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Research Article

The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products

Colin D. Kay[1], Paul A. Kroon[2] and Aedin Cassidy[1]

[1] School of Medicine, Health Policy and Practice, University of East Anglia, Norwich, UK
[2] Institute of Food Research, Norwich Research Park, Norwich, UK

To date the *in vitro* mechanistic bioactivity of anthocyanins has been exclusively explored using aglycones and glycoside conjugates, despite a lack of evidence establishing these as the biologically available forms. We conducted intestinal epithelial cell (Caco-2 cells) culture experiments, which indicated that after a 4 h incubation of anthocyanins in cell-free culture media (DMEM), 57% of the initial cyanidin-3-glucoside (C3G) and 96% of cyanidin had degraded. The level of degradation was not statistically different from that of cultured cell incubations, suggesting that degradation was spontaneous. Degradation products were identified as protocatechuic acid (PCA) and phloroglucinaldehyde (PGA), and were confirmed in two other buffer matrices (phosphate and Hank's buffers). In cultured cell media the degradation products PCA and PGA were metabolised to glucuronide and sulphate conjugates, as indicated by both enzyme hydrolysis (sulphatase and glucuronidase treatments) and MS (PCA and PGA m/z = 155; sulphate = 235; glucuronide = 331). These data suggest a significant proportion of intestinal metabolites of anthocyanins are likely to be conjugates of their degradation products. Future efforts to establish the biological activities of anthocyanins should therefore include the investigation of phenolic acid and aldehyde products of degradation, along with their respective metabolites.

Keywords: Anthocyanin / Degradation / Flavonoid / Metabolite / Phenolic

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1 Introduction

Advances in molecular and cellular biology have greatly enhanced efforts to understand the cellular mechanisms underlying the health effects of phytochemicals. However, the mechanism of action of the parent phytochemical/compound, as commonly investigated under *in vitro* conditions, likely differs from that of the forms present in the human body; as evidence for extensive metabolism is apparent in the literature [1–3]. Polyphenols in particular can undergo a great deal of structural transformation both *in vivo* and *in vitro* that will undoubtedly alter their activity. This is particularly apparent for the flavonoid subclass, anthocyanins.

Anthocyanins impart blue and red colour to many plant tissues and are present in relatively high quantities in flowers, berries, grapes, red wine and grape juice, blood orange juice and red apple skins [4, 5]. Studies have estimated that the daily consumption of anthocyanins is anywhere from 3 to 215 mg/day (3 mg/day [6], 12 mg/day [4], 82 mg/day [7], 80–215 mg/day [8]); however, it should be noted that these data are derived from food frequency questionnaires and dietary recalls, which often underestimate intakes, as anthocyanins are poorly represented in available food composition databases. In addition, doses ranging from 400 to 500 mg can be obtained from a single serving of many commonly consumed berries and juices, such as blackberries, blueberries, black currents and blood orange juice (blackberries: 353–433 mg/serving; blueberry: 579–705 mg/serving; black currant: 533 mg/serving [4]; blood orange juice: 500 mg/serving [5]).

It has been reported that those in the population who consume the highest amounts of anthocyanin-rich foods have a reduced risk of developing CVD [9]. In addition, anthocyanins have consistently displayed effects associated with cardiovascular benefits, where numerous *ex vivo* and *in vitro* experimental studies provide evidence for vascular protection [10–19], through reduced immune cell activated radical [16, 18], cytokine [19] and adhesion molecule [18] pro-

Correspondence: Dr. Colin Kay, Diet and Health Group, Biomedical Research Centre, School of Medicine, Health Policy and Practice, MED 1, Floor 2, University of East Anglia, Norwich NR4 7TJ, UK
E-mail: Colin.Kay@uea.ac.uk
Fax: +01603-593752

Abbreviations: C3G, cyanidin-3-glucoside; **DAD,** diode array detector; **GIT,** gastro-intestinal tract; **PCA,** protocatechuic acid; **PGA,** phloroglucinaldehyde

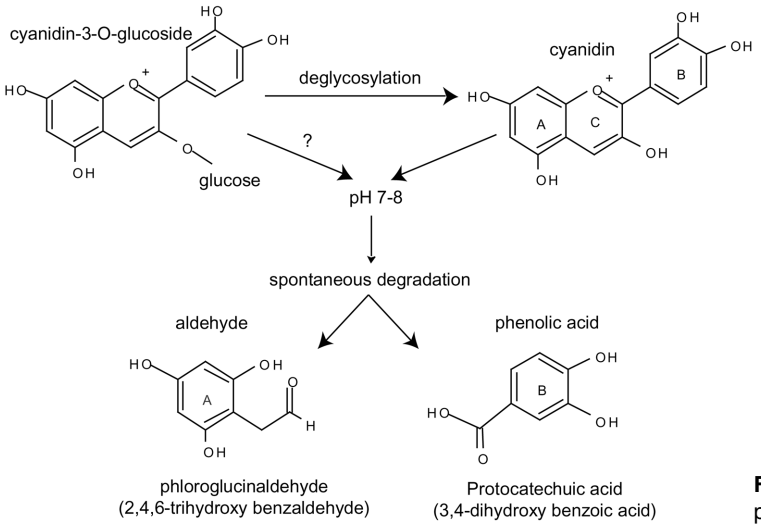

Figure 1. Structures of cyanidin and its degradation products.

