**Bioavailability of Phenolic Compounds and Antioxidant Effects of Wine Pomace Seasoning After Oral Administration in Rats.**

Running title:

**Bioavailability and Bioactivity of a Wine Pomace Seasoning.**

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**Abstract**

The bioavailability of phenolics contained in a powdered red wine pomace seasoning (RWPS) and its beneficial effects after acute and short-term supplementation were investigated. First, a single oral-dose of RWPS (300 mg/kg body weight) was administered to healthy Wistar rats (n=6) and several phenolic acids were determined post-RWPS intake in plasma (0h, 2h and 4h) and urine (-3–0h, 0–3h and 3–6h). Higher prevention of lipid peroxidation (plasma and urinary $F_2$-isoprostanes) and improved nitric oxide bioavailability were observed in samples collected at 4h and between 3–6h ($p<0.05$), suggesting an important antioxidant role of the metabolites generated by the action of colonic microbiota. In contrast, following 4-weeks of daily RWPS consumption, no significant differences were detected between supplemented and control groups for any of the biomarkers assessed, which indicates that acute beneficial effects of RWPS intake might be only transient under normal physiological conditions for this rat model.

**Keywords:**

Bioavailability; Isoprostanes; Nitric oxide; Polyphenols; Oxidative stress; Wine pomace.

**Abbreviations**

**ABTS**, 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; **eNOS**, endothelial nitric oxide synthase; **$F_2$-IsoPs**, $F_2$-isoprostanes; **FC**, Folin-Ciocalteu; **FRAP**, ferric reducing ability of plasma; **GC-NCI-MS**, gas chromatography/electron capture-negative chemical ionization mass spectrometry; **GC-EI-MS**, gas chromatography/electron ionization mass spectrometry; **$NO_2^-$**, nitrite; **$NO_3^-$**, nitrate; **ROS**, reactive oxygen species; **RWPS**, red wine pomace seasoning; **TAC**, total antioxidant capacity.

## 1. Introduction

Wine pomace potentially constitutes an abundant and relatively inexpensive source of a wide range of grape derived phenolic compounds, including phenolic acids, stilbenes, flavan-3-ols (mono-, oligo- and polymers) and anthocyanins (Kammerer, Claus, Carle, & Schieber, 2004). While most wine pomace-derived products are obtained applying extractive processes, those produced avoiding any kind of extraction present several advantages as carriers of dietary polyphenols (Saura-Calixto, 2011).

Red wine pomace seasonings (RWPSs) are value-added products obtained directly from winemaking by-products. The capacity of the phenolic compounds contained in these powdered seasonings to prevent detrimental effects of oxidative reactions in biological systems represents an interesting opportunity for nutraceutical and food industries to use RWPSs as dietary supplements and functional food ingredients (García-Lomillo, González-Sanjosé, Del Pino-García, Rivero-Pérez, & Muñiz, 2014; Yu & Ahmedna, 2013). In addition, the potential for beneficial properties of RWPSs is supported by their high content in dietary fiber (García-Lomillo et al., 2014). RWPSs show high *in vitro* antioxidant capacities (Del Pino-García, García-Lomillo, Rivero-Pérez, González-Sanjosé, & Muñiz, 2015), and promising antioxidant protective effects demonstrated *ex vivo* in endothelial cell cultures (Del Pino-García et al., 2016). However, when dietary polyphenols are consumed, their bioavailability *in vivo* should be always considered.

The actual bioactivity and antioxidant function of plant-derived phenolic compounds in the organism is clearly affected by their chemical structural changes due to the extensive digestive and metabolic transformations, and by the interactions with other molecules in the plant or food matrices that determine their intestinal absorption (Bohn, 2014; Fernández-Panchón, Villano, Troncoso, & García-Parrilla, 2008; Heim, Tagliaferro, & Bobilya, 2002). As such, health effects consequent of polyphenol intake depend on their bioavailability (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Certain phenolic compounds are highly bioavailable whereas others are poorly or not absorbed in the small intestine (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005) and reach the colon, where they are metabolized by the intestinal microbiota into various phenolic acids, mainly phenylpropionic, phenylacetic, and benzoic acid derivatives (Aura, 2008; Rechner et al., 2002). Nevertheless, long-term consumption of polyphenol-rich foods does not generally result in accumulation of significant amounts of these metabolites in plasma (Lotito & Frei, 2006). In fact, half-lives of circulating phenolic compounds and metabolites are short, usually in the range of a few hours, with their levels varying

from low to high nanomolar concentrations in plasma and within the micromolar range in urine (Aura, 2008; Del Rio et al., 2013; Lafay & Gil-Izquierdo, 2008; Manach et al., 2005). Consequently, the antioxidant efficacy of phenolic compounds *in vivo* may be limited by their digestive and metabolic alterations, rather low bioavailability, and rapid elimination from the organism.

In light of the above, expanding our knowledge towards the metabolic fate of wine pomace phenolic compounds is critical to understand the actual benefits of these potential bioactive compounds in preventing and protecting against oxidative stress. Therefore, the current study aimed to quantitatively investigate the phenolic acid metabolites that circulate in plasma and are excreted in urine after administration to healthy rats of a single-dose of RWPS. The effect of this dietary supplement, obtained directly from seedless red wine pomace, to modulate biomarkers of oxidative stress and endothelial function under normal physiological conditions in both acute and short-term intervention experiments was also investigated.

