Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry 13 (2005) 5195–5205
Bioorganic & Medicinal Chemistry

Metabolism of anthocyanins and their phenolic degradation products by the intestinal microflora

Katrin Keppler and Hans-Ulrich Humpf *

Institut für Lebensmittelchemie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 45, 48149 Münster, Germany

Received 31 March 2005; revised 3 May 2005; accepted 3 May 2005
Available online 15 June 2005

Abstract—Anthocyanins are suggested to be responsible for protective effects against cardiovascular diseases and certain forms of cancer. Although previous studies have implicated that intact anthocyanidin glycosides were decreased extensively by interactions in the gastrointestinal tract, only few data are available concerning the metabolism by the intestinal microflora. Using a new in vitro model, we have investigated the microbial deglycosylation and degradation of six anthocyanins exhibiting three different aglycones with mono- or di-β-D-glycosidic bonds using high-performance liquid chromatography-diode array (HPLC-DAD) and gas chromatography–mass spectrometry (GC–MS) detection. We have found that all anthocyandin glycosides were hydrolysed by the microflora within 20 min and 2 h of incubation depending on the sugar moiety. Due to the high instability of the liberated aglycones at neutral pH, primary phenolic degradation products were already detected after 20 min of incubation. Further metabolism of the phenolic acids was accompanied by demethylation. Because of their higher chemical and microbial stability, phenolic acids and/or other, not yet identified, anthocyanin metabolites might be mainly responsible for the observed antioxidant activities and other physiological effects in vivo.

1. Introduction

Anthocyanins are part of the widespread flavonoids and are responsible for the red and blue pigmentation of many vegetables and fruits. Naturally occurring anthocyanins are polyhydroxy or polymethoxy derivatives of 2-phenyl-benzopyrylium often with β-3-*O*-glycosidic or β-3,5-*O*-diglycosidic bonds.[1,2] The free aglycones, called anthocyanidins, are rarely found in dietary plant materials. The average intake of anthocyanins has been estimated at up to 180–215 mg/day, which is higher than that for other flavonoids such as flavonols.[3–5] Within the last two decades there has been an increasing interest in phenolic phytochemicals due to their protection against cardiovascular diseases and certain forms of cancer.[6,7] Like other polyphenols, anthocyanins are potent antioxidants.[8–12] This function is related to their antiatherosclerotic,[13,14] anticarcinogenic[15,16] and anti-inflammatory[17] properties.

The bioavailability of the intact phenolic phytochemicals is a fundamental factor for their physiological functions. Recent studies reported a very low absorption and urinary excretion of the intact anthocyanidin glycosides between 0.016% and 0.11% of the dosage in humans and different animal models.[18–21] Also, the recovery of anthocyanins in faeces is very low. These results indicate an extensive biotransformation of the anthocyanins after oral ingestion and absorption. Methyl derivatives of the anthocyanins were found in plasma or different tissue samples at very low concentrations;[22–24] glucuronic acid conjugates were detected only in a few studies.[25,26] Sulfate conjugates and the corresponding aglycones were not established in all studies, which is in contrast to most flavonoids.[7,27] Therefore, besides the degradation at neutral pH in the small and large intestines or the metabolism by the gut microflora other factors must contribute to the low bioavailability of anthocyanins. Recent studies have focused on the metabolism of flavonoids by intestinal microorganisms using single bacteria species, mixed culture models or human faecal samples.[4,6,7] The identification and quantification of the degradation products such as aglycones and phenolic acids have confirmed that the microflora has an enormous hydrolytic potential and even ring scission properties. This suggests that the bacterial cleavage

Keywords: Anthocyanins; Pig caecum; Intestinal microflora; Metabolism; Phenolic acids.
* Corresponding author. Tel.: +49 251 8333867; fax: +49 251 8333396; e-mail: humpf@uni-muenster.de

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.05.003

of glycosidic bonds also occurs in anthocyanins. After hydrolysis of the protective 3-glycosidic linkage, the released aglycons are stable under acidic but unstable under physiological conditions at neutral pH. The formed α-diketone is very reactive and decomposes easily to phenolic acids and aldehydes[1,28] (Fig. 1). Phenolic acids have been studied with comparable results for their antioxidant activity and free radical scavenging capacity.[8,13,29–31] Even inhibitory effects on carcinogenesis have been shown in different model systems.[32] 3,4-Dihydroxybenzoic acid (protocatechuic acid PA, **10**) has been detected in plasma, tissue and the jejunum after oral ingestion of cyanidin 3-glucoside in concentrations higher than that of the anthocyanin itself.[22] These results show that the spontaneous degradation of anthocyanidins after bacterial deglycosylation is one limiting factor in the bioavailability of anthocyanins. Besides these data, there are only a few experimental studies incubating anthocyanins with the human gut microflora.[33,34]

However, most in vitro incubation experiments are not comparable to in vivo conditions. For example, incubation experiments with single bacterial species[35,36] or a mixed culture model[37] represent only a small section of the complex gut microflora with its more than 400 bacterial species.[38] Furthermore, incubation with human faecal samples[33,34,39] is critical due to the strict anaerobiosis in the intestine. For a better reproducibility of the in vivo conditions, we have developed a new in vitro model system[40,41] according to Sarlikiotis et al.[42] Here, the gut microflora is directly isolated from the caecum of freshly slaughtered pigs and incubation experiments were performed under strict anaerobic conditions on a microscale. The pig is an excellent model for human metabolism studies because of the similarities in physiology and nutrition.[43] The animals used in our model were produced by biodynamic agriculture (ecofarming) excluding the influence of drugs, especially antibiotics. In the meantime, this model system has been adopted by other research groups.[44]

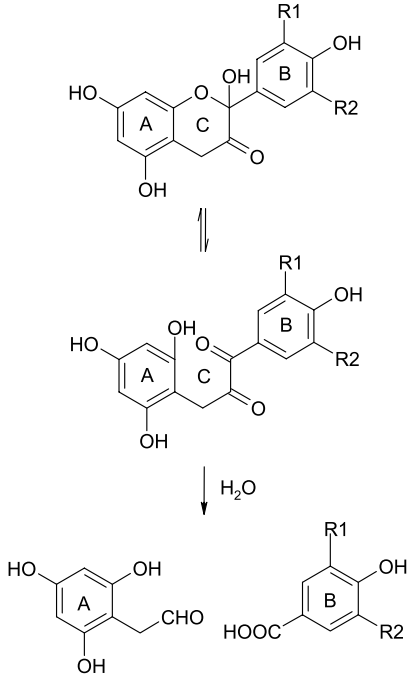

Figure 1. Degradation of an anthocyanidin (pH > 4) into an α-diketone and monomeric phenolic compounds (modified according to Jurd[1]).

In the present study, we investigated the microbial metabolism and chemical stability of six anthocyanins **1**–**6** based on the aglycones cyanidin **7**, malvidin **8** and peonidin **9** (Fig. 3). Glucose or rutinose was linked 3-mono- or 3,5-di-β-D-*O*-glycosidic to the aglycones. The hydrolysis of the β-glycosidic bonds and the decay of the aglycones were measured during the incubation. The resulting degradation products were identified and quantified. Additionally, the microbial and chemical fate of the phenolic degradation products were investigated to determine their physiological importance. Vitality and metabolic efficiency of the microflora was investigated by microbial marker enzymes such as α-D-galactosidase and β-D-glucosidase.

2. Results and discussion

2.1. Activity of α-D-galactosidase and β-D-glucosidase

To control the vitality and metabolic efficiency of the microorganisms from each caecum, we investigated the activity of α-D-galactosidase. The α-D-galactosidase is an exoglycosidase, which is exhibited by the intestinal microflora and not by the pig or human digestive system. Therefore, this microbial marker enzyme is an excellent tool to assay the activity of the intestinal microflora. Due to the β-D-glycosidic linkage of all the investigated anthocyanins, we also measured the β-D-glucosidase activity of each inoculum as control. Not only the gut microflora, but also intestinal cells express some β-glucosidases such as the cytosolic β-glucosidase (CBG)[45] or lactase-phlorizin hydrolase (LPH).[46] LPH occurs on the outside of the brush-border membrane and can hydrolyse flavonoids before absorption.[47] Due to the localization of the CBG and LPH, the measured β-D-glucosidase activity in the inoculum was attributed to the intestinal microflora.

