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ORIGINAL CONTRIBUTION

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Stability and biotransformation of various dietary anthocyanins *in vitro*

Summary *Background* Anthocyanins, which are found in high concentrations in fruit and vegetable, may play a beneficial role in retarding or reversing the course of chronic degenerative diseases. However, little is known about the biotransformation and the metabolism of anthocyanins so far. *Aim of the study* The aim of the study was to investigate possible transformation pathways of anthocyanins by human faecal microflora and by rat liver microsomes as a source of cytochrome P450 enzymes as well as of glucuronyltransferases. *Methods* Pure anthocyanins, an aqueous extract of red radish as well as the assumed degradation products were incubated with human faecal suspension. The incubation mixtures were purified by solid-phase extraction and analysed by HPLC/DAD/MS and GC/MS. Quantification was done by the external standard method. Furthermore the biotransformation of anthocyanins by incubation with rat liver microsomes in the presence of the cofactor NADPH (as a model for the phase I oxidation) and in the presence of activated glucuronic acid (as a model for the phase II glucuronidation) was investigated. *Results* Glycosylated and acylated anthocyanins were rapidly degraded by the intestinal microflora after anaerobic incubation with a human faecal suspension. The major stable products of anthocyanin degradation are the corresponding phenolic acids derived from the B-ring of the anthocyanin skeleton. Anthocyanins were not metabolised by cytochrome P450 enzymes, neither hydroxylated nor demethylated. However they were glucuronidated by rat liver microsomes to several products. *Conclusions* The gut microflora seem to play an important role in the biotransformation of anthocyanins. A rapid degradation could be one major reason for the poor bioavailability of anthocyanins in pharmacokinetic studies described so far in the literature. The formation of phenolic acids as the major stable degradation products gives an important hint to the fate of anthocyanins in vivo.

Key words anthocyanins – biotransformation – human gut microflora – degradation – stability – phenolic acids

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Abbreviations

aA1	Pelargonidin-3-sophorosid-5-glucoside acylated with ferulic
aA2	Pelargonidin-3-sophorosid-5-glucoside acylated with ferulic and malonic acid
API-ES	atmospheric pressure ionisation
Cy	cyanidin
Cy3 glu	cyanidin-3-glucoside
DAD	diode array detection
DP	degradation product
DP2	Pg-glu/soph
DP3	Pg-soph

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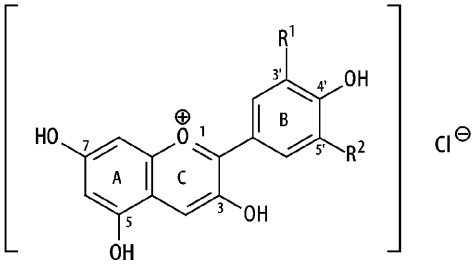
Dp	delphinidin
ESI	electrospray ionisation
HFM	human faecal microflora
HPLC	high performance liquid chromatography
MS	mass spectrometry
Mv3 glu	malvidin-3-glucoside
Mv	malvidin
Pn3 glu	peonidin-3-glucoside
Pg	pelargonidin
Pg-glu/soph	pelargonidin-3-sophorosid-5-glucoside
Pn	peonidin
Pt	petunidin
UDPGA	uridindiphosphate glucuronic acid

Introduction

Anthocyanins belong to a large group of secondary plant metabolites collectively known as flavonoids. They are widely distributed in fruit and vegetable but are also found in flowers and other plant materials and are responsible for the red, blue and purple colours. From the chemical point of view, anthocyanins are glycosides of 2-phenylbenzopyrylium (flavylium) salts derivatives, which differ in the number of hydroxy and/or methoxy groups as well as in the nature and number of the attached sugar moieties. The most common naturally occurring anthocyanins are the 3-*O*-glucosides and the 3,5-*O*-diglucosides of malvidin (Mv), cyanidin (Cy), pelargonidin (Pg), delphinidin (Dp), petunidin (Pt) and peonidin (Pn). The chemical structure of the most important anthocyanins and their aglycones, which are termed anthocyanidins, are shown in Fig. 1.

Studies on the biological activity demonstrated that anthocyanins and anthocyanidins are powerful antioxidants in various test systems [1–4]. Furthermore, anti-inflammatory actions, as well as antimicrobial and anti-tumor activities have been described [5–7]. Because of these multiple biological properties, anthocyanins – like many other flavonoids – may contribute to the prevention of diseases related to oxidative stress, such as coronary heart diseases and cancer.

So far, most of the research on the biological activities of anthocyanins with regard to their potential health effects has been performed *in vitro*. For that reason, research in the last few years focused on the bioavailability and biotransformation of anthocyanins, which could be major determinants of the biological activity of these compounds *in vivo*. Until now, the bioavailability of anthocyanins was mostly investigated in humans after the consumption of anthocyanin-rich food like blackcurrant juice [8], red grape juice and red wine [9], elderberries and blueberries [10] as well as strawberries [11]. One consistent result of these studies is that the systemic bioavailability of anthocyanins is very poor. Only 0.02–1.80 % of the ingested anthocyanin doses were excreted unchanged or as phase II metabolites in the urine of the volunteers. For example, Felgines et al. [11] recently found that Pg-3-glucoside, which was ingested by volunteers with a meal containing 200 g of strawberries, was metabolized to three monoglucuronides, one sulfoconjugate and the aglycone Pg itself. The total urinary excretion of all detected anthocyanin metabolites was 1.8 % of Pg-3-glucoside ingested.



	R^1	R^2	abbr. of the 3-glucoside
cyanidin	OH	H	**Cy3glu**
malvidin	OCH_3	OCH_3	**Mv3glu**
pelargonidin	H	H	**Pg3glu**
peonidin	OCH_3	H	**Pn3glu**

Fig. 1 Chemical structures of common anthocyanidins

However, the question what happened to more than 98 % of the anthocyanins in the human body is still unanswered. In the present study, we investigated the biotransformation of anthocyanins by using two *in vitro* models. Firstly, we determined the metabolism of anthocyanins in the gut by incubation of pure anthocyanins with suspensions of human faecal microflora under anaerobic conditions. Secondly, we incubated pure anthocyanins with rat liver microsomes to evaluate the phase I metabolism by cytochrome P450 enzymes and the phase II metabolism by glucuronyltransferases. The analysis of the incubation extracts was carried out by HPLC/DAD and HPLC/ESI-MS techniques.

