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# Anthocyanin Absorption and Antioxidant Status in Pigs

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The effect of a simultaneous intake of food or flavonoids on anthocyanins 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 anthocyanins 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 anthocyanins in the BC extract were detected. The total amount of anthocyanins 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 anthocyanin levels had returned to baseline. The present study demonstrates that the simultaneous intake of food or other flavonoids delays the absorption profile for anthocyanins. Our results also suggest that the increase in antioxidant capacity is not due to dietary anthocyanins but may be due to metabolites that result from anthocyanin consumption.

**KEYWORDS: Anthocyanins; antioxidant; blackcurrant; FRAP; ORAC**

## INTRODUCTION

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

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

While anthocyanins are strong antioxidants in vitro (*17*−*21*), it is uncertain whether consumption of anthocyanin-containing food significantly augments antioxidant levels in vivo. The bioavailability of anthocyanins has been shown to be extremely low, and the proportion 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 anthocyanins (*4*, *5*, *23*−*26*), and few have investigated the effects of an additional food matrix on anthocyanin absorption (*6*, *27*, *28*). As anthocyanins 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 anthocyanin absorption as well as on antioxidant levels.

While a study using an in vitro digestion procedure that mimics the physiochemical and biochemical conditions encountered in the gastrointestinal tract found little effect from a variety

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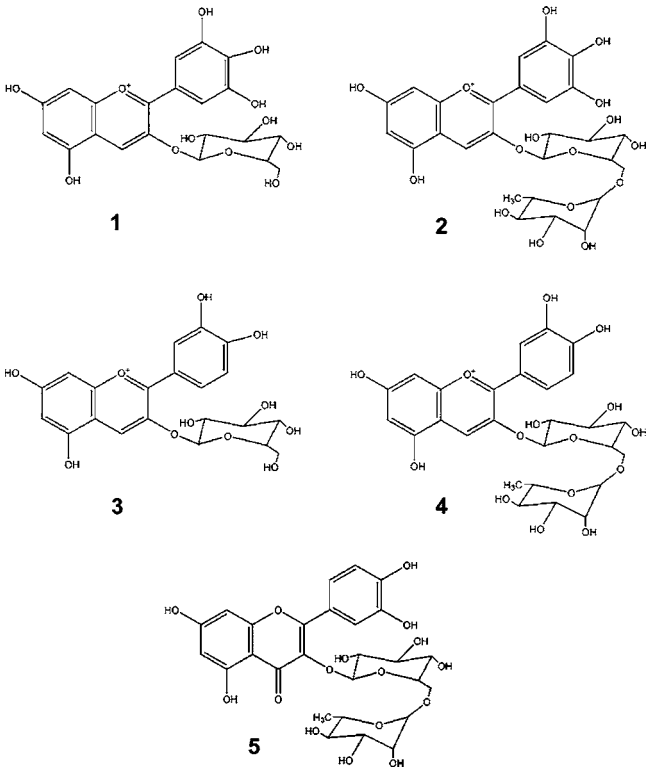


**Figure 1.** Chemical structures of the four major anthocyanins in blackcurrants: delphinidin-3-glucoside (**1**), delphinidin-3-rutinoside (**2**), cyanidin-3-glucoside (**3**), and cyanidin-3-rutinoside (**4**) and the flavonol rutin (**5**).

of food matrices on anthocyanin 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 anthocyanins from BC in rats. Absorption of anthocyanins 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 anthocyanin (**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 anthocyanin 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 anthocyanin metabolism (*8*) .

## MATERIALS AND METHODS

**Anthocyanins.** A blackcurrant powdered concentrate (Currantex 30, total anthocyanin 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 days prior to surgery. The animals were provisioned at 10% of their metabolic body weight (MBWT = $kg^{0.75}$) with water available ad libitum. All pigs were fed twice daily; 2% of MBWT provided as the 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 anthocyanin or other flavonoids. Animals were fasted every night between 6 p.m. and 8 a.m.