duction, as well as direct effects on NO bioactivity [20, 21]. Furthermore, animal studies have confirmed these vascular activities in endothelial cells and vascular tissues [11 – 13]. Unfortunately, doses of anthocyanins utilised in many *in vitro* and *ex vivo* studies commonly exceed 10 μM (10 – 100^{+} μM) [11, 13, 14], which despite a high dietary consumption of anthocyanins, does not appear achievable in human serum. Human pharmacokinetic studies generally identify less than 1% (<0.2 μM) of the ingested parent anthocyanins in biological fluids, despite the consumption of doses exceeding 500 mg [3, 10, 22]. Furthermore, significant questions remain regarding the biologically active form of anthocyanins present in the blood, as previous *in vitro* studies were conducted almost exclusively using parent (un-metabolised) compounds. As spontaneous degradation of anthocyanins to phenolic acids and aldehydes (Fig. 1) is reported to occur under experimental [23] and biological conditions [24, 25], it is likely that the absorption of anthocyanins has been underestimated and the degradation products of anthocyanins contribute significantly to their alleged benefits. This incongruity between bioavailability and reported bioactivity has undoubtedly hindered the field of anthocyanin research [10, 26].

The overall objective of the present study was to establish the chemical fate of anthocyanins and the nature of their metabolism in the gut. Specifically, we investigated the fate of cyanidin-3-glucoside (C3G), the most representative dietary anthocyanin [11 – 14]. We aimed to characterise the loss of C3G and the appearance of its degradation products under conditions relevant to the gastro-intestinal tract (GIT). Additionally, we aimed to identify the products formed during breakdown, establish their recovery and determine if these products were further metabolised by the intestine during absorption using an *in vitro* intestinal epithelial cell (caco-2) model.

2 Materials and methods

2.1 Experimental procedure

Due to the instability of anthocyanins at physiological pH, control samples taken throughout the experiment were instantly pH adjusted (to pH = 2) in order to stabilise the anthocyanins, thus arresting degradation. These pH adjusted samples were then used as references to determine the amount of degradation that had occurred at each experimental stage (cell culture and enzyme-hydrolysis incubations). The experimental procedure is illustrated in Fig. 2. All chemicals and reagents utilised within the experiments were purchased from Sigma (UK), unless otherwise stated.

2.2 Degradation in phosphate buffer

Degradation experiments were conducted in triplicate in 0.1 M phosphate buffer (37°C, pH = 7.4). The primary samples (t = 0; 720 μg C3G or 1000 μg/mL cyanidin) (Extrasynthase, Genay, France) were buffered (pH = 7.4) 1 min prior to the initial HPLC injection. Ten sequential injections were preformed every 1.5 h, using an auto-sampler at 37°C and a HPLC mobile phase with a stepwise gradient consisting of a 5% v/v formic acid in water and ACN. Anthocyanins and their products of degradation were identified and quantified relative to pure standards. All reported values were expressed relative to controls, controlled for temperature, time and pH across three replicates. C3G, cyanidin and their degradation products were identified in phosphate buffer incubations using HPLC with diode array detector (DAD) detection. The HPLC system was comprised of an Agilent 1100 series HPLC with DAD detector scanning at 290, 325, 520 nm. Separation was preformed on a Luna C18 (4 μm, 250 × 4.6 mm) RP-HPLC column (Phenominex, UK) with security guard and column temperature of

