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Red wine anthocyanins are rapidly absorbed in humans and affect monocyte chemoattractant protein 1 levels and antioxidant capacity of plasma☆

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Abstract

Epidemiological studies suggest that a moderate consumption of anthocyanins may be associated with protection against coronary heart disease. The main dietary sources of anthocyanins include red-coloured fruits and red wine. Although dietary anthocyanins comprise a diverse mixture of molecules, little is known how structural diversity relates to their bioavailability and biological function. The aim of the present study was to evaluate the absorption and metabolism of the 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin in humans and to examine both the effect of consuming a red wine extract on plasma antioxidant status and on monocyte chemoattractant protein 1 production in healthy human subjects. After a 12-h overnight fast, seven healthy volunteers received 12 g of an anthocyanin extract and provided 13 blood samples in the 24 h following the test meal. Furthermore, urine was collected during this 24-h period. Anthocyanins were detected in their intact form in both plasma and urine samples. Other anthocyanin metabolites could also be detected in plasma and urine and were identified as glucuronides of peonidin and malvidin. Anthocyanins and their metabolites appeared in plasma about 30 min after ingestion of the test meal and reached their maximum value around 1.6 h later for glucosides and 2.5 h for glucuronides. Total urinary excretion of red wine anthocyanins was 0.05±0.01% of the administered dose within 24 h. About 94% of the excreted anthocyanins was found in urine within 6 h. In spite of the low concentration of anthocyanins found in plasma, an increase in the antioxidant capacity and a decrease in MCP-1 circulating levels in plasma were observed.

Keywords: Red wine; Anthocyanins; Absorption; Antioxidant activity; MCP-1

1. Introduction

Anthocyanins are a group of natural occurring pigments responsible for the red-blue colour of many fruits and vegetables. They are glycosides of the anthocyanidins which consist of various polyhydroxy or polymethoxy derivates of 2-phenylbenzopyrylium or flavilium salts [1]. The intake of anthocyanins in humans, mainly ingested in the form of cyanidin derivatives from fruits and malvidin and related compounds from red wine, has been estimated to be between 12 and 215 mg/day [2–4], which is higher than other flavonoids including quercetin, kaempferol, myricetin, apigenin and luteolin [5]. The main sources of anthocyanins in the human diet are to be found in many red-coloured fruits, such as black currants, blueberries, red plums or cherries, cereals and vegetables, as well as in wine. Additionally, there is an increasing interest in the use of this group of natural colorants as food additives due to the negative attitude of

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consumers towards artificial alternatives. This growing use of anthocyanins in the food industry is likely to result in an increase in overall population consumption.

However, anthocyanin bioavailability is low with <1% absorption of the ingested dose. Following absorption, they appear in the blood and urine as glycosilated, methylated, glucuro-and/or sulfoconjugate forms. The recovery of anthocyanins and anthocyanins metabolites in urine has been estimated to be between 0.004% and 0.11% of dose [6]. However, most of the available data on anthocyanin bioavailability is based on studies carried out examining the metabolism of cyanidin derivatives, from black currant or elderberry, pelargonidin glycosides from strawberries and delphinidin glycosides from blueberries. To date, there is little information available on the absorption and subsequent metabolism and excretion of malvidin and other red wine/grape anthocyanidins, which are main contributors to the total anthocyanin intake in Mediterranean countries.

Epidemiological evidences as wells as in vitro and animal studies suggest that a moderate consumption of these anthocyanins may be associated with protection against cardiovascular diseases [7,8], chronic inflammation [9] and cancer [10]. Anthocyanins exhibit moderate free radical scavenging activity and have been shown to decrease tumour necrosis factor-alpha-induced monocyte chemoattractant protein 1 (MCP-1) production in cultured endothelial cells and endothelioma cells [7,9,10]. MCP-1 is a CC chemokine shown to, in part, mediate the recruitment of macrophages to sites of infection or inflammation sites. Therefore, the role of anthocyanidins as antioxidants and regulators of MCP-1 expression are likely to make a significant contribution to their antiatherogenic effects.

However, in vivo data regarding the effect of red wine anthocyanins on MCP-1 levels in humans are missing. Furthermore, it is unclear if and to what extend red wine anthocyanins may affect the antioxidant capacity of human plasma. Thus, the objective of the present work was to study the bioavailability of red grape anthocyanins and to clarify the effect of their consumption on both plasma antioxidant status and MCP-1 concentrations.

