Bioavailability of Red Wine Anthocyanins As Detected in Human Urine†

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Anthocyanins, which are natural plant pigments from the flavonoid family, represent substantiated constituents of the human diet. Many foods but especially red grapes and wines contain large amounts of flavonoids, which are mostly anthocyanins. The aim of our study was to determine the potential bioavailability, in human, of several anthocyanins from red wine. Six healthy, fasting volunteers, having a polyphenols-free diet, drank 300 mL of water every hour for 12 h and collected urine. Several weeks later, the same volunteers repeated the same procedure but replaced the water of the fourth drinking dose with white wine. Two weeks later, they repeated the procedure with red instead of white wine. In the 300 mL dose of red wine, the subjects received 218 mg of anthocyanins, which were detected in their urine by HPLC analysis with a photodiode array detector. Two of the compounds found among the wine anthocyanins were found unchanged in the urine. Other anthocyanin compounds, which seemed to have undergone molecular modifications, were detected in the urine after incubation with HCl. The anthocyanin level in the urine reached a peak within 6 h of the wine drinking. Within 12 h of the wine drinking, we found 1.5−5.1% of the ingested anthocyanins, in the urine.

Keywords: *Red wine; anthocyanins; antioxidants; absorption; urine; human*

INTRODUCTION

Flavonoids, which occur both in edible plants and in foodstuffs derived from plants, form substantial constituents of the human diet. The daily intake of flavonoids in western countries has been estimated to be between 0.5 and 1.0 g (Kuhnau, 1976; Pierpoint, 1986). However, it seems that the intake is frequently lower (Hertog et al., 1993). Red grapes and wines contain large amount of flavonoids, mostly anthocyanins, at concentrations of 1000−3200 and 200−2000 mg/L, respectively, (Singelton, 1982; Macheix et al., 1990; Kanner et al., 1994).

Flavonoids and other plant phenolics have been reported to have multiple biological effects, such as antioxidant activity (Chipault et al., 1956; Chang et al., 1977; Hudson and Lewis, 1983; Bors and Saran, 1987; Kanner et al., 1994), antiinflammatory action (Lands and Hanel, 1982; Landolfi et al., 1984; Moroney et al., 1988), inhibition of platelet agregation (Van-Wauwe and Gossenec, 1983), antimicrobial activities (Dugan, 1980; Pratt and Hudson, 1990), and antitumor activities (Formica and Regelson, 1995).

Recently, much attention has been paid to the antioxidant properties of flavonoids, which seem to protect tissues against oxygen free radicals and lipid peroxidation. Circulating low-density lipoproteins (LDL) are one of the fundamental targets of deleterious oxidation, resulting in the accumulation of atherogenic lipoprotein in human in vivo.

The so-called "French paradox" is characterized by the fact that the inhabitants of some regions of France have a lower than average rate of coronary heart disease (CHD) despite their consumption at high levels of saturated fatty acids and cholesterol, which are positively correlated with increased risk of CHD. This paradox has been attributed by us to the nonalcoholic compounds, the phenolic antioxidants, which are very abundant in red wines. These phenolic antioxidants act protectively via prevention of the oxidation of LDL and the inhibition of platelet aggregation (Frankel et al., 1993; Kinsella et al., 1993; Kanner et al., 1994). The presence of significant amounts of anthocyanins in red wine contributes to its powerful antioxidative activity. Indeed, anthocyanins have been found to be strong antioxidants, in varied in vitro studies (Tamura and Yamagami, 1994; Tsuda et al., 1994; Kanner, 1997). However, in humans, the bioavailability of the flavonoids present in foods and especially in wines is an unclear important issue.

Most recently, Hollman et al. (1995), in a human study with ileostomy subjects, demonstrated the absorption of orally administrated quercetin aglycon and, especially, the high absorption from the intestine of onion quercetin glycosides. Several studies in rats showed that citrus flavonols and flavones and their glycosides may reach the blood stream after oral administration (Nieder, 1991; Cova et al., 1992). The absorption of the citrus flavanone, naringin, in humans has also been studied recently (Fuhr and Kummert, 1995; Ameer et al., 1996). The absorption properties of anthocyanins have been evaluated in the rat (Morazzoni et al., 1991), but no such study has been performed in humans. New evidence for the possible presence of anthocyanins in human plasma has been demonstrated (Paganga and Rice-Evans, 1997).

