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ORIGINAL CONTRIBUTION

# In vitro metabolism of anthocyanins by human gut microflora

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**Summary** *Background* Only a small part of the dietary anthocyanins are absorbed. Thus large amounts of the ingested compounds are likely to enter the colon. *In vitro* and *in vivo* studies have shown that colonic bacteria transform various flavonoids to smaller phenolic acids. However, there is very little information on bacterial transformations of anthocyanins. *Aim of the study* was to explore if anthocyanin glycosides were deglycosylated, whether the resulting aglycones were degraded further to smaller phenolic compounds by colonic bacteria, and to characterise metabolites. *Methods* Isolated cyanidin-3-glucoside and -rutinoside were fermented *in vitro* using human faecal microbiota as an inoculum. Metabolites were analysed and characterised by HPLC-DAS and LC-MS. They were identified by comparing their characteristics with those of available standards, and semi-quantified using the amount of substrate analysed from samples at initial timepoint. *Results* Cyanidin-3-glucoside and cyanidin aglycone could be identified as intermediary metabolites of cyanidin-3-rutinoside. At early timepoints (before 2 h), the formation of protocatechuic acid as a major metabolite for both cyanidin glycosides and detection of lower molecular weight metabolites show that anthocyanins were converted by gut microflora. Furthermore, reconjugation of the aglycone with other groups, non-typical for dietary anthocyanins, was evident at the later (after 2h) timepoints. *Conclusions* Bacterial metabolism of anthocyanins involves the cleavage of glycosidic linkages and breakdown of the anthocyanidin heterocycle.

**Key words** anthocyanins – deglycosylation – alpha, L-rhamnosidase – beta, D-glycosidase – bacterial metabolism – heterocycle breakdown

## Abbreviations

| | |
|---|---|
| Cy3 g | cyanidin-3-glucoside |
| Cyrut | cyanidin-3-rutinoside, cyanidin-3-rhamnoglucoside |
| Cy-1 | bacterial metabolite from cyanidin glycosides: aglycone, cyanidin. |
| Cy-2 | bacterial breakdown product from cyanidin glycosides: protocatechuic acid (3,4-dihydroxybenzoic acid) |
| Cy-3 | unidentified bacterial metabolite from cyanidin glycosides |
| Cy-4 | bacterial conjugate of cyanidin possibly containing nitrogen or sulphur |
| HPLC-DAS | high-performance liquid chromatography-photodiode array spectrometry |

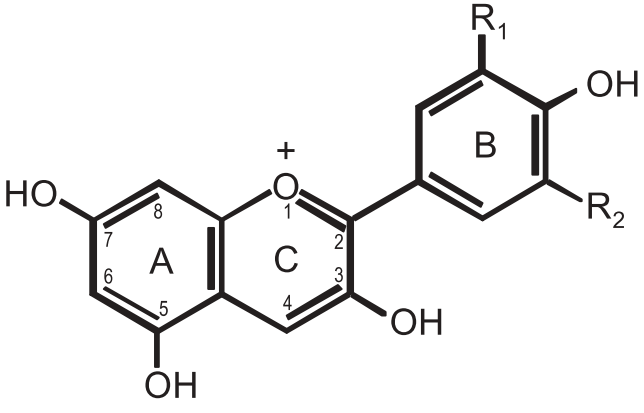
| | |
|---|---|
| LC-MS | liquid chromatography-mass spectrometry |
| UV | ultraviolet |

## Introduction

Plant flavonoids are common dietary compounds having potential health effects as antioxidants [1]. Flavonoids have also been shown to induce apoptosis or to be anticarcinogenic [2, 3]. On the other hand, they can act as antimutagens/promutagens and antioxidants/pro-oxidants, which is largely dependent upon levels consumed as well as on physiological conditions. Exposure to very high (non-dietary) levels of flavonoids may potentially lead to formation of reactive oxygen species, and ultimately DNA damage [4]. Thus intake and bioavailability are important in estimating beneficial effects of flavonoids on human health.

Anthocyanins are flavonoids which produce blue, purple red and intermediate colours in plants. They are present as glycosides with an anthocyanidin C6-C3-C6 skeleton (Fig. 1), whose structure is dependent on pH. In aqueous media, anthocyanins appear as flavylium cation (red) at acidic pH [0–2], and as a colourless pseudobase with a small amount of colourless or slightly yellow chalcone structures (Fig. 2) between pH values 2–6 [5]. pH is thus one of the key factors affecting anthocyanin structure.

Availability of anthocyanins may vary according to the source and may also be influenced by the matrix of the source [6]. In order to evaluate the health potential of anthocyanins, their bioavailability including absorption, metabolism, distribution in the tissues and excretion must be established. Several studies have demonstrated that intact anthocyanins are very poorly absorbed and are excreted only to a small extent in urine [6–8]. Anthocyanins also undergo metabolism, since part of the absorbed anthocyanins have been found as methylated, sulphated or glucuronidated forms [9, 10]. Flavonoid metabolites are delivered again to the GI tract via enterohepatic circulation and after reaching the colon are subjected to bacterial metabolism by colon flora. Little is still known about the biological effects of the metabolites [11]. Bacterial transformations affect the mutagenicity of the ingested compounds, since for example bacterial metabolites of quercetin have been demonstrated to be less mutagenic than the original flavonoid [12]. Thus bacterial metabolites in the colon may have a different biological role than that demonstrated for the parent compound.