2. Methods and materials

2.1. Materials

All reagents were obtained from Sigma Aldrich (Madrid, Spain) unless otherwise stated and were of analytical or high-performance liquid chromatography (HPLC) grade where applicable. Water was purified via a Milli Q plus system (Millipore, Bedford, MA, USA). Enocianin (E-163), a *Vitis vinifera* grape peel anthocyanidin-rich extract, was kindly supplied by Sensient Food Colors Italy (Reggio Emilia, Italy). The 3-glucosides of pelargonidin, delphinidin, cyanidin, petunidin, peonidin and malvidin used as external standards were from Polyphenols (Sandnes, Norway).

2.2. Chemical characterization of the anthocyanin extract

All analyses were carried out using a Hewlett-Packard Agilent 1100 Series liquid chromatograph with a quaternary pump and photodiode array detector (DAD). The HPLC system was equipped with a Phenomenex Aqua C18 column (5 μm; 200 Å; 4.6×150 mm), which was set thermostatically at 35°C. Solvents used were aqueous 0.1% trifluoracetic acid (A) and HPLC-grade acetonitrile (B) at a flow rate of 0.5 mL/min. Starting isocratically with 10% B up to 20 min the gradient was 13% B from 20 to 35 min, 15% B from 35 to 55 min, 35% B from 55 to 65 min and 10% B from 65 to 70 min. Detection wavelengths were 280, 360 and 520 nm. Analyses were carried out in triplicate.

Mass spectrometry (MS) was performed using a Finnigan LCQ equipped with API surface, using an electrospray ionization interface. The HPLC system was connected to the mass spectrometer probe via the diode array detector cell outlet using polyethyletherketone tubing. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium at flow rates of 1.2 and 6 L/min, respectively. The capillary temperature was 195°C, and the capillary voltage was 4 V. The MS detector was programmed to perform a series of three consecutive scans: a full scan from 120 to 1500 amu, an MS^2 scan of the most abundant ion in the full mass and MS^3 of the most abundant ion in the MS^2. The normalized energy of collision was 45%. Spectra were recorded in the positive ion mode.

Total anthocyanins in the grape extract were quantified at 520 nm as malvidin-3-glucoside by integrating all the peaks. The concentration of 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin in the sample was determined using commercially available standards.

2.3. Human study

2.3.1. Human subjects

Seven healthy subjects were asked to participate in the study. Selection criteria were as follows: 26–36 years old and body mass index (BMI) between 20 and 28 kg/m^2. Individuals with diagnosed cardiovascular disease, liver disease or those with gastrointestinal disorders were not included. Furthermore, individuals who consumed more than the recommended alcohol intakes (>14 and >21 units per week for females and males) were ineligible to participate. The study was approved by the University of Reading Ethics and Research Committee (03/14), and the subjects were recruited from within the School of Food Biosciences. The single oral bolus dose acute study was conducted on seven healthy volunteers (two males, five females; average age 31 years, average BMI 23.2 kg). All subjects provided their written consent to participate in this study.

2.3.2. General procedure

Subjects were asked to refrain from consumption of anthocyanin-rich products such as wine, red fruits or derived products and other products rich in antioxidant polyphenols

such as tomatoes, onions or tea for 24 h prior to the acute study. Participants were required to come to the clinical investigation unit (CIU) at 8 a.m. in a 12-h fasted state. Upon arrival at CIU, an intravenous cannula was inserted into a forearm (antecubital) vein and a 10-ml baseline blood sample was then taken into a heparin tube (BD Vacutainer plasma tubes, Becton Dickinson, Franklin Lakes, NJ, USA). The subjects then consumed 12 g of the anthocyanin extract together with 125 g of sugar-sweetened yogurt and a standard breakfast consisting of one and a half toast with butter and a glass of water. Standardised meals were also provided at lunch (cheese and salad sandwich, cheeps and chocolate bar) and dinner (pizza and banana) times (4 and 10 h after anthocyanin consumption, respectively).

Blood samples (10 mL) were taken every 30 min for the first 3 h and every hour from the third hour onwards up to 10 h, and the final fasted sample taken at the 24-h time point. Blood samples were centrifuged at 3000×*g* for 10 min at 4°C and aliquots of serum (2×0.5 mL) stored at −70°C for analysis of MCP-1 and antioxidant activity. Plasma samples used for the quantification of anthocyanins and their metabolites were acidified by the addition of 0.2 mL of 0.44 mM trifluoroacetic acid (TFA) to 0.7 ml of plasma and then stored at −70°C until further analysis.

Urine collections were made over the 24-h study period at the following intervals: 0–3 h, 3–6 h, 6–10 h and 10–24 h after anthocyanin consumption. Urine was collected in 2.5 L flasks containing 2-g boric acid; 4×25 mL aliquots from each time interval collection were stored at −70°C until further analysis.

