JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simulated Digestion and Antioxidant Activity of Red Wine Fractions Separated by High Speed Countercurrent Chromatography

MARIA NOGUER,[†] ANA B. CEREZO,[†] MICHAEL RENTZSCH,[‡] PETER WINTERHALTER,[‡] ANA M. TRONCOSO,[†] AND MARIA CARMEN GARCÍA-PARRILLA*,[†]

Nutrición y Bromatología, Facultad de Farmacia, c/ P García González no. 2, Sevilla 41012, Spain, and Institut für Lebensmittelchemie, Technische Universität Braunschweig, Schleinitzstrasse 20, 38106 Braunschweig, Germany

Wine is an important source of dietary antioxidants because of its phenolic compound content. The antioxidant activity (AA) of pure monomer substances present in wines, such as phenolic acids, flavanols, and anthocyanins, has already been described, but the AA of polymeric phenols is still unknown. In this study, we have fractionated a red wine by countercurrent chromatography (CCC) into four fractions: fraction 1, made up of polymeric compounds; fraction 2, containing malvidin-3-glucoside; fraction 3, containing peonidin-3-glucoside; and fraction 4, containing vitisin A. The AA of these fractions was determined by oxygen radical absorbance capacity and ferric reducing ability assays. The weight of fraction 1 was the largest, so this was the largest contributor to the AA of the wine. However, the antioxidant powers (μM Trolox/g fraction) of fractions 2−4 were similar and higher than that of fraction 1. We also determined AA before and after in vitro gastric and intestinal digestions. After gastric digestion, the AA was 100−1000 times higher than the original fraction values. Gallic acid was determined in gastric and intestinal digested fractions. After intestinal digestion, the concentrations of simple phenols, such as caffeic acid, *p*-coumaric acid, and protocatechualdehyde, increased as they were released from the fractions under our conditions. Protocatechuic acid was determined in more intestinal digested fractions than in gastric digested fractions. These results partly explain the increase in AA after the digestion and indicate the relevance of polymeric polyphenolic compounds as precursors of smaller molecules with biological activity.

KEYWORDS: Antioxidant activity; wine; phenol; polymeric; CCC; in vitro digestion

INTRODUCTION

The relationship between diet and health has encouraged intensive research into bioactive compounds found in foods and beverages. Polyphenolic compounds have been widely determined in wines because of their effect on organoleptic properties, their differences in varieties that are useful taxonomic markers, and their changes during wine production processes. Recent interest in their biological properties (antioxidant, antiinflammatory, and antimutagenic activities) is intended to help explain the health effects of wine consumption (*1*).

Polyphenolic chemical structures are extremely diverse. The main classes are nonflavonoids (benzoic and hydroxycinnamic acids and aldehydes, their tartaric esters and derivatives, and stilbenes) and flavonoids (flavan-3-ols, anthocyanins, and flavonols). Liquid chromatography−mass spectrometry can be used to describe new polyphenolic type molecules, thus enlarging the group to hundreds of individual compounds: Current knowledge has recently been updated (*2*).

The antioxidant properties of wines depend on their polyphenolic composition, and several approaches exist to determine the relationships between the polyphenols. One is to analyze individual phenols by high-performance liquid chromatography (HPLC) coupled with diode array (DAD) or mass spectrometry detectors (*3*). The in vitro antioxidant activities (AAs) of phenolic standards and wines can also be assessed (*4*). Linear functions obtained from phenolic compound concentrations and their AAs correlate well with the AAs of white wines and sherry (*5*). In addition, the contribution of the main classes of phenolic compounds to the AAs of wine can also be estimated. As red wine age increases, polymeric fractions formed by malvidin- and peonidin-3,5-diglucosides, as well as acetyl, coumaroyl, and caffeoyl derivatives of anthocyanins, are formed. Furthermore, condensed red wine pigments formed from malvidin-3-glucoside

* To whom correspondence should be addressed. Tel: 34954556760. Fax: 34954233765. E-mail: mcparrilla@us.es.
[†] Facultad de Farmacia.
[‡] Technische Universität Braunschweig.

10.1021/jf8007376 CCC: $40.75
Published on Web 09/09/2008

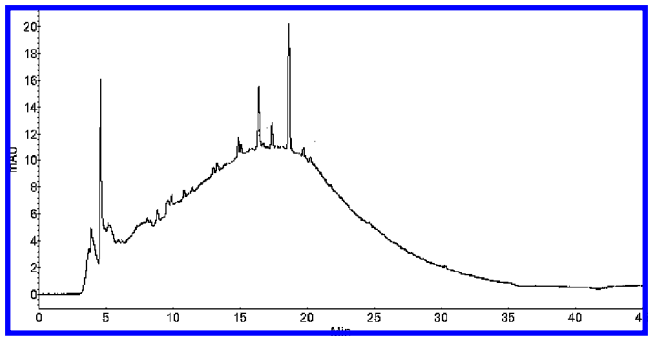

Figure 1. HPLC chromatogram profile of fraction 1.

(vitisin A and acetylvitisin A) have been isolated on a preparative scale (*6*).

The enormous complexity of red wines requires another design that takes into account not only benzoic and hydroxycinnamic phenols but also anthocyanic and polymeric fractions. Indeed, polymeric polyphenolic compounds represent 65−85% of total polyphenolic content depending on the age and origin of the wine (*7*). Solid phase extraction is a way to separate wine polyphenols into fractions and then determine their AAs. However, C-18 cartridges can retain polymeric polyphenols and leave their AAs largely unknown. Highly polymerized polyphenols can be bound to macromolecules with a molecular mass of over 12000 Da (*8*). Indeed, nonextractable polyphenols are high molecular weight proanthocyanidins, anthocyanins, and other polyphenols associated with dietary fiber and indigestible compounds (*9*).