α-D-Galactosidase and β-D-glucosidase activities were assayed using *p*-nitrophenyl α-D-galactopyranoside (PNP α-gal) or *p*-nitrophenyl β-D-glucopyranoside (PNP β-glc) as substrates. Microbial enzymes hydrolyse the substrates into the corresponding sugar and *p*-nitrophenol (PNP), which is measured photometrically. The highest amounts of PNP, which has been released from PNP α-gal, were measured after 40 min of incubation (Fig. 2a). For β-D-glucosidase, the highest activity was reached after 20 min of incubation (Fig. 2b). After reaching the maximum, the amount of *p*-nitrophenol decreased due to the further degradation by the intestinal microflora. For this reason, the theoretically maximum value (1 mM) of *p*-nitrophenol was not traceable. The degradation of *p*-nitrophenol is also a marker for the vitality and metabolic efficiency of the microflora.

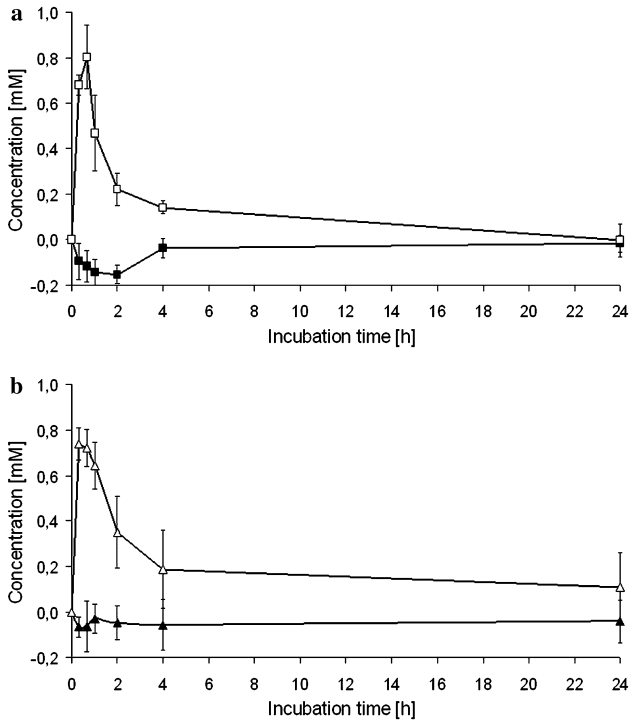

Figure 2. Time-dependent release of *p*-nitrophenol (PNP) (□), (△) after incubation of: (a) PNP α-D-galactopyranoside (1 mM) and (b) PNP β-D-glucopyranoside (1 mM) with the pig inoculum filtrate. Non-sterilized inoculum filtrate without substrate was measured as blank for matrix background absorption. PNP α-D-galactopyranoside (1 mM) (■) and PNP β-glucopyranoside (1 mM) (▲) were incubated additionally in sterilized inoculum filtrate for chemical degradation and as control for sterilization. Absorption was measured at 405 nm with a microplate reader ($n = 3$, mean ± SD).

2.2. Microbial metabolism and chemical stability of anthocyanins 1–6 and anthocyanidins 7–9

For the incubation experiments, anthocyans **1**–**9** (Fig. 3) were incubated at 100 μM for 24 h (for details see Section 4.3). Samples were analyzed by HPLC-DAD from 20 min to 24 h. Anthocyanins **1**–**6**, liberated anthocyanidins **7**–**9** and phenolic degradation products **10**–**13** were identified by a comparison of HPLC retention times and UV–vis spectra with those of authentic reference compounds. Anthocyanins **1**–**6** and anthocyanidins **7**–**9** were quantified, using HPLC-DAD detection. Aliquots of the gut microflora inoculum filtrates were measured additionally by GC–MS to confirm and quantify phenolic degradation products using 3,4-dihydroxyphenylacetic acid as internal standard. Non-sterilized inoculum aliquots without substrate were also analyzed by HPLC-DAD and GC–MS as matrix blank.

2.2.1. Microbial metabolism of anthocyanins 1–6. To study the microbial metabolism of anthocyanins, cyanidin 3-β-*O*-glucoside (C3G) **1**, malvidin 3-β-*O*-glucoside (M3G) **2**, cyanidin 3,5-β-*O*-diglucoside (C35G) **3**, malvidin 3,5-β-*O*-diglucoside (M35G) **4**, cyanidin 3-β-*O*-rutinoside (C3R) **5** and peonidin 3-β-*O*-glucoside (P3G) **6** were incubated with the non-sterilized inoculum filtrate of the pig caecum. We asked the question whether the aglycones and/or the kind of sugar moiety have an influence on hydrolysis. Figures 4a–f show the decreasing amounts of anthocyanins **1**–**6** and increasing amounts of the degradation products **10**–**13** in the deglycosylation experiments using the gut microflora. All anthocyanins were hydrolysed by the microflora within 20 min and 2 h of incubation. During this time, the released aglycones cyanidin **7**, malvidin **8** and peonidin **9** were measured using HPLC-DAD in very small amounts <10 μM (data not shown) eluting at 18.5, 20.0, and 19.5 min, respectively. After 20 min of incubation, C3G **1** and M3G **2** were almost completely hydrolysed by the microflora. In the case of P3G **6**, the rate of hydrolysis differed from the behaviour of other monoglucosidic anthocyanins **1**, **2**, although the same inoculum filtrates were applied. As expected, the microflora converted (C35G) **3** and (M35G) **4** not as fast as the monoglucosidic derivatives **1** and **2**. Figure 5 shows HPLC-chromatograms of the M35G **4** deglycosylation. A monoglucosidic anthocyanin metabolite of both **3** and **4** was detectable after 20 min of incubation. Retention times were 15.0 min for the monoglucosidic cyanidin metabolite and 16.6 min for the monoglucosidic malvidin metabolite. The peak signals were very low (hydrolysis of **3** and **4** led to 4.0 ± 0.9 and 5.2 ± 1.9 μM, respectively) and decreased after 40 min due to further metabolism. Unfortunately, we were not able to distinguish between the 5-β-glucosidic or the 3-β-glucosidic derivative of cyanidin or malvidin. C3R **5** was metabolized very slowly, as considerable amounts of intact anthocyanin were still detectable after 2 h of incubation. The decreased rate of the hydrolysis indicated a steric hindrance of the β-glucosidase because of the complex sugar moiety rutinose. Although an α-L-rhamnosidase activity of the intestinal microflora has been reported,[48] C3G **1** was not released under the applied experimental conditions. By comparing the hydrolysis of the monoglucosidic **1**, **2** or the diglucosidic **3**, **4** cyanidin and malvidin derivatives a very close resemblance was found. Besides the results of P3G **6**, the aglycones apparently had almost no influence on the hydrolysis of the glycosides. In contrast, a different sugar moiety, as shown with the rutinose in **5**, did influence the rate of the hydrolysis considerably.

Because of the high instability of the released anthocyanidins **7**–**9** at neutral pH, primary phenolic degradation products **10**–**13** were already detectable by HPLC-DAD after 20 min in all incubation experiments of the anthocyanins **1**–**6**. Figure 6 shows HPLC-chromatograms of the M35G **4** degradation. Due to the identical A-ring of the applied anthocyanins, phloroglucinol aldehyde (PHA) **13** (retention time, $t_R = 12.6$ min) was formed in all incubation experiments. Thus, in agreement with Piffaut et al.[49] and Seeram et al.[50] we confirmed that the A-ring led to a benzoic derivative and the loss of a carbon after anthocyanidin degradation and not to a phenylacetic derivative as reported by Jurd.[1] The decay of **7** additionally led to protocatechuic acid (PA) **10** ($t_R = 6.0$ min); **8** degraded into syringic acid (SA) **11** ($t_R = 13.5$ min), and vanillic acid (VA) **12** ($t_R = 11.5$ min) was detected as the characteristic degradation product of **9**. The concentrations of phenolic degradation products **10**–**13** were calculated using GC–MS