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The aim of the present study was to determine the potential bioavailability of several anthocyanins from red wine, in humans.

MATERIALS AND METHODS

Materials. Dihydrogen ammonium phosphate and orthophosphoric acid were obtained from Riedel de-Haen, Germany, and ethanol (HPLC grade) and hydrochloric acid from Bio Lab Ltd., Israel. The white wine was Sauvignon Blanc from the latest harvest, that had not been stored in oak barrels (Carmel Mizrahi, Israel), and the red wine was Israeli commercial Cabernet Sauvignon, 1.5 years old which had matured in French oak barrels for 8 mo. (Tzora Winery, Israel). Malvidin, cyanidin, delphinidin, and pelargonidin standards were provided by Roth, Germany. The malvidin-3-glucoside standard was purified as follows: The skins of 40 g of Argaman variety red grapes were blended for 1 min in 100 mL of 1:1 methanol/water solution (commercial blender, Waring, U.S.A.). The sample was then stirred for 10 min with a magnetic stirrer and centrifuged for 10 min at 2000 rpm (GLC-2 centrifuge, Sorvall, U.S.A.). The precipitate was extracted and centrifuged again with absolute methanol until the extract was colorless. The supernatants were concentrated together under vacuum at 40 °C and acidified to 0.1 M HCl with concentrated HCl. The concentrated extract was separated by the high-pressure-liquid-chromatography (HPLC) method described below, with 100 μL injection volume. The main anthocyanin, malvidin-3-glycoside was collected with the mobile phase into tubes at the exit of the detector. Purification of the pigment from the mobile phase was done through C18 cartridges (Sep-Pak Classic, Waters, U.S.A.): After loading 1 mL of the solution, the cartridge was washed with purified water until the pH values of the water entering and leaving the cartridge were equal. The adsorbed pigment was eluted with methanol acidified to pH 1.5 with HCl, the methanolic eluate was evaporated under vacuum, and the malvidin-3-glycoside was obtained as a dry powder. The purification was checked again by the HPLC analysis.

Subjects and Study Design. Six healthy fasting volunteers (three women and three men) aged 25−45, participated in our study, which was carried out at our lab. On each of the experiment days, they did not consume any kind of product rich in polyphenols (vegetables, fruits, tea, etc.) On the control day, every hour for 12 h, they drank 300 mL of water and collected urine into sterile tubes, which were frozen immediately (−20 °C). Several weeks later, on the first test day, they repeated the same procedure but replaced the fourth dose of water with white wine (the fourth dose was taken 4 h from the beginning of the experiment and 1 h after breakfast). After a further 2 weeks, on the second test day, the same volunteers repeated this experiment with 300 mL of red wine, containing 218 mg of anthocyanins, in place of the white wine.

