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# Dose-response effect on polyphenols bioavailability after intake of white and red wine pomace products by Wistar rats

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**Abstract**

Wine pomace by-products are an important source of phenolic acids with significant health benefits. However, phenolic acid bioavailability *in vivo* has been little studied and there are few comparative studies on bioavailability between red and white wine pomaces and the effect of different doses of intake. The qualitative and quantitative profile of phenolic acid metabolites in plasma and urine samples from Wistar rats was performed by gas chromatography/mass detection, after oral administration of four doses (50, 100, 150, and 300 mg) of both the red and the white wine pomace products (rWPP and wWPP, respectively). The antioxidant capacity of the plasma samples assessed by both the ABTS and the FRAP levels were also evaluated. The results showed that neither the bioavailability nor the antioxidant capacity *in vivo* of the rWPP increased at high doses. However, both parameters were dependent on the intake of the wWPP.

**Keywords:** Phenolic acids, bioavailability, red wine pomace, white wine pomace, antioxidant.

## 1 Introduction

Agroindustrial food wastes and by-products such as grape pomace products can be used to the development of functional foods. The polyphenol content of grape pomace and its beneficial effects depends on different factors such as grape variety, winery process, type of grape pomace (skins, seeds or entire grape pomace, pure polyphenol extract or grape pomace products), and polyphenol bioavailability [1,2].

The properties of polyphenols have been associated with antioxidant, anti-inflammatory [2], anti-aging [3], and anti-cancer [4] effects, as well as the prevention of different diseases [5,6]. Nevertheless, it is important to consider polyphenol bioavailability; it is known that not all of the polyphenols present in grape pomace will be necessarily bioactive in the organism. Their intrinsic activity is dependent on the intestinal absorption and bioactive metabolites are the result of digestive and hepatic metabolic processes and differ from native polyphenols [7]. In this sense, phenolic acids of wine pomace include polymers, esters, and glycosides that are hydrolysed by gastrointestinal enzymes and further modified by the intestinal microbiota [8]. The metabolization of these compounds increases their hydrophilicity and facilitates urinary and/or biliary elimination [7]. Furthermore, some studies reported that wine pomace by-products are a good source of dietary fiber and polyphenols [9,10].

However, very few studies have evaluated the effect of the intake of different doses of wine pomaces and the differences between red and white wine pomaces bioavailabilities. In previous studies, we observed that high levels of phenolic acids in plasma are associated with a high prevention of lipid peroxidation and a high nitric oxide bioavailability in Wistar rats after oral administration of a single oral dose of 300 mg/kg BW of red wine pomace product (rWPP) [11]. Considering that the health effects of the polyphenols could depend on their dietary intake, is important to evaluate if a higher intake of WPP will increase the polyphenol bioavailability. In this sense, the aims of our study were to fill that gap by investigating the bioavailability and the antioxidant capacity of different oral doses of red and white pomace products (rWPP and wWPP, respectively) following oral administration to rats in a 6 hour study.

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## 2 Materials and methods

### 2.1 Chemicals

ABTS, 1-hidroxy-2-naphtoic acid (internal standard), 2,4,6-Tris (2-pyridyl)-S-Triazine (TPTZ), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), β-glucosidase from almonds (≥ 2 U/mg), β-glucuronidase from Helix pomatia (≥ 100000 U/mL), ethyl acetate, gallic acid, formic acid, methanol, N,O-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA+TMS), Pyridine anhydrous (99.8%), and all phenolic compound standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Folin-Ciocalteau reagent, FeCl3, FeSO4, Na2CO3 were obtained from Panreac Química, S.L.U. (Barcelona, Spain).

### 2.2 Red and White Wine Pomace Products

Red and white wine pomace-derived products from the vinification of *Vitis vinífera L. cv. Tempranillo* and *Verdejo* (rWPP and wWPP, respectively) were prepared at the University of Burgos according to a previously described method [12,13].

### 2.3 Animal experiments

Experimentation with live animals was approved by the Ethics Committee for Experimental Animal Care at the University Hospital of Burgos (ref. CEBA 13) and it was carried out in accordance with the Spanish and European laws (Royal Decree 53/2013 of the Spanish Ministry of agriculture, Food and Environment and Ministry of Economy and Competitiveness, and European Directive 2010/63/EU).

#### 2.3.1 Animals

Male Wistar rats (*Rattus norvegicus; n* = 30; age, 12 weeks; weight: 423 ± 42 g) were obtained from the Animal Research and Welfare Service of Valladolid (SIBA, Valladolid, Spain). The rats were left to acclimatize for 2 weeks. The room temperature was maintained at 21 ± 2 ºC and humidity at 40 ± 10 %, with a 12:12-h light:dark cycle and free access to food (A04 Safe Diet) and water. The animals were placed for 1 hour in metabolic cages and, for adaptation purposes, were subjected to human manipulation during the weeks before the experiments. All procedures were designed to limit the number of animals used by leaving the animals to rest for 2 wk between experiments.

#### 2.3.2 Experimental Design

rWPP and wWPP doses at 50, 100, 150, and 300 mg/kg of body weight (2 mL of water) was administrated to the rats by oral gavage after food deprivation for 8h with free access to water. Rats were individually placed

in metabolic cages and urine was collected both before and after WPP administration in 2-hourly intervals (-2-0h, 0-2h, 2-4h and 4-6h). Aliquots of these urine samples were stored frozen at -80ºC until analysis. Blood samples were collected at baseline (0h) and post-WPP administration (2h, 4h, and 6h). Blood samples were taken by lateral saphenous venipuncture with a 23G needle and held in Vacutainer tubes containing sodium/lithium heparin as anticoagulant and were centrifuged at 1500g for 10 minutes to recover the plasma. The volume of blood taken for each rat was never in excess of 1.2 mL according to the NC3Rs recommendations (4.4% blood volume removed) [14].

**2.4 Antioxidant Capacity**

Total antioxidant capacity of the rWPP and wWPP products were determined by QUENCHER-methods described previously [13]. Additionally, total antioxidant capacity of urine and plasma samples was measured by FRAP and ABTS methods [11].

**2.4.1 Quencher Total Antioxidant Capacity of the Wine Pomace Products (WPPs)**

QUENCHER-TAC (Q-) versions [13] of three conventional total antioxidant capacity (TAC) assays were performed (Folin-Ciocalteu index, ABTS and FRAP were selected to evaluate the TAC of each fraction). For all determinations a sample mass of WPP (1 ± 0.005 mg) was used.

- Quencher Folin-Ciocalteu assay (Q-FC): WPPs were mixed with equal volumes (0.2 mL) of Milli-Q water and Folin-Ciocalteu reagent and, after 5 min, 4 mL of 0.7 M $Na_2CO_3$ solution was added and the mixture made up to a final volume of 10 mL with Milli-Q water. After incubation for 1 h in the dark with continuous stirring, the supernatant was separated and the absorbance at 750 nm was measured in a UV-vis spectrophotometer U- 2000 (Hitachi, Ltd., Hubbardston, MA, USA). A dose-response curve was plotted using different quantities of gallic acid as the standard.

- Quencher ABTS assay (Q-ABTS): WPPs were mixed with 10mL of the ABTS·+ working solution and incubated 30 min in the dark in an orbital shaker. Then, the supernatant was separated and the absorbance was measured at 734 nm. A linear calibration curve was obtained with different amounts of Trolox as per the relevant standard.