The implications of this chemical complexity on antioxidant effects and the consequences of digestion on polymers deserve investigation. As previously stated, other biologically active molecules can be released from the complex polymers along the gastrointestinal tract.

In vitro digestion methods are useful for evaluating dietary polyphenol changes under gastric or intestinal conditions, as previously described for wine (*10*), orange juice (*11*), and chokeberry (*12*). Individual phenols were monitored, but the effect of these conditions on the polymeric fraction remains unexplored. One prerequisite for studying them is to test the polymeric compounds in isolation from others. In this paper, we overcome this prerequisite by using high speed countercurrent chromatography (CCC).

CCC is used to determine AAs directly in several wine fractions (including polymers). We chose this technique as an all-liquid chromatographic method that works without any solid stationary phase and separation is solely based on the partition of compounds between two immiscible liquid phases. The main advantages is that no irreversible adsorption occurs (*13*). Anthocyanic fractions and polymeric compounds are isolated in optimum conditions for further analysis.

In this paper, we assess the in vitro AAs of red wine polyphenolic fractions (including polymers) isolated by CCC. We also test whether AA changes when these fractions are subjected to gastrointestinal conditions and explore whether there is any release of small phenolic compounds.

MATERIALS AND METHODS

Sample. The sample under study was a 2000 vintage Monastrell variety red wine from a vineyard in Jumilla (SE Spain).

Reagents. The reagents used in the antioxidant assays were 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid (Trolox), and fluorescein (FL) from the Cayman Chemical Co., Aldrich, and Fluka, respectively, and ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) and acetic acid from Sigma. To perform CCC, we used acetonitrile, butanol, methyl-*tert*-butyl-ether (MTBE), trifluoroacetic acid (TFA), methanol, and acetic acid provided by Fluka. To perform gastric and intestinal digestions, we used sodium chloride, hydrochloric acid, sodium hydroxide, and potassium phosphate monobasic from Panreac; pancreatin (P-1500), lipase (L-3126), bile salts (B-8631), α-amylase (A-3176), and trizma maleate (T-3128) were from Sigma; amyloglucosidase (Roche 11065721) and pepsine (107190) were from Roche and Merck.

Table 1. $[M - H]^-$ and MRM Transitions of Phenolic Compounds under Study

standard	$[M - H]^-$	Q1/Q3	collision energy (CE) (V)	T_R (min)
protocatechualdehyde	137.0	137.0 → 108	−35	24.70
3-hydroxybenzoic acid	137.0	137.0 → 93.1	−20	31.63
4-hydroxybenzoic acid	137.0	137.0 → 93.1	−20	28.53
p-hydroxyphenylacetic acid	151.1	151.1 → 107.0	−25	28.73
		151.1 → 151.1	−10	
protacatechuic acid	153.1	153.1 → 109.0	−25	19.77
p-coumaric acid	163.1	163.1 → 119.0	−25	34.87
3-(4-hydroxyphenyl)-propionic acid	165.1	165.1 → 121.1	20	34.89
		165.1 → 165.1	−10	33.08
gallic acid	169.1	169.1 → 125.0	−20	12.60
caffeic acid	179.0	179.0 → 135.1	−25	30.32
homovanillic acid	181.1	181.1 → 137.0	−15	30.34
(+)-catechin	289.1	289.1 → 245.2	−20	22.22
		289.1 → 289.1	−10	

Apparatus. Spectrophotometric determinations were performed on a UV/vis U-2800 Digilab Hitachi spectrophotometer. Fluorimetric measurements were recorded with an F-2500 Hitachi fluorimeter equipped with a microcuvette (10 mm path length) provided by Hellma and connected to a device that maintained the temperature at 37 °C. A CCC-1000 high speed countercurrent chromatograph (Pharma-Tech Research Corporation, Baltimore, MD) equipped with three coils connected in series (inner diameter of tubing, 2.6 mm; total volume, 850 mL) was used. The HPLC system consisted of a System 1100 Binary Pump G1312A (Agilent, Boblingen, Germany), a Rheodyne 7725i injection valve with a 20 μL loop (Techlab, Erkerode, Germany), and a Lichrograph L-4000 UV/vis detector (Merck Hitachi, Tokyo, Japan). UV chromatograms were recorded with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan). The LC part of the system was controlled by ChemStation version A.06.01. MS data were processed by esquire NT 4.0 software (Bruker Daltonik, Bremen, Germany). HPLC analysis of digested fractions was performed on an Agilent Series 1100 system equipped with a quaternary pump (Series 1100 G1311A), automatic injector (Series 1100 G1313A), and on line degasser (Series 1100 G1379A). Detection was carried out with a UV/vis (Series 1100 G1315B) coupled to a Chemstation HP A.10.02 (HP/Agilent). HPLC-MS of digested fractions was performed using a Perkin-Elmer Series 200 HPLC system (Wellesley, United States) coupled to an Applied Biosystems QTRAP LC/MS/MS system (Foster City, United States) comprising a hybrid triple quadrupole linear ion trap (QqQ_{LIT}) mass spectrometer equipped with an electrospray ion source. Gastric and intestinal digestions were carried out in a P-Selecter Tectron Bio that kept the temperature at 37 °C.