Material and methods

Chemicals

Cyanidin-3-glucoside (Cy3glu), malvidin-3-glucoside (Mv3glu), peonidin-3-glucoside (Pn3glu), peonidin (Pn), pelargonidin (Pg) and delphinidin (Dp) were purchased from Polyphenols AS (Sandnes, Norway), cyanidin-3,5-diglucoside (cyanin), malvidin-3,5-diglucoside

(malvin), cyanidin (Cy) and malvidin (Mv) as well as vanillic, syringic, ferulic and caffeic acid were obtained from Roth (Karlsruhe, Germany), protocatechuic and coumaric acid, uridindiphosphate glucuronic acid (UDPGA) and $NADP^+$ were from Fluka (Buchs, Switzerland). All other chemicals were purchased from Merck (Darmstadt, Germany). To prepare the red radish extract, the pared surface material of red radish (*Raphanus satius L.*) was extracted with 1 % trifluoroacetic acid in methanol. The extract was then filtered and concentrated under reduced pressure.

Stability studies

Different anthocyanidins (Cy, Mv, Pg, Pn, Dp) and various anthocyanins (Cy3glu, Mv3glu, cyanin, malvin) were dissolved in a 0.05 M potassium phosphate buffer (pH 7.4) as well as in a cell culture medium (Dulbeccos's modified Eagle's medium, DMEM, without any supplements) at 37 °C. The final concentration of the test compounds was about 160 µM. After defined periods of time (0, 0.5, 1, 2, 3, and 5 h) an aliquot was taken from the incubation mixture. The aliquot was mixed with 10 % (v/v) phosphoric acid (85 %) to prevent further degradation. The reaction mixture was analysed with HPLC/DAD and HPLC/ESI-MS.

Preparation of human faecal suspension

The human faecal suspension was prepared as described [12]. In brief, fresh faecal samples (approx. 2.5 g), obtained from two healthy male volunteers, who had not taken any antibiotics for at least the previous six months, were homogenised with 25 mL potassium phosphate buffer (100 mM, pH 7.3, degassed with ultrasonic and flushed with N_2 to eliminate O_2) and filtered through gauze to remove insoluble particles. The filtrate was diluted up to 50 mL with the same buffer, aliquoted and stored at –80 °C until further use.

Incubation with human faecal microflora (HFM) and sample clean-up

The substrate (different anthocyanins, different phenolic acids and red radish extract) was dissolved in a total volume of 2 mL of phosphate buffer (100 mM, pH 7.3) containing 300 µL of a reducing medium (240 mg $Na_2S \cdot 7H_2O$ in 40 mL H_2O with 1700 µL 1N NaOH) and 10 µL of a solution of different trace elements (3.3 g $CaCl_2 \cdot 2H_2O$, 2.5 g $MnCl_2 \cdot 4H_2O$, 0.25 g $CoCl_2 \cdot 6H_2O$, 2 g $FeCl_3 \cdot 6H_2O$ in 250 mL bidestilled water). This solution was incubated with 3 mL of faecal suspension in an anaerobic chamber at 37 °C. The anaerobic conditions were realised with anaerocult A (Merck, Darmstadt, Germany) and controlled with anaerotest stripes (Merck, Darmstadt, Germany). After defined periods of time (0, 0.5, 1, 2, 3, 4, 24 and 48 h) an aliquot of 0.5 mL was taken from the incubation mixture. The fermentation was stopped by adding 50 µL phosphoric acid (85 %). Negative control incubations were performed with autoclaved faecal suspension (121 °C, 30 min) and treated in the same way. To check our incubation conditions we used the soy isoflavone daidzein as a positive control. Daidzein was transformed under the described conditions to dihydrodaidzein and equol. The samples were centrifuged at 4000 g for 5 min and the supernatants were applied to an Oasis HLB-cartridge (1 ml, 30 mg; Waters, Milford, USA) preconditioned with 2 mL methanol and 2 mL of an aqueous hydrochloric acid solution (0.01 %, v/v). The column was then washed with 1 mL aqueous hydrochloric acid solution (0.01 %, v/v) and the anthocyanins as well as the assumed degradation products were eluted with 2 mL of hydrochloric acid in methanol (0.01 %, v/v). The solid phase extraction step had a recovery rate of more than 86 % for the anthocyanins and more than 91 % for the used phenolic acids. The eluate was dried under nitrogen. The dry residue was dissolved in 125 µL aqueous trifluoroacetic acid (1 %, v/v), vortex-mixed and analysed by injecting 50 µL into the HPLC system.

Incubation with rat liver microsomes and NADPH

The microsomes were prepared from rat livers as described [13]. Standard incubation mixtures contained 2 mg microsomal protein, anthocyanidin-3-glycosides (Cy3glu, Mv3glu, Pn3glu) at a final concentration of 50 µM or anthocyanin aglycones (Pg, Cy and Pn at a final concentration of 1 mM) and a NADPH-generating system (3 mM $MgCl_2$, 1 mM $NADP^+$, 8 mM D,L-isocitrate and 0.5 U isocitrate dehydrogenase) in a final volume of 2 mL 0.05 M potassium phosphate buffer pH 7.4. After 2 min of preincubation at 37 °C in a shaking water bath, the reaction was started by adding the NADPH-generating system and stopped after 30 min by adding 100 µL H_3PO_4 (85 %). Due to their instability, the anthocyanin aglycones were added together with the NADPH-generating system after preincubation. After centrifugation (5 min at 7000 g), the samples were immediately analysed by HPLC. Negative controls were carried out by omitting the NADPH-generating system or by using heat-inactivated microsomes. As a positive control we used the known reaction of ethoxycoumarin to umbelliferone.

Microsomal glucuronidation of anthocyanins and anthocyanidins

Cy as a representative for anthocyanidins (at a final concentration of 1 mM), Cy3 glu as an anthocyanidin-3-glycoside (at a final concentration of 100 µM) and anthocyanidin-3,5-diglydosides (malvin, cyanin at a final concentration of 100 µM) were each incubated with rat liver microsomes in a final volume of 100 µl of 100 mM sodium phosphate buffer, pH 7.4, containing 100 mM $MgCl_2$, 5 mg/mL microsomal protein and 1 mM UDPGA at 37 °C for 30 min. The reaction was stopped by adding 20 µL TCA solution (20 % in water), followed by neutralisation with 25 µL 1M NaOH. After centrifugation (5 min at 7000 g), the samples were immediately analysed by HPLC/DAD and HPLC/MS. Negative controls were carried out by omitting UDPGA or by using heat-inactivated microsomes.