- Quencher FRAP assay (Q-FRAP): WPPs were mixed with 10 ml of the FRAP solution and incubated at 37 ºC for 30 min with continuous stirring. Absorbance was measured at 593 nm. The results were expressed as µmol of iron (II) equivalents/g of product (Fe(II)E/g) using linear calibration obtained with different amounts of FeSO4.

**2.4.2 Total antioxidant capacity of plasma and urine samples**

The total antioxidant capacity of both the plasma and the urine samples was assayed using two independent determinations: the ferric reducing antioxidant power (FRAP) and the ABTS methods previously described above [11]. Briefly, a volume of 980 µL of the FRAP solution was added to 20 µL of plasma or urine samples and incubated at 37 ºC for 30 min and absorbance was measured at 593 nm. The results were expressed as mM of iron (II) equivalents (Fe(II)) using a linear calibration obtained with different amounts of FeSO4. For the ABTS method, 10 µL of plasma or urine sample was mixed with 960 µL of the ABTS·+ working solution and after 4 min the absorbance at 734 nm was measured.

**2.5 Quantification of phenolic compounds in WPPs and plasma and urine samples**

The concentration of phenolic acids was measured using gas chromatography coupled to a triple quadrupole mass spectrometry detection (GC/MS/MS) in WPPs, plasma and urine samples according to a method previously described [11]. The identification and quantification of stilbenes, flavanols, and flavonols were measured in the WPPs by High-performance liquid chromatography-diode-array detection (HPLC-DAD) as previously described [15]. Briefly, plasma samples (75 µL) were acidified (pH 4.6) and incubated with 10 µL of β-glucuronidase (1000 U) with sulfatase activity (75 U) and 10 µL of β-glucosidase (10 U) during 4h at 37ºC to hydrolyze glucuronide, sulfate and glucoside metabolites. Urine samples (75µL) followed the same procedure as the plasma samples, but β-glucosidase was not added to the samples. The mixture was further acidified (pH < 3) and extracted with ethyl acetate. After addition of 5% NaHCO3, the bottom layer was acidified and extracted again with ethyl acetate, and then dried under nitrogen. In addition, 1 mg of each WPPs was extracted in methanol:formic acid (97:3, 25ºC, 24h) and then dried under nitrogen. The dried extracts were derivatized and then analyzed by GC-MS/MS for the determination of the phenolic acids.

**2.5.1 Gas chromatography–triple quadrupole mass spectrometry detection (GC/MS/MS)**

The samples (1mg of WPP or 75 µL of plasma or urine) were derivatized with 50 µL of BSTFA and 50 µL of dry pyridine, mixed and heated at 40 ºC for 30 min. The trimethylsilyl (TMS) derivatives were analyzed with an Agilent 7890B GC System (Agilent Technologies, Inc., Palo Alto, CA) coupled to an Agilent 7010 GC/MS TripleQuad detector and fitted with an DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies) using helium as the carrier gas. The calibration curves were established by measuring peak areas versus the responses in comparison with the internal standard 1-Hydroxy-2-naphthoic acid over a range of analyte concentrations. The concentration of phenolic acids was finally expressed as µg/g of both

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red and white pomace product (rWPP or wWPP), μM for plasma samples and μmol/mmol creatinine for urine samples. Representative chromatograms are showed in Supplementary Figure 1 and Supplementary Figure 2.

**2.5.2 Area under curve (AUC) calculation**

The area under the curve (AUC) of the graph of total phenolic acid content in the plasma samples over time was calculated by the Trapezoid method [16].

**2.5.3 High-performance liquid chromatography-diode-array detection (HPLC-DAD)**

The WPPs was submitted to a liquid extraction (MeOH:Formic acid 97:3, 25ºC, 24h) according to a previously described method [15]. The determinations were performed using analytical reversed-phase HPLC on an Agilent 1100 series HPLC system (Agilent Technologies Inc., Palo Alto, CA, USA) coupled to a diode array detector. A Spherisorb3® ODS2 reversed phase C18 column (250 mm x 4.6 mm, 3 μm particle size; Waters S.A., Barcelona, Spain) was used for the determination of stilbens, flavanols and flavonols and, a Nova-Pak reverse phase C18 column (250 mx x 4.6 mm, 4 μm particle size; Waters LTd., Elstree, U.K.) was used for the anthocyanins analysis. For the stilbens, flavanols and flavonols analysis, the eluent was monitored at 254, 280, 320, and 360, with the compound spectra between 220 and 600 nm. For the anthocyanins assay, the eluent was monitored at 520 nm, with the compound spectra between 220 and 600 nm. Peak identification was performed by comparison of retention times and diode array spectral characteristics with the standards. The results were expressed in μg/g of WPP.

**2.6 Creatinine determination**

Levels of urinary creatinine were measured with the DetectX Urinary Creatinine Detection Kit (Arbor Assays, Michigan, USA).

**2.7 Data presentation and statistical analysis**

The results were expressed as means ± standard deviation of independent samples (n=3). Statistical analysis was performed using Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies, Inc., Warranton, VA, USA). Significant differences between rWPP and wWPP were determined with the Student-*t* test. One-way analysis of variance (ANOVA), using Fisher's least significant difference (LSD) test, was used to determine significant differences between data from the plasma and urine concentrations of phenolic acids and antioxidant capacity at the different doses and 2 hourly intervals. Linear correlations between the plasma phenolic acid content and the antioxidant capacity after each assay and dose were evaluated by

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Pearson's correlation coefficients, with the correlation significance determined by the Student-*t* test. A value of $p < 0.05$ was applied to all analyses.

## 3 Results and discussion

Identification of phenolic compounds using GC/MS/MS and HPLC/DAD and antioxidant capacity (Q-FC, Q-ABTS and Q-FRAP) were performed in the WPPs. Four different concentrations (50, 100, 150 and 300 mg/kg BW) of each WPP were orally administered to Wistar rats, in order to study their bioavailability, and four sets of plasma and urine samples were collected at 2 h intervals (-2_0, 0_2, 2_4 and 4_6 hours). The phenolic acids were identified and quantified and the antioxidant capacity was measured. The concentrations evaluated in the present work were selected by the authors according to previous studies. The human equivalent dose (HED) was also considered due to the potential use of the WPPs as a dietary supplement. All doses were in the range of 0.5 – 3 g/day.

### 3.1 Antioxidant capacity and phenolic composition of both the red Wine Pomace Product (rWPP) and the white Wine Pomace Product (wWPP)

As shown in the table 1, important differences in the antioxidant capacity and phenolic composition of both the rWPP and the wWPP were observed. In agreement with other studies of grapes, wines, and wine pomace extracts [17–19], the antioxidant capacity, assayed by Q-FC, Q-ABTS and Q-FRAP, was significantly higher (2.1, 1.8, and 1.4 fold) in the rWPP than in the wWPP (Table 1).

A total of 16 phenolic acids were identified and quantified by GC/MS/MS and 5 anthocyanins, 5 flavanols, 4 flavonols, and 2 stilbenes were identified and quantified by HPLC/DAD. The sum of total phenolic composition was about 2176 μg/g for the rWPP and of 781 μg/g for the wWPP (Table 1). In this sense, the main difference between the two WPPs was the anthocyanins, since the sum of the rest of phenolic compounds was 658 μg/g for rWPP and 781 μg/g for wWPP.

