and followed the procedures set by the Animal Ethics Committee of the Massey University (Palmerston North, New Zealand) (23). A total

of 10 male Swiss mice (age, 6-8 weeks) were used for the experiments. The mice were obtained from the Small Animal Production Unit at Massey University. They were housed in rodent cages and kept in a room with controlled temperature ($21 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (12 h light dark cycles with dawn-and-dusk transitional periods). The mice were fed a balanced standard rodent diet (Table 1) prepared at the Food Processing Unit, Massey University and were given access to both food and demineralised water *ad libitum* until the day of the experiment.

Table 1. Ingredient composition of the diet.

Ingredient	Amount (g/kg diet)
Wheat	403.5
Barley	300
Broll	50
Lucerne	50
Meat meal	60
Fish meal	70
Skim milk powder	50
Soybean oil	10
Premix ^a	5
Methionine	1
NaCl	0.5

^aContained (g/kg mix): Vitamin A (1.4 MIU); Vitamin D3 (0.20); Vitamin E (10.0); Vitamin B₁ (0.80); Vitamin B₂ (1.0); Vitamin B₆ (1.2); Vitamin B₁₂ (0.004); Vitamin B₅ (5.0); biotin (0.02); Vitamin B₃ (4.0); folic acid (0.20); cholin (50.0); Fe (16.0); Zn (10.0); Mn (10.0); Cu (1.0); I (0.10); Co (0.14); Sn (0.04); Mg (100.0); Ca (147.8); antioxidant (0.026); K (100.1); S (10.3); Cl (9.1).

Experimental setup. On the day of the experiments, mice were deprived of food for 2-3 h, anaesthetized with 6% halothane and euthanased by cervical dislocation. Within 1 min of euthanasia, each mouse's abdomen was opened by a midline incision; the intestine was dissected out and immersed in Ringer's solution (RS; see below) at room temperature. Two-centimeter-long pieces of intestine from each region (duodenum, jejunum, ileum, and colon) were opened longitudinally along the mesenteric border and mounted in individual Ussing chambers. The exposed surface of the tissue was 0.67 cm^2 . The composition of the RS was as follows (in mmol/L): 120 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 1.8 NaH₂PO₄, 0.2 Na₂HPO₄, 25 NaHCO₃, 2 glutamine, and 2 sodium pyruvate. The pH was adjusted with 1M HCL to between 4.5 and 7.0, depending on the region from which the intestinal piece was taken (24-28). The tissues were kept at 37.5°C and the RS aerated with Carbogen (95% O₂/5% CO₂). All tissues were voltage clamped throughout the experiments at 0 mV (29)

using automatic clamp units (Campus Electronics and Mechanical, University of Otago, Dunedin, New Zealand). The RS was replaced at least three times at the start of each experiment to wash the tissues and remove any luminal contents. The volume of the RS on each side of the chambers was 8 mL. The tissues were left to equilibrate after washing for at least 30 min before the ACN extract was added to the mucosal RS.

Chambers without tissue were used as controls to measure ACN degradation due to experimental conditions during the 120 min of the experiment. Therefore, for each intestinal region, control chambers were set up and bathed with a RS of the same pH value as the respective intestinal segment.

ACNs. Frozen Boysenberries (*Rubus loganbaccus x baileyanus* Britt, 600g) were extracted twice with 1.5 L acetone/water/acetic acid (70:29.5:0.5). Extracts were evaporated to a 500-mL total volume at 40°C and lipids were removed by extracting (three times) with 500 mL of hexane. The aqueous layer was evaporated and then diluted to 20% methanol/water using methanol. Subsequently, the aqueous fraction was applied onto a large LH-20 Sephadex column (50-mm Ø x 370-mm height, 450 g LH-20) and washed with 20 % methanol/water. Boysenberry ACNs were eluted with 60% methanol/water, evaporated to dryness, and stored in the dark at 4°C until use.

ACNs of Boysenberries have previously been characterized as cyanidin-3-sophoroside, cyanidin-3-glucoside (C3G), cyanidin-3-glycosylrutinoside, and cyanidin-3-rutinoside (17, 30). The extract used in the present study contained predominantly C3G (**Figure 1**), the major monosaccharide ACN of Boysenberries (17).

For each experiment, a stock solution containing 5 mg Boysenberry extract per 4 mL of RS at the appropriate pH value was prepared. This stock solution was used to prepare a standard for each pH value as well as application of the extract to the mucosal solution (8 mL) with an average C3G concentration of $2.67 \pm 0.15 \mu\text{mol/L}$.

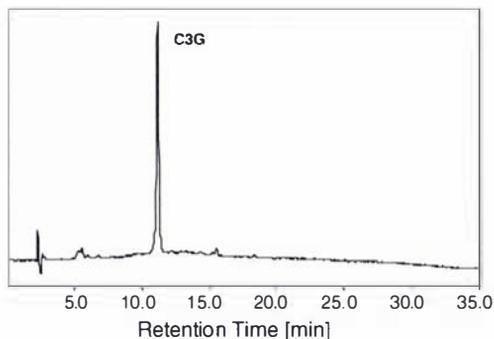


Figure 1. Representative HPLC chromatogram of purified Boysenberry C3G.

Sample preparation. Samples (400 μL) from the mucosal and serosal compartments of the Ussing chambers were taken at 10, 40, 80 and 120 min. Mucosal samples were immediately acidified (1:1) with 5% formic acid/ H_2O and stored at 4°C until analysis. Serosal samples were stored at 4°C until solid-phase extraction was performed, usually not later than 24 h. IST Isolute C18 (EC) cartridges were conditioned with 5% formic acid/methanol (5 mL) and 5% formic acid/ H_2O (10 mL) before the serosal samples (400 μL) were loaded onto the columns. Subsequently the cartridges were washed with 5% formic acid/ H_2O (10 mL) and ethyl acetate (5 mL) before the columns were dried by drawing air for approximately 15 s to remove ethyl acetate. ACNs were eluted into glass tubes with 5% formic acid/methanol (2 mL). The extracts were evaporated close to dryness under a stream of N_2 at $\leq 35^\circ\text{C}$ and the residue was re-dissolved in 5% formic acid/ H_2O (200 μL). Samples were transferred into HPLC vials and stored at 4°C until analysis.

HPLC analysis. C3G-concentrations in mucosal and serosal samples were determined by reversed-phase HPLC with photodiode array detection (PDA). The column used was a LiChrosphere 100 RP-18 end-capped 5 μm column (250 x 4 mm). Solvents A (1.5 % o-phosphoric acid) and B (acetic acid/acetonitrile/phosphoric acid/ H_2O , 20:24:1.5:54.5) were run at a flow rate of 1 mL/min. The solvent gradient started with a composition at 80 % A, 20 % B and changed to reach 30 % A, 70 % B at 25 min. The composition then changed to reach 10 % A, 90 % B by 30 min and held this composition until 35 min, before returning to the starting conditions at 40 min for 5 min with a total run time of 45 min. The sample injection volume was 10 μL for the mucosal samples and 50 μL for the serosal samples,

respectively. The PDA was used to collect spectral data (250-600 nm) and chromatograms were extracted at 520 nm. Chromatography data were collected and processed using a Water Millennium Chromatography Manager (version 4.0). C3G-concentrations were calculated using an authentic standard of cyanidin-3-O-galactoside with known concentration.

Statistical analysis. Data are presented as means \pm SEM. Statistical analysis was carried out using SAS System for Windows (version 8). The residues of each analysis were tested for normality. The significance of differences was assessed by one-way ANOVA for comparison of individual means. Differences with $p \leq 0.05$ were considered significant.

RESULTS

After adding C3G to the RS of the mucosal compartment of the Ussing chambers, C3G disappearance from the mucosal solution and C3G appearance in the serosal solution were measured by HPLC. **Figure 2 (A-D)** shows C3G concentrations in the mucosal solution of tissue-mounted chambers, together with each respective control during the 120 min of the experiment.

With the duodenal segment, a significant decrease of C3G over time could be seen with the intestinal tissue (27.6%), whereas there was no significant effect over time with the control throughout the experiment (**Figure 2 A**). Significant differences between the control and the intestinal tissue, indicating absorption, were observed at 80 and 120 min.

For the jejunal tissue, there was a significant decrease in C3G concentration in the mucosal solution over time (initial concentration: 1.26 $\mu\text{g/mL}$; concentration after 2 h, $0.57 \pm 0.03 \mu\text{g/mL} = 54.8\%$) (**Figure 2 B**). This decrease was not due to degradation as the control showed that C3G was stable in RS throughout the experiment (concentration 2 h after mucosal addition, $1.23 \pm 0.03 \mu\text{g/mL} = 98.6\%$). A significant difference ($p < 0.001$) between the control and the jejunal tissue, indicating absorption, appeared already after 10 min.

With the ileal and the colonic segments, both the controls and the respective intestinal tissues showed a significant C3G decrease over time (ileum-control, 31.8%; ileum-tissue: 34.8%; colon-control, 16.5%; colon-tissue, 18.2%; **Figure 2 C, D**). However, no significant

difference between the controls and the intestinal tissues, representing absorption, was observed at any time point.

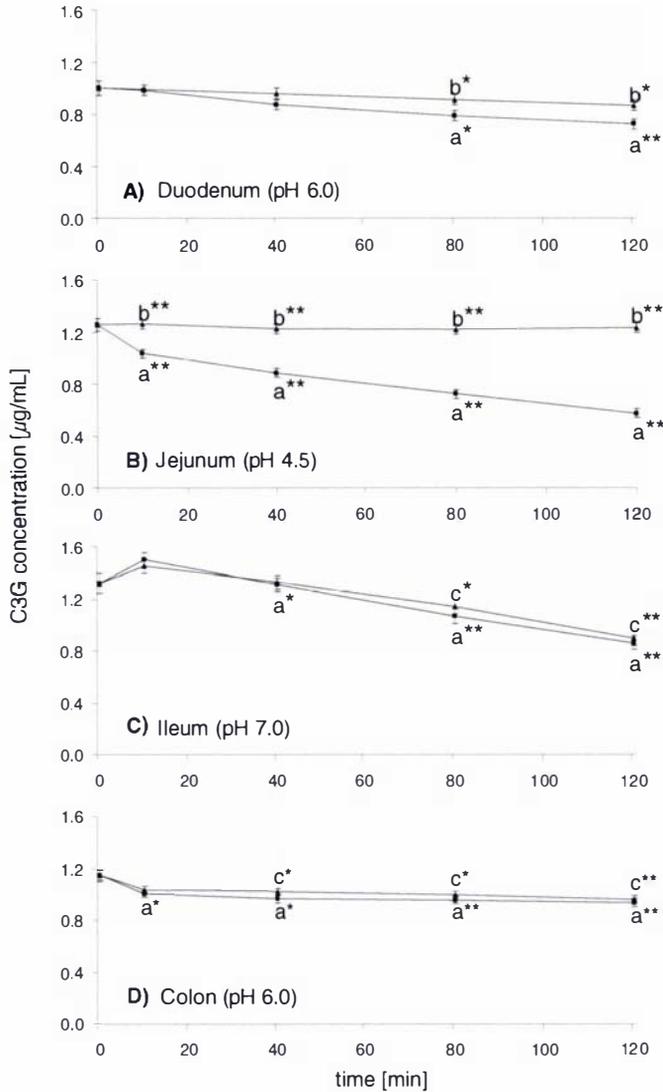


Figure 2. C3G concentration over time in mucosal solution of Ussing chambers mounted with duodenal (A), jejunal (B), ileal (C), and colonic (D) tissue. ▲ Control; ■ intestinal tissue. Values are the means \pm SEM ($n = 6-8$). The letter *a* indicates significant difference over time (intestinal tissue); *b* significant difference between intestinal tissue and control at the respective time point; *c* significant difference over time (control). * $p < 0.05$; ** $p < 0.001$.

C3G was not detected in any serosal solution (data not shown), which implies, that the adjacent tissue layers, that were still present in preparations, may have hindered diffusion across the tissue.

The differences between C3G degradation with each control and C3G decrease with the respective intestinal tissue (as seen in **Figure 2 A-D**) were calculated and labeled as ACN absorption. The C3G absorption rate over time is shown in **Figure 3**.

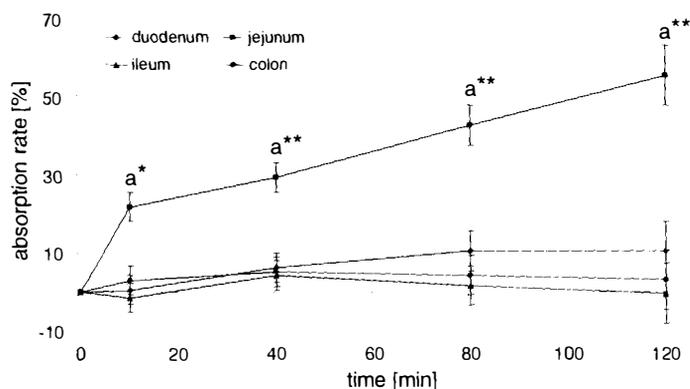


Figure 3. C3G absorption rate over time. Values are the means \pm SEM ($n = 8$). The letter *a* indicates significant difference between the jejunum and the other intestinal segments at the respective time point; * $p < 0.01$; ** $p < 0.001$.

The jejunal segment showed a linearly increasing C3G absorption over time, with a maximum absorption rate of $55.3 \pm 7.6\%$ at 2 h. Its absorption rate was significantly higher at each time point compared to the duodenal, ileal and colonic segment. A slight absorption rate of $10.4 \pm 7.6\%$ could be seen for the duodenal segment after 80 min.

The amount of C3G that was absorbed during the 2 h of the experiment was $5.37 \pm 0.78 \mu\text{g C3G}/\text{cm}^2/2 \text{ h}$ for the mouse jejunal tissue, significantly more than the other intestinal segments ($\leq 1.10 \mu\text{g C3G}$) (**Figure 4**).

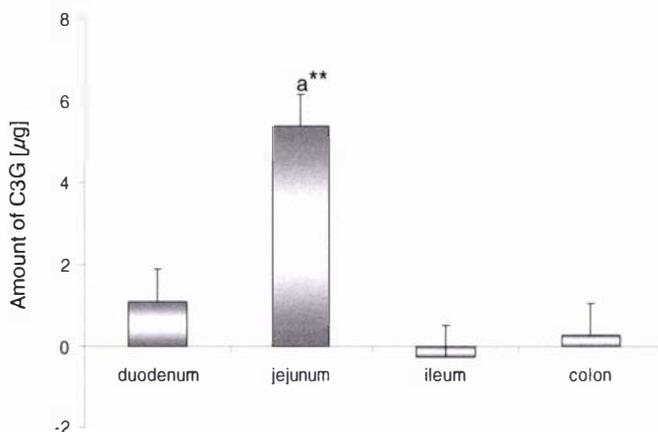


Figure 4. Total amount C3G absorbed per cm^2 intestinal tissue ($\mu\text{g}/\text{cm}^2/2\text{h}$). Values are the means \pm SEM ($n = 8$). The letter *a* indicates significant difference between the jejunum and the other intestinal segments. $**p < 0.001$.

DISCUSSION

ACNs are polyphenolic compounds that belong to secondary plant metabolites and are a component of our daily diet. There has been increasing interest on their biologic activities as they are claimed to enhance health by protecting against some chronic diseases (31, 32). However, to investigate and maximize beneficial effects in humans, their bioavailability including absorption, metabolism and excretion, needs to be understood. Although there are many studies investigating the occurrence of ACNs and their metabolites in body fluids such as blood plasma or urine, there is a lack of studies investigating the fate of ACNs within the GIT. The purpose of the present study was to investigate the absorption of ACNs from a berry fruit extract within the GIT and, in particular, to evaluate the main absorption site of ACNs using the Ussing chamber as an *in vitro* system and mouse intestine as the model tissue. The berry fruit extract mainly contained the ACN C3G.

We have demonstrated that C3G is absorbed from the intestine in mice at different rates depending on location within the GIT. The main absorption site for C3G was shown to be the jejunum. C3G removal from the mucosal compartment of the Ussing chamber was not due to degradation influenced by experimental conditions as the control showed that C3G was stable throughout the experiment (**Figure 2 B**). The decrease in concentration of C3G

was rather due to the physiological action of the tissue, and most likely indicates C3G absorption or metabolism within the respective intestinal segment. The fact that C3G absorption was found to predominantly take place in one intestinal region (i.e., the jejunum) suggests the involvement of an active transport mechanism. The hypothesis that an active transport mechanism is involved in ACN absorption is also supported by other studies. Mulleder *et al.* (33) found reduced excretion of ACNs in urine after simultaneous ingestion with sucrose, implying that ACN absorption is blocked by sucrose and therefore associated with intestinal sugar transport systems. Other studies have shown that monoglucosides of quercetin, a flavonoid, interact with an active transport mechanism, namely the intestinal sodium-dependent glucose transporter (SGLT1) (34, 35), and are taken up into the epithelium, where they are rapidly deglycosylated and then glucuronidated (34). Tsuda *et al.* (12) investigated C3G and its metabolites in jejunal tissue of rats after direct stomach intubation. C3G, the aglycone cyanidin (CY) and protocatechuic (PC) acid (oxidation product of C3G) were detected in the jejunal tissue. Tsuda *et al.* (12) showed that C3G is rapidly detected in plasma and that PC can be detected at concentrations eight times higher compared with C3G while CY is not present in plasma. The latter authors hypothesized that C3G is partly hydrolyzed by β -glucosidase to CY in the intestine. CY is unstable at physiological conditions and rapidly degraded to PC, which accumulates at high concentrations in plasma (12). Further investigations are needed to establish if the C3G disappearance in the present study is due to metabolism along the mucus layer or due to accumulation or metabolism within the epithelial tissue. Besides the jejunum, some disappearance of C3G was observed with the duodenal tissue (mean absorption rate \pm SEM, $10.4 \pm 7.6\%$), indicating that the duodenum also contributes to absorption or metabolism of C3G in mice. Disappearance of C3G from the Ussing chambers mounted with ileal and colonic tissues was not significantly different from their respective controls (**Figure 2 C, D**), indicating that no C3G absorption or metabolism occurs in the lower small and large intestines of mice.

In the present study, the acidity of the RS was adjusted to levels normally found in the intestine. In humans, the pH in the duodenum, jejunum, ileum and colon range from 6.1 to 6.7, 4.4 to 6.6, 6.8 to 8.0 and 6.0 to 7.2, respectively (25-28). A similar value (6.47) was found by De Lisle *et al.* (24) for the duodenum in mice. The acidity within the intestinal tract can vary between the fasted and fed state (27) and is most likely influenced by the

diet. As ACNs are pH sensitive, it was the purpose of the present study to simulate the different pH values of the different intestinal regions to investigate ACN absorption. The results show that C3G is more stable at a lower pH (**Figure 2**). It is uncertain, if the absorption rate in the duodenum could be increased, or if any absorption could be seen in the ileum or colon if the pH of the RS is lowered to 4.5. It seems unlikely, based on the results of the present study, that lowering the pH of the RS in Ussing chambers mounted with colonic tissues would result in absorption, as some absorption was observed with the duodenal tissue at the same pH (**Figure 2 A, D**). Nevertheless, pH values as low as 4.5 are not normally (25-28) to be expected in the ileum and colon, as there is a pH gradient, with pH becoming less acidic at more distal locations (27).

Recent studies have demonstrated the possibility of ACN absorption from the stomach (36-38). It has been suggested that an organic anion membrane carrier, bilitranslocase, expressed in epithelial cells of the gastric mucosa, could be involved in the absorption of ACNs (36). As several studies have demonstrated that ACNs are rapidly absorbed following oral administration (12-14, 39), absorption of ACNs through the gastric wall (38) may provide an explanation. pH values for the stomach have been reported to be between 1.4 and 2.1 (40). As ACNs are more stable at lower pH, this could play a role in ACN absorption from the stomach. Whether ACN absorption throughout the GIT is pH dependent remains to be determined.

We have demonstrated for the first time differences in C3G absorption by different intestinal segments from mice, with the highest absorption occurring in the jejunum, minor absorption occurring in the duodenum, and practically no absorption occurring in the ileum or colon. The identification of the main absorption site for ACNs is a step for further investigations regarding bioavailability. Future research should focus on a possible way to maximize absorption of ACNs and consequently to increase their beneficial health effects with a normal human diet.

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ABBREVIATIONS USED

ACNs, anthocyanins; C3G, cyanidin-3-glucoside; CY, cyanidin; GIT, gastrointestinal tract; mV, millivolt; PDA, photodiode array; RS, Ringer's solution.

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ERRATUM

Erratum to: THE JEJUNUM IS THE MAIN SITE OF ABSORPTION FOR ANTHOCYANINS IN MICE

[Journal of Nutritional Biochemistry 2006, 17, 31-36]

The present paper states that the jejunum is the main absorption site for anthocyanins (ACNs) in mice. However, several points have been risen with concern to the validity of the results:

- Each intestinal section has only been tested at one pH-value. To rule out a pH effect on ACN absorption, each section should have been tested at several pH-values.
- The stomach as part of the gastrointestinal tract has not been tested under the present conditions (Ussing chambers), due to the thickness of the gastric lining. However, the possibility of ACN absorption from the stomach was mentioned in the discussion, and should be considered in the conclusion.
- The pH values for each intestinal region may seem unphysiological; although they have been chosen according to previous reports. However, we admit that the pH value of the serosal bathing solution was incorrectly chosen for all intestinal regions, as it represents 'blood', and should have been at the appropriate pH (~7.4).

Changes to the conclusion:

The difference regarding ACN absorption between the selected intestinal regions may have been due to a pH effect rather than tissue and needs to be further investigated. Under the present conditions, it was shown that of the four chosen intestinal regions, the jejunum had the greatest ACN absorption. However, a contribution of gastric absorption should be considered.

CHAPTER 3

THE FLAVONOL QUERCETIN-3-GLUCOSIDE INHIBITS CYANIDIN-3-GLUCOSIDE ABSORPTION *IN VITRO*

As anthocyanins are mainly absorbed from one part of the gastrointestinal tract (the jejunum), the question arises if they might be absorbed by an active transport mechanism, rather than passive diffusion. The aim of the study reported in this chapter was to determine further detail in the mechanisms of anthocyanin absorption.

ABSTRACT

At present, little is known about the mechanisms responsible for intestinal absorption of anthocyanins (ACNs). For example, it has not yet been established if ACNs are absorbed through an active transport mechanism, such as the sodium-dependent glucose transporter (SGLT1), or by passive diffusion. Previously, we found that the absorption of ACNs differs between regions of the digestive tract and is maximal in the jejunum, suggesting that an active transport mechanism is involved. In the present study, we examined the effect of D-glucose (main substrate of SGLT1), phloridzin (inhibitor of SGLT1), and quercetin-3-glucose (Q3G, a flavonol) on the absorption of cyanidin-3-glucoside (C3G; $\sim 5 \mu\text{mol/L}$) by mouse jejunum mounted in Ussing chambers. We found that the presence of either D-glucose (10, 20, and 40 mmol/L) or phloridzin (50, 100, and 200 $\mu\text{mol/L}$) resulted in a small but insignificant inhibition of C3G disappearance from the mucosal solution (decrease of disappearance with glucose, 33%; with phloridzin, 18%; NS). However, when the flavonol Q3G (50 $\mu\text{mol/L}$) was added to the mucosal solution together with the C3G, the disappearance of C3G was significantly decreased (74%; $p < 0.001$), and Q3G disappeared instead. In addition, we found phloretin and quercetin, the aglycones of phloridzin and Q3G, respectively, present in the mucosal solution and tissue extracts, indicating hydrolysis of these compounds by the enterocytes of the jejunum. In contrast, the aglycone cyanidin was not detected at all. Our results show that in the mouse small intestine, ACN absorption is not solely dependent on the activity of the SGLT1 transporter, as D-glucose and phloridzin had only a slight effect on uptake. Q3G, however, clearly inhibited C3G disappearance. These results suggest that there might be a competitive inhibition between C3G and Q3G absorption. It is possible that an absorption mechanism other than the SGLT1 is involved, which has a structural preference towards flavonols.

INTRODUCTION

Anthocyanins (ACNs) belong to a large and widespread group of water-soluble plant constituents, known collectively as flavonoids (1). They are glycosides and acylglycosides of anthocyanidins, which are polyhydroxyl and polymethoxyl derivatives of 2-phenylbenzopyrylium (flavylium) cation (2). ACNs are the red and blue pigments present in a large number of plant tissues including leaves, flowers, and fruits and, therefore, are

consumed as normal components of the diet. The daily ACN consumption in humans has been estimated to be as much as 180-215 mg (3). Although the intake of ACNs seems reasonably high as compared to most other flavonoids (23 mg/day), including quercetin, kaempferol, myricetin, apigenin, and luteolin (4), their bioavailability, as indicated by the recovery in the plasma and urine after ingestion, has been shown to be extremely low (5-11). However, ACNs are rarely ingested on their own but rather as part of a fruit, vegetable, or meal, containing various other polyphenols and ingredients. Therefore, the amount of ACNs that is eaten together with other foods might be rather low. The ratio of flavonoids to ACNs in apples for example has been shown to be 10:1 (12)

More and more research has concentrated on the biological activities and possible health effects of ACNs. In particular, their antioxidant properties have been reported to protect against oxidative damage involved in a variety of diseases, such as diabetes (13), arteriosclerosis (14), neurodegeneration (15), and cancer (16-18). However, because the bioavailability, including absorption and metabolism, of ACNs has been shown to be very low, claimed health benefits of ACNs are questionable. To obtain a better understanding of the health-enhancing properties of ACNs, it is necessary to increase our knowledge of ACN absorption and metabolism. Once the absorption mechanisms for ACNs are better understood, their bioavailability could be increased and potential health benefits enhanced.

