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ORIGINAL CONTRIBUTION

Achim Bub
Bernhard Watzl
Daniel Heeb
Gerhard Rechkemmer
Karlis Briviba

Malvidin-3-glucoside bioavailability in humans after ingestion of red wine, dealcoholized red wine and red grape juice

Summary *Background & Aims* Dietary polyphenols, including anthocyanins, are suggested to be involved in the protective effects of red wine against cardiovascular diseases. Very little data are available concerning the bioavailability of anthocyanins, major sources of red pigmentation in red wine. The aim of this study was to compare changes in plasma malvidin-3-glucoside (M-3-G), a red wine anthocyanin, and its urinary excretion after ingestion of red wine, dealcoholized red wine and red grape juice. *Design* Six healthy male subjects were studied in a randomized cross over setting in a human nutrition research unit under controlled conditions. All subject consumed 500 mL of each beverage on separate days providing the following M-3-G quantities: red wine 68 mg, dealcoholized red wine 58 mg, and red grape juice 117 mg. M-3-G was measured by HPLC and photodiode detection. *Results* M-3-G was found in plasma and urine after ingestion of all the beverages studied. The aglycon, sulfate or glucuronate conjugates of M-3-G were not detected in plasma and urine. Increases in plasma M-3-G concentrations were not significantly different after the consumption of either red wine or dealcoholized red wine and were about two times less than those measured after consumption of red grape juice. This difference may be caused by the about two times higher M-3-G concentration determined in red grape juice. Area under the plasma concentration curves were as follows: 288 ± 127 nmol × h/L (red wine), 214 ± 124 nmol × h/L (dealcoholized red wine) and 662 ± 210 nmol × h/L (red grape juice) and showed a linear relationship with the amount of anthocyanin consumed (mean ± SD). *Conclusions* M-3-G is poorly absorbed after a single ingestion of red wine, dealcoholized red wine, or red grape juice and seems to be differentially metabolized as compared to other red grape polyphenols. Our results suggest that not anthocyanins such as M-3-G themselves but rather not yet identified anthocyanin metabolites and/or other polyphenols in red wine might be responsible for the observed antioxidant and health effects *in vivo* in subjects consuming red wine.

Key words Anthocyanins – Malvidin-3-glucoside – Red wine – Dealcoholized red wine – Red grape juice – Bioavailability – Urine

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A. Bub · B. Watzl · D. Heeb · G. Rechkemmer · K. Briviba (✉)
Institute for Nutritional Physiology
Federal Research Centre for Nutrition
Haid-und-Neu-Strasse 9
76131 Karlsruhe, Germany
Tel.: +49-721/6625-407
Fax: +49-721/6625-404
E-Mail: karlis.briviba@bfe.uni-karlsruhe.de

Introduction

It has been proposed that regular consumption of red wine in moderate amounts reduces the risk for coronary heart disease via protection of LDL against oxidative damage and via inhibition of platelet aggregation [4, 10, 13]. Red wine polyphenols such as anthocyanins are thought to be partially responsible for these beneficial effects. Antioxidant activity of anthocyanins including the protection of LDL against oxidation was demonstrated in a number of different *in vitro* systems [14]. M-3-G (Fig. 1) is one of the anthocyanins present in red wine causing the red pigmentation in red grapes and red wine [8]. However, the data on the bioavailability of M-3-G in humans are so far very limited [1, 7]. It is also not

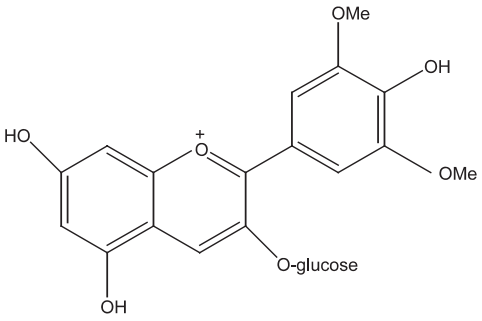

Fig. 1 Structure of malvidin-3-glucoside.

known whether or not alcohol in red wine can affect bioavailability and excretion of anthocyanins. It has been proposed that alcohol can modify bioavailability of polyphenols by increasing intestinal absorption or by modulation of xenobiotic metabolism. In order to investigate the effect of alcohol and of the wine making process on the bioavailability of malvidin-3-glucoside in humans, we compared anthocyanin bioavailability in subjects consuming red wine or dealcoholized red wine. Profile and concentrations of individual polyphenols including anthocyanins in red grapes can vary greatly with grape species, variety, and production area [8]. To avoid this variance, we used red grape juice, red wine and dealcoholized red wine made from grapes (variety 'Lemberger') which all were obtained from the 1998 harvest from one vineyard in Germany (State Winery, Weinsberg).