## 2. Materials and methods

### 2.1. Chemicals

Acetonitrile anhydrous (99,8%), 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), N,O-*bis*(trimethylsilyl)trifuoroacetamide with 1% trimethylchlorosilane (BSTFA+TMCS), butylated hydroxytoluene (BHT), bovine serum albumin (BSA), diethylene triamine pentaacetic acid (DTPA), N,N-diisopropylethylamine (DIPEA), 2,4-dinitrophenylhydrazine (DNFH), ethanol, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), gallic acid (GA), glutathione (GSH), guanidine hydrochloride, hydrochloric acid (HCl), hydrogen peroxide ($H_2O_2$), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), iodine ($I_2$), isooctane, methanol, 2,3,4,5,6-pentafluorobenzyl bromide (PFB-Br), phosphoric acid solution ($H_3PO_4$), potassium hydroxide (KOH), pyridine anhydrous (99,8%), potassium iodide (KI), sodium acetate, sodium bicarbonate ($NaHCO_3$); sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), sodium [$^{15}$N] nitrate, sodium [$^{15}$N] nitrite, 1,1,3,3-tetramethoxypropane (TMP), tetraoctylammonium bromide (TOA-Br), 2,4,6-Tris (2-pyridyl)-S-triazine (TPTZ), and all phenolic acids standards were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Acetone, hexane, and glacial acetic acid were provided by Univar Australia Pty. Ltd. (Ingleburn, NSW, Australia). Certify II cartridges for solid phase extraction (SPE) were from Varian Australia, Pty. Ltd. (Mulgrave, VIC, Australia). Ferric(III)-chloride acid ($FeCl_3$), ferrous(II)-

sulphate ($FeSO_4$), Folin-Ciocalteau (FC) reagent, potassium persulfate ($K_2O_8S_2$), sodium acetate ($NaC_2H_3O_2$), and sodium carbonate ($Na_2CO_3$) were obtained from Panreac Quimica S.L.U. (Barcelona, Spain). 8-iso-$PGF_{2\alpha}$-$d_4$ (Cat. No. 316351), 8-iso-$PGF_{2\alpha}$ (Cat. No. 16350), and 8,12-iso-$iPF_{2\alpha}$-VI-$d_{11}$ (Cat. No. 10006878) standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). All solvents were HPLC grade.

### 2.2. Composition of red wine pomace seasoning (RWPS)

The wine pomace-derived product used as a dietary supplement in this study was prepared in the pilot plant of the Food Technology Department of University of Burgos (Spain) (González-Sanjosé, García-Lomillo, Del Pino-García, Muñiz-Rodríguez, & Rivero-Pérez, 2013). Red wine pomace from the vinification of *Vitis vinifera* L. *cv Tempranillo* was kindly supplied by several wineries situated in Burgos (Spain). Seedless wine pomace was used as raw material to produce the powdered seasoning, which was finally submitted to a heat treatment as a microbial stabilization step for its safety use as a food ingredient. The nutrient composition, total antioxidant capacity, and phenolic contents of this seedless red wine pomace seasoning (RWPS) have been previously reported (García-Lomillo et al., 2014) and are summarized in **Table 1**.

### 2.3. Animals and experimental design

The protocol for this rat study was approved by the Ethics Committee for Experimental Animal Care at the University Hospital of Burgos and the Ethics Committee at the University of Burgos. All animal procedures were carried out in accordance with the current 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).

Male Wistar rats weighing 307±12 g were purchased from the Animal Research and Welfare Service of Valladolid (SIBA, Valladolid, Spain). Animals were housed at 23ºC with an alternating light/dark cycle of 12h, and they received a standard rodent diet (A04 Safe Iberica-Panlab, Barcelona, Spain) and drinking water *ad libitum* during the experiments.

#### 2.3.1. Study 1: Acute supplementation.

RWPS at a dose of 300 mg/kg of body weight (2 mL in water) was administered by oral gavage to Wistar rats (n = 6) after food deprivation for 12h with free access to water. Rats were placed in metabolic cages and

cumulative urine was collected in 3 hour-periods before (-3–0h) and post-RWPS administration (0–3h and 3–6h). Aliquots of these urine samples were stored frozen at -80 ºC until biochemical analyses. Blood samples were withdrawn at baseline (0h) and post-RWPS consumption (2h and 4h). These samples were collected by tail venipuncture into Vacutainer® tubes containing sodium and lithium heparin (17 I.U./mL blood) as anticoagulant (Becton Dickinson, Co., Madrid, Spain). In order to prevent oxidation of lipids on storage, those blood samples for $F_2$-IsoP analysis were collected in ice-cold tubes containing a GSH/BTH/EDTA mixture, as previously suggested (Barden, Mas, Croft, Phillips, & Mori, 2014). To recover plasma, all blood samples were centrifuged at 1,500 g for 10 min at 4ºC. Plasma aliquots were kept frozen at -80 ºC until analyses.

**2.3.2. Study 2: Short-term supplementation.**

Wistar rats were randomly divided into two groups (n = 5): the control (C) group and the RWPS-supplemented (+RWPS) group. During 4 weeks, rats were given a daily single dose of the seasoning (300 mg/kg of body weight) dissolved in 2 mL water (+RWPS group) or 2 mL of water (C group), which was orally administered by gavage between 9 and 10 a.m. Food and drink consumption and the animal body weights were recorded every day. There were no significant differences among the groups for any of these determinations (data not shown). Two days before the end of the study, rats were placed in metabolic cages 6h post-RWPS intake and urine was collected during 5h. The last day of the experiment, 12-h fasted animals (which had received the last RWPS dose approximately 24h prior to biological sample collection) were anesthetized and sacrificed to collect blood samples by cardiac puncture. Urine and blood samples were collected and treated as described above (Study 1) and urine and plasma aliquots stored at -80ºC for further analyses.