At present, the mechanisms of ACN absorption are still not fully understood. Whether ACNs are absorbed through passive diffusion or via an active transport mechanism remains to be determined. Several studies have shown that ACNs are absorbed as glycosides in humans and rats (11, 19-24), and the intact forms have been recovered in plasma and urine after oral administration. The fact that ACNs are recovered in plasma and urine as the glycosidic forms implies that they are also absorbed as such. It is possible that the sugar moiety of the ACN molecule interacts with a sugar transport system, such as the intestinal sodium-dependent glucose transporter (SGLT1) and that ACNs are therefore absorbed via an active transport mechanism. We have recently shown that ACNs (cyanidin-3-glucoside; C3G) are mainly absorbed from the jejunum in mice (25). The fact that ACNs are not absorbed equally throughout the gastrointestinal tract, suggests that an active transport mechanism is involved. In the case of other flavonoids, some reports indicate that quercetin-glucosides, as present in onions, show a higher bioavailability as compared to quercetin (aglycone) or quercetin-glucorhamnoside (rutin) (26-29). These results suggest

that, due to the glucose moiety in the quercetin-3-glucoside (Q3G) molecule, the SGLT1 could be involved in Q3G absorption. Indeed, Wolfram *et al.* (30) have shown that the SGLT1 is involved in the uptake of the flavonol Q3G across the brush border membrane of rat small intestine. As Q3G and C3G have a similar chemical structure (**Figure 1**), it is possible that both compounds share the same transport mechanism.

The aim of the present study was to investigate a possible interaction of the ACN C3G with the SGLT1. Similar to our previous report, we used mouse jejunum mounted in Ussing chambers to investigate if D-glucose, phloridzin, and Q3G, all substrates of the SGLT1, affect C3G absorption. Inhibition of C3G absorption by any of these substances would provide evidence for an interaction between the SGLT1 and C3G.

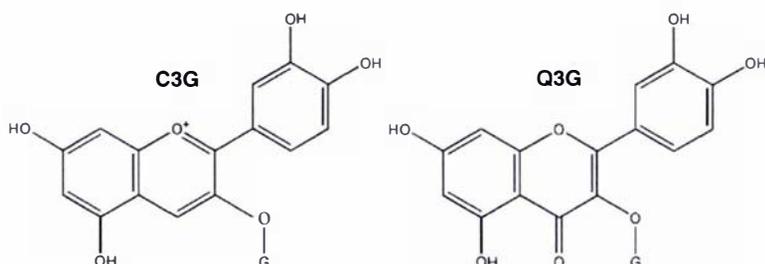


Figure 1. Chemical structures of C3G and Q3G.

MATERIALS AND METHODS

Animals. The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee (31).

Sixteen male Swiss mice (age 6-8 weeks) obtained from the Small Animal Production Unit, Massey University (Palmerston North, New Zealand) were used. They were housed in rodent cages and kept in a room with controlled temperature ($21 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (12 h light and dark cycles with dawn and dusk transitional periods). The mice were fed a balanced standard rodent diet (as previously described elsewhere, (25)), prepared by the Food Processing Unit at Massey University, and were given access to both food and demineralised water *ad libitum* until the day of the experiment.

Experimental setup. On the day of the experiments, mice were deprived of food for 2-3 h, anaesthetized with 6% halothane, and euthanized by cervical dislocation. Within 1 min of euthanasia, the abdomen was opened by a midline incision, the intestine dissected out and immersed in Ringer's solution (RS) (RS-A, **Table 1**) at room temperature.

Table 1. Composition of RSs (mmol/L)^a.

compound	RS-A ^b	RS-B ^c
NaCl	120.00	114.00
KCl	5.00	5.00
CaCl ₂	2.00	1.25
MgCl ₂	1.00	1.10
NaH ₂ PO ₄	1.80	0.30
Na ₂ HPO ₄	0.20	1.65
NaHCO ₃	25.00	25.00
Glucose	0.00	10.00/20.00/40.00
Glutamine	2.00	0.00
Pyruvate Na	2.00	0.00

^aCompositions are based on ^bRef. (32) (without glucose), and ^cRef. (33) (with glucose), with minor modifications.

Two centimeter long pieces of the jejunum were opened longitudinally along the mesenteric border and mounted in individual Ussing chambers, which were then filled with RS. To investigate the effects of D-glucose on C3G absorption, the Ussing chambers were filled with RS containing either no D-glucose (RS-A, **Table 1**), or different concentrations of D-glucose (RS-B, **Table 1**). To investigate the influence of phloridzin and Q3G on C3G absorption, the Ussing chambers were filled with RS containing no D-glucose (RS-A, **Table 1**) and the phloridzin or Q3G was added simultaneously with the C3G at the start of each experiment. As ACNs are pH sensitive, all solutions were adjusted to pH 4.5 to stabilize the ACN during the experiments. pH values in the jejunum have been reported to be between 4.4 and 6.6 (34). For stabilizing reasons, we have chosen the lower range of these reported pH values. The area of jejunal tissue exposed to the RS was 0.5 cm². The tissues were kept at 37.5°C and the RS was aerated with Carbogen (95% O₂/5% CO₂). All tissues were voltage clamped throughout the experiments at 0 mV (35) using automatic clamp units (Campus Electronics and Mechanical, University of Otago, Dunedin, New Zealand). The RS was replaced at least three times before the start of each experiment to wash the tissues and remove any luminal contents. The volume of the RS on each side of the chambers was 6 mL.

After an equilibration period of at least 30 min, the test compounds C3G (dissolved in RS-A; final concentration, $5.01 \pm 0.26 \mu\text{mol/L}$), phloridzin (dissolved in RS-A; final concentrations, 50, 100 or 200 $\mu\text{mol/L}$), or Q3G [dissolved in dimethyl sulfoxide (DMSO); final concentration, 50 $\mu\text{mol/L}$; final concentration of DMSO in the Ussing chamber 0.25%] was added to the mucosal compartment. To keep the mucosal and the serosal compartments at an equal volume, the same amount of just RS-A was added to the serosal compartment at the beginning of each experiment. Chambers without tissue were used as controls to measure degradation of the test compounds due to experimental conditions during the 120 min of the experiment.

ACNs. The ACNs were prepared as previously described (25). Briefly, 600 g frozen Boysenberries (*Rubus loganbaccus x baileyanus* Britt) were extracted twice with 1.5 L of acetone/water/acetic acid (70:29.5:0.5). Extracts were evaporated to 500 mL total volume at 40°C and lipids were removed by extraction three times with 500 mL hexane. The aqueous layer was evaporated and then diluted to 20% methanol/water using methanol. Subsequently, the aqueous fraction was applied to a large LH-20 Sephadex column (50 mm \varnothing x 370 mm height, 450 g of LH-20) and washed with 20% methanol/water. Boysenberry ACNs were eluted with 60% methanol/water, evaporated to dryness, and stored in the dark at 4°C until use.

ACNs of Boysenberries have previously been characterized as cyanidin-3-sophoroside, (C3G), cyanidin-3-glucosylrutinoside, and cyanidin-3-rutinoside (19, 36). The extract used in the present study contained predominantly C3G, the major monosaccharide ACN of Boysenberries (19). For each experiment, a stock solution containing 2.5 mg Boysenberry extract/2 mL RS (RS-A, **Table 1**) was prepared.

Sample preparation. Samples (400 μL) of RS from the mucosal and serosal compartments of the Ussing chambers were collected immediately after the addition of the C3G to the mucosal RS (0 min) and at 30, 60, 90, and 120 min. The mucosal RS samples were immediately acidified (1:1) with 5% formic acid/ H_2O and stored at 4°C until analysis. Serosal RS samples were not analysed, as it has been found that test compounds do not enter the serosal compartment due to the presence of submucosal tissue layers (subepithelial tissue, muscle tissue), which hinder diffusion into the serosal bathing solution (30). This is consistent with our previous observations that ACNs were not detected in the

serosal RS using similar experimental conditions (25). At the end of each experiment, the intestinal tissues were removed from the chambers and trimmed using iris scissor to leave only the part exposed to the test compounds (0.5 cm^2). The tissues were washed with ice-cold RS-A and kept at -20°C until analysed (usually not longer than 24 h). Determination of test compounds in the tissues was performed according to Tsuda *et al.* (20) with minor modifications. Frozen samples were blended with an Ultra-Turrax basic homogenizer in 2 mL of 0.4 M sodium phosphate buffer (pH 4.2), containing 0.1% (ethylenedinitrilo) tetraacetic acid (EDTA). Aliquots (400 μL) of homogenate were mixed with 2 mL acetone, containing 0.1% trifluoroacetic acid (TFA), and centrifuged at $3000 \times g$ for 5 min at 4°C . The supernatant was collected, 20 μL of TFA added, and the samples evaporated under a stream of N_2 at $\leq 35^\circ\text{C}$. The dried extracts were redissolved in 200 μL methanol, containing 1% TFA, and transferred into a high-performance liquid chromatography (HPLC) vial for analysis.

HPLC analysis. Concentrations of the test compounds in the mucosal RS and tissue extracts were determined by reversed phase HPLC with photodiode array (PDA) detection. The separation column used was a Phenomenex Aqua C_{18} (3 μm , 125 \AA , 2.0 x 150 mm). Solvents A (5% formic acid/ H_2O) and B (acetonitrile) were run at a flow rate of 1 mL/min. The solvent gradient started with a composition at 95% A, 5% B (for 5 min) and changed to reach 50% A, 50% B at 30 min (for 5 min). The composition then changed to reach 20% A, 80% B at 40 min (for 5 min), before returning to the starting conditions with a total run time of 50 min.

The sample injection volume was 10 μL for the mucosal RS samples and 50 μL for the tissue extracts. The PDA detector was used to collect spectral data (250-600 nm) and chromatograms were extracted at 520 and 280 nm. Chromatography data were collected and processed using a Waters Millennium Chromatography Manager version 4.0. C3G (Extrasynthese, Genay, France), and Q3G and phloridzin (Sigma, Sydney, Australia) concentrations were calculated using authentic standards with known concentrations.

Statistical analysis. Data are presented as means \pm SEM. Statistical analysis was carried out using the SAS System for Windows (version 8). The residuals of each analysis were tested for normality. The significance of differences was assessed by repeated measures

ANOVA and the least significant difference was used for comparison of individual means. Differences with $p \leq 0.05$ were considered significant.

RESULTS

After C3G was added to the mucosal RS of Ussing chambers containing mice jejunal tissue, the effect of the additional presence of D-glucose, phloridzin, or Q3G on C3G disappearance (indicative for absorption) from the mucosal RS was monitored over a period of 2 h. A control (Ussing chamber without tissue) was run with each experiment to determine the stability of C3G, Q3G, and phloridzin during the experimental period. The control experiments (no tissue) showed that there were no significant decreases in concentrations of C3G (**Figures 2, 4, and 8**), Q3G (**Figure 10**), or phloridzin (data not shown) during the 2 h experimental period, indicating no breakdown or destruction of these compounds due to the experimental conditions. The results of the controls verify that all three compounds were stable throughout the experiments and that any observed disappearance in tissue-mounted Ussing chambers indicates physiological activity of the tissue, such as absorption or metabolism.

Figure 2 shows the disappearance of C3G from the mucosal RS over time, in the presence of varying concentrations of D-Glucose. Without D-Glucose in the RS (0 mmol/L D-Glucose), there was a significant ($p < 0.05$) reduction in mucosal concentration of C3G after 30 min. The reduction became greater as the experiment progressed ($p < 0.01$ at 60 min; $p < 0.001$ at 90 and 120 min). The addition of D-Glucose to the mucosal RS at all concentrations reduced, but did not completely block C3G disappearance; a significant decrease in C3G concentration was still apparent at 90 min (10, 20 mmol/L, $p < 0.01$; 40 mmol/L, $p < 0.05$), and 120 min (10, 20 mmol/L, $p < 0.001$; 40 mmol/L, $p < 0.01$).

The C3G disappearance over the experimental period of 2 h without D-glucose was $44.8 \pm 11.3\%$, with 40 mmol/L glucose $30.1 \pm 6.5\%$, resulting in a 32.8% reduction (NS) of disappearance with the high D-glucose concentration (**Figure 3**). The slope (-0.33) of the regression equation between C3G disappearance and D-Glucose concentration in the RS after 2 h was not significantly different from zero ($p = 0.255$).

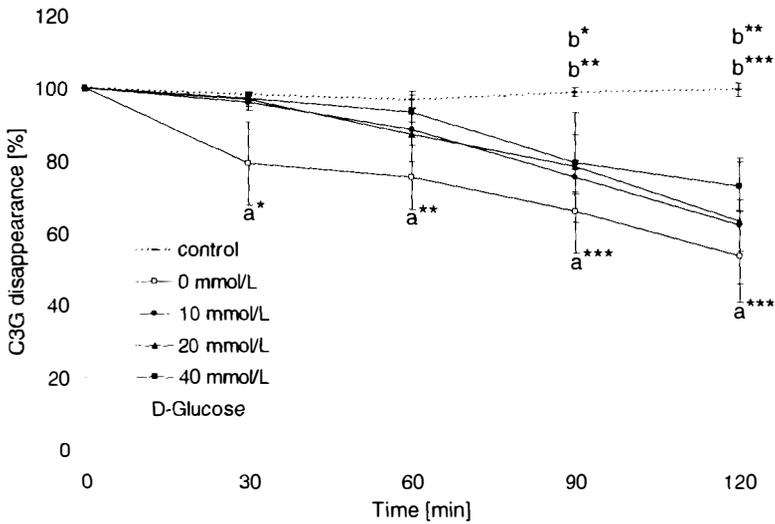


Figure 2. C3G disappearance from the mucosal solution over time without (0 mmol/L) and with the additional presence of D-glucose (10, 20, and 40 mmol/L). Values are means \pm SEM ($n = 3$). The letter *a* indicates the significant difference to time point 0 without glucose; the letter *b* indicates the significant difference to time point 0 with D-glucose. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

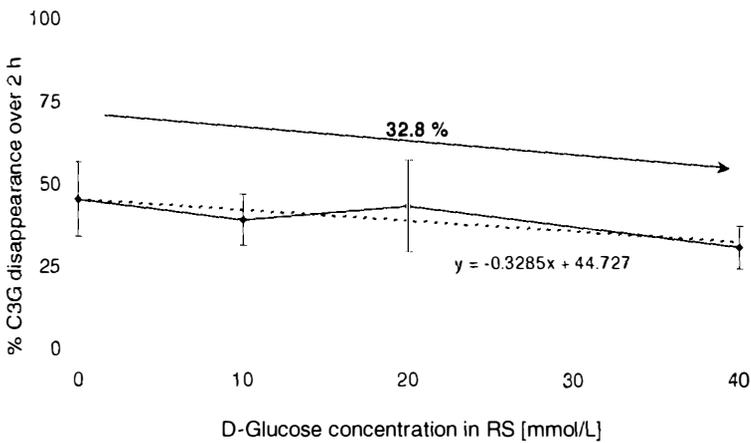


Figure 3. Percent C3G disappearance over 2 h without (0 mmol/L) and with the additional presence of D-glucose in the RS (10, 20, and 40 mmol/L). Values are means \pm SEM ($n = 3$).

The results for the experiments to determine the effects of phloridzin on the disappearance of C3G from the mucosal RS over time are shown in **Figure 4**. When no phloridzin was present in the RS (0 $\mu\text{mol/L}$ phloridzin), C3G disappeared significantly from the mucosal solution between 60 and 120 min ($p < 0.001$). The addition of phloridzin did not appear to inhibit the disappearance of C3G; significant reductions in C3G concentrations in the mucosal RS were still apparent at 90 (50, 100 $\mu\text{mol/L}$, $p < 0.05$; 200 $\mu\text{mol/L}$, $p < 0.01$) and 120 min ($p < 0.001$), even in the presence of the highest concentration of phloridzin.

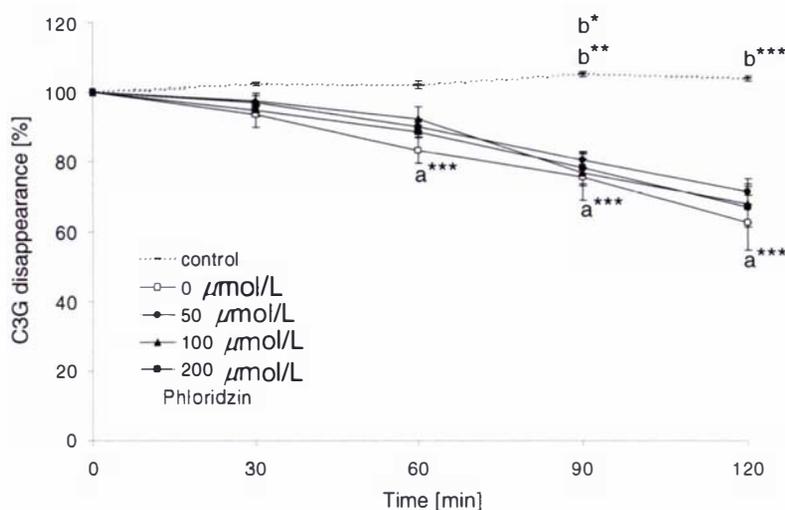


Figure 4. C3G disappearance from the mucosal solution over time without (0 $\mu\text{mol/L}$) and with the additional presence of phloridzin (50, 100, and 200 $\mu\text{mol/L}$). Values are means \pm SEM ($n = 3$). The letter *a* indicates the significant difference to time point 0 without phloridzin; the letter *b* indicates the significant difference to time point 0 with phloridzin. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Increasing concentrations of phloridzin in the RS showed an insignificant effect on C3G disappearance over the experimental period of 2 h (without phloridzin, $41.7 \pm 6.5\%$; with 200 $\mu\text{mol/L}$ phloridzin, $34.2 \pm 9.3\%$), resulting in a reduction (NS) of the disappearance by 18% (**Figure 5**). The slope (-0.03) of the regression equation between C3G disappearance and phloridzin concentration in the RS after 2 h was not significantly different from zero ($p = 0.489$). In addition, we detected phloretin, the aglycone of phloridzin in the mucosal RS, as well as in tissue extracts (**Figure 6 C, D**).

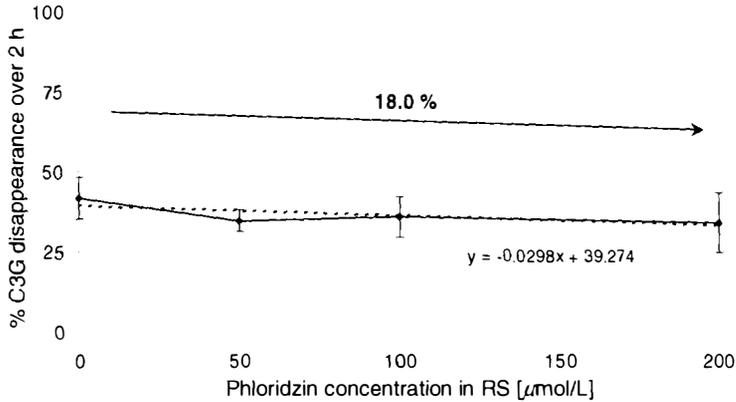


Figure 5. Percent C3G disappearance over 2 h without (0 $\mu\text{mol/L}$) and with the additional presence of phloridzin in the RS (50, 100, and 200 $\mu\text{mol/L}$). Values are means \pm SEM ($n = 3$).

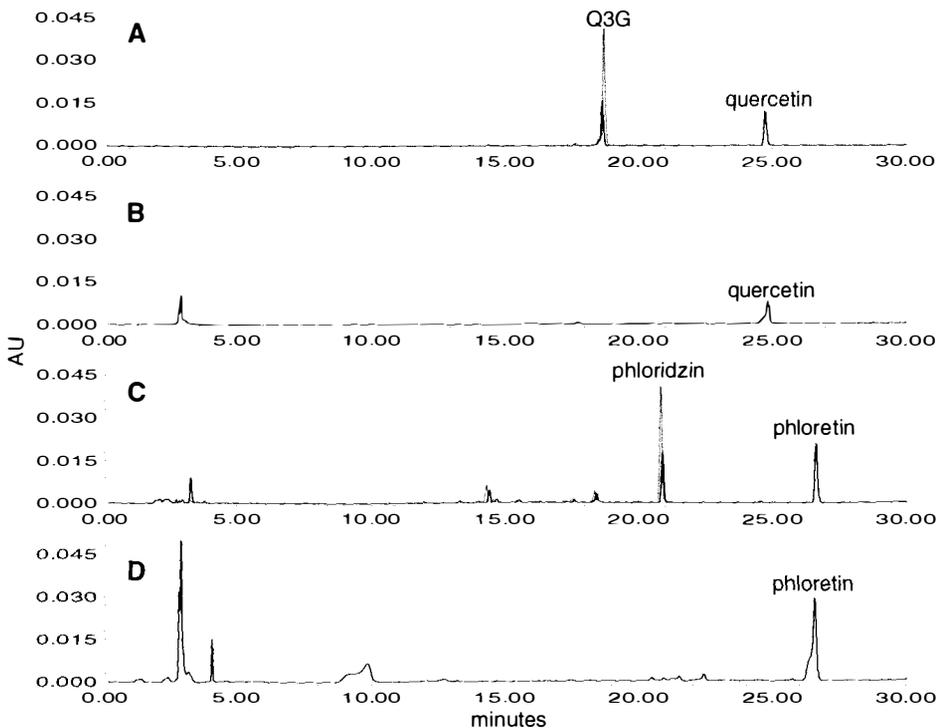


Figure 6. HPLC Chromatograms of mucosal solutions (A and C) and tissue samples (B and D). Dotted lines represent the start of the experiments (0 min), showing the original compounds Q3G and phloridzin; continuous lines represent the end of the experiments (120 min), showing the respective aglycones quercetin and phloretin. Detection is at 280 nm.

The disappearance of phloridzin from the mucosal RS over time ($> 60\text{min}$, $p < 0.001$) was paralleled by a significant increase of the aglycone phloretin in the mucosal RS ($> 60\text{min}$, $p < 0.001$) (**Figure 7**). Furthermore, the percentage conversion of phloridzin to phloretin was noticeably lower the higher the initial concentrations of phloridzin were.

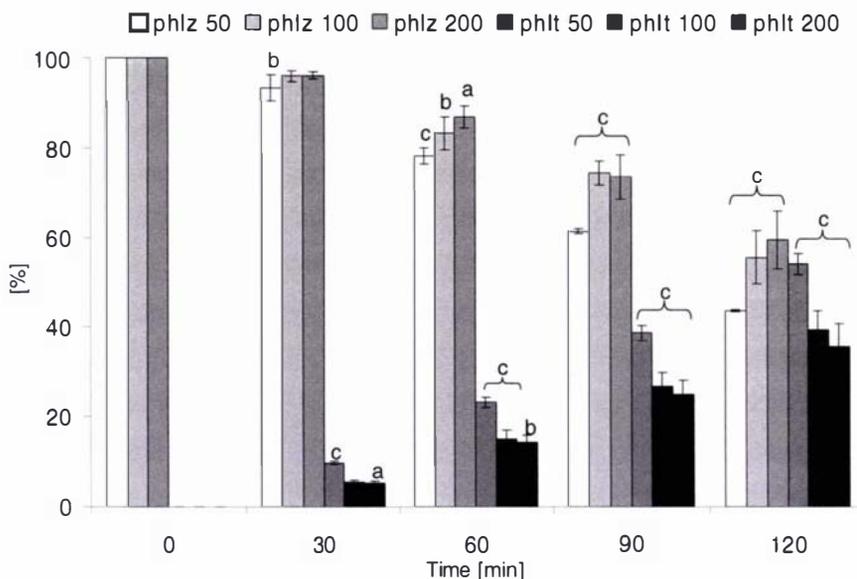


Figure 7. Phloridzin disappearance and phloretin appearance in mucosal solution over time. The bars show phloridzin (phlz) disappearance as well as phloretin (phlt) appearance after the simultaneous addition of either 50, 100, or 200 $\mu\text{mol/L}$ phloridzin to the RS. Values are means \pm SEM ($n = 3$). Shown is the significant difference to time point 0. a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$.