| | $R_1$ | $R_2$ |
|---|---|---|
| Pelargonidin | H | H |
| Cyanidin | OH | H |
| Peonidin | $OCH_3$ | H |
| Delphinidin | OH | OH |
| Petunidin | $OCH_3$ | OH |
| Malvidin | $OCH_3$ | $OCH_3$ |

**Fig. 1** Structures of the main anthocyanidins in food

Colon bacteria have a number of deconjugating enzyme activities, e. g. β,D-glucuronidases, β,D-glucosidases and α,L-rhamnosidases, which release aglycones of flavonoids from their glycosides and glucuronides [13–17]. However, the appearance of aglycones is only transient, since a rapid ring fission occurs, producing a number of small phenolic acids: hydroxyphenylacetic acids are formed from myricetin and quercetin [16–19]; hydroxyphenyl propionic acids from catechins, e. g. procyanidins [20], and phenylpropionic acid, cinnamic acid and benzoic acids from chlorogenic acid [21]. Pathways for quercetin (flavonol), (+)-catechin (flavanol) and hesperetin (flavanone) metabolism have been proposed by Hollman and Katan [22] including bacterial and tissue metabolites found in urine or bile. Bacterial metabolism of several flavonoid groups has been well demonstrated both by *in vivo* and *in vitro* data, but scientific evidence for bacterial metabolites of anthocyanins is scarce.

The aim of this study was to study the bacterial metabolism of anthocyanins isolated from edible fruits and berries. The occurrence of deglycosylation and further metabolism of anthocyanins by faecal flora *in vitro* is demonstrated together with a time course of metabolite formation.

## Materials and methods

### Phenolic compounds

Rutin (quercetin-3-*O*-rhamnoglucoside) was purchased from Extrasynthese (Genay, France). Protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxybenzaldehyde, 4-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid were from Aldrich (Steinheim, Germany). Cyanidin-3-glucoside (Cy3 g) and cyanidin-3-rutinoside

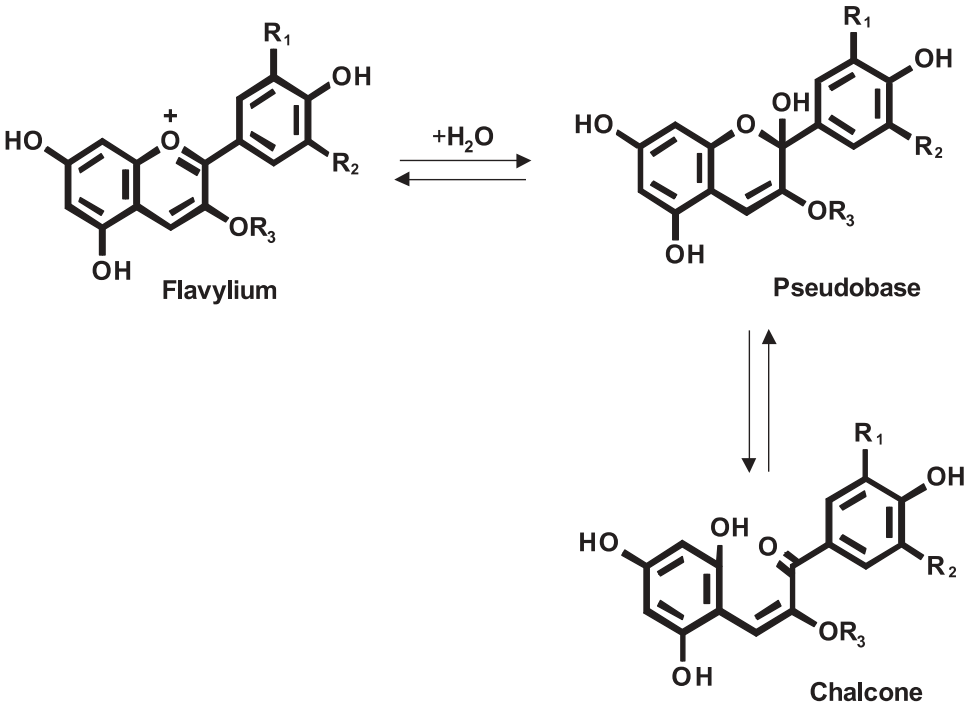



**Fig. 2** Anthocyanidin equilibria in aqueous media

(Cyrut) were obtained from the skins of red plum (*Prunus domestica* L.). Malvidin-3-glucoside, delphinidin-3-glucoside, petunidin-3-glucoside and peonidin-3-glucoside were isolated from the skins of red grapes (*Vitis vinifera* L.).

## Anthocyanin isolation

In order to isolate anthocyanins from plant material, it was submitted to repeated extractions with methanol containing 0.1 % HCl until no relevant red colour, indicating the presence of anthocyanins as flavylium cations, remained in the residue. Methanol phases were mixed and centrifuged (2500 rpm at 4 °C for 10 min), the supernatant was collected, a volume of water was added and the methanol evaporated in vacuo; the aqueous extract was washed with n-hexane and then placed on a column filled with a mixed stationary phase of Silicagel 60 and Polyclar AT [80:20] activated by boiling in HCl; the more polar substances were flushed with water and then the anthocyanins eluted with acidified methanol (0.1 % HCl). The methanol was evaporated in vacuo after addition of water and the concentrated aqueous extract obtained was subjected to semipreparative HPLC for further purification of anthocyanins as described by de Pascual-Teresa et al. [23]. Chromatographic solvents were removed in vacuo and the compounds transferred to water and freeze-dried. The purity and identity of the anthocyanins isolated were checked by HPLC using diode array spectroscopy (HPLC-DAS) and mass spectrometry detection (LC-MS).