Flavonoids include different family subgroups (i.e. anthocyanins, flavanols, flavonols) that differ in the degree of oxidation of their oxygenated heterocycle. Anthocyanins, the most abundant group in the rWPP (1518 μg/g rWWP), were absent in the wWPP. This observation is important for the study of bioavailability, because the anthocyanins can be metabolized by microbiota into phenolic acids such as syringic, vanillic, protocatechuic, and coumaric acids, thereby contributing to the total content of phenolic acids in the plasma samples [20]. In the second place, the flavonols were the major compounds detected in both WPPs,

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representing 48% and 36%, respectively, of all phenolic compounds (excluding anthocyanins), followed by the flavanols (33% and 35% of the total) (Table1). Flavonols and flavanols are known to be present in wine pomace, mainly from skins and seeds [21]. Furthermore, the procyanidins that are not extracted during the winemaking process remain in the wine pomace and enrich its content of phenolic compounds. In our study, the main flavonol was kaempferol-3-O-rutinoside at concentrations of 211 and 194 μg/g for the rWPP and the wWPP, respectively, followed by other flavonols: myricetin-3-O-rhamnoside, kaempferol-3-O-glucoside and quercetin-3-O-rutinoside. With respect to flavanols, epigallocatechin was the most abundant in both WPPs (146 and 76.2 μg/g for rWPP and wPPP), followed by catechin, procyanidin B1, epicatechin, and procyanidin B2. It is known that both flavonols and flavanols contribute to the total phenolic acid content of plasma, and the procyanidins reach the colon intact, before they are degraded by the colon microbiota resulting in the release of the flavanols monomers. Catechin and epicatechin (and their gallate esthers, epigallocatechin and epigallocatechin gallate) suffer further degradation by the colon microbiota including hydrolysis of gallic acids from the galloylated forms, and C-ring fission producing dihydrophenyl-γ-valerolactone and phenylvaleric acids. The progressive microbial catabolism of these two acids releases different forms of phenyl and benzoic acids such as hydorxyphenylacetic, hydroxybenzoic, protocatechuic, and gallic acids. Minor catabolites of flavanols by colon microbiota include hippuric, p-coumaric, vanillic, homovanillic, 3-O-methylgallic, and syringic acids, among others [20,22,23].

A significant difference in the content of stilbenes and phenolic acids was observed between the rWPP and the wWPP (Table 1). The most abundant stilbene was resveratrol, significatively higher in rWPP (4.58 μg/g product) than in the wWPP (1.21 μg/g product), while the total of phenolic acids was more abundant in the wWPP (224 μg/g product) than in the rWPP (115 μg/g product). The most abundant phenolic acids were gentisic and protocatechuic acids for the rWPPP (27.9 and 18.1 μg/g product) and homoprotocatechuic and caffeic acids for the wWPPP (52.3 and 35.7 μg/g product). These phenolic compounds were also found in other wine pomaces [24,25], but in different concentrations, which may be due to the grape variety, winery process, and even the presence of an extraction process [1].

**3.2 Phenolic metabolite bioavailability in plasma after rWPP and wWPP intake**

The plasma levels of phenolic acid metabolites were assayed by GC/MS/MS and collected for each dose of rWPP and wWPP at basal, 2, 4, and 6 hour post intake (Supplementary table 1).

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The most abundant phenolic acids in the plasma samples after rWPP intake were hydroferulic, p-hydroxyphenylacetic, syringic, and vanillic acids (12.0 μM, 6.7 μM, 5.2 μM and 4.8 μM, respectively) for the four doses (50, 100, 150 and 300 mg/kg BW) (Figure 1A-D) and the highest levels were observed after the dose of 100 mg/BW (Figure 1B). The high concentration of hydroferulic acid in plasma proceeds from the colonic metabolism of several phenolic compounds present in the wine pomace principally anthocyanins, ferulic and other cinnamic acids [23,26]. Moreover, the principal type of phenolic compounds in the rWPP, the anthocyanins, are metabolized in the upper gastrointestinal tract, and their metabolites include 4-hydroxyhippuric and ferulic acids derivates that reach their maximum in plasma at 1-1.5 hours after consumption [27]. In another hand, the highest levels observed for syringic and vanillic acids could be a consequence of the microbial metabolism, in the large intestine, of the high content of malvidin and peonidin present in the rWPP [23,28]. Significant concentrations of m-hydroxyphenylacetic, protocatechuic, homovanillic, gentisic, and caffeic acids were also observed after rWPP intake, but the rest of phenolic acids were maintained at concentrations below 1μM (Figure 1A-D and Supplementary table 1). It must be highlighted that 80% of the metabolites under study increased in plasma when the intake was higher than 100 mg/kg BW compared to basal levels, while the intake of 50 mg/kg BW resulted in increases of 50% of the metabolites after 2h (Supplementary table 1).

The main phenolic acids assayed in the plasma post-intake of wWPP showed that were m-hydroxyphenylacetic, p-hydroxyphenylacetic, protocatechuic, and 4-O-methylgallic acids with values of 7.5 μM, 4.8 μM, 4.7 μM, and 4.4 μM (Figure 1E-H). In this case, m-hydroxyphenylacetic and protocatechuic acids could derivate from hydrophenylvalerolactones and dihydroxyphenylpropionic acids produced by the microbial catabolism of flavanols such as catechin, epicatechin, and procyanidin B2 that were predominant in the wWPP [22,23]. Significant concentrations of ferulic, homovanillic, vanillic, homoprotocatechuic, syringic, and hydroferulic acids were observed, although all other metabolites had concentrations of less than 1μM (Supplementary table 1).

An increase of total phenolic acid metabolites in the plasma samples for all doses (50, 100, 150 and 300 mg/kg BW) of rWPP was observed at 2h, 4h, and 6h after their intake (figure 2A). In this sense, 43% of the phenolic metabolites after 2h of rWPP intake had their highest concentrations in the plasma samples, and 55% at 4h (figure 2A), suggesting absorption in the first part of the gastrointestinal tract and small intestine [8]. Only 2% of the metabolites increased later on, suggesting that they were formed in the large intestine by

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microbial metabolism [29]. Furthermore, for the intake of 150 and 300 mg/kg BW doses of rWPP about a 44% and 56% of all metabolites studied remained in the plasma after 4h until to 6h, while only 19% was observed for doses of 50 and 100 mg/kg BW. In this respect, it is known that the half-life of phenolic metabolites depends on both their stabilization by conjugation with plasma proteins and their eventual elimination through of the biliary or urinary pathways. Urinary excretion is usually very low and in some cases showed a second maximum peak in plasma, due to an enterohepatic circulation [30]. In this case, a possible cumulative effect observed for some flavonoid metabolites could be responsible for the maintenance of the high plasma concentrations of phenolic acids.