The presence of Q3G in the mucosal RS significantly inhibited C3G disappearance. **Figure 8** shows the C3G disappearance in mucosal RS in the absence and presence of Q3G over time. While C3G decrease was highly significant over time without Q3G (60-120 min; $p < 0.001$), the amount of decrease was strongly reduced by the presence of 50 $\mu\text{mol/L}$ Q3G (90 min, $p < 0.05$; 120 min, $p < 0.001$). Adding Q3G to the mucosal RS significantly decreased C3G disappearance over the experimental period of 2 h (without Q3G, $65.9 \pm 2.3\%$; with 50 $\mu\text{mol/L}$ Q3G, $17.2 \pm 1.7\%$), resulting in a 73.9% reduction ($p < 0.001$) of the C3G disappearance (**Figure 9**). Interestingly, a significant decrease of Q3G from the mucosal RS over time (30 min, $p < 0.01$; 60-120 min, $p < 0.001$) could be detected instead (**Figure 10**). Additionally, like with phloridzin, the aglycone quercetin was detected in the

mucosal RS and tissue extracts (**Figure 6 A, B**). However, the aglycone cyanidin was not detected in mucosal RS, or in tissue extracts.

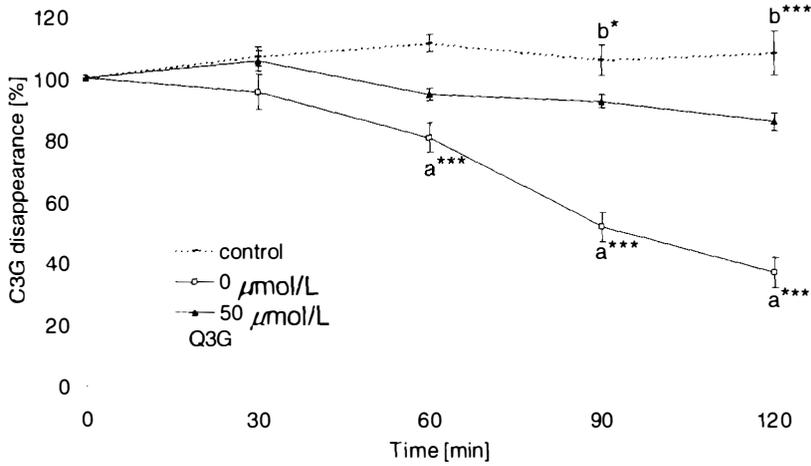


Figure 8. C3G disappearance from the mucosal solution over time without (0 $\mu\text{mol/L}$) and with the additional presence of Q3G (50 $\mu\text{mol/L}$). Values are means \pm SEM ($n = 2-4$). The letter a indicates the significant difference to time point 0 without Q3G; the letter b indicates the significant difference to time point 0 with Q3G. * $p < 0.05$; *** $p < 0.001$.

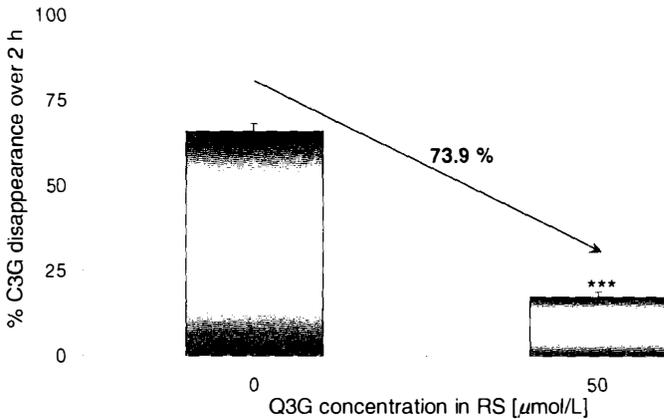


Figure 9. Percent C3G disappearance over 2 h without (0 $\mu\text{mol/L}$) and with the additional presence of Q3G in the RS (50 $\mu\text{mol/L}$). Values are means \pm SEM ($n = 2-4$). *** $p < 0.001$.

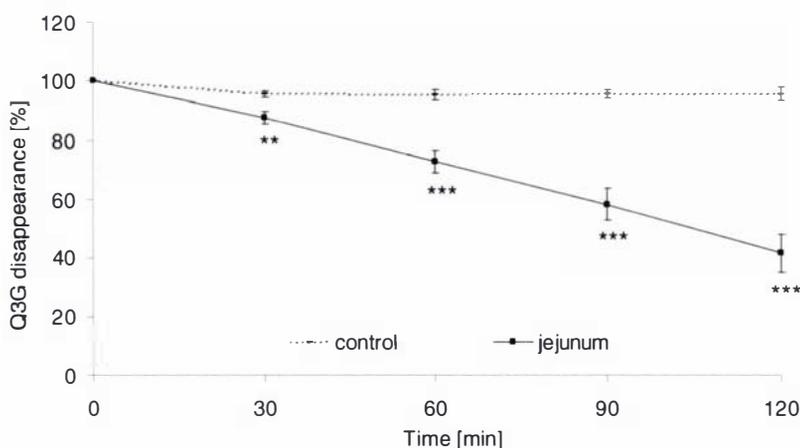


Figure 10. Q3G disappearance from the mucosal solution over time. Values are means \pm SEM ($n = 4$). Indicated are significant differences to time point 0. ** $p < 0.01$; *** $p < 0.001$.

DISCUSSION

There is increasing interest in the health-related effects of ACNs as they exhibit a range of biological activities and their frequent consumption may help to improve or at least maintain human health (3, 8, 24). However, to exert health effects, ingested ACNs need to be efficiently absorbed, circulate in sufficient concentrations in the human body, and reach the target tissues at concentrations sufficiently high to generate a biological effect. Further knowledge regarding absorption mechanisms is necessary to help understand details and to increase bioavailability of ACNs.

In our previous study we have shown that ACNs are predominantly absorbed from the jejunal tissue, which suggests the involvement of an active transport mechanism (25), rather than passive diffusion throughout the entire intestinal tract. As an example, the SGLT1 has been shown to be involved in the absorption of flavonols (30, 37-39). This transporter is generally responsible for the “active” accumulation of sugars into cells. It has been established that there is one major SGLT1 that handles all hexoses, with D-glucose and D-galactose being the natural substrates (40). The function of the SGLT1 and its presence in the intestinal epithelium has been confirmed for numerous domestic animals and for humans (41). Rat SGLT1 is 87% identical to human SGLT1 (42).

Because of the close similarity of the chemical structure (**Figure 1**) between flavonols (Q3G) and ACNs (C3G), we expected a similar absorption mechanism for both compounds. If the SGLT1 were involved in ACN absorption, the simultaneous presence of D-glucose and ACN in the intestinal tract would cause a competition of the compounds for the binding site of the SGLT1. In the Ussing chambers used for these experiments, competition for a single transporter would result in less ACN disappearance from the mucosal RS. In the present study, up to 40 mmol/L D-glucose showed a slight, but not significant, inhibition of C3G disappearance (**Figure 2, 3**). The SGLT1 has been shown to saturate at 30-50 mmol/L glucose *in vivo* (43), and it is therefore likely, that at the concentration of 40 mmol/L glucose the SGLT1 was saturated in the present study. If C3G would interact with the SGLT1, a saturation of the transporter would result in less disappearance of C3G. However, C3G did still significantly decrease from the mucosal RS, even with the high glucose concentration present (**Figure 2**), indicating no competition between the sugar and the ACN compound for the binding site at the transporter. Thus, it seems likely that C3G is not a substrate for the SGLT1. This is in contrast to a study by Mulleder *et al.* (44), where it was observed that urinary excretion of ACNs in humans was reduced when sucrose was ingested together with an elderberry concentrate, indicating that ACNs are associated with an intestinal sugar transport system. However, it is difficult to compare the two studies, as the present study used an *in vitro* model to investigate the effect of D-glucose on ACN absorption at a molecular basis, whereas Mulleder *et al.* (44) conducted an *in vivo* study investigating urinal excretion of ACNs after oral administration to humans. In another study, Wolfram *et al.* (30) investigated the involvement of the SGLT1 on flavonol-absorption (Q3G) in rat jejunum mounted in Ussing-type chambers. They found that a simultaneous addition of only 10 mmol/L D-glucose significantly reduced the disappearance of the flavonol Q3G from the mucosal solution, indicating competition for the transporter. It has further been shown, that Q3G inhibits glucose uptake by SGLT1 in a specific and competitive manner, suggesting that Q3G is partly absorbed by SGLT1 (45). This implies, that although flavonols and ACNs have some structural similarity, their absorption mechanisms appear to differ.

Phloridzin is a useful compound to study intestinal D-glucose transport, because it competitively binds to SGLT1 and inhibits its activity (46). In fact, Wolfram *et al.* (30) used phloridzin as a SGLT1 inhibitor and observed a significant reduced disappearance of

the flavonol Q3G from the mucosal compartment when phloridzin (100 $\mu\text{mol/L}$) was simultaneously present. Thus, if ACNs are absorbed via the SGLT1 mechanism, the simultaneous presence of phloridzin would result in less disappearance of ACNs. We therefore investigated the effect of phloridzin (50-200 $\mu\text{mol/L}$) as an inhibitor of SGLT1 on C3G absorption. We found that phloridzin did not affect C3G disappearance, which supports the assumption, that C3G is not a substrate and is not transported by the SGLT1.

In the present study, we also detected the aglycones phloretin and quercetin in the mucosal RS, indicating deglycosylation, whereas the aglycone cyanidin was not detected. The observed disappearance of Q3G and phloridzin from the mucosal RS, and the appearance of the aglycones quercetin and phloretin in the mucosal RS, is most likely due to lactase phloridzin hydrolase (LPH) activity, a mammalian β -glucosidase present in the brush border of the small intestine (47). The appearance of the aglycone phloretin was expected, as phloridzin is a primary substrate for LPH. Interestingly, increasing phloridzin concentration resulted in a decreased appearance of phloretin (**Figure 6**), which could imply a saturation of the enzyme. Besides phloridzin, LPH has been shown to be capable of hydrolyzing various flavonol and isoflavone glucosides (47). Deglycosylation by LPH and subsequent diffusion of the aglycone is also claimed to be the major route for Q3G absorption (37). Our detection of the aglycone quercetin in the mucosal RS as well as in jejunal tissue confirm the conclusions of Day *et al.* (37). The Q3G disappearance observed by Wolffram *et al.* (30) could therefore also be due to the activity of the LPH, as these authors detected a paralleled appearance of the aglycone quercetin in the mucosal solution. It is possible that the absorption mechanism for ACNs might be similar to the absorption of flavonols as suggested by Day *et al.* (37), involving deglycosylation by the LPH along the brush border membrane with subsequent diffusion into the enterocyte. In this study, the aglycone cyanidin was not detected in the mucosal solution or tissues extracts suggesting that deglycosylation does not occur. However, ACN aglycones have low stability and could therefore easily escape detection.

LPH has two distinct catalytic active sites. One for lactase activity, which has a broad substrate specificity with several glycosides as substrate, the other for phloridzin hydrolase activity (48, 49). The SGLT1 also possesses a sugar-binding site, with affinity to hexoses, such as D-glucose, as well as phloridzin (40). Thus, it is likely that in a system where both

proteins (LPH and SGLT1) are present, a substrate for LPH is also attracted by the SGLT1. Indeed, the two SGLT1 substrates used in the present study (D-glucose and phloridzin) have also been reported to inhibit LPH (50, 51). Therefore, the reduced Q3G disappearance observed by Wolfram *et al.* (30) could well be due to the inhibition of LPH instead of SGLT1. In our study however, neither glucose nor phloridzin had an effect on C3G disappearance, eliminating the assumption of an interaction between C3G and LPH. Furthermore, this could explain the lack of detection of the aglycone cyanidin in the present study.

Interestingly, we found that C3G disappearance was significantly inhibited by the presence of the flavonol Q3G (**Figure 8, 9**), and Q3G disappeared preferentially from the mucosal RS (**Figure 10**). These results show for the first time that a simultaneous ingestion of ACNs and flavonols might cause a competition for absorption, resulting in a reduced absorption of ACNs in the presence of flavonols. The competition between ACNs and flavonoids for intestinal absorption suggests that both compounds share the same transport mechanism. Moreover, our results indicate that this transport mechanism obviously has a preference towards flavonols instead of ACNs.

In conclusion, our results show that D-glucose and phloridzin had no effect on C3G disappearance, which proves that neither SGLT1 nor LPH are involved in ACN absorption. However, we have demonstrated for the first time that the flavonol Q3G significantly inhibited C3G absorption. Our results indicate that ACNs and flavonols share a common absorption mechanism, with an obvious preference for the latter. We suggest that it is important to investigate ACN absorption together with other flavonoids or nutrients, as ACNs are rarely ingested entirely on their own. The occurrence of other flavonoids in fruit, vegetables, and wine or of other nutrient compounds in a meal might have a significant effect on ACN absorption, and should be taken in account in future studies regarding ACN bioavailability. Further investigations are necessary to clarify interactions of different flavonoids regarding their absorption.

ABBREVIATIONS USED

ACNs, anthocyanins; C3G, cyanidin-3-glucoside; DMSO, dimethyl sulfoxide; LPH, lactase phloridzin hydrolase; PDA, photodiode array; Q3G, quercetin-3-glucoside; RS,

Ringer's solution; SGLT1, sodium-dependent glucose transporter; TFA, trifluoroacetic acid.

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ERRATUM

Erratum to: THE FLAVONOL QUERCETIN-3-GLUCOSIDE INHIBITS CYANIDIN-3-GLUCOSIDE ABSORPTION *IN VITRO*

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The following issues regarding the validity of the present paper have been raised:

- The control for Q3G (0 $\mu\text{mol/L}$ Q3G) did not contain DMSO. Therefore, there was no real control for Q3G, and possible effects of DMSO on C3G absorption were not taken into account. Furthermore, the effect of DMSO at a concentration of 0.25% for a biological preparation is relatively high, which might have altered an effect on absorption.
- The pH value for all Ringer solutions was adjusted according to a previous paper. However, the pH value of the serosal Ringer solution would represent the 'blood' stream and should have been adjusted to the respective pH value (~7.4), in order to simulate a physiological situation with the experimental setup.
- The experimental conditions with a pH value of 4.5 might have altered the transport function of the SGLT1 in acidic conditions, and should have been taken into account in the discussion.

Changes to the conclusion:

The strong inhibition of C3G disappearance from the mucosal Ringer solution may have been due to an effect of the vehicle DMSO and not Q3G. In addition, DMSO at a concentration of 0.25% may have caused an effect on absorption and should be taken into account. The chosen pH value for Ringer solutions represents rather unphysiological conditions, which question the functionality of the SGLT1, and therefore its possible involvement in ACN absorption.

CHAPTER 4

A VISCOUS FOOD MATRIX INFLUENCES ABSORPTION AND EXCRETION BUT NOT METABOLISM OF ANTHOCYANINS FROM BLACKCURRANTS IN RATS

Anthocyanins are rarely ingested on their own, but rather in combination with other food components. The effect of such combinations on ACN bioavailability has not been investigated so far. The aim of the study reported in this chapter was to investigate the influence of a simultaneously ingested food matrix on anthocyanin absorption.

ABSTRACT

The aim of the present study was to investigate the effect of a simultaneous intake of food and anthocyanins (ACNs) on ACN absorption, metabolism and excretion. Blackcurrant (BC) ACNs were dissolved in water with or without the addition of oatmeal and orally administered to rats providing approximately 250 mg total ACNs/kg BW. Blood, urine, digesta and tissue samples of the stomach, jejunum and colon were subsequently collected at 0.25, 0.5, 1, 2, 3, 7, and 24 h. Identification and quantification of ACNs was carried out by RP-HPLC and LC-MS. Four major ACNs were present in the BC extract: delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside. In plasma, the four ACNs of BC were identified and quantified. Time to reach maximal total ACN plasma concentration ($C_{\max \text{ BC/water}} = 0.37 \pm 0.07 \mu\text{mol/L}$; $C_{\max \text{ BC/oatmeal}} = 0.20 \pm 0.05 \mu\text{mol/L}$) occurred faster after BC/water ($t_{\max} = 0.25 \text{ h}$), compared to BC/oatmeal administration ($t_{\max} = 1.0 \text{ h}$). In digesta and tissue samples, the four original BC ACNs were detected. The rutinosides slightly increased during their passage through the gastrointestinal tract, while the glucosides decreased. Maximum ACN excretion in urine occurred later after BC/oatmeal compared to BC/water administration (3 vs. 2 h). The four original ACNs of BC in their unchanged form, as well as several metabolites were identified in the urine samples of both groups.

The simultaneously intake of food affects ACN absorption and excretion in the urine but not metabolism. Further investigations regarding the effect of nutrient interactions on ACN absorption are required.

INTRODUCTION

Anthocyanins (ACNs) are a group of flavonoid compounds that are mainly responsible for the red, blue and purple pigments in many fruits, fruit juices, wines, leaves and flowers. They are glycosylated polyhydroxyl or polymethoxyl derivatives of the 2-phenylbenzopyrylium (flavylium) cation (1). ACNs are of great nutritional interest, because of the marked daily intake of 180 to 215 mg in the United States (2), which is much higher than the intake (23 mg/day) of most other flavonoids, including quercetin, kaempferol, myricetin, apigenin, and luteolin (3). Berry fruits in particular are rich dietary sources of ACNs, and some can contribute 100 to 300 mg in a single serving (4). Blackcurrants (BC;

Ribes nigrum L.) for example contain as much as 250 mg/100 g fresh fruit (5). The berries of BC contain four major ACNs, delphinidin-3-glucoside (D3G), delphinidin-3-rutinoside (D3R), cyanidin-3-glucoside (C3G), and cyanidin-3-rutinoside (C3R) (Figure 1), which were first identified by Chandler and Harper (6). BC extracts have been widely used to investigate ACN absorption in humans and animals (7-11).

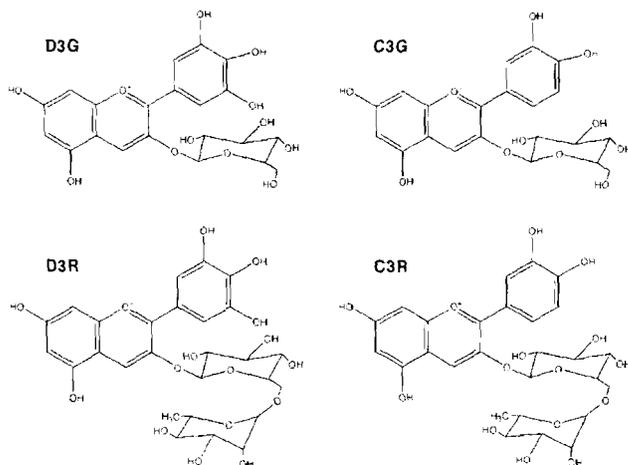


Figure 1. Chemical structures of the four major ACNs in blackcurrants: delphinidin-3-glucoside (D3G), delphinidin-3-rutinoside (D3R), cyanidin-3-glucoside (C3G), and cyanidin-3-rutinoside (C3R).

There is great interest in exploring the health benefits of ACNs found in fruits and vegetables. Indeed, the number of studies investigating ACN bioavailability has increased over the last few years, due to their proposed health-related effects. ACNs are natural compounds known to act as powerful antioxidants (12) and have been shown to have antioxidant activity *in vitro* (12-21) and *in vivo* (22-27). However, the majority of studies focusing on ACN absorption, have shown that the bioavailability of ACNs is extremely low (10, 27-32). So far most of these studies have only administered purified ACNs or berry fruit extracts dissolved in water. A few studies administered the ACNs to humans as part of a meal consisting of sugar, bread and butter (33), or with a high fat meal (27). However, those studies did not focus on the effect of the additional food matrix on ACN absorption. Nielsen *et al.* (9) investigated the effect of a BC juice compared to an aqueous citric acid mix on ACN absorption in rabbits, as well as the additional ingestion of a rice cake on ACN absorption in humans. ACNs were slightly better absorbed when administered as

juice, but no influence by the intake of a rice cake was observed. One study has investigated the co-digestion of an ACN extract with commonly combined food stuffs, such as bread, breakfast cereal, ice cream and cooked minced beef in an *in vitro* digestion model (34). ACN absorption was unaffected or slightly increased (minced beef) by co-incubation with the foodstuffs. However, there are insufficient data regarding the effect of the food matrix on ACN absorption and metabolism *in vivo*. The latter is important in regards to ACN intake and maximising the potential health-related effects. As ACN bioavailability has been shown to be extremely low, ways to increase their absorption could assist in enhancing possible health benefits.

The aim of the present study was to investigate the effect of the simultaneous ingestion of ACNs and a food matrix on absorption and metabolism of ACNs. Rats were orally administered either BC mixed in water, or BC mixed in water and oatmeal, and blood, urine, as well as digesta and tissue samples of the stomach, jejunum and colon were collected after several time intervals, and analysed for ACN content.

MATERIAL AND METHODS

ACNs. A commercial blackcurrant powdered concentrate (Currantex 30[®], total ACN content 32.9%, w/w) was kindly provided by Just the Berries Ltd., Palmerston North, New Zealand. The ACN composition of the BC concentrate was 15.7% D3G, 37.7% D3R, 7.1% C3G, and 34.4% C3R.

All other nutrients, reagents, and chemicals used were purchased from commercial sources.

Animals and Diets. Male Sprague Dawley rats ($n = 70$, ~340 g) were bred and raised at the Small Animal Production Unit, Massey University, Palmerston North, New Zealand. They were housed in groups of 4 in polycarbonate rodent cages and kept in a room with controlled temperature ($21 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$) and lighting (12 h light and dark cycles with dawn and dusk transitional periods). The animals had free access to tap water and a standard rodent chow (35), which was prepared at the Food Processing Unit, Massey University. One week before the experiment, rats were individually housed for adaptation under the same conditions.

The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee of Massey University (protocol 04/59), New Zealand (36).

Study design. After the adaptation period, food was withdrawn, but water was continued *ad libitum* over-night (~15 h). The following morning, the rats were randomly assigned to 2 groups containing 7 subgroups (0.25, 0.5, 1, 2, 3, 7, 24 h), each consisting of 5 rats. One group received the BC powder dissolved in 0.1 M citric acid buffer (pH 3.6) (BC/water) while the other group received BC powder dissolved in 0.1 M citric acid buffer containing 6.7% (w/w) finely ground oatmeal (Uncle Toby's milk oaties, Goodman Fielder Ltd. Auckland, New Zealand) (BC/oatmeal). Both treatments were prepared to provide 250 mg total ACNs/kg BW, and administered to the rats by stomach intubation (2.15 ± 0.02 mL/rat), using an infant polyvinyl chloride feeding tube (W/X-ray line, Fg 8; Unomedical, Australia). After administration, rats were placed into individual metabolic cages (Tecniplast, Italy) with free access to tap water until sampling. Samples of the treatments were stored at -20°C until analysed.

Sampling. At the respective time points (0.25, 0.5, 1, 2, 3, 7, 24 h), animals were anaesthetised with isoflurane, containing 3% oxygen, and blood samples obtained by cardiac puncture into 10 mL evacuated glass tubes (Vacutainer, Becton Dickinson). The blood samples were kept on ice until they were centrifuged at $4,165 \times g$ for 20 min at 4°C for plasma collection. Aliquots of plasma (1 mL) were acidified with 0.2 mL of 5% trifluoroacetic acid and stored at -20°C until analysis.

Within 1 min of euthanasia after blood sampling, the abdomen of the animals was opened by a midline incision, and the stomach and intestinal tract dissected out. The digesta of the entire stomach, and a sub-sample of the jejunum and colon (~4 cm each) were collected by finger drainage into glass tubes and stored at -20°C until analysis. The emptied tissue samples were thoroughly washed with cold tap water to remove any ACNs that adhered to the surface of the mucosa, collected into microfuge tubes, and stored at -20°C until analysis.

Urine samples were collected at 2, 3, 7, and 24 h using metabolic cages (Tecniplast, Italy), and the volumes recorded. Aliquots (1 mL) were acidified with 0.2 mL of 5% trifluoroacetic acid and stored at -20°C until analysis.

ACN concentration of treatments. Frozen samples of the treatments were thawed at RT, diluted with 5% formic acid/H₂O, and a 20 μ L portion analysed together with the plasma and urine samples by HPLC.

ACN extraction from plasma. Frozen, acidified plasma samples were thawed quickly in a water bath at 37°C and treated with 10% TCA to precipitate proteins. Therefore, an aliquot (500 μ L) of acidified plasma was mixed 1:1 with 10% TCA, centrifuged for 5 min at 10,000 x g at RT and 800 μ L of the supernatant (SN) collected. The pellet was re-suspended and washed two more times with 800 μ L 10% TCA and the resulting SNs combined to a final volume of 2.4 mL. The extracts were evaporated close to dryness under N₂ (< 35°C) and the residues re-dissolved in 300 μ L 5% formic acid/H₂O. The samples were centrifuged for 10 min at 3,040 x g at RT and a 20 μ L portion of the SN subjected to HPLC analysis. The recoveries of ACNs with this method were tested with spiking experiments using an authentic standard of C3G (Extrasynthase, Genay, France) and found to be $94.9 \pm 2.8\%$.