## Fermentation protocol

Fermentations of anthocyanins and rutin were performed according to the method described by Aura et al. [17]. Faecal slurry was prepared under strictly anaerobic conditions with the use of faeces from four healthy donors, who usually ingested a normal diet, presented no digestive diseases and had not received antibiotics for at least three months. Freshly passed faeces were immediately taken in an anaerobic chamber. Equal amounts of faecal samples were pooled, homogenised and diluted to 1 % (w/v) or to 5 % (w/v) with carbonate-phosphate buffer (0.11 M carbonate – 0.02 M phosphate; pH 5.5) and filtered through 1 mm sieve. Part of the slurry was autoclaved (121 °C for 20 min) to produce heat-inactivated flora. The phenolic compound was added to empty head-space bottles in methanol, which was spontaneously evaporated. Bottles were inoculated either with active or inactive human faecal slurry and incubated at 37 °C with magnetic stirring (250 rpm) for 0, 10, 20, 30, 60, 90, and 120 min in order to study the deglycosylation of cyanidin-3-glucoside, cyanidin-3-rutinoside and rutin using a 1 % faecal suspension, and for 0, 2, 4, 6, 8 or 24 h in order to study the further metabolism of cyanidin-3-rutinoside. Also, cyanidin-, delphinidin-, malvidin-, petunidin- and peonidin-3-glucosides were incubated with an active faecal suspension (5 %,

w/v) for 0, 6 and 24 h. The initial concentration of the phenolic compound was in all assays 100 μM. Faecal blanks without added substrate were incubated simultaneously. Time courses of compound disappearance and metabolite formation were followed. Samples were freeze-dried and stored at –30 °C prior to the analyses. All the assays were carried out in triplicate.

### Analyses of the anthocyanins and their metabolites

Extraction of the samples was carried out as follows: 10 ml of 0.2 % HCl in methanol was added, the samples were vortex-mixed using a Polytron® PT 10–35 (Kinematica GmbH, Switzerland), placed in an ultrasonic bath (Branson Ultrasonic Corporation, USA) for 10 min and centrifuged (13000 g, 4 °C, 10 min; Sorvall® RC-5B, DuPont). Supernatant was collected and extraction of the residue was repeated four times. The collected methanol phases were concentrated under vacuum to approximately 0.5 ml. Volume was adjusted to 1 ml with acidified water (pH 1.5; HCl) for analysis by HPLC using photodiode array spectrophotometry (HPLC-DAS) and mass detection (LC-MS).

HPLC-DAS was performed using a 3 μm Spherisorb ODS2 column (150 × 4.6 mm). The solvents were 4.5 % formic acid (A) and acetonitrile (B), establishing a linear gradient from 10 to 20 % B over 20 min, 20 to 25 % B over 10 min, 25 to 35 % B over 10 min and 35 % B isocratic 10 min at a flow rate of 1.5 ml/min. Chromatograms were recorded at 280 nm and 520 nm. Anthocyanins were quantified from their chromatographic peak areas recorded at 520 nm by comparison with calibration curves prepared from the anthocyanin standards previously isolated and characterised.

LC-MS analyses were performed by using an AQUA® (Phenomenex, USA) C18, 5 μm (150 × 4.6 mm) column at 35 °C. The solvents were 0.1 % trifluoracetic acid (A) and acetonitrile (B), establishing the following gradient: isocratic 10 % B over 5 min, from 10 to 15 % B over 15 min, isocratic 15 % B over 5 min, from 15 to 18 % B over 5 min, and from 18 to 35 % B over 20 min at a flow rate of 0.5 ml/min. The HPLC system was connected to the probe of the mass spectrometer via the diode array detector cell outlet. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. The capillary temperature was 180 °C and the capillary voltage 3V. Spectra were recorded in positive ion mode. The mass spectrometer was a Finnigan LCQ equipped with an API source, using an electrospray ionisation (ESI) interface.

### Analysis of rutin and quercetin

Samples were extracted with methanol containing ascorbic acid (1 mmol/L) and apigenin (60 μmol/L) as an internal standard and analysis of quercetin derivatives was performed by HPLC-DAS according to Aura et al. [17]. Comparisons of flavonol standards with metabolites formed in the faecal slurries were based on retention time and UV spectra.

### Assays of (α, L)-rhamnosidase, (β, D)-glucosidase and (β, D)-glucuronidase enzyme activities

For the study of the bacterial enzyme activities, the pH of the suspension of faecal inoculum (1 %, w/v) was adjusted to 6.0 by addition of *ortho*-phosphoric acid, the cells were lysed with lysozyme (Sigma L-6876, 1 g/L at 37 °C for 1 h) and the reaction was stopped with sodium azide (2 mg/L). Samples were centrifugated (13000 g for 5 min) to obtain cell-free extract. Enzyme activities were measured from the cell-free extract as described by Aura et al. [17]. Protein concentration was measured according to the method of Bradford [24].