CCC. Six liters of wine diluted with water (1:1) was loaded onto an Amberlite column [Amberlite XAD7 (Fluka) 1 kg, column dimensions: 100 cm × 7 cm] to eliminate proteins, organic acids, residual sugars, and ions. The flow rate was 1 drop/s. The Amberlite column was conditioned with 2 L of methanol and then 2 L of water. Diluted sample (1:1) was loaded and cleaned with 3 L of water and then eluted with 2 L of mixture (methanol/acetic acid, 19:1). The extract (2.5 g/L wine) was concentrated with a rotary evaporator under vacuum. This extract was fractioned with a CCC-100 high-speed countercurrent chromatograph equipped with three coils connected in series. The conditions were previously optimized (*14*). The solvent system consisted of MTBE/*n*-butanol/acetonitrile/water (2/2/1/5) acidified with 0.1% trifluoroacetic acid, with the lighter (organic) phase acting as the stationary phase and the aqueous phase acting as the mobile phase.

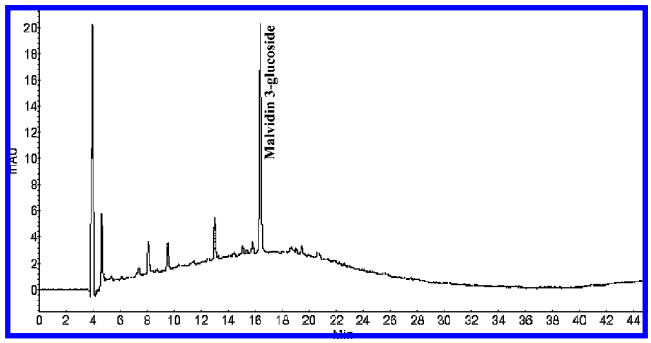

Figure 2. HPLC chromatogram profile of fraction 2.

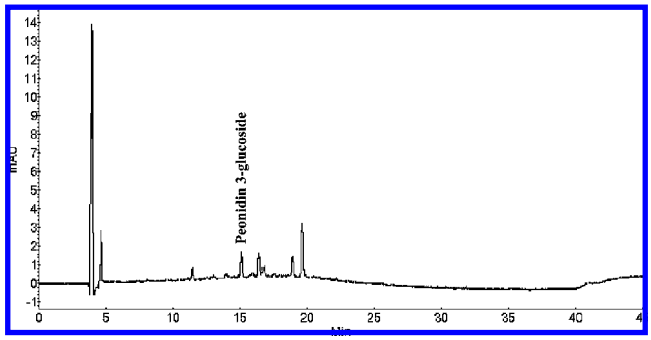

Figure 3. HPLC chromatogram profile of fraction 3.

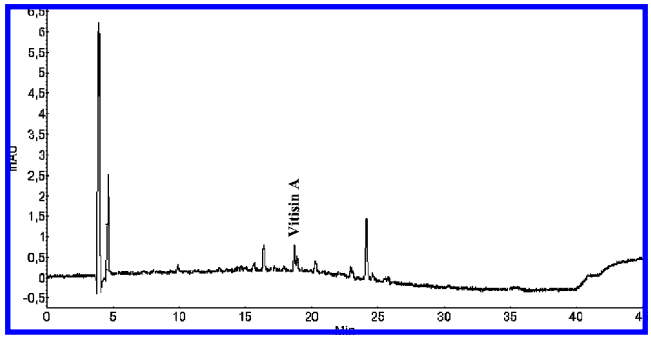

Figure 4. HPLC chromatogram profile of fraction 4.

The elution mode was head to tail with a 3 mL/min flow rate. One gram of the wine extract was dissolved in 26 mL of a mixture of the upper (organic) and lower (aqueous) phases (50:50; v/v) before injection. Four different fractions and the organic stationary phase were collected according to the profile of the chromatogram; after the evaporation of organic solvents, the fractions were freeze-dried.

HPLC/DAD/MS. Wine fractions were analyzed by HPLC/DAD/MS. Solvents and gradient conditions were as proposed by the Office International de la Vigne et du Vin (*15*). Solvents were water−formic acid−acetonitrile [solvent A (87:10:3, v/v/v) and solvent B (40:10:50, v/v/v)], and the flow rate was 0.8 mL/min. The linear gradient was from 6 to 30% B at 0−15 min; from 30 to 50% B at 15−30 min; from 50 to 60% at 30−35 min; and from 60 to 6% at 35−45 min. For DAD detection, the optimum wavelength was 510 nm. MS parameters were as follows: positive mode; capillary, −2500 V; end plate offset, −500 V; capillary exit, 70 V; skim 2, 10 V; dry gas, 325 °C; gas flow, 11 L/min; and nebulizer, 60 psi.

Gastric and Intestinal in Vitro Digestion. Gastric-simulated fluid was made according to United States Pharmacope (USP) (*15*) using 2 g of NaCl, 3.2 g of pepsin, 7.0 mL of HCl, and enough distilled water to make 1 L. This test solution had a pH of 1.2. We followed the USP (*16*) recipe to prepare intestinal simulated fluid: 6.8 g of potassium phosphate monobasic was diluted in 250 mL of water and mixed with 190 mL of 0.2 N NaOH; 400 mL of water and 10 g of pancreatin were added; the pH was adjusted to 7.5 ± 0.1; and the mixture was diluted up to 1000 mL with water. α-Amylase dilution (120 mg/mL) was made in Trizma-maleate buffer (470% w/v in distilled water). The digestion procedure (*16*), which required shaking throughout at 37 °C, was as follows: Samples (0.5 g of each fraction) were treated with 28.7 mL of gastric simulated fluid; the pH was adjusted to 4.5 ± 0.2 with 0.5 M NaOH; 0.15 mL of amyloglucosidase dilution (10 mg/mL) was added, and we waited for 30 min; the pH was adjusted to 6.9 ± 0.2 with 0.5 N NaOH, and 1.66 mL of α-amylase dilution was added; this reacted for 45 min, and then, a 2 mL aliquot was taken; after centrifugation (10 min, 3000 rpm), it was kept in the freezer (−20 °C) until further antioxidant analysis; the rest of the gastric digested sample was subjected to 28.7 mL of intestinal simulated fluid for 30 min; a solution containing lipase (0.023 g) and bile extract (0.058 g) in 3.61 mL of phosphate buffer (pH 7.5) was added; after 30 min and centrifugation (10 min, 3000 rpm), the supernatant was taken and frozen for further analysis.