Methods. The concentration of each urine tube was determined with a creatinine test kit (Sigma Diagnostics, U.S.A.). The urine was concentrated by freeze-drying, and the dried samples were kept at −20 °C until preparation for HPLC analysis. The dried urine samples were dissolved in a solution of 10% ethanol in water, at a volume which gave a creatinine concentration of 150 mg/dL (=13.26 mmol/L) and then centrifuged for 3 min at 14 000*g* in a cold centrifuge (Sigma, 201M). One part of the supernatant was acidified with 10% (v/v) of concentrated HCl, to bring its pH to 1. First HPLC analysis was carried out immediately after the acidification and a second analysis, following incubation of the sample with the HCl for 24 h in the dark, at room temperature. The same procedure was carried out with the wine and malvidin-3-glucoside standard solution. All the samples were filtered through a 0.45 μm nylon filter (Lida, USA). HPLC analysis was carried out according to Lamuela-Raventos and Waterhouse (1994), with modification as described below. The HPLC system used (LC-10A, Shimadzu, Germany) consisted of an auto injector, a photodiode array detector (SPD-M10Avp), and a software system which controlled all the equipment and carried out data integration and processing (CLASS-VP, Shimadzu). The injection volume was 20 μL, and a precolumn (Nova-Pak C18 Guard-Pak Waters) and cartridge column (Nova-Pak C18 60 Å 4 μM, 3.9 × 150 mm, Waters) were used. The linear gradient for the separation was Solvent A: 0.05 M dihydrogen ammonium phosphate adjusted to pH 2.6 with orthophosphoric acid; Solvent B: 20% A with 80% ethanol; Solvent C: 0.2 M orthophosphoric acid (pH 1.6). The total time for the separation was 80 min, with a flow of 0.7−1 mL/min. The identification of anthocyanins in wine and urine was according to anthocyanins standards with comparison to Lamuela-Raventos and Waterhouse (1994). An injection of 1 M HCl was used as a control for each series of samples. Samples were analyzed in duplicate. The wines were analyzed by the same HPLC method. Spectral scanning of urine samples and of anthocyanin solutions was preformed with an HP 8452A diode array spectrophotometer. Quantitative calculation of anthocyanins in wine and urine was based on the area under the peaks from the HPLC analysis, according to standard curve. The standard curve was based on eight different concentrations, between 0 and 40 μM, of malvidin-3-glycoside, each processed in duplicate. The malvidin-3-glycoside powder was dissolved in 1 M HCl and scanned by spectrophotometer. The concentration of the pigment solution was determined according to $E_{518\ nm} = 33\ 000\ L \cdot mol^{-1} \cdot cm^{-1}$ (Moscowitz and Hrazdina, 1981). The R^2 of the standard curve was 0.9934.

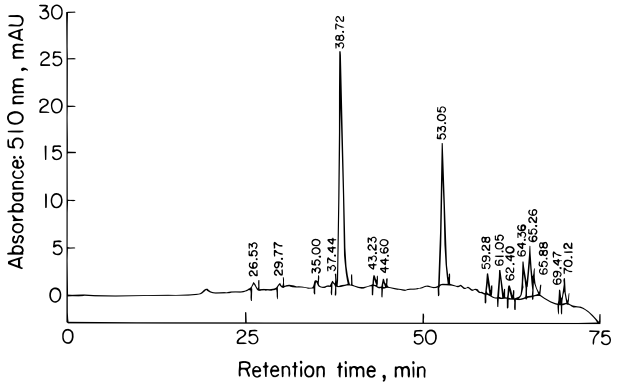

Figure 1. HPLC chromatogram of Cabernet Sauvignon wine. Detection at 510 nm.

RESULTS

The Cabernet Sauvignon red wine chromatogram (Figure 1) shows the presence of four main groups of anthocyanins: anthocyanidin-3-glycosides, with the aglycon identified as delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively (peaks 1−5, retention time (RT) 26.53−38.72 min); anthocyanidin-3-glycoside-acetate (peaks 6−8, RT 43.23−53.05 min). The third group (peaks 9−12, RT 61.05−64.36 min) seems to be comprised of anthocyanidin-3-glycoside-coumarates, according to Lamuela-Raventos and Waterhouse (1994). The peaks with RT of 65.26 and 65.88 min seem to be anthocyanin dimers, characterized by their specific absorption at 431 nm (Figure 4a,b) (Somers, 1971; Mazza, 1995).

After ingestion of red wine, human urine does not contain any pigments, which could be identified as anthocyanins. However, concentrated urine adjusted to pH 1.0 by HCl addition, immediately developed a pink pigment, which absorbed at 500 nm (Figure 2). This pink pigment was observed in the urine only after red wine ingestion. The pink pigment disappeared when the urine pH was adjusted back to 5.0. Such characteristic changes are very similar to those of anthocyanins, which convert from flavylium cation to a colorless

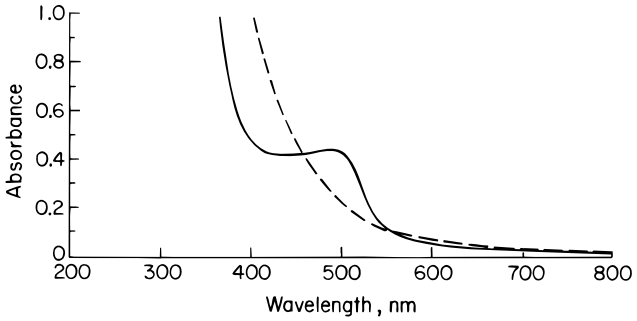

Figure 2. Spectra of human urine after red wine drinking: (- - -) urine and (—) urine at pH 1.0.