ACN extraction from urine. Frozen, acidified urine samples were thawed quickly in a water bath at 37°C and centrifuged for 5 min at 10,000 x g at RT. The SNs were transferred into HPLC vials for analysis ('SN-sample', 20 μ L). ACNs bound to proteins were extracted by washing the remaining pellet five times with 500 μ L 10% TCA as described for the plasma samples above. A portion (1 mL) of the combined SNs (2.5 mL) was transferred into HPLC vials for analysis ('wash-sample', 20 μ L). The ACN concentration was calculated as a sum of the 'SN-' and the 'wash-sample'. The recovery of ACNs with this method was tested with spiking experiments using C3G and was found to be $91.2 \pm 3.0\%$.

Urinary creatinine excretion was measured using a commercial diagnostic kit (Roche Diagnostic, New Zealand Ltd; kit-no. 11 489 291 216) for creatinine analysis by the Jaffe procedure. The samples were analysed on a Flexor E clinical chemistry analyser (Vital Scientific, Dieren, The Netherlands).

ACN extraction from digesta. Frozen digesta samples were blended twice with 6 volumes of a solution of 50/50/10 methanol/H₂O/formic acid using an Ultra-Turrax basic homogenizer. Samples were then centrifuged at 5,070 x g for 10 min at RT and the resulting SNs combined. A 1 mL portion of the SN was then passed through a membrane filter (MILLEX-GP Filter Unit; 22 μ m pore size; Millipore). The samples were 1:10 diluted

with the 50/50/10 methanol/H₂O/formic acid solution, transferred into HPLC vials, and stored at -20°C until HPLC analysis (20 μ L). Recovery of C3G-spiked digesta samples was $97.3 \pm 2.3\%$.

ACN extraction from tissues. Frozen tissue samples were manually cut into small pieces and blended twice with 6.6 (stomach), 15 (jejunum), and 8.6 (colon) w/v of 5% formic acid/H₂O using an Ultra-Turrax basic homogenizer. Samples were then centrifuged at 5,070 x g for 8 min at 4°C, the resulting SNs combined and centrifuged again at 4,050 x g for 10 min at RT. A 2 mL portion of the SN was evaporated under N₂ (< 35°C), re-dissolved with 1 mL 5% formic acid/H₂O, and sonicated for 30 sec. Finally the samples were passed through a membrane filter (MILLEX-GP Filter Unit; 22 μ m pore size; Millipore) into HPLC vials, and stored at -20°C until HPLC analysis (20 μ L). Recovery of C3G-spiked tissue samples was $97.9 \pm 3.4\%$.

HPLC analysis. ACN concentrations of the treatments, as well as in plasma, urine, digesta and tissue samples were determined by reversed-phase (RP) HPLC with photodiode array (PDA) detection as previously described (35). The sample injection volume was 20 μ L for all samples. D3G-, D3R-, C3G-, and C3R-concentrations were calculated as C3G-equivalents, using an authentic standard of C3G (Extrasynthese, Genay, France) with known concentration.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The LC-MS analysis was performed as previously described by Cooney *et al.* (37), with minor modifications. Solvents were (A) 5:3:92 (v/v/v) acetonitrile:formic acid: H₂O and (B) acetonitrile + 0.1% formic acid and the flow rate was 200 μ L/min. The initial mobile phase, 100% A was ramped linearly to 83% A at 17 min, 80% A at 20 min, 70% A at 26 min, 50% A at 28.5 min, 5% A at 32 min and held for 3 min before resetting to the original conditions. Sample injection volume was 20 μ L. Detection was by absorbance at 530 nm.

MS data were acquired in the positive mode using a data-dependent LC-MSⁿ method with dynamic exclusion enabled and a repeat count of 2. The ESI spray voltage, capillary temperature, sheath gas pressure and auxiliary gas were set at 27 V, 300°C, 45 psi, and 10 psi, respectively.

Statistical analysis. Data are presented as means \pm SEM. Statistical analysis was carried out using SAS System for Windows, version 8. The residuals of each analysis were tested

for normality. Where not normally distributed, data were corrected by natural log transformation. Area under the plasma vs. time curve (AUC_{0-7h}) was calculated using the linear trapezoidal rule. The significance of differences was assessed by one-way ANOVA for comparison of individual means. Differences with $p \leq 0.05$ were considered significant.

RESULTS

The actual mean \pm SEM administered amount of total ACN to the rats in both treatments were 275.8 ± 5.2 and 203.9 ± 4.7 mg/kg BW for the BC/water and BC/oatmeal, respectively.

The individual and the total ACN concentration in rat plasma over the first 7 hours after administration of the BC/water and BC/oatmeal are shown in **Figure 2**. The concentration of ACNs in the plasma had returned to baseline after 7 h. The two different treatments showed a different pattern of the individual ACNs in plasma over time (**Figure 2 A, B**). After administration of BC/water, all four individual ACNs reached maximum plasma concentration after 0.25 h (**Figure 2 A, Table 1**), whereas after BC/oatmeal administration, the maximum concentration was reached between 0.5 and 1 h (**Figure 2 B, Table 1**). Furthermore, the absorption half-live ($t_{1/2 \text{ abs}}$) for both rutinoides was longer after BC/oatmeal administration (D3R: 0.36 ± 0.26 h, NS; C3R: 0.50 ± 0.24 h, $p < 0.05$) compared to BC/water administration (D3R: 0.06 ± 0.08 h; C3R: < 0.08 h). The total amount of individual ACNs absorbed between administration and 7 h (AUC_{0-7h}) was smaller after administrating BC/oatmeal, than BC/water (**Table 1**). However, the maximum concentrations of the individual ACNs were not significantly different between the two treatments (**Table 1**). Interestingly, after BC/water administration there was another increase in the plasma concentration after 1 h for the rutinoides D3R and C3R (**Figure 2 A**), which was not observed after BC/oatmeal administration.

The maximum total ACN concentration (**Figure 2 C**) was reached quicker after BC/water administration ($t_{\text{max}} = 0.25$ h, $t_{1/2 \text{ abs}} < 0.08$ h; $p < 0.05$) compared to BC/oatmeal administration ($t_{\text{max}} = 1.0$ h, $t_{1/2 \text{ abs}} = 0.43 \pm 0.21$ h) (**Table 1**) and was significantly higher ($p < 0.001$) at that time point (0.25 h, **Figure 2 C**).

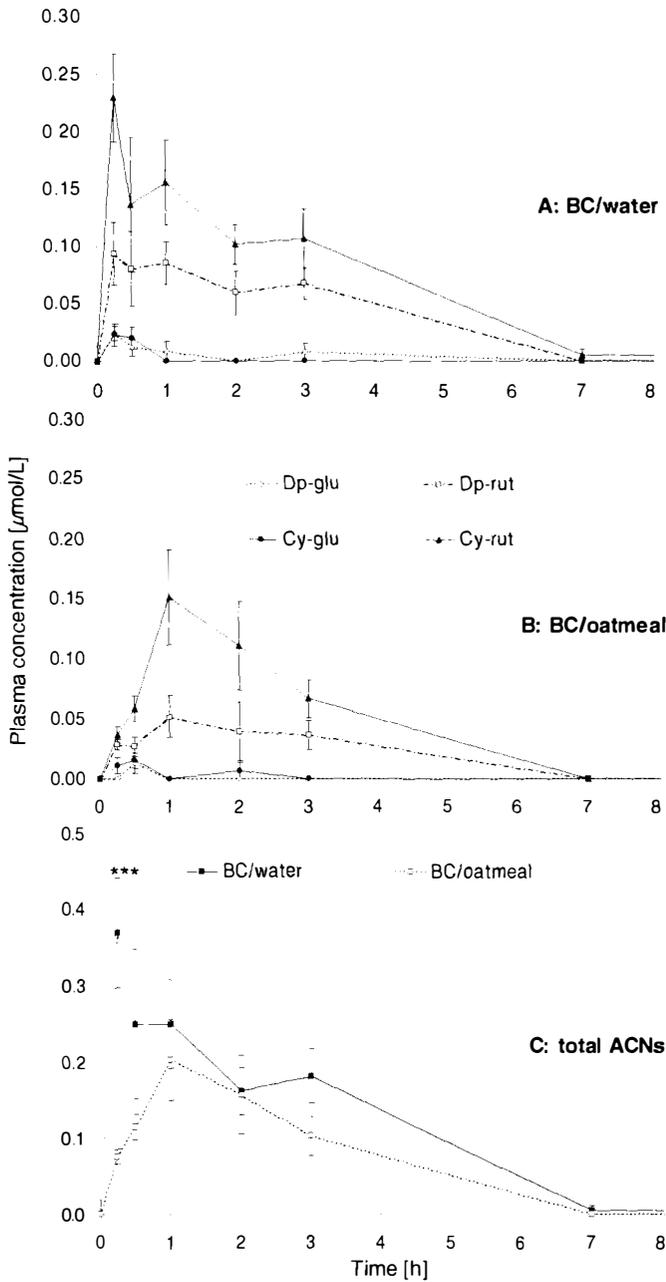


Figure 2. ACN concentration in rat plasma. Profile of individual ACNs in rat plasma after BC/water administration (A), BC/oatmeal administration (B), and total ACNs in rat plasma (C). Mean \pm SEM, $n = 5$. Significant difference between the treatments at 0.25 h after administration, $***p < 0.001$. Data were only analysed between 0 and 3 h, as the concentrations reached baseline values from 7 h onwards.

In addition, the maximum total ACN concentration in plasma was significantly higher after BC/water administration ($0.37 \pm 0.07 \mu\text{mol/L}$; $p < 0.05$), than after BC/oatmeal administration ($0.20 \pm 0.05 \mu\text{mol/L}$) (**Table 1**). Furthermore, the total amount of total ACNs absorbed between 0 and 7 h ($\text{AUC}_{0-7\text{h}}$) was significantly higher after BC/water administration ($\text{AUC}_{0-7\text{h}}$: $1.00 \pm 0.11 \mu\text{mol}^*\text{h/L}$, $p < 0.05$), compared to BC/oatmeal administration ($\text{AUC}_{0-7\text{h}}$: $0.63 \pm 0.09 \mu\text{mol}^*\text{h/L}$, **Table 1**).

Table 1. Pharmacokinetic parameters of ACNs in rat plasma after a single oral dose of approximately 250 mg total ACN/kg BW^a.

	C_{max} ($\mu\text{mol/L}$)†		t_{max} (h)‡		$\text{AUC}_{0-7\text{h}}$ ($\mu\text{mol h/L}$)§		$t_{1/2\text{abs}}$ (h)	
	BC/ Water	BC/ oatmeal	BC/ water	BC/ oatmeal	BC/ water	BC/ oatmeal	BC/ water	BC/ oatmeal
D3G	0.02 ± 0.01	0.01 ± 0.01	0.25	0.5	0.04 ± 0.02	0.01 ± 0.00	NA	NA
D3R	0.09 ± 0.03	0.05 ± 0.02	0.25	1.0	0.35 ± 0.04	0.19 ± 0.04^a	0.06 ± 0.08	0.36 ± 0.26
C3G	0.02 ± 0.01	0.02 ± 0.01	0.25	0.5	0.01 ± 0.00	0.02 ± 0.01	NA	NA
C3R	0.23 ± 0.04	0.15 ± 0.04	0.25	1.0	0.60 ± 0.08	0.42 ± 0.06	< 0.08	0.50 ± 0.24^a
Total	0.37 ± 0.07	0.20 ± 0.05^a	0.25	1.0	1.00 ± 0.11	0.63 ± 0.09^a	< 0.08	0.43 ± 0.21^a

* Values are means \pm SEM, $n = 5$.

† Maximal plasma concentration.

‡ Time to reach C_{max} .

§ Area under the plasma vs. time curve.

|| absorption half-life.

^a Significant difference between treatments, $p < 0.05$.

NA – not applicable.

The HPLC chromatograms of digesta and tissue samples of the stomach, the jejunum and the colon showed only the four original ACNs of BC, with no additional peaks indicating the presence of ACN metabolites (data not shown). The ratios of total ACN concentration found in the tissue samples ($\mu\text{g/g}$ wet tissue) to total ACN concentration found in the corresponding digesta samples ($\mu\text{g/g}$ digesta as-is) over the entire experimental period (0-24 h) are shown in **Figure 3**. This ratio represents the efficiency of absorption of the respective tissue. With both treatments, the jejunum showed a higher ratio ($p < 0.001$) compared to the stomach and the colon.

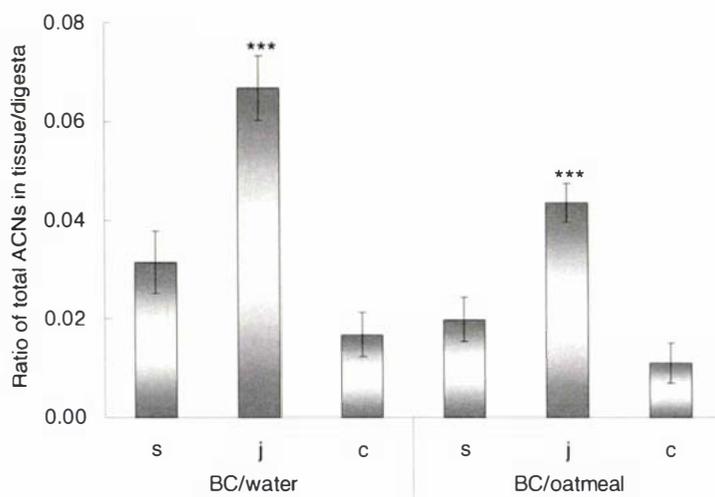


Figure 3. Ratio between the total ACN concentration found in tissue samples ($\mu\text{g/g}$ wet tissue) to the total ACN concentration found in digesta samples ($\mu\text{g/g}$ digesta as-is) of the stomach (s), the jejunum (j), and the colon (c), after administration of BC/water or BC/oatmeal over 24 h. Mean \pm SEM, $n = 26$ -29 (stomach), $n = 13$ -15 (jejunum), $n = 13$ (colon). Significant difference within treatments between the intestinal regions, *** $p < 0.001$.

Figure 4 presents the total ACN concentration in tissue samples of the stomach, the jejunum and the colon after BC/water or BC/oatmeal administration over time. After BC/water administration, a high mean concentration of ACNs was found in the stomach tissue after 0.25 h, which gradually decreased over time (**Figure 4 A**). After BC/oatmeal administration however, there was an initial increase with the highest mean concentration recorded at 0.5 h, before the ACN concentration also gradually decreased (**Figure 4 B**). The jejunal tissue samples contained the highest mean ACN concentration at 1 h for both, the BC/water (**Figure 4 A**) and BC/oatmeal treatment (**Figure 4 B**). ACNs were also detected in colon tissues of the rats in both treatments. The maximum mean concentration was reached at 2 h for the BC/water, and at 3 h for the BC/oatmeal treatment (**Figure 4 A, B**).

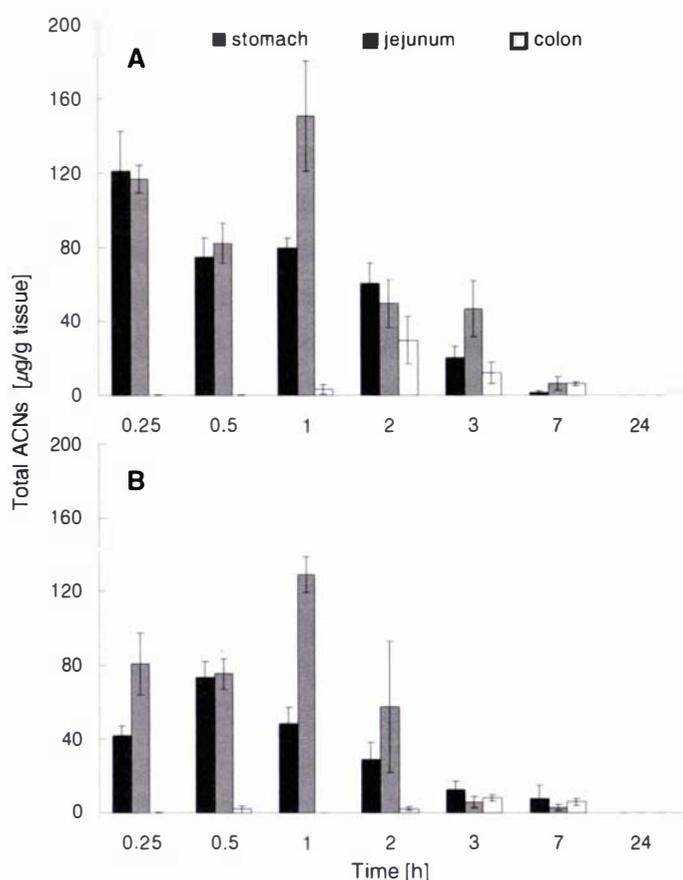


Figure 4. ACN concentration in tissue samples of the stomach, the jejunum, and the colon, after administration of BC/water (A), or BC/oatmeal (B). Mean \pm SEM, $n = 5$.

The ratio of individual BC ACNs expressed as a percentage of the total ACN content in the treatments, digesta and tissue samples are shown in **Figure 5 A** and **B**, respectively. The relative percentage of both glucosides (D3G, C3G) in the digesta decreased numerically (NS) during their passage through the gastrointestinal tract (GIT). The percentage of the rutinoid D3R in the digesta remained similar throughout the GIT, while the percentage of the rutinoid C3R was increased in the jejunum ($p < 0.05$) and colon ($p < 0.001$) digesta (**Figure 5 A**). Similar to the digesta samples, the percentage of both glucosides decreased (D3G: $p < 0.01$, C3G: NS) in the tissue samples during the passage of digesta through the GIT (**Figure 5 B**). However, the percentage of the rutinoid D3R in the various tissue

samples of the GIT was not different (NS), while C3R was increased in the stomach, jejunum ($p < 0.05$) and colon ($p < 0.001$) compared to the treatment.

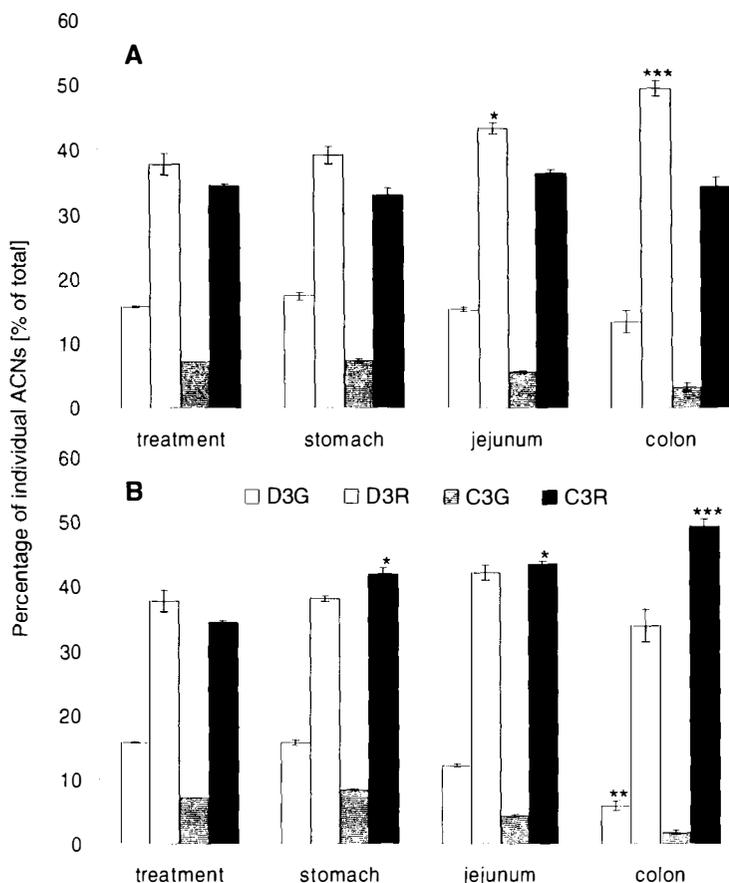


Figure 5. Percentage of individual ACNs of BC in digesta (A) and tissue (B) samples over 24 h. Mean \pm SEM, $n = 3$ (treatment), $n = 55-56$ (stomach), $n = 30-31$ (jejunum), $n = 22-28$ (colon). Significant difference of the individual ACNs in the different intestinal regions (within the respective sample), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The HPLC chromatogram (**Figure 6**) of a urine sample taken 2 h after administration of BC/water to a rat, showed the four original ACNs of BC (peak 1-4), and 4 additional metabolites (peak 5-8). HPLC chromatograms of urine samples taken at other time points as well as after administration of BC/oatmeal showed similar profiles (data not shown). Additional peak identification was performed by LC-MS analysis, and the RP-HPLC and LC-MS results are shown in **Table 2**.

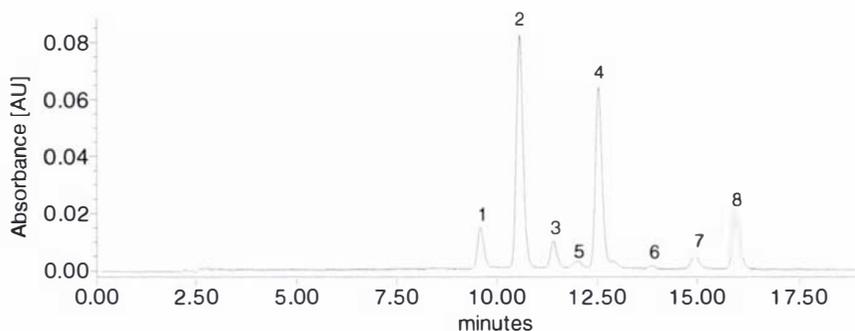


Figure 6. HPLC chromatogram of a rat urine sample, taken 2 h after administration of BC/water, and showing the original ACNs of BC (peak 1-4) and 4 additional metabolites (peak 5-8). Detection is at 520 nm.

TABLE 2. Peak identification of urine samples by RP-HPLC and LC-MS.

Compound	RP-HPLC peak	LC-MS m/z
<u>Original ACNs</u>		
D3G	1	465/303
D3R	2	611/303
C3G	3	449/287
C3R	4	595/287
<u>Methylated forms</u>		
methylated D3G	5	479/317
methylated D3R	6	625/317
peonidin-3-glucoside	7	463/301
peonidin-3-rutinoside	8	609/301
<u>Diglycuronidated forms</u>		
delphinidin diglucuronide	ND	655/303
cyanidin diglucuronide	ND	639/287
petunidin diglucuronide	ND	669/317
peonidin diglucuronide	ND	ND

ND – not detected.

The identities of the four original ACNs of BC were confirmed by LC-MS analysis. Peaks 5-8 were identified as the methylated forms of the four original ACNs, methylated D3G, methylated D3R, peonidin-3-glucoside, and peonidin-3-rutinoside. In addition, traces of the diglycuronidated forms of delphinidin, cyanidin, and petunidin were detected. The concentration of ACNs excreted per unit of urinary creatinine between 2 and 24 h is shown in **Figure 7**. Sufficient urine samples were only collectable from animals sacrificed between 2 and 24 h after administration of the treatments.

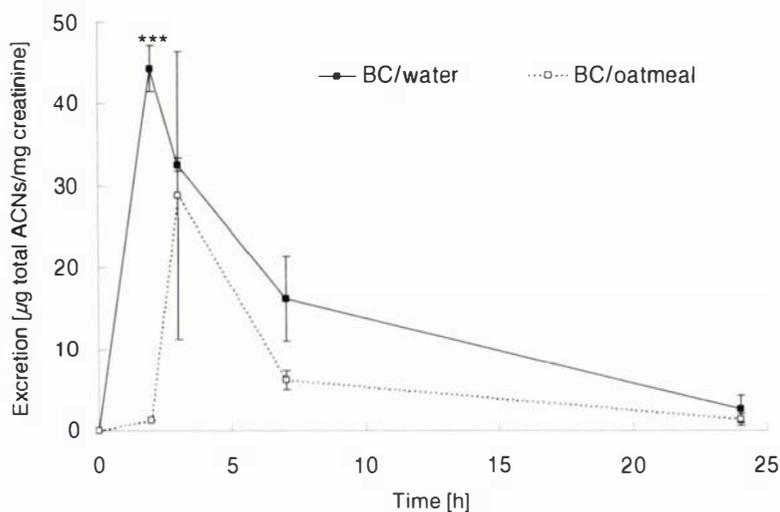


Figure 7. ACN excretion in urine. Mean \pm SEM, $n = 1-5$. Significant difference of urinary ACN excretion between the two treatments at 2 h. *** $p < 0.001$.

