JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Effect of Pressurized Hot Water Extraction on Antioxidants from Grape Pomace before and after Enological Fermentation

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ABSTRACT: Grape pomace was extracted with pressurized hot water at laboratory scale before and after fermentation to explore the effects of fermentation and extraction temperature (50−200 °C) and time (5 and 30 min) on total extracted antioxidant levels and activity and to determine the content and recovery efficiency of main grape polyphenols, anthocyanins, and tannins. Fermented pomace yielded more total antioxidants (TAs), antioxidant activity, and tannins, than unfermented pomace but fewer anthocyanins. Elevating the extraction temperature increased TA extraction and antioxidant activity. Maximum anthocyanin extraction yields were achieved at 100 °C and at 150 °C for tannins and tannin−anthocyanin adducts. Using higher temperatures and longer extraction times resulted in a sharp decrease of polyphenol extraction yield. Relevant proanthocyanidin amounts were extracted only at 50 and 100 °C. Finally, TA recovery and activity were not directly related to the main polyphenol content when performing pressurized hot water grape pomace extraction.

KEYWORDS: *pressurized hot water extraction, grape pomace, Vitis vinifera, polyphenols, thermal degradation*

INTRODUCTION

Grapes (*Vitis* spp.) are one of the largest fruit crops in the world[1] and are among the highest antioxidant-containing fruits.[2] In 2009, world grape production reached approximately 66.9 million tons, of which 71% corresponded to grapes for winemaking. Consequently, grape byproducts are produced in massive quantities, especially by the winemaking industry. Pomace, a winery byproduct that consists of skins, seeds, and stems remaining after enological fermentation, represents 20% of grapes by weight.[3] Pomace is currently used as a crop fertilizer although with limited success because of its inhibitory effect on plant seed germination due to the high content of polyphenolics.[4] However, the latter contains anthocyanins and condensed tannins (including pigmented polymers and non-pigmented proanthocyanidins), which are high valuable compounds that could significantly benefit health[5] and sensory quality of wine.[6]

Anthocyanins from grape skins are protective against diverse potentially damaging cellular oxidants through different biological mechanisms.[7] Anthocyanins are also used as natural food colorants.[8] Additionally, condensed tannins (proanthocyanidins) are one of the most abundant polyphenols in grapes.[9] Many pharmacological and therapeutic features of grape products such as antioxidant, anti-inflammatory, and antimicrobial activities, as well as cardio-, hepato-, and neuroprotective properties have been primarily attributed to grape tannins content.[10] Moreover, the antioxidant action of low molecular weight polyphenols has been recently questioned because of their low bioavailability.[11] Proanthocyanidins, which are not absorbed and remain in the gut due to their polymeric nature, may have direct effects on the stomach[12] and intestinal mucosa, protecting these tissues from oxidative stress or carcinogen action.[13] Therefore, even though most of the health benefits of wine have been attributed to polyphenols, it is not known how much of the grapes' original polyphenolic content remains in the pomace after enological fermentation. It is known, however, that fermentation favors the breakdown of the cell walls in grapes tissues.[14] Hence, significant amounts of valuable bioactive phenolic compounds could be recovered by applying a clean and effective extraction process after fermentation.

Organic solvents are commonly used to efficiently extract polyphenols from raw plant materials on a large scale.[15] These processes are not environmentally friendly, however, because it is difficult to eliminate all solvent traces from the resulting extracts. In addition, organic solvents substantially increase extraction process costs.