Material and methods

Materials

Unless otherwise stated, all chemicals were purchased from Merck (Darmstadt, Germany). Malvidin-3-glucoside, cyanidin-3-glucoside, pelargonodin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and delphinidin-3-glucoside were purchased from Polyphenols AS (Sandnes, Norway). The dry red wine grape variety ('Lemberger', vintage 1998) and red grape juice (1998) were provided by the State Winery Weinsberg (Weinsberg, Baden-Württemberg, Germany). Dealcoholization of the red wine was achieved by a vacuum rectification process (Center for Dealcoholization EAZ Petershans GmbH, Waiblingen, Germany). All three beverages were produced from red grapes (variety 'Lemberger', vintage 1998) grown in the same vineyard (State Winery, Weinsberg, Germany).

Subjects and study design

Six non-smoking male volunteers participated in this study (Table 1). They were in good health as determined by a screening history and medical examination. They refrained from taking vitamin supplements or any medication two months before and during the study. The study was approved by the ethical committee of the Landesärztekammer Baden-Württemberg and all participants gave written consent. Body fat was calculated by bioelectrical impedance analysis (Biodynamics 310, Seattle, Washington, USA). Resting energy expenditure was determined after an overnight fast by indirect calorimetry (SensorMedics Vmax 229, Bilthoven, The Netherlands).

The study was performed under controlled conditions at the human nutrition unit of the Federal Research Center for Nutrition within a period of 4 weeks. A randomized cross over design was used. During the study the men adhered to their usual diet, but were instructed to avoid food products containing anthocyanins or products with a high polyphenol content (Table 2). Each volunteer had 4 experimental treatments: 500 mL of red wine (12 % ethanol v/v), dealcoholized red wine, red grape juice, or ethanol (12 % ethanol v/v), with a washout period of one week between each experimental day. After an overnight fast, volunteers took 500 mL of one of the 4 beverages together with 2 white rolls (150 g). Then 200 min later, subjects consumed a complete meal.

Table 1 Anthropometric data of the six subjects under study

	Mean*	S. D.
Age (y)	31	4
Height (cm)	182	6
Body mass (kg)	78	12
BMI (kg/m^2)	24	4
Body fat (%)	16	6

*: n=6

Table 2 Food from which the volunteers had to abstain from

Alcoholic beverages
Fruit juice
Coffee, tea
Red and blue colored berries
Citrus fruits
Apples
Apricots, peaches
Guava, mango
Cantalupe, watermelon

Blood and urine samples

Fasting venous blood samples were taken into EDTA-containing tubes (Sarstedt-Monovette, Nümbrecht, Germany) before, 20, 40, 60, 90, 120, 180, and 360 min and 24 h after beverage consumption and immediately placed on ice. Plasma was collected after centrifugation at 1500 × g for 10 min at 4 °C. For serum measurements, blood was collected in a "Serum-Z-Monovette" (Sarstedt-Monovette) and after complete clotting separated by centrifugation at 1500 × g for 10 min at room temperature. Plasma and serum were stored at –80 °C until analysis.

During the experimental day, urine samples were collected for 2 periods after beverage consumption each lasting 3 h (0–180 min, 180–360 min). Urine samples were stored in plastic bottles at –80 °C. For control measurements, urine samples were collected on the day before and on the day after the experimental day during the same day time for a period of 3 h each. Total cholesterol, HDL cholesterol, triglycerides, glucose, γ-glutamyl-transferase, and ethanol were analyzed by commercially available enzymatic kits (Roche, Mannheim, Germany). Urinary creatinine was analyzed enzymatically (Labor+Technik Lehmann, Berlin, Germany).