2.4. Plasma analysis

For determination of the pharmacokinetic profile of anthocyanins, plasma samples were analysed by HPLC-DAD-MS after solid phase extraction using SPE C18 cartridges. Pelargonidin-3-glucoside (35 pmol in 0.44 mM TFA), used as the internal standard, was added to acidified plasma samples (0.65 mL). Anthocyanins and their metabolites were extracted from plasma samples by using an octadecylsilane cartridge (Sep-pack C18, Waters, MA, USA). Prior to sample elution, the solid-phase cartridge was equilibrated with 7 mL methanol and then 7 mL of 0.1% TFA. Once the acidified plasma sample was deposited in the cartridge a first wash was done by eluting 7 mL of 0.1% TFA. Finally, the compounds of interest were eluted by adding 0.44 mM TFA in methanol. Samples were dried in a vacuum, taken up in 0.15 mL 0.44 TFA and centrifuged at 15000×*g* for 10 min at room temperature. The supernatant (100 μL) was directly injected into the HPLC-DAD-MS system.

HPLC, DAD and MS conditions were as required for the chemical characterization of the anthocyanin extract, as detailed above, except that the MS was programmed in eight different segments according to the anthocyanin elution in order to improve the sensitivity of the method. Each of the segments was programmed to record a different selected reaction monitoring corresponding to the breakup of the anthocyanin or metabolite of interest. Quantification of the different anthocyanins and their metabolites was done by integrating the MS-chromatograms and substitution in standard curves of the five anthocyanins against the area of the internal standard. In the case of the glucuronides, the quantification was done referred to their corresponding glucosides. Standard solutions were injected every day at the beginning and end of each HPLC sequence. The method was optimized, and percent recoveries of 60±6%, 87±5%, 65±4%, 93±5% and 107±10%, respectively, were evident for the 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Interassay precision was determined by performing 10 independent analyses from a given sample showing a variation coefficient of 17%. Good linearity for the assay (r^2>0.995) was found over the investigated calibration range of 1–1000 ng/mL for all the anthocyanins. The detection and quantification limits were in every case below 0.1 ng/mL.

2.5. Urine analysis

Urine samples were analysed by HPLC-DAD-MS after solid phase extraction using SPE C18 cartridges. HCl 0.17 mL was added to 10 ml urine together with the internal standard, pelargonidin-3-glucoside (1.4 nmol in 0.44 mM TFA). Anthocyanins and their metabolites were extracted from urine samples by using the same procedure as that used for the plasma samples themselves, but with the exception that the final elution was carried out with 2 mL methanol: 0.44 TFA in water (75:25, v:v). The supernatant (100 μl) was directly injected into the HPLC-DAD-MS system under the same conditions as those for plasma samples. The method was optimized, and percent recoveries of 90±8%, 73±4%, 84±4%, 70±5% and 100±9%, respectively, were observed for the 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin and malvidin. Interassay precision was determined in the same way as for plasma samples and showed a variation coefficient of 9%. Good linearity for the assay (r^2>0.993) was found over the investigated calibration range of 1–1000 ng/mL for all the anthocyanins.

2.6. Ferric reducing ability of plasma

The ferric reducing ability of plasma (FRAP) assay was performed as previously described [11]. FRAP reagent was freshly prepared each day by mixing together 10 mM 2,4,6-tripyridyl-*s*-triazine and 20 mM iron (III) chloride in 0.25 M acetate buffer, pH 3.6. The absorbance of plasma samples as compared to Trolox was read at 593 nm (Power Wave XS, Biotek Instruments) 6 min after incubation at room temperature against a FRAP reagent blank and distilled water. FRAP values are expressed as Trolox equivalents.

2.7. Trolox equivalent antioxidant capacity

Sixteen hours prior to the assay being performed, the 2,2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS)