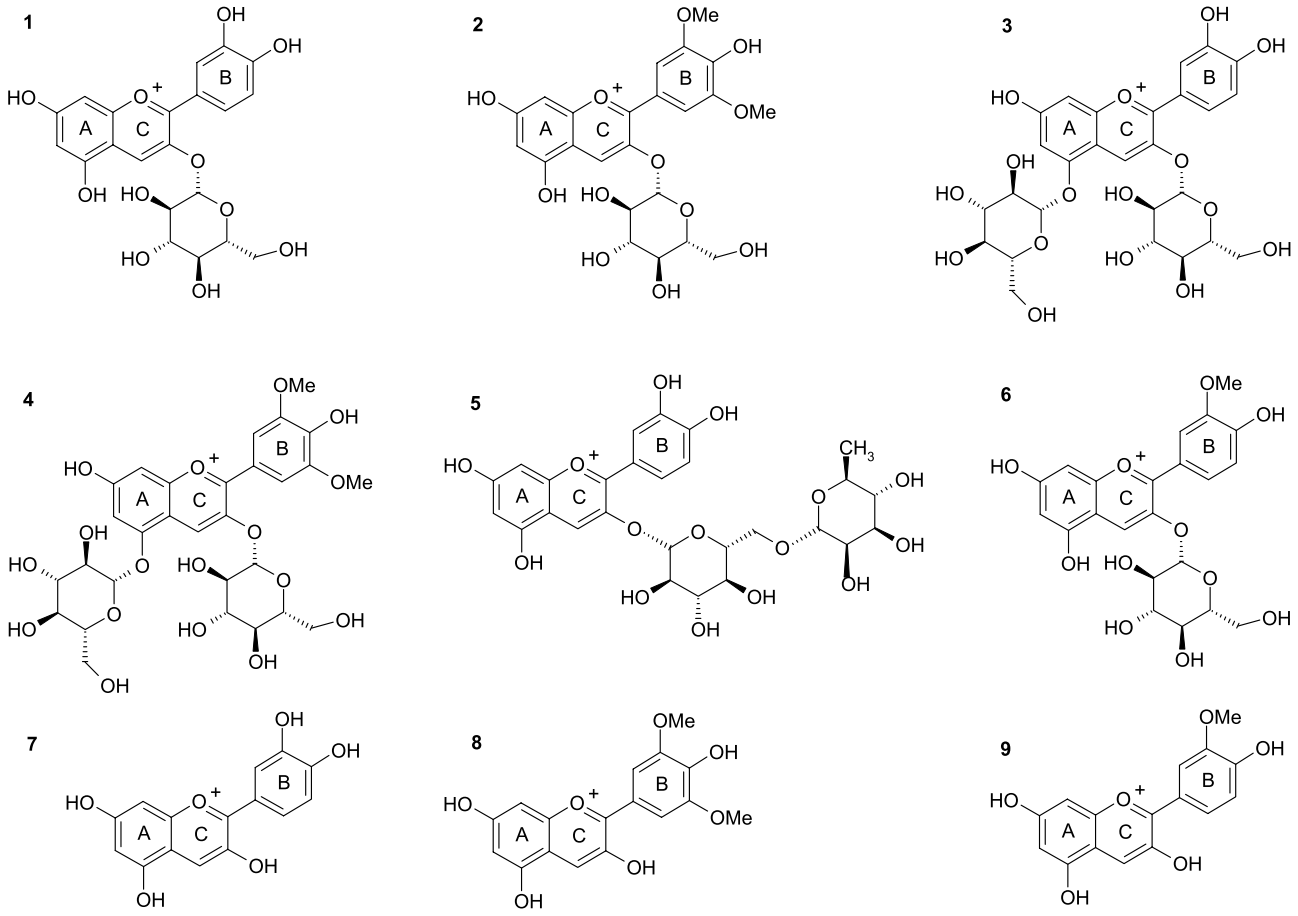

Figure 3. Structures of the anthocyanins used in this study: cyanidin 3-β-D-*O*-glucoside (C3G) **1**, malvidin 3-β-D-*O*-glucoside (M3G) **2**, cyanidin 3,5-β-D-*O*-diglucoside (C3G) **3**, malvidin 3,5-β-D-*O*-diglucoside (M35G) **4**, cyanidin 3-β-D-*O*-rutinoside (C3R) **5** and peonidin 3-β-D-*O*-glucoside (P3G) **6**. Structures of the anthocyanidins: cyanidin **7**, malvidin **8** and peonidin **9**.

detection in the selected ion monitoring mode (for details see Section 4.4.3). Structures of the phenolic degradation products **10**–**13** are depicted in Figure 7. The number and kind of glycosidic bonds linked to the aglycone had no influence on the concentrations (see Fig. 4) of the formed primary phenolic degradation products **10**–**13**. In contrast, the timepoint, where maximum amounts were reached, depended on the rate of the anthocyanin hydrolysis. Therefore, the phenolic degradation products of the fast hydrolysed monoglucosidic anthocyanins **1**, **2** and **6** increased compared to the diglucosidic anthocyanins **3**, **4** and the cyanidin 3-rutinoside **5**. However, concentrations of phenolic compounds decreased continuously after reaching the maximum. This observation indicated further chemical or microbial degradation of the phenolic degradation products. We detected phloroglucinol acid **14** as the oxidation product of phloroglucinol aldehyde **13** in small amounts (not quantified) using GC–MS detection.

2.2.2. Chemical stability of anthocyanins 1–6. The chemical stability of anthocyanins **1**–**6** under physiological conditions in the intestine (37 °C, reductive milieu, pH 6.4) was determined by incubation of the compounds with the sterilized inoculum filtrate. Figures 4a–f (■) show the time–concentration degradation profiles of anthocyanins **1**–**6** incubated in the sterilized inoculum. The monoglucosidic cyanidin and malvidin, and the anthocyanidin **7**–**9** metabolites (see Section 2.2.1) were not detected by HPLC-DAD in these control samples with sterilized inoculum. These results are in agreement with other studies[51,52] showing that a non-enzymatic deglycosylation of flavonoids under physiological conditions, even at acid pH of the stomach, does not occur. On the other hand, anthocyanins **1**–**6**, especially the monoglucosidic derivatives, were not stable in the sterilized control samples. Phenolic degradation products such as protocatechuic acid **10**, syringic acid **11** and vanillic acid **12** were measured after 24 h in small amounts <5 μM (data not shown) using GC–MS detection. Phloroglucinol aldehyde **13** or any other phenolic aldehyde was not detected. We assume that under the experimental conditions in the sterilized control samples, ring fission and the formation of phenolic acids **10**–**12** even occur to the anthocyanins. The decay of cyanidin glycosides under similar conditions has been reported.[50] Besides this minor degradation pathway, anthocyanins will form chalcone and coumarin glycosides or other transformation products.[1,28]

2.2.3. Chemical stability of anthocyanidins 7–9. To distinguish between metabolic or chemical degradation, we also incubated the free anthocyanidins **7**–**9** in the supplemented PBS (pH 6.4) at 37 °C to exclude the matrix