Sample preparation for analysis of polyphenols in beverages, plasma and urine

Red wine, dealcoholized red wine and red grape juice were diluted with HPLC mobile phase (solution A (4/4/92 CH_3OH/CH_3CN/1 % H_3PO_4 in H_2O (v/v/v))) and centrifuged at 14 000 × g for 10 min. A two hundred microliter sample was used for HPLC analysis. For the analysis of beverages, the gradient cycle consisted of an initial 15-min isocratic segment (solution A 100 %). Then the linear gradient was changed progressively by increasing solution B (100 % CH_3CN) to 11 % at 25 min, 14 % at 32 min. It was maintained at 86 % solution A and 14 % solution B from 32 to 40 min and then solution B was increased progressively to 20 % at 50 min and finally changed back to 0 % solution B. Polyphenol content in beverages is shown in Table 3.

Polyphenols in plasma and urine were extracted using solid-phase extraction cartridge as described by Cao and Prior (1999) with slight modifications. Briefly, the cartridge (Sep-Pak C18, Waters, Milford, MS) was washed with 10 mL of methanol and equilibrated with 10 mL of 1 % H_3PO_4 in water. Two hundred microliters of ortho-phosphoric acid (85 %) was added to 2 ml of EDTA plasma. The samples were centrifuged at 3660 × g for 10 min at 4 °C and the supernatant was applied to the equilibrated cartridge. The water-soluble compounds were removed by water containing 1 % H_3PO_4. Anthocyanins were eluted with 10 mL 1 % H_3PO_4 in methanol. The methanol phase was collected and solvent was removed under N_2. The samples were redissolved in 300 µL of the HPLC mobil phase (84 % water containing 1 % H_3PO_4, 16 % CH_3CN (v/v)) and 200 µL were injected into the HPLC.

Anthocyanins in plasma were analyzed using the mobile phase (84 % water containing 1 % H_3PO_4, 16 % acetonitrile). After each HPLC-run, the column was washed with 100 % acetonitrile for 10 min. For urine, 800 µL of ortho-phosphoric acid (85 %) was added to 8 ml urine and the samples were centrifuged at 3660 × g for 10 min at 4 °C. The supernatant was applied to the equilibrated cartridge and anthocyanins were extracted using solid-phase extraction cartridge as described above. The methanol phase was collected and methanol was removed under N_2. The samples were dissolved in 800 µL of the HPLC mobile phase (solution A (4/4/92 CH_3OH/CH_3CN/1 % H_3PO_4 in H_2O (v/v/v))) and 200 µL were injected into the HPLC. The gradient cycle consisted of an initial 3-min isocratic segment (92 % A, 8 % B). Solution B was 100 % CH_3CN. Then the linear gradient was changed progressively by increasing solution B to 10 % at 35 min, and 25 % at 45 min. Then the column was washed with 100 % B for 5 min and finally changed back to 8 % solution B.

For anthocyanin measurements in urine, individual samples were treated with β-glucuronidase and sulfatase before solid phase extraction. A slightly modified version of a previously described method was used (Donovan et al. 1999). Briefly, 200 µL phosphate buffered ascorbic acid (PBA, 200 g/L ascorbic acid, 0.4 M NaH_2PO_4, pH 3.6) was mixed with 8 mL urine and incu-

Table 3 Contents of anthocyanins, catechins and resveratrol in beverages

Beverage	Anthocyanins (mg/L)						Catechins (mg/L)		Resveratrol (mg/L)
	M-3-G	Pt-3-G	D-3-G	C-3-G	Po-3-G	∑ Acy	(+)-Catechin	(–)-Epicatechin	
Red wine	136.9 ± 1.3	18.4 ± 0.1	13.0 ± 0.2	0.31 ± 0.01	2.50 ± 0.1	171.1	35.5 ± 0.4	27.9 ± 0.4	4.94 ± 0.04
Dealcoholized red wine	115.4 ± 1.3	15.7 ± 0.1	11.3 ± 0.1	0.24 ± 0.02	2.18 ± 0.1	144.8	27.4 ± 0.7	19.6 ± 0.4	5.15 ± 0.17
Red grape juice	233.6 ± 0.5	41.4 ± 0.1	46.3 ± 0.1	3.28 ± 0.01	14.0 ± 0.1	338.6	37.5 ± 1.1	26.8 ± 1.1	3.75 ± 0.05