HPLC analysis

HPLC analysis was performed on a Shimadzu system equipped with a photodiode array detector. Separation was carried out on a Nova-Pak C18 (250 × 4.6 mm; 4 µm) reversed-phase column (Waters, Milford, USA) using aqueous trifluoroacetic acid (1 %) as mobile phase A and acetonitrile as mobile phase B with the following linear gradient: From 0 min to 15 min 100 % A, changing then in 10 minutes to 11 % B, then in 7 minutes to 14 % B followed by holding there for 8 minutes, changing in 10 minutes to 20 % B, and in further 10 minutes to 50 % B and finally in 10 min to 100 % B. The flow rate was 0.8 mL/min and the eluent was recorded with a diode array detector at 520 nm for the anthocyanins and 260 nm for the degradation products. Detected peaks were scanned between 200 and 600 nm. The concentrations of Cy3glu, Mv3glu, Pn3glu, malvin, cyanin, syringic acid, protocatechuic acid and vanillic acid were calculated from calibration curves with standard solutions. Components were identified according to retention time, UV/visible spectra and spiking with standards.

HPLC/MS analysis

HPLC/MS experiments were carried out on an HP 1100 series HPLC instrument (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler, a binary HPLC pump, a column heater, a diode array detector, as well as a HP ChemStation for data collection. The column was a Waters Nova Pak C18 HPLC column, 4 µM, 250/4.6 mm (Waters, Milford, USA). The solvents were (A) 5 % formic acid in water and (B) methanol, with a gradient of % B: initial, 5 %; 10–15 min, 20 %; 30–35 min, 30 %; 50–55 min, 45 %; 65–68 min, 5 % (run time 68 min). Flow rate was 0.7 ml/min. The HPLC apparatus was interfaced to an HP series 1100 mass-selective detector with an atmospheric pressure ionization electrospray chamber. Conditions for analysis in the positive mode included a capillary voltage of 2.5 kV, a nebulising pressure of 30 psig, a drying gas temperature of 350 °C at a drying gas flow of 12 l/min. The fragmentor voltage was varied from 70 to 200 V. Data were collected on an HP ChemStation using scan mode over a mass range of *m/z* 100–1500 at 0.7 s per cycle.

Results

Stability of anthocyanidins and anthocyanin-glucosides

Anthocyanidins are unstable compounds in neutral media. To determine their stability in detail, we incubated the anthocyanidins Mv, Cy, Pg, Dp and Pn at concentrations of 160 µM in phosphate buffer (pH 7.4) at 37 °C and tracked the degradation by HPLC/DAD/MS analysis. Mv, Cy, Pn and Dp had almost completely disappeared after 60 min and several new peaks were detected. To give an example the representative chromatograms of the degradation process of Cy are shown in Fig. 2. Peaks 1, 2, 3, 4 and 5 in Fig. 2B have an anthocyanidin-like absorption spectrum revealing that the anthocyanidin skeletal structure still exists. The positive HPLC/API-ES spectra, which were taken with fragmentor voltages of 100 and of 200 V to detect both the molecular ions and characteristic fragment ions, are shown in Fig. 3. The peaks 1, 3 and 4 exhibited (quasi-) molecular ions at m/z 589, peak 2 and 5 molecular ions at m/z 605 and m/z 587, respectively. We assume that these peaks were dimerisation products of two Cy units, because a major pH form of the anthocyanidins in neutral media is the reactive quinoid base. Beside these dimers, which were also detected in the case of all other anthocyanidin aglycones, the corresponding phenolic acid (in case of Cy protocatechuic acid in Fig. 2C) of each anthocyanidin was detected. The mechanism and the formed degradation products are shown in Fig. 4. The stability of the anthocyanidins is influenced by the B-ring substituents. Additional hydroxy or methoxy groups decrease the stability of the aglycone in neutral media. Therefore Pg is the most stable anthocyanidin. In comparison to the other aglycones only about 20 % of Pg disappeared during a 30 min incubation period.

In contrast to the aglycones, the anthocyanin-monoglucosides and even more the anthocyanin-diglucosides are rather stable under neutral pH conditions. The reason for the different behaviour is that the sugar moieties prevent the degradation of the highly unstable α-diketone intermediates to the phenolic acid and the

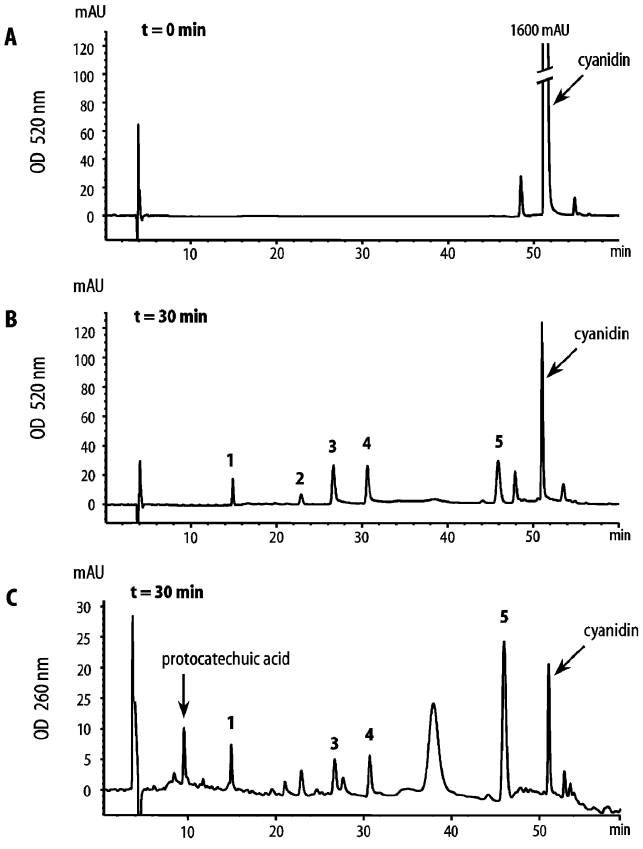


Fig. 2 HPLC chromatograms of the degradation process of cyanidin monitored at 520 and 260 nm

aldehyde component. Furthermore it is most likely that dimerisation of the anthocyanidins is prevented by the steric demanding sugar moieties. The various pH forms of the anthocyanidins and anthocyanins are summarised in Fig. 5.

Degradation of anthocyanin-3-glucosides by HFM

After anaerobic incubation of Mv3glu, Pn3glu and Cy3glu as three representatives for anthocyanin-monoglucosides, the incubation mixture was purified by solid phase extraction with Oasis HBL cartridges. This procedure allows the isolation of the non-degraded anthocyanins as well as the isolation of assumed degradation products like phenolic acids. The incubation of anthocyanin-monoglucosides with human faecal flora resulted in a rapid decrease of the parent compounds (data not shown). After 2 hours, more than 90 % of all three anthocyanin-3-glucosides were degraded. In control incubations with heat-inactivated faecal suspension only a slight decrease could be observed suggesting that the microflora is primarily responsible for the degradation process. During the incubations new metabolites were formed and identified as the respective corresponding phenolic acids emanating from the B-ring of the anthocyanidins. In case of Mv3glu and Pn3glu syringic and vanillic acid were identified as the major degradation products respectively. In accordance to our previously described results Cy3glu was degraded to protocatechuic acid [14]. Therefore the degradation of anthocyanin-3-glucosides to the corresponding phenolic acids seems to be a general pathway for these substances.