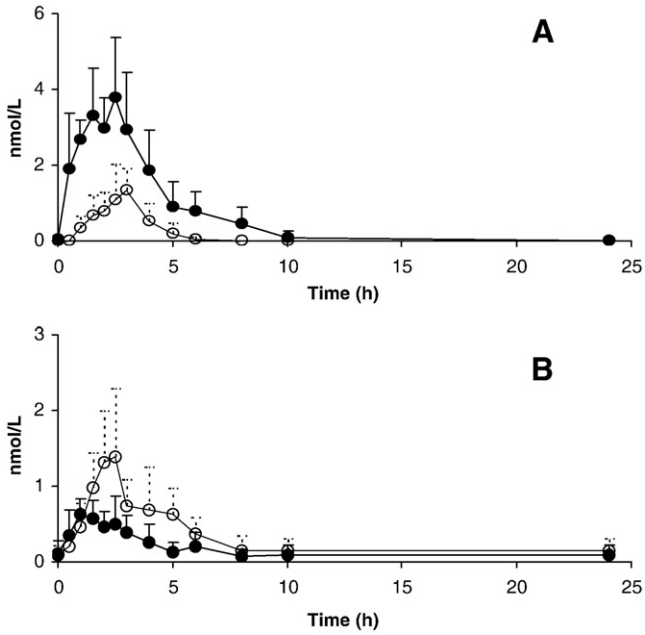

Fig. 1. Plasma levels of the 3-glucoside (●) and 3-glucuronide (○) of malvidin (A) and peonidin (B) throughout the 24 h of study. Values are means±S.D. (n=7).

radical was prepared by adding 5 mL of a 4.9-mM potassium persulfate solution to 5 mL of a 14-mM ABTS solution [11]. This solution was diluted in distilled water to yield an absorbance of 0.70 at 734 nm (Perkin Elmer UV/Vis Lambda Bio 20). The final reaction mixture contained 10 μL of standard or plasma in 1-mL ABTS solution. The samples were vortexed for 10 s, and 6 min after the addition, the absorbance at 734 nm was recorded and compared to the ABTS radical solution plus distilled water. A standard curve was obtained by using Trolox as an internal standard (range, 0–100 μM). Trolox equivalent antioxidant capacity (TEAC) values express the micromoles of Trolox equivalent to the antioxidant capacity of a 1.0-μmol solution of the test substance [12].

2.8. Monocyte chemoattractant protein 1

The levels of MCP-1 in plasma were measured by using a commercially available ELISA kit (R & D Systems Europe, Abingdon, UK). Sera were diluted 1 in 3 for the quantification of MCP-1. The concentration of this molecule was calculated with reference to a standard curve performed with the corresponding recombinant molecule. All serum samples were tested in duplicate.

2.9. Pharmacokinetic and statistical analysis

Curves were generated for each subject using a one-compartment model. The maximum plasma concentration (C_{max}), and the time taken to reach maximum plasma concentration (t_{max}) were calculated from the observed values. The area under the curve (AUC) from 0 to 24 h was calculated by the linear trapezoidal method. The elimination rate constant (k_{el}) was calculated by using the formula $Ct=C_0\times e^{-k_{el}t}$ and half-life ($t_{1/2}$) with the formula $t_{1/2}=0.693/k_{el}$. Values are expressed as means±S.E.M. of the seven subjects unless otherwise noted. Statistical analysis was carried out using analysis of variance and Student's t test (SPSS for Windows version 15.0).

3. Results

3.1. Characterization of the anthocyanin extract

Anthocyanidin monoglucosides represented 71.2% of the anthocyanins present in the anthocyanin extract used in this study. Acylated anthocyanins made up 23.8% and the pyranoanthocyanin derivatives 5.0% of the total amount of identified compounds. The total amount of anthocyanin monoglucosides in the 12 g of extract consumed in the test meal was 183.8 mg, of which the most abundant anthocyanin was malvidin-3-glucoside (89.9 mg), followed by delphinidin-3-glucoside (45.0 mg), petunidin-3-glucoside (33.8 mg), peonidin-3-glucoside (12.4 mg) and cyanidin-3-glucoside (2.7 mg).

3.2. Absorption of anthocyanins: plasma levels

No anthocyanins were evident in the baseline plasma samples of the seven participants. After ingestion of the anthocyanin extract, the malvidin and peonidin monoglucosides, as well as their glucuronides, could be detected and quantified in the plasma. The 3-monoglucosides of delphinidin and petunidin could be detected but were under the detection limit to allow accurate quantification. No detectable cyanidin-3-glucoside was present in any of the plasma samples collected follow anthocyanin administration.

Fig. 1 shows the kinetics of appearance of the different quantified pigments in the plasma over the study period (24 h). The large errors bars demonstrate considerable interindividual variations in the levels detected. In the case of the malvidin and peonidin glucuronides, the large S.D. is in part due to the lack of detection of these metabolites in the plasma of two of the subjects participating in the study. As will be discussed later, these same subjects presented very low levels of malvidin-glucuronide in their urine (<5 μg).