Water is a nonflammable, nontoxic, and readily available solvent. It is safer, cheaper, and more environmentally friendly than organic solvents for grape pomace extraction. Moreover, it is possible to manipulate water's solvent properties to optimize phytochemical extraction by changing the temperature.[16] This involves raising the water temperature to between 100 and 374 °C while applying sufficient pressure to maintain water in a liquid state (i.e., pressurized hot water). Water polarity declines dramatically with increasing temperature due to hydrogen bond dissolution and reaches values comparable to organic solvent−water mixtures.[16] The lower viscosity and surface tension of hot water also increase mass transfer rates of compounds from the

Received: March 5, 2013
Revised: June 3, 2013
Accepted: June 24, 2013
Published: June 24, 2013

dx.doi.org/10.1021/jf4010143 | J. Agric. Food Chem. 2013, 61, 6929−6936

plant tissue matrix.[16] Both temperature and pressure play significant roles in disrupting water surface equilibrium, thereby lowering the activation energy required for desorption processes.[17] This molecular behavior underscores the basis for using pressurized hot water to replace organic solvents in phytochemicals extraction processes.[16] Furthermore, pressurized hot water extraction (PHWE) is a straightforward scalable process from data gathered in small-scale equipments.[18] Despite several pressurized extraction system designs proposed by researchers[18] and specialized companies,[19] most industrial-scale units are proprietary and therefore detailed information regarding their design and operation are not available in the open literature.[18]

Several studies on PHWE of polyphenols from winery byproducts have been reported. Garcia-Marino et al.[9] studied catechin and proanthocyanidin recovery from grape seeds obtained as winery byproducts, using PHWE in a temperature range of 50−150 °C (at 102 atm) for 30 min. Using pressurized hot water, 2-fold more catechin and epicatechin was recovered versus conventional methanol extraction processes. Aliakbarian et al.[20] studied the effects of different extraction temperatures (100, 120, and 140 °C) and pressures (79, 113, and 148 atm) on total polyphenol and flavonoid recovery, and the radical scavenging capacity, of grape pomace extracts. PHWE was more efficient than a hydroalcoholic mixture at atmospheric pressure for extracting these compounds. However, in these and other similar studies,[21,22] no comparison was made of the polyphenol recovery amount and activity in extracts derived from grape pomace before versus after fermentation. Additionally, previous studies paid little or no attention to the effect of extraction time. During the PHWE of polyphenols from plant materials, diverse phenomena occur including thermal degradation, selective polyphenol extraction, and formation of neo-antioxidant compounds, all of which are highly dependent on extraction temperature and duration.[23,24] Depending on the PHWE conditions used, it is possible to obtain extracts with different chemical compositions and activities and, consequently, different bioactive properties.

In this work, we characterized the polyphenolic content of extractable grape pomace, before and after fermentation, as a preliminary step in determining the content and types of bioactive compounds remaining in this abundant byproduct. We also evaluated the impact of extraction conditions on extract antioxidant activity and on the recovery of total antioxidants (TAs), major polyphenols, anthocyanins, and condensed tannins.

MATERIALS AND METHODS

Chemicals. Reagents and standards used were analytical grade. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, and sodium carbonate were purchased from Merck (Germany). Tripyridyl triazine (TPTZ), $FeCl_3(6H_2O)$, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,5-dihydroxybenzoic acid, ascorbic acid, gallic acid, maleic acid−sodium dodecyl sulfate, triethanolamine, iron(III) chloride, bovine serum albumin, sodium hydroxide, hydrochloric acid (37%), glacial acetic acid, and sodium chloride were obtained from Sigma (MO, USA).

Grape Pomace. Cabernet Sauvignon pomace was obtained from Carmen Vineyard, Region Metropolitana, Chile. The prefermentation process was performed at 18 °C for 10 days, and the must was loaded into a 10 m^3 fermentation tank. Fermentation was conducted between 25 and 30 °C for 21 days without pectolytic enzymes. Two samples of the same pomace were taken at different stages of the winemaking process. The first sample was taken at the beginning of the winemaking process just after the must was introduced into the fermentation tank (unfermented pomace). The second sample was taken after the fermentation process had finished (fermented pomace). The samples were dried at ambient temperature for 2 days to reach equilibrium humidity (10% w/w) in a drying cabinet with forced ventilation. Each sample was reduced to a particle size lower than 1 mm diameter by an Oster blender (Sunbeam Products, Inc., Boca Raton, FL) and was frozen to −20 °C until extraction.