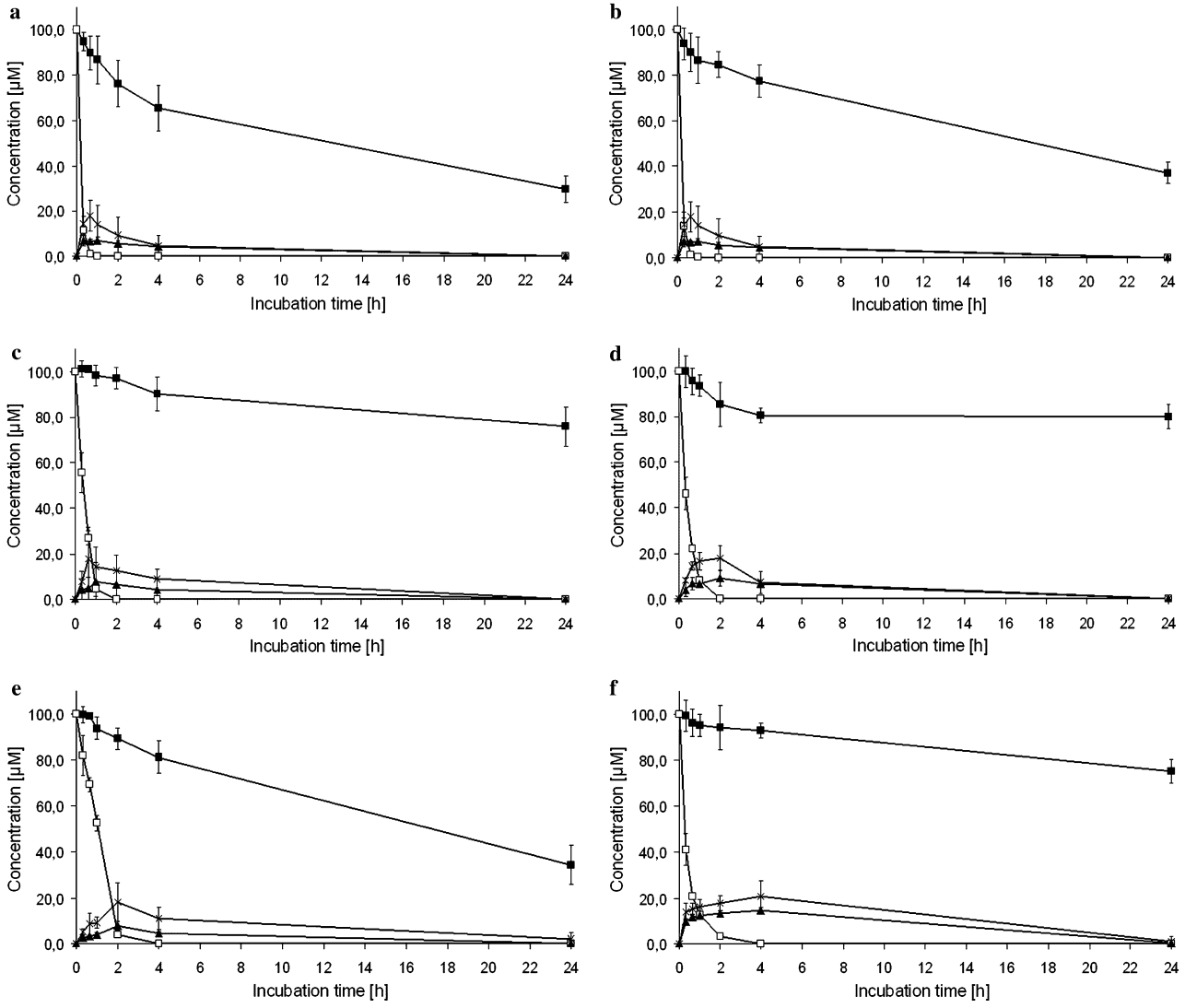

Figure 4. Time–concentration degradation profiles of anthocyanins (**1**–**6**) (measured by HPLC-DAD at 515 nm) and release of phenolic degradation products (measured by GC–MS with SIM using 3,4-dihydroxyphenylacetic acid as internal standard after incubation with pig inoculum filtrates (□) for metabolism experiments or sterilized pig inoculum filtrates (■) for measuring the chemical stability. (a) Formation of PA **10** (∗) and PHA **13** (▲) from C3G **1** (□); C3G **1** (■) in sterilized filtrate; (b) formation of SA **11** (∗) and PHA **13** (▲) from M3G **2** (□); M3G **2** (■) in sterilized filtrate; (c) formation of PA **10** (∗) and PHA **13** (▲) from C35G **3** (□); C35G **3** (■) in sterilized filtrate; (d) formation of SA **11** (∗) and PHA **13** (▲) from M35G **4** (□); M35G **4** (■) in sterilized filtrate; (e) formation of PA **10** (∗) and PHA **13** (▲) from C3R **5** (□); C3R **5** (■) in sterilized filtrate; (f) formation of VA **12** (∗) and PHA **13** (▲) from P3G **6** (□); P3G **6** (■) in sterilized filtrate ($n = 3$, mean ± SD).

effects of the inoculum. The recoveries of cyanidin **7**, peonidin **9** and malvidin **8** were 53.3 ± 3.2, 27.9 ± 4.4, and 0.0 µM ($n = 3$, mean ± SD), respectively, after 1 h of incubation. Chemical breakdown seemed to increase with the number of methoxyl substituents in the B-ring (**8** > **9** > **7**). Incubation of anthocyanidins **7**–**9** in PBS and incubation of anthocyanins **1**–**6** in non-sterilized inoculum (see Section 2.2.1) led to the same pattern of primary phenolic degradation products **10**–**14**. The amounts of PA **10** (28.6 ± 5.2 µM), VA **11** (31.2 ± 6.4 µM), SA **12** (53.1 ± 4.9 µM) and PHA **13** (23,6 ± 3.7, 32.7 ± 7.1 and 44.68 ± 5.2 µM for cyanidin, peonidin and malvidin, respectively) reflected the decay of the aglycones. The concentrations of PHA **13** in these matrix-free incubation experiments were in accordance with the concentrations of the referring phenolic acids **10**–**12**. This is in contrast to the anthocyanin incubation experiments where the aldehyde concentration was much lower. Because of the high rate of the chemical degradation, it cannot be distinguished whether the ring fission of the anthocyanidins was caused primarily by the microbial cleavage or by chemical decay. Likely, both pathways are responsible for the poor recoveries of anthocyanidins.

However, the measured amounts of phenolic degradation products **10**–**13** could not explain the starting concentration of anthocyanins **1**–**6** applied in the deglycosylation experiments with the gut microflora (see Section 2.2.1). Anthocyanidins **7**–**9** incubated in PBS without inoculum (see Section 2.2.2) did not lead to the expected concentrations of phenolic acids **10**–**12** and phloroglucinol aldehyde **13**. We assume that an unknown, but more stable, anthocyanidin intermediate, such as a polymerized chal-

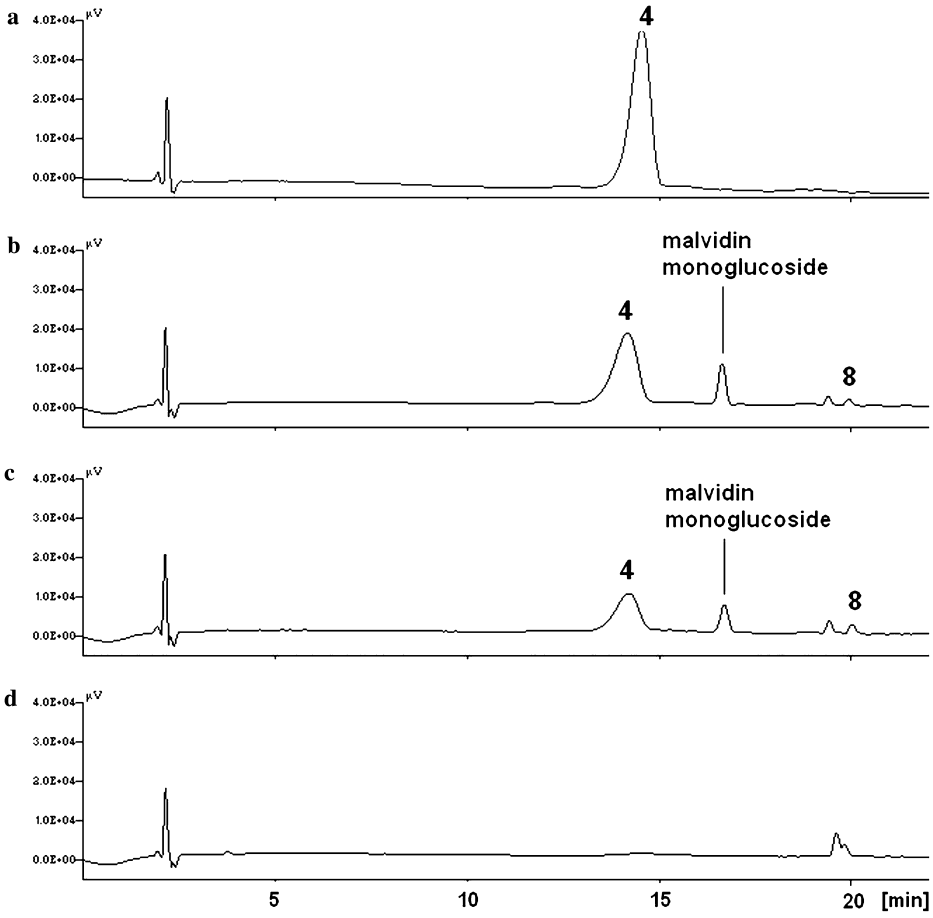

Figure 5. HPLC-chromatograms of M35G **4** (100 μM) after: (a) incubation with sterilized pig inoculum filtrate for 20 min; (b) incubation with non-sterilized pig inoculum filtrate for 20 min; (c) incubation with non-sterilized pig inoculum filtrate for 40 min; (d) non-sterilized pig inoculum filtrate without **4** was measured as blank after 20 min of incubation. The free aglycone malvidin **8** and a monoglucosidic malvidin metabolite were only found in chromatograms (b and c) (HPLC-chromatograms were monitored at 515 nm).

cone,[1] is formed or phenolic compounds are limited due to the chemical decay or further microbial metabolism in the case of the active inoculum.