Table 1 shows plasma kinetic parameters of the quantified pigments. The average time required to reach maximum plasma concentration (t_{max}) for the glucosides was found to be 1.6 h. This is in agreement with published data where averages times were 1.5 h when anthocyanins are ingested together with solid foods as in our study [13]. Glucuronides reached their maximum concentration over a longer period of around 2.3 h, thus indicating that anthocyanin glucuronidation may take place, at least to some extent, in the liver. In general, the rapidity with which anthocyanins appear in the plasma may be explained by their ability to permeate the gastric mucosa as shown by Passamonti et al. [14] in a rat model.

Table 1
Plasma kinetic parameters for the anthocyanin and their metabolites

Pigment	Ingested dose (mg)	t_{max} (h)	C_{max} (nmol/L)	AUC (nmol h/L)	$t_{1/2}$ (h)
Peonidin-3-glucoside	15.2±1.5	1.4±0.8	0.8±0.2	3.8±1.6	3.7±2.5
Peonidin glucuronide		2.4±0.2	1.7±0.8	7.0±3.2	2.0±1.1
Malvidin-3-glucoside	80.2±3.1	1.8±0.6	4.2±1.3	15.0±5.8	2.0±0.6
Malvidin glucuronide		2.6±0.5	1.2±0.9	2.3±2.0	1.3±0.7

Maximum plasma concentrations (C_{max}) reached after anthocyanin consumption corresponded with those described in other bioavailability studies using similar anthocyanin doses [15]. In the present work, following ingestion of 90 mg malvidin-3-glucoside, the average maximum plasma levels reached were 4.2±1.3 nM in its intact form and 1.2±0.9 nM as its conjugate metabolite with glucuronic acid.

C_{max} decreased in the following order: malvidin-3-glucoside, peonidin glucuronide, malvidin glucuronide and peonidin-3-glucoside. However, when normalising the C_{max} with respect to the ingested doses of malvidin and peonidin glucosides, the decreasing order of the four pigments changed to peonidin glucuronide, peonidin glucoside, malvidin glucoside and malvidin glucuronide. The C_{max} for the two metabolic forms of peonidin together was 2.5 times greater than that of the two metabolic forms of malvidin together. In the same way, when normalizing the AUC with respect to the doses of peonidin and malvidin glucosides ingested, AUC decreased as follows: peonidin glucuronides (0.46 nmol h/L×mg^{-1}), peonidin glucoside (0.25 nmol h/L mg^{-1}), malvidin glucoside (0.19 nmol h/L mg^{-1}) and malvidin glucuronide (0.03 nmol h/L mg^{-1}); with the AUC for the total of peonidin metabolites three times higher than that of malvidin and its metabolite.

In every case, anthocyanins presented short half-lives. Glucuronic acid conjugates of the anthocyanins showed half-lives of 2.0±1.1 and 1.3±0.7 h for peonidin and malvidin glucuronides respectively. Again, anthocyanin half-lives showed large interindividual variations. The maximum variability was that of peonidin glucoside with a variation coefficient of 67.6%, and half-life values of between 0.9 and 7.6 h. In contrast, in the case of malvidin-3-glucoside, this variability was much lower with half-life values between 1 and 2.8 h.