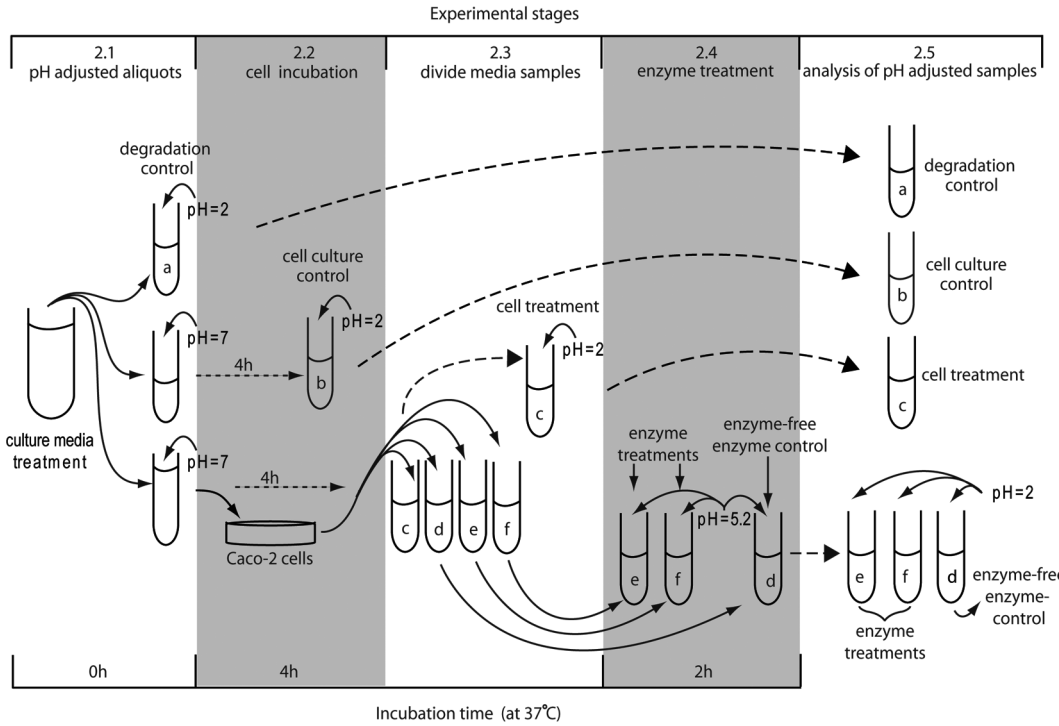

Figure 2. Flow diagram depicting the experimental procedure. Cell treatments consisted of either, C3G, cyanidin, PCA or PGA spiked media (DMEM and Hank's buffer). Superscripts (a–f) correspond to sample identifiers. [a]Degradation/primary control: treated media (cell-free) acidified to pH = 2 with 0 h incubation. [b]Control media: treated culture media (cell-free) buffered to pH 7 with 4 h incubation. [c]Cell treatment: treated culture media buffered to pH 7, cultured with caco-2 cells for 4 h. [d]Enzyme-free enzyme hydrolysis control: treated culture media buffered to pH 7, cultured with caco-2 cells for 4 h, followed by 2 h under enzyme-free, enzyme hydrolysis conditions. [e]Sulphatase treatment: treated culture media buffered to pH 7, cultured with caco-2 cells for 4 h, prior to sulphatase treatment (additional 2 h incubation at pH = 5.2). [f]Glucuronidase treatment: treated culture media buffered to pH 7, cultured with caco-2 cells for 4 h prior to glucuronidase treatment (additional 2 h incubation at pH = 5.2). All samples were acidified to pH 2 directly after the required incubation periods. Statistical comparisons of change in relative concentration as presented throughout the experiments were as follows: a to b, degradation in cell-free media; b to c, change in cultured caco-2 cells; c to d, degradation during enzyme-free incubation at pH = 5.2; d to e and f, metabolism in cultured cells. Refer to Fig. 4 for graphical representation of results.

37°C, injector temperature of 4°C, injection volume of l00 µL, flow rate of 1.0 mL/min and run time of 45 min.

Anthocyanins and their degradation products were identified in phosphate buffer utilising pure standards and HPLC with DAD detection, whereas HPLC/DAD coupled with MS was employed for the identification of anthocyanins, degradation products and respective conjugates in culture media samples, as described in Section 2.6.

2.3 Degradation in caco-2 cell incubations

In order to characterise the loss of C3G and the appearance of its degradation products under conditions relevant to the GIT, we utilised a caco-2 cell model, treating the cells with C3G and cyanidin (separate experimental incubations), and each dissolved in DMEM media. Parallel incubations were conducted in both cultured cells and in cell-free media (DMEM) to control for degradation in the media matrix. In order to establish the extent of degradation in cultured caco-2 cells, cells were subcultured in 10 cm dishes (passage 40–50), seeded at a density of 2×10^4 in phenol-free DMEM (10% FCS) and grown to 100% confluence. Cells were treated with C3G, cyanidin, PCA and PGA (dissolved in DMSO) at a final concentration of 100 µM in media (<1% DMSO/mL media). Triplicate treatment incubations (4 h) were performed on two separate occasions in serum-free DMEM (six replicates total). For sample processing, the media was removed and transferred to vials, while the cells were rinsed 3× with Hank's buffer prior to removal with a cell scraper. All samples were acidified when aliquoted (10–20 µL TFA; cells and media, respectively), vortexed (30 s), sonicated (10 min) and stored at −80°C overnight. Samples were centrifuged the following day, capturing the supernatant for further processing or analysis. Parallel cell-free media incubations were conducted as detailed in Section 2.2 and treated as degradation controls. All samples were immediately acidified (pH = 2.3) after their required incubation times as in Fig. 2. All reported values

were expressed relative to controls, controlled for temperature, time, pH and extraction procedure across six replicates.

2.4 Recovery in caco-2 cell incubations

We characterised the loss of C3G and cyanidin, and the appearance of the degradation products in cultured cells incubated in Hank's buffer as an alternative to DMEM. Again, parallel incubations were conducted in both cultured cells and in cell-free buffer media, to control for degradation in the media matrix. The cell incubations with Hank's buffer were necessary to identify differences between the DMEM and Hank's buffer treatments; establishing the effects of matrix on recovery. In addition, treating with Hank's media provided a 'cleaner' environment for the identification of the products of caco-2 cell metabolism.

2.5 Stability of degradation products

In order to establish the stability of degradation products, degradation experiments were conducted (as above) treating caco-2 cells and cell-free media with pure PCA and PGA (at a concentration of 100 μM).