The maximum urinary ACN excretion per unit creatinine after BC/oatmeal administration occurred later compared to the BC/water administration ($t_{\max} = 3$ vs. 2 h). The mean concentration of ACNs excreted at 2 h was higher ($p < 0.001$) after administration of BC/water. In addition, the maximum urinary concentration of ACNs excreted was lower after administration of BC/oatmeal compared to BC/water (28.8 ± 17.6 vs. 44.3 ± 2.8 μg total ACNs/mg creatinine; $p = 0.06$). The 24 h ACN recovery (% of the initial dose) of the total ACNs in the urine of the rats receiving the BC/water and BC/oatmeal treatment was 0.03 ± 0.02 and $0.02 \pm 0.01\%$, respectively.

DISCUSSION

A number of studies have been conducted to investigate ACN absorption by animals, such as rats and pigs, and humans. However, most of these studies administered purified ACNs or berry fruit extracts dissolved in water (4, 7, 8, 29, 37-41), thereby not taking into account possible interactions of ACNs with other dietary components. With the exception of one study, neither of the previous studies investigating ACN bioavailability has compared the effect of different administration forms of ACNs *in vivo*.

The present study investigated the effect of a food matrix on ACN absorption, metabolism, and excretion in rats, by comparing two different administration forms of ACNs. The animals were either given a BC concentrate dissolved in acidified water (BC/water), or the same mixture containing additional oatmeal as a food source (BC/oatmeal). The latter was chosen to represent a breakfast type meal that was simple to administer to rats via stomach intubation. In the plasma samples of the rats in both groups we detected the four original ACNs of BC, as they were present in the BC concentrate. No difference was observed in the plasma profile of the four ACNs measured between the two treatments, indicating that the food matrix had no effect on the metabolism of ACNs. The present results show that ACNs are absorbed in their intact form as glycosides, as has been shown in previous rat and human studies (8, 27, 31, 42-44). More recently, the metabolism of ACNs has been described to include methylation and glucuronidation, and the respective methylated and glucuronidated forms have been reported in the digestive area of rats, including the stomach, jejunum, liver and kidney, as well as in plasma and urine of humans and rats (29, 30, 45, 46). In the present study, we only detected metabolites of the BC ACNs in the urine samples, but not in plasma, digesta or tissue samples.

The ACN concentration in rat plasma was different between the two administration forms (**Figure 2 A, B**). After administration of BC/water, all four ACNs of BC were absorbed and reached maximum concentrations within the first 0.25 h (**Figure 2 A, Table 1**). In a previous study, it was shown that ACNs are mainly absorbed from the jejunum in mice (35). The rapid absorption in the present study in rats is therefore not surprising, as the aqueous BC/water treatment would have quickly reached the jejunum after 0.25 h, where the ACNs were readily absorbed into the jejunal tissue (**Figure 4 A**). The relatively rapid absorption can also be explained by potential absorption of ACNs from the stomach, as has been shown in rats by Talavera *et al.* (47). In the present study, we found substantial concentrations of ACNs in the stomach tissue, which would support the latter. However, the ratio of the ACN concentration (0-24 h) of the tissue to the digesta samples of the stomach and the jejunum indicates that the absorption by the jejunum was significantly more efficient (**Figure 3**). However it should be noted that determination of the absorption site by calculating this ratio does not take into account the movement of digesta in the GIT, and the method needs to be further validated in additional *in vitro* systems. Interestingly, we detected another numerical increase in the concentrations of the rutosides D3R and

C3R in plasma at 1 h after administration of the BC/water (**Figure 2 A**). This second increase was also detected in the jejunal tissue (**Figure 4 A**). An earlier study also identified two peaks, one at 0.25 and one at 1 h in rat plasma after oral administration of 100 mg D3G/kg BW (38). As has been previously suggested by Ichiyangi *et al.* (7), the second increase in the concentration of these compounds could be the result of the enterohepatic cycle. Absorbed ACNs may be transported to the hepatocytes, and subsequently excreted with the bile into the duodenum, where a re-absorption of the ACNs in the jejunum could take place. Interestingly, this second increase of ACNs in plasma was not observed in the rats administered the BC/oatmeal.

The maximum plasma ACN concentration after BC/oatmeal administration was reached after 1 h (**Figure 2 C, Table 1**) compared to 0.25 h for the BC/water treatment. These data are supported by the maximum concentration of ACNs in the jejunal tissue, which occurred at the same time the maximum total ACN concentration was observed in the plasma samples. The reason for the delayed absorption of ACNs from the BC/oatmeal diet can be explained by the higher viscosity of the BC/oatmeal treatment, the longer transit time of the chyme through the GIT, and the potential binding of ACNs to other food components. The latter results support our previous *in vitro* finding (35) that the main absorption site of ACNs in the GIT of animals is the jejunum. A longer transit time of ACNs through the GIT would increase their potential for absorption, and optimise possible health-related benefits of ACN consumption. However, a lower mean ACN concentration in the plasma (0.203 ± 0.053 vs. 0.369 ± 0.072 $\mu\text{mol/L}$), and a smaller total amount of ACN absorbed between 0 and 7 h ($\text{AUC}_{0-7\text{h}}$: 0.63 ± 0.09 vs. 1.00 ± 0.11 $\mu\text{mol}^*\text{h/L}$) after BC/oatmeal administration compared to BC/water administration was observed (**Table 1**) which would seem to contradict the latter. It has to be mentioned however, that the lower amount of ACNs administered to the rats in the BC/oatmeal group (74% of the BC/water treatment group) partly explains this difference observed between the two treatments. The animals fed the BC/oatmeal therefore only received 203.9 ± 4.7 mg total ACNs/kg BW compared to the BC/water group which received 275.8 ± 5.2 mg total ACNs/kg BW. Although the two dietary treatments were prepared to have identical ACN concentrations, the 26% lower total ACN concentration of the BC/oatmeal treatment may have been due to thermal instability of ACNs during preparation of the BC/oatmeal treatment. The BC material was added

before cooking the oatmeal, as the mixture became too viscous to successfully and homogeneously mix in the BC material afterwards. In addition, it is possible that the rats fed the BC/oatmeal had a lower ACN absorption due to the binding of ACN to components in the oatmeal, such as fibre. McDougall *et al.* (34) have suggested, that polyphenols transiently bind to food matrices during digestion. It is likely that ACNs have different affinities to different food components, and in our case, the ACNs might have been partly bound to the fibre in oatmeal and escaped absorption. The ACN-fibre complex would be transported further down the GIT, reducing the amount of ACNs available for absorption by the jejunum. However others have reported that when ACNs are co-digested (*in vitro*) with cooked minced beef, the absorption of ACNs is slightly increased (34).

ACNs, which are not absorbed in the small intestine, appear further down the GIT. The colon digesta samples collected between 2 and 7 h in the present study showed a dark purple colour and contained ACNs (BC/water: 1.17 ± 0.23 mg/g digesta; BC/oatmeal: 0.70 ± 0.20 mg/g digesta). This confirms the observation made by He *et al.* (48), who investigated ACNs in intestinal contents after feeding rats a berry-enriched diet for 14 days, and reported an intense coloration of the fecal and ceecal contents. Surprisingly, we also detected ACNs in the colon tissues, which would indicate that ACNs are absorbed by colonocytes. This is in contrast to our previous *in vitro* study, where ACNs were found not to be absorbed by mice colon tissue (35). The present study however used a relatively high dosage rate of ACNs (~250 mg total ACNs/kg BW) in comparison to the *in vitro* study.

Regarding the proportion of the individual ACNs in the GIT, it was interesting to observe a decrease in the relative concentration of glucosides during their transit through the GIT, whereas the relative concentrations of rutosides increased. The variation of the different ACNs in different segments of the GIT from pigs was recently reported by Wu *et al.* (49), who found that except for C3G, the profile of four other major ACNs in black raspberry was very similar to that in the berry. The instability of glucosides in the gut content has previously been shown by He *et al.* (48), and has been suggested to be the result of lactase phloridzin hydrolase activity, a mammalian β -glucosidase present in the brush border of the small intestine, which is also responsible for flavonoid deglycosylation in sheep and humans (50, 51). However, the latter would not explain why the rutoside concentration in the digesta of the rats in the present study increased. It has been suggested

that the overall stability of ACN rutinoides is increased in the body, as the rutinoid moiety appears to decrease the metabolism of ACNs (11).

Besides the four major ACNs of BC, several additional peaks were detected in urine samples, after administration of either BC/water or BC/oatmeal. LC-MS analysis identified the additional peaks as the methylated and diglucuronidated forms of the original four ACNs. A number of studies have reported the methylation and glucuronidation of ACNs in rats, pigs and humans (11, 33, 40, 45, 52). It has been shown that the methylation of D3G occurs at the 4'OH position in rats (38), therefore the two methylated forms of D3G and D3R in the present study may be the 4'-O-methyl-D3G and 4'-O-methyl-D3R metabolites. A previous study on BC absorption and metabolism (11) presented a similar HPLC chromatogram of pig urine after administering BC ACNs by gastric intubation. Compared to our study however, the authors identified isopeonidin metabolites and reported the monoglucuronides of cyanidin, isopeonidin, and peonidin, whereas we only detected diglucuronides. These differences could indicate a species-specific metabolism of ACNs. However, monoglucuronides of C3G have also been reported in the urine of rats (52). The fact that we did not observe a difference between the two treatments in regards to ACN metabolites in the urine indicates that the addition of a food matrix does not influence metabolism of ACNs. The low urine recoveries of total ACNs in the present study (0.03% BC/water; 0.02% BC/oatmeal), confirm the previously reported low bioavailability of ACNs when assessed by urinary excretion. Most previous studies on ACN bioavailability in rats and humans reported ACN recoveries in urine between 0.004 and 0.11% (8, 30, 31, 42). Three more recent studies by Wu *et al.* reported recoveries in pigs of 0.067, 0.073, and 0.088%, respectively (11, 40, 49). As the urine samples in the present study were only acidified at collection, the lower recoveries found may be partly due to the delayed (between voiding and collection) acidification of the 24 h urine samples. As ACNs are more stable at a lower pH, degradation of ACNs at physiological urinary pH could have occurred to some extent, which would result in a lower overall bioavailability estimate.

In conclusion, the present study demonstrates that a simultaneous intake of a food source affects ACN absorption and urinary excretion. ACNs were faster absorbed and excreted, when administered in an acidified water solution compared to an acidified oatmeal solution. The administration of ACNs mixed only in acidified water resulted in a higher ACN concentration in blood plasma, compared to when oatmeal was simultaneously

ingested. The additional food matrix does not appear to have an effect on ACN metabolism. In addition, the results of the present study support the finding that ACNs are mainly absorbed from the jejunum.

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ABBREVIATIONS USED

ACNs, anthocyanins; AUC, area under the curve; BC, blackcurrant; C3G, cyanidin-3-glucoside; C3R, cyanidin-3-rutinoside; D3G, delphinidin-3-glucoside; D3R, delphinidin-3-rutinoside; ESI, electrospray interface; GIT, gastrointestinal tract; LC-MS, liquid chromatography mass spectrometry; PDA, photodiode array; RP, reversed-phase; SN, supernatant; $t_{1/2\text{ abs}}$, absorption half-life.

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CHAPTER 5

ANTHOCYANIN ABSORPTION AND ANTIOXIDANT STATUS IN PIGS

Chapter 3 has demonstrated that the simultaneous occurrence of different flavonoid groups result in an inhibition of ACN absorption *in vitro*. The additional ingestion of a food matrix has also been shown to affect ACN absorption in rats (Chapter 4). Based on these results, the aim of the study presented in the following chapter was to investigate the effect of the simultaneous intake of ACNs with a food matrix, and another source of flavonoids on ACN absorption and antioxidant capacity in pigs.

ABSTRACT

The effect of a simultaneous intake of food or flavonoids on anthocyanin (ACN) absorption and antioxidant status in pigs was investigated. Twelve male pigs at 27.1 ± 0.7 kg BW fitted with jugular venous cannulae were maintained in individual metabolic crates. The animals were each given one of three dietary treatments in random order: blackcurrant powder (BC) to give a dose of 100 mg total ACNs/kg BW mixed either with water and sugar (Diet A), cereal (Weet-Bix), milk, and sugar (Diet B), or cereal, milk, sugar, and an additional flavonol (rutin, ~ 100 mg/kg BW) (Diet C). The four major ACNs of BC, delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside were identified and quantified by HPLC-PDA in all three diets. In the pig plasma, four peaks with a reversed pattern to those of ACNs in the BC extract were detected. The total amount of ACNs absorbed was not significantly different between the three diets, but the rate of absorption and subsequent decline was slower following administration of diet B and C than diet A. All three diets increased antioxidant capacity when measured by the FRAP assay but not when measured by the ORAC and non-protein ORAC assay. However, the increase was delayed and did not appear until 4 h after ingestion, at a time when plasma ACN levels had returned to baseline. The present study demonstrates that the simultaneous intake of food or flavonoids delays the absorption profile for ACNs. Our results also suggest that the increase in antioxidant capacity is not due to dietary ACNs but may be due to metabolites that result from ACN consumption.

INTRODUCTION

Anthocyanins (ACNs) are part of a common class of phytochemicals known collectively as flavonoids, which are responsible for the red, purple and blue colours displayed in many vegetables and fruits (1). They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylum salts. Berry fruits are particularly rich dietary sources, and some can contribute to 100-300 mg of ACNs in a single serving (2). Blackcurrants (BC) for example, may contain up to 250 mg ACNs/100 g fresh fruit (3). Berry extracts of BC have been widely used to investigate ACN absorption in humans as well as in rats and rabbits (4-8). The berries of BC contain four major ACNs

(**Figure 1**), delphinidin-3-glucoside (**1**), delphinidin-3-rutinoside (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-rutinoside (**4**), which were first reported by Chandler and Harper (9).

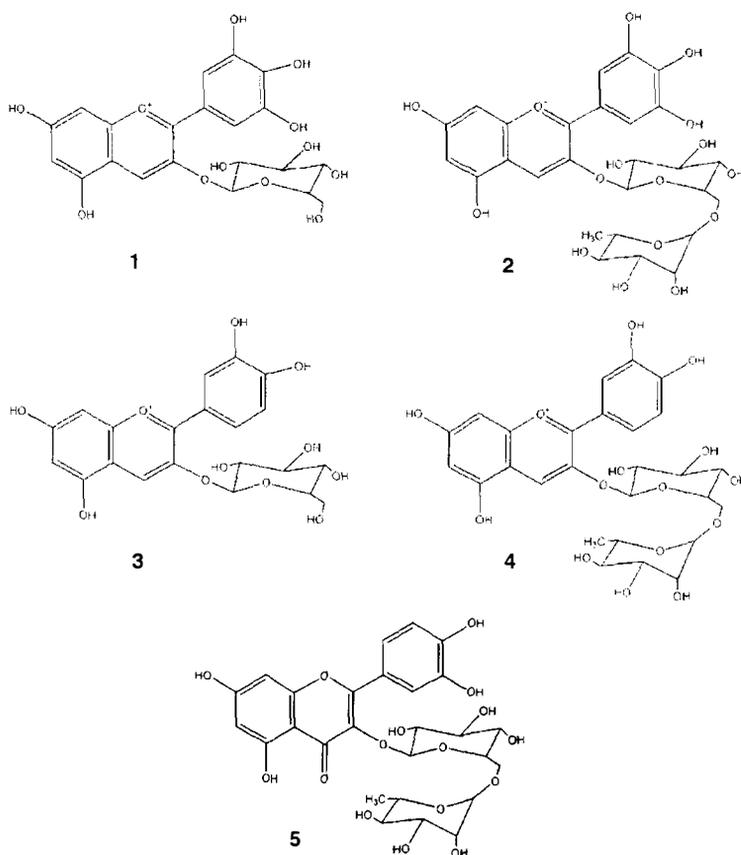


Figure 1. Chemical structures of the four major ACNs in blackcurrants; delphinidin-3-glucoside (**1**), delphinidin-3-rutinoside (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-rutinoside (**4**) and the flavonol rutin (**5**).

Consumption of fruits and vegetables is known to reduce the risk of cancer and cardiovascular disease (10-14), and ACNs may contribute to this effect. Among all common fruits and vegetables in the diet, dark blue and red coloured berries especially were reported to have the highest antioxidant capacities (15). Indeed, fruits with a high ACN content have a high antioxidant capacity (1, 16, 17), and their beneficial effects may be related to their potent antioxidant activity.

While ACNs are strong antioxidants *in vitro* (17-21), it is uncertain whether the consumption of ACN containing food significantly augments antioxidant levels *in vivo*. The bioavailability of ACNs has been shown to be extremely low, and the proportion of ACNs absorbed and excreted in urine may be less than 0.1% of the ingested dose (22). However, most of these studies used purified aqueous extracts of ACNs (4, 5, 23-26), and few have investigated the effects of an additional food matrix on ACN absorption (6, 27, 28). As ACNs are rarely ingested entirely on their own, but rather in combination with other food matrices and other flavonoids, it is important to evaluate their influence on ACN absorption as well as on antioxidant levels.

While a study using an *in vitro* digestion procedure that mimics the physiological and biochemical conditions encountered in the gastrointestinal tract found little effect from a variety of food matrices on ACN absorption (29), an *in vivo* study showed absorption to be significantly increased when consumed with a food matrix (6). Conversely, in a previous unpublished *in vivo* study we found addition of a food matrix (oatmeal) significantly delayed absorption of ACNs from BC in rats. Absorption of ACNs may also be influenced by co-consumption of other flavonoids. Thus, we found in a previous *in vitro* study using segments of mice jejunum mounted in Ussing chambers that the flavonol quercetin-3-glucoside strongly inhibited ACN (3) absorption, and was preferentially absorbed (30).

The purpose of the present study was to determine the effect of an additional food, a wheat-based cereal with milk and sugar, and an additional flavonoid source, namely, rutin (**Figure 1**), a flavonol glycoside comprised of the flavonol quercetin and the disaccharide rutinose, on ACN absorption and antioxidant status in the pig. The pig was chosen as a model since it is metabolically similar to the human in regard to ACN metabolism (8).

MATERIAL AND METHODS

ACNs. A blackcurrant powdered concentrate (Currantex 30, total ACN content 32.9%.) was kindly provided from Just the Berries Ltd., Palmerston North, New Zealand. The ACN composition of the BC concentrate was 15.5% **1**, 39.0% **2**, 7.3% **3**, and 36.0% **4**.

Animals and Diets. Twelve male pigs (PIC Camborough 22 x PIC 331) approximately 11 weeks of age and a body mass of 27.1 ± 0.7 kg were purchased from a commercial piggery, (Aorere Farms Wanganui, New Zealand). The animals were maintained on a 12/12

light cycle in individual metabolic crates at the Animal Physiology Unit, Massey University (Palmerston North, New Zealand) for an acclimation period of 10 d prior to surgery. The animals were provisioned at 10% of their metabolic body weight (MBWT = $\text{kg}^{0.75}$) with water available *ad libitum*. All pigs were feed twice daily: 2% of MBWT provided as a test diet (**Table 1**) in the morning and 8% of MBWT provided as commercial pig food (Denver Stock Feed, Palmerston North, New Zealand) in the afternoon to adapt the animals to a feeding routine. During the acclimation and washout periods the test diet was administered without ACN or other flavonoids. Animals were fasted every night between 6 pm and 8 am.

The study reported here was approved and followed the procedures set out by the Massey University Animal Ethics Committee of Massey University (protocol 05/84), New Zealand (31).

Table 1. Composition of the test diets.

Diet	Ingredient	%
A "juice"	BC ^a	4
	sugar	3
	water	93
B "breakfast"	BC ^a	4
	sugar	3
	milk	68
	Weet-bix ^b	25
C "breakfast + flavonol"	BC ^a	4
	sugar	3
	milk	67
	Weet-bix ^b	25
	rutin ^c	1

^a BC = blackcurrant; administered dose of total ACNs: 100.1 ± 0.4 mg/kg BW

^b Wholegrain wheat-based cereal (Sanitarium, New Zealand).

^c Administered dose of rutin (Sigma Aldrich, New Zealand): 93.2 ± 2.4 mg/kg BW

Study design. Immediately following acclimation an intravenous cannula (IV Catheter extra long, 14 g x 75 cm, Genia, France) was inserted into the external jugular vein of each pig under general anaesthesia (isoflurane) with the external opening secured to the skin and connected to an extension piece for ease of blood collection. Each cannula and extension was flushed with normal saline (sodium chloride 0.9%, Baxter Healthcare PTY LTD, NSW, Australia), and subsequently filled with heparinized saline (heparin sodium, CP Pharmaceuticals Ltd., Wrexham, UK) in order to prevent *in situ* coagulation. The cannulae were flushed 3 times daily with normal saline following withdrawal of the previously administered heparinized saline and subsequently refilled with either 10 IU/mL heparinized

saline (in the morning and afternoon), or 50 IU/mL heparinized saline (overnight). The experimental period commenced 2 days after installation of the cannulae and comprised a crossover study with consecutive randomised 3 experimental days interspersed with 3-day washout periods (**Table 2**).

Table 2. Experimental Design.

Pig	ED ^a I	W/O ^b (3 d)	ED II	W/O (3 d)	ED III
1	A ^c	↓	B ^d	↓	C ^e
2	A		B		C
3	A		C		B
4	A		C		B
5	B		A		C
6	B		A		C
7	B		C		A
8	B		C		A
9	C		A		B
10	C		A		B
11	C		B		A
12	C		B		A

^a Experimental day

^b Washout period

^c Diet A "juice"

^d Diet B "breakfast"

^e Diet C "breakfast + flavanol"

Each pig was weighed on the day prior to each experimental day and the MBWT recalculated. On each experimental day baseline venous blood samples (0 h) were withdrawn 30 min prior to the feeding of the test diet (**Table 1**) at 2% of their MBWT, providing a dose of approximately 100 mg/kg BW total ACNs and 100 mg/kg BW rutin. The diets were administered at the same time each day (8 am) in order to avoid confounding chronological effects. The animals had free access to the test diets, and the meals were usually eaten within a few minutes. Additional venous blood samples (~7 mL) were drawn at 0.25, 0.5, 0.75, 1, 2, 4, and 8 h, and immediately stored at 0°C. The samples were centrifuged at 2,500 x g for 15 min at 4°C, and plasma aliquots were stored at -20°C. Plasma samples for ACN analysis were acidified with 20% of 5% trifluoroacetic acid prior to storage. After the last blood sample was withdrawn (4 pm), the pigs were fed the remaining 8% of their MBWT in the form of commercial pig food.

ACNs in test diets. A portion (0.5 g) of each diet was blended with an Ultra-Turrax basic homogenizer in 5 ml of ethanol/H₂O/acetic acid (80/20/1). The samples were subsequently stored for 48 h at 4°C, centrifuged at 3040 x g for 20 min at RT, and appropriate dilutions with 5% formic acid/H₂O analysed by HPLC.

ACNs in plasma. Each frozen, acidified plasma sample was thawed at 37°C in a water bath. Protein was precipitated from each sample with 10% trichloroacetic acid (TCA). An aliquot (500 µL) of acidified plasma was mixed 1:1 with 10% TCA and centrifuged for 5 min at 10,000 x g at RT. The supernatant (800 µL) was collected, and the pellet was resuspended and rewashed twice each with 800 µL 10% TCA. The supernatants were combined for each sample and evaporated almost to dryness under N₂ (< 35°C). The residue was resuspended in 150 µL of 1% aq. HCl and centrifuged for 10 min at 3,040 x g at RT. A 30 µL portion of the supernatant was taken for HPLC analysis. The recoveries of ACNs in plasma with this method were tested with spiking experiments using an authentic standard of **3** (Extrasynthase, Genay, France) and found to be 90.5 ± 3.9%.

HPLC Analysis. ACN concentrations in plasma samples were determined by reversed-phase HPLC with photodiode array (PDA) detection. The HPLC system comprised a Waters Alliance 2690 HPLC equipped with a 996 PDA. The separation column was a 250 mm x 4.6 mm i.d., 4 µm, Synergi Hydro-RP Column (Phenomenex, New Zealand). Solvents A (5 % formic acid/H₂O) and B (acetonitrile) were run at a flow rate of 1 mL/min. The solvent gradient started with a composition at 95% A and changed to reach 70% A at 25 min. The composition then changed to 20% A by 30 min and was held at this composition until 35 min, before returning to the starting conditions at 40 min for 5 min with a total run time of 45 min. The sample injection volume was 30 µL. Spectroscopic data from the PDA were collected at 250-600 nm, and chromatograms extracted at 520 nm. Chromatography data were collected and processed using a Water Millennium Chromatography Manager version 4.0. **1**, **2**, **3**, and **4** concentrations were calculated as cyanidin-3-glucoside-equivalents using an authentic standard of cyanidin-3-glucoside (Extrasynthase, Genay, France) with known concentration.

Antioxidant status in plasma. Two commonly used assays to analyse antioxidant status in pig plasma were employed, the ORAC (oxygen radical absorbance capacity) and the FRAP (ferric reducing ability of plasma) assay.