Pressurized Hot Water Extraction (PHWE). Fermented and unfermented grape pomace were subjected to PHWE. A 5 g sample (dry weight) of grape pomace was mixed with 100 g of quartz sand to completely fill the 100 mL stainless steel extraction cell and avoid filter clogging. The grape pomace was extracted in an accelerated solvent extraction device (ASE 150, Dionex) with 50 mL of distilled and filtrated (0.22 μm) water to obtain a matrix/extractant ratio of 1:10. A full factorial design with two factors was performed in triplicate at 102 atm. The factors assessed were extraction temperature (50, 100, 150, and 200 °C) and extraction time (5 and 30 min); these values were selected based on previous studies.[24] After extraction, the cell contents were rinsed with 100 mL of distilled and filtrated (0.22 μm) water and purged for 360 s by applying pressurized nitrogen (10.2 atm). Finally, the collected extracts were freeze-dried and stored in amber vials at −20 °C until analysis. Extract solutions of 1 g/L were prepared for analysis.

DPPH Radical Scavenging Activity Determination. Pomace extract antiradical capacity was determined using the DPPH radical-scavenging method.[25] First, 50 μL volumes of extract solutions at different concentrations were mixed with 2 mL of DPPH working solution (50 μM). Bleaching of DPPH was measured at 516 nm (DR 2000 Spectrophotometer; Hach Company, Loveland, CO) until the absorbance remained unchanged (∼30 min) in the dark and at room temperature. The effective pomace extract concentration needed to inhibit 50% of DPPH radical absorption (IC_{50}; mg/L) was calculated. The extract antioxidant capacity was compared with Trolox, using the Trolox equivalent antioxidant capacity (TEAC) equation: TEAC = IC_{50} Trolox/IC_{50} sample.[26] DPPH values were expressed as mg of Trolox equivalent (TE) per gram of dry mass of pomace (dp).

Ferric-Reducing Antioxidant Power (FRAP) Determination. The FRAP test offers a putative index of antioxidant reducing capacity in a sample.[27] A working solution was prepared by mixing 300 mM of acetate buffer (pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and a freshly prepared 20 mM $FeCl_3(6H_2O)$ solution in 10:1:1 (v/v/v) proportion. For the assay, 3 mL of working reagent was mixed with 100 μL of sample or calibration standard (ascorbic acid), and absorbance was measured at 593 nm after a 30 min reaction time.[28] A calibration curve was constructed using ascorbic acid (0.1−0.8 mM). The regression coefficient of ascorbic acid was 0.9989. Results were expressed as ascorbic acid equivalent (AAE) per gram of dp.

Total Antioxidant (TA) Determination by Folin Assay. Total antioxidants were determined by Folin assay. Although this method is commonly considered for polyphenol analysis, it indeed determines all compounds in the sample with antioxidant capacity and not only polyphenols.[29] A mixture of 4.25 mL of phenolic extract (1 mg/mL) and 0.25 mL of Folin−Ciocalteu reagent were diluted 1:1 (v/v) with distilled water and mixed with 0.5 mL of a 10% sodium carbonate solution (w/v). Absorbance was measured at 765 nm after a 1 h reaction time at room temperature. A calibration curve was constructed using gallic acid as the calibration standard (20−90 mg/L). The regression coefficient of gallic acid was 0.9987. Results were expressed as gallic acid equivalent (GAE) per g of dp.

Polymeric Pigment and Tannin Assay by Harbertson−Adams. Anthocyanins, condensed tannin, and small and large polymeric pigments (SPPs and LPPs, respectively) content in grape pomace extracts were determined with the Harbertson−Adams assay adapted from the Hagerman and Butler method.[30] Results were expressed as malvidin 3-*O*-glucoside equivalents per g of dp, catechin equivalents (CEs) per g of dp, and absorbance units for anthocyanins, total tannins, and polymeric pigments, respectively.