**2.4. Identification and quantification of phenolic metabolites**

**2.4.1 Phenolic acids in plasma**

Concentration of phenolic acids were measured in plasma samples by using a gas chromatography coupled to electron ionization mass spectrometry (GC-EI-MS) method previously described (Caccetta, Croft, Beilin, & Puddey, 2000), with slight modifications. Plasma samples collected at all-time points from each rat were assayed together on the same day. Briefly, 100 µL of plasma were acidified with 2 mL of 0.1 M acetate buffer (pH 4.6) and then 50 ng of the internal standard (IS: 1-hydroxy-2-naphthoic acid), 20 µL of β-glucuronidase

(4,000 U) with sulfatase activity (200 U), and 10 µL of β-glucosidase (16 U) were added. After acidification, extraction and derivatization, the trimethylsilyl derivatives obtained were analyzed on an Agilent 6890N Network Gas Chromatograph (Agilent Technologies, Inc., Palo Alto, CA) coupled to an Agilent 5973B Series Mass Selective Detector and fitted with an DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies). Mass chromatograms in the full scan mode were recorded in the mass range of 50-500 atomic mass units. Selected ion monitoring (SIM) was carried out to monitor the major characteristic ion for each compound, and peak identification was based on retention time and mass spectra compared with authentic standards. Control samples in which plasma was substituted by Milli-Q water were also analyzed for noise signal correction. For quantification, calibration curves were established by measuring peak areas versus response in comparison with the internal standard over a range of each analyte concentrations. The concentration of phenolic acids in plasma was finally expressed in µM.

**2.4.2. Phenolic acids in urine**

Phenolic acids present in urine samples were quantified according to a previously reported protocol (Ward, Croft, Puddey, & Hodgson, 2004). This method follows almost the same procedure as described above for plasma samples, but β -glucosidase was not added to the samples and 200 µL of urine was used for each analysis. To normalize all urine determinations, urinary creatinine concentration was determined for each urine sample at the Core Clinical Laboratory at Royal Perth Hospital, using a Technicon Axon analyser (Bayer Diagnostics, Scoresby, VIC, Australia). Levels of phenolic acids in urine were hence expressed in µmol /mmol creatinine.

**2.5. Assessment of plasma total antioxidant capacity (TAC)**

Three different colorimetric methods were used to estimate plasma TAC, following the protocols as previously reported (Santiago-Arteche et al., 2012).

*Folin-Ciocalteu (FC) method:* This assay determines the total reducing capacity of samples and is generally used to estimate the total phenols in foods and biological samples. The blue-colored phosphomolybdic/phosphotungstic acid complexes generated following reaction absorbs light at 750 nm. Gallic acid (GA) was used as the standard to prepare the calibration lines. The results were expressed as millimolar of GA equivalets (mM GAE).

*Ferric reducing ability of plasma (FRAP) method:* This assay also evaluates the existence of plasma reducing agents but is based on their ability to reduce Fe(III) to Fe(II). The absorbance at 593 nm is determine as the blue-colored Fe(II)-TPTZ complex formed absorbs light at this wavelength. Aqueous solutions of $FeSO_4$ at different concentrations were prepared for calibration. The results were finally expressed as micromolar of Fe(II) equivalents (µM Fe(II)E).

*ABTS method:* The ability of plasma antioxidants to scavenge free radical was assessed by their capacity to quench the $ABTS^{\cdot+}$ radical, which absorbs at 734 nm. A linear dose-response curve was obtained using Trolox as the standard, thus expressing final results as millimolar of Trolox equivalets (mM TE).

A PowerWave XS2 microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) was used for the absorbance measurements.

## 2.6. Determinations of $F_2$-isoprostanes ($F_2$-IsoPs)

### 2.6.1. $F_2$-IsoPs levels in plasma

The concentration of $F_2$-IsoPs in plasma samples were determined by gas chromatography/electron capture-negative chemical ionization mass spectrometry (GC-NCI-MS), following a previously published method (Mori, Croft, Puddey, & Beilin, 1999) with slight modifications. In brief, an IS solution (0.1 mg/L in methanol) of the deuterium-labeled standard (8-iso-$PGF_{2\alpha}$-$d_4$, also known as 15-$F_{2t}$-IsoP-$d_4$) was firstly obtained. Plasma samples were thawed and a 0.2 mL aliquot mixed with 50 µL of the IS solution. Then, 250 µL of KOH (1 M in methanol) was added to the samples, vortexed, incubated at 40 ºC for 30 min, acidified to a pH 4.5-4.7 with HCl (1 M), and applied to SPE cartridges. The isolated $F_2$-IsoPs were then derivatized, dried under nitrogen, reconstituted in isooctane, and analyzed by GC-NCI-MS using an Agilent 6890N Network Gas Chromatograph coupled to an Agilent 5975B Series Mass Selective Detector and fitted with a DB5-MS column (25 m x 0.20 mm, 0.33 µm film thickness, Agilent Technologies). Selected ion monitoring (SIM) was performed to monitor the carboxylate anion (M-181; loss of pentafluorobenzyl group) at m/z 569 and m/z 573 for $F_2$-IsoPs and the IS (8-iso$PGF_{2\alpha}$-$d_4$), respectively. Peak identification was based on comparison of retention times with the standard 8-iso-$PGF_{2\alpha}$. A 5-point calibration curve obtained from different solutions of this standard was used for quantitative analysis. Plasma $F_2$-IsoPs results were expressed as concentration values in nM.

220

221 **2.6.2. $F_2$-IsoPs concentrations in urine**

222 The concentration of $F_2$-IsoPs in urine samples was also assessed by GC-NCI-MS according to almost the
223 same method as indicated above for plasma, but the IS was 8,12-iso-iP$F_{2\alpha}$-VI-$d_{11}$ (*m/z* 580) and alkaline
224 hydrolysis was not performed. Urinary $F_2$-IsoPs results were normalized with respect to the creatinine
225 concentration in urine, expressing $F_2$-IsoPs concentration values in nmol/mmol creatinine.