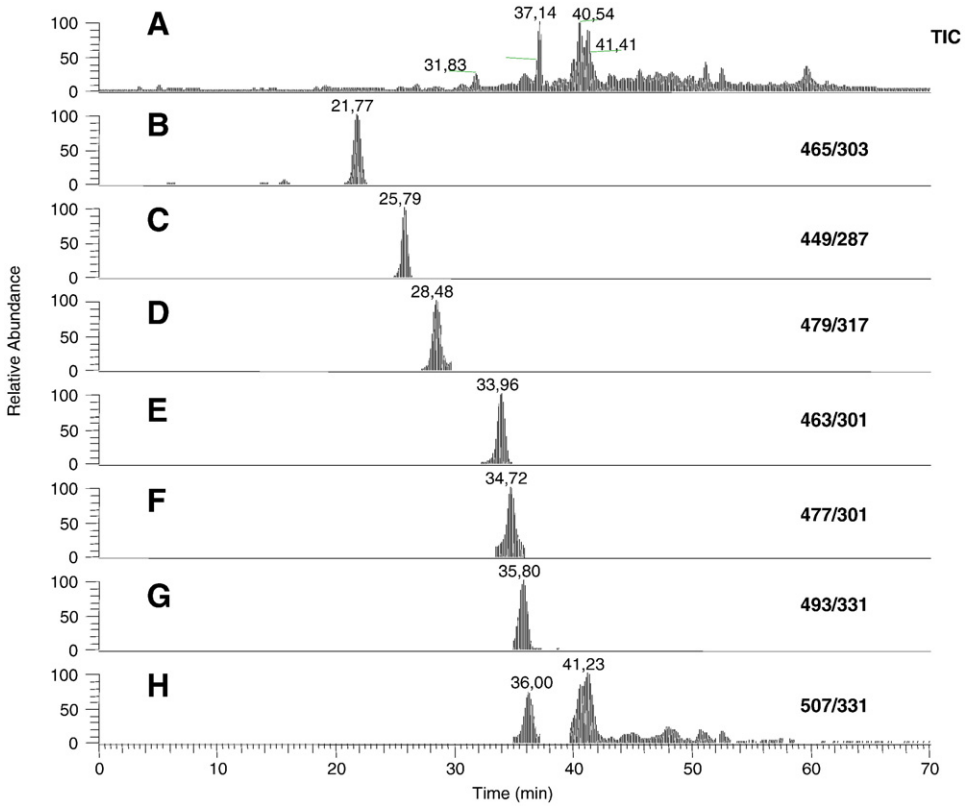

Fig. 2. MS chromatograms of the monoglucosides of anthocyanidin and their corresponding metabolites detected in the urine of one of the volunteers. Total ion current (A), delphinidin-3-glucoside (B), cyanidin-3-glucoside (C), petunidin-3-glucoside (D), peonidin-3-glucoside (E), peonidin glucuronide (F), malvidin-3-glucoside (G) and malvidin glucuronide (H).

3.3. Urinary excretion profiles of the anthocyanins and their metabolites

After ingestion of the enocyanin containing the five anthocyanidin glucosides, urinary excretion of the different metabolites was monitored for 24 h by HPLC-DAD-MS analysis. Cyanidin-3-glucoside was the only anthocyanin that could not be quantified in urine, even though it was detected in the urine of all seven subjects. Fig. 2 shows, as an example, the HPLC-MS analysis of all the anthocyanins and their metabolites detected in the urine of the subjects.

The total amount of anthocyanins in the urine, including the glucosides and glucuronides, after the 24-h study was 0.074 mg (0.060–0.102 mg). Again, a marked interindividual variation was shown in the excreted amounts of each of the quantified pigments. The average amount of delphinidin-3-glucoside excreted was 0.013 mg with individual values between 0.008 and 0.017 mg. For petunidin-3-glucoside, the amount excreted in urine was 0.006 mg (0.002–0.010 mg). The greatest interindividual variations were to be found in the pigments peonidin-3-glucoside and malvidin glucuronides. In some of the volunteers, the amounts of peonidin-3-glucoside excreted in urine were under the detection limit. On the other hand, in the urine of some of the other subjects, the excretion was 0.007 mg. Malvidin glucuronide was present in very low amounts in some cases (0.003-0.005 mg), while in others, the calculated amount of excretion went up to 0.041 mg. In the case of peonidin glucuronide, the amount excreted in urine was relatively constant (0.005±0.001 mg), and the same occurred for malvidin-3-glucoside (0.030±0.007 mg).

The average amount of total anthocyanins excreted in urine represented only 0.05±0.01% of the total anthocyanins ingested with a range of 0.04–0.07% evident in the participants. Within the pigments quantified in urine, petunidin-3-glucoside was the one presenting the lowest percentage of excretion, 0.03%. Delphinidin-3-glucoside, peonidin-3-glucoside and malvidin-3-glucoside showed identical percentages (0.06%) of urinary excretion, in spite of the fact that for peonidin and malvidin, both the glucuronide and the glucoside forms were taken into account when calculating the percentage of excretion.

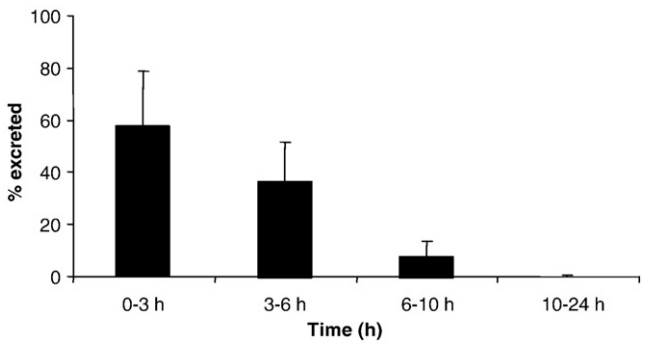

Fig. 3. Percentages of total anthocyanins excreted in the 24-h study period.