Oxygen Radical Absorbance Capacity (ORAC) Method. A 150 μL amount of digested fraction, 150 μL of FL solution (2.93 mg/L), and 75 μL of AAPH (221.25 mM) were mixed in a fluorimetric cuvette, as previously described (*17*).

Ferric Reducing Ability (FRAP) Method. Three milliliters of FRAP reactive (10:1:1) acetate buffer (300 mM, pH 3.6), TPTZ (10 Mm in HCl 40 Mm), and $FeCl_3 \cdot 6H_20$ (20 mM) were used; 100 μL of problem solution and 300 μL of Milli Q water were also used. The absorbance was measured after 8 min at 593 nm. An aqueous solution of $FeSO_4 \cdot 7H_2O$ in the 0−1000 μmol/L range was used for calibration. Results are expressed as μmol/L of ferric reducing antioxidant power (FRAP value) (*18*).

Chromatographic Analysis of Digested Fractions. The column was a Merck LiChroCART (250 mm × 4 mm) Superspher 100 RP-18 (5 μm) protected by a Merck LiChroCART 4-4 guard cartridge. The syringe filters were Millex-GV_{13} (0.22 μm) SLGV T13 NL. The chromatographic conditions are described (*5*) elsewhere. The method uses a binary gradient: A (glacial acetic acid/water pH 2.65), B (20% A + 80% acetonitrile) programmed in a gradient as follows: 0 min (100% A); 5 min (98% A + 2% B); 10 min (96% A + 4% B); 15 min (90% A + 10% B); 30 min (80% A + 20% B); 35 min (70% A + 30% B); 40 min (100% B); 45 min (100% A); and 60 min (100% A). The flow rate was 1.5 mL/min, and the temperature was set at 40 °C. The injection volume was 50 μL.

The photodiode array was programmed to record data from 240 to 450 nm. Unknown spectra were identified by comparing them with those of pure standards and were quantified by external calibration. Analyses were carried out in triplicate.

HPLC/MS analyses were performed on a 250 mm × 4.6 mm Zorbax SB-C18 reversed-phase column with a particle size of 3.5 μm (Agilent). The flow rate was 0.4 mL min^{-1}. Chromatographic separation was performed using a binary gradient consisting of (A) water and (B) methanol. Both components contained 0.1% formic acid (v/v). The elution profile was 20% B (2 min), and a linear gradient was as follows: 20−21−30−37−39.5−39.6− 45 min, 35−40−60−80−85−100% of B, followed by 5 min of re-equilibration of the column before the next run (*19*). The concentration for phenolic compound standards was 10 μg/mL in 0.1% formic acid, 50% methanol, and diluted with water.

The injection volume was 20 μL. A multiple reaction monitoring (MRM) experiment was applied in which the parent ions and fragmented ions were monitored at Q1 and Q3, respectively. For HPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas, 35 psi; ion spray voltage, −4500 V; source temperature, 350 °C; source gas, 20 psi; declustering potential, −60 V; and entrance potential, −10 V. MRM transitions were performed with the parameters shown in **Table 1**. A dwell time of 100 ms was set for each transition.

RESULTS AND DISCUSSION

Separation of Phenolic Fractions. The wine extract obtained as described in the Materials and Methods was fractionated by CCC. Three separations, each of 1 g of extract, were performed. Four fractions were obtained. The weights (mg/g extract) of the polyphenolic fractions obtained by CCC were in the following ranges: fraction 1, from 469.5 to 609.3 mg/g extract; fraction 2, from 62.1 to 90.73 mg/g

Table 2. AA of Red Wine Phenolic Fractions before and after Digestion Expressed as μM Trolox/g Fraction

	FRAP			ORAC		
samples	before digestion (mM Trolox/ g fraction)	after gastric digestion (mM Trolox/g fraction)	after pancreatic digestion (mM Trolox/g fraction)	before digestion (mM Trolox/g fraction)	after gastric digestion (mM Trolox/g fraction)	after pancreatic digestion (mM Trolox/g fraction)
(F11) fraction 1 1st injection	2.97 ± 0.08	134.7 ± 1.4	56.8 ± 1.4	26.3 ± 0.9	931.7 ± 42.6	145.6 ± 7.4
(F12) fraction 1 2nd injection	1.97 ± 0.06	134.7 ± 1.4	56.8 ± 1.4	12.53 ± 0.00	1234.5 ± 24.1	1755.3 ± 1.5
(F13) fraction 1 3rd injection	2.59 ± 0.05	127.9 ± 0.7	53.4 ± 7.6	6.33 ± 0.05	869.9 ± 18.0	906.5 ± 33.7
(F21) fraction 2 1st injection	6.00 ± 0.00	1050.0 ± 3.1	584.4 ± 4.7	9.00 ± 1.20	8082.0 ± 128.0	4144.5 ± 295.5
(F22) fraction 2 2nd injection	3.30 ± 0,11	860.0 ± 17.3	577.8 ± 1.6	6.60 ± 0.30	8301.4 ± 879.4	6235.1 ± 324.0
(F23) fraction 2 3rd injection	3.22 ± 0.16	1021.1 ± 6.3	500.0 ± 4.7	4.80 ± 0.50	7080.8 ± 46.7	26804.9 ± 206.1
(F31) fraction 3 1st injection	3.90 ± 0.00	1319.4 ± 62.9	527.8 ± 19.6	48.83 ± 0.19	1600.5 ± 281.2	2251.8 ± 195.3
(F32) fraction 3 2nd injection	6.51 ± 0.00	1191.7 ± 70.7	591.7 ± 39.3	3.30 ± 1.30	3296.1 ± 638.4	31726.4 ± 335.5
(F33) fraction 3 3rd injection	2.61 ± 0.00	1155.6 ± 11.8	641.7 ± 7.9	26.10 ± 1.00	6887.8 ± 125.3	45795.1 ± 321.0
(F41) fraction 4 1st injection	4.20 ± 0.00	1942.6 ± 17.0	675.0 ± 66.8	46.60 ± 8.50	20833.3 ± 771.1	18608.9 ± 230.9
(F42) fraction 4 2nd injection	3.70 ± 0.00	1727.8 ± 29.4	633.3 ± 23.6	26.00 ± 1.40	3969.8 ± 107.8	11351.7 ± 576.1
(F43) fraction 4 3rd injection	12.19 ± 0.00	1350.0 ± 94.3	727.8 ± 15.7	6.10 ± 0.60	38039.2 ± 154.8	39039.1 ± 203.6