Although the degradation to the phenolic acid is an important pathway, it cannot explain the total disappearance of the anthocyanin-3-glucosides during incubation. Calculated on the basis of our *in vitro* fermentation studies the amount of the formed phenolic acid reached up to a maximum of 62 % of the parent compound. Thus, reactions other than the shown breakdown pathway must also play a role in the degradation of anthocyanins.

Degradation of anthocyanin-3,5-diglucosides by HFM

In addition, we studied the degradation of the anthocyanin-3,5-diglucosides cyanin and malvin. In accordance with our results for the anthocyanin-monoglucosides we found a distinct decrease of both diglucosides after incubation with HFM (Fig. 6A). During the incubation time of cyanin a new peak was detected by HPLC analysis at 520 nm which had the same retention time and absorption spectra as Cy3glc. This monoglucoside reached its highest concentration after 30 min and decreased to a zero-level after four hours (Fig. 6B). Simultaneously we observed a clear increase in the concentration of protocatechuic acid, the decomposition product of Cy (Fig. 6C). Analogous results were obtained for malvin. In the first step Mv3glu was formed, which was further degraded to syringic acid, the corresponding phenolic acid derived from the B-ring of Mv. In all control incubations with autoclaved faecal suspension both diglucosides were very stable. Neither a decrease of the substrate concentration nor a formation of new products was observed.

Degradation of acylated anthocyanins by HFM

To study the degradation process of acylated anthocyanins we used an anthocyanin extract of red radish. The main anthocyanin compounds in red radish are – corresponding to own HPLC/MS analysis as well as to data from the literature [15] – derivatives of pelargonidin-3-sophorosid-5-glucoside (Pg-glu/soph), which are acylated at different alcohol groups of the sugar moieties with *p*-coumaric acid, ferulic acid, caffeic acid and malonic acid. As shown for the anthocyanin mono- and diglucosides the acylated anthocyanins are also de-

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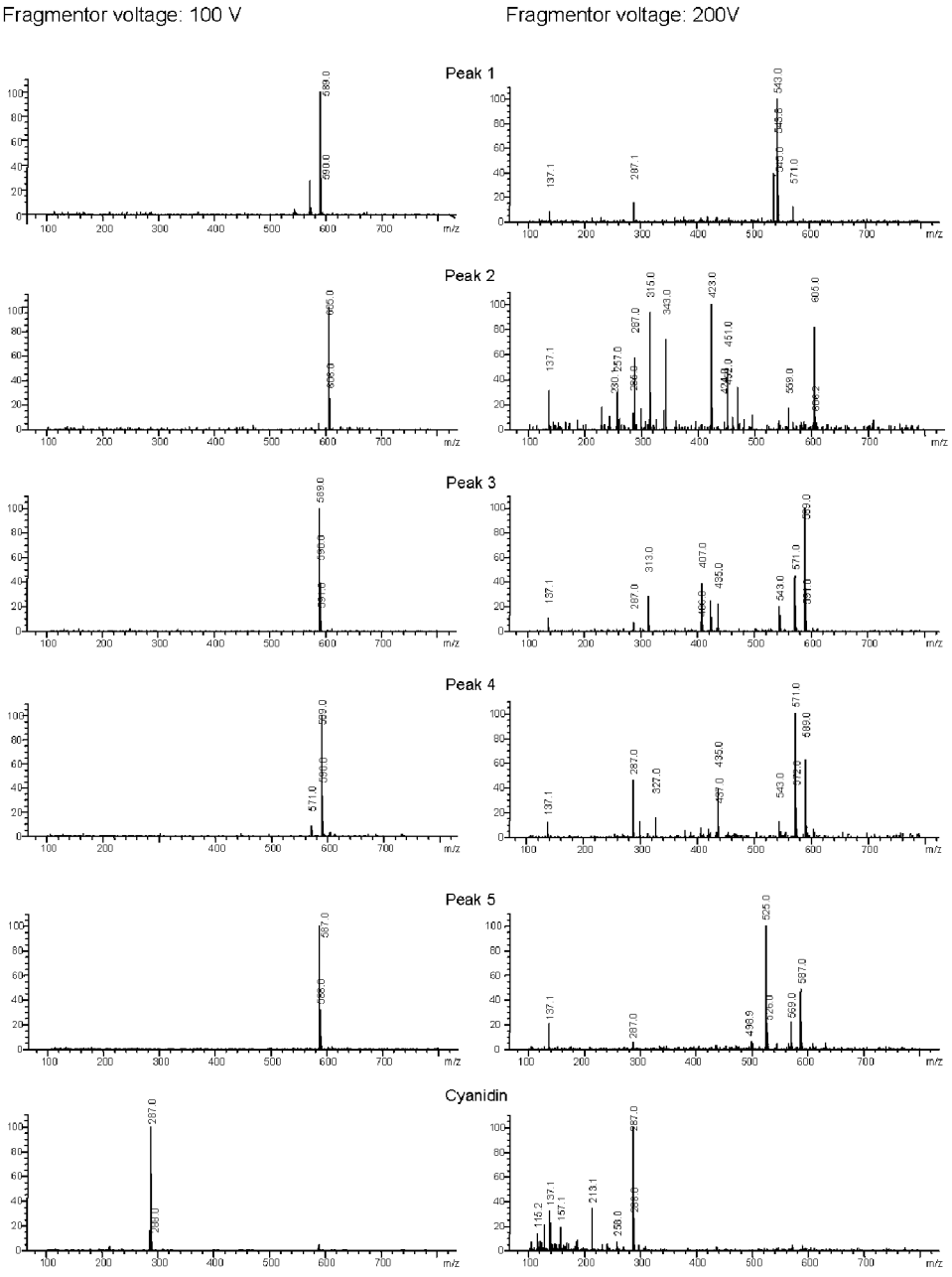
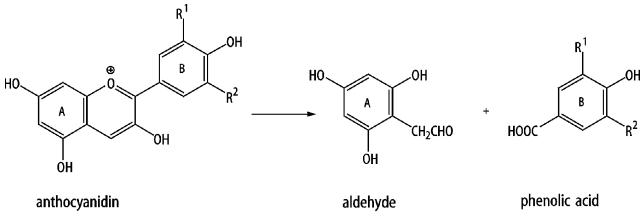


Fig. 3 HPLC/API-ES mass spectra of the degradation products of cyanidin formed under neutral pH conditions

graded by HFM. The red radish anthocyanins aA1 (Pg-glu/soph acylated with ferulic acid) and aA2 (Pg-glu/soph acylated with ferulic and malonic acid) were degraded to different intermediates with an anthocyanin skeleton (Fig. 7). Two main intermediate degradation products (DP) were identified as Pg-glu/soph (DP2) and Pg-soph (DP3) by retention time and HPLC/MS analysis; the structure of a further product is yet unknown (DP1). The end products of the acylated Pg-anthocyanins are the decomposition product of the anthocyanidin Pg, which is 4-hydroxybenzoic acid (Fig. 7B and C, detection at 260 nm), as well as the hydroxycinnamic acids *p*-coumaric, ferulic and caffeic acid (Fig. 7B and C, detection at 320 nm). The proposed formation pathway of the different metabolites is summarised in Fig. 8.