2.6 Characterisation of metabolic conjugates

To determine the products formed during breakdown and establish if these products were further metabolised by the intestine during absorption, samples from the caco-2 cell incubations were subject to enzyme hydrolysis and compounds were identified pre and postenzyme treatment using HPLC/MS. Aliquots (1 mL) of the cell treatment samples were buffered to pH 5.3 using a phosphate buffer prior to enzyme hydrolysis (100 U sulphatase or 1000 U B-glucuronidase) for 2 h at 37°C. Samples were subsequently re-acidified for HPLC/MS analysis (as detailed Section 2.3). Cell-free media and enzyme-free samples were processed as above and used as degradation controls (Fig. 2). HPLC/ESI-MS was performed on an Agilent HPLC/DAD coupled to an Agilent LC/MSD SL mass spectrometer. The HPLC consisted of an Agilent 1100 series HPLC and DAD monitoring absorbance at 290, 325 and 550 nm. The separation was preformed using a Synergi Polar-RP (4 μM, 250 × 4.6 mm; Phenomenex, UK) RP column with Synergi security guard column. The column temperature was set at 37°C and the injector temperature at 4°C, with an injection volume of l00 μL. The flow rate was set at 1.0 mL/min with a mobile phase consisting of 0.5% v/v formic acid in water and 0.5% v/v formic acid in MeOH. A stepwise gradient was preformed with a run-time of 90 min. MS ESI was initially performed in full scan mode with a mass range of 100 – 1000 Da, drying gas flow rate of 13 L/min at 350°C, and nebuliser pressure of 50 Psig. Anthocyanins were resolved in positive ionisation mode at a capillary voltage of 4000 V and a fragmentation intensity of 100; while phenolics were identified using negative ionisation mode utilising a capillary voltage of −3000 V with fragmentation intensity of 70. Higher resolution scans were preformed in SIM mode scanning at 155, 235, 287, 331, 367 and 463 *m*/*z* for confirmation, once initial structures were elucidated.

2.7 Statistical analysis

All values are expressed relative to controls. Data are presented as mean ± SD, with differences between treatments and controls evaluated using Microsoft Excel. Statistical comparisons were conducted using two-tailed *t*-tests at a significance level of $p < 0.05$.

3 Results

The series of experimental procedures utilised were necessary to establish if the low reported bioavailability of anthocyanins is the consequence anthocyanin disappearance resulting from incomplete recovery and/or degradation of parent structures and/or subsequent metabolism of degradation products.

3.1 Anthocyanin stability in biological buffers and cell-culture media

In order to establish the chemical fate of anthocyanins and the nature of the breakdown process, degradation was first characterised in a simple medium (phosphate buffer). Quantification in phosphate buffer was necessary to establish the identities and rates of formation of the degradation products at physiological pH and temperature, without the contribution of the cell media matrix or the caco-2 cells themselves. At 37°C in phosphate buffer, cyanidin and its 3-glycoside were stable at low pH (0% degradation over 6 h; pH 2.3); however, at physiological pH (pH 7.4), 15% of the original C3G was degraded within the first 3 h ($p < 0.01$) (Fig. 3A). In addition, the rate of degradation increased over the first three hours of incubation (68.4 ± 0.8 μmol/h at 1.5 h to 76.2 ± 3.2 μmol/h at 3.0 h), but steadily decreased over the remaining 10.5 h (58.9 ± 3.7 μmol/h at 4.5 h to 23.6 ± 2.1 μmol/h at 13.5 h). In contrast, cyanidin was extremely unstable with an instantaneous loss of 42% of the parent structure at pH 7.4 ($p < 0.0001$), and less than 10% initial levels remaining after 3 h ($p < 0.005$) (Fig. 3B).

In 4 h Hank's buffer incubations (cell-free media) under the same conditions (temperature and pH), there was a 15% loss of C3G and a 96% loss of cyanidin ($p < 0.0001$) (Fig. 4A). However, in DMEM media incubations (cell-free), there was a 57% loss of C3G and a 96% loss of cyanidin ($p < 0.0001$) (Fig. 4A). This translated to 42% greater loss of C3G in DMEM than Hank's buffer over 4 h, while the