2.3. Microbial metabolism and chemical stability of phenolic degradation products 10–13

As shown above, protocatechuic acid (PA) **10**, syringic acid (SA) **11**, vanillic acid (VA) **12** and phloroglucinol aldehyde (PHA) **13** were released as primary degradation products of anthocyanidins **7**–**9** (see Section 2.2.3) and anthocyanins **1**–**6** after deglycosylation (see Section 2.2.1). However, as results of the microbial metabolism of anthocyanins **1**–**6** show (see Section 2.2.1), the concentrations of the formed phenolic compounds were not stable and decreased throughout the incubation time. Especially phloroglucinol aldehyde **13** did not reach the expected concentrations. Because of their strong antioxidative and free radical scavenging properties, we investigated their chemical and microbial stability. For this, phenolic acids **10**–**12** and the phenolic aldehyde **13** were incubated at 100 μM with sterilized (Section 2.3.1) and non-sterilized (Section 2.3.2) inoculum filtrate for 24 h. Samples were analyzed by HPLC-DAD from 20 min to 24 h to follow the decrease of **10**–**13**. Aliquots of the non-sterilized inoculum filtrates and the sterilized inoculum filtrates were additionally analyzed by GC–MS to identify further metabolites or chemical degradation products. Non-sterilized inoculum aliquots without substrate were also analyzed by HPLC-DAD and GC–MS as matrix blank.

2.3.1. Chemical stability of phenolic degradation products 10–13. The chemical stability of phenolic degradation products **10**–**13** under physiological conditions in the intestine (37 °C, reductive milieu, pH 6.4) was determined by incubation of the compounds in the sterilized inoculum filtrate. As can be seen from Figures 8a–c, phenolic acids **10**–**12** were quite stable in the sterilized control samples. In contrast, phloroglucinol aldehyde **13** (Fig. 8d) was degraded extensively, as only very small amounts were detectable after 24 h. Because of the high reactivity of aldehydes, this observation is not surprising. Condensation with free NH_2-groups of amino acids or proteins and formation of imine are the possible pathways of aldehyde degradation.

2.3.2. Microbial metabolism of phenolic degradation products 10–13. To study the microbial metabolism, phenolic degradation products **10**–**13** were incubated with the non-sterilized inoculum filtrate of the pig caecum. As Figure 8d shows, PHA **13** was degraded

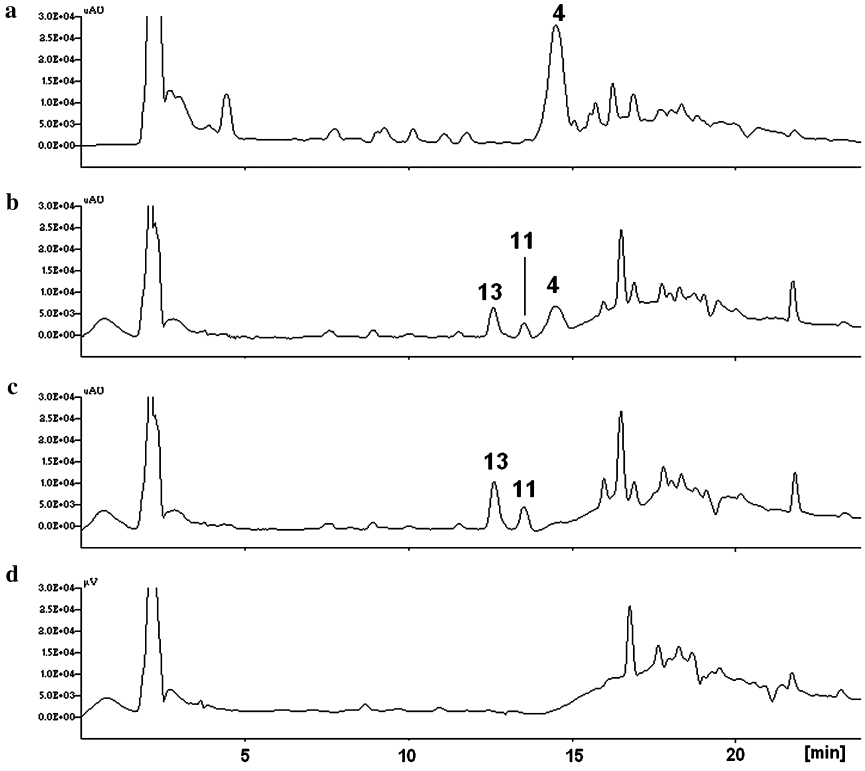

Figure 6. HPLC-chromatograms of M35G **4** (100 μM) after: (a) incubation with sterilized pig inoculum filtrate for 20 min; (b) incubation with non-sterilized pig inoculum filtrate for 40 min; (c) incubation with non-sterilized pig inoculum filtrate for 1 h; (d) non-sterilized pig inoculum filtrate without **4** was measured as blank after 20 min of incubation. Phenolic degradation products **11** (syringic acid) and **13** (phloroglucinol aldehyde) were detectable in chromatograms (b and c). (HPLC-chromatograms were monitored at 291 nm.)

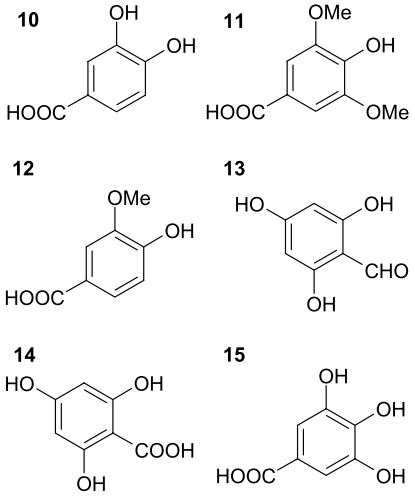

Figure 7. Structures of the phenolic degradation products of anthocyanidins (for details see text): protocatechuic acid (PA) **10**, syringic acid (SA) **11**, vanillic acid (VA) **12**, phloroglucinol aldehyde (PHA) **13**, phloroglucinol acid **14** and gallic acid (GA) **15**.

by the intestinal microflora very similar in comparison to the sterilized control samples (see Section 2.3.1). Therefore, it was not possible to distinguish between chemical or microbial transformation of the aldehyde. Despite the reductive conditions, we detected phloroglucinol acid **14** as the oxidation product of **13** in very low amounts (data not shown) only in the non-sterilized inoculum filtrate. The microflora converted the phenolic acids PA **10**, SA **11** and VA **12** slowly in very close resemblance (Figs. 8a–c). After 24 h of incubation, 12.15 ± 1.72, 9.34 ± 8.48, and 7.67 ± 4.05 μM ($n = 3$, mean ± SD) were still measured for **10**, **11** and **12**, respectively. This indicates that phenolic acids were utilized as a source of energy by the intestinal microflora.

The decrease of SA **11** and VA **12** was accompanied by the formation of two further metabolites: **11** was *O*-demethylated to gallic acid (GA) **15**, and **12** was converted to protocatechuic acid **10** by demethylation (for structures see Fig. 7). Because of this additional metabolic pathway, the degradation of SA **11** and VA **12** was a little faster than the metabolism of PA **10**. The O-demethylated metabolites GA **15** and PA **10** were only detectable from 2 h up to 4 h of incubation due to the further metabolism by the bacterial microflora. Because of the very small concentrations, these intermediates were not quantified. Figure 9 shows a GC–MS-chromatogram of the degradation of VA **12**. However, the O-demethylation products were not detected in the matrix blank without substrate and in the sterilized control samples of all incubation experiments. Therefore, this is a clear indication that demethylation is caused by the gut microorganisms.

The microbial pathway of O-demethylation is contrary to the enzymatic activity of intestinal and hepatic tissues, which exhibited catechol-*O*-methyltransferase.[27]