Chemicals and Apparatus. Fluorescein, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,4,6-Tripyridyl-s-Triazine (TPTZ) were obtained from Sigma Aldrich (New Zealand). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Merck (New Zealand). Standard laboratory chemicals were all of analytical grade.

ORAC and FRAP analyses were conducted using a BMG FLUOstar Optima fluorescent plate reader, which was purchased from BMG Labtech (through Alphatech, New Zealand). The 96-well black plates for ORAC analysis were purchased from BMG Labtech, and the clear polystyrene 96-well plates (NUNC) for FRAP analysis obtained from Sigma Aldrich.

ORAC sample preparation. Plasma ORAC was measured using a method similar to that described by Ou *et al.* (32). A fluorescein stock solution (48 nM) was prepared by dissolving 0.018 g of fluorescein in 10 mL of 75 mM potassium phosphate buffer (pH 7.4), and a second dilution of 10 μ L of that into 1 mL phosphate buffer. Aliquots of the fluorescein stock were stored at -20°C until use. Aliquots of 0.1 M AAPH, prepared by dissolving 0.271 g AAPH in 10 mL phosphate buffer, were also stored at -20°C until use. A Trolox stock solution (200 μ M) was prepared by dissolving 5 mg Trolox in 100 mL phosphate buffer and stored at -20°C until use. For each analysis, a fresh Trolox standard series was prepared with phosphate buffer to yield Trolox concentrations of 50, 100, 150, and 180 μ M. Plasma samples were diluted 125-fold with phosphate buffer immediately prior to analysis. To measure the plasma ORAC in non-protein fraction (NP-ORAC), protein was precipitated using 0.5 N perchloric acid (1:1; v:v; plasma:acid) and removed by centrifugation at $10,000 \times g$ for 5 min. The supernatant (non-protein fraction) was diluted 6.67-fold with phosphate buffer immediately prior to analysis.

ORAC experimental conditions. The automated ORAC assay was carried out on the BGM FLUOstar plate reader with a fluorescence detector. The fluorescent filters were set to pass the light with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The FLUOstar plate reader is equipped with an incubator and two injection pumps. The temperature of the incubator was set to 40°C .

Prior to each experiment, the first injection pump was primed with fluorescein stock (fluorescent probe). Subsequently, a black 96-well plate was prepared by injecting 160 μ L of fluorescein (48 nM) into each well, followed by reading the fluorescence. Then the

plates were manually loaded with phosphate buffer (blank, 10 μL), Trolox standard series (control antioxidant standard, 10 μL), and plasma samples (10 μL) into the respective wells, and the plates were placed back into the plate reader to warm up back to 40°C. The second injection pump was then primed with AAPH (peroxyl radical generator), 25 μL injected into each well, and readings taken every min for 91 min.

ORAC Calculations. The final ORAC values were calculated according to Ou *et al.* (32). The relative fluorescence for each standard was determined to give the area under the curve (AUC), and a standard curve was plotted. The final ORAC values were determined by linear regression equation of Trolox concentrations against the net area under the fluorescein decay curve and are expressed as Trolox equivalents (TE) in mmol/L. Data were analysed by Microsoft Excel.

FRAP sample preparation. Plasma FRAP was measured using a method based on that by Benzie and Strain (33). A sodium acetate solution (300 mM, pH 3.6) was prepared by dissolving 3.1 g sodium acetate trihydrate in 950 mL H₂O, adding 16 mL glacial acetic acid, and brought to a total volume up to 1000 mL. This solution was stored at room temperature until use. A TPTZ solution (10 mM) was prepared by dissolving 0.312 g TPTZ in 100 mL HCl (40 mM) and stored dark at room temperature. A FRAP reagent was prepared fresh prior to each analysis by combining TPTZ solution with a 20 mM ferric chloride solution (1:1), which was prepared by dissolving 0.270 g ferric chloride hexahydrate in 50 mL H₂O (total volume). A Trolox standard series was prepared fresh prior to analysis from a frozen 10 mM Trolox stock, to yield Trolox concentrations between 25 and 250 μM . Plasma samples were analysed undiluted.

FRAP experimental conditions. The automated FRAP assay was carried out on the BGM FLUOstar plate reader, and absorbance readings taken at 593 nm. Prior each experiment, the first injection pump was primed with sodium acetate solution and 140 μL of the solution injected into each well of a NUNC 96-well plate. Subsequently, the plates were manually loaded with Trolox standards (15 μL), sodium acetate buffer (blank; 15 μL), and plasma samples (15 μL) into the respective wells, and reinserted into the plate reader. The second injector was primed with freshly prepared FRAP reagent, 60 μL of that mixture injected into each well, and readings taken 4 min thereafter.

FRAP calculations. Blank values were subtracted from sample and standard values, and a linear regression for the Trolox standards (absorption against concentration) was constructed using Microsoft Excel. The regression equation was used to calculate the FRAP values of the samples and were expressed as Trolox equivalents in $\mu\text{mol/L}$. Data were analysed by Microsoft Excel.

Statistical analysis. Data are presented as means \pm SEM. The significance of differences between the baseline (0 h) and the following time points among treatments was assessed by repeated measures ANOVA for comparison of individual means. Area under the plasma concentration vs. time curve ($\text{AUC}_{0-8 \text{ h}}$) was calculated with the linear trapezoidal rule (ORIGIN, version 7.5; GENSTAT, version 8.1). Differences between treatment groups for $\text{AUC}_{0-8 \text{ h}}$ were tested by one-way ANOVA. Statistical analysis was carried out using SAS System for Windows, version 8. The residuals of each analysis were tested for normality. Differences with $p \leq 0.05$ were considered significant.

RESULTS

The HPLC-chromatogram of diet A (**Figure 2A**) shows the four major anthocyanins of BC: **1**, **2**, **3**, and **4**. The pattern comprised two small peaks representing the glucosides and two large peaks representing the rutosides. Similar HPLC chromatogram profiles were produced by diets B and C (data not shown). All plasma samples collected before feeding the diets (baseline; $t = 0 \text{ h}$) showed no detectable anthocyanin profiles in the HPLC chromatogram (**Figure 2B**). The HPLC chromatogram of a plasma sample taken 2 h after feeding diet A (**Figure 2C**) showed four peaks, of which two had retention times identical to the corresponding rutosides, while the other two peaks had retention times somewhat later than the corresponding glucosides of the BC extract. Furthermore, in plasma, the two rutoside peaks were reduced in size relative to the rutoside peaks in the BC extract, where they dominate (**Figure 3**). HPLC chromatograms of all plasma samples showed similar anthocyanin profiles (data not shown) regardless of diet and sampling time.

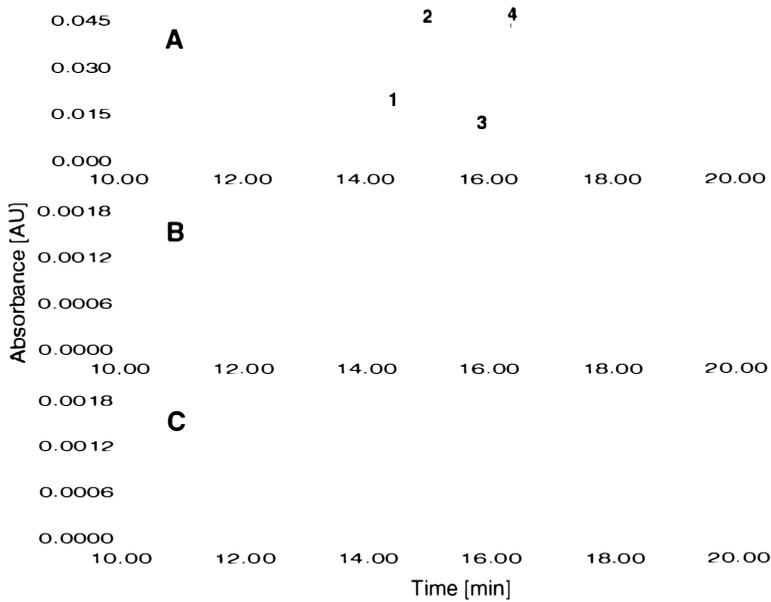


Figure 2. HPLC chromatogram of diet A (A), baseline plasma sample (B), and a postfeeding plasma sample (C). Detection is at 520 nm.

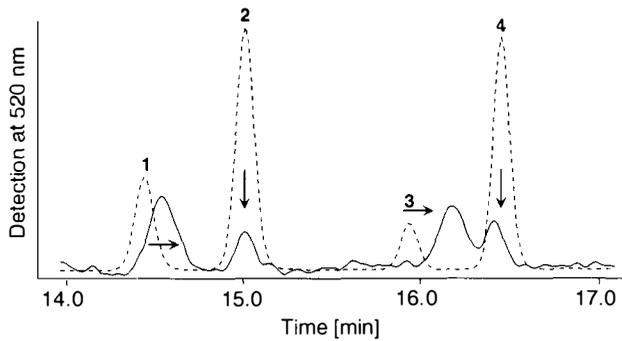


Figure 3. Overlay chromatogram of diet A (---) and a postfeeding plasma sample (—). Arrows indicate the slight shift in retention times of the corresponding glucosides of the blackcurrant ACNs present in the diets and the significant reduction of the rutinoids. Detection is at 520 nm.

The total ACN concentration in plasma over time is shown in **Figure 4**. ACNs from diet A were absorbed at significantly faster rates ($p < 0.05$ at 0.25 h; $p < 0.001$ between 0.5 – 1 h), than those from diet B and C. Thus, the maximum concentration in plasma occurred sooner after administration of diet A ($t_{max} = 2$ h) than that following administration of diet B or C ($t_{max} = 4$ h) (**Table 3**). However, there were no significant differences in maximum plasma concentration (0.08-0.09 $\mu\text{g}/\text{mL}$; **Table 3**) between the three diets or in the total amount absorbed over the period of 8 h ($\text{AUC}_{0-8\text{h}} = 0.49 - 0.61 \mu\text{g}\cdot\text{h}/\text{mL}$) (**Table 3**).

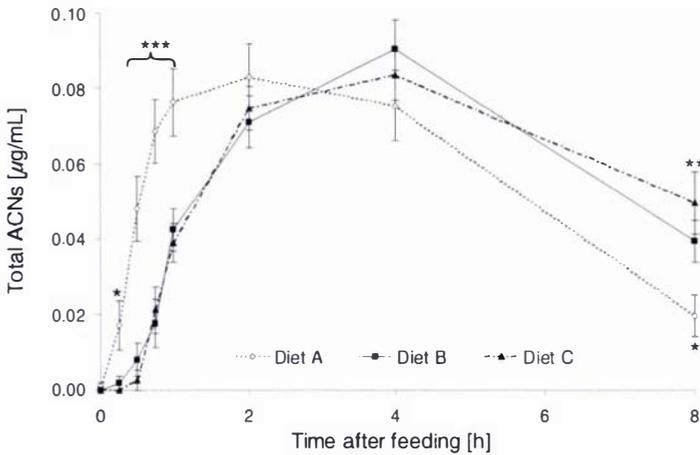


Figure 4. ACN concentration in pig plasma over time after feeding diet A, B, or C. Mean \pm SEM, $n = 10$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Pharmacokinetic parameters of ACNs in pig plasma after a single oral dose of 100 mg of total ACNs/kg BW.

Diet	C_{max} ($\mu\text{g}/\text{mL}$)	t_{max} (h)	$\text{AUC}_{0-8\text{h}}$ ($\mu\text{g}\cdot\text{h}/\text{mL}$)
A	0.08 ± 0.01	2	0.49 ± 0.06
B	0.09 ± 0.01	4	0.54 ± 0.05
C	0.08 ± 0.01	4	0.61 ± 0.08

In addition, there was no significant difference in ACN plasma concentrations following the consumption of diet B or C at any time during the experiment. However, 8 h after the

administration of the diets, the plasma ACN concentration was significantly lower after feeding diet A than that after the administration of either diet B ($p < 0.05$), or diet C ($p < 0.01$).

Regarding the antioxidant capacity of plasma samples, we did not detect any increase in plasma ORAC following administration of any of the diets. On the contrary, we observed a slight decrease in antioxidant capacity at 4 ($p < 0.01$) and 8 h ($p < 0.05$) compared to the baseline with diet B. Similarly, no effect of any of the diets was observed when plasma samples were analysed using the NP-ORAC assay (**Figure 5**).

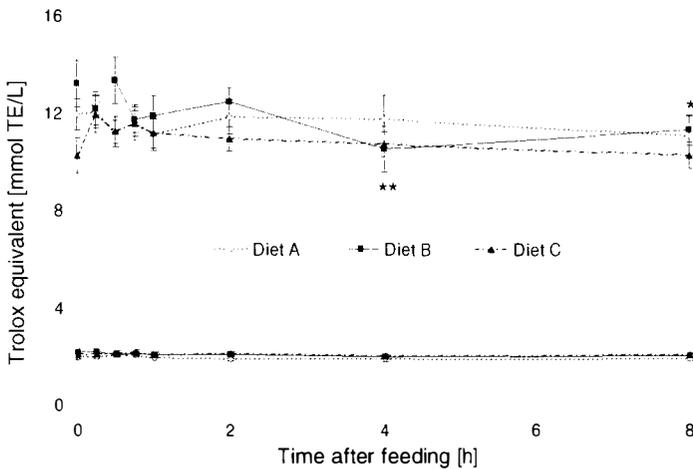


Figure 5. Plasma ORAC (top array of curves) and NP-ORAC (bottom array of curves) over time after feeding diet A, B, or C. Mean \pm SEM, $n = 10$. * $p < 0.05$, ** $p < 0.01$.

Plasma FRAP significantly decreased ($p < 0.001$) from 39.66 ± 2.07 , 39.58 ± 1.75 , and 37.19 ± 20.12 $\mu\text{mol/L TE}$ at baseline for diet A, B, and C, respectively, to 25.70 ± 2.56 , 22.40 ± 2.66 , and 23.23 ± 2.67 $\mu\text{mol/L TE}$, 0.25 h following consumption of diet A, B and C respectively (**Figure 6**). However, from 4 h onward, the antioxidant capacity increased and was significantly higher (diet A: 46.88 ± 1.64 , $p < 0.05$; diet B: 42.84 ± 3.06 , NS; diet C: 46.86 ± 1.67 , $p < 0.001$) compared to baseline levels. All three FRAP curves showed a continuous increase, with time up to the final time of sampling at 8 h (**Figure 6**).

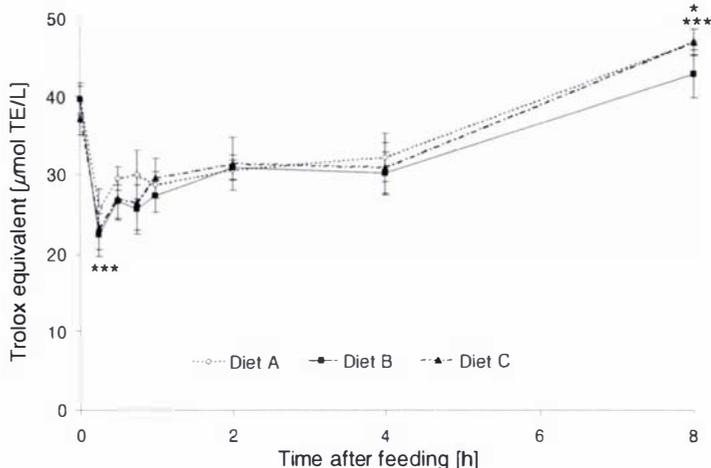


Figure 6. Plasma FRAP over time after feeding diet A, B, or C. Mean \pm SEM, $n = 10$. * $p < 0.05$; *** $p < 0.001$.

DISCUSSION

In the present *in vivo* study, we investigated the effect of a simultaneous intake of ACNs and a food matrix as well as an additional flavonoid on ACN absorption and antioxidant capacity in pigs. The animals were fed three different diets, and blood samples were taken at several time intervals. All plasma ACN profiles were broadly similar to those in the diets with four peaks eluting close to the corresponding retention times of the four major ACNs of BC. However, the height of the first and third peak was greater than that of the BC ACN profile, while the height of the second and fourth peak was lower than that of the corresponding peaks in the BC ACN profile (**Figure 3**). To our knowledge, none of the previous studies on ACN absorption using BC material have reported a similar ACN profile in plasma samples. The two detected peaks, which eluted slightly later than the glucosides of those in the dietary BC, may indicate that a proportion of dietary glucosides underwent metabolic modification following absorption, perhaps by methylation or glucuronidation. These results are concordant with those of a recent study showing that **3** was methylated or glucuronidated following administration of a freeze-dried BC powder (8). Similarly, our results are concordant with the results of studies in which single ACNs from BC (**1**) were administered to rats and showed that delphinidin ACNs were methylated to 4'-*O*-methyl-**1**

(23). However, it is noteworthy that our results conflict with those of a number of earlier studies in which BC ACNs were found unmodified in plasma and urine (5-7).

The lack of any subsidiary peaks to the rutinoides **2** and **4** that could indicate chemical modification of these compounds show that these ACNs do not undergo any significant metabolic conversion. These results are concordant with previous work, showing that ACNs with a disaccharide as sugar moiety (such as rutinoides) are less readily metabolised and appear in their intact forms in urine (8). The lower peak height of the rutinoides in plasma than those in dietary BC may indicate that absorption of these compounds is less efficient than those of the corresponding glucosides (**1** and **3**). However, Matsumoto *et al.* (24) reported the metabolism of the rutinoides **2** *in vivo*.

The present study showed no significant effect of an additional food matrix or flavonol on the total amount of ACNs absorbed ($AUC_{0-8h} = 0.49-0.61 \mu\text{g}^* \text{h/mL}$) over the period of 8 h. A previous study investigating the absorption of ACNs from red wine and red grape juice in humans reported an absorption (AUC) of 0.1 and 0.17 $\mu\text{g}^* \text{h/mL}$ with an administered amount of ~4.1 mg/kg BW (34). These different findings may indicate species-specific differences in ACN absorption. However, the two studies have used different ACN sources with different ACN profiles, which may have resulted in dissimilar absorption rates.

In our study ACNs were absorbed more promptly when only dissolved in water ($t_{\text{max}} = 2$ h; diet A) compared to when mixed with milk and a wheat-based cereal ($t_{\text{max}} = 4$ h; diets B and C). The result is similar to that of a previous study in rats in which we found addition of oatmeal to dietary ACNs delayed ACN absorption ($t_{\text{max}} = 1$ h) compared to when the ACNs were administered in water alone ($t_{\text{max}} = 0.25$ h) (unpublished results). A similar observation has been made in humans by Nielsen *et al.* (6) following the ingestion of a rice cake with BC juice.

The significantly lower plasma ACN concentrations at 8 h in the group that was fed diet A, compared with that fed diet B and C, may result from more prompt absorption with consequent more rapid excretion. Thus, the intake of additional food has an effect on ACN absorption and should therefore be taken into account when investigating ACN bioavailability.

We found no inhibitory effect of rutin on the absorption of ACNs in the present study. This result was surprising as we had previously demonstrated *in vitro* that the flavonol quercetin-3-glucoside strongly inhibits absorption of **3** (30). This finding may have resulted

from weaker competition between the BC ACNs and rutin absorption *in vivo*, compared to that observed between ACNs and quercetin-3-glucoside *in vitro*, due to the different sugar moieties of rutin and quercetin-3-glucoside. However, these results highlight important differences between *in vitro* findings and actual effects of dietary components on human health *in vivo*.

Although there are numerous published methods for measuring total antioxidant capacity *in vitro*, there is a lack of a validated assay that can adequately measure 'antioxidant capacity' for all foods and biological samples (35). To measure the antioxidant capacity of pig plasma we selected two different assays, which assess either the radical-scavenging (hydrogen atom transfer, ORAC), or -reducing (electron transfer, FRAP) capacity of the biological fluid under investigation (35).

The ORAC assay, developed by Cao et al. (36) is an assay where an added antioxidant competes with a substrate (fluorescein) for the radicals generated by thermal decomposition of azo-compounds, like 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH), and inhibits or retards the substrate oxidation. This assay uses a biologically relevant radical source and is the only method that combines both inhibition time and degree of inhibition into a single quantity (36, 37).

The FRAP assay, developed by Benzie and Strain (33), measures the ferric-to-ferrous reduction of iron by antioxidants. In this assay the probe itself is an oxidant that abstracts an electron from the antioxidant, causing colour changes of the probe, which is proportional to the antioxidant concentration (35).

We did not find any significant increase in plasma antioxidant capacity measured by ORAC and NP-ORAC following the administration of any of the diets. In fact, plasma antioxidant capacity significantly decreased after administration of diet B. However, the assessment of plasma antioxidant capacity by FRAP yielded different results. After an initial decrease in plasma antioxidant capacity, all plasma FRAP levels increased significantly between 4 and 8 h. The interpretation of plasma antioxidant capacity depends on the particular antioxidant parameter (hydrogen atom or electron transfer) being measured and the conditions of the assay (38). Since the two assays, ORAC and FRAP, were selected to measure different aspects of 'antioxidant capacity' it is not surprising that different results were found. Several studies also report that different methods for antioxidant capacity may give conflicting results for plasma antioxidant capacity. For

example, Mazza *et al.* (28) found that antioxidant capacity increased in humans following the consumption of a high-fat meal with a freeze-dried blueberry powder when measured by the ORAC assay but did not increase when measured by TEAC (Trolox equivalent antioxidant capacity), an assay based on electron transfer. The fact that plasma FRAP increased at a time when the concentrations of plasma ACNs were decreasing may indicate that the fluctuations in FRAP values were not directly related to the detected ACNs but variation in the concentration of metabolites of ACNs, which were not detected in plasma samples under the present conditions. It has been suggested that a significant proportion of dietary ACNs may undergo metabolism and that the metabolites are responsible for many of the reported health effects (39). Lotito *et al.* (40) on the other hand, have shown that the increase of plasma antioxidant capacity measured as FRAP in humans after apple consumption was mainly due to the metabolic effect of fructose, not apple-derived flavonoids. However, the fructose content of the BC material in the present study was 0.03%, considerable lower than the fructose content (6%) of apples and other fruits (41). A recent review concluded that increased plasma antioxidant capacity in humans after consumption of flavonoid-rich food is not caused by the flavonoids themselves, but is likely the consequence of increased uric acid levels (42). Therefore the consistent increase of plasma FRAP in the present study may be due to potential sources of endogenous urate production, such as fructose, sucrose, sorbitol, and lactate in all three diets (42).

In conclusion, our results have shown that the addition of other food components to a dietary source of ACNs (BC) delayed the absorption profile but did not decrease the total absorption of ACNs. Furthermore, we found a delayed increase of antioxidant capacity as measured by FRAP in plasma from all three dietary groups, which was probably not due to dietary ACNs, but possibly due to their metabolites or an increased urate concentration induced by other food components in the diets. Further studies on the effects of food matrix on ACN absorption and antioxidant status are necessary to understand the relevant contribution of ACNs in a complete healthy diet for humans.

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ABBREVIATIONS USED

AAPH, 2,2'-Azobis(2-amidinopropane) dihydrochloride; ACNs, anthocyanins; BC, blackcurrant; FRAP, ferric reducing ability of plasma/antioxidant power; MBWT, metabolic body weight; ORAC, oxygen radical absorbance capacity; PDA, photodiode array; TCA, trichloric acid; TE, trolox equivalents; TPTZ, 2,4,6-Tripyridyl-s-Triazine.

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CHAPTER 6

BERRY FRUIT AND OXIDATIVE STRESS A HUMAN INTERVENTION STUDY

In addition to the investigation on anthocyanin absorption and mechanisms thereof, the aim of this thesis was to evaluate antioxidative effects of anthocyanins. The following chapter represents my contribution to a human intervention study, which was conducted at Massey University, Palmerston North, New Zealand and funded jointly by the New Zealand Foundation for Research Science and Technology and a number of berry fruit industry organisations. My role involved the investigation of the effects of berry fruit juice consumption on oxidative DNA damage, as measured by the comet assay.

ABSTRACT

Polyphenolic compounds including flavonoids, and anthocyanins (ACNs) are potent antioxidants *in vitro*. Some evidence supports the view that the antioxidant properties of fruit and vegetable phytochemicals may be responsible for their proposed health-related effects. Oxidative damage to DNA, lipid and protein increases with age and is believed to contribute to the progression of some cancers and cardiovascular disease. The human intervention study was undertaken to determine if daily supplementation with either a blackcurrant or Boysenberry drink could improve oxidative stress in an elderly population. The study design used was a fully blinded parallel intervention with a placebo control. Treatments were 1) synthetic drink (placebo); 2) blackcurrant; 3) Boysenberry. The study contained 51 participants and the intervention period lasted for 12 weeks. The placebo control was formulated from synthetic colours and flavours, and had essentially zero antioxidant capacity and no ACNs. The blackcurrant drink was formulated as 20% single strength juice fortified with Currantex 20[®] blackcurrant powder, containing approximately 500 mg ACNs/serve. The Boysenberry drink was formulated as 100% single strength juice, containing approximately 250 mg ACNs/serve. A number of parameters of oxidative stress, including lipid oxidation, protein oxidation, as well as DNA oxidation and DNA strand break were measured at baseline, 6 weeks and 12 weeks after commencement of the intervention. In addition, the antioxidant capacity in plasma was analysed (ORAC_{FL}). Several measures of oxidative stress improved during the study. Oxidised plasma lipids and proteins significantly decreased over the 12-week period, while the antioxidant capacity significantly increased. This improvement occurred equally in the two berry fruit treatments and in the placebo control. The origin of this improvement in oxidative stress is unknown but may have resulted from changes in lifestyle (e.g. diet) as study participants became more aware of nutrition and health. However, changes in some measures (ORAC_{FL} and plasma lipid oxidation) show that both the Boysenberry and blackcurrant drinks reduced oxidative stress.