## Results

### Recoveries of the anthocyanins from faecal matrix

In order to study the yield of extraction from faecal matrix, freeze-dried faecal blanks were spiked with malvidin-3-glucoside corresponding to the initial concentration of 100 μM in the incubations. From the initial dose of malvidin-3-glucoside, 66 % ± 8 % (n = 6) was recovered. Incubations with other anthocyanins (i. e., pelargonidin-3-glucoside, delphinidin-3-glucoside and petunidin-3-glucoside, each n = 2) showed an average recovery of 54 % ± 9 %. Recoveries of cyanidin-3-glucoside from 1 % (w/v) active and inactive faecal matrix were 75 % ± 2 % and 64 % ± 8 %, respectively, and those of cyanidin-3-rutinoside from active and inactive faecal matrix were 83 % ± 6 % and 63 % ± 3 %, respectively. When using higher (5 %; w/v) faecal slurry concentration, recovery of cyanidin-3-rutinoside was 42 % ± 3 % and heat-inactivation of the faecal suspension did not change the recovery (43 % ± 15 %).

### α,L-rhamnosidase and β,D-glucosidase activities

The activities of the enzymes, α,L-rhamnosidase and β,D-glucosidase, required for the deglycosylation of flavonoid glycosides, were measured from active and inactive 1 % faecal suspensions. The analysis was performed using *p*-nitrophenyl-(α, L)-rhamnopyranoside and –(β,D)-glucopyranoside as substrates for corresponding enzymes. The specific activities of α,L-rhamnosidase were 0.23 ± 0.05 and 0.12 ± 0.06 nmol/(min·mg protein) for active and inactive faecal slurry, respec-

tively. The specific activities for β,D-glucosidase were 0.63 ± 0.09 and 0.14 ± 0.02 nmol/(min·mg protein) for active and inactive faecal slurry, respectively.

## Deglycosylation of anthocyanins and rutin by gut microflora

The disappearance of the phenolics was followed by HPLC-DAS. Most of the anthocyanin glycosides disappeared within the first hour in the presence of 5 % faecal slurry containing active gut microflora, and only traces of the different anthocyanins were still detectable at that first time point. In the assays carried out with 1 % faecal slurry containing active gut microflora, cyanidin-3-rutinoside was degraded more slowly than cyanidin-3-glucoside (Fig. 3). Small decrease of anthocyanin glycosides occurred also when samples were incubated with inactive flora (Fig. 3). Formation of cyanidin-3-glucoside was detected as an indication of partial deglycosylation of cyanidin-3-rutinoside during incubation with active faecal flora (1 %, w/v) *in vitro* (Fig. 4.). Rutin deglycosylation was studied for comparison, because quercetin is a more stable compound than anthocyanins in aqueous media. Deglycosylation of rutin and appearance of quercetin aglycone were linear using a 1 % faecal flora and occurred at the same rate. Deglycosylation of rutin was not shown in the presence of inactive gut flora (Fig. 5).

## Identification of metabolites

HPLC-DAS chromatograms of the samples incubated with 1 % faecal slurry (active flora) were screened for new peaks that could be attributed to anthocyanin metabolites. The rapid formation of three possible metabolites (Cy-1, Cy-2 and Cy-3 in Fig. 6A) was detected in the chromatograms recorded at 280 nm for both cyanidin-3-rutinoside (Cyrut) and cyanidin-3-glucoside (Cy3g). Another metabolite (Cy-4) was also detected in the chromatograms recorded at 520 nm (Fig. 6B) in the latest time points (120 min). UV-visible spectra of the observed metabolites are shown in Fig. 7. None of these metabolites or any additional ones were observed from anthocyanin glycosides incubated with inactive flora.

LC-MS analysis (a positive molecular ion at m/z 287), UV-visible spectra (Fig. 7) and chromatographic retention verified that Cy-1 was cyanidin, aglycone. No mass spectra could be obtained for Cy-2 and Cy-3, owing to

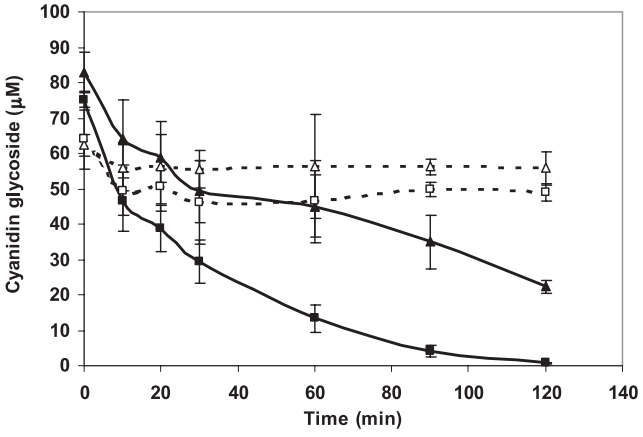



**Fig. 3** Degradation of cyanidin-3-rutinoside (—▲—; ---△---) and cyanidin-3-glucoside (—■—; ---□---) in vitro in the presence of active and inactive gut flora, respectively. Initial concentration of anthocyanin glycosides in the faecal slurries (1 %, w/v) was 100 μM

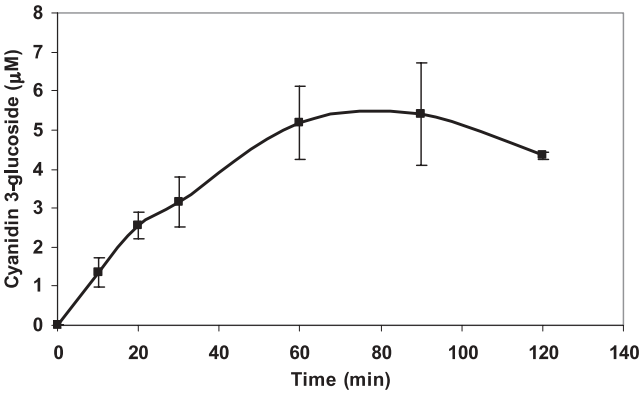



**Fig. 4** Formation of cyanidin-3-glucoside (—■—) from cyanidin-3-rutinoside in vitro in the presence of active gut flora (1 %, w/v). Initial concentration of cyanidin-3-rutinoside was 100 μM