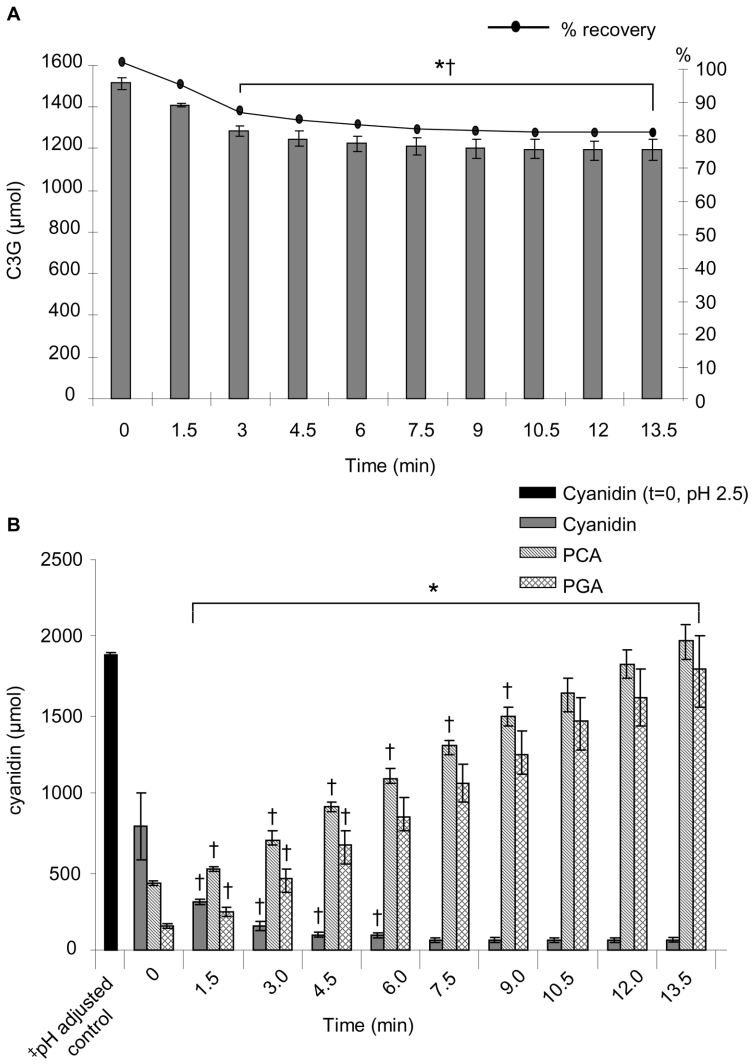

Figure 3. Degradation of C3G and cyanidin in phosphate buffer at pH = 7.4 and 37°C. A, degradation of C3G; B, degradation of cyanidin and relative formation of PCA and PGA. *Mean values were significantly different from those of the initial control sample ($t = 0$ h; paired *t*-test; $p < 0.01$). † Mean values were significantly different from those of the previous time point (within a given compound: cyanidin, PCA or PGA; paired *t*-test, $p < 0.05$). ‡ The pH adjusted control sample represents a sample at pH 2, which was not buffered to pH 7.4 prior to injection onto the HPLC column ($t = 0$). The data are presented as means ± SD of three independent experiments.

degradation of cyanidin was not different between the two matrices. Similarly, a greater degradation of C3G and cyanidin was also observed during enzyme-hydrolysis incubations with DMEM, where significant decreases in the level of C3G and cyanidin occurred in incubations with DMEM, but no decreases were observed in Hank's buffer.

3.2 Formation of phenolic products during anthocyanin breakdown

The products formed from the degradation of C3G and cyanidin during incubation in phosphate buffer and cell-culture media were identified (using pure standards *via* retention time, UV–Vis and MS data) as protocatechuic acid (PCA: 155 *m*/*z*; 247, 294 λ_{max}) and phloroglucinaldehyde (PGA: 155 *m*/*z*; 293 λ_{max}) derived from the A- and B-rings of the parent structure (Fig. 1). In phosphate buffer, the recovery of the degradation products was equivalent to that of the loss in parent structure (on the basis that stoichiometric conversion of one mole of anthocyanin will give rise to one mole each of the phenolic acid and aldehyde) (Fig. 3B).

In 4 h Hank's buffer incubations (cell-free), losses of C3G were accompanied by significant increases in PCA, which were six times that of levels identified in $t = 0$ h incubations, relative to the pH adjusted controls ($p < 0.0001$); while a 10-fold increase in the level of PGA was observed ($p < 0.001$). In addition, at the end of 4 h incubation of C3G in DMEM media (cell-free), PCA was identified at levels three times that observed in 0 h control incubations ($p < 0.0001$) (Fig. 4A), while no increase in PGA was observed above $t = 0$ levels ($p < 0.001$).

For the aglycone structure, after 4 h incubation of cyanidin in Hank's media, there was ten times the level of PCA relative to $t = 0$ h levels ($p < 0.0001$), while increases in the level of PGA were nonsignificant as a result of high variations in recovery (±58%, $p > 0.1$). Additionally, 4 h DMEM