Table 3. AA of Red Wine Phenolic Fractions before and after Gastric and Intestinal Digestion Determined by FRAP and ORAC Methods Expressed as mM Trolox/g Extract[a]

	FRAP			ORAC		
samples	before digestion (mM Trolox/ g extract)	after gastric digestion (mM Trolox/g extract)	after pancreatic digestion (mM Trolox/g extract)	before digestion (mM Trolox/g extract)	after gastric digestion (mM Trolox/g extract)	after pancreatic digestion (mM Trolox/g extract)
(F11) fraction 1 1st injection	1.40 ± 0.04	63.3 ± 0.6	26.6 ± 0.6	13.3 ± 0.4	439.5 ± 20.1	68.7 ± 3.5
(F12) fraction 1 2nd injection	1.20 ± 0.04	100.9 ± 1.4	49.7 ± 1.04	7.64 ± 0.00	857.3 ± 14.7	1070.3 ± 0.9
(F13) fraction 1 3rd injection	1.80 ± 0.04	88.3 ± 0.4	36.8 ± 5.2	4.40 ± 0.04	604.1 ± 12.5	629.5 ± 23.4
(F21) fraction 2 1st injection	0.40 ± 0.00	69.3 ± 0.6	38.8 ± 0.3	0.60 ± 0.08	538.8 ± 85.4	276.3 ± 19.7
(F22) fraction 2 2nd injection	0.30 ± 0.01	76.9 ± 0.1	52.4 ± 0.1	0.60 ± 0.03	753.3 ± 79.8	565.8 ± 29.4
(F23) fraction 2 3rd injection	0.20 ± 0.01	63.4 ± 0.4	31.5 ± 0.3	0.30 ± 0.03	439.8 ± 2.9	1664.9 ± 12.8
(F31) fraction 3 1st injection	0.20 ± 0.00	70.1 ± 0.4	27.0 ± 1.0	2.50 ± 0.01	82.0 ± 14.4	115.3 ± 10.0
(F32) fraction 3 2nd injection	0.20 ± 0.00	36.6 ± 2.1	18.1 ± 1.2	0.10 ± 0.04	101.2 ± 19.6	974.1 ± 10.3
(F33) fraction 3 3rd injection	0.10 ± 0.00	44.3 ± 0.4	24.9 ± 0.6	1.00 ± 0.04	263.9 ± 4.8	1754.6 ± 12.3
(F41) fraction 4 1st injection	0.10 ± 0.00	45.8 ± 0.4	16.5 ± 2.4	1.1 ± 0.2	491.7 ± 18.2	439.2 ± 54.5
(F42) fraction 4 2nd injection	0.10 ± 0.00	46.4 ± 0.7	17.0 ± 0.6	0.70 ± 0.04	106.8 ± 2.9	305.4 ± 15.5
(F43) fraction 4 3rd injection	0.20 ± 0.00	22.1 ± 1.5	11.7 ± 0.2	0.10 ± 0.01	623.9 ± 25.4	640.3 ± 33.4

[a] Codes in the samples F (fraction), number of fraction, and number of injection replicates.

extract; fraction 3, from 30.7 to 51.23 mg/g extract; and fraction 4, from 16.4 to 26.93 mg/g extract. Wine fractions were analyzed by HPLC/DAD/MS (*14, 15*).

Fraction 1 consisted mostly of a colored hump. A similar fraction, referred to as polymeric pigments, was earlier obtained after the CCC separation of wine (**Figure 1**). Fraction 2 presented malvidin 3-glucoside (m/z = 493) as the main identified component (**Figure 2**). Fraction 3 contained peonidin 3-glucoside (m/z = 463) below the quantification limit (**Figure 3**). Fraction 4 contained vitisin A (m/z = 561) (**Figure 4**).