The evaluation of the total phenolic plasma content over time in wWPP showed that 55% of the metabolites reached the maximum level in the plasma samples at 2h after their intake (figure 2B), suggesting that absorption was principally in the first part of the gastrointestinal tract and approximately 24 and 20% of the metabolites had the peak plasma at 4h and 6h after wWPP intake. Likewise, the results of metabolite evolution over time in the plasma sample after intake of wWPP showed differences between the lower dose (50mg/kg BW) and the other doses (100, 150 and 300 mg /kg BW) (Supplementary table 1). For the intake of 50 mg/BW occurs a decrease of 50% in the levels of plasma phenols at 2 and 4h, but the decrease for the doses of 100, 150, and 300 mg/kg BW was lowest with percentages of 44%, 31%, and 31% respectively. More importantly, after 2 h post-intake of doses of 150 and 300 mg/kg BW of wWPP a 38% of the phenolic metabolites showed their highest levels in plasma, and approximately 25% of the metabolites continued to increase at 4 h and 6 h for the dose of 150 mg/kg BW.

It indicates that the total phenolic acids in the plasma samples showed different behaviours for both the red and the white WPPs (Supplementary table 1). In addition, with the purpose of comparing the total phenolic acid content in the plasma for each dose, the area under the curve (AUC) of the total phenolic acid content in the plasma samples over time was calculated (Figure 3A). A significant increase of the total metabolites of the rWPP was observed for the dose of 100 and 150 mg/kg BW, but it was lower for the 300 mg/kg BW dose. These results suggest that an increased intake of rWPP will not imply an increase of the total phenol content in the plasma samples. In contrast, the metabolites significantly increased in the plasma samples at higher doses of wWPP.

In summary, these results demonstrated that the bioavailability of wWPP phenolic acid in the plasma samples was dose-dependent, but not for the rWPP. These results suggested an apparent saturation

mechanism for rWPP and not for wWPP, which was also reported in other studies for some polyphenols after blueberry consumption [31]. These differences can be explained by considering the different absorption, excretion, and metabolic pathways of the WPPs, including different matrix compositions, which depend on the major polyphenols of the WPP and possible interaction with plasma proteins.

**3.3 Phenolic metabolite elimination in urine after rWPP and wWPP intake**

p-hydroxyphenylacetic and vanillic acids were the phenolic acids excreted at the highest concentrations (2.2 and 1.9 μmol/mmol creatinine) in the urine samples (Supplementary table 2), corresponding to the rWPP doses of 100 and 150 mg/kg BW, respectively. These results agree with the observed for rWPP in plasma where both polyphenols showed the highest values. Urine concentrations up to 0.1μM were observed for hydrocaffeic, hydroferulic, 4-O-methylgallic, 3,5-dihydroxy-4-methoxybenzoic, homovanillic, m-hydroxyphenylacetic, isoferulic, protocatechuic, and ferulic acids principally for the 100 mg/kg BW dose. Approximately 43% of the maximum urinary levels corresponds to the elimination at 4-6h post rWPP intake (Figure 3B), which was in line with the appearance of metabolites in the plasma samples. At this point, it is important to recall that polyphenol elimination can be through two pathways: renal and biliary. Taking these into account, the levels of different phenolic acids in urine are dependent not only on their urinary excretion ratio, but they also depend on their capacity to bind plasma proteins and the amount eliminated by biliary excretion [32].

Maximum phenolic concentrations post wWPP intake were found for p-hydroxyphenylacetic acid (0.9μmol/mmol creatinine) at a dose of 300 mg/kg BW dose (Supplementary table 1). Interestingly, this phenolic acid was one of the highest concentrations of metabolites in the plasma samples. Urine levels of dihydro-3-coumaric, gentisic, vanillic, ferulic, m-hydroxyphenylacetic, homovanillic, and protocatechuic acids were higher than 0.1 μM, principally for the dose of 150 mg/kg BW. It is important to consider that phenolic compounds underwent an intense phase II metabolism at intestinal ephitelium and/or hepatic level [33]. The highest excretion of the metabolites occurred at 2-4h post wWPP intake (Figure 3B) in agreement with the peak plasma concentration of wWPP metabolites.

Thus, the differences between rWPP and wWPP were not only found in the profile of phenolic acids in the plasma, but also they differed with regard to the time of maximum urinary elimination, which was probably affected by the duration of the highest levels on plasma. The rWPP metabolites reached their maximum in

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the plasma samples at 4h and in the urine samples at 4-6h, while the wWPP metabolites reached their maximum earlier, at 2h in the plasma samples and at 2-4h in the urine samples.

In addition, the metabolites increased in both plasma and urine, but neither plasma nor urine is dose-dependent for rWPP intake. Furthermore, not all individual metabolites exhibit dose relationship with intake of WPP either in plasma or urine. Some metabolites (4-O-methylgallic, 3-O-methylgallic, hidrocaffeic, dihydro-3-coumaric, gentisic, syringic acids) correlate with the amount of WPP ingested showing a linear dose response. However, this does not apply to all polyphenol metabolites such as m-hydroxyphenylacetic, vanillic, hydroferulic or p-hydroxyphenylacetic acids .Other studies of the polyphenol dose relationship with metabolites neither showed dose relationship with metabolites analysis [31,34,35].

**3.4 Total Antioxidant capacity of plasma**

The biological properties of polyphenols present in the WPPs and their antioxidant activity depend on their absortion, distribution, stability and metabolism [2]. An important point to consider in the case of the wine pomace products used in this study, is that they underwent no extraction.

The increase of ABTS values post wWPP (Figure 4B) was significative for all doses, while there was no significative increase in the ABTS values for the rWPP (Figure 4A) during the time of the experiment. A possible explanation could be found in the different phenolic acid profiles of the plasma samples after red or white WPP intake. For example, the wWPP metabolites had higher contents of m-hydroxyphenylacetic, protocatechuic, 4-O-methylgallic, homovanillic, and ferulic acids than rWPP and metabolites that can act as a good scavenger of ABTS radicals. On the other hand, it is important to consider that some of the metabolites can be conjugated, which might affect their antioxidant capacity [7].

The ABTS values increased (between 8-30% with respect to the baseline) at 2h and 4h post wWPP intake for all doses and at 6h only for the 150 and 300 mg/kg BW doses. The highest increase of ABTS values (30%) was at 2h post intake of 300 mg wWPP /kg BW. This result is in agreement with the time of the highest level of phenolic acid in plasma.

The ferric reducing capability of plasma (FRAP) increased post intake of 100 (at 2, 4 and 6h), 150, and 300 (4h) mg/kg BW of wWPP (Figure 4D) and no significant increase of FRAP was observed for the rWPP (Figure 4C). The highest increase of FRAP values (20%) was at 4h post intake of 300 mg wWPP/kg BW.

**3.5 Correlations between the antioxidant capacity and the phenolic metabolites content in plasma**

There was a positive correlation (Table 2) between the antioxidant capacity of the plasma assayed by ABTS and the total phenolic acids in the plasma samples after wWPP intake. A significative correlation ($p<0.05$) between the FRAP levels and the metabolites in the plasma was observed for the intakes of 100 and 300 mg of wWPP/kg BW. In contrast, there was no significant correlation between the antioxidant capacity and the metabolite levels in the plasma samples for the rWPP intake (table 2) where the highest level of phenolic acids will not necessarily imply a higher antioxidant capacity, probably because of the metabolite profiles and their bioactive form. A numerous of active mechanisms were not assayed, which could include modulations of gene expression, enzymatic activities, and possible antioxidant activities inside the cells [36–38]. In summary, the bioavailability of phenolic metabolites after rWPP intake in plasma and urine from Wistar rats showed no dose effect. However, the intake of wWPP exhibited a dose effect, with major concentrations of phenolic acid in plasma and urine metabolites after intake of 150 and 300 mg/BW. Furthermore, the bioavailability of the wWPP phenolic acids occurs earlier than for the rWPP phenolic acids, as indicates the maximum peak observed in plasma at 2 h post-intake for the wWPP and at 4 h post-intake for the rWPP. Similarly, the urine levels of the wWPP observed in the plasma samples increased with the dose and the metabolites of the rWPP decreased at the highest dose. The dose-dependent effect of the wWPP was accompanied by a significative correlation between the phenolic metabolites in the plasma samples and their antioxidant capacity.