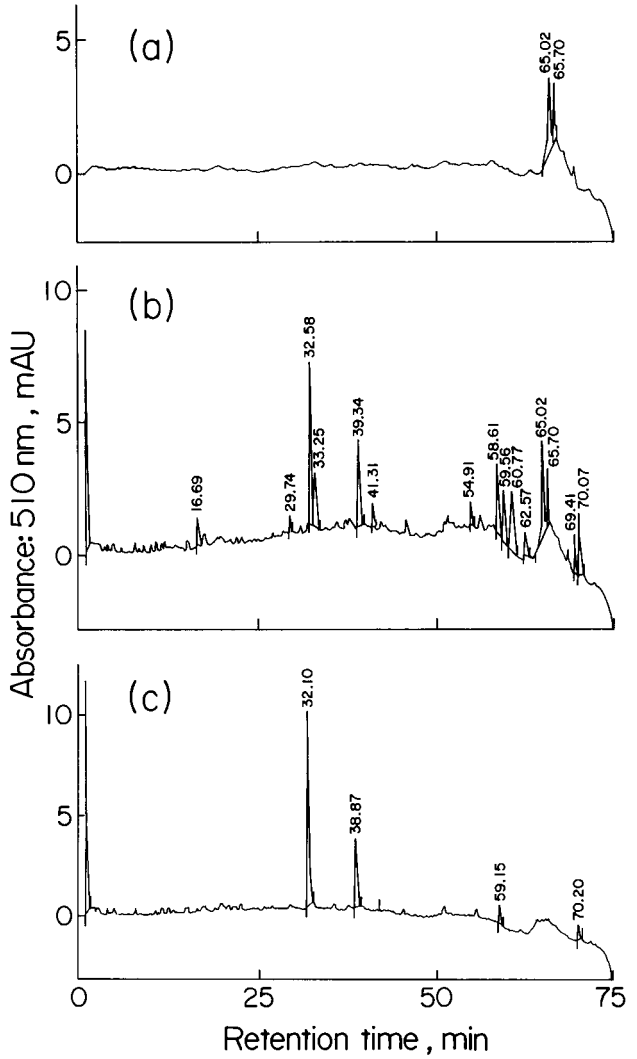

Figure 3. HPLC chromatograms of human urine after wine drinking: (a) urine after red wine drinking adjusted to pH 1; (b) urine after red wine drinking incubated with HCl, pH 1, for 24 h; (c) urine after white wine drinking incubated with HCl, pH 1, for 24 h.

pseudobase and vice versa, in response to pH changes (Brouillard and Delaporte, 1977).