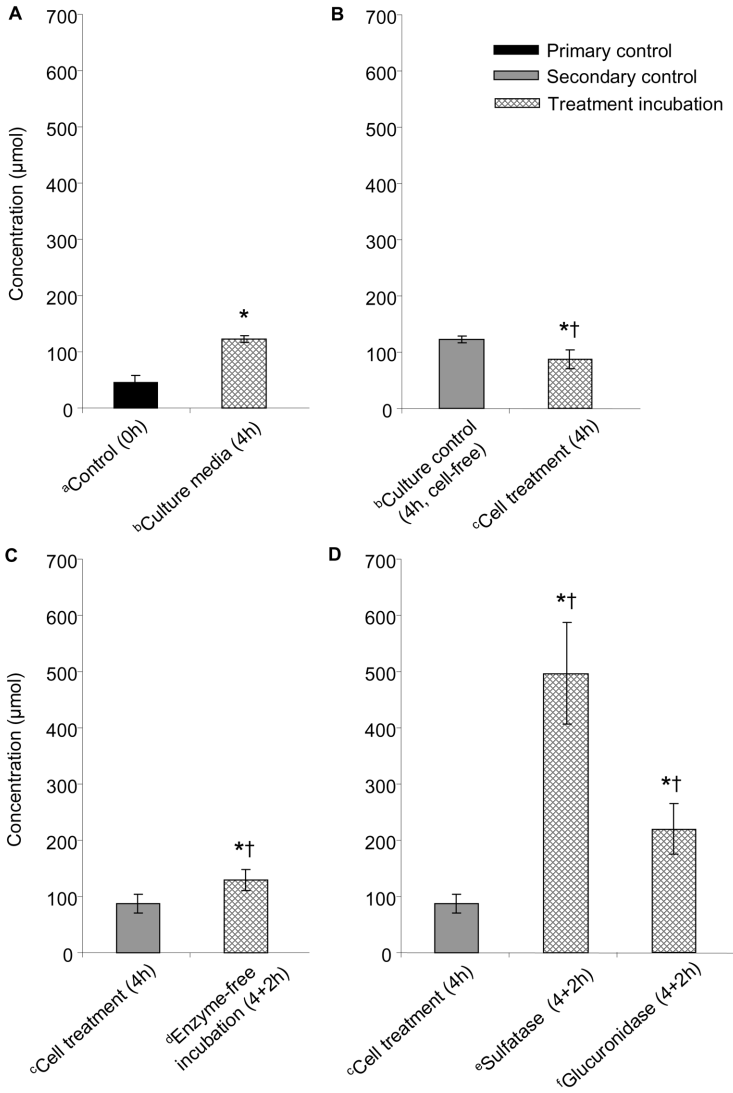

Figure 4. Change in the concentration of PCA (3,4-dihydroxybenzoic acid) during cell-free culture media (DMEM) incubations, caco-2 cell culture incubations and enzymatic hydrolysis incubations with cyanidin-3-glucoside. A, formation of PCA during cell-free, culture media control incubation with C3G (0 h, pH 2 primary control *vs.* 4 h cell-free media incubations at 37°C and pH = 7); B, loss of PCA during 4 h caco-2 cell incubations with C3G (cell-free media incubations of 4 h at 37°C and pH = 7 *vs.* caco-2 cell incubations under the same conditions); C, formation of PCA during enzyme-free control incubation (cultured media adjusted to pH 2 *vs.* enzyme-free cultured media incubated an additional 2 h at pH = 5 and 37°C); D, liberation of PCA following enzyme-hydrolysis incubations with sulphatase and glucuronidase (cultured media adjusted to pH 2 *vs.* 2 h enzyme incubations at pH = 5 and 37°C). All samples were acidified to pH 2 directly after the incubation periods. Superscripts (a – f) correspond to samples in the experimental sampling procedure as illustrated in Fig. 2. *Mean values were significantly different from those of the initial/primary control sample, which was adjusted to pH 2 upon spiking with C3G (paired *t*-test; $p < 0.05$). † Mean values were significantly different from those of the previous sample/secondary control (paired *t*-test; $p < 0.05$).

media incubation (cell-free) with cyanidin resulted in a 50% increase in the levels of PCA (over $t = 0$ h levels, $p < 0.0001$) with again, no significant increase in PGA ($p > 20.1$).

3.3 Stability and recovery of phenolic degradation products

When parallel experimental incubations (4 h) were conducted using pure standards of PCA and PGA in all buffers and media, there was no significant loss of the phenolic compounds observed relative to 0 h controls ($p > 0.1$).

3.4 Anthocyanin losses in cultured cells

There was a 20.5% loss of C3G ($p = 0.02$) in cells cultured with Hank's buffer in contrast to an 85.9% loss of cyanidin ($p < 0.0001$). In addition, when cells were cultured with DMEM, the loss was 51.7% for C3G and 97.2% for cyanidin ($p < 0.0001$); representing a 31.2% greater loss of C3G in DMEM cultured cells over Hank's buffer, while the loss of cyanidin was only 11.3% greater in DMEM. There was no significant difference in the levels of C3G between 4 h cell-culture treatments and cell-free control incubations for DMEM or Hank's incubations; suggesting degradation was spontaneous. As well, there was no additional loss of cyanidin observed for cell-culture incubations with Hank's buffer *versus* cell-free incubations; however, there was 18.1% less cyanidin ($p = 0.05$) observed in the cell incubations with DMEM relative to cell-free incubations.

3.5 Stability during enzyme hydrolysis

There were significant decreases in the level of C3G (17.4%; $p = 0.003$) and cyanidin (32.5%; $p = 0.006$) during

the enzyme-free, enzyme hydrolysis control incubations with DMEM, but no decrease was observed for either C3G or cyanidin during enzyme-free, enzyme-hydrolysis incubations with Hank's buffer; indicating a reduced recovery from the DMEM media matrix. The enzyme hydrolysis control incubation had no effect on the recovery of PCA or PGA in cell treatments incubated with pure PCA or PGA ($p > 0.1$).

3.6 Evidence of metabolites resulting from anthocyanin incubations

Levels of PCA and PGA formed during 4 h cell-culture incubations with C3G were 23.7% ($p \leq 0.0005$) lower than those formed in cell-free media incubations suggesting the difference resulted from the metabolism of degradation products (Fig. 4B). In addition, when cell-culture media from 4 h C3G incubations was treated with sulphatase or glucuronidase, levels of PCA and PGA increased above those observed in both 4 h cell-culture incubations and enzyme-free control incubations ($p< 0.0001$)(Figs. 4C and D), suggesting liberation of metabolic conjugates. These associations were observed for both DMEM and Hank's buffer experiments.