**4 Abbreviations:** AUC, area under the curve; BW, body weight; WPPs, wine pomace products; rWPP, red wine pomace product; wWWP, white wine pomace product.

**5 Chemical compounds studied in this article:** 3-O-methylgallic acid (PubChem CID: 19829); 4-O-methylgallic acid (PubChem CID: 78016); caffeic acid (PubChem CID:689043); dyhydro-3-coumaric acid (PubChem CID: 91); ferulic acid (PubChem CID:445858); gentisic acid (PubChem CID:3469); homovanillic acid (PubChem CID: 1738); homoprotocatechuic acid (PubChem CID: 547); hydrocaffeic acid (PubChem CID: 348154); hydroferulic acid (PubChem CID: 14340); isoferulic acid (PubChem CID: 736186); m-hydroxyphenylacetic acid (PubChem CID: 12122); p-hydroxyphenylacetic acid (PubChem CID:127); protocatechuic acid (PubChem CID: 72); syringic acid (PubChem CID: 10742); vanillic acid (PubChem CID: 8468).

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## 6 Conflicts of interest

The authors declare no conflict of interest.

## 7 Acknowledgments

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## 8 Supporting Information

Supplementaty table 1, concentration (μM) of phenolic acid metabolites assessed by GC/MS/MS in plasma after rWPP and wWPP intake. Supplementary table 2, concentration (μmol/mmol creatinine) of phenolic acid metabolites assessed by GC/MS/MS in urine after rWPP and wWPP intake. Supplementary Figure 1, Representative GC/MS/MS chromatograms and list of analyzed compounds by multiple reaction monitoring (MRM) segments, retention times, and precursor and product ions. Supplementary Figure 2, Representative GC/MS/MS chromatograms and list of analyzed compounds in the red (A) and white (B) wine pomace products.

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## TABLES

**Table 1. Phenolic composition and antioxidant capacity of the rWPP and wWPP.**

| | | µg/g rWPP | µg/g wWPP | |
|---|---|---|---|---|
| ***PHENOLIC COMPOUNDS*** | ***Phenolic acids*** | | | |
| | m-Hydroxyphenylacetic acid | 3.10 ± 0.17 | 4.10 ± 0.45 | |
| | p-Hydroxyphenylacetic acid | 3.19 ± 0.23 | 14.9 ± 0.46 | * |
| | Vanillic acid | 3.19 ± 0.16 | 11.5 ± 0.56 | * |
| | Homovanillic acid | 2.45 ± 0.01 | 2.89 ± 0.06 | * |
| | Protocatechuic acid | 18.1 ± 1.50 | 7.79 ± 0.13 | * |
| | Homoprotocatechuic acid | 13.9 ± 0.10 | 52.3 ± 2.46 | * |
| | Gentisic acid | 27.9 ± 0.01 | 29.4 ± 0.06 | * |
| | Syringic acid | 3.08 ± 0.34 | 14.0 ± 3.39 | * |
| | 4-O-methylgallic acid | 1.16 ± 0.01 | 2.48 ± 0.31 | * |
| | 3-O-methylgallic acid | 7.19 ± 0.08 | 20.1 ± 1.35 | * |
| | Dihydro-3-coumaric acid | 1.95 ± 0.02 | 2.17 ± 0.03 | * |
| | Hydroferulic acid | 2.24 ± 0.01 | 5.19 ± 0.09 | * |
| | Hydrocaffeic acid | 9.50 ± 0.01 | 11.2 ± 0.04 | * |
| | Isoferulic acid | 3.87 ± 0.01 | 4.52 ± 0.14 | * |
| | Ferulic acid (trans-) | 3.68 ± 0.47 | 5.76 ± 0.69 | * |
| | Caffeic acid (trans-) | 10.7 ± 0.40 | 35.7 ± 2.42 | * |
| | *Total Phenolic acids*[a] | 115 ± 1.08 | 224 ± 7.00 | * |
| | ***Stilbenes*** | | | |
| | t-piceid | 0.64 ± 0,11 | 0.63 ± 0.02 | |
| | t-resveratrol | 4.58 ± 0,55 | 1.21 ± 0.01 | * |
| | *Total Stilbenes*[a] | 5.22 ± 0.65 | 1.85 ± 0.01 | * |
| | ***Flavanols*** | | | |
| | Catechin | 11.5 ± 3.11 | 68.7 ± 5.67 | * |
| | Epigallocatechin | 146 ± 28.4 | 76.2 ± 23.1 | * |
| | Epicatechin | 15.6 ± 2.25 | 44.5 ± 5.67 | * |
| | Procyanidin B1 | 13.3 ± 0.06 | 54.6 ± 17.7 | * |
| | Procyanidin B2 | 33.5 ± 0.77 | 32.8 ± 3.18 | |
| | *Total Flavanols*[a] | 220 ± 24.5 | 277 ± 5.28 | * |
| | ***Flavonols*** | | | |
| | Myricetin-3-O-rhamnoside | 50.9 ± 2.76 | 27.9 ± 2.43 | * |
| | Kaempferol-3-O-rutinoside | 211 ± 19.7 | 194 ± 31.9 | |
| | Kaempferol-3-O-glucoside | 32.8 ± 2.98 | 29.8 ± 3.47 | |
| | Quercetin-3-O-rutinoside | 23.1 ± 5.59 | 26.6 ± 4.81 | |
| | *Total Flavonols*[a] | 318 ± 28.0 | 278 ± 32.3 | |
| | ***Anthocyanins*** | | | |
| | Delphinidin-3-O-glucoside | 244 ± 22.3 | ND | * |
| | Cyanidin-3-O-glucoside | 14.9 ± 2.85 | ND | * |
| | Petunidin-3-O-glucoside | 263 ± 17.4 | ND | * |
| | Peonidin-3-O-glucoside | 41.6 ± 1.12 | ND | * |
| | Malvidin-3-O-glucoside | 954 ± 1.98 | ND | * |
| | *Total Anthocyanins*[a] | 1518 ± 1.21 | ND | * |
| | ***Total Phenolic compounds***[a] | **2176 ± 66.6** | **781 ± 36.0** | * |
| ***TAC (Total Antioxidant Capacity)*** | *Q-FC (mg GAE/g WPP)* | 20.2 ± 0.08 | 9.50 ± 0.05 | * |
| | *Q-ABTS (mmol TE/g WPP)* | 0.16 ± 0.02 | 0.09 ± 0.02 | * |
| | *Q-FRAP (mmol Fe(II)Equivalent/g WPP)* | 0.39 ± 0.01 | 0.28 ± 0.01 | * |