A typical chromatogram of urine from a volunteer after ingestion of red wine, before incubation with HCl, is presented in Figure 3a. The chromatogram shows two peaks, at retention times of 65.02 and 65.70 min. These peaks seem identical to those of the compounds from red wine that appeared at the retention times of 65.26 and 65.88 min, respectively. The spectra of those peaks from red wine and urine are presented in Figure 4a,b. The absorption spectra of these two compounds did not show strong pH sensitivity as the spectra of the rest of the pigments, which are described below. This fact supports the characterization of these compounds as anthocyanin dimers (Boulton et al., 1996). When the urine was incubated with 1 M HCl for 24 h at room temperature, several other peaks were derived after HPLC analysis, as presented in Figure 3b and Table 1. The peaks that appeared at RT 33.25, 41.31, and 54.91 min showed typical spectra of anthocyanins (Table 1). These peaks did not appear at the urine chromatograms after white wine ingestion. The rest of the peaks, which appeared after incubation with HCl, showed spectra similar to those of anthocyanins but with a hypsochromic shift in the absorption maximum toward 487 nm (Table 1). After white wine ingestion urine adjusted to pH 1.0 with 1 M HCl did not contain any pigment, but after incubation at room temperature for 24 h it showed some of the pigments (Figure 3c) with the specific absorbance at 487 nm (Table 1). After water drinking urine did not contain any pigment even after 24 h of incubation with HCl. No changes were detected in the anthocyanins profile when the red wine or malvidin-3-glucoside standard solution were incubated for 24 h with HCl, by the same procedure as for the urine incubation. Each of the compounds derived at RT 32.58 and 39.34 min from urine after red wine drinking, and which had an absorption maximum at 487 nm, was collected with the mobile phase, into sterile tubes, after the separation by the HPLC. Those solutions having pH 1.9 were scanned by spectrophotometer and showed spectra maxima at 280 and 487 nm, as found by the HPLC detector. When the solutions were adjusted to pH 5.0, the absorption at 487 nm disappeared; when the pH was adjusted back to 1.9, the maximum at 487 nm would rise immediately.

The anthocyanins and the "anthocyanin-like" compounds (with an optical absorption maximum at 487 nm) were absorbed from the gut into the blood system, and it was possible to identify them in the urine after 1−3 h. The excretion kinetics during 12 h of the experiment was altered with the volunteers. Three representative diagrams are presented in Figure 5. Each of the volunteers ingested 218 mg of anthocyanins, calculated on the basis of malvidin-3-glycoside molecular weight. The calculated amount of anthocyanins and anthocyanin-like compounds in the urine was between 1.0 and 6.7% of that ingested. The amount of anthocyanins collected in the urine, as calculated from the peaks at 65.02 and 65.70 min, was 1.5−5.1% of the amount of those anthocyanins ingested by the volunteers.

DISCUSSION

Anthocyanins are natural pigments of the flavonoid family. They are responsible for the red, purple, and blue colors displayed in many vegetables and fruits. Our study presents data demonstrating the absorption in part of the anthocyanins in humans, after ingestion of a normal amount of red wine (two glasses).

The compounds seem to be eluted from the blood to the urine in a pseudobase form, the colorless form of the anthocyanins that appears in solutions at natural pH. These compounds form the red pigment, the flavylium cation, at low pH, as was demonstrated in our study when the urine excreted after red wine ingestion was acidified; the chemistry of these transformation has