Degradation of phenolic acids by HFM

Syringic and vanillic acid showed no alteration after incubation with HFM. For protocatechuic acid a slight de-



anthocyanidin	corresponding phenolic acid
cyanidin	**protocatechuic acid**
malvidin	**syringic acid**
peonidin	**vanillic acid**
pelargonidin	**4-hydroxybenzoic acid**

Fig. 4 Degradation of anthocyanidins and formation of the corresponding phenolic acids

crease in the substrate concentration was observed after a long incubation period of 24 hours (data not shown). In all cases no metabolites were detected by HPLC/DAD.

Incubation with rat liver microsomes in the presence of NADPH

Rat liver microsomes in the presence of NADPH are a well known *in vitro* model to investigate the oxidative phase I metabolism of xenobiotics catalysed by cytochrome P450 enzymes. In all incubations with different anthocyanins there was no detectable reaction, leading to the conclusion that anthocyanins (monoglucosides as well as anthocyanidins) are not substrates for these enzymes.

Incubation with rat liver microsomes in the presence of UDPGA

The reaction of Cy as a representative for anthocyanin aglycones with a system consisting of microsomes and UDPGA, was used as an *in vitro* model for the glucuronidation reaction, an important phase II metabolising pathway. We identified two Cy-monoglucuronides by HPLC/DAD/MS analysis. The absorption and mass spectra are shown in Fig. 9. Beside these phase II prod-

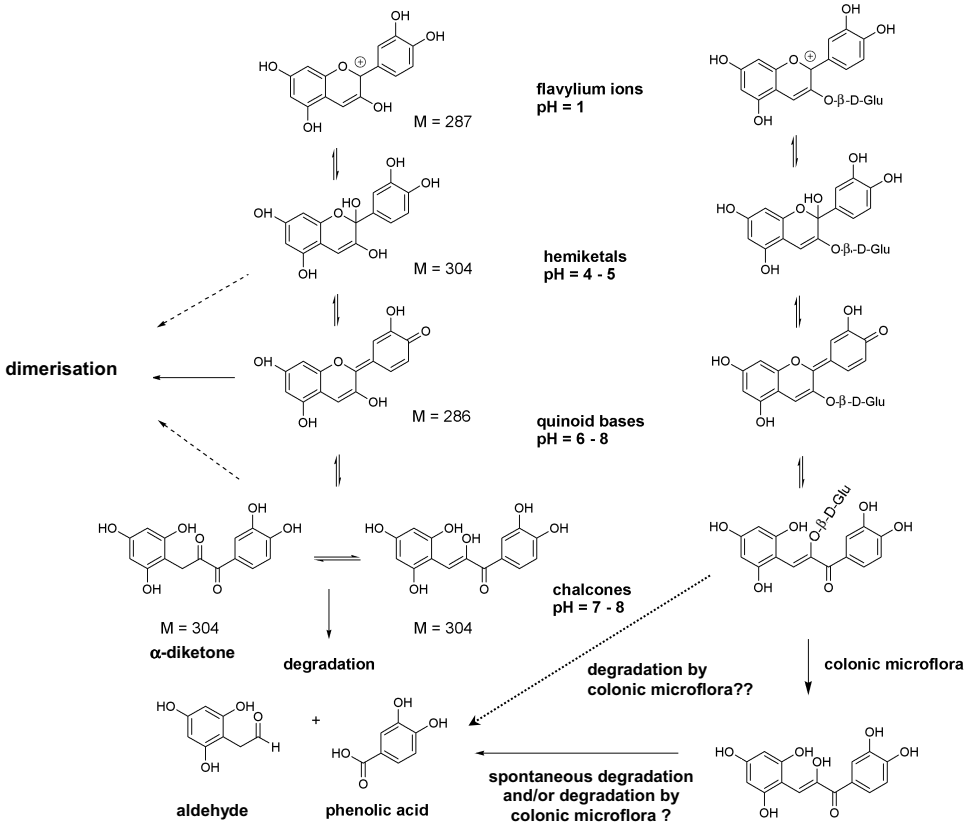


Fig. 5 pH-dependent structural changes and degradation of cyanidin and cyanidin-3-glucoside

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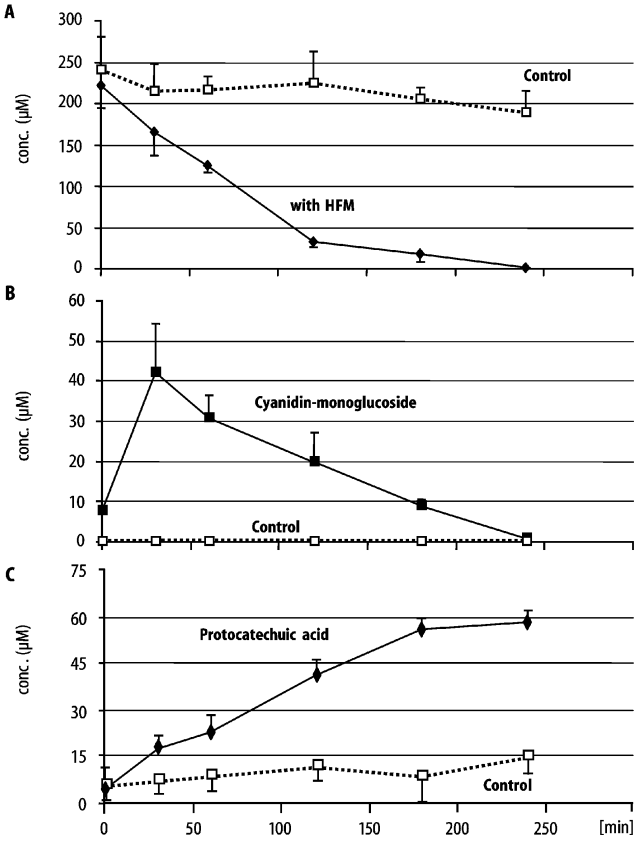