Fraction 1, corresponding to polymer compounds, was the most abundant. The wine had been aged for 7 years, and the concentrations of total anthocyanins and monomers were therefore lower than those previously described for young wines (*20*). This was expected because of the polymerization reactions. Malvidin 3-glucoside is the most abundant anthocyanin in many varieties, and its levels in Monastrell grapes are 2401.5−3229.9 μg g^{-1} (*21*). Monastrell grapes have a high percentage of other nonacylated anthocyanins such as malvidin 3-glucoside, which was identified in our analysis (*21*). Monastrell grapes from Jumilla have high levels of total anthocyanins (8.66 mg/g skin), while those of others such as Syrah, Merlot, or Cabernet Sauvignon are 4.78−8.20 mg/g skin. Previous studies (*22*) have shown that the other concentrations in Monastrell wine after 9 months of aging are malvidin 3-glucoside (70.69 mg/L), peonidin 3-glucoside (10.17 mg/L), petunidin-3-glucoside (9.94 mg/L), delphinidin-3-glucoside (6.16 mg/L), and cyanidin-3-glucoside (3.04 mg/L). In this study, using a wine aged for 7 years, we detected malvidin 3-glucoside and peonidin 3-glucoside.

AAs of Polyphenolic Fractions: In Vitro Digestion Effects. Simulated digestion solutions without the CCC fractions were assessed as controls. The AAs of controls after the digestion procedure had the following values: gastric digestion, 0.02 ± 0.00 mM Trolox (FRAP value) and 2.5 ± 0.3 mM Trolox (ORAC value); intestinal digestion, 0.02 ± 0.00 mM Trolox (FRAP value) and 5.90 ± 0.22 mM Trolox (ORAC value). Fractions 2−4 had similar AAs in terms of weights of fractions (**Table 2**). If we take into account the weights of fractions in **Table 3**, the contribution of the polymers in fraction 1 to the overall AA of wine is the largest. The AA of fractions was determined before and after gastric and pancreatic digestions. Remarkably higher values were found after gastric digestion (100−1000 times original fraction values). These data agree with those reported in ref *8*, where the authors subjected several foods (cereals, vegetables, legumes, and fruits) to a similar in vitro physiological procedure and verified that after this simulated digestion the AA was higher than the initial AA obtained after the antioxidants had been extracted from a food matrix with chemical solvents.

Our results for intestinal treatment depended on the method used to assess AAs. The results of the FRAP method were similar for all fractions: After intestinal digestion, the AA

Table 4. Phenolic Compounds Released from Each Fraction after Gastric and Intestinal Digestion (mg/g)[a]

	gallic acid	*p*-coumaric acid	caffeic acid	protocatechualdehyde	protocatechuic acid
F11G	0.03 ± 0.00	−	−	−	+
F12G	−	−	−	−	+
F21G	4.05 ± 0.07	+	+	+	+
F22G	2.02 ± 0.07	+	+	+	+
F23G	3.49 ± 0.04	+	+	+	+
F31G	−	−	−	−	−
F32G	−	−	−	−	−
F33G	−	−	−	−	−
F41G	−	−	−	−	−
F42G	−	0.02 ± 0.00	−	−	−
F43G	−	0.02 ± 0.00	−	−	−
F11I	−	−	−	−	−
F12I	+	+	−	+	+
F13I	−	−	−	−	+
F21I	3.54 ± 0.03	+	+	+	+
F22I	0.38 ± 0.01	+	+	+	+
F23I	1.97 ± 0.03	+	+	+	+
F31I	+	1.71 ± 0.01	+	1.00 ± 0.06	+
F32I	+	3.38 ± 0.10	1.24 ± 0.00	2.10 ± 0.02	+
F33I	+	+	1.06 ± 0.00	1.49 ± 0.02	+
F41I	0.09 ± 0.00	+	0.28 ± 0.01	+	+
F42I	0.26 ± 0.00	1.96 ± 0.00	0.18 ± 0.00	+	+
F43I	+	+	−	+	+

[a] −, Not detected; +, detected under quantification limits. Codes in the samples F (fraction), number of fraction, and number of injection replicate G (after gastic digestion) I (after intestinal digestion).

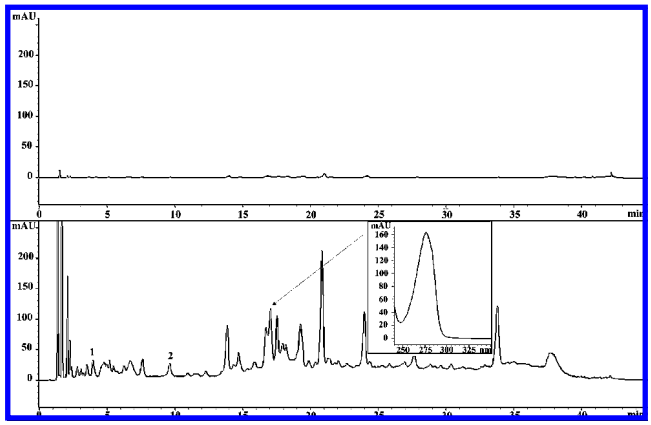

Figure 5. HPLC profile recorded for fraction 3 and 1st injection at 280 nm after gastric (upper chromatogram) and intestinal digestion (chromatogram at the bottom). Peaks: 1, gallic acid; 2, protocatechualdehyde. A DAD spectrum for unknown peak similar to 3-(4-hydroxyphenyl)propionic acid is displayed.

decreased to 50% of the values obtained after gastric digestion. This result agrees with the dilution effect after addition of the intestinal medium. On the other hand, after intestinal digestion, the ORAC values were considerably higher than the initial ones, although no clear conclusion can be drawn.

Because three preparative fractionations with HSCCC have been carried out, slight changes in the composition of the combined fraction are obtained that are likely to explain the differences observed in the AA. In **Table 2**, for example, the three injections for fraction 2 yielded very similar results after intestinal digestion determined by the FRAP method, similar results after gastric digestion determined by the ORAC method, and completely different results after intestinal digestion determined by the ORAC method.