Clear differences were shown in the excretion profile of anthocyanins. For instance, from the seven subjects in this study, two excreted most of the anthocyanins in their intact forms, as glucosides (87.1±0.08%). Two other participants in the study excreted 28.6±3.8% as glucuronides, and only one of the subjects eliminated up to 50% of the anthocyanins in urine as glucuronide. Moreover, differences were shown in the form in which the monoglucosides of peonidin and malvidin were excreted in urine. In the case of the 24-h excretion of peonidin-3-glucoside, 66±25% of it was in its conjugated form, whereas in the case of malvidin-3-glucoside only 32±17% was in its glucuronidated form.

Anthocyanins and their metabolites were rapidly eliminated in urine. In the first 6 h after anthocyanin ingestion, 94 ±7% of the total anthocyanins eliminated had been excreted (Fig. 3). All the pigments quantified in the urine of the subjects had a period of maximum excretion of between 0 and 3 h, with the exception of malvidin glucuronide, which presented a slight delay in its period of maximum excretion, which was between 3 and 6 h (49±15%). In this second period of time, between 3 and 6 h, the elimination of peonidin glucuronide was also relatively high (43±34%).

Since malvidin-3-glucoside represented approximately 60% of the ingested anthocyanins, the possible existence of other metabolites derived from this anthocyanin was carefully analysed. Other metabolites such as the sulphates, di-glucuronides, sulphates-glucuronides and malvidin aglycon were monitored by HPLC-MS in the select ion monitoring (SIM) mode for each individual ion. However, none of these metabolites were detected in any of the urine samples analysed.

3.4. Antioxidant status

Most authors have described maximum effects in the increment of the antioxidant power of plasma after approximately one hour of the ingestion of red wine [16-18]. Given this, together with the fact that anthocyanidins reached their maximum plasma concentrations within the first 3 h, antioxidant status measurements were only taken at every time point for the first 4 h after the acute anthocyanin dose.

The values for antioxidant power obtained for the basal sample of each subject at 0 h (722–940 μmol of Trolox/L of plasma) were used as control values in order to evaluate the antioxidant effect of anthocyanin ingestion. Modest increases in FRAP values of the plasma samples were evident for every subject during the analysed period. However, the observed increases did not reach statistical significance. In most of the subjects, the maximum effect was observed after 0.5 h of treatment (4–15% increase in FRAP value), and only in two of them was the maximum observed after 2 h (4–22% increase in FRAP value). In the case of TEAC values, only two of the volunteers showed an increase in the antioxidant status, with no significant difference from baseline observed in the group as a whole.

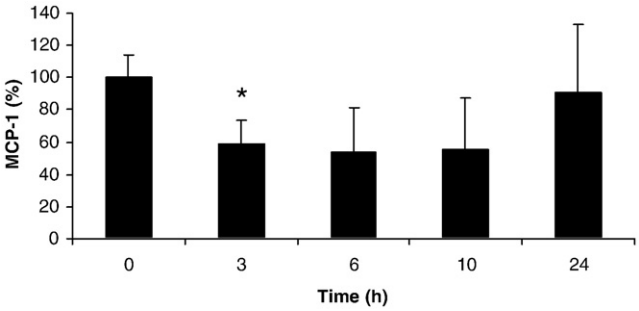

Fig. 4. Circulating MCP-1 levels expressed as percentages relative to the basal sample at time 0 h. Data are the mean±standard deviation of the seven subjects.

3.5. Monocyte chemoattractant protein 1

Previous work has demonstrated that red wine anthocyanins have an inhibitory effect on MCP-1 secretion in different endothelial cell models [7,19,20]. Moreover, in a study carried out in rabbits ingesting a high-cholesterol diet together with red wine for 6 weeks, a significant decrease in the expression of MCP-1 and its mRNA was shown [21].

In this work, the circulating levels of MCP-1 have been monitored throughout the 24-h study time at 0, 3, 6, 10 and 24 h after anthocyanin ingestion. Basal circulating levels of MCP-1 were considered in the normal range for all the volunteers (165±43 pg/mL). Fig. 4 shows the relative changes of MCP-1 in plasma samples for all the volunteers in the study. Only at 3 h was the decrease on the circulating levels of MCP-1 (96±42 pg/mL) statistically significant. After 24 h, the levels of MCP-1 were again in the normal range (199±118 pg/mL).

4. Discussion

Anthocyanin absorption after a single dose of red grape extract is low (<10 nmol/L) with a marked interindividual variation in the maximum concentration reached for total anthocyanins and individual metabolites. The maximum concentrations in plasma for glucosides and glucuronides were reached at 1.6 and 2.5 h, respectively.