Levels of PCA and PGA formed during 4 h cell-culture incubations with cyanidin were 41.4% ($p \leq 0.001$) lower than those formed in cell-free media incubations, again, suggesting that the difference resulted from the metabolism of degradation products. When cell-culture media from 4 h cyanidin incubations was treated with sulphatase, levels of PCA rose above those observed for the 4 h cell-culture incubations ($p < 0.0001$), indicating liberation of metabolic conjugates. There was however no difference in the levels of PCA observed following the glucuronidase treatment of the cyanidin incubated samples; nor was there any statistically significant difference in the level of PGA resulting from either enzyme treatment. These associations were observed for both DMEM and Hank's buffer experiments with cyanidin.

In the media of cells incubated with pure C3G, glucuronide (m/z 155/331 = loss 176; glucuronide residue) and sulphate (m/z 155/235 = loss 80; sulphate residue) derivatives of PCA (m/z = 155) were identified utilising LC/MS. Additionally cyanidin was identified (cyanidin m/z = 287) along with glucuronide (m/z 287/463 = loss 176) and sulphate (m/z 287/367 = loss of 80) derivatives of cyanidin. Conjugates were also identified in cells incubated with pure cyanidin, PCA and PGA, as well as in caco-2 cell lysates themselves. It should be noted that the sulphatase enzyme used here had limited specificity, resulting in hydrolysis of both sulphate and glucuronide conjugates. This resulted in an elevated level of PCA liberation in the sulphatase treated cell media in proportion to the glucuronidase treated media, as depicted in Fig. 4D.

3.7 Effects of cell-culture conditions on metabolite formation

As detailed in Section 3.6, the cells appeared to produce greater amounts of metabolites when treated with DMEM compared to Hank's buffer. This was to be expected as DMEM contains substrates necessary for cell growth. However, the DMEM conditions gave rise to increased degradation, reduced recovery and higher MS S/Ns. Increased recovery and cleaner chromatograms resulting from the Hank's buffer treatments were therefore important in the confirmation of DMEM results; however, as Hank's is not an optimal environment for cells, incubations of longer than 4 h in Hank's buffer are not recommended as the cells begin to lose viability. As a result, although recovery and detection were improved in Hank's buffer, cellular performance and therefore rates of metabolism were reduced. These factors likely contributed to the differences in degradation, recovery and metabolism observed between the DMEM and Hank's treated cells in the presented experiments.

4 Discussion

The difficulty in investigating anthocyanin metabolism or bioactivity results from both their structural instability and their poor recovery resulting from matrix binding. Significant losses of anthocyanins during sample preparation are frequently reported [27] and believed to be in part responsible for the low levels identified in the blood and urine [28]. Although high losses of anthocyanins are commonly reported in experimental and biological matrices, in most studies, both degradation and recovery are reported interchangeably and quantified only by the disappearance of anthocyanins. This has made it difficult to establish the extent of losses arising from poor experimental recovery or from degradation, as products of degradation are rarely identified. In addition, degradation studies of fruit extracts containing anthocyanins or pure standards have been conducted in the past, utilising similar experimental conditions as presented herein (pH and temperature), but with different culture medium and cell lines [29, 30]. However, in these instances, it was not possible to establish if the loss of anthocyanins observed was the result of degradation or poor recovery and none investigated metabolism. The overall objective of the present study was to establish the chemical fate of anthocyanins and the nature of their metabolism in the gut.

In this report, we have shown that C3G degrades to yield cyanidin, which rapidly and spontaneously degrades to yield PCA and PGA for all the tested media. This was best demonstrated in the phosphate buffer experiments under physiological temperature and pH conditions. This degradation of cyanidin to PCA and PGA has been previously reported in thermal and bacterial degradation experiments

[31 – 33]. In our experiments, it was necessary to first use a simple buffer (phosphate buffer) to establish modifications to structural conformation, as it is a 'clean' medium, which allows the distinction between degradation and recovery by limiting potential matrix binding compounds. Once this was established, the relationship between recovery, degradation and metabolism was explored under standard cell-culture conditions, using a caco-2 cell model of intestinal epithelial cell metabolism. The ability of anthocyanins to complex with proteins, metals and other flavonoids, thus effecting recovery, is well documented [27, 34]. In our experiments, we established that the loss of C3G and cyanidin in a simple phosphate buffer was primarily the result of spontaneous degradation rather than poor recovery, as the disappearance of C3G and cyanidin was proportionate to the appearance of PCA and PGA (Fig. 3). It should however be noted that there was a lag in the formation of the ultimate phenolic degradation products (see Fig. 4B), where in the early time points the formation of PCA and PGA was not equivalent to the disappearance of cyanidin. We obtained evidence from additional peaks in the HPLC chromatograms (data not presented) which suggests this delay results from the presence of ring-open intermediates, which may have varying degradation rates under differential experimental conditions [27, 35]. Ultimately, complete and stoichiometric degradation to PCA and PGA occurred. It is also worth mentioning that in Fig. 3B, the relative amount of cyanidin at time zero was considerably less (~50%) than that of the initial level of cyanidin in spiked control samples. We attributed this difference in the level of cyanidin to an instantaneous loss of the coloured form of cyanidin at neutral pH, which did not occur in parallel samples where cyanidin was dissolved in a buffer at pH 2. Further experiments allowing for equilibrium at various pHs in both buffers and in water, confirmed this observation (unpublished data).