Values are means ± SD (n > 5). Polyphenol concentrations were determined as described in the Materials and methods. *M-3-G* malvidin-3-glucoside; *Pt-3-gluc* petunidin-3-glucoside; *D-3-G* delphinidin-3-glucoside; *C-3-G* cyanidin-3-glucoside; *Po-3-G* peonidin-3-glucoside; *Acy* Anthocyanin

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bated with 400 U sulfatase and 10,000 U β-glucuronidase at 37 °C for 45 min. Polyphenols were extracted using a solid-phase extraction cartridge as described above. The gradient cycle consisted of an initial 15-min isocratic segment (100 % solution A). Solution A was 4/4/92 CH_3OH/CH_3CN/1 % H_3PO_4 in H_2O (v/v/v). Then the linear gradient was changed progressively by increasing solution B (100 % CH_3CN) to 11 % at 25 min, and 14 % at 32 min. It was maintained at 86 % solution A and 14 % solution B from 32 to 40 min and then solution B was increased progressively to 20 % at 50 min and finally changed back to 0 % solution B.

Detection of polyphenols by HPLC

Polyphenols were determined by reverse-phase HPLC using a Nova-Pak-C18, column (4 µm, 4.6 × 250 mm) from Waters. Samples were analyzed using a Shimadzu photodiode detector at 280, 350 and 520 nm and a Shimadzu fluorescence detector (λ_{ex}280/λ_{em}320 nm) for catechins. A binary gradient and a total flow rate of 1 mL/min was used. Polyphenolic compounds were identified by comparing their retention time and UV-vis spectra with those of standards. Catechin and epicatechin were also identified by fluorescence detection. The concentrations of polyphenols were calculated from the calibration curves made with standard solutions.

Statistics

Results are reported as means ± standard deviation (SD) or standard error of the mean (SEM). Changes between the baseline (0 h) and the following time points among treatment groups were tested for significance by repeated-measures ANOVA with Fischer's test for comparison of individual means when appropriate. Area under the plasma concentration vs. time curve (AUC_{0-6h}) was calculated with the linear trapezoidal rule (SigmaPlot 4.0, SPSS Inc., Chicago, USA). Differences between treatment groups for AUC_{0-6h} as well as for urinary excretion of anthocyanins were tested by ANOVA. The time of mean maximum plasma concentrations for M-3-G was taken from the time course given in Fig. 3. The time of individual C_{max} was taken to calculate the mean time of C_{max}. Linear regression analysis was performed and the coefficient of correlation (r) was calculated. Significance was accepted at the $p < 0.05$ level. All statistical procedures were performed using StatView 5.0 (SAS Institute Inc., Cary, USA).

Results

All participants tolerated the intervention with red wine, dealcoholized red wine, red grape juice, and ethanol well and completed the study. Serum total cholesterol, HDL cholesterol, triglycerides, glucose, and γ-glutamyl-transferase were within the normal range, and base line values were not significantly different among the treatment groups (data not shown). Serum γ-glutamyl-transferase was not affected by any treatment during the entire study.

Malvidin-3-glucoside in plasma

The plasma samples collected before ingestion of beverages contained no compounds with an absorption maximum at 510–530 nm. After consumption of beverages a new peak was detected at 520 nm (Fig. 2). This compound showed the elution time and absorption spectra between 400–600 nm, which is typical for M-3-G. The aglycone of M-3-G, malvidin, was not detected in any plasma sample.

Time courses of M-3-G concentrations in plasma are shown in Fig. 3. Consumption of red wine caused a rapid increase in the M-3-G concentration and a mean maximum of 1.38 ± 0.36 nM was detected after 20 min. The mean maximum plasma concentrations of M-3-G after consumption of dealcoholized red wine and red grape juice were reached within 90 min and 180 min, respectively. There was no significant difference between maximum plasma concentrations of M-3-G after ingestion of red wine or dealcoholized red wine. The calculated time of C_{max} from individual C_{max} values were 50 ± 39 min (red wine), 90 ± 27 min (dealcoholized red wine), and 120 ± 50 min (red grape juice), with the time for C_{max} of red wine and red grape juice being significantly different ($p=0.008$). Also, the area under the curve after consumption of red wine and dealcoholized red wine was similar; 288 ± 127 nmol × h/L and 214 ± 124 nmol × h/L, respectively. This is in line with similar concentrations of M-3-G in both beverages (Table 1). These data indicate that alcohol did not affect the amount of M-3-G absorbed. However, alcohol may slightly modify the time course of increase in M-3-G plasma concentration (Fig. 3) by enhancing the early absorption of M-3-G. The increase in plasma M-3-G concentrations for red wine at 20 min tended to be higher than the dealcoholized red wine plasma concentration ($p=0.056$). Plasma ethanol concentrations were not different after red wine and ALC consumption with the maximum at 40 min (red wine: 0.73 ± 0.17‰, ALC: 0.69 ± 0.13‰).