Simulated digestions were previously applied to determine the stability of the phenols along the gastrointestinal tract. After

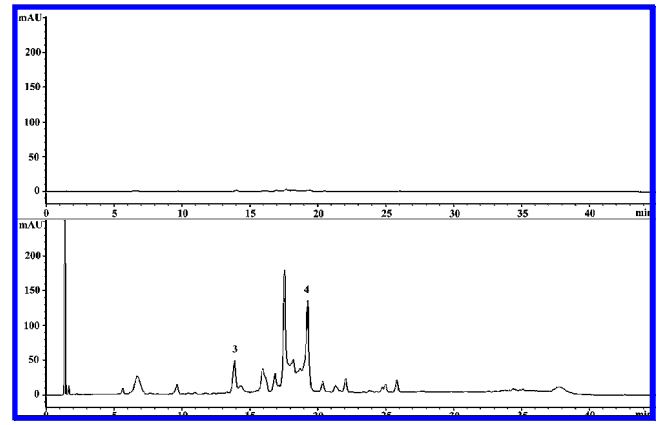

Figure 6. HPLC profile recorded for fraction 3 and 1st injection at 320 nm after gastric (upper chromatogram) and intestinal digestion (chromatogram at the bottom). Peaks: 3, caffeic acid; 4, *p*-coumaric acid.

gastric digestion, the concentration of anthocyanins, flavanols, and phenolic acids did not change significantly (*12*). On the other hand, data after intestinal digestion show that the concentration of anthocyanins, flavanols, and phenolic acid decreased (*12, 23*). Explanations for this behavior take into account the effect of pH 7 (intestinal conditions) on phenols' stability. The interaction between digestive proteins and monomers [(+)-catechin and (−)-epicatechin] and dimers (procyanidins B2 and B3) from grape seed extract during pancreatic digestion also may explain their disappearance after intestinal incubation (*24*). Our experimental design is intended to explore the role of polyphenols with higher degrees of polymerization. These are more likely to interact with proteins (such as enzymes in our experiment) than monomeric phenols (*25*).

Using HPLC-DAD and HPLC-MS, we analyzed the digested fractions to determine whether this chemical and enzymatic digestion could decompose polymers into small phenolic acids. **Table 4** summarizes the amount of phenolic compounds expressed in mg/g of fraction after gastric and intestinal digestions. We can see that each fraction yields different substances as breakdown products. After gastric digestion, we could assess gallic acid above quantification limits in fractions 1 and 2 and *p*-coumaric acid in fraction 4. A mass detector confirmed the occurrence of *p*-coumaric acid, caffeic acid, protocatechualdehyde, and protocatechuic acid, but these were below the quantification limit. On the other hand, intestinal digestion led to the release of these substances in sufficient quantity to be quantified by DAD (**Figures 5** and **6**). The phenolic acid AAs have been reported previously (*4*). The concentration of gallic acid (fraction 2) decreased as expected due to a pH effect. We identified one peak as 4-hydroxy-3-propionic acid from the retention time and from perfect matching with the standard DAD spectrum (**Figure 5**). On the other hand, a peak with identical $[M - H]^-$ and MRM transition appears at 29 min, which is 5 min earlier in the chromatogram profile. This merits further research since it is released in larger amounts in fractions 1 and 2 where polymers are most abundant. Colonic flora degrade flavonoids and other polyphenols in a wide variety of simple phenylpropanoic acids (*26*). Our study shows that small phenolic acids can also be released just after chemical and enzymatic treatments, thus contributing to the overall effect of wine intake.

The release of protocatechualdehyde in fraction 3 is also interesting since this compound presents biological activity. Recently, it was shown to reduce advance glycation products formed in diabetic human lens epithelial cells (*27*). These results

reinforce the idea that to evaluate the health effects of a food, it is necessary to assess compounds formed during digestion that are not necessarily present in the food itself. In conclusion, gastric and intestinal digestions do affect the polymeric fractions of red wine by increasing their AAs with regard to the initial values and releasing phenolic compounds with small molecular weights.

ABBREVIATIONS USED

AA, antioxidant activity; CCC, countercurrent chromatography; FRAP, ferric reducing ability; ORAC, oxygen radical absorbance capacity; FL, fluorescein; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; TPTZ, 2,4,6-tripyridyl-*s*-triazine; AAPH, 2,2′-diazobis amidine propane dihydrochloride; USP, United States Pharmacope.

ACKNOWLEDGMENT

We also thank the Mass Spectrometry Facility of the University of Seville for the QTRAP instrument. We are extremely grateful to Dr. M. E. Soria-Díaz for her effective assistance with HPLC/MSMS analysis.

LITERATURE CITED

(1) Rivero-Perez, M. D.; Muñiz, P.; González-Sanjosé, M. L. Antioxidant profile of red wines evaluated by total antioxidant capacity, scavenger activity, and biomarkers of oxidative stress methodologies. *J. Agric. Food Chem.* **2007**, *55*, 5476–5483.

(2) Monagas, G.; Bartolomé, B.; Gómez-Cordovés, C. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 85–118.

(3) Landrault, N.; Poucheret, P.; Ravel, P.; Gasc, F.; Cros, G.; Teissedre, P. L. Antioxidant capacities and phenolics levels of French wines from different varieties and vintages. *J. Agric. Food Chem.* **2001**, *49*, 3341–3348.

(4) Villano, D.; Fernandez-Pachon, M. S.; Troncoso, A. M.; Garcia-Parrilla, M. C. Comparison of antioxidant activity of wine phenolic compounds and metabolites in vitro. *Anal. Chim. Acta* **2005**, *538*, 391–398.