226

227 **2.7. Measurement of the concentrations of nitrates ($NO_3^-$) and nitrites ($NO_2^-$) in urine**

228 The nitric oxide breakdown products $NO_3^-$ and $NO_2^-$ were quantified in urine samples as an indirect method to
229 estimate the nitric oxide production. Both nitric oxide metabolites were converted to their stable
230 pentafluorobenzyl derivatives directly from aqueous acetone medium, using TOA-Br as catalyst, and then
231 analyzed using GC-NCI-MS in the selected-ion monitoring (SIM) mode ($NO_3^-$ *m/z* = 62; $NO_2^-$ *m/z* = 46), as
232 previously reported (Yang, Bondonno, Indrawan, Hodgson, & Croft, 2013). The equipment used was an
233 Agilent 6890N Network Gas Chromatograph coupled to an Agilent 5973B Series Mass Selective Detector and
234 fitted with a DB-225MS column (30 m x 0.25 mm, 0.25 µm film thickness, Agilent Technologies). Peak
235 identification was based on retention time and mass spectra compared with $^{15}$N-labeled internal standards
236 ($^{15}NO_3^-$ *m/z* = 63; $^{15}NO_2^-$ *m/z* = 47). Each nitric oxide breakdown product was quantified using calibration
237 lines obtained from authentic $NO_3^-$ and $NO_2^-$ standards and labeled standards. Final results for $NO_3^-$, $NO_2^-$,
238 and the sum of both $NO_3^-$+$NO_2^-$ were normalized with the urinary levels of creatinine and expressed in
239 µmol/mmol creatinine.

240

241 **2.8. Data presentation and statistical analysis**

242 All results from Studies 1 and 2 are expressed as mean values ± standard error of the mean (SEM). The
243 Statgraphics® Centurion XVI, version 16.2.04 (Statpoint Technologies Inc., Warranton, VA, USA) software
244 was used for statistical analyses. In the acute experiment (Study 1), data obtained at the three determination
245 times were subjected to paired-samples Student's t-test to detect whether the results at one time could be
246 paired with the values of the rest. Linear correlations between each individual phenolic acid and the
247 biomarkers studied at each collection time were also established by determining Pearson's correlation
248 coefficients, with the significance of the correlation being determined using Student's t-test. In the short-term

experiment (Study 2), a standard Student's t-test was used to determine significant differences between experimental groups. A value of $p < 0.05$ was considered for all analyses.

## 3. Results

### 3.1. Phenolic metabolic fate in plasma and urine after acute RWPS supplementation

A total of fourteen phenolic acids were identified and quantified, which are shown in **Table 2** ordered according to their time of maximum concentration in plasma, as indicative of which compounds may be preferentially absorbed at either the gastrointestinal or the colonic phases.

In plasma samples, dihydroferulic, followed by 4-hydroxyphenylacetic, were the phenolic acids present at the highest concentration post-RWPS consumption, considerable levels of these metabolites were also present under basal conditions. Substantial concentrations of homovallinic, protocatechuic, syringic, gentisic, caffeic, dihydro-3-coumaric and ferulic acids were also detected following the seasoning administration, whereas the concentrations of the rest of phenolics were within the nM range. Plasma concentration of phenolic acids at 2h and 4h was dependent on the compound. Some metabolites reached maximum levels 2h post-RWPS intake, and then tended to decrease or leveled out at 4h. The most marked fall at 4h was observed for 4-*O*-methylgallic, whereas more variability was found for gentisic and caffeic acids, and similar concentrations at 2h and 4h were obtained for 4-hydroxyphenylacetic, homovallinic, vallinic, syringic and isoferulic acids. Interestingly, plasma levels of dihydroferulic, protocatechuic and 3-hydroxyphenylacetic acids gradually increased post-RWPS consumption. Concentration of dihydro-3-coumaric, ferulic and homoprotocatechuic acids did not change significantly following the seasoning intake.

In urine samples, 4-hydroxyphenylacetic and dihydroferulic were also the phenolic acids excreted in the highest concentrations after RWPS consumption (they reached low μmol/mmol creatinine values). High contents of dihydro-3-coumaric, vallinic, ferulic and syringic acids were also found in urine. Levels of several phenolics were increased in samples collected between 0–3h post-RWPS administration, but their concentration slightly (dihydro-3-coumaric and caffeic acids) or significantly (4-hydroxyphenylacetic, protocatechuic, vallinic and syringic acids) decreased between 3–6h, with levels of syringic acid returning to basal values. For dihydroferulic, isoferulic, gentisic and 3-hydroxyphenylacetic acids, similar concentrations were detected between samples collected at 0–3h and 3–6h intervals following RWPS intake. As regards

278 homovallinic, 4-*O*-methylgallic, and ferulic acids, no significant increase in their cumulative excretion was
279 detected with time in comparison with their basal urinary levels.
280
281 **3.2. Plasma lipid peroxidation and total antioxidant capacity (TAC) following acute RWPS**
282 **supplementation**
283 Following the acute administration of RWPS to rats (Study 1), lipid peroxidation was estimated from the
284 concentration of plasma $F_2$-isoprostanes ($F_2$-IsoPs), and total antioxidant capacity (TAC) of plasma was
285 assessed using the FC index, FRAP and ABTS assays (**Fig. 1**). Plasma $F_2$-IsoPs levels decreased by 23% at 4h
286 post-RWPS intake compared to samples collected before the intervention. A significant increase of around
287 10% in the FC index was observed in plasma collected both at 2h and 4h post-RWPS consumption compared
288 to basal conditions, whereas FRAP and ABTS values were only significantly higher in the latest collected
289 samples. The increment was considerably more marked for FRAP results (about 24%) than for ABTS values
290 (about 3%).
291
292 **3.3. Urinary markers of oxidative damage to lipids and nitric oxide bioavailability following acute**
293 **RWPS consumption.**
294 Excretion of $F_2$-IsoPs in urine was lower at both collection times after acute RWPS intake than at basal
295 conditions ($p < 0.05$), with no difference between the levels of this biomarker excreted at 0– 3h and 3–6h post-
296 RWPS consumption (**Fig. 2**, A). Urinary concentrations of both nitric oxide metabolites ($NO_3^-$ and $NO_2^-$)
297 followed similar trends, showing a marked raise post-RWPS intake only in the urine accumulated between 3–
298 6h ($p < 0.01$) (**Fig. 2**, B).
299
300 **3.4. Correlations between biomarkers and phenolic acid contents in the acute study**
301 In plasma collected during the acute study (Study 1), significant ($p < 0.05$) and positive correlations between
302 the FC index and the concentration of three phenolic acids were detected (FC:dihydroferulic, r = 0.496; FC:4-
303 hydroxyphenylacetic, r = 0.487; FC:gentisic, r = 0.478).
304 In urine, $F_2$-IsoPs values were significantly ($p < 0.05$) and negatively correlated with the contents of seven
305 phenolic acids ($F_2$-IsoPs: gentisic, r = -0.765; $F_2$-IsoPs:3-hydroxyphenylacetic, r = -0.739; $F_2$-IsoPs:

306 dihydroferulic, r = -0.697; $F_2$-IsoPs:4-hydroxyphenylacetic, r = -0.613; $F_2$-IsoPs:syringic, r = -0.593; $F_2$-
307 IsoPs:protocatechuic, r = -0.577; $F_2$-IsoPs:vallinic, r = -0.547).
308
309 **3.5. Plasma and urinary biomarkers of oxidative stress and endothelial function following short-term**
310 **RWPS consumption.**
311 As shown in **Table 3**, none of the biomarkers (plasma $F_2$-IsoPs and TAC; urinary $F_2$-IsoPs and nitric oxide
312 metabolites) that were evaluated in biological samples collected from 12h-fasted rats at the end of the Study 2
313 (daily RWPS consumption during 4 weeks) showed significant differences between control (C) and
314 supplemented (+RWPS) rats.
315
316 **4. Discussion**
317
318 The high phenolic contents of wine pomace suggests its potential to positively influence the redox
319 environment and vascular function following consumption of foods or dietary supplements derived from this
320 product (Pérez-jiménez et al., 2009; Yu & Ahmedna, 2013).
321 The metabolic fate of phenolic compounds following acute administration of a seedless red wine pomace
322 seasoning (RWPS) was first investigated in plasma and urine of healthy rats. The phenolic acids determined in
323 this study represent both the free forms and the phenolic backbones of phase II metabolites, as the conjugating
324 moieties of such metabolites were cleavage during the phenolic extraction protocol by the incubation of
325 biological samples with different phase II deconjugation enzymes. Most of the phenolic compounds analyzed
326 have been proposed to arise from the biotransformation of dietary polyphenols once ingested (Aura, 2012;
327 Manach et al., 2005; Rechner et al., 2002).
328 Acute RWPS intake significantly altered plasma and urinary profiles of phenolic acids. A reasonable
329 relationship between the absorption and excretion of most metabolites was observed. In general, phenolic acid
330 levels increased in plasma at 2h and/or 4h post-intake, and concentrations found in urine were higher in the
331 first collection interval (0–3h samples) and then tended to fall (3–6h samples), with a few exceptions for which
332 a maintained excretion with time was observed.
333 The phenolic composition of the seedless RWPS has been described in a previous work (Del Pino-García et
334 al., 2016). Some of these compounds may be directly released from the wine pomace matrix throughout the

entire gastrointestinal tract. Free phenolic acids and those linked weakly to the cell wall or contained in vacuoles were probably liberated and rapidly absorbed in the stomach and the upper intestine (Bohn, 2014), being thereby present at high concentrations in the first samples collected post-RWPS administration (2h for plasma and 0–3h for urine). In contrast, those phenolics physically trapped within the matrix microstructure but released in the lower intestine most likely peaked primarily in samples collected at 4h in plasma and at 3–6h in urine.

In addition, numerous new phenolic acids are generated following cleavage of ester and glycoside bonds, ring-fission, and extensive biotransformations of more complex precursors carried out by gut microbiota. As microbial population density is much higher in the large than the small intestine, most of these conversions are known to occur once non-assimilated compounds reach the colon and/or following the return to the lumen, via bile, of previously absorbed molecules (enterohepatic recirculation) (Aura, 2008; Landete, 2012). The resultant metabolites are thus expected to reach maximum concentrations primarily in the latest collected samples. However, microbial transformations were certainly much faster for extractable phenolics than for those compounds strongly attached to insoluble components of the wine pomace matrix (Aura, 2012; Rechner et al., 2002). This fact may partly explain the different intensity and timing of each metabolite appearance, and why the concentrations of some phenolics were largely maintained over time. The latter was observed for dihydroferulic, isoferulic, gentisic, protocatechuic and 3-hydroxyphenylacetic acids (in both plasma and urine), as well as for 4-hydroxyphenylacetic, vallinic, homovallinic and syringic acids (only in plasma).

Health benefits of phenolic metabolites can be ascribed to their pleotropic bioactivities, which may include antioxidant properties due to modulation of the endogenous antioxidant defenses (enzymatic and non-enzymatic). The resultant protective effects prevent oxidative damage of such vulnerable biomolecules as lipids (Fraga, Galleano, Verstraeten, & Oteiza, 2010; Pandey & Rizvi, 2009).