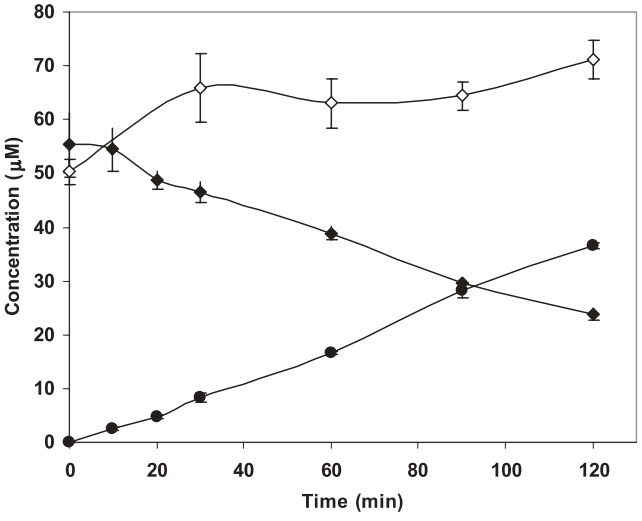



**Fig. 5** Deglycosylation of rutin in vitro in the presence of active (—◆—) and inactive (—◇—) faecal slurry and appearance of quercetin (—●—) in the presence of active flora. Initial concentration of rutin in the faecal slurries (1 % w/v) was 100 μM

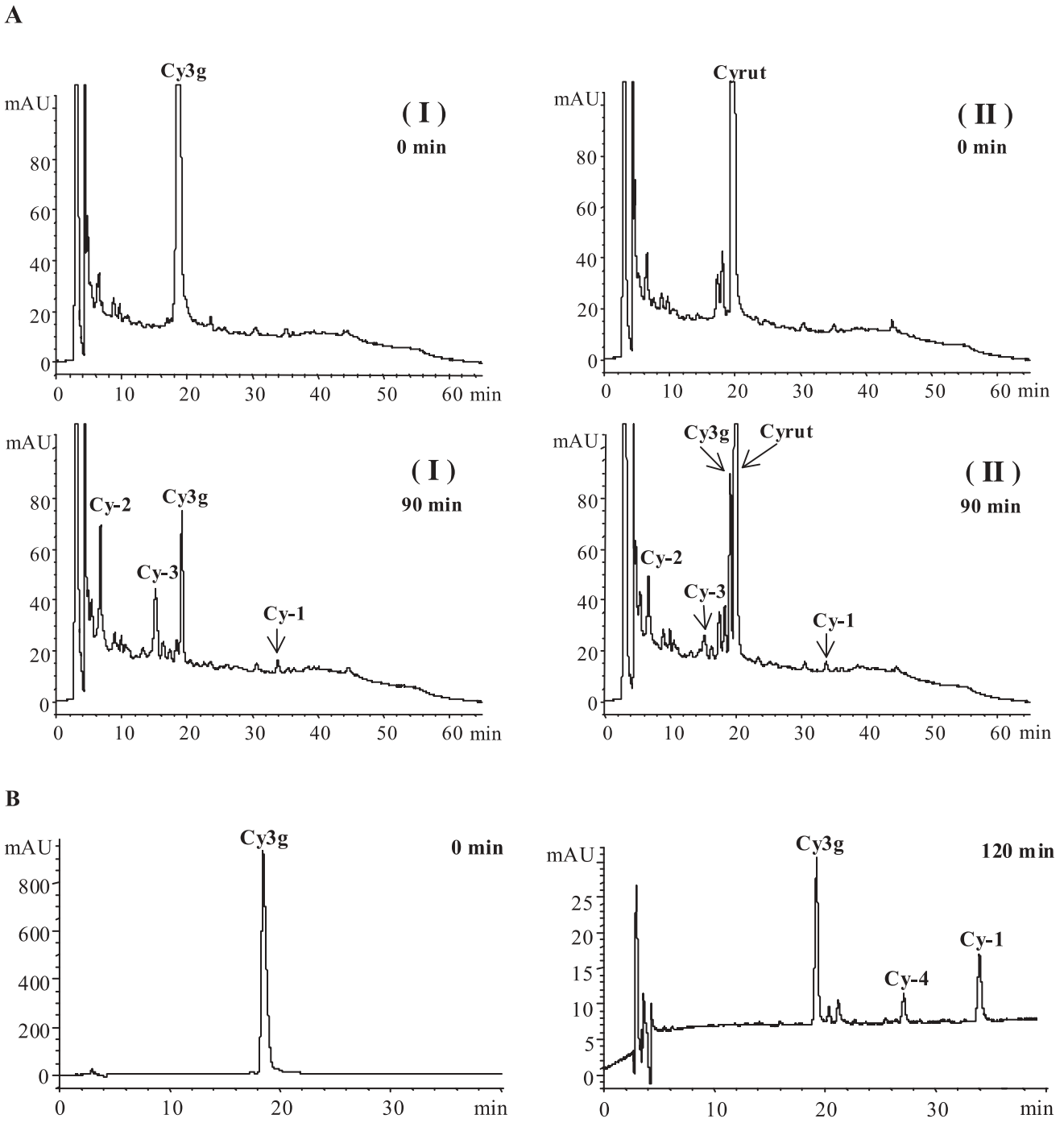



**Fig. 6** HLPC chromatograms recorded at (**A**) 280 nm corresponding to the samples of cyanidin-3-glucoside (Cy3g; I) and cyanidin-3-rutinoside (Cyrut; II) incubated in the presence of active gut flora from a 1 % (w/v) faecal slurry collected at 0 min and 90 min and (**B**) 520 nm corresponding to the samples of cyanidin-3-glucoside (Cy3g) incubated in the presence of active gut flora from a 1 % faecal slurry collected at 0 min and 120 min