**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.

**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 + flavonol".

**Study Design.** Immediately following acclimation an intravenous cannula (IV catheter extra long, 14 g × 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, U.K.) 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 randomized 3 experimental days interspersed with 3-day washout periods (**Table 2**). 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 anthocyanins and 100 mg/kg BW rutin. The diets were administered at the same time each day (8 am) in order to avoid confounding chronobiological effects. The animals had free access to the test diets, and the meals were usually eaten within a

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*) .

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 2500*g* for 15 min at 4 °C, and plasma aliquots were stored at −20 °C. Plasma samples for anthocyanin 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.

**Anthocyanins in Test Diets.** A portion (0.5 g) of each diet was blended with an Ultra-Turrax basic homogenizer in 5 mL of ethanol/$H_2O$/acetic acid (80/20/1). The samples were subsequently stored for 48 h at 4 °C, centrifuged at 3040*g* for 20 min at room temperature, and appropriate dilutions with 5% formic acid/$H_2O$ analyzed by HPLC.

**Anthocyanins in Plasma.** Each frozen, acidified plasma sample was thawed at 37 °C in a water bath. Protein was precipitated from each sample with 10% trichloracetic acid (TCA). An aliquot (500 $\mu$L) of acidified plasma was mixed 1:1 with 10% TCA and centrifuged for 5 min at 10 000*g* at room temperature. The supernatant (800 $\mu$L) was collected, and the pellet was resuspended and rewashed twice each with 800 $\mu$L of 10% TCA. The supernatants were combined for each sample and evaporated almost to dryness under $N_2$ (<35 °C). The residue was resuspended in 150 $\mu$L of 1% aq. HCl and centrifuged for 10 min at 3040*g* at room temperature. A 30 $\mu$L portion of the supernatant was taken for HPLC analysis. The recoveries of anthocyanins in plasma with this method were tested with spiking experiments using an authentic standard of **3** (Extrasythase, Genay, France) and found to be 90.5 ± 3.9%.

**HPLC Analysis.** Anthocyanin 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 × 4.6 mm i.d., 4 $\mu$m, Synergi Hydro-RP Column (Phenomenex, New Zealand). 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 of 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 $\mu$L. Spectroscopic data from the PDA were collected at 250−600 nm, and chromatograms were extracted at 520 nm. Chromatography data were collected and processed using a Water Millennium Chromatography Manager V 4.0. **1**, **2**, **3**, and **4** concentrations were calculated as cyanidin-3-glucoside-equivalents using an authentic standard of cyanidin-3-glucoside (Extrasynthese, Genay, France) with known concentration.

**Antioxidant Status in Plasma.** Two commonly used assays to analyze antioxidant status in pig plasma were employed: ORAC (oxygen radical absorbance capacity) and 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 were 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 $\mu$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 of AAPH in 10 mL of phosphate buffer, were also stored at −20 °C until use. A Trolox stock solution (200 $\mu$M) was prepared by dissolving 5 mg of Trolox in 100 mL of 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 $\mu$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*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 $\mu$L of fluorescein (48 nM) into each well, followed by reading the fluorescence. Then the plates were manually loaded with phosphate buffer (blank, 10 $\mu$L), Trolox standard series (control antioxidant standard, 10 $\mu$L), and plasma samples (10 $\mu$L) 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 $\mu$L injected into each well, and readings taken every minute 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 analyzed 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 of sodium acetate trihydrate in 950 mL of $H_2O$, adding 16 mL of 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 of TPTZ in 100 mL of HCl (40 mM) and stored in the 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 of ferric chloride hexahydrate in 50 mL of $H_2O$ (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 $\mu$M. Plasma samples were analyzed undiluted.

**FRAP Experimental Conditions.** The automated FRAP assay was carried out on the BGM FLUOstar plate reader, and absorbance readings were taken at 593 nm. Prior to each experiment, the first injection pump was primed with sodium acetate solution and 140 $\mu$L of the solution was injected into each well of a NUNC 96-well plate. Subsequently, the plates were manually loaded with Trolox standards (15 $\mu$L), sodium acetate buffer (blank; 15 $\mu$L), and plasma samples (15 $\mu$L) into the respective wells and reinserted into the plate reader. The second injector was primed with freshly prepared FRAP reagent, 60 $\mu$L 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 expressed as Trolox equivalents in $\mu$mol/L. Data were analyzed by Microsoft Excel.