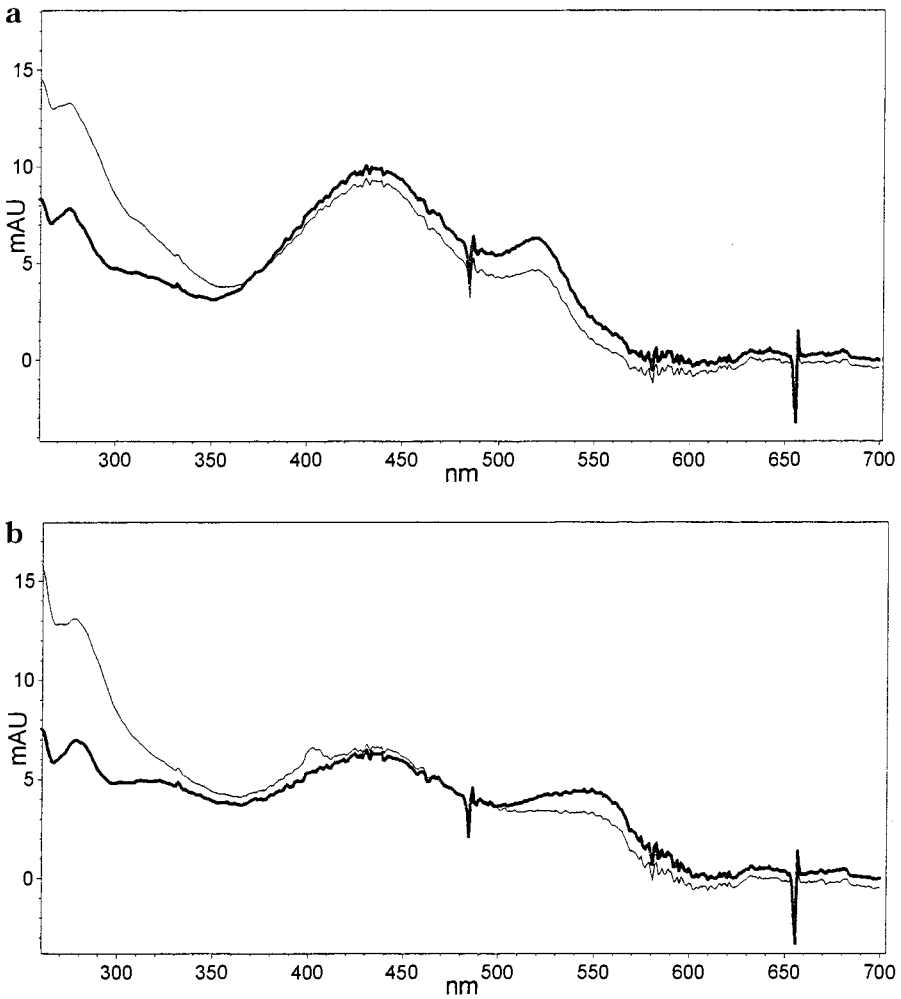

Figure 4. Spectra of pigments as derived from HPLC analysis, scanned by diode array detector: (a) (bold spectrum) compound at peak RT 65.26 min, from red wine, and (light spectrum) compound at peak RT 65.02 min, excreted in urine after red wine ingestion; (b) (bold spectrum) compound at peak RT 65.88 min, from red wine, and (light spectrum) compound at peak RT 65.70 min, excreted in urine after red wine ingestion.

Table 1. Pigments in Urine after Wine Ingestion As Derived on HPLC Analysis

	pigments found in urine by HPLC analysis	
treatment	RT	absorption max (nm)
red wine	32.58	279, 487
	33.25	272, 500
	39.34	262, 487
	41.31	262, 500
	54.91	277, 505
	58.61	280, 491
	59.56	280, 487
	60.77	260, 372, 490
	62.57	279, 487
	65.02[a]	276, 431, 520
	65.70[a]	277, 431, 545
white wine	32.10	270, 486
	38.87	270, 486
	59.15	270, 486

[a] Pigments which were found in the urine with and without incubation with HCl.

been very well described (Brouillard and Delaporte, 1977). The compounds in red wine that appeared at RT 65.26 and 65.88 min were most probably identical to the compounds in the urine that appeared at RT 65.02 and 65.70 min, as indicated by the similar RT and the same spectra. The small change in the RT is at the level of the instrument deviation. It seems that those

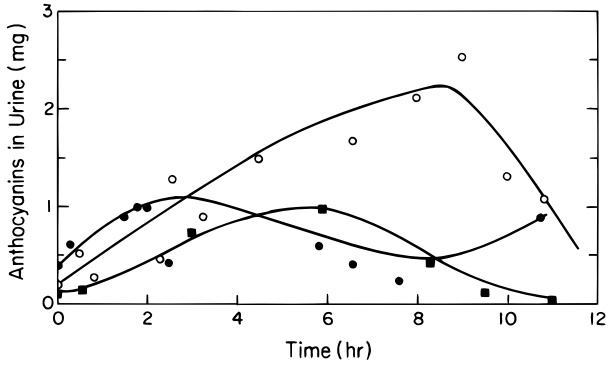

Figure 5. Anthocyanins excreted in urine of volunteers after consumption of 300 mL of red wine: (○) volunteer 1, (●) volunteer 2, (■) volunteer 3.

compounds in the urine had not gone through molecular changes in the volunteer's body, although we found only 1.5−5.1% of the amount ingested.