Acute RWPS supplementation (Study 1) led to a significant protection of lipids from oxidative damage, as evidenced by the $F_2$-IsoPs levels in both the plasma and the urine samples. These products of polyunsaturated fatty acids (arachidonic acid) are recognized as a sensitive and reliable index of *in vivo* lipid peroxidation (Montuschi, Barnes, & Roberts, 2004; Mori et al., 1999). $F_2$-IsoPs in plasma have a short half time (approximately 18 min) and are rapidly excreted in urine, which means that they must be formed constantly to maintain a steady-state concentration (Griffiths et al., 2002). Since the concentration of $F_2$-IsoPs decreased in plasma from 2h to 4h post-intake, whereas levels of this biomarker were similar in urine collected at both 3-

hour-intervals post-administration, the maximum level of lipid protection might be achieved between 2h and 4h following RWPS consumption. In addition, the concentration of $F_2$-IsoPs in urine was negatively correlated with several phenolic acids, and a number of phenolic metabolites detected in both plasma and urine followed an opposite trend than $F_2$-IsoPs levels. These findings support the important role that those metabolites generated principally by colonic microbiota might play following acute RWPS intake in the prevention and/or protection against lipid peroxidation.

The assessment of changes in plasma TAC after the intake of food antioxidants is a non-specific approach to examine their effects in the redox balance of the organism. Although it has been proposed as a simple biomarker of the overall absorption of such antioxidants (Fernández-Panchón et al., 2008; Prior & Cao, 1999), it is unlikely to represent true *in vivo* direct antioxidant actions of phenolic compounds (Croft, 2016; Hollman et al., 2011; Sies, 2007). Hence, the changes observed in TAC, especially at 4h post-RWPS intake, might be partly due to indirect antioxidant effects of phenolic metabolites although could be also influenced by fluctuations in other circulating antioxidants such as uric acid following RWPS consumption.

Oxidative stress appears to be a common denominator underlying endothelial dysfunction, a pathological condition which is a target for prevention of numerous cardiovascular diseases. The fundamental feature of endothelial dysfunction is the impaired bioavailability of nitric oxide, a vasodilator molecule that exerts several vasoprotective effects. Nitric oxide can be inactivated by reactive oxygen species (ROS) or can be produced at lower levels by the endothelial nitric oxide synthase (eNOS), which are both the most frequent causes of endothelial dysfunction (Versari, Daghini, Virdis, Ghiadoni, & Taddei, 2009). Therefore, dietary approaches that enhance nitric oxide bioavailability are regarded as beneficial strategies to protect the endothelium and prevent cardiovascular events (Andriantsitohaina et al., 2012; Chistiakov, Revin, Sobenin, Orekhov, & Bobryshev, 2015; Papageorgiou et al., 2013). The potential ability of RWPS consumption to increase nitric oxide production is supported by the increased excretion of urinary nitrate and nitrite observed. Our results further revealed that such increase in nitric oxide bioavailability was not noticeable until, at least, 3h post-RWPS intake. These findings evidence that colonic metabolites derived from RWPS may play a substantial direct and/or indirect role enhancing nitric oxide bioavailability. Direct effects resulting in higher nitric oxide could be due to the ability of phenolic compounds to scavenge superoxide anion, which is the main ROS that reacts with nitric oxide to generate peroxynitrite, a potent vasoconstrictor and oxidant molecule that targets mainly lipid oxidation (O'Donnell & Freeman, 2001). However, indirect effects are more likely,

these include: i) activation/up-regulation of eNOS and inhibition of eNOS uncoupling); ii) inhibition of ROS generation (such as inactivation/down-regulation of NADPH oxidases and xanthine oxidases); iii) reduction of ROS levels (such as the activation/up-regulation of the antioxidant defense enzymes) (Montezano & Touyz, 2011; Zinkevich & Gutterman, 2011). Nitric oxide and ROS themselves can also induce several redox signaling pathways that control the expression of vascular antioxidant defense enzymes, including heme oxygenase-1and extracellular superoxide dismutase (Finley et al., 2011; Gewaltig & Kojd, 2002; Zinkevich & Gutterman, 2011).

In contrast to acute treatment, longer term (4-weeks) of RWPS consumption (Study 2), did not improve oxidative stress and endothelial function biomarkers analyzed in plasma and urine of 12h-fasted rats. This finding may be due to the disappearance of many phenolic metabolites during the fasting period and suggests a transient effect of these compounds. These findings need to be confirmed in humans, in agreement with previous authors (Hollman et al., 2011; Pérez-Jiménez & Saura-Calixto, 2008; Rodrigo, Castillo, Carrasco, Huerta, & Moreno, 2005), our results using animal models support the hypothesis that healthy individuals, which in general are not submitted to high chronic oxidative insults, might be less susceptible to benefit from dietary antioxidant supplements than patients with pathological conditions associated to oxidative stress.

In summary, acute consumption of a seasoning product derived from seedless red wine pomace (RWPS) temporally improved the vascular redox status, decreased lipid peroxidation, and improved nitric oxide bioavailability in healthy rats. These potentially beneficial effects were parallel in time with significant increments in the concentrations of several plasma and urinary phenolic metabolites derived from the seasoning but, in general, they could not be ascribed to specific compounds as indirect effects of bioactive metabolites may prevail over their direct antioxidant activities. The phenolic acids derived from the action of gut microbiota (mainly dihydroferulic, followed by gentisic, 3- and 4-hydroxyphenylacetic, syringic, protocatechuic and vallinic acids) may have a predominant role.

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## Disclosure statement

The authors declare no competing financial interest

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**Figure captions**

**Fig. 1. Plasma $F_2$-isoprostanes levels (A) and total antioxidant capacity, as determined using the FC index (B), FRAP (C) and ABTS (D) assays, in rats from Study 1**. These oxidative stress biomarkers were evaluated in plasma samples collected at basal conditions (0h) and following acute administration of seedless red wine pomace seasoning (RWPS) (2h and 4h). $F_2$-IsoPs: $F_2$-isoprostanes; FC: Folin-Ciocalteu; FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid. Data expressed as mean ± SEM values (n = 6). Letters indicate significant changes ($p < 0.05$) between hours pre- and post-administration.