**Statistical Analysis.** Data are presented as means ± standard errors of the mean (SEM). The significance of differences between the baseline (0 h) and the following time points among treatments was assessed by repeated measures analysis of variance for comparison of individual means. The area under the plasma concentration vs time curve ($AUC_{0-8\ h}$) was calculated with the linear trapezoidal rule (ORIGIN, version 7.5; GENSTAT, version 8.1). Differences between treatment groups for $AUC_{0-8\ h}$ were tested by one-way analysis of variance. Statistical analysis was carried out using the SAS System for Windows (version

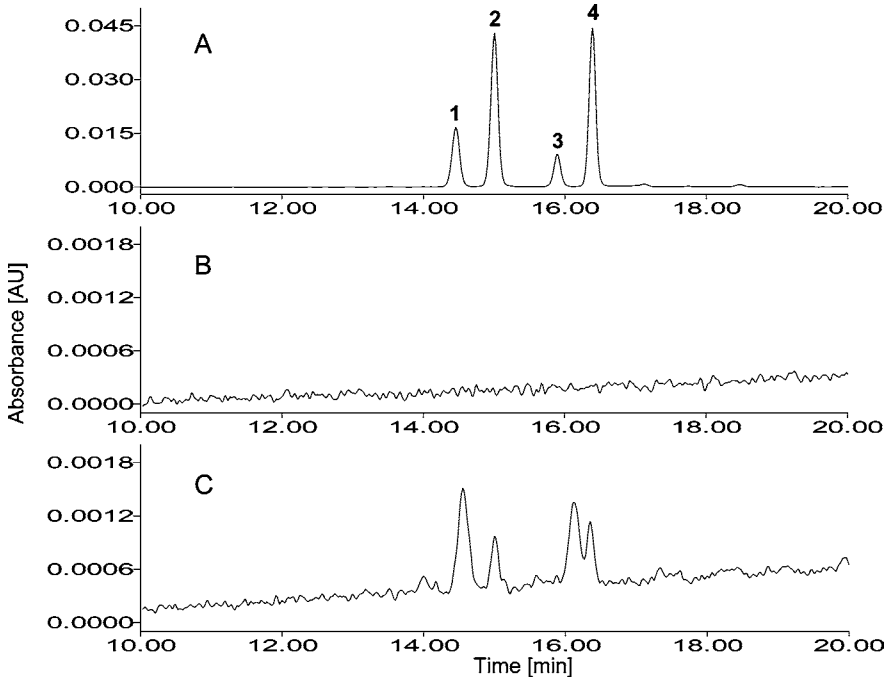


**Figure 2.** HPLC chromatogram of diet A (A), baseline plasma sample (B), and a postfeeding plasma sample (C). Detection is at 520 nm.

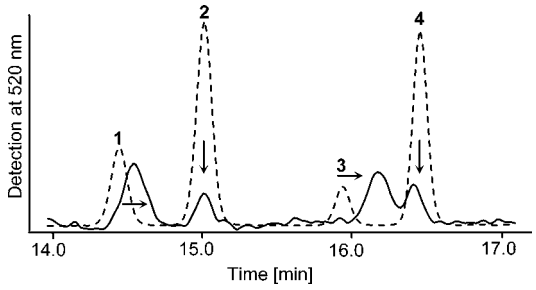


**Figure 3.** Overlay chromatogram of diet A (- - -) and a postfeeding plasma sample (–). Arrows indicate the slight shift in retention times of the corresponding glucosides of the blackcurrant anthocyanins present in the diets and the significant reduction of the rutinosides. Detection is at 520 nm.

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 rutinosides. Similar HPLC chromatogram profiles were produced by diets B and C (data not shown). All plasma samples collected before feeding the diets (baseline; $t = 0$ 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 rutinosides while the other two peaks had retention times somewhat later than the corresponding glucosides of the BC extract. Furthermore, in plasma, the two rutinoside peaks were reduced in size relative to the rutinoside 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.