INTRODUCTION

There is increasing evidence that the consumption of fruit and vegetables promotes good health by providing protection against various degenerative diseases such as

cardiovascular disease, some cancers and the onset of dementia (1-5). Fruits and vegetables contain a range of phytochemicals that may act individually or in concert to produce disease protective effects (6). In fact, it has been suggested that the combination of phytochemicals in fruits and vegetables is critical to their powerful antioxidant and anticancer activity (7, 8). Among the compounds thought to act in this way are carotenoids, vitamins, folate, potassium, iron, calcium, selenium and polyphenols, including flavonoids and anthocyanins (ACNs). The actual constituents responsible for these beneficial effects are not known and the mechanisms are not well understood. However, some evidence supports the view that the antioxidant properties of fruit and vegetable phytochemicals may be responsible for some of the disease protective effects, since oxidative damage to key cellular components is linked with cancer, and cardiovascular disease (9, 10).

The cells of the human body are continuously attacked by both exogenous and endogenous reactive oxygen species (ROS) (11), including superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^\bullet), and singlet oxygen (1O_2), which are generated as by-products of normal cell aerobic respiration that is essential to life (12). Cells of the human body have developed a complex network of antioxidant defence systems to counteract the generation of ROS and to protect against the oxidation of macromolecules by scavenging ROS (13). However, increased levels of these ROS or free radicals may overwhelm these defence mechanisms generating oxidative stress, which may impair metabolism, cause oxidative damage to essential cellular components such as lipids, proteins and nucleic acids (DNA), and eventually result in cell death (14, 15). Oxidatively damaged DNA is believed to contribute to the progression of cancers (9) whereas development and progress of atherosclerosis results from lipid peroxidation (10). Furthermore, oxidative stress has been associated with the development of neuronal degeneration such as Alzheimer's (16) and Parkinson's disease (17), as well as being involved in the process of aging (18).

Cell culture systems and animal models have provided a wealth of information on the biological effects of phytochemicals from fruits and vegetables and on the mechanisms by which diets high in fruits and vegetables may reduce the risk of chronic diseases (2, 19, 20). Polyphenols, the major phytochemicals in fruits and vegetables, function as antioxidants, scavenging free radicals (21), inhibiting or activating enzymes, or operating as metal chelators (22, 23), thus preventing damage to lipids, proteins and DNA. Vitamin C, E, and

carotenoids are thought to be responsible for most of the antioxidant activity in foods (24, 25). Previous studies with antioxidant-rich extracts of blackcurrant and Boysenberry showed that these berry fruit have the ability to reduce oxidative stress in *in vitro* cell experimental systems (26-28). These experiments show that both Boysenberry and blackcurrant polyphenolics behave as a biological antioxidant by reducing oxidative stress. Red and dark coloured fruits and berries contain large quantities of ACNs. A number of studies have shown that ACNs act as powerful antioxidants (29) *in vitro* (15, 30-33) and *in vivo* (34-37). However, only a few human trials have been conducted to investigate the specific effects of ACNs on oxidative DNA damage (11, 38-41). The single cell gel electrophoresis assay (comet assay), has been used successfully for evaluating DNA damage, and has been suggested as an excellent technique for use with biological markers in the detection, monitoring, and prognosis of chronic degenerative diseases, including certain types of cancer (42).

Since various degenerative diseases, such as cardiovascular disease and cancer appear to be related to oxidative damage, this human intervention study was undertaken to determine if regular consumption of either a blackcurrant or Boysenberry drink would enhance the antioxidative status in an elderly population. In this study, oxidative damage to lipid (plasma malondialdehyde), protein (carbonylated plasma protein), and DNA (urinary excretion of 8-oxo-deoxyguanosine, 8-OHdG; DNA strand breaks, comet assay), were selected as measures of oxidative stress. In addition, measurements of plasma antioxidant capacity (ORAC_{FL} and non-protein ORAC_{FL}) were also included as indicators of antioxidant status.

MATERIALS AND METHODS

POPULATION AND TREATMENTS

Study Population. The study population was composed of 52 elderly (≥ 65 years), community-dwelling people. **Table 1** shows anthropometric parameters of the subjects. Volunteers were recruited from the Palmerston North area through advertisements in the local media, selection from existing participant databases maintained by the Institute of Food, Nutrition, and Human Health, Massey University (Palmerston North, New Zealand) and direct contact with retirement establishments. Study participants were selected from the volunteers.

Table 1. Anthropometric parameters of the study subjects^a.

Group	Gender	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m ²)
Placebo	M	70 ± 1	174.3 ± 1.9	86.8 ± 4.1	28.6 ± 1.4
	F	72 ± 2	157.5 ± 2.7	68.3 ± 4.8	27.4 ± 1.3
Blackcurrant	M	72 ± 2	175.1 ± 2.3	77.5 ± 2.8	25.3 ± 0.8
	F	75 ± 3	159.2 ± 2.5	67.2 ± 4.2	26.4 ± 1.1
Boysenberry	M	73 ± 2	171.2 ± 1.9	78.0 ± 6.0	26.5 ± 1.6
	F	69 ± 2	161.6 ± 1.8	73.9 ± 5.9	28.2 ± 2.0

^a Values are means ± SEM.

The following criteria were used to exclude potential participants:

- diagnosis of cancer (any form), vascular disease, diabetes mellitus, or mental illness
- smokers
- alcohol abuse
- endocrine disease – myxidemia
- conditions leading to amyloidosis
- anaemia
- malnutrition from malabsorption (B12, B6, folate, iron)
- neurological disorders – multiple sclerolosis, Parkinson's-, Alzheimer's Disease
- renal impairment
- treatment with tranquillisers, antidepressants, anticonvulsants
- impaired immune status – AIDS, multiple myeloma, chronic lymphatic leukaemia
- visual impairment – intact visual fields

To be included in the study participants had to:

- have a BMI between 18 and 34 kg/m²
- be able and willing to provide written informed consent
- have a reliable informant/carer
- have been recommended by their regular General Practitioner to be suitable for the study.

Study Design. Study participants were randomised into three parallel arms: one arm was provided with placebo control drink, one arm received a blackcurrant drink, and the final

arm received a Boysenberry drink. All participants were blinded with respect to the treatment they received. The study continued for 12 weeks with assessments of the selected parameters on oxidative stress at baseline, 6 weeks and 12 weeks.

Non-fasting plasma and urine samples were collected in the mornings at Medlab Central, Palmerston North. The time between consuming the daily drink and collection was not controlled or recorded.

Treatment Drinks. All three drinks were manufactured by Barkers Fruit Processors Ltd. (Geraldine, New Zealand). Following formulation, each drink was pasteurised and packed into 100 mL sachets. These were then frozen at -20°C until used. Each participant was instructed to consume 2 sachets (total 200 mL) of their allocated drink during the day while maintaining their normal diet. The ingredients used to prepare each drink are provided in **Table 2**. The treatments were:

- 1) *Placebo control*: synthetic berry fruit drink (200 mL) containing synthetic colours and flavours, granulated food grade sugar and citric acid to simulate a generic berry fruit drink.
- 2) *Blackcurrant drink*: freshly produced by reconstitution of blackcurrant juice concentrate (variety 'Ben Ard') commercially manufactured by the New Zealand blackcurrant Co-Operative Ltd. and diluted to simulate 20% pure single strength blackcurrant juice. The drink contained added granulated food grade sugar and a blackcurrant powdered concentrate (Currantex 20[®], manufactured and marketed by Just the Berries Ltd., Palmerston North, New Zealand). Currantex 20[®] was added to adjust the ACN concentration of the drink to approximately 500 mg/200 mL.
- 3) *Boysenberry drink*: freshly produced by reconstitution of a berry fruit Export New Zealand Ltd. juice concentrate and diluted to simulate a pure single-strength Boysenberry juice.

Prior to manufacture and packaging, test formulations were prepared and the palatability and acceptability determined by a panel of eight study participants. All the formulations were found to be acceptable as follows:

- 1) *Placebo control*: No one identified this drink as a synthetic but many thought it was a little too sweet. The placebo is clear and has a much lighter colour than the other two drinks but was believed to be real fruit by all the participants.
- 2) *Blackcurrant drink*: Two formulations were prepared at 14° and 11.6° Brix (essentially percentage sugar). Both were well liked by the 8 participants and they were described as smooth drinks. A slight musty taste was apparent that probably was derived from the Currantex 20[®]. During preparation of these test drinks there were some problems in dissolving the Currantex 20[®] that were not present during the manufacture of the final test drinks.
- 3) *Boysenberry drink*: Seven of the eight participants found this drink acceptable and were prepared to consume 200 ml per day. A number expressed a preference for the Boysenberry drink although it has a 'tart' flavour compared with the other drinks.

Table 2. Ingredients and specifications of the drinks used as treatments and the placebo control.

Ingredients	Placebo % w/w	Blackcurrant % w/w	Boysenberry % w/w
Water	87.9	85.8	84.7
Sugar	11.6	9.7	
Citric acid anhydrous	0.40		
Currantex 20 ^s		1.0	
Boysenberry Conc. N4003			15.3
Blackcurrant Conc. N4037		3.55	
Flavour	0.50		
Ponceau (124)	0.15		
Colour (123+132)	0.07		
Total	99.9	100.0	100.0
Target Specifications			
Boysenberry Juice (ssj ^a) %w/w			100.00
Blackcurrant Juice (ssj ^a) %w/w		20.00	
°Brix (calculated)	11.60	12.00	9.98
Specific Gravity (g/mL)	1.048	1.048	1.040
Acidity % (eq citric acid anhydrous)	0.40	0.38	

^a ssj – single strength juice.

* Brix – essentially percentage sugar.

Subjects were questioned at the beginning of the study regarding their daily total fruit and berry fruit intake, and a summary is shown in **Figure 1**.

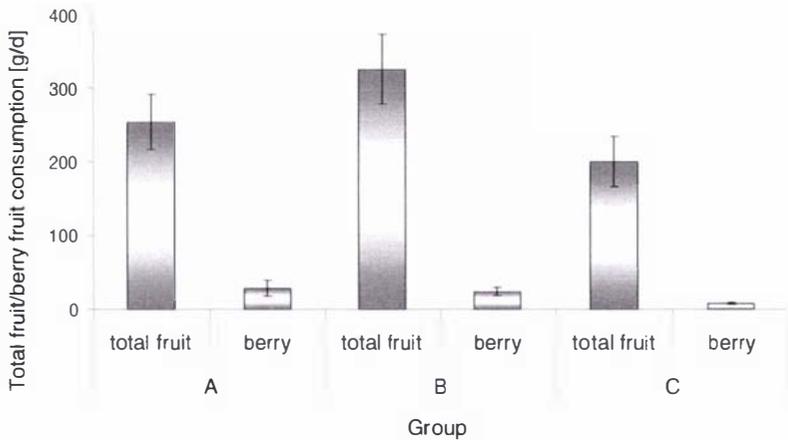


Figure 1. Total fruit and berry fruit consumption (grams per day; average serving size 80 g) of the participants during the 12-week study period.

MEASUREMENTS OF OXIDATIVE STRESS

Lipid oxidation (plasma malondialdehyde). Plasma malondialdehyde (MDA) was measured by a gas chromatograph mass spectrometer (GC-MS) based method (43). Briefly, MDA standards were prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma Inc, Sacramento, CA) at 40°C. Sample plasma (500 μL) was mixed with 350 μL of 10% trichloroacetic acid to precipitate the protein and extracted with 0.8% butylated hydroxytoluene in hexane. After discarding the upper hexane layer, MDA was derivatised with phenylhydrazine (Sigma Inc, Sacramento, CA) at RT. Derivatised MDA was extracted with hexane, an internal standard (benzophenone) was added and the MDA concentration determined by selected ion monitoring GC-MS (Shimadzu GCMS-QP5050A, Shimadzu Corporation, Kyoto, Japan; equipped with J & W Scientific DB™ – 5MS 30 m, 0.25 mm ID, 0.25 μm film thickness). MDA and the internal standard were recorded at mass to charge ratios (m/z) of 76.90, 104.85, 143.90 and 182.05, concentrations were calculated from a calibration curve (0-450 ng/mL) and presented as ng/mL of plasma.

Protein oxidation (protein carbonylation). Plasma protein carbonyl content was measured by an enzyme-linked immunosorbent assay (ELISA) supplied by Zenith Technology (Dunedin, New Zealand). The assay was performed according to the instructions of the manufacturer.

DNA oxidation (8-OHdG). 8-OHdG was measured in urine using an ELISA. '8-OHdG Check' commercial kits were supplied by the Genox Corporation (Japan Institute for the Control of Aging, Fukuroi City, Japan). 8-OHdG is a result of oxidation of bases in nuclear DNA. The oxidatively damaged bases are excised by DNA repair mechanisms and excreted in the urine. The concentration of the 8-OHdG in urine is a result of both the level of oxidative stress and the rate at which the damaged bases are repaired. The ELISA used is a competitive *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of 8-OHdG in urine, plasma and serum

Antioxidant capacity (ORAC_{FL}). Plasma ORAC_{FL} was measured using a method similar to that described by Ou *et al.* (44). Plasma samples were diluted 125-fold with 75 mmol/L potassium phosphate buffer, pH 7.4. For non-protein ORAC_{FL} (NP-ORAC_{FL}) analysis, plasma proteins were precipitated with an equal volume of 0.3% perchloric acid, centrifuged at 12,000 x g and the supernatant diluted 6.67-fold with 75 mmol/L potassium phosphate buffer, pH 7.4. Standard curves were prepared using 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid, a water-soluble analogue of Vitamin E, at 50-200 $\mu\text{mol/L}$. Each sample or standard was analysed in triplicate in a 96-well format. Plates were prepared by adding 10 μL of sample and 160 μL of fluorescein (4.8×10^{-8} mol/L) to each well. 2,2'-Azobis(2-amidinopropane) dihydrochloride (25 μL , 0.1 mol/L) was added to each well and the fluorescence measured every minute for 90 min. The relative fluorescence for each standard was calculated, to give the area under the curve, and a standard curve was plotted.

DNA oxidation (comet assay). DNA damage in lymphocytes was measured by the alkaline comet assay according to Singh *et al.* (45) with little modification.

Materials and Solutions. Dimethyl sulfoxide (DMSO), (ethylenedinitrilo) tetraacetic acid disodium salt (Na₂ EDTA), ethidium bromide, HISTOPAQUE[®] 1077, and tris[hydroxymethyl] aminomethane hydrochloride (Trizma hydrochloride) were purchased from Sigma Aldrich, Australia. Triton[®] X-100 was obtained from Riedel-de Haen AG, Germany. Low melting agarose (UltraPure[™] low melting point agarose, LMA), and normal melting agarose (UltraPure[™] Agarose, NMA) were both obtained from Invitrogen, New Zealand. Pre-cleaned microscope slides (26 x 76 mm) were purchased from Lomb

Scientific, Australia, coverslips (Esco 22 x 22 and 22 x 50 mm) from Biolab Scientific, Australia. The electrophoresis tank was obtained from Bio-Rad, New Zealand.

Phosphate buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 , 1.15 g Na_2HPO_4 , dissolved in 1 L H_2O , pH 7.4); Lysis solution (Stock: 146.1 g NaCl, 37.2 g Na_2EDTA , 1.2 g Trizma Base, dissolved in 1 L H_2O , pH 10; Assay-solution: 89% Stock, 1% Triton-X, 10% DMSO); Electrophoresis buffer (Stock A: 10 M NaOH; Stock B: 200 mM Na_2EDTA ; Assay-solution: 96.5% H_2O , 3% Stock A, 0.5% Stock B); Neutralising buffer (Stock: 4 M Trizma Base; Assay-solution: 90% H_2O , 10% Stock, pH 7.5); Staining solution (Stock: 10 mg ethidium bromide/50 mL; Assay-solution: 1:10 dilution of Stock with H_2O).

Lymphocyte isolation. Lymphocytes were isolated by mixing 3 mL whole blood with 3 mL phosphate buffered saline (PBS). A layer of HISTOPAQUE[®] 1077 (3 mL) was subsequently carefully placed underneath the blood-PBS mixture, and the sample centrifuged at 2030 x g for 30 min at RT. Approximately 2 mL of the lymphocyte-containing 'buffy coat' was mixed with PBS (8 mL) and centrifuged at 1200 x g for 10 min at RT, to remove any remaining HISTOPAQUE[®] 1077. The resulting cell pellet was subsequently resuspended in 1 mL PBS, and the cell number adjusted to $2 \times 10^6/\text{mL}$ (cell suspension).

Comet assay. Prior to performing the comet assay, microscope slides were pre-coated with 2 layers. The first one consisted of 20 μL NMA, which was placed on one end of the microscope slide, spread across, and dried at 60°C. As second layer, a line of 85 μL NMA was placed on top of the first layer, covered with a cover slip and placed on an icebox for 10 min to set the agarose. The precoated slides were stored at 4°C in a box with moist tissue until use.

100 μL of the cell suspensions were added to 1 mL PBS without (to measure endogenous DNA damage; controls) and with the addition of hydrogen peroxide (H_2O_2 -induced DNA damage, 100 μM), incubated for 5 min on ice and centrifuged at 1700 x g for 3 min at 4°C. The resulting cell pellet was resuspended in 35 μL LMA, the mixture placed on a precoated microscope slide, covered with a small coverslip, and placed on ice to set. Duplicates were placed on one microscope slide. After removing the small coverslips, a fourth layer of agarose (75 μL LMA) was placed across the slide, covered with a big coverslip, and placed on ice to set.

The in agarose embedded cells were then placed into a lysis solution (4°C, ~1 h) to remove cellular proteins and leave the DNA as ‘nucleoids’. Next, the slides were arranged in a horizontal electrophoresis and covered with electrophoresis buffer (4°C for 20 min, pH > 13) for alkaline treatment, to produce single stranded DNA and to express alkali-labile sites as single strand breaks. After alkali unwinding, the single stranded DNA in the agarose gels was electrophoresed under alkaline conditions (breaks in the DNA molecule disturb its supercoiling, allowing free DNA to migrate towards the anode, producing a ‘comet’). Subsequently, microscope slides were neutralized with neutralising buffer and stained with ethidium bromide.

DNA-damage was visualized by fluorescence microscopy (Zeiss Axiolab Compound Light Microscope, 50 W Short Arc Mercury Lamp Epi-Fluorescence Unit, emissions filter 580-590 nm). Cells with damaged DNA display increased migration of DNA fragments from the nucleus. The length of the migration indicates the amount of DNA damage (45). Quantification of DNA damage was performed using a visual analysis. One hundred comets per sample were classified into one of 5 classes according to the relative intensity of fluorescence in the ‘comet’-tail (0 = no tail; 4 = almost all DNA in tail) with the total score per sample being between 0 and 400 “arbitrary units”.

Statistical analysis. Data are presented as means \pm SEM. The significance of differences was assessed by repeated measures ANOVA for comparison of individual means. Statistical analysis was carried out using SAS System for Windows, version 8. The residuals of each analysis were tested for normality. Differences with $p \leq 0.05$ were considered significant.

RESULTS

Fifty-one of the original 52 participants completed the study. Generally the drink formulations were well tolerated and liked by the participants. A questionnaire administered to the participants at the end of the study showed that 5/12 participants described the Boysenberry drink as unpleasant and 15/16 described the blackcurrant drink as pleasant. Ages ranged from 65-92 years with most participants in the 65-74 age groups. Approximately equal numbers of males and female were enrolled in the study (Table 3). The original intention was to recruit 60 participants, however this target was not achieved.

The phytochemical components of each treatment drink were measured at the start of the study (baseline), at 6 weeks, and at the end of the study (12 weeks, **Table 4**). The composition changed little during the study and no significant differences were detected.

Table 3. Number of participants completing the study according to treatment, gender and age.

		Age	65-69	70-74	75-79	80-84	85-89	90-95	Sum
Placebo	Gender								
	Male		3	5	0	1	0	0	9
	Female		2	3	2	1	0	0	8
	Sum		5	8	2	2	0	0	17
Blackcurrant	Male		4	2	3	1	0	0	10
	Female		2	2	2	0	0	1	7
	Sum		6	4	5	1	0	1	17
Boysenberry	Male		3	2	1	3	0	0	9
	Female		6	1	0	1	0	0	8
	Sum		9	3	1	4	0	0	17
All	Male		10	9	4	5	0	0	28
	Female		10	6	4	2	0	1	23
	Sum		20	15	8	7	0	1	51

Table 4. Phytochemical composition and antioxidant properties of the treatments.

Component	Treatment	Baseline	6 Weeks	12 Weeks
Total anthocyanins (HPLC mg/serve)	Placebo			
	Blackcurrant ^a	742 (557)	741 (556)	754 (565)
	Boysenberry ^b	274	241	235
Total phenols (mg GAE/mL) (Folin C)	Placebo			
	Blackcurrant	4.1	3.9	3.8
	Boysenberry	4.5	3.7	3.8
Vitamin C (mg/serve)	Placebo			
	Blackcurrant	30	34	33
	Boysenberry	n.d.	n.d.	n.d.
Antioxidant Activity				
ORAC (mmol/L TE)	Placebo	n.a.	n.a.	n.a.
	Blackcurrant	88	121	96
	Boysenberry	90	107	101
FRAP (mmol/L TE)	Placebo	n.a.	n.a.	n.a.
	Blackcurrant	23.7	20.9	24.3
	Boysenberry	23.1	25.2	25.3

^acalculated using relative response factors for blackcurrant anthocyanins. Cyanidin-glucoside equivalents in brackets.

^bcalculated as cyanidin-glucoside equivalent.

GAE – gallic acid equivalents.

TE – trolox equivalents.

n.d. – not detected.

n.a. – not analysed.

Results of parameters for oxidative stress are shown in **Figure 2-4** and **Table 5**. The concentration of plasma MDA, the marker for lipid peroxidation, was significantly reduced in all three groups over the 12-week period ($p < 0.001$). Likewise, the concentration of oxidised plasma protein (protein carbonylation) decreased significantly in all three groups over time ($p < 0.01$).

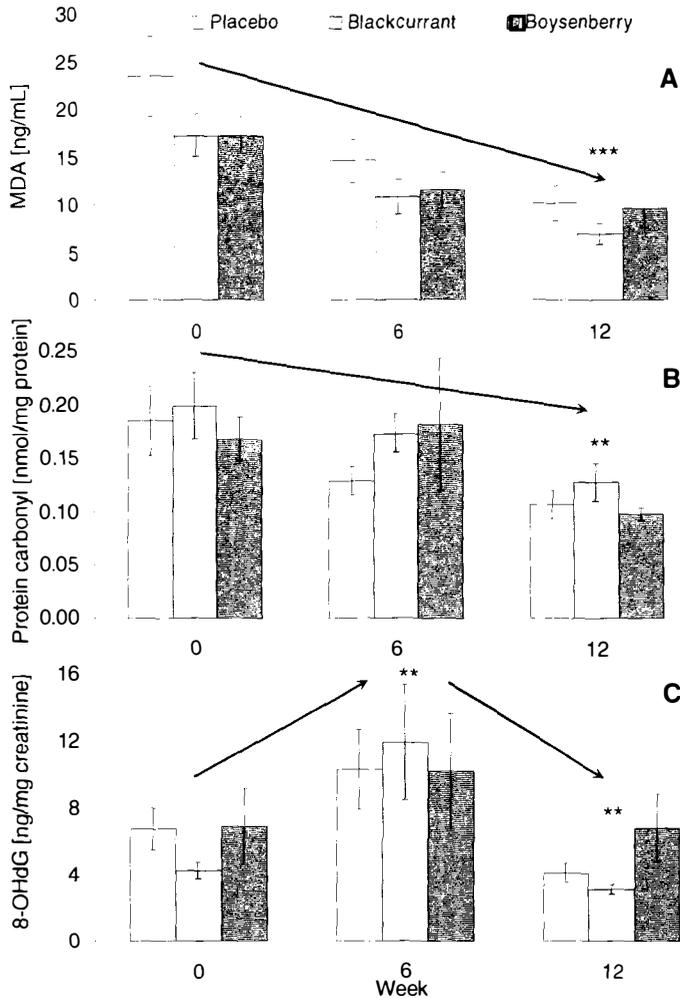


Figure 2. Plasma MDA (A), protein carbonyl (B), and urine 8-OHdG (C) levels over the 12-week study period. Values are means \pm SEM ($n = 17$). Shown is the significant difference of all three groups over time. ** $p < 0.01$; *** $p < 0.001$.