Fig. 6 Time-dependent degradation of cyanin and the formation of the degradation product protocatechuic acid during the anaerobic incubation with human faecal flora. Values are expressed as means ± SD, n = 3

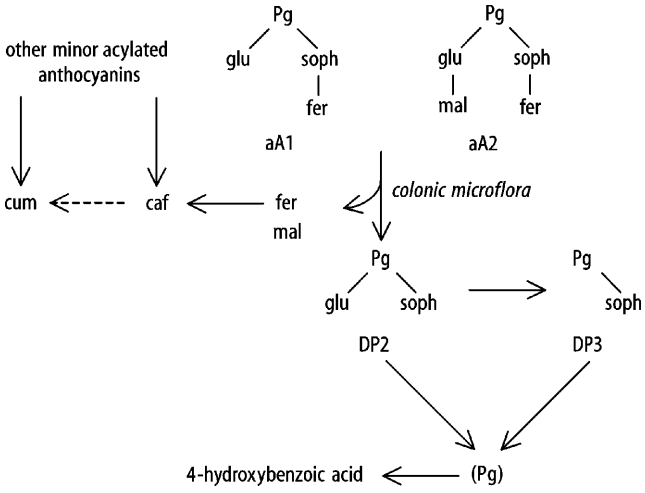

Fig. 8 Proposed formation pathway for the degradation of the acylated anthocyanins of red radish (*glu* glucose, *soph* sophorose, *fer* ferulic acid, *mal* malonic acid, *caf* caffeic acid, *cum* cumaric acid, *aA1* pelargonidin-3-sophorosid-5-glucoside acylated with ferulic acid; *aA2* pelargonidin-3-sophorosid-5-glucoside acylated with ferulic and malonic acid)

ucts, we detected two degradation products (DP) due to the instability of the aglycones. Interestingly, the anthocyanidin monoglucosides are also substrates for UGT-glucuronyltransferases. In the case of Cy3glu we detected four different glucuronidated products by HPLC/DAD (Fig. 10) which were identified as mixed monoglucoside-monoglucuronides by HPLC/MS (Fig. 11). In contrast to the aglycones and the monoglucosides, the anthocyanin-diglucosides were no substrates for the glucuronyltransferases. Glucuronidated products were not found.

Discussion

Incubations of anthocyanin aglycones in buffer solution and common cell culture media show that these substances are highly unstable under neutral pH condi-

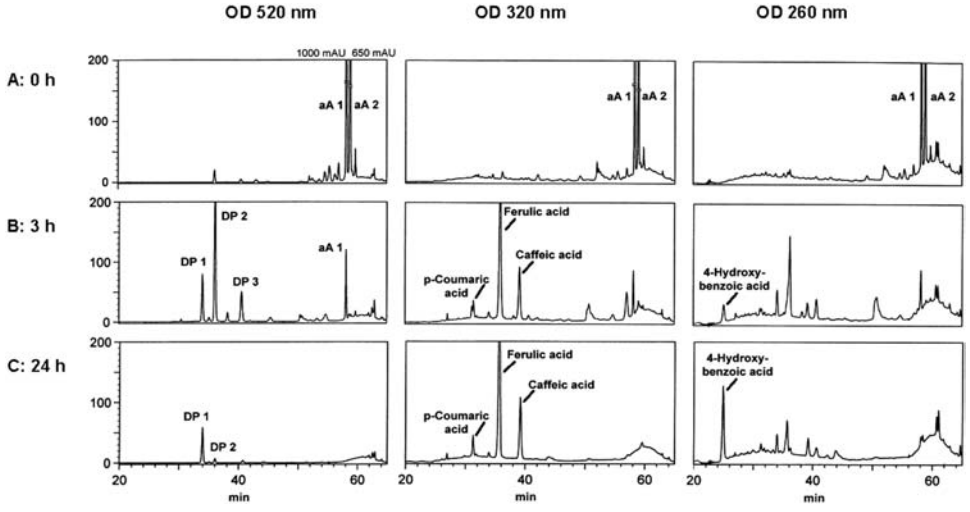

Fig. 7 Representative HPLC chromatograms of the degradation of acylated anthocyanins isolated from red radish. **A** shows the chromatogram before the incubation, **B** 3 hours and **C** 24 hours after reaction beginning. The chromatograms are shown at three different wavelengths

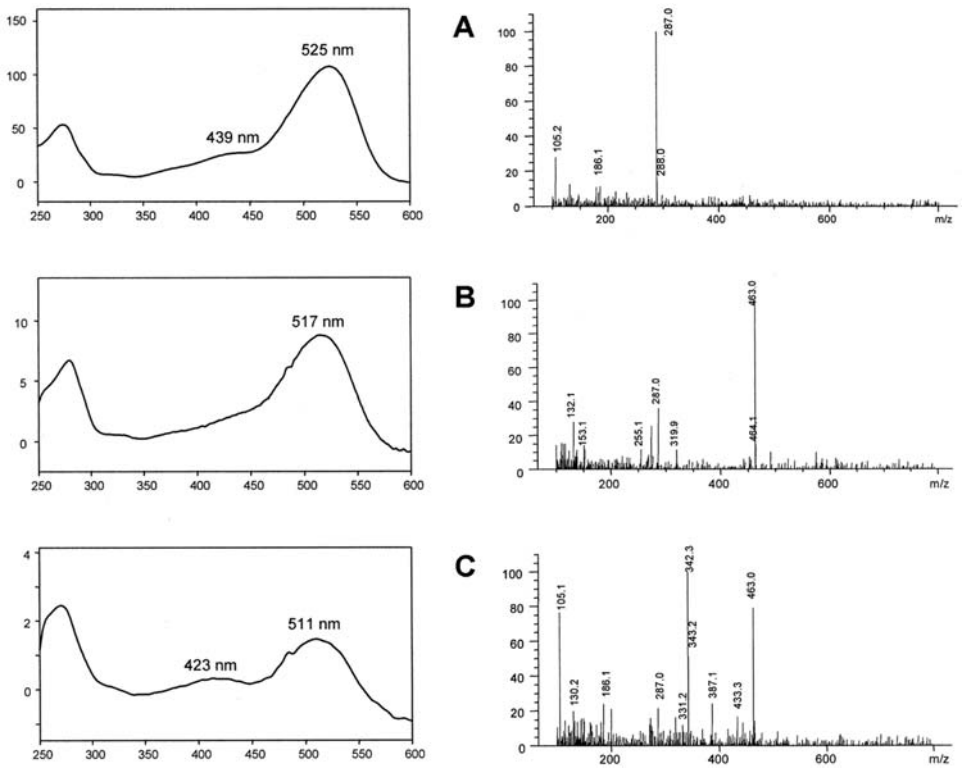

Fig. 9 Absorption spectra and mass spectroscopic spectra of cyanidin (**A**) and the two monoglucuronides (**B**, **C**)