the soft ionisation conditions used (typical for anthocyanins). Their UV spectra (Fig. 7) suggested that they could be phenolic acids from the anthocyanin breakdown. Comparison of UV spectra and elution characteristics (co-chromatography) with phenolic standards, allowed positive identification of Cy-2 as protocatechuic acid (i. e., 3,4-dihydroxybenzoic acid). Similar comparison showed that Cy-3 did not correspond to following putative metabolites: vanillic acid, 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 2,4,6-trihydroxyben-

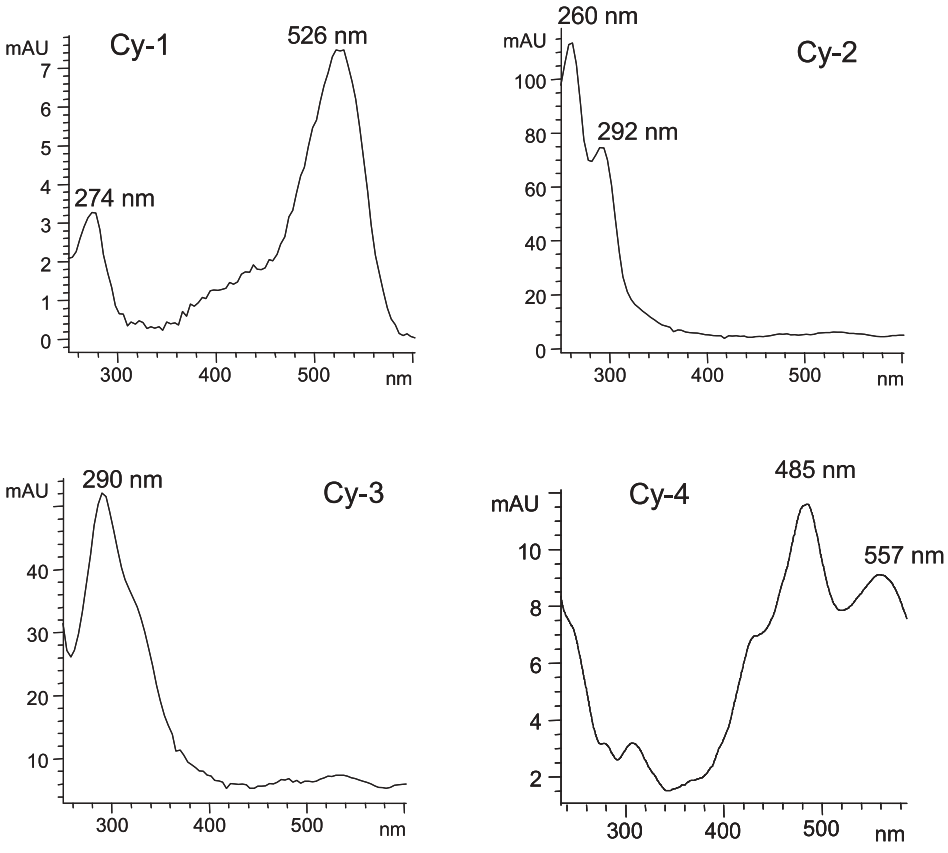



**Fig. 7** UV-visible spectra of possible anthocyanin metabolites produced by gut microflora

zaldehyde, 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid. Cy-2 or Cy-3 were not detected in the samples incubated with 5 % (w/v) faecal slurry in the presence of cyanidin-3-rutinoside at any of the time points, nor were such breakdown products found for any of the other anthocyanins incubated with 5 % (w/v) faecal slurry.

Cy-4 was detected in the incubations carried out either with 1 % or 5 % faecal slurry. Cy-4 showed a positive molecular ion at m/z 372 and its UV-visible spectrum (Fig. 7) was characterised by the presence of two maxima in the visible region at 485 nm and 557 nm. Metabolites with absorption spectra similar to Cy-4 were also observed for all the anthocyanins studied in the fermentation assays carried out with 5 % active faecal slurry; all these metabolites showed the same differences (85 mass units) between their molecular masses and those of the parent aglycones (Table 1).

The time courses of the metabolite formation from cyanidin-3-glucoside using 1 % faecal slurry are shown in Fig. 8A. Cy-1, cyanidin aglycone, appeared only transiently and to a small extent. The major metabolite was Cy-2 protocatechuic acid. Cy-3, a minor unidentified metabolite, appeared at 2 h. Cy-4, the other unidentified conjugate, was formed only at the later time points with cyanidin-3-glucoside and corresponding rutinoside as substrates (Figs. 8A and B). The maximum extent of Cy-4 formation were 0.31 ± 0.08 μM at 2 h and 0.21 ± 0.03 μM at 4 h time point, for cyanidin-3-glucoside with 1 % faecal slurry and cyanidin-3-rutinoside with 5 % faecal slurry, respectively.