Caco-2 cells incubated with pure C3G resulted in significant losses of C3G followed by increases in PCA and PGA (Fig. 4). However, this also occurred in cell-free incubations, and there was no significant difference between the losses of C3G and the appearance of degradation products observed in cell incubations over the 4 h cell-free incubations (control), suggesting that C3G degradation was exclusively due to spontaneous chemical breakdown and not due to Caco-2 cell induced enzymatic deglycosylation followed by chemical degradation. These findings were corroborated by the results of the cyanidin treated cells where we observed no difference in the rapid and near complete degradation of cyanidin and subsequent formation of PCA and PGA between the cell and cell-free incubations. Together these findings indicate that C3G spontaneously degrades to PCA and PGA prior to caco-2 cell metabolism, lending support to the notion that spontaneous degradation of C3G is likely to occur in the small intestine in humans. Nonenzymatic degradation of anthocyanin glycosides has previously been reported in experiments of thermal degradation [23] as well as degradation using McCoy's 5A medium under similar culture conditions [29]. We were not able to determine if C3G is deglycosylated (forming cyanidin) prior to degradation to PCA and PGA, or if 'ring-open' intermediates are initially formed causing destabilisation of the chalcone structure, and subsequently deglycosylation.

In humans, it is possible that an even greater level of degradation occurs as a result of deglycosylation *via* LPH and CBG within the intestinal epithelium as suggested for other flavonoids of similar structure [36 – 38]. Enzymatic deglycosylation was not observed to contribute significantly to the degradation of anthocyanins in the present caco-2 cell experiments, which is likely due to the low expression of LPH in cultured caco-2 cells [36]. Overall, these data suggest that anthocyanins will degrade continuously while in the digestive tract and during the processes of absorption and metabolism in the small intestine, *i.e*. anthocyanins will degrade within the GIT, at the brush border (after hydrolysis by LPH), within the intestinal cells (if hydrolysed by CBG), within the hepatic portal blood, during liver metabolism and within the systemic circulation [28, 36]. Therefore, the numerous existing reports describing low concentrations of anthocyanins in human blood and urine samples are consistent with these compounds undergoing continuous degradation *in vivo*, in addition to spontaneous degradation occurring during analytical extraction and processing.

The concentration of degradation products (PCA and PGA) was lower in the caco-2 cell incubations with C3G, compared to the cell-free control incubations, despite there being no statistical difference in the levels of un-metabolised C3G between the treatments. This provided initial indirect evidence that the PCA and PGA had been metabolised in the cultured caco-2 cells. The presence of glucuronide and sulphate conjugates was confirmed by LC/MS through use of enzymatic incubations with sulphatase or glucuronidase. Glucuronide conjugates were also identified by LC/MS when cells were incubated with pure PCA and PGA, thus establishing PCA and PGA as substrates for caco-2 cell UDP glucuronosyltransferases. Interestingly, sulphate conjugates were identified by LC/MS in cells treated with C3G; but these conjugates could not be detected following incubation with pure PCA and PGA, suggesting metabolism may be required prior to degradation. However, further experiments are required in order to support this notion. These data demonstrate that caco-2 cells can produce phenolic acid and aldehyde metabolites of C3G, and it is likely that intestinal metabolism of anthocyanins to phenolic acid and aldehyde conjugates occurs *in vivo*. Glucuronidation and sulphation of other phenolic acids [30], phenols [39] and flavones [40] has previously been reported in caco-2 cells.

In accordance with the findings of Kern *et al*. [31], the additive molar recovery of phenolic compounds was not consistent with the disappearance of parent anthocyanins in

cultured cell media, suggesting either limited recovery and/or the formation of uncharacterised intermediates. The degradation of C3G theoretically should yield equal molar equivalents of PCA and PGA (as observed in the phosphate buffer experiments) while in the present Caco-2 cell experiments the recovery of degraded C3G as PCA was only 60.1 ± 29.9 µmol, while the loss of C3G was 199.3 ± 13.1; representing a recovery of 30% degraded C3G as PCA. In addition, the level of PGA recovery was considerably lower, accounting for only 9.4% of the loss of C3G. The extremely poor recovery of PGA was most likely as a result of aldehyde reactivity, which could result in differential matrix binding effects and/or the formation of hydrates, hemiacetals and acetols under the acidic aqueous and alcohol environments that are routinely used in RP HPLC. The inability to characterise the aldehyde derivative of anthocyanin breakdown products has been alluded to in several published reports [23, 25, 31, 41]. Therefore, PCA and its glucuronide and sulphate derivatives are likely to contribute to the metabolite pool of ingested C3G in humans, although the contribution of PGA to the metabolite pool is difficult to quantify because of its reactivity and extremely low recovery in biological matrices. In addition, as this study only utilised one anthocyanidin species (cyanidin), future studies are required to establish the extent of metabolism, degradation and recovery using the other anthocyanidins.

In conclusion, we have shown that anthocyanins and anthocyanidins undergo rapid degradation and metabolism and are often poorly recovered from biological samples, which may have compromised previously reported studies concerned with anthocyanin bioavailability. We have shown that C3G and cyanidin degrade under simulated intestinal conditions to yield PCA and PGA, which can be further metabolised to glucuronide and sulphate conjugates. Therefore, the biological activities of dietary anthocyanins are likely in part to be caused by degradation products and their metabolised derivatives, and future research into anthocyanin bioactivity should include these products.

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