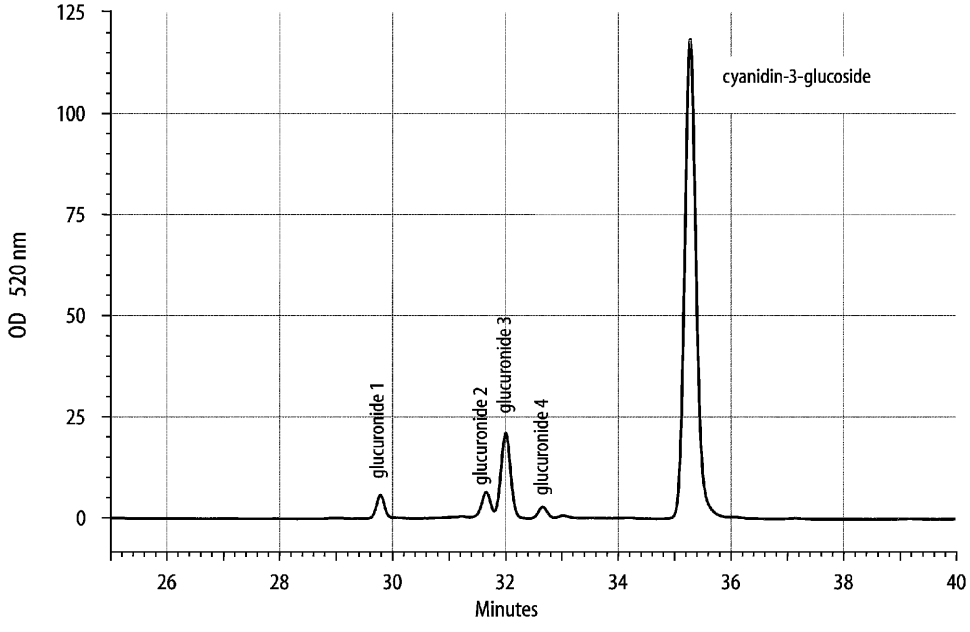

Fig. 10 Representative HPLC chromatogram of the glucuronidation products of cyanidin-3-glucoside formed by liver microsomes in the presence of UDPGA. In case of negative control incubation, no metabolites are formed (data not shown)

tions. On the one hand they degrade via the α-diketone intermediate with the formation of a phenolic acid and an aldehyde. On the other hand, they could dimerise via the quinoid anhydrobase, especially at higher concentrations (Fig. 5). The different molecular weights of the detected Cy dimers (Fig. 3) indicate that their structures may consist of two different Cy monomer units (flavylium cation, hemiketal quinoid base and α-diketone), which possess different molecular weights (see Fig. 5).

The chemical instability of anthocyanidins must therefore be taken into consideration when the biologi-

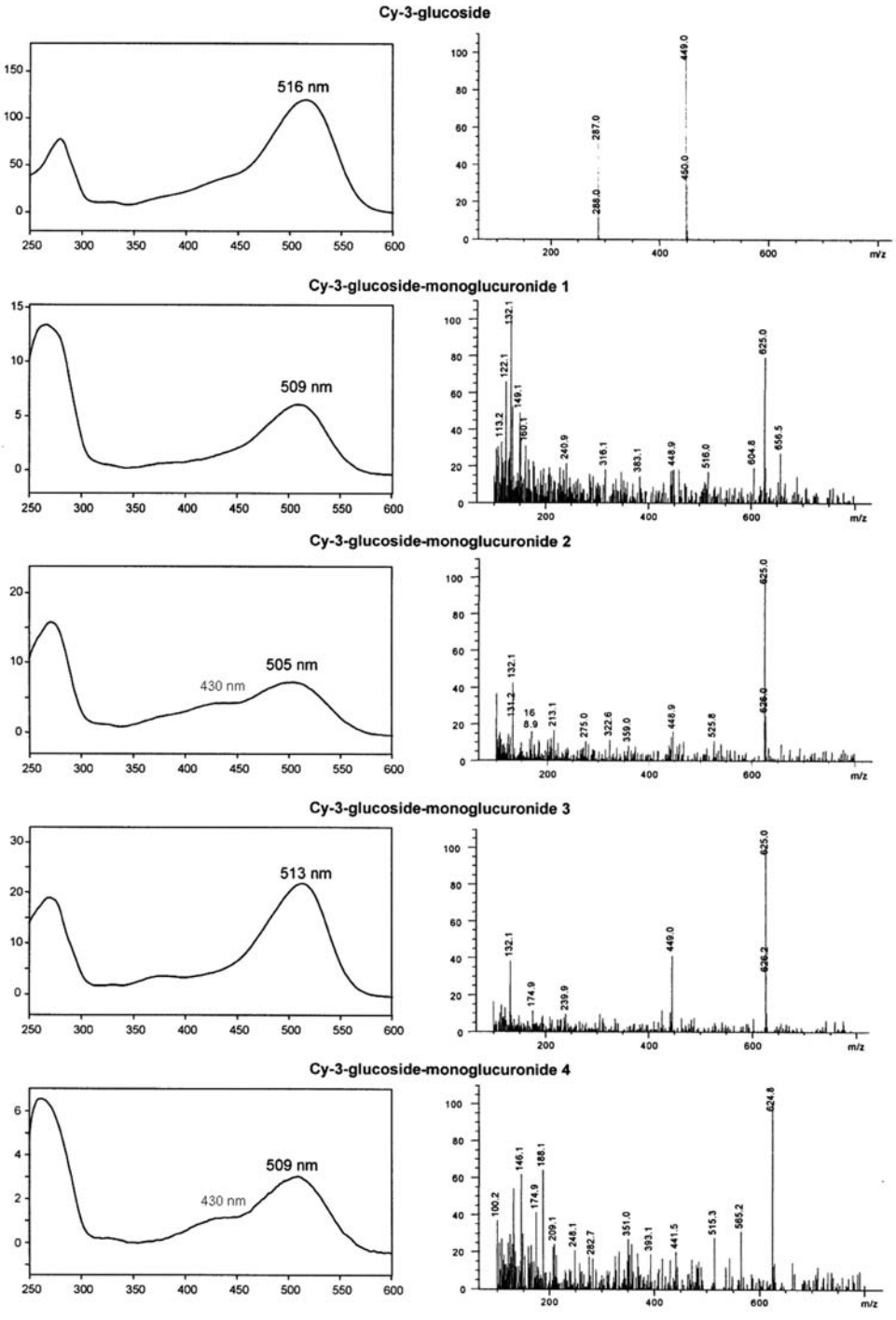

Fig. 11 Absorption spectra and mass spectroscopic spectra of cyanidin-3-glucoside and its glucuronidated products

cal properties of these aglycones are investigated in cell culture systems and in *in vitro* studies, because it can be assumed that the degradation and/or reaction products also contribute to the observed biological effects. In contrast to the anthocyanidins the anthocyanin mono- and diglucosides and even more so the acylated anthocyanins are rather stable over a wide pH range.