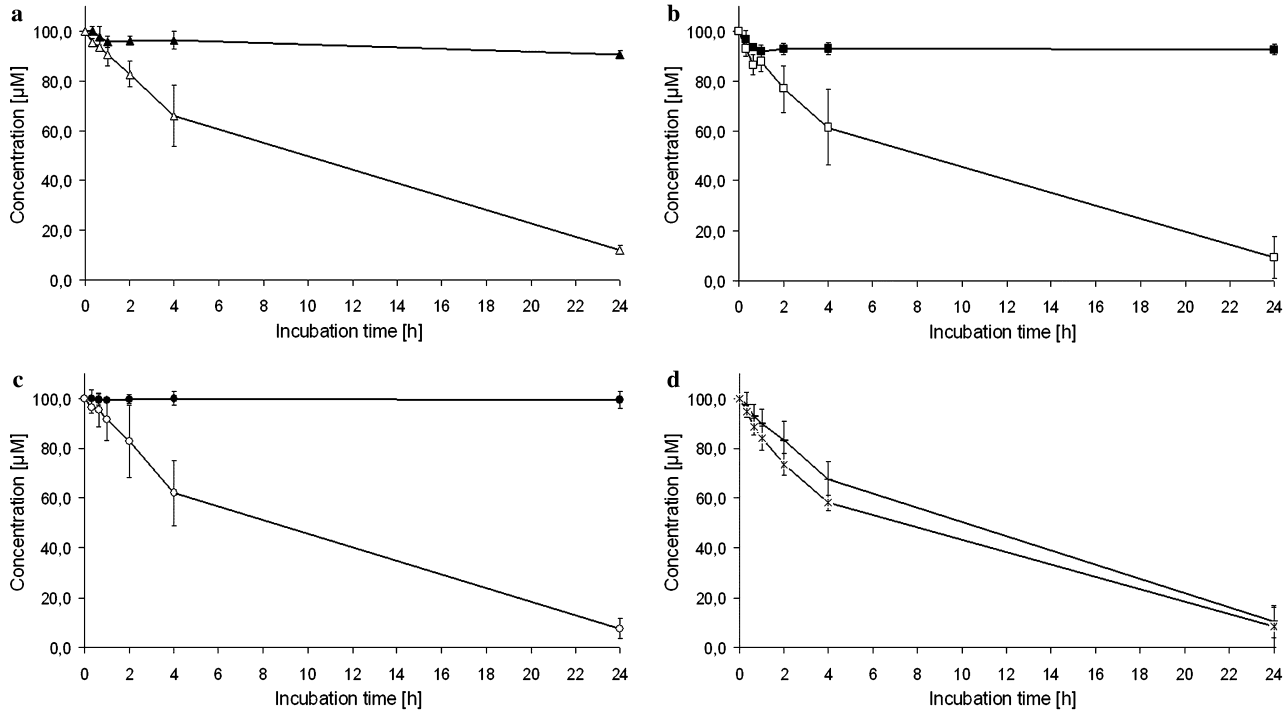

Figure 8. Time–concentration degradation profiles of phenolic compounds **10**–**13** (measured by HPLC-DAD at 265 nm for **10**, 275 nm for **11** and 291 nm for **12** and **13**) after incubation with pig inoculum filtrates for metabolism experiments or sterilized pig inoculum filtrates for measuring the chemical stability. (a) Degradation of PA **10** (△); PA **10** (▲) in sterilized filtrate; (b) degradation of SA **11** (□); **11** (■) in sterilized filtrate; (c) degradation of VA **12** (○); VA **12** (●) in sterilized filtrate; (d) degradation of PHA **13** (*); PHA **13** (–) in sterilized filtrate ($n = 3$, mean ± SD).

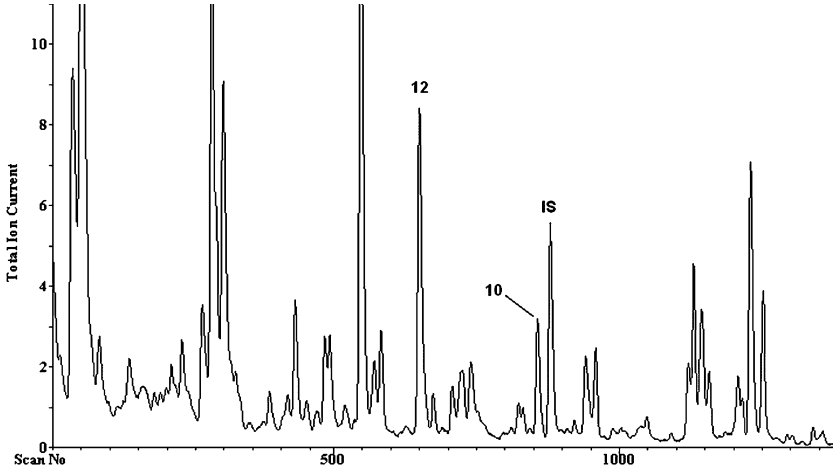

Figure 9. Representative GC–MS chromatogram (monitored as total ion chromatogram, TIC) of vanillic acid VA **12** (100 µM) after incubation with pig inoculum filtrate for 4 h. Protochatechuic acid (PA) **10** could be detected as demethylation product of VA **12**; internal standard **IS**, 3,4-dihydroxyphenylacetic acid. Unlabelled peaks are caecum constituents.

O-Methylation plays a crucial role in the transformation of polyphenols and other drugs after absorption. However, O-demethylation influences the antioxidative properties and absorption of phenolic acids in the small intestine. Antioxidant activity depends on the number and position of free hydroxyl groups in the molecule, because the monophenolic ring is not such an effective hydrogen donor. As reported by Rice-Evans et al.,[8] gallic acid (GA) **15**—the O-demethylation product of syringic acid (SA) **11**—reveals a threefold higher antioxidant capacity towards SA **11**; in comparison to the intact anthocyanidin, malvidin **8**, the antioxidant properties have even increased by one unit. GA **15** exhibits anticarcinogenic activity.[53,54] In contrast, the decay of peonidin **9** into vanillic acid **12** and the further metabolism to protocatechuic acid **10** decreased the antioxidant capacity.

Altogether, phenolic acids as degradation products of anthocyanins still possess antioxidant activities in a modified way. Because of their high-chemical stability and only moderate metabolism by the intestinal micro-

flora, they can act as local as well as systemic antioxidants. Common dietary phenolic acids are transported via the paracellular pathway and via the monocarboxylic acid transporter (MCT) into intestinal Caco-2 cell monolayers depending on their polarity and hydroxylation pattern.[55–58]

3. Conclusion

Antioxidant activities of anthocyanins in vitro are not transferable to health effects observed in vivo. To understand their impact on human health, it is necessary to know their bioavailability. Metabolism by the intestinal microflora is one important factor controlling the bioavailability. To investigate the questions of deglycosylation, ring scission and other bacterial degradation pathways of anthocyanins, we have applied a new in vitro model system, which has been developed by our group.[40,41] Here, the naturally composed gut microflora is directly isolated from the caecum of freshly slaughtered pigs excluding any aerobic atmosphere. Our results clearly show that anthocyanidin glycosides are hydrolysed extensively by the intestinal microflora depending on the sugar moiety. After cleavage of the protective 3-glycosidic linkage, the released aglycones are very unstable under physiological conditions in the intestine at neutral pH and degraded spontaneously into phenolic acids **10**–**12** and aldehydes **13**. As our data confirm previous studies, it is rather unlikely that intact anthocyanins **1**–**6** and especially anthocyanidins **7**–**9** are able to act as local or systemic antioxidants or anticarcinogens due to their complicated fate, which is influenced by deglycosylation and degradation into monomeric phenolic molecules **10**–**13**, dependent on both pH and microbial metabolism. Recent studies reported that anthocyanidin glucosides are efficiently absorbed from the stomach through a bilitranslocase-mediated mechanism and appeared rapidly in the plasma.[59,60] The gastric absorption might improve the possibility of anthocyanins to act as systemic drugs but the main antioxidative properties would be preserved by the more stable phenolic degradation products. We have shown that the released phenolic acids **10**–**12** were decreased by the intestinal microflora in a moderate way. Metabolism of the methoxyl derivatives as syringic acid **11** and vanillic acid **12** was accompanied by O-demethylation. The cleavage of methyl groups and liberation of free hydroxyl groups modulate the antioxidant properties of these phenolic compounds. Because of their higher chemical and microbial stability, phenolic acids and/or other not yet identified anthocyanin metabolites might be mainly responsible for the observed antioxidant activities and other physiological effects in vivo and not the anthocyanins themselves.

4. Experimental

4.1. Chemicals

Anthocyans **1**–**9**, 2,4,6-trihydroxybenzoic acid (phloroglucinol acid **14**) and 2,4,6-trihydroxybenzaldehyde (phloroglucinol aldehyde **13**) were purchased from Extrasynthese (Genay, France), 3,4,5-trihydroxybenzoic acid (gallic acid **15**), 3,4-dihydroxybenzoic acid (protocatechuic acid **10**), 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid **11**) and 4-hydroxy-3-methoxybenzoic acid (vanillic acid **12**) were obtained from Roth (Karlsruhe, Germany), 3,4-dihydroxyphenylacetic acid, *p*-nitrophenyl α-D-galactopyranoside, *p*-nitrophenyl β-D-glucopyranoside and *p*-nitrophenol were provided by Sigma–Aldrich (Taufkirchen, Germany). Because it is more practical, general names are used in the manuscript for the phenolic degradation products **10**–**15**. Solvents for HPLC as well as all other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich in gradient or reagent grade quality. Water was purified with a MilliQ Gradient A10 (Millipore, Schwalbach, Germany) system.