In the case of 8-OHdG, one marker for DNA oxidative damage, an initial increase ($p < 0.01$) at 6 weeks was observed, which was followed by a significant decrease between 6 and 12 weeks ($p < 0.01$) (**Figure 2**). However, no difference was observed between the treatment groups for plasma MDA, protein carbonylation or 8-OHdG. Results for the antioxidant capacity (ORAC_{FL} and NP-ORAC_{FL}) are shown in **Figure 3**. A significant increase of plasma antioxidant capacity ($p < 0.001$) was found over time for both biomarkers with all three groups. However, the NP-ORAC_{FL} showed an initial decrease at 6 weeks ($p < 0.01$), before it increased after 12 weeks ($p < 0.001$). No differences were observed between the treatments for the total ORAC_{FL}, but a slight difference ($p = 0.049$) between the two treatment juices was observed for the NP-ORAC_{FL}.

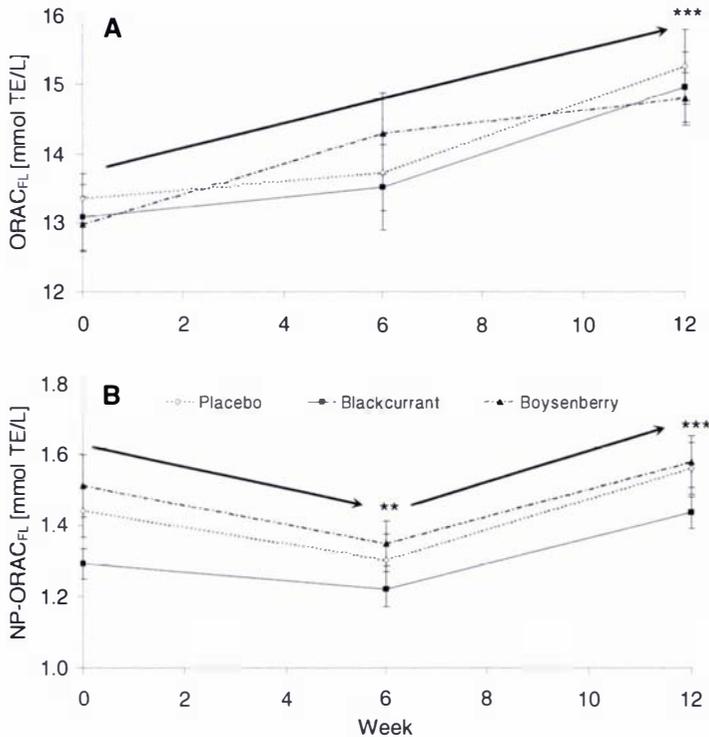


Figure 3. Plasma ORAC_{FL} (A), and NP-ORAC_{FL} (B) over the 12-week study period. Values are means \pm SEM ($n = 17$). Shown is the significant difference compared to baseline. ** $p < 0.01$; *** $p < 0.001$.

Regarding DNA strand breaks (endogenous and H₂O₂-induced), there were no significant differences between the treatments at baseline, 6 weeks or 12 weeks (**Table 5**). Looking at each treatment over time, a slight decrease of endogenous DNA damage in the placebo group was observed (**Figure 4 A**). Both groups consuming the treatment juices showed an initial increase of endogenous DNA damage at 6 weeks, which decreased back to baseline after 12 weeks (**Figure 4 B, C**).

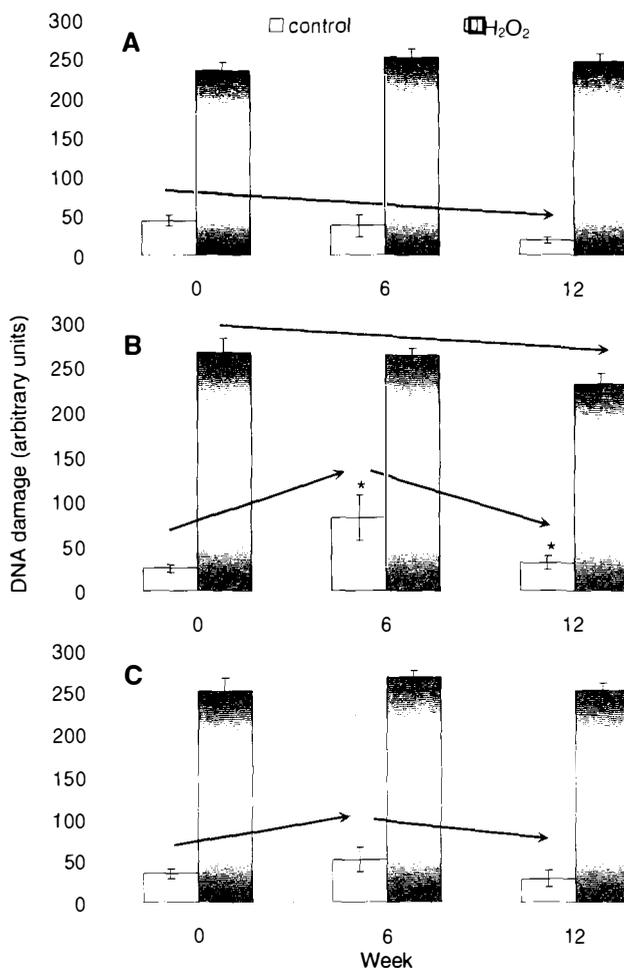


Figure 4. Endogenous (control) and H₂O₂-induced DNA damage in isolated lymphocytes, after consumption of the placebo (A), blackcurrant (B), or Boysenberry (C) juice, over the 12-week study period. Values are means \pm SEM ($n = 17$). Shown is the significant difference over time. * $p < 0.05$.

In case of H₂O₂-induced DNA damage, a trend for protection (NS) was found in the blackcurrant group (**Figure 4 B**). However, no effect was found in the Boysenberry group.

Table 5 represents a summary of the statistical analysis. Besides a slight difference between the treatment juices for the NP-ORAC_{FL}, no statistically significant effect of the treatments on oxidative stress was found for any of the measured parameter. However, there was a reduction in measures of oxidative stress over time course of the study. All but one parameter of oxidative damage (H₂O₂ induced DNA damage) showed a significant decrease of oxidative stress related measures over time.

Table 5. Summary of differences between treatments, time or time and treatments on parameters of oxidative stress. Differences with $p \leq 0.05$ were considered significant.

Parameter	Treatment Differences ^a	Time Differences ^b	Treatment/time Differences ^c
Lipid peroxidation (malondialdehyde)	0.105	<0.001	0.758
Protein oxidation (protein carbonyls)	0.623	<0.01	0.675
Oxidised DNA (8-OHdG)	0.672	<0.001	0.584
Antioxidant capacity			
Total ORAC _{FL}	0.797	<0.001	0.729
NP-ORAC _{FL}	0.049	<0.001	0.848
DNA strand breaks			
(DNA control)	0.389	<0.01	0.079
(DNA H ₂ O ₂)	0.375	0.101	0.232

^asignificant difference between treatments.

^bsignificant difference during the time course of the study.

^csignificant difference between treatments during the time course of the study.

DISCUSSION

During the past 20 years, understanding of the role of nutrition in health promotion and disease prevention has improved (11). Many flavonoids in fruit and vegetables have been shown *in vitro* to have strong antioxidant properties. However, little is known from human experimental studies about the physiological effects of phytochemicals. This study attempted to determine if daily supplementation with either a blackcurrant or Boysenberry drink by an elderly population could improve the level of oxidative stress.

The antioxidant capacities of dietary antioxidants are often determined by *in vitro* measurements, such as the oxygen radical absorbance capacity (ORAC) (46-48) and ferric-reducing antioxidant power (FRAP) assays (49, 50). However, although these measures are valid indicators of the antioxidant potential of a dietary substance (51), *in vitro* assays of

this type do not provide evidence that a specific substance acts as an *in vivo* antioxidant when consumed. *In vivo* measurement of antioxidant capacity requires confirmation that oxidative stress is actually reduced and such studies are much less common.

In the present study 6 different measures of oxidative stress and damage were investigated: plasma MDA, protein carbonyls, ORAC_{FL}, NP-ORAC_{FL}, lymphocyte DNA strand breaks (comet assay), and 8-OHdG. A slight difference was observed between the blackcurrant and Boysenberry treatment for the NP-ORAC_{FL}. However, no statistically significant differences between the treatments in other measures of oxidative stress were found, but there was a reduction of oxidative stress over the time course of the study. The reason for this trend over time is unclear but the changes in each measure reflect an improved antioxidant status. For example, both plasma total ORAC_{FL} and NP-ORAC_{FL} significantly increased ($p < 0.001$), whereas the levels of oxidised protein and lipid both significantly decreased ($p < 0.01$, and $p < 0.001$, respectively) in the plasma. Thus, over the course of the study there was a reduction in oxidative stress. This may have been due to seasonal variation in oxidative stress attributed to factors such as environment and diet, but such effects have not been reported. Previous studies of this type have used a randomised, cross-over design rather than the parallel, placebo-controlled design used for this study and such effects would not have been detected. Alternatively enrolment in the study may have changed awareness and encouraged a change in dietary, or other lifestyle patterns (e.g. exercise), which had a greater effect than the effect induced by the treatments.

A number of other studies have reported reductions of oxidative stress in humans and animals following the consumption of dietary antioxidants. For example a highly enriched ACN extract of *Abies koreana* improved plasma antioxidant capacity in Vitamin-E deficient rats and reduced the concentration of hydroperoxides and 8-OHdG in the liver (36). When oxidative stress was induced in rats by treatment with paraquat, ACNs isolated from eggplant and red cabbage were able to provide some protection and reduced the oxidative damage as measured by several indices (52, 53). Furthermore ACN-containing berry fruit incorporated into juice mixtures increased both antioxidant status (plasma TEAC – trolox equivalent antioxidant capacity) and reduced oxidative damage (plasma MDA) in human studies (39, 54). In contrast, a study in which human subjects consumed 750, 1000 or 1500 mL of a blackcurrant/apple juice combination, found no change in plasma antioxidant status, a decrease in plasma MDA, and an increase in plasma protein oxidation

(55, 56). A recent intervention study in which an elderly population was given a phenolic-rich dessert composed of grape, cherry, blackberry, blackcurrant and raspberry for two weeks, found no changes in antioxidant status (56).

Regarding DNA damage, there have been several *in vivo* and *in vitro* studies, which investigated the effect of polyphenols on oxidative DNA damage. Moller *et al.* (38) have shown that even a large amount of dietary antioxidants (397 mg blackcurrant ACNs/d, or 365 mg ACN drink/d, 3 weeks), did not decrease endogenous oxidative DNA damage in healthy humans. In another study, involving the supplementation of a polyphenol-rich juice (~230 mg polyphenols/d, 2 weeks), the juice intervention had no effect on endogenous DNA strand breaks, but significantly reduced oxidative DNA damage in lymphocytes (39). A further study found the consumption of grape juice (480 mL/d for 8 weeks) to be associated with decreased levels of endogenous DNA damage (11). However, the last two studies did not include a control group or treatment, which hampers the reliability of their results. Furthermore, Pool-Zobel *et al.* (57) have shown that, although ACNs are potent antioxidants, they do not decrease endogenous oxidative DNA damage *in vitro*. In the present study we observed a slight decrease of endogenous DNA damage in the placebo group (weeks 0-12) and in both treatment groups (weeks 6-12). However, we did not detect any effect on H₂O₂-induced DNA damage. In contrast, *in vitro* studies using flavonoids have shown that quercetin and myricetin protect against H₂O₂-induced DNA of human colon cells and lymphocytes (58, 59). An *in vivo* study investigating DNA protective effects of flavonoid glycosides from onions reported an increased resistance of lymphocyte DNA damage, as well as a significant decrease of 8-OHdG (60). On the other hand, two *in vivo* studies on ACN and oxidative damage have both shown no effect on 8-OHdG, endogenous, or H₂O₂-induced DNA damage (40, 41). These previous studies indicate that flavonoids may have an effect on DNA damage, whereas ACNs have not.

In summary, this study in which an elderly population was given a blackcurrant, Boysenberry, or placebo drink, has shown a positive effect on oxidative stress. Five out of six measures of antioxidant status showed a reduction in oxidative damages during the 12-week period of the study. However, the observed improvement of oxidative stress was found equally in both berry juice treatment groups and in the placebo control, which might have resulted from changes in lifestyle and nutrition while participating in the study.

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ABBREVIATIONS USED

ACNs, anthocyanins; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; ELISA, enzyme-linked immunosorbent assay; H₂O₂, hydrogen peroxide; LMA, low melting agarose; MDA, malondialdehyde; NMA, normal melting agarose; ORAC_{FL}, oxygen radical absorbance capacity (FL, fluorescein); PBS, phosphate buffered saline; ROS, reactive oxygen species.

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CHAPTER 7

GENERAL DISCUSSION

Anthocyanins (ACNs) are one of the most abundant phenolic compounds, widely distributed in fruits and vegetables, and are ingested by humans in substantial amounts with the daily diet. Over the last decade, there has been an increased interest in the health effects of ACNs as they exhibit a range of *in vitro* biological activities. Many flavonoids that are found in fruits and vegetables have been shown to have antioxidant properties, both *in vitro* and *in vivo*. However, for ACNs to act as health promoting compounds *in vivo*, they first have to be successfully absorbed from the ingested food, and be present in sufficient concentrations within the human body. Details regarding the absorption of ACNs, including the absorption site within the gastrointestinal tract (GIT) are not fully understood. To date, all studies on ACN absorption, metabolism, and excretion agree on the low apparent bioavailability of these compounds. Further knowledge on the absorption mechanism could make it possible to enhance ACN absorption and subsequently improve proposed health-related effects for humans.

This thesis presents further investigations on the details of ACN absorption *in vitro* and *in vivo* (Chapter 2-5), as well as additional information about the antioxidant activity of ACNs (Chapter 5, 6).

A number of studies on ACN absorption have been conducted and the occurrence of ACNs and their metabolites has been investigated in body fluids, such as blood plasma, and urine. However, the fate of ACNs within the GIT has not been examined yet. It has been suggested, due to the rapid appearance of ACNs in the blood circulation, that ACNs are absorbed from the stomach (1, 2), including the involvement of an organic anion membrane carrier (bilitranslocase) expressed in epithelial cells of the gastric mucosa (3). It has further been proposed, that flavonoids, which reach the large intestine, are hydrolysed by the action of β -glycosidase in anaerobic enterobacteria to release the aglycone (4), which might subsequently be absorbed. However, no data regarding the absorption of ACNs within the small and large intestine of the GIT has been available so far. *In vitro* investigations, using Ussing chambers mounted with several regions of mouse GIT tissues (duodenum, jejunum, ileum and colon), clearly showed the jejunum to be a strong absorption site for ACNs in mice (Chapter 2). However, the difference regarding ACN absorption between the selected intestinal regions may have been due to a pH effect rather than tissue and needs to be further investigated. As most studies on ACN absorption administered the compounds as an aqueous solution via stomach intubation, the fast appearance of ACNs in the blood is not

necessarily a result of ACN absorption from the stomach alone, but may be because of the rapid transit of the administered dose into the jejunum with its subsequent contribution to ACN absorption. No absorption was found with the ileal or colon tissue, indicating that no ACN absorption occurs in the lower small and the large intestine of mice (Chapter 2). The results of the *in vitro* study in Chapter 2 were supported with a further *in vivo* study, where the jejunum was found to be a strong absorption site for ACNs in rats (Chapter 4). The maximum plasma concentrations of ACNs occurred at the same time as maximum concentrations were measured in the jejunal tissues. In addition, the ratio of the ACN concentration in tissue to digesta samples was significantly higher in the jejunum, compared with the stomach or colon, indicating a more efficient absorption mechanism of this particular intestinal region. However, substantial amounts of ACNs were found in the rats' stomach tissue, supporting the contribution of ACN absorption by the stomach, as shown by Talavera et al. (2). The fact that ACNs are mainly absorbed from one particular region of the GIT indicates the involvement of a specific transport mechanism, rather than passive diffusion of the compounds throughout the entire GIT. It has been proposed that the sodium-dependent glucose transporter (SGLT1) contributes to the absorption of flavonoids, such as the flavonol quercetin-3-glucoside (Q3G) (5). However, *in vitro* experiments have shown that ACNs are not necessarily a substrate for this respective transport mechanism (Chapter 3). As with other flavonoids, it is not known yet, if ACNs are absorbed in their intact forms as glycosides, or subsequent to hydrolysis to the respective aglycone. Lactase phloridzin hydrolase (LPH) is a mammalian β -glucosidase, present in the brush border of the small intestine, which has been reported to be capable of hydrolysing various flavonol and isoflavone glucosides (6). Therefore, ACNs may be hydrolysed by LPH and the aglycone subsequently absorbed from the GIT. Yet, similar with the SGLT1, no interaction was found between ACNs and the LPH in *in vitro* experiments using Ussing chambers (Chapter 3). These results indicate that ACNs are neither hydrolysed to their respective aglycone by LPH, nor absorbed by the SGLT1. A strong inhibition of ACN absorption, induced by the flavonol Q3G, was found with a simultaneous absorption of the flavonol itself instead (Chapter 3). However, this inhibition may have been due to an effect of the vehicle DMSO and not Q3G. This first report of inhibition of ACN absorption by another flavonoid group *in vitro* provides important information in understanding details of ACN bioavailability. The simultaneous presence of ACNs and flavonols in the GIT lumen

may result in a competition for a shared absorption mechanism, which has a clear preference for the flavonols. ACNs are rarely ingested on their own, but rather in a mixture of flavonoids as they naturally occur in fruits and vegetables. For example, the ratio between flavonols and ACNs in apples has been shown to be about 10:1 (7). In addition, a number of commercial fruit juices are apple-based. It is therefore of importance to take interactions between different flavonoids into account when investigating ACN bioavailability. Based on the results of Chapter 3, the effect of a simultaneous intake of a flavonoid source on ACN absorption *in vivo* was investigated using the pig model (Chapter 5). Compared with the *in vitro* study however, the simultaneous ingestion of the flavonol rutin (quercetin rutinoside) did not show any inhibitory effect on ACN absorption *in vivo* (Chapter 5). The reason why the *in vivo* study results did not support the *in vitro* results could be because of the different flavonols administered in the studies (Q3G, Chapter 3; quercetin rutinoside, Chapter 5), which were chosen for availability reasons.

Additionally to interactions between ACNs and other flavonoid groups, the simultaneous ingestion of other food sources, such as breakfast cereals, bread and berry fruit jam or fruit based deserts, together with ACNs need to be considered regarding ACN bioavailability. Again, almost every study on ACN absorption administered purified ACNs in aqueous solution, without taking into account possible interactions between the compound and other food ingredients ingested with a normal healthy diet. The aim of the present thesis was to investigate berry fruit ACNs in human nutrition, and to examine the effect of a food matrix on ACN absorption. Two *in vivo* studies were conducted to investigate the effect of a food matrix on ACN absorption (Chapter 4, 5). When ACNs from blackcurrants were administered to rats by stomach intubation with or without the addition of oatmeal, the food matrix delayed the absorption profile of the ACNs in plasma, and urinal excretions were slower compared with the aqueous administration form (Chapter 4). However, the administration of ACNs in water resulted in a higher ACN absorption, compared with the ACN/oatmeal combination. A possible reason for that might be an interaction between ACNs and components of the oatmeal, such as fibre, depriving the ACNs from absorption in the jejunum. McDougall *et al.* (8) have suggested, that polyphenols may transiently bind to food matrices during digestion, therefore ACN-food interactions should be considered when investigating ACN bioavailability. In addition to the absorption profile, the rat study in Chapter 4 has shown that ACNs are absorbed in their

intact forms and are metabolised mainly by methylation and glucuronidation, as shown by metabolites excreted in the urine. These findings are concordant with most previous ACN absorption studies. However, the food matrix did not have an effect on ACN metabolism, as no difference was found in plasma and urine compounds between treatments. A further study investigated the effect of a food matrix (wheat-based cereal mix) on ACN absorption in pigs (Chapter 5). Compared to the rats, the treatments were not administered via stomach intubation, but normally eaten by the pigs. Similar to the rat study, the absorption profile of ACNs in the plasma was delayed due to the presence of a food matrix. However, the total amount of ACNs absorbed was not significantly different between the treatments. Also in contrast to the rat study, some of the ACNs appeared as metabolites in plasma samples, indicating possible species-specific differences in ACN absorption and metabolism.

In addition to discovering details about ACN absorption, the aim of the present thesis was to investigate the antioxidative activity of ACNs, which has been shown in a number of previous studies. Some evidence supports the hypothesis that the antioxidant properties of fruits and vegetables may be responsible for some of the disease protective effects, since oxidative damage to key cellular components is linked with cancer, and cardiovascular disease (9, 10). Antioxidants can reduce oxidative molecular and cellular damage by preventing the attack of biomolecules by free radicals or by interrupting the perpetuation of free radical species (11), and therefore play an important role in the prevention of oxidative damage. The daily supplementation of an elderly population with an ACN-containing berry juice over a period of 12 weeks showed a positive effect on several parameters of oxidative stress (Chapter 6). However, the improvement of the oxidative stress was observed in both the placebo and treatment groups, and could be a result from changes in lifestyle and nutrition of the participants during the study. The protective effect of flavonols, such as quercetin, Q3G, myricetin, and kaempferol, on oxidative damage like induced DNA strand breaks has been shown *in vitro* and *in vivo* (12-14). On the other hand, ACNs have been reported to have no effect on oxidative DNA damage *in vitro* (15) or *in vivo* (16, 17). A slight protection of endogenous DNA damage was observed, but the overall conclusion was that berry juice supplementation did not reduce induced DNA damage as measured by the comet assay.

The effect of a simultaneous ingestion of other flavonoids or food sources on the antioxidant activity of ACNs has not been shown yet. The pig study (Chapter 5) also

investigated the effect of the additional intake of a flavonoid source and food source on the antioxidant capacity of plasma. An increase of antioxidant capacity, as measured by the FRAP assay, was observed regardless of the treatments. Nevertheless, this increase only occurred at times, when plasma ACN levels were already decreasing back to baseline levels. These results strongly suggest that antioxidant effects observed after ACN consumption are not related to the original ingested compounds, but to metabolites thereof. However, analysis by the ORAC_{FL} assay showed no effect on antioxidant capacity in pig plasma, which may have been due to the different experimental conditions compared to the FRAP assay. As the ORAC_{FL} assay was run at neutral pH and higher temperatures, ACNs in plasma may have undergone structural changes to compounds with less antioxidant activity, resulting in no response when analysed with the ORAC_{FL} assay.

In conclusion, the jejunum contributes strongly to ACN absorption, and the absorption most likely involves an absorption mechanism, with a preference towards other groups of flavonoids, resulting in a competition for absorption between different classes of flavonoids. Furthermore, ACN absorption is delayed, and to some extent reduced by the simultaneous ingestion of a food matrix. As ACNs are rarely ingested entirely by themselves, but rather in combination with other flavonoids present in the fruits, or other nutrients present in foodstuffs, the effect of the associated ACN-flavonoid, or ACN-nutrient interactions is crucial for the bioavailability of ACNs. The results of the present thesis aid to formulate future recommendations regarding ACN intake. A possible enhancement of ACN absorption due to the right form and time of administration may be the first step for the enhancement of the proposed health-related effects of ACNs.

FUTURE DIRECTIONS

Further Ussing chambers experiments may provide the necessary information to evaluate the exact transport mechanism involved with ACN absorption. The *in vitro* investigations could include experiments to determine whether ACNs are absorbed:

- by passive or facilitated diffusion
- by active transport
- trans-, or paracellular

and moreover, how the absorption of ACNs is affected by:

- other flavonoids
- other polyphenols
- other food components (nutrients)

The outcomes of the present thesis need to be assessed in further *in vivo* studies, to develop a possible increase of ACN absorption. In addition, a possible enhancement of their antioxidant capacity needs to be further studied *in vivo*, to fully understand the contribution of ACNs in a healthy human diet. Future studies could involve:

- research on the effect of an additional food matrix or other flavonoid intake on ACN bioavailability and antioxidant status in humans
- investigations on the effect of the administration form of ACNs (aqueous, viscous, encapsulated) and the timing of ACN intake (before, together with, or after a meal) on ACN bioavailability in humans

ABBREVIATIONS USED

ACNs, anthocyanins; GIT, gastrointestinal tract; LPH, lactase phloridzin hydrolase; Q3G, quercetin-3-glucoside; SGLT1, sodium-dependent glucose transporter.

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CONFERENCE PRESENTATIONS

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