The total anthocyanin concentration in plasma over time is shown in **Figure 4**. Anthocyanins from diet A were absorbed

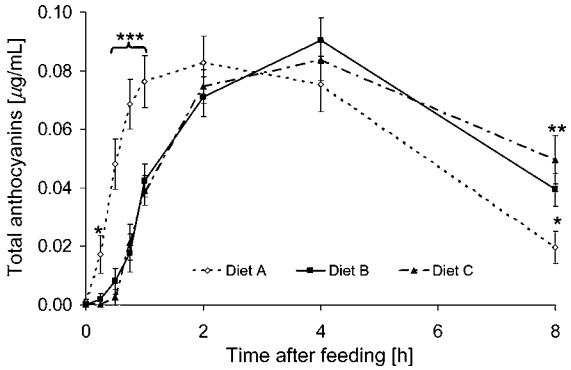


**Figure 4.** Anthocyanin concentration in pig plasma over time after feeding diet A, B, or C. Mean ± SEM, $n = 10$. *$p < 0.05$, **$p < 0.001$, ***$p < 0.001$.

**Table 3.** Pharmacokinetic Parameters of Anthocyanins in Pig Plasma after a Single Oral Dose of 100 mg of Total Anthocyanins/kg BW

| diet | $C_{max}$ ($\mu$g/mL) | $t_{max}$ (h) | $AUC_{0-8\,h}$ ($\mu$g·h/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 |

at significantly faster rates ($p < 0.05$ at 0.25 h; $p < 0.001$ between 0.5 and 1 h) than those from diets 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 $\mu$g/mL; **Table 3**) between the three diets or in the total amount absorbed over the period of 8 h ($AUC_{0-8\,h} = 0.49-0.61$ $\mu$g·h/mL) (**Table 3**). In addition, there was no significant difference in anthocyanin plasma concentrations following consumption of diet B or C at any time during the experiment. However, 8 h after the administration of the diets, the plasma anthocyanin concentration was significantly lower after feeding diet A than that after administration of either diet B ($p < 0.05$) or diet C ($p < 0.01$).

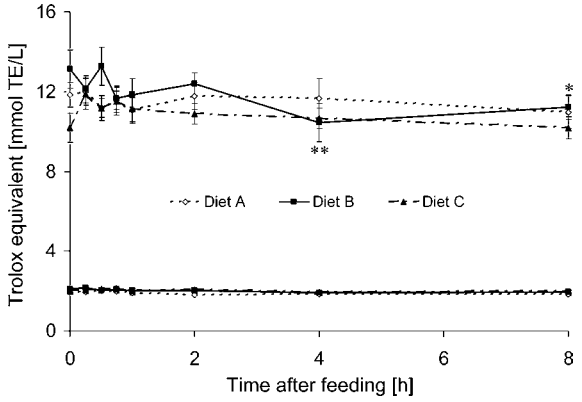


**Figure 5.** Plasma ORAC (top array of curves) and NP-ORAC (bottom array of curves) over time after feeding diet A, B, or C. Mean ± SEM, $n = 10$. *$p < 0.05$, **$p < 0.01$.

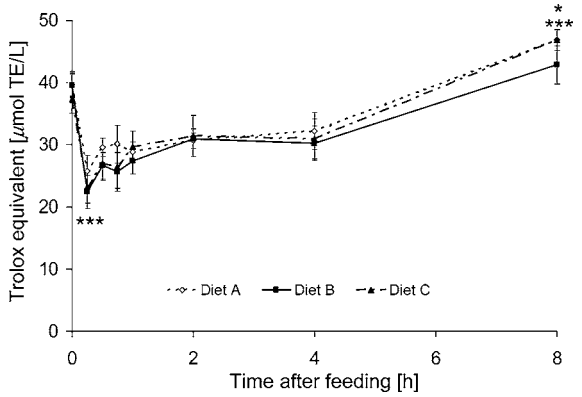


**Figure 6.** Plasma FRAP over time after feeding diet A, B, or C. Mean ± SEM, $n = 10$. *$p < 0.05$, ***$p < 0.001$.

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 analyzed using the NP-ORAC assay (**Figure 5**). Plasma FRAP significantly decreased ($p < 0.001$) from 39.66 ± 2.07, 39.58 ± 1.75, and 37.19 ± 20.12 μmol/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 μmol/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**).