4.2. Preparation of inoculum

The caeci were isolated from freshly slaughtered pigs, immediately ligated and removed in an anaerobic jar containing Anaerocult® A (Merck, Darmstadt, Germany) to retain the anaerobic atmosphere. In the laboratory, all experiments with the caeci were performed in a Sekuroka®-Glove Bag (Roth) flushed with CO_2, excluding O_2 completely. The isolated inoculum of each caecum was suspended in the same volume (w/w) of 0.15 M PBS (pH 6.4) containing a trace element solution 0.0125% (13.2 g/100 ml $CaCl_2{\cdot}2H_2O$, 10.0 g/100 ml $MnCl_2{\cdot}4H_2O$, 1.0 g/100 ml $CoCl_2{\cdot}6\,H_2O$ and 8.0 g/100 ml $FeCl_3{\cdot}6H_2O$) and a Na_2S-solution 11.11% (575.9 mg/100 ml of 0.037 M NaOH) according to Seefelder.[40] All buffers, solutions and vessels were flushed with a mixture of N_2 and CO_2 (5:1; v/v) before use. To remove larger particles, the inoculum suspensions were filtered through a coarse material net. The filtrates were used for the incubation experiments. An aliquot of the filtrates was inactivated by sterilization at 121 °C for 15 min at 1.1 bar (AMB240 autoclave, Astell, Kent, UK). The inactivated faecal suspensions were used as control for chemical degradation and matrix effects. Each incubation experiment was performed with three different caeci.

4.3. Incubation experiments

The preparation of the incubation experiments was performed in the glove bag maintaining anaerobiosis. Stock solutions of the analytes were prepared: anthocyanidins **7**–**9** (1 mM) were dissolved in methanol containing 1% HCl (concn 37%); anthocyanins **1**–**6** (1 mM), phloroglucinol aldehyde **13** (1 mM), phenolic acids **10**–**12** (1 mM), *p*-nitrophenyl α-D-galactoside (10 mM) and *p*-nitrophenyl β-D-glucoside (10 mM) were dissolved in methanol/water (1:1; v/v) containing 1% HCl (concn 37%). For the incubation experiments, 0.1 ml of the stock solution was added to 0.9 ml vital or sterilized inoculum filtrate in a 2 ml plastic vial. For each incubation time, a separate vial was used. Aliquots of the non-sterilized inoculum filtrate with solvent of the referring stock solution were prepared as matrix blank samples. The maximum methanol concentration in the incubation experiments reached 5% (v/v), which did not have any negative effect on the bacterial vitality. For the chemical degradation of

anthocyanidins **7**–**9**, 0.1 ml of the stock solutions was incubated with 0.9 ml supplemented PBS (pH 6.4). The sealed vials were placed in an incubator (37 °C) for 20, 40 min, 1, 2, 4 and 24 h. The microbial metabolism was stopped by placing the vessels in a freezer at −80 °C.

4.4. Sample preparation and analysis

4.4.1. α-D-Galactosidase and β-D-glucosidase activities. The frozen samples were thawed quickly in a water bath at 37 °C. The samples were centrifuged immediately at 15,000*g* for 20 min (4 °C). Aliquots of the supernatants were diluted with 0.5 M KOH (1:20) in a 96-well microplate. The absorbance of each well was measured with a microplate reader (FLUOstar Optima, BMG LabTechnologies, Jena, Germany) at 405 nm, calculating the released *p*-nitrophenol (PNP) using a standard calibration curve in the range of 0.1–15 μg/ml. Because of the matrix background absorption, active inoculum without substrate was applied as blank.

4.4.2. HPLC-DAD analysis (1–9, 10–13). The frozen samples were thawed quickly in a water bath at 37 °C. For inactivating and extraction, 1 ml methanol containing 10% HCOOH was added immediately. After shaking, the samples were centrifuged at 15,000*g* for 20 min (4 °C). Supernatants were filtered (GHP Ascrodisc 0, 2 μm, Pall Life Sciences, Ann Arbor, USA) and aliquots of the filtrate were injected into the HPLC. The compounds were separated on an analytical Eurospher 100 column (250 × 4.6 mm i.d., 5 μm; Knauer, Berlin, Germany) using a binary gradient delivered by a Jasco PU-2089 low-pressure gradient HPLC pump (Jasco, Groß-Umstadt, Germany) with 5% (v/v) formic acid as solvent A and methanol as solvent B. The following gradient was used: 0 min, 15% solvent B; 5 min, 25% solvent B; 10 min, 30% solvent B; 15 min, 60 % solvent B; 24 min, 90% solvent B; 27 min, 90% solvent B; 30 min, 15% solvent B; 32 min, 15% solvent B. The flow rate was 1 ml/min. For injection (40 μl), a Jasco autosampler AS-2057 Plus was used. A Jasco diodearray detector MP-2010 Plus was applied over the wavelength range of 220–600 nm for peak detection. Data acquisition was carried out with the software Borwin-PDA 1.5 (Jasco). Compounds were identified by comparison of the UV spectra and the retention times with authentic references. Concentrations were calculated using calibration curves ranging from 1 to 120 μM for anthocyans **1**–**9** and 5 to 120 μM for phenolic acids **10**–**12** and phloroglucinol aldehyde **13**. The following wavelengths were monitored for quantitative analysis: 515 nm for anthocyans **1**–**9**, 291 nm for phloroglucinol aldehyde **13** and vanillic acid **12**, 275 nm for syringic acid **11** and 265 nm for protocatechuic acid **10**.

4.4.3. GC–MS analysis (12–17). Aliquots of the HPLC filtrates were supplemented with 3,4-dihydroxy-phenylacetic acid (end concentration 25 μM) as internal standard **IS** and dried under a stream of nitrogen. The residues were derivatized with *N*,*O*-bis(trimethylsilyl)-acetamide for 20 min at 55 °C. After centrifugation (15,000*g*, 10 min), the supernatants were injected into the GC–MS system. Electron impact (EI) gas chromatography–mass spectrometry (GC-MS) data were acquired on a HP6890 series gas chromatograph and HP5973 mass spectrometer (Hewlett–Packard/Agilent, Böblingen, Germany). Data acquisition was carried out with the Chemstation software (Agilent). Chromatographic separation was performed on a 60 m × 0.25 mm i.d. fused silica, 0.25 μm methyl silicone coating J&W Scientific DB-1 column (Agilent) using 1 ml/min helium as carrier gas. The injector temperature was set at 280 °C, while the injection volume was 1 μl splitless. The column temperature was held initially at 160 °C for 1 min, then programmed at 4 °C/min to 260 °C, then with 15 °C/min to 320 °C, which was held isothermally for 10 min. The transfer line was heated at 320 °C. The mass spectrometer was operated in the electron impact mode (EI, 70 eV electron energy) with a source temperature of 230 °C and the quadrupole was heated at 150 °C. Mass spectra were acquired in the full scan mode ranging from *m*/*z* 40 to 800 with a scan rate of 2.0 scans/s. The signals were identified by comparison of the retention times and mass spectral data with silylated authentic reference compounds and a mass spectra library (NIST, Gaithersburg, USA). The mass spectrometer was operated in the selected ion monitoring mode detecting ions at *m*/*z* 312 (vanillic acid **12**, t_R 13.4 min), *m*/*z* 342 (syringic acid **11**, t_R 16.6 min), *m*/*z* 355 (phloroglucinol aldehyde **13**, t_R 17.1 min), *m*/*z* 370 (protocatechuic acid **10**, t_R 15.2 min) and *m*/*z* 384 (3,4-dihydroxyphenylacetic acid **IS**, t_R 15.3 min). Concentrations were calculated using calibration curves ranging from 1 to 75 μM for **10**–**13**.

Acknowledgment

We thank B. Kurzen for supplying us with the caeci.