**Fig. 2. Urine levels of $F_2$-isoprostanes (A) and nitric oxide metabolites (B) in rats from Study 1.** These biomarkers were determined in urine samples collected at basal conditions (-3–0h) and at the indicated time intervals following acute administration of seedless red wine pomace seasoning (RWPS) (0–3h and 3–6h). $F_2$-IsoPs: $F_2$-isoprostanes; Nitrites: $NO^-_2$; Nitrates: $NO^-_3$; Nitrates and Nitrites: $NO^-_3$+$NO^-_2$). Data expressed as mean ± SEM values (n = 6). Letters indicate significant changes ($p < 0.05$) between time intervals pre- and post-administration.

## Tables

**Table 1. Composition and antioxidant capacity of seedless red wine pomace seasoning (RWPS).**

| Nutrient/Compound | Concentration |
|---|---|
| **Moisture** (%) | 67.8 ± 4.3 |
| **Dietary fiber** (mg/g) | **453** ± 7 |
| **Fat** (mg/g) | 34.4 ± 0.7 |
| **Protein** (mg/g) | 134 ± 8 |
| **Ash** (mg/g) | 134 ± 3 |
| **Minerals** (mg/g) | |
| Potassium | **40.4** ± 2.4 |
| Sodium | 1.22 ± 0.10 |
| Calcium | 1.70 ± 0.10 |
| Phosphorus | 1.80 ± 0.10 |
| **Phytochemicals** | |
| Total phenolic compounds (mg GAE/g) | **24.4** ± 0.2 |
| Total anthocyanins (mg Malv-3GE/g) | 2.47 ± 0.10 |
| Total proanthocyanidins (mg P-B1E/g) | 39.6 ± 1.03 |
| Total catechins (mg D-CatE/g) | 8.78 ± 0.22 |
| **Total antioxidant capacity** [a] | |
| FRAP (μmol Fe(II)E/g) | **244** ± 5 |
| ABTS (μmol TE/g) | **103** ± 4 |
| ORAC (μmol TE/g) | 215 ± 6 |
| DPPH (μmol TE/g) | 39.4 ± 1.5 |

Concentration results expressed per gram of RWPS as mean values ± standard deviation (n=3). GAE: Gallic acid equivalents; Malv-3GE: Malvidin-3-*O*-glucoside equivalents; P-B1E: Procyanidin B1 equivalents; D-CatE: D-Catechin equivalents; Fe(II)E: Iron(II) equivalents; TE: Trolox equivalents.

*a)* Total antioxidant capacity (TAC) determined using the following assays: FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; ORAC: Oxygen radical absorbance capacity; DPPH: 2,2-Diphenyl-1-picrylhydrazyl.

568 **Table 2. Concentration of phenolic acids in plasma and urine of rats from Study 1[a], following acute administration of a red wine pomace seasoning (RWPS).**

| | PLASMA (μM) | | | | URINE (μmol/mmoL creatinine) | | | |
|---|---|---|---|---|---|---|---|---|
| **Phenolic acids** | **0h** | **2h** | **4h** | **Trend[b]** | **-3–0h** | **0–3h** | **3–6h** | **Trend[b]** |
| 4-*O*-methylgallic acid | **0.056** ± 0.031 a | **0.094** ± 0.020 b | **0.061** ± 0.029 a |  | **0.041** ± 0.032 a | **0.379** ± 0.227 a | **0.092** ± 0.041 a |  |
| Gentisic acid | **0.717** ± 0.196 a | **1.19** ± 0.24 b | **1.25** ± 0.28 ab |  | **0.044** ± 0.041 a | **0.705** ± 0.157 b | **0.584** ± 0.125 b |  |
| Caffeic acid (*trans*-) | **0.564** ± 0.141 a | **0.972** ± 0.268 b | **1.30** ±0.44 ab |  | **0.081** ± 0.073 a | **1.01** ± 0.17 b | **0.460** ± 0.183 ab |  |
| 4-Hydroxyphenylacetic acid | **3.07** ± 0.55 a | **4.95** ± 0.68 b | **5.35** ± 0.85 b |  | **1.38** ± 0.74 a | **6.60** ± 0.84 c | **4.37** ± 0.76 b |  |
| Homovallinic acid | **0.876** ± 0.249 a | **1.67** ± 0.33 b | **1.61** ± 0.32 b |  | **1.09** ± 0.36 a | **1.14** ± 0.44 a | **0.698** ± 0.233 a |  |
| Vallinic acid | **0.162** ± 0.092 a | **0.590** ± 0.217 b | **0.539** ± 0.202 b |  | **0.249** ± 0.166 a | **3.01** ± 0.50 c | **0.876** ± 0.158 b |  |
| Syringic acid | **0.464** ± 0.097 a | **1.00** ± 0.23 b | **0.765** ± 0.149 b |  | **0.144** ± 0.087 a | **1.34** ± 0.24 b | **0.501** ± 0.167 a |  |
| Isoferulic acid | **0.091** ± 0.042 a | **0.296** ± 0.109 b | **0.286** ± 0.117 b |  | **0.038** ± 0.023 a | **0.477** ± 0.152 b | **0.148** ± 0.030 b |  |
| Dihydroferulic acid | **2.02** ± 0.54 a | **5.75** ± 0.93 b | **8.59** ± 1.44 c |  | **0.421** ± 0.320 a | **4.24** ± 0.85 b | **3.50** ± 0.80 b |  |
| Protocatechuic acid | **0.526** ± 0.198 a | **1.57** ± 0.41 b | **2.21** ± 0.62 c |  | **0.048** ± 0.028 a | **0.329** ± 0.042 c | **0.197** ± 0.037 b |  |
| 3-Hydroxyphenylacetic acid | **0.376** ± 0.064 a | **0.468** ± 0.064 ab | **0.569** ± 0.100 b |  | **0.594** ± 0.189 a | **0.849** ± 0.237 b | **0.857** ± 0.241 b |  |
| Dihydro-3-coumaric acid | **0.774** ± 0.219 a | **0.707** ± 0.246 a | **1.04** ± 0.38 a |  | **0.169** ± 0.086 a | **2.58** ± 0.79 b | **1.59** ± 0.57 ab |  |
| Ferulic acid (*trans*-) | **0.452** ± 0.082 a | **0.571** ± 0.133 a | **0.508** ± 0.086 a |  | **0.176** ± 0.125 a | **1.70** ± 0.12 a | **0.620** ± 0.153 a |  |
| Homoprotocatechuic acid | **0.149** ± 0.067 a | **0.266** ± 0.082 a | **0.339** ± 0.102 a |  | **0.138** ± 0.047 a | **0.157** ± 0.062 a | **0.093** ± 0.032 a |  |

569 Results expressed as mean ± SEM values (n = 6). Letters indicate significant differences ($p < 0.05$) between either plasma or urine samples for each phenolic acid.