**Table 1.** Phenolic composition and antioxidant capacity of the red (rWPP) and white (wWPP) wine pomace products. Phenolic acid composition was assayed by GC/MS/MS. Stilbene, flavanol, flavonol and anthocyanin content were assayed by HPLC/DAD. The totals are the sum of each of the individual phenolic compounds. Values are expressed as µg/g rWPP or wWPP. Antioxidant capacity was assayed by QUENCHER methods (Q-FC, Q-ABTS and Q-FRAP). Values are expressed as mg GAE/g WPP (Q-FC),

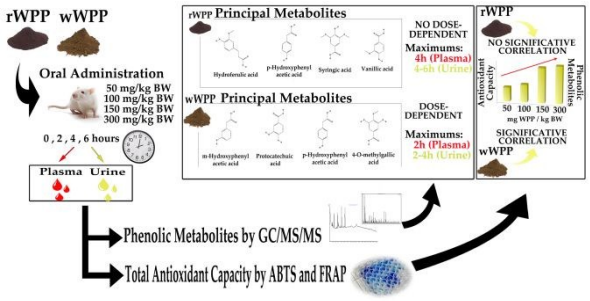
mmol TE/g WPP (Q-ABTS) and mmol Fe(II)E/g WPP (Q-FRAP). Data are presented as mean ± SD (n=3). Significant differences ($p < 0.05$) between rWPP and wWPP are indicated with an asterisk (*). ND: not detected, Q-FC: QUENCHER-Folin-Ciocalteu, Q-ABTS: QUENCHER-ABTS, Q-FRAP: QUENCHER-FRAP, TAE: Tannic acid equivalent, TE: Trolox equivalent. rWPP: red wine pomace product, wWPP: white wine pomace product.



**Table 2. Correlations between the total phenolic content in plasma and the Total Antioxidant Capacity**

| | | | ABTS | | FRAP | |
|---|---|---|---|---|---|---|
| | Dose (mg WPP/kg BW) | | rWPP | wWPP | rWPP | wWPP |
| **TOTAL PHENOLIC ACIDS** | 50 | r | NSC | 0.7314 | NSC | NSC |
| | | p | | 0.0069 | | |
| | 100 | r | NSC | 0.6267 | NSC | 0.7563 |
| | | p | | 0.0292 | | 0.0044 |
| | 150 | r | NSC | 0.7719 | NSC | NSC |
| | | p | | 0.0033 | | |
| | 300 | r | NSC | 0.7369 | NSC | 0.5783 |
| | | p | | 0.0063 | | 0.0489 |

**Table 2.** Correlation between the total phenolic content in the plasma samples and the antioxidant capacity assayed by the ABTS and the FRAP methods for both the red and white wine pomace products (rWPP and wWPP, respectively). The values represented the correlation coefficient (r) and probability ($p>0,05$) between the total content of phenolic acids in plasma samples following oral administration to rats of 50, 100, 150 or 300 mg of both rWPP and wWPP/kg of body weight (BW), and the total antioxidant capacity of the plasma samples assayed by the ABTS and the FRAP methods. NSC: not significant correlation observed. rWPP: red wine pomace product. wWPP: white wine pomace product.



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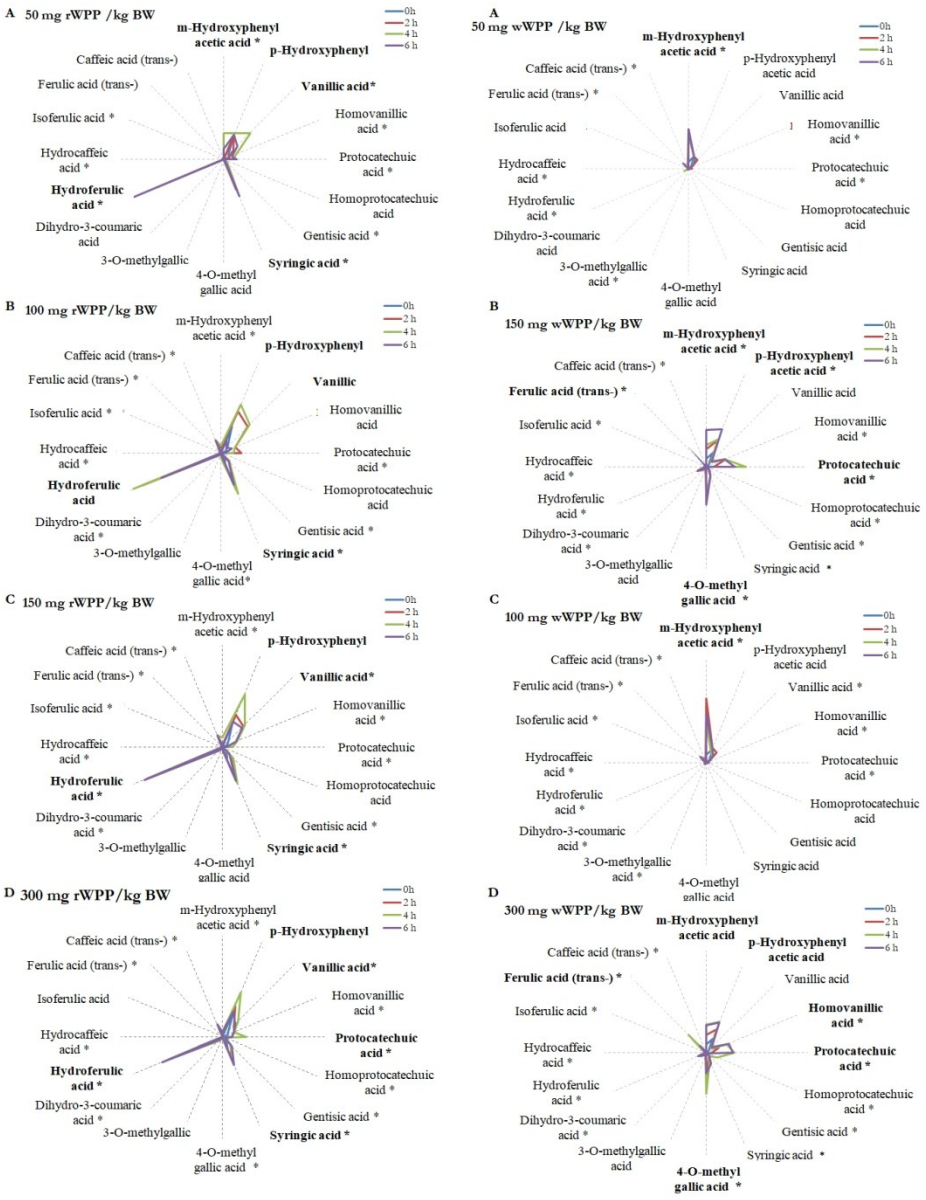


Figure 1. Radial representation of red (rWPP) and white (wWPP) pomace metabolites in plasma at basal and after 2h, 4h and 6h post intake for the doses of 50, 100, 150 and 300 mg/kg BW. (A-D) Concentration of the phenolic acid metabolites in the plasma samples (n=3) after red wine pomace product (rWPP) intake assayed by GC/MS/MS. Samples were collected at the indicated hours (plasma) pre- and post-administration of rWPP to ratsat doses of 50 (A), 100 (B), 150 (C) or 300 (D) mg/kg of body weight (BW). Significant differences ($p < 0.05$) in the total phenolic content in plasma between basal, 2h, 4h and 6h for each dose are indicated with an asterisk (*). The main phenolic acid metabolites for each dose are shown in bold font. (E-H) Concentration of the phenolic acid metabolites in the plasma samples (n=3) after white wine pomace product (wWPP) intake assayed by GC/MS/MS. Samples were collected at the indicated hours (plasma) pre- and post-administration of wWPP to rats at doses of 50 (E), 100 (F), 150 (G) or 300 (H) mg/kg of body weight (BW). Significant differences ($p < 0.05$) in the total phenolic content in plasma between basal, 2h, 4h and 6h for each dose are indicated with an asterisk (*). The main phenolic acid metabolites for each dose are shown in bold font. rWPP: red wine pomace product, wWPP: white wine pomace product.