Among the compounds that appeared in the post-wine-urine after incubation with HCl, we distinguish the compounds at RT 33.25, 41.31, and 54.91 min. Those compounds appeared in urine only after red wine ingestion, and their spectra were very typical of anthocyanins. The explanation could lie in the chemistry of the anthocyanins: at natural pH, as in the blood or urine, the anthocyanin molecules seem to be present in

two main forms: pseudobase and chalcone. Both of these forms are colorless, but, whereas the pseudobase, when acidified, transforms rapidly to red flavylium-cation, the chalcone transforms back to the red pigment much more slowly. That could explain the length of time needed, in our experiment, to transform the colorless anthocyanins in the urine to a red pigment with optical absorption at 500 or 510 nm.

Proanthocyanidins are significantly abundant in both white and red wines. The reaction between proanthocyanidins and HCl is well-known (Weinges and Nader, 1982), and its products are anthocyanins. Such reaction could easily have taken place in our study during the incubation of the urine with HCl for 24 h at room temperature, if the urine contained proanthocyanidins or their derivatives. The occurrence of such reactions could explain the group of pink pigments that developed and showed optical absorbency maxima at 280 and 485 nm. Not only was the spectrum of those compounds similar to that of anthocyanin but also the color changes of the main compounds in the group (RT 32.10−32.58 and 38.87−39.34 min) elicited by pH changes exactly resembled the behavior of anthocyanins. Recently, vitisin, a new kind of anthocyanin-like pigment with an absorbance maximum at 482 nm was reported in red wine (Bakker and Timberlake, 1997).

The anthocyanins and the "anthocyanins-like" compounds were detected in the urine of the volunteers 1−3 h after the wine drinking. The main anthocyanins in the red Cabernet Sauvignon used in our experiment were malvidin-3-glycoside and malvidin-3-glycoside-acetate. They were not found with their original molecular structure in the urine of the volunteers. The pigments at retention time 39.34 and 38.87 min, which were found in the urine chromatograms, were not malvidin-3-glucoside, as indicated by the different spectra. In addition, spiking malvidin-3-glucoside standard into the urine sample gave two different peaks, though very close, instead of only one larger peak (of the malvidin-3-glucoside) (data not shown). In our opinion it is reasonable to suggest that those compounds were absorbed through the gut but underwent modifications which changed the chromophoric center and its elution pattern from the HPLC coloumn. The absorption of quercetin glycosides in human was demonstrated by Hollman et al. (1995). Some glycosides of flavonoids were detected in human plasma by Paganga and Rice-Evans (1997). Morazzoni et al. (1991) studied the pharmacokinetics of Vaccinium Myrtillus anthocyanin-glycosides, including malvidin-3-glucoside among them, in rats; they had given the rats 400 mg/kg of anthocyanins orally and detected about 5% of the administrated dose in the urine within 24 h, without taking modification of the molecules into account. In our study, the anthocyanins dose was about 3.5 mg/kg only. This 100-fold difference in the administered dose could explain the absence of malvidin-3-glucoside in the urine, in our study. The antioxidative action of the absorbed anthocyanins in vivo could lead to their molecular modification and destruction of the original form. In vitro experiments have shown that anthocyanin bleaches after its reaction with superoxide ($O_2^{\bullet -}$), a phenomenon which indicates a specific molecular modification (Yamasaki et al., 1996). Similar results were obtained by us during inhibition of lipid peroxidation by anthocyanins (unpublished results). Glucuronidation and methylation seem to be part of the metabolism of these compounds in humans. These modifications could start in the small intestine, liver, or kidney (Fuhr and Kummert, 1995; Ameer et al., 1996). The flavonoid, diosmetin, was found to be absorbed in rats mainly after glucuronidation and was detected in the rat's plasma 2−4 h after treatment (Boutin et al., 1993). The flavonoid, naringin, from grapefruit was found to be excreted in human urine mainly as naringenin glucuronide (Fuhr and Kummert, 1995).

The study reported here presents a method to detect anthocyanins in human urine and to follow the kinetics of their excretion after normal consumption of wine. The final identification of the anthocyanins in the urine as well as their detection in the plasma is now being studied in our laboratory.

Our study also presents, in part, data on the bioavailability in humans of several anthocyanins which are dietary antioxidants found in wines and in many fruits and vegetables.

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