Part of the interindividual variation in the plasmatic levels of anthocyanin metabolites may be explained by biochemical factors such as differences in the expression or activity of enzymes involved in anthocyanin metabolism. Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs). To date, more than 20 UGT isoforms have been described, showing differences in their regulation, substrate selectivity and tissue distribution. In the gastrointestinal tract, members of the UGT1A are mainly expressed and, among them, UGT1A1, which presents a high allelic variability is considered to be responsible, at least partially, for the marked interindividual variation in the oral bioavailability of a number of drugs [22]. Furthermore, it is likely that this genetic polymorphism makes a significant change in the rate of malvidin conjugation with glucuronic acid and its subsequent metabolism.

The fact that the AUC for peonidin is three times higher than that of malvidin could indicate that peonidin is more bioavailable than malvidin. On the other hand, peonidin in plasma may also come from the physiological metabolism of other anthocyanins. For instance, cyanidin-3-glucoside methylation in its 3′ position would produce a peonidin-3-glucoside molecule. This interconversion has already being described by other authors in human studies [23,24]. However, the marked differences between malvidin and peonidin AUCs may not be fully explained by this interconversion since the amount of cyanidin present in the anthocyanin extract was very low. Therefore, these differences in bioavailability of individual pigments indicates that the anthocyanin structure influences absorption, as has already being described for the petunidin, delphinidin and malvidin galactosides [25] and pelargonidin and cyanidin glucosides [24].

Total anthocyanins excreted in urine represented 0.05% of the ingested amount. From this excreted amount, 72.4±13.6% was in its intact glucosylated form and 27.6±13.4% in its glucuronidated form. No data on the glucuronidation rate of peonidin and malvidin has been published so far. However, Felgines et al. [26] have reported an 80% excretion of pelargonidin-3-glucoside in its glucuronidated form. After consumption of different glycosides of cyanidin, only 6–14% [4,27] were excreted in their glucuronidated forms. These data may indicate that the process of conjugation with glucuronic acid could be conditioned by the anthocyanidin structure. For this reason, pelargonidin, with only one hydroxyl group in the B-ring, would impede to a lesser degree its conjugation with glucuronic acid than cyanidin, with two hydroxyl groups.

Even if we could not detect any malvidin glucuronides in the urine or plasma of some of the subjects in this study, it should be noted that the glucuronide conjugate of peonidin was found in every subject, both in plasma and urine. Moreover, the levels of peonidin glucuronide were higher for longer than malvidin glucuronide, despite the fact that the 12 g extract provided doses of peonidin approximately sevenfold lower than malvidin. This could be explained by the structural differences of the two anthocyanins, which may have an influence on the affinity of theses molecules for the enzymes involved in flavonoid glucuronidation. It might be that the molecule of malvidin with three substituents in the B-ring presents a greater steric impediment to interaction with the aforementioned enzyme than peonidin, with only two substituents in the B-ring.

Even though the concentration of anthocyanins and their metabolites in plasma were, in every case, below 10 nmol/L for the given anthocyanin ingestion, a modest trend towards improvement in oxidant status and a decrease in the circulating levels of monocyte chemoattractant protein-1 was shown after ingestion of the anthocyanin extract for all the participating subjects. The chemokine MCP-1 is

involved in atherogenesis through different mechanisms: it promotes the adhesion of blood circulating monocytes across the endothelium, participates in their recruitment into the subendothelium space and contributes to macrophage foam-cell differentiation. MCP-1 gene expression is in part mediated by the redox-sensitive transcription factors nuclear factor (NF)-κB. Furthermore, in cultured monocytes, anthocyanins isolated from bilberries and black currants efficiently suppressed LPS-induced activation of NF-κB in vitro [28]. Since MCP-1 circulating levels in plasma were decreased by red wine anthocyanins in the current study, an inhibition of NF-κB signalling may be one potential mechanism by which anthocyanins may mediate antiatherogenic properties.

Furthermore polyphenols do not only work as antioxidants per se but can also induce the gene expression of other antioxidants via Nrf2-dependent signal transduction pathway [29], which may be also true for anthocyanidins.

In a recent study by Gorelic et al. [30] it has been reported that wine polyphenols may inhibit the absorption of lipid peroxidation products, which may, in turn, decrease cellular oxidative stress.

No doubt, polyphenols exert their beneficial effect by a combination of mechanisms, all of them related to their well-known properties as antioxidants, metal chelators and their impact on cell signalling. However, more human intervention studies, with larger populations and longer periods of treatment, are needed in order to assess the potential benefits of red wine polyphenols on vascular health.

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