## Discussion

Anthocyanins were not recovered completely even from spiked faecal background samples nor from 0 h samples. Since an increase in the faecal slurry concentration decreased the recovery, it is possible that the loss is due to irreversible attachment to the faecal matrix. Protein denaturation and cell disruption might further facilitate the adsorption of anthocyanins and thus explain the greater losses of anthocyanins found at the initial timepoint incubated with heat-inactivated flora.

Both the rate of deglycosylation of rutin and the rate of appearance of quercetin aglycone were linear using a 1 % faecal flora. Formation of cyanidin-3-glucoside from the corresponding rutinoside and the identification of Cy-1 as the cyanidin aglycone appearing from both cyanidin-3-glycoside incubations confirmed the deglycosylation of anthocyanins by gut flora and the presence of deconjugating enzymes. Deglycosylation of

European Journal of Nutrition (2005) Vol. 44, Number 3


**Table 1** Molecular ions of the aglycones and of their Cy-4-like metabolites, size difference between the molecular ions and absorption characteristics of the metabolites

| Anthocyanin | Molecular ion [$M^+$] of the corresponding aglycones (m/z) | Molecular ion [$M^+$] of Cy-4-like metabolites (m/z) | Difference between the molecular ions $\Delta$ [$M^+$] (m/z) | $\lambda_{max}$ in the metabolites spectra in the visible region (nm) |
|---|---|---|---|---|
| Cyanidin-3-glucoside and cyanidin-3-rutinoside | 287 | 372 | 85 | 432, 485, 557 |
| Delphinidin-3-glucoside | 303 | 388 | 85 | 430, 486, 558 |
| Petunidin-3-glucoside | 317 | 402 | 85 | 434, 490, 562 |
| Pelargonidin-3-glucoside | 271 | 356 | 85 | 398, 474, 550 |
| Malvidin-3-glucoside | 331 | 416 | 85 | 432, 494, 566 |

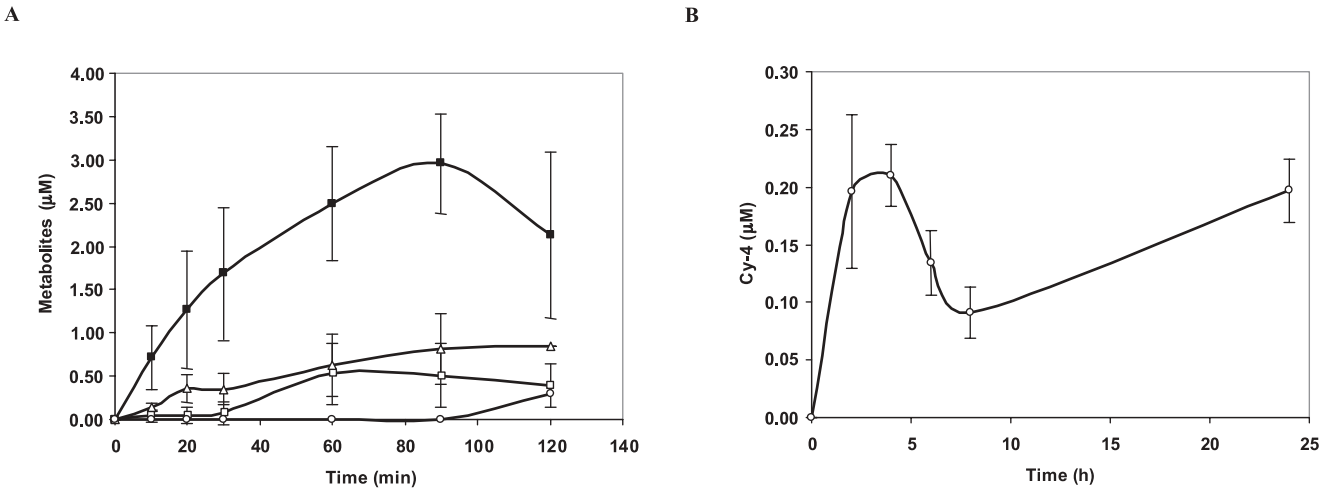



**Fig. 8** Time courses of the formation of the metabolites in the presence of active gut flora. (**A**) Cyanidin-3-glucoside incubated with 1 % (w/v) faecal slurry. (**B**) Cyanidin-3-rutinoside incubated with 5 % (w/v) faecal slurry. Symbols: Cy-1: (—□—) cyanidin aglycone; Cy-2: (—■—) protocatechuic acid; Cy-3: (—△—) Unidentified metabolite; Cy-4: (—○—) Unidentified conjugate

cyanidin-3-rutinoside occurred more slowly than that of cyanidin-3-glucoside, being in agreement with lower levels of α,L-rhamnosidase than β-D-glucosidase in 1 % (w/v) faecal inoculum. This in turn is in agreement with the earlier observation of Aura et al. [17] using a 5 % (w/v) faecal inoculum. Slurry (1 %) was diluted and the specific activities of α,L-rhamnosidase and β-D-glucosidase activities measured were near the background caused by the enzyme source or substrate. The small specific activities measured from the inactive faecal slurry were at the background level.

Cy-2 was identified as protocatechuic acid. Such a compound would result from the fragment corresponding to cyanidin ring-B released after breakdown of the anthocyanidin heterocycle [25]. Various hydroxylated and non-hydroxylated benzoic, phenylacetic or phenylpropionic acid derivatives have also been identified as microbial metabolites from other flavonoids [17–20, 22, 26, 27]. Thus identification of Cy-2 was in agreement with earlier findings.