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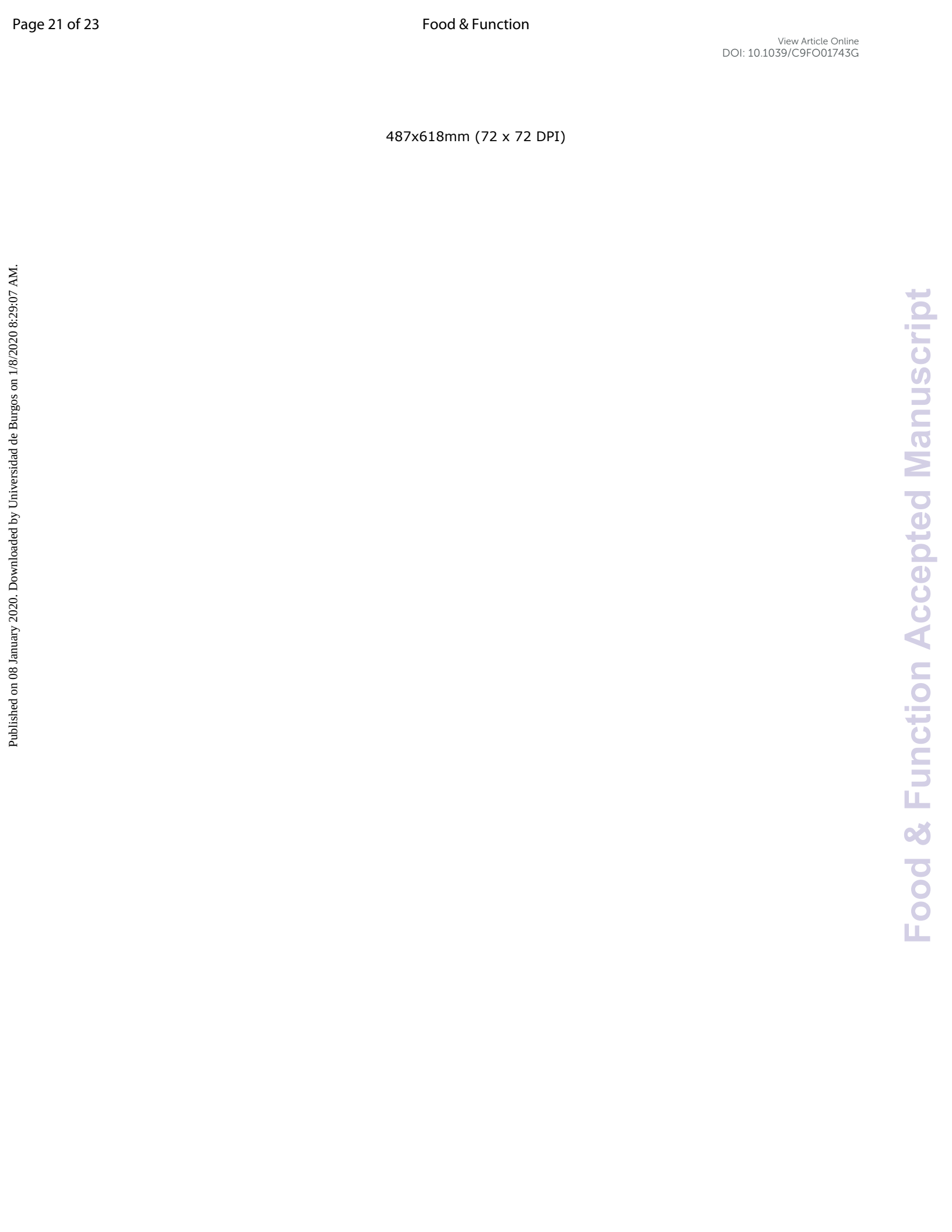

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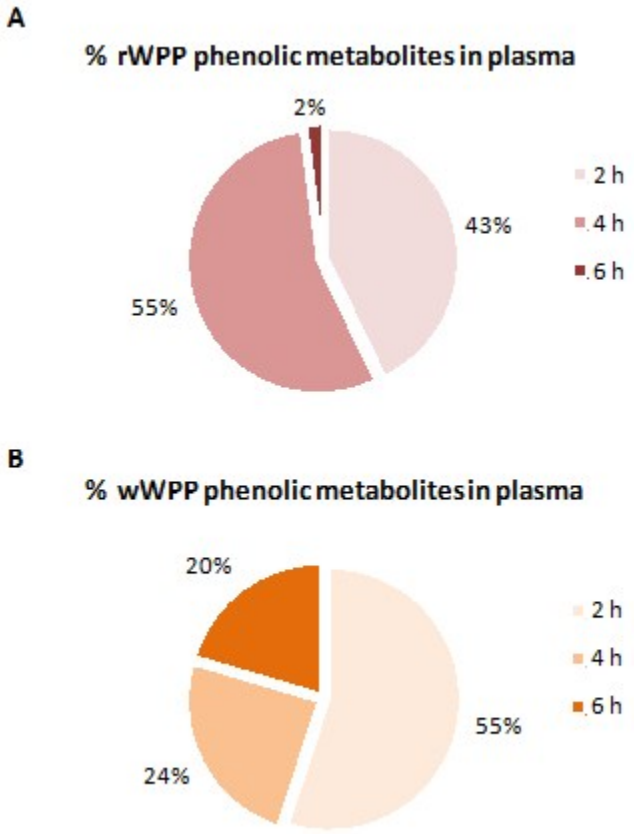


Figure 2. (A) Percentage of red wine pomace (rWPP) metabolites in the plasma samples (n=3) with their highest concentration after 2, 4 or 6 hours post intake. The percentages represent the number of the individually rWPP metabolites that have a maximum level in plasma at 2h, 4h or 6h for all doses. (B) Percentage of white wine pomace (wWPP) metabolites in the plasma samples (n=3) with their highest concentration after 2, 4 or 6 hours post intake. The percentages represent the number of the individually rWPP metabolites that have a maximum level in plasma at 2h, 4h or 6h for all doses. rWPP: red wine pomace product, wWPP: white wine pomace product.