The concentration of M-3-G in the red grape juice was about 2-fold higher than that in red wine or in dealcoholized red wine. Also the maximum plasma concen-

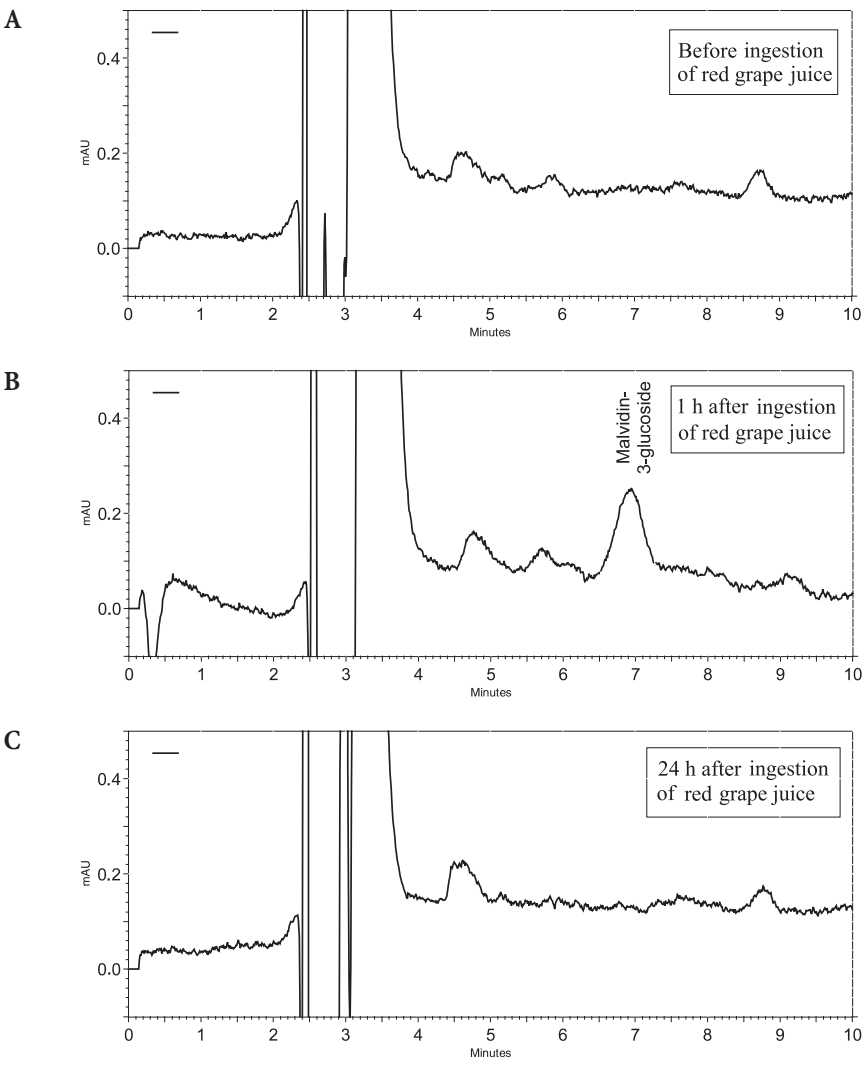

Fig. 2 Representative HPLC chromatograms of plasma samples collected before and after ingestion of 500 mL of red grape juice containing 117 mg malvidin-3-glucoside. The absorbance was monitored at 520 nm. **A** Plasma was collected before, **B** 60 min, and **C** 24 hours after ingestion of red wine.