570 *a*) Samples were collected at the indicated hours (plasma) or time intervals (urine) pre- and post-supplementation of rats with RWPS (300 mg/kg of body weight).

571 *b)* Trends of each phenolic acid concentration along time are represented by sparklines.

**Table 3. Levels of biomarkers of oxidative stress and endothelial dysfunction in plasma and urine of rats from Study 2 [a].**

| Biological Sample | Biomarker [b] | Units [c] | C [d] | +RWPS [e] |
|---|---|---|---|---|
| Plasma | **$F_2$-IsoPs** | nM | **2.27** ± 0.16 | **2.15** ± 0.17 |
| Plasma | **FC index** | mM GAE | **9.47** ± 0.29 | **9.27** ± 0.16 |
| Plasma | **FRAP** | mM Fe(II)E | **0.874** ± 0.041 | **0.810** ± 0.030 |
| Plasma | **ABTS** | mM TE | **8.01** ± 0.19 | **7.99** ± 0.15 |
| Urine | **$F_2$-IsoPs** | nmol/mmol creatinine | **3.69** ± 0.25 | **3.63** ± 0.48 |
| Urine | **$NO^-_3$ + $NO^-_2$** | µmol/mmol creatinine | **57.9** ± 4.3 | **57.0** ± 2.0 |
| Urine | **$NO^-_3$** | µmol/mmol creatinine | **46.6** ± 3.6 | **46.4** ± 1.5 |
| Urine | **$NO^-_2$** | µmol/mmol creatinine | **11.3** ± 0.7 | **10.6** ± 0.5 |

Results expressed as mean ± SEM values (n = 5).

*a)* Biomarkers were analyzed in samples collected from 12h-fasted rats at the end of 4-week-administration of red wine pomace seasoning (RWPS).

*b)* $F_2$-IsoPs: $F_2$-isoprostanes; FC: Folin-Ciocalteu; FRAP: Ferric reducing ability of plasma; ABTS: 2,2'-Azinobis 3-ethylbenzothiazoline-6-sulfonic acid; $NO^-_2$: Nitrites; $NO^-_3$: Nitrates; $NO^-_3$+$NO^-_2$: Nitrates and Nitrites.

*c)* GAE: gallic acid equivalents; Fe(II)E: iron(II) equivalents; TE: Trolox equivalents.

*d)* C: control rats.

*e)* +RWPS: Rats supplemented with RWPS.

No significant differences ($p < 0.05$) were detected between C and +RWPS groups.

**Fig 1.** (Two-column figure)

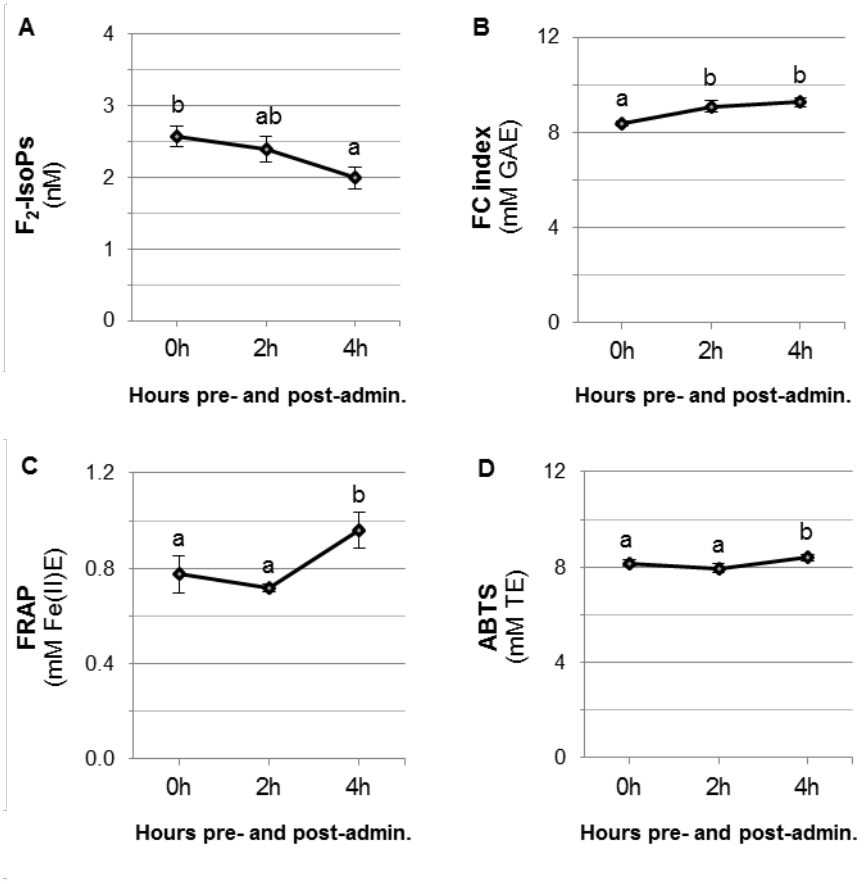

**Fig 2.** (Two-column figure)