References and notes

1. Jurd, L. *Adv. Food Res. Suppl.* **1972**, *3*, 123.
2. Harborne, J. B. *The Flavonoids: Advances in Research Since 1986*; Chapman & Hall: London, 1994.
3. Kühnau, J. *World Rev. Nutr. Diet* **1976**, *24*, 117.
4. Scalbert, A.; Williamson, G. *J. Nutr.* **2000**, *130*, 2073.
5. Clifford, M. N. *J. Sci. Food Agric.* **2000**, *80*, 1063.
6. Rice-Evans, C. *Curr. Med. Chem.* **2001**, *8*, 797.
7. Hollmann, P. C. H. *J. Sci. Food Agric.* **2001**, *81*, 842.
8. Rice-Evans, C. A.; Miller, N. J.; Paganga, G. *Free Radical Biol. Med.* **1996**, *20*, 933.
9. Tsuda, T.; Shiga, K.; Kawakishi, S.; Osawa, T. *Biochem. Pharmacol.* **1996**, *52*, 1033.
10. Wang, H.; Cao, G.; Prior, R. L. *J. Agric. Food Chem.* **1997**, *45*, 304.
11. Pool-Zobel, B. L.; Bub, A.; Schroder, N.; Rechkemmer, G. *Eur. J. Nutr.* **1999**, *38*, 227.
12. Matsumoto, H.; Nakamura, Y.; Hirayama, M.; Yoshiki, Y.; Okubo, K. *J. Agric. Food Chem.* **2002**, *50*, 5034.
13. Ghiselli, A.; Nardini, M.; Baldi, A.; Scaccini, C. *J. Agric. Food Chem.* **1998**, *46*, 361.
14. Satué-Gracia, M. T.; Heinonen, M.; Frankel, E. N. *J. Agric. Food Chem.* **1997**, *45*, 3362.
15. Kamei, H.; Kojima, T.; Hasegawa, M.; Koide, T.; Umeda, T.; Yukawa, T.; Terabe, K. *Cancer Invest.* **1995**, *13*, 590.

16. Meiers, S.; Kemény, M.; Weyand, U.; Gastpar, R.; von Angerer, E.; Marko, D. *J. Agric. Food Chem.* **2001**, *49*, 958.
17. Wang, H.; Nair, M. G.; Strasburg, G. M.; Chang, Y.-C.; Booren, A. M.; Gray, J. I.; DeWitt, D. L. *J. Nat. Prod.* **1999**, *62*, 294.
18. Netzel, M.; Strass, G.; Janssen, M.; Bitsch, I.; Bitsch, R. *J. Environ. Pathol. Toxicol. Oncol.* **2001**, *20*, 89.
19. Bub, A.; Watzl, B.; Heeb, D.; Rechkemmer, G.; Briviba, K. *Eur. J. Nutr.* **2001**, *40*, 113.
20. Matsumoto, H.; Inaba, H.; Kishi, M.; Tomingag, S.; Hirayama, M.; Tsuda, T. *J. Agric. Food Chem.* **2001**, *49*, 1546.
21. Nielsen, I. L. F.; Dragsted, L. O.; Ravn-Haren, G.; Freese, R.; Rasmussen, S. E. *J. Agric. Food Chem.* **2003**, *51*, 2813.
22. Tsuda, T.; Horio, F.; Osawa, T. *FEBS Lett.* **1999**, *449*, 179.
23. Miyazawa, T.; Nakawaga, K.; Kudo, M.; Muraishi, K.; Someya, K. *J. Agric. Food Chem.* **1999**, *47*, 1083.
24. Felgines, C.; Texier, O.; Besson, C.; Fraisse, D.; Lamaison, J.-L.; Rémésy, C. *J. Nutr.* **2002**, *132*, 1249.
25. Wu, X.; Cao, G.; Prior, R. L. *J. Nutr.* **2002**, *132*, 1856.
26. Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Manach, C.; Lamaison, J.-L.; Remesy, C. *J. Nutr.* **2004**, *134*, 2275.
27. Rice-Evans, C. A.; Spencer, J. P. E.; Schroeter, H.; Rechner, A. *Drug Metab. Drug Interact.* **2000**, *17*, 291.
28. Markakis, P. *CRC Crit. Rev. Food Technol.* **1974**, *4*, 437.
29. Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C. *J. Agric. Food Chem.* **1999**, *47*, 1453.
30. Kawabata, J.; Okamoto, Y.; Kodama, A.; Makimoto, T.; Kasai, T. *J. Agric. Food Chem.* **2002**, *50*, 5468.
31. Sroka, Z.; Cisowski, W. *Food Chem. Toxicol.* **2003**, *41*, 753.
32. Ohnishi, M.; Yoshimi, N.; Kawamori, T.; Ino, N.; Hirose, Y.; Tanaka, T.; Yamahara, J.; Miyata, H.; Mori, H. *Jpn. J. Cancer Res.* **1997**, *88*, 111.
33. Aura, A.-M.; Martin-Lopez, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K.-M.; Poutanen, K.; Santos-Buelga, C. *Eur. J. Nutr.* **2005**, *44*, 133.
34. Fleschhut, J.; Rechkemmer, G.; Kulling, S. E. *Lebensmittelchemie* **2003**, *57*, 25.
35. Miyake, Y.; Yamamoto, K.; Osawa, T. *J. Agric. Food Chem.* **1997**, *45*, 3738.
36. Braune, A.; Engst, W.; Blaut, M. *J. Agric. Food Chem.* **2005**, *53*, 1782.
37. Winter, J.; Moore, L. H.; Dowell, V. R.; Bokkenheuser, V. D. *Appl. Environ. Microbiol.* **1989**, *55*, 1203.
38. Finegold, S. M.; Sutter, V. L.; Mathisen, G. E. In *Human Intestinal Microflora in Health and Disease*; Hentges, D. J., Ed.; Academic Press: London, 1983, pp 3–31.
39. Steer, T. E.; Johnson, I. T.; Gee, J. M.; Gibson, G. R. *Br. J. Nutr.* **2003**, *90*, 635.
40. Seefelder, W. Ph.D. Dissertation, University of Würzburg, Germany, 2002.
41. Keppler, K.; Humpf, H.-U. *Lebensmittelchemie* **2004**, *58*, 60.
42. Sarlikiotis, A. W.; Betzing, J.; Wohlschlegel, C.; Bauer, K. H. *Pharm. Pharmacol. Lett.* **1992**, *2*, 62.
43. Pond, W. G.; Houpt, K. A. *The Biology of Pig*; Cornell University Press: New York, 1978.
44. Labib, S.; Erb, A.; Kraus, M.; Wickert, T.; Richling, E. *Mol. Nutr. Food Res.* **2004**, *48*, 326.
45. Daniels, L. B.; Coyle, P. J.; Chiao, Y.; Glew, R. H. *J. Biol. Chem.* **1981**, *256*, 13004.
46. Dahlqvist, A.; Borgström, B. *Biochem. J.* **1961**, *81*, 411.
47. Day, A. J.; Canada, F. J.; Diaz, J. C.; Kroon, P. A.; Mclauchlan, W. R.; Faulds, C. B.; Plumb, G. W.; Morgan, M. R. A.; Williamson, G. *FEBS Lett.* **2000**, *468*, 166.
48. Aura, A.-M.; O'Leary, K. A.; Williamson, G.; Ojala, M.; Bailey, M.; Puuponen-Pimiä, R.; Nuutila, A. M.; Oksman-Caldentey, K.-M.; Poutanen, K. *J. Agric. Food Chem.* **2002**, *50*, 1725.
49. Piffaut, B.; Kader, F.; Girardin, M.; Metche, M. *Food Chem.* **1994**, *50*, 115.
50. Seeram, N. P.; Bourquin, L. D.; Nair, M. G. *J. Agric. Food Chem.* **2001**, *49*, 4924.
51. Gee, J. M.; Rhodes, M. J. C.; Johnson, I. T. *Free Radical Biol. Med.* **1998**, *25*, 19.
52. Pérez-Vicente, A.; Gil-Izquiero, A.; García-Viguera, C. *J. Agric. Food Chem.* **2002**, *50*, 2308.
53. Inoue, M.; Suzuki, R.; Koide, T.; Sakaguchi, N.; Ogihara, Y.; Yabu, Y. *Biophys. Res. Commun.* **1994**, *204*, 898.
54. Inoue, M.; Suzuki, R.; Sakaguchi, N.; Li, Z.; Takeda, T.; Ogihara, Y.; Jiang, B. Y.; Chen, Y. *Biol. Pharm. Bull.* **1995**, *18*, 1526.
55. Konishi, Y.; Kubo, K.; Shimuzu, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2014.
56. Konishi, Y.; Kobayashi, S.; Shimuzu, M. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 2317.
57. Konishi, Y.; Kobayashi, S. *J. Agric. Food Chem.* **2004**, *52*, 2518.
58. Konishi, Y.; Kobayashi, S. *J. Agric. Food Chem.* **2004**, *52*, 6418.
59. Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. *FEBS Lett.* **2003**, *544*, 210.
60. Talavera, S.; Felgines, C.; Texier, O.; Besson, C.; Lamaison, J.-L.; Remesy, C. *J. Nutr.* **2003**, *133*, 4178.