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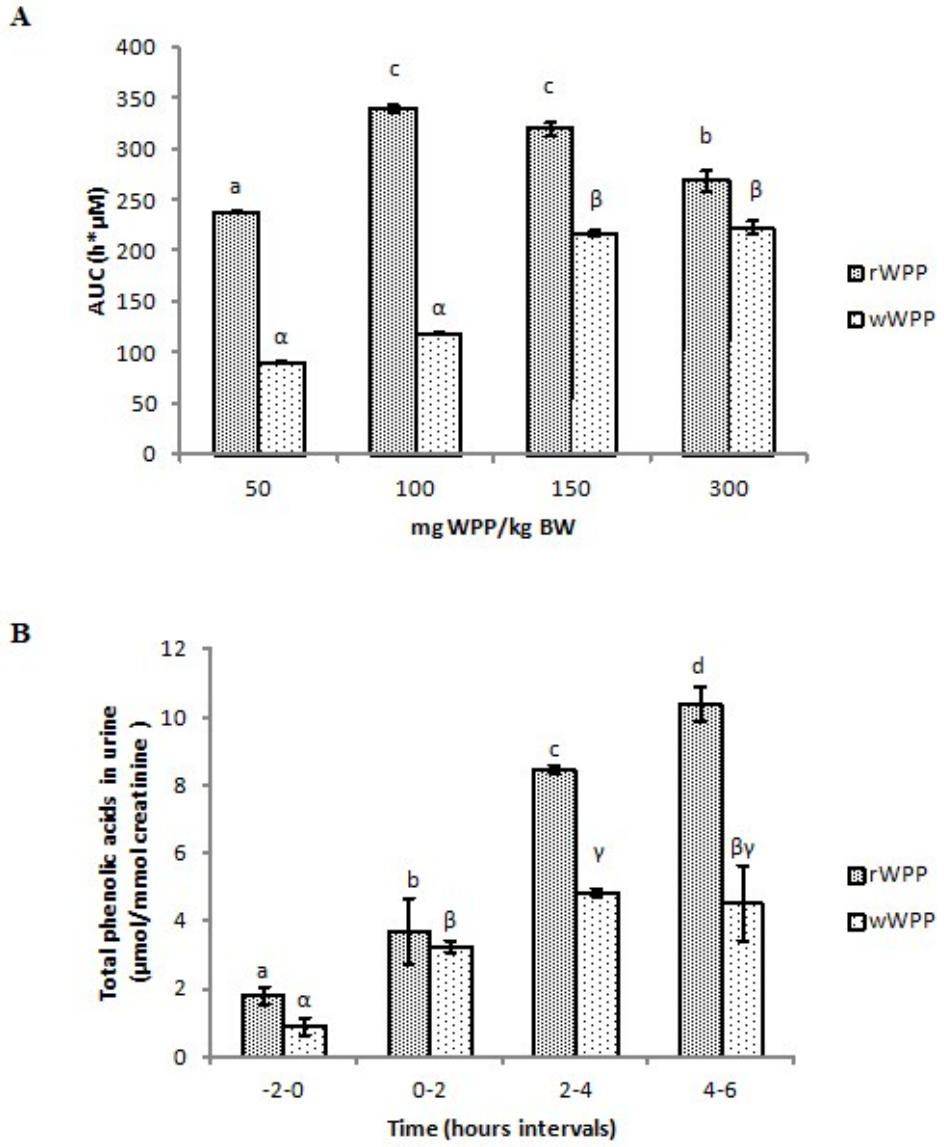


Figure 3. (A) Area under the curve (AUC) of total phenolic acid content in plasma. The AUC values were calculated from the curves of the total phenolic metabolites present in the plasma samples in the 0-6h interval after 50, 100, 150 or 300 mg/kg of body weight (BW) administration to rats of the red (rWPP) and white (wWPP) pomace products. Data are presented as mean ± SD (n=3). Significant differences ($p < 0.05$) in the AUC values between 50, 100, 150 and 300 mg of WPP/kg of body weight (BW) are indicated with Latin letters for the rWPP and with Greek letters for the wWPP. (B) Total phenolic metabolites in the urine samples of rats at basal (0h) and 2, 4 and 6 hours post intake of red (rWPP) or white (wWPP) wine pomace products. Data are presented as mean ± SD, n=3. Significant differences ($p < 0.05$) are expressed in Latin letter for the rWPP and Greek letter for the wWPP. rWPP: red wine pomace product, wWPP: white wine pomace product.

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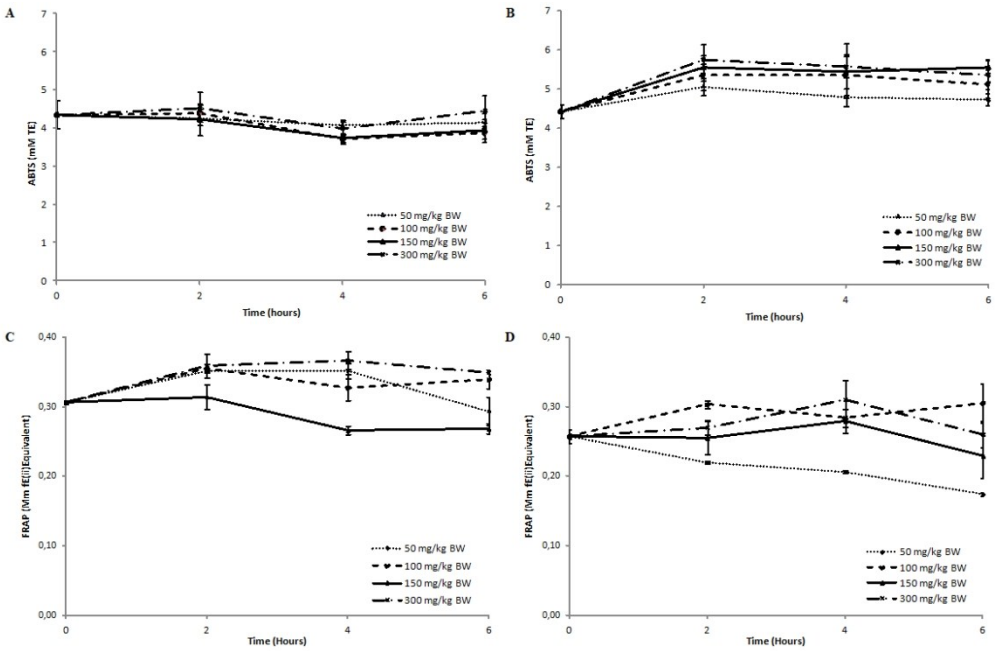


Figure 4. (A) Plasma ABTS values for the different doses (50, 100, 150, and 300 mg/kg Body Weight) administered to rats at basal (0h) and 2, 4 and 6 hours post intake of red wine pomace product (rWPP). (B) Plasma ABTS values for the different doses (50, 100, 150 and 300 mg/kg Body Weight) administered to rats at basal (0h) and 2, 4 and 6h post intake of white wine pomace product (wWPP). (C) Plasma FRAP values for the different doses (50, 100, 150 and 300 mg/kg BodyWeight) administered to rats at basal (0h), 2, 4 and 6 hours post intake of red wine pomace product (rWPP) in rats. (D) Plasma FRAP values for the different doses (50, 100, 150 and 300 mg/kg Body Weight) at baseline (0h), 2, 4 and 6 hours post intake of white wine pomace product (wWPP) in rats. Values are expressed as mmol TE/g WPP (ABTS) and mmol Fe(II)E/g WPP (FRAP). Data are presented as mean ± SD (n=3). rWPP: red wine pomace product, wWPP: white wine pomace product, TE: Trolox equivalent.

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