(5) Fernandez-Pachón, M. S.; Villaño, D.; Troncoso, A. M.; García-Parrilla, M. C. Determination of the phenolic composition of sherry and table white wines by liquid chromatography and their relation with antioxidant activity. *Anal. Chim. Acta* **2006**, *563*, 101–108.

(6) Degenhardt, A.; Hofmann, S.; Knapp, H.; Winterhalter, P. Preparative isolation of anthocyanins by high-speed countercurrent chromatography and application of the color activity concept to red wine. *J. Agric. Food Chem.* **2000**, *48*, 5812–5818.

(7) De Beer, D.; Harbertson, J. F.; Kilmartin, P. A.; Roginsky, V.; Barsukova, T.; Adams, D. O.; Waterhouse, A. L. Phenolics: A comparison of diverse analytical methods. *Am. J. Enol. Vitic.* **2004**, *55*, 389–400.

(8) Saura-Calixto, F.; Serrano, J.; Goñi, I. Intake and bioaccessibility of total polyphenols in a whole diet. *Food Chem.* **2007**, *101*, 492–501.

(9) Saura-Calixto, F.; Diaz-Rubio, M. E. Polyphenols associated with dietary fiber in wine: A wine polyphenols gap. *Food Res. Int.* **2007**, *40*, 613–619.

(10) Martínez-Ortega, M. V.; García-Parrilla, M. C.; Troncoso, A. M. Changes in phenolic composition of wines submitted to in vitro dissolution tests. *Food Chem.* **2001**, *73*, 11–16.

(11) Gil-Izquierdo, A.; Gil, M. I.; Ferreres, F.; Tomas-Barberan, F. A. In vitro availability of flavonoids and other phenolics in orange juice. *J. Agric. Food Chem.* **2001**, *49*, 1035–1041.

(12) Bermúdez-Soto, M. J.; Tomas-Barberán, F. J.; García-Conesa, M. T. Stability of polyphenols in chokeberry (*Aronia melocarpa*) subjected to in vitro gastric and pancreatic digestion. *Food Chem.* **2007**, *102*, 865–874.

(13) Winterhalter, P. Application of countercurrent chromatography (CCC) to the analysis of natural pigments. *Trends Food Sci. Technol.* **2007**, *18*, 507–513.

(14) Salas, E.; Dueñas, M.; Schwarz, M.; Winterhalter, P.; Cheynier, V.; Fulcrand, H. Characterization of pigments from different high speed countercurrent chromatography wine fractions. *J. Agric. Food Chem.* **2005**, *53*, 4536–4546.

(15) Office International de la Vigne et du Vin. HPLC determination of nine major anthocyanins in red and rosé wine. Resolution OENO 22/2003.

(16) United States Pharmacopeia (USP 23), The National Formulary (NF 18) United States Pharmacopeial Convention, 1995.

(17) Fernandez-Pachon, M. S.; Villano, D.; Garcia-Parrilla, M. C.; Troncoso, A. M. Antioxidant activity of wines and relation with their polyphenolic composition. *Anal. Chim. Acta* **2004**, *513*, 113–118.

(18) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

(19) Gu, L.; House, S. E.; Rooney, L.; Prior, R. L. Sorghum bran in the diet dose dependently increased the excretion of catechins and microbial-derived phenolic acids in female rats. *J. Agric. Food Chem.* **2007**, *55*, 5326–5334.

(20) Salas, E.; Dueñas, M.; Schwarz, M.; Winterhalter, P.; Cheynier, V.; Fulcrand, H. Characterization of pigments from different high speed countercurrent chromatography wine fractions. *J. Agric. Food Chem.* **2005**, *53*, 4536–4546.

(21) Ortega-Regulés, A.; Romero-Cascales, I.; Ros-García, J. M.; Gómez-Plaza, E. Anthocyanin fingerprint of grapes: environmental and genetic variations. *J. Sci. Food Agric.* **2006**, *86*, 1460–1467.

(22) Lorenzo, C.; Pardo, F.; Zalacaín, A.; Alonso, G. L.; Salinas, M. R. Effect of red grapes co-winemaking in polyphenols and color of wines. *J. Agric. Food Chem.* **2005**, *53*, 7609–7616.

(23) McDougall, G. J.; Fyffe, S.; Dobson, P.; Stewart, D. Anthocyanins from red cabbage—Stability to simulated gastrointestinal digestion. *Phytochemistry* **2007**, *68*, 1285–1294.

(24) Laurent, C.; Besançon, P.; Caporiccio, B. Flavonoids from a grape seed extract interact with digestive secretions and intestinal cells as assessed in an in vitro digestion/Caco-2 cell culture model. *Food Chem.* **2007**, *100*, 1704–1712.

(25) De Freitas, V.; Mateus, N. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* **2001**, *49*, 940–945.

(26) Russell, W. R.; Labat, A.; Scobbie, L.; Duncan, S. H. Availability of blueberry phenolics for microbial metabolism in the colon and the potential inflammatory implications. *Mol. Nutr. Food Res.* **2007**, *51*, 726–731.

(27) Kim, Y. S.; Kim, N. H.; Lee, S. W.; Lee, Y. M.; Jang, D. S.; Kim, J. S. Effect of protocatechualdehyde on receptor for advanced glycation end products and TGF-β1 expression in human lens epithelial cells cultured under diabetic conditions and on lens opacity in streptozotocin-diabetic rats. *Eur. J. Pharmacol.* **2007**, *569* (3), 171–179.

Received for review March 10, 2008. Revised manuscript received July 25, 2008. Accepted July 31, 2008. We thank the Spanish Government (Ministry of Education and Science) for financial support (Research Project 2007-64622) and for a research fellowship.

JF8007376