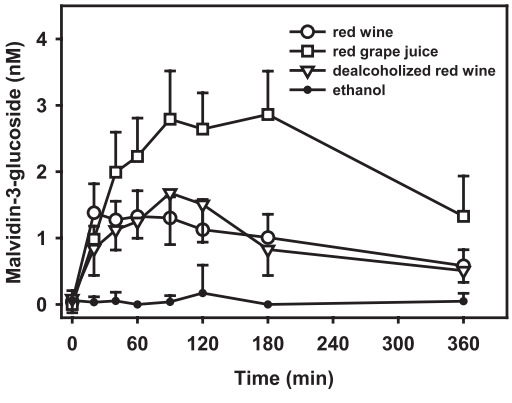

Fig. 3 Plasma malvidin-3-glucoside concentrations after ingestion of 500 mL of red wine, dealcoholized red wine and red grape juice. Data are presented as mean ± SEM of 6 subjects.

tration and area under curve for M-3-G was about 2-fold higher than those observed after ingestion of red wine ($p < 0.01$) or dealcoholized red wine ($p < 0.01$). There is a linear relationship ($r=0.837$) between the ingested amount of M-3-G and the AUC of M-3-G in plasma (Fig. 4).

Polyphenols such as catechins are often detected in the form of glucuronide or sulfate conjugates. In previous studies no glucuronates or sulfates of anthocyanins were found [1, 9]. Also, here, neither glucuronates nor sulfates of M-3-G were detected in the urine samples of 2 subjects after ingestion of 0.5 L of red grape juice (Fig. 5).

Malvidin-3-glucoside in urine

Consumption of red grape beverages increased the concentration of M-3-G in the urine samples collected dur-

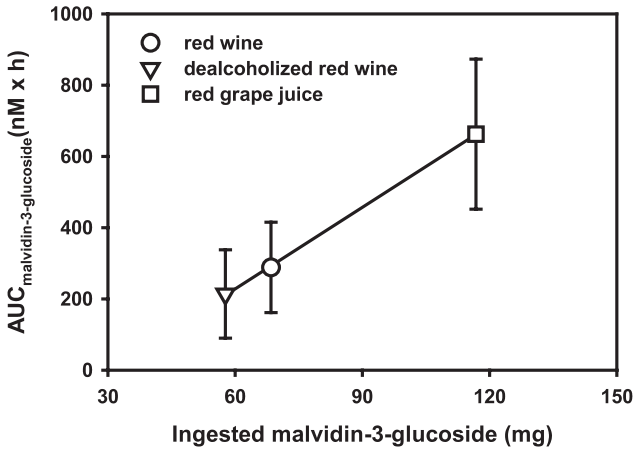

Fig. 4 Dose-response plot for the ingested amount of malvidin-3-glucoside and area under the plasma concentration curve. Data are presented as mean ± SD of 6 subjects.

ing the first six hours (Table 4). The aglycone malvidin was not detected in the urine samples. Similar to the plasma data, the urine samples showed the highest concentrations of M-3-G in the samples collected during the first 3 hours. Twenty four hours after ingestion of beverages, M-3-G was not detected in the urine samples, indicating a fast excretion of M-3-G. Although the dose of M-3-G ingested was almost twice as high during the red

Table 4 Total excretion of malvidin-3-glucoside (µg) in urine after ingestion of red wine, dealcoholized red wine and red grape juice

	0–3 hours	4–6 hours	Σ
Red wine	10.9±4.4	5.5±2.5	16.4
Dealcoholized red wine	8.2±2.9	5.1±1.7	13.3
Red grape juice	22.4±27.5	4.6±2.4	27.0

Data are presented as mean ± SD of 6 subjects.