Cy-3 was not identified. Its UV spectrum, and the fact that it was not ionised in the mild conditions used, indicate that the fragment could be a phenoxy acid or an aldehyde. However, comparison with standard compounds that could be also expected after the anthocyanidin breakdown ruled out 2,4,6-trihydroxybenzaldehyde, 4-hydroxybenzoic acid, 2,4,6-trihydroxybenzoic acid, 4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid as structures for Cy-3.

Cy-4 and similar metabolites derived from the other anthocyanins could not be structurally identified. However, some structural features could be characterised from UV-visible and mass spectra data (Table 1). No loss of a glucose residue (–162 mass units) was observed in the MS-MS fragmentation of any of the Cy-4-like metabolites, indicating that they lack the glucose moiety. On the other hand, the shape of the absorption spectra, showing maxima at 470–570 nm, suggested that the basic flavonoid structure and ring conjugation characteristics of the anthocyanidins were maintained. Also, the increased absorption at 400–430 nm might indicate that the C-4 position of anthocyanidin is substituted. All

the Cy-4-like metabolites derived from each of the anthocyanin glycosides tested showed molecular ions, which were 85 mass units higher than their parent aglycones. All these characteristics indicate that their formation might involve anthocyanin deglycosylation and further conjugation of the aglycone with a still unknown residue, which may contain nitrogen (or sulphur) based on their even molecular ions.

Nitrogen containing metabolites were formed from iridoid glycoside in the presence of ammonium ions by human faecal β-glucosidase [28]. Ammonia is released in the colon from undigested proteins and amino acids by bacterial fermentation [29]. Thus both the enzyme and its co-substrate were present in the faecal fermentation *in vitro,* and nitrogenous metabolites could be present in the anthocyanin experiments. In addition, arylsulphotransferases have been detected from human faecal material, when sulphated metabolites were formed from phenolic antibiotics [30–32]. Thus it is possible that the putative reconjugation of the anthocyanin aglycones may contain a nitrogenous or sulphated group.

The time courses of the formation of bacterial metabolites from cyanidin-glycosides were semi-quantified using the substrate concentration at the initial timepoint. The levels of all metabolites were low compared to the initial dose of the substrate anthocyanin. Cyanidin aglycone (Cy-1) appeared only transiently, because it most likely was converted to the other metabolites. The profile of the main metabolite Cy-2, protocatechuic acid, also appeared transiently and it was not present in the incubations carried out with a denser bacterial population (5%), just as Cy-3, the unidentified metabolite. The late appearance of Cy-4 was confirmed by the experiment with the denser bacterial population (5%) and longer incubation time. The order of the magnitude of its amount was reproducible in both concentrations of the faecal slurry. However, the levels of all metabolites were very low (less than 5%) considering the initial amount of substrate added to the incubations (100 μM).

The low recoveries of the substrate indicate that the capacity of the faecal matrix to bind flavonoids and their possible metabolites are high. Anthocyanins could be converted to as yet undetected metabolites, such as gases, as shown by Walle et al. [33] for radiolabelled $^{14}$C-(C [4])-quercetin. The anthocyanins can also be degraded before the bacterial conversions take place due to the instability of flavylium cation in mildly alkaline pH values [5].

The *in vitro* fermentation method was modified for studying the bioconversion of pure flavonoids [17]. In the *in vitro* fermentation method faeces was diluted to 5% (w/v), which corresponded to caecal concentration of faecal material in humans (5–13%) calculated from the volumes of faeces and chyme entering caecum [34]. In the present study 1% faecal slurry was diluted to be non-physiological in order to verify a rapid deglycosylation of anthocyanins. The initial pH of the medium 5.5 corresponded nicely to the pH 5.43 radiotelemetrically measured from transverse colon in humans [35]. According to McBurney and Thompson [36] it was recommendable to use several, at least three, donors of faecal samples to improve the accuracy of *in vitro* estimates of colonic fermentation. The number of the donors was four and the individual variation was not measured in our study; however, it has been reported for enterolactone production [37] and for equol production [38], production of bacterial metabolites in humans from plant lignans and isoflavonoids, respectively. According to Sawai et al. [26] the serum concentration of bacterial metabolites had returned to the baseline values within 20–35 h after consumption. Thus the end-point of fermentation was set to 24 h, which was also used as an endpoint in the fermentation experiments with quercetin derivatives [17] and non-digestible carbohydrates [39].

## Conclusions

Cyanidin-3-glucoside and cyanidin aglycone could be identified as intermediary metabolites of cyanidin-3-rutinoside. Also, cyanidin-3-glucoside was deglycosylated. The identification of protocatechuic acid as the major metabolite and formation of two unidentified metabolites not present in the faecal background, or in the samples incubated with inactive flora, indicates that anthocyanins are converted by gut microflora. Furthermore, reconjugation of the aglycone with other groups, non-typical for dietary anthocyanins, is evident. Bacterial metabolism involves the cleavage of glycosidic linkages and breakdown of the anthocyanidin heterocycle.

It is not adequate to predict the health effects of anthocyanins by their intact structures. According to the findings presented above it is evident that anthocyanins can be transformed by bacteria in the colon to smaller phenolic compounds or conjugates of the aglycone, of which health implications are poorly characterised. Both the local effects in the colon and systemic effects in human body should be studied taking into consideration the changes in the flavonoid structures by bacterial action in the colon.

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