Our incubation studies with HFM show that intestinal bacteria were able to degrade mono- and diglucosylated and even acylated anthocyanins with very complex structures. A major pathway of this degradation process is the formation of the phenolic acid descending from the B-ring of the anthocyanin skeleton. We conclude that the first step of the bacterial biotransformation is the

cleavage of the sugar moiety leading to the formation of the anthocyanin aglycon. The aglycon could be further metabolised by the bacteria or degraded by a chemical reaction without the action of bacteria via the quinoid anhydrobase to the phenolic acid as pointed out before. However, this pathway cannot explain the whole fate of the anthocyanin, which disappeared during the incubation with HFM. In our opinion another conceivable pathway is the reaction of the quinoid anhydrobase with reactive groups in macromolecules like free thiol and amino groups of proteins. This type of reaction has often been described for various quinone structures.

In contrast to anthocyanins, phenolic acids are rather stable against further metabolism by human gut microflora. We have not observed any degradation in the case of syringic and vanillic acid and only a slight degradation of protocatechuic acid. Our findings are in line with results in the literature where phenolic acids were incubated with single bacterial strains. Hsu et al. [16] for example found a degradation of protocatechuic acid during incubation with *Clostridium thermoaceticum* while syringic acid was not affected. In this experiment the decarboxylation and dehydroxylation products of protocatechuic acid, catechol and phenol, were identified as metabolites.

It is conceivable that the observed *in vitro* reactions are also important for the *in vivo* situation and could help to understand and explain the poor bioavailability of anthocyanins observed in human intervention studies. We assume that anthocyanins (mono-, diglucosides as well as acylated anthocyanins) were degraded by enzymes of the gut bacteria in the colon releasing the aglycones. The pH of the intestine lies between 6 and 7, the pH in the colon can reach 8.0. Therefore the aglycone does not exist as a flavylium cation in the gut but undergoes a pH dependent structural change to the unstable quinoid base and to the chalcone. Via the formation of these two intermediates the same reactions as *in vitro* could occur, i. e. the degradation to the phenolic acid and the aldehyde as well as the reaction of the quinoid intermediate with thiol and amino groups of macromolecules. Thus it can be expected that the identified metabolites and degradation products can also be formed *in vivo* after ingestion of anthocyanins.

A further possible reaction in competition with the intestinal and/or chemical degradation at neutral pH in the gut is the phase II glucuronidation, which could take place in the gut or – after absorption of the aglycone or the intact glucoside – in the liver. Since the bioavailability of anthocyanins is poor, only a small amount of the compound will reach the liver. Therefore we assume that the phase II glucuronidation mainly takes place in the intestinal wall.

In the model used, the Cy aglycone as well as the Cy-3-monoglucoside were transformed to several glucuronidated products. In comparison to other flavonoids, e. g. isoflavonoids, which have been tested in our laboratory (results not shown), the rate of glucuronidation in the case of the anthocyanins is low, assuming that anthocyanidins as well as anthocyanins are only poor substrates for UDP glucuronosyltransferases. This may be one reason why intact anthocyanins are found in the plasma.

After incubation of Cy with rat liver microsomes in the presence of UDPGA two Cy-monoglucuronides were identified, whereas in the case of the Cy-3-monoglucoside four mixed glucoside-glucuronides have been found. A feasible explanation for this surprising result is based on the fact that some of the reaction products are not stable. The formation of four different mixed monoglucoside-monoglucuronides shows that glucuronidation takes place at any free hydroxyl position in the Cy molecule which are the hydroxyl positions at C5 and C7 of the A-ring and the hydroxyl groups at C3' and C4' of the B-ring. In all cases stable end products were formed. In contrast to the monoglucoside and its glucuronidated products the aglycone itself and in some cases also the glucuronidated products are unstable under the reaction conditions at neutral pH. If the glucuronidation takes place at the hydroxyl group in position C3 of the C-ring and the C4'-position of the B-ring, stable end products are formed whereas the glucuronidation at all other positions could not prevent the degradation process described in Fig. 5. The UV spectrum of the Cy-monoglucuronide in Fig. 9B is nearly identical with that of the Cy-3-monoglucoside in Fig. 11 assuming that the glucuronide in 9B is the 3-monoglucuronide. Thus we assume that the UV spectrum of the Cy-glucuronide shown in Fig. 9C, which possesses maxima at 511 nm and 423 nm, is the Cy-4'-monoglucuronide. This assumption is in accordance with the fact that the UV spectra of anthocyanidins with a sugar moiety in the 5-position of the A-ring do not have a shoulder peak around 420–440 nm [15]. This information is also important for the interpretation of the UV spectra of the four mixed monoglucuronide-monoglucosides of Cy, which are shown in Fig. 11. Two of the monoglucuronides possess only one maximum whereas the other two possess one clear maximum plus a marked shoulder peak around 430 nm. Therefore we assume that Cy-3-glucoside-monoglucuronide 1 and 3 are glucuronidated at the A-ring, whereas Cy-3-glucoside-monoglucuronide 2 and 4 are B-ring glucuronides.

In contrast, no phase I metabolites could be observed, neither hydroxylated nor demethylated products indicating that neither anthocyanidins nor anthocyanin-monoglucosides were substrates for the cytochrome P450 enzyme family.

Against the background of the poor bioavailability of the anthocyanins found *in vivo* and our *in vitro* findings, the question is raised whether the anthocyanins themselves or their metabolites are responsible for the de-

scribed biological effects. For example, it is described that protocatechuic acid – the degradation product of Cy3 glu – shows a strong antioxidative effect [17] and protects DNA against oxidative damage [18]. Furthermore, protocatechuic acid shows potent chemopreventive effects on colon and oral carcinogenesis in rats even at a concentration of 100 ppm [19–22] and inhibits tumourigenesis induced by 7,12-dimethylbenz(a)anthracene [23] and 12-O-tetradecanoylphorbol-13-acetate [24]. Therefore we conclude that a final and complete evaluation of the biological effects of anthocyanins will have to take all metabolites and degradation products of these compounds into account.

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