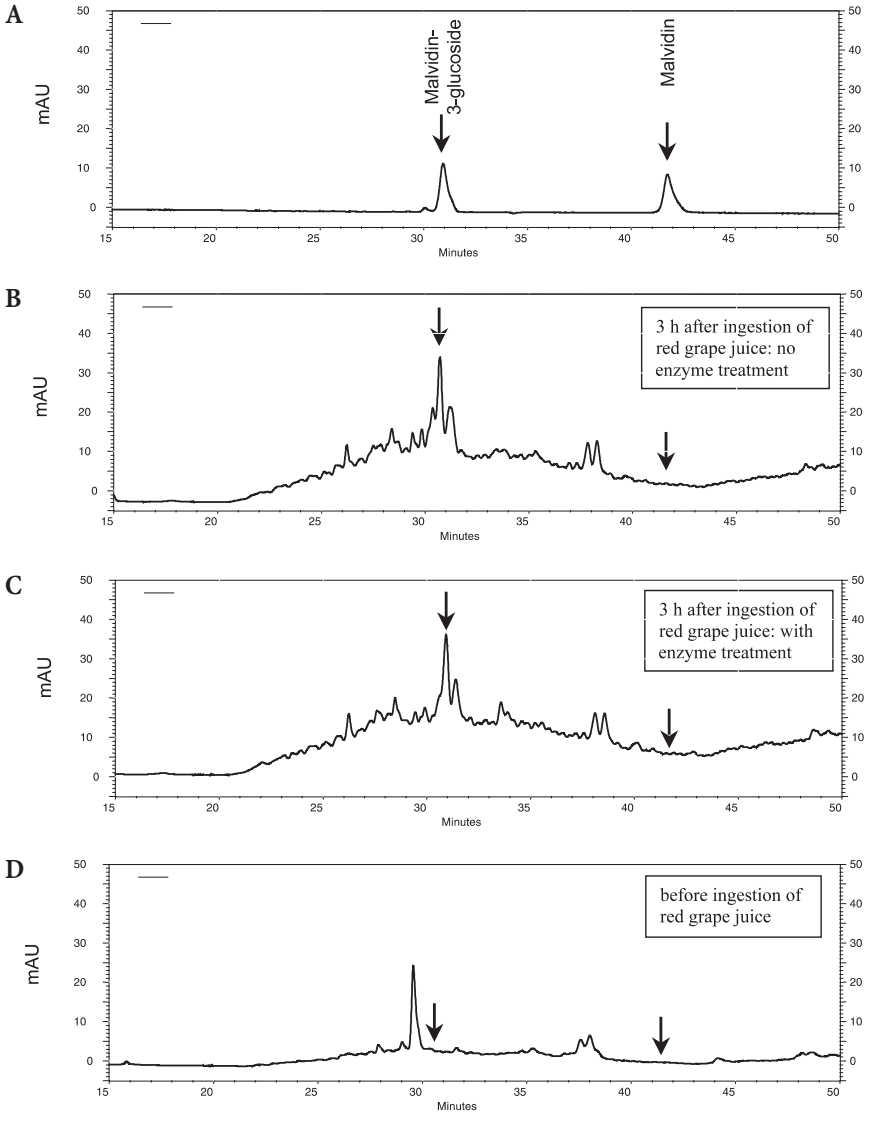

Fig. 5 Representative HPLC chromatograms of an urine sample treated with glucuronidase and sulfatase. The absorbance was monitored at 520 nm. **A** malvidin-3-glucoside and malvidin, **B** urine collected during the first 3 hours after ingestion of red grape juice, **C** urine collected during the first 3 hours was treated with glucuronidase and sulfatase as described in the Materials and methods and **D** urine collected before ingestion of red grape juice.

grape juice intervention, no significant differences in M-3-G excretion was found between the 3 beverages. The total amount of M-3-G excreted by urine during the first 6 hours after ingestion of red wine, dealcoholized red wine and red grape juice was 10.9 ± 4.3, 8.2 ± 2.9 and 22.4 ± 27.5 µg, respectively. This is less than 0.03 % of the ingested amount. Like in plasma, a linear relationship was found for the total excretion of M-3-G and the dose ingested (r=0.386). After red grape juice consumption, one subject had a very high excretion of M-3-G (77.8 µg) which is responsible for the extreme variation. So far, we have no explanation for this finding, since kidney function of this volunteer was not altered. This suggests high interindividual differences in bioavailability of anthocyanins.