## DISCUSSION

In the present in vivo study we investigated the effect of a simultaneous intake of anthocyanins and a food matrix as well as an additional flavonoid on anthocyanin absorption and antioxidant capacity in pigs. The animals were fed three different diets, and blood samples were taken at several time intervals. All plasma anthocyanin profiles were broadly similar to those in the diets with four peaks eluting close to the corresponding retention times of the four major anthocyanins 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 anthocyanin profile (**Figure 3**). To our knowledge, none of the previous studies on anthocyanin absorption using BC material have reported a similar anthocyanin 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 anthocyanins from BC (**1**) were administered to rats and showed that delphinidin anthocyanins 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 anthocyanins were found unmodified in plasma and urine (*5*−*7*) .

The lack of any subsidiary peaks to the rutinosides **2** and **4** that could indicate chemical modification of these compounds show that these anthocyanins do not undergo any significant metabolic conversion. These results are concordant with previous work, showing that anthocyanins with a disaccharide as sugar moiety (such as rutinoside) are less readily metabolized and appear in their intact forms in urine (*8*). The lower peak height of the rutinosides 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 rutinoside **2** in vivo.

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

In our study anthocyanins were absorbed more promptly when only dissolved in water ($t_{max} = 2$ h; diet A) compared to when mixed with milk and a wheat-based cereal ($t_{max} = 4$ 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 anthocyanins delayed absorption ($t_{max} = 1$ h) compared to when the anthocyanins were administered in water alone ($t_{max} = 0.25$ h) (unpublished results). A similar observation has been made in humans by Nielsen et al. (*6*) following ingestion of a rice cake with BC juice.

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

We found no inhibitory effect of rutin on the absorption of anthocyanins 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*, *35*). This finding may have resulted from weaker competition between the BC anthocyanins and rutin absorption in vivo,

compared to that observed between anthocyanins 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 (*36*). 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 (*36*) .

The ORAC assay, developed by Cao et al. (*37*), 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 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 (*37, 38*) .

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 color changes of the probe, which is proportional to the antioxidant concentration (*36*) .

We did not find any significant increase in plasma antioxidant capacity measured by ORAC and NP-ORAC following 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 (*39*). Since the two assays, ORAC and FRAP, were selected to measure different aspects of 'antioxiant 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 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 anthocyanins were decreasing may indicate that the fluctuations in FRAP values were not directly related to the detected anthocyanins but variation in the concentration of metabolites of anthocyanins, which were not detected in plasma samples under the present conditions. It has been suggested that a significant proportion of dietary anthocyanins may undergo metabolism and that the metabolites are responsible for many of the reported health effects (*40*). Lotito et al. (*41*), 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%, considerably lower than the fructose content (6%) of apples and other fruits (*42*). 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 (*43*). 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 (*43*) .

In conclusion, our results have shown that the addition of other food components to a dietary source of anthocyanins (BC) delayed the absorption profile but did not decrease the total absorption of anthocyanins. 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 anthocyanins 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 anthocyanin absorption and antioxidant status are necessary to understand the relevant contribution of anthocyanins in a complete healthy diet for humans.

## ABBREVIATIONS USED:

AAPH, 2,2′- azobis(2-amidino-propane) dihydrochloride; BC, blackcurrant; FRAP, ferric reducing ability of plasma/antioxidant power; MBWT, metabolic body weight; ORAC, oxygen radical absorbance capacity; PDA, photodiode array; TCA, trichloroacetic acid; TE, Trolox equivalents; TPTZ, 2,4,6-tripyridyl-*s*-triazine.

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