Discussion

Previous studies on bioavailability of anthocyanins in humans using cyanidin glucosides from elderberry extract or concentrated juice of elderberries and black currants showed poor absorption of cyanidin glucosides [1, 9]. This is in line with our data for M-3-G after ingestion of red grape beverages. Consumption of 0.5 L of red wine containing 68.6 mg M-3-G resulted in a C_{max} for M-3-G in plasma of 1.4 nmol (0.6 µg)/L. Also, the amount of M-3-G found in urine was very low. During the first 6 hours, 16 µg M-3-G were excreted which is less than 0.03 % of the ingested amount. Lapidot et al. (1998) reported the urinary excretion of anthocyanins from red wine in 3 healthy volunteers to be 1.5–5 % of the ingested amount of 218 mg anthocyanins. However, they did not identify single anthocyanins, e. g., M-3-G, and therefore their results are not comparable to ours. Nevertheless, the excretion rate of anthocyanins is rather low and the fate of anthocyanins and especially of M-3-G in the human body remains unclear. Theoretically, several factors could contribute to the low bioavailability of anthocyanins, e. g., excretion with feces, decay at neutral pH in the small and large intestine, metabolism by the large intestinal microflora, rapid accumulation in different tissues or metabolism in intestinal mucosa and liver resulting in ring fission.

It was proposed that anthocyanins, similar to other flavonoids such as quercetin, are absorbed in the large intestine after deglucosylation by bacterial enzymes. β-Glucosidase activity is described in human small intestine. Lactase phlorizin hydrolase was also thought to be responsible for deglucosylation of quercetin glucosides [2]. However, also direct absorption of quercetin glucosides across the intestinal epithelium has been described [5, 6]. In accordance with previous studies, in our study, neither aglucons nor glucuronates/sulfates were found in plasma and urine samples, indicating that M-3-G is absorbed in its glucosylated form. This is in contrast to other wine constituents such as (+)-catechin, which is highly metabolized and present in human plasma after red wine consumption as methylate, sulphate and glucuronate/sulphate conjugates [3]. This indicates that polyphenols from red wine may have different metabolism routes in humans.

In the case of (+)-catechin, alcohol did not affect its bioavailability from red wine [3]. This is in line with our findings for malvidin-3-glucoside. The AUC for plasma M-3-G was not different in red wine and dealcoholized red wine. The ingested amount of M-3-G from each beverage shows a linear relationship with the AUC of plasma M-3-G concentrations (Fig. 4). Therefore, ethanol in red wine does not seem to affect the absolute uptake and plasma concentrations of M-3-G.

Tissue and cell culture experiments indicate that monoglucosides of the flavonol quercetin are transported across the apical membrane of enterocytes by the sodium-dependent glucose transporter 1 (SGLT1) [5, 15]. The glucose content in the intestinal lumen after ingestion of flavonoid-containing food might influence flavonoid uptake. However, data relating this issue in humans are not available so far. In our study, the volunteers received on each experimental day the same "breakfast" and a similar lunch with the beverages. Differences in the total sugar consumption were only due to the sugar content of the beverages (red wine: 1 g/L; dealcoholized red wine: 1 g/L; red grape juice: 206 g/L). As a result, serum glucose after red grape juice consumption peaked at t=20 min (142 ± 22 g/dL), whereas red wine (121 ± 24 g/dL) and dealcoholized red wine (131 ± 23 g/dL) showed C_{max} for glucose at t=60 min. Mean time of individual C_{max} for plasma M-3-G after RGJ consumption was delayed (t=120 min) as compared to red wine (t=50 min, p=0.008) and dealcoholized red wine (t=90, p=0.10). This could point to a time delaying effect of the sugar present in RGJ, which could result from a competitive action of glucose and M-3-G on SGLT1. The difference between red wine and dealcoholized red wine was not statistically significant. However, a role of ethanol in early M-3-G absorption cannot be totally excluded, since the increase in plasma M-3-G concentrations for red wine at 20 min tended to be higher than the increase after dealcoholized red wine consumption (p=0.056).

Anthocyanins are potent antioxidants in *in vitro* systems [11, 12, 16]. *In vivo*, however, their contribution to the antioxidant activity in humans is questionable. It is rather unlikely that anthocyanins at such low plasma and urine concentrations as reported in this and some other papers [1, 9] would be able to compete effectively with endogenous or dietary antioxidants such as thiols, alpha-tocopherol, ascorbate, carotenoids and antioxidant enzymes, since their concentrations are 3–5 orders of magnitude higher in plasma. Thus, not yet identified and detected anthocyanin metabolites and/or other

polyphenols but not anthocyanins themselves might be responsible for the observed antioxidant activities and health effects *in vivo* related to the consumption of anthocyanin rich beverages, extracts, or food.

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