Qualitative Proanthocyanidin Analysis by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass

Spectrometry (MS). *Sample Conditioning.* The DHB matrix (2,5-dihydroxybenzoic acid, 10 mg) and the cationizing agent (sodium chloride, 1 mg) were dissolved in 1 mL of 1% aqueous trifluoroacetic acid. First, 1 μL of this solution was mixed with 1 μL of sample solution (1 mg lyophilized pomace extract dissolved in 1 mL of 1% aqueous trifluoroacetic acid), which was then homogenized and deposited (2 μL) on a target plate. After drying at room temperature, the crystals were irradiated in the spectrometer.[31]

Analytical Conditions. A MALDI-TOF/TOF mass spectrometer (AutoFLEX III; Bruker Daltonics GmBH., Bremen, Germany) equipped with a pulsed N_2 laser (337 nm) controlled by the flexControl 1.1 software package (Bruker Daltonics) was used to obtain MS and tandem MS/MS data. The voltage was 20 kV and the reflectron voltage 21 kV. Spectra are the sum of 500 scans with a frequency of 200 Hz.[31] The positive mode was chosen in agreement with the literature for these types of compounds.[32] Proanthocyanidin molecular weights were calculated according to the following equation: $[M + Na^+] = 290.08 \times EC + 274.08 \times AFZ + 306.07 \times EGC + 152.01 \times GAL - 2.02 \times B - 4.04 \times A + 22.99$ where EC, AFZ, EGC, and GAL correspond to the number of (epi)catechin, (epi)afzelechin, (epi)gallocatechin, and galloyl moieties, respectively, and A and B correspond to the number of A and B linkages, respectively.

Statistical Analyses. Extractions and analyses were performed in triplicate with the data presented as mean value ± SD. Statgraphics Plus for Windows, version 4.0 (StatPoint Technologies, Inc., Herndon, VA) was used for statistical analyses. To study the effects of fermentation stage, extraction temperature, and extraction time on overall extraction performance, analysis of variance (factorial) and least significant difference tests were applied to the response variables with p-values ≤ 0.05 considered indicative of statistically significant differences between comparator groups.

RESULTS AND DISCUSSION

To have a common basis for comparison, results of all the analyses of the extracts are expressed in terms of dry mass of pomace (dp) before extraction.

Effect of Fermentation and Extraction Temperature and Time on Total Antioxidant Recovery and Antioxidant Activity. Grape pomace antioxidant activity measured with the FRAP assay was only affected by extraction temperature ($p < 0.001$). The FRAP value increased as the temperature increased, reaching a maximum at 150 °C for unfermented pomace (4.4 mg AAE/g dp) and 200 °C for fermented pomace (4.6 mg AAE/g dp; Figure 1a). Similar studies have shown this positive effect of temperature on the reducing/antioxidant capability of plant extracts.[33] Unexpectedly, for unfermented pomace, a slight decrease in the reducing capacity with increasing temperature from 150 to 200 °C was observed, although this effect was not observed with fermented pomace. Fermentation process degrades the pomace cell structure, increasing the release of numerous pomace-derived compounds including polysaccharides, mannoproteins, seed cuticle, and certainly polyphenols.[14] Therefore, the type and amount of antioxidants extracted could be different when using fermented versus unfermented grape pomace.

Pomace extract antiradical activity assessed with the DPPH assay was significantly affected by the three factors assessed: fermentation, extraction temperature, and extraction time (all p-values <0.001). Fermented pomace extracted at 200 °C for 5 min presented the highest value (184 mg TE/g dp). In most extraction conditions, fermented pomace showed higher antiradical activity than unfermented pomace (Figure 1b). Due to cell wall polysaccharides degradation, the extractability of phenolic compounds in the fermented pomace is enhanced,[34] resulting in extracts with higher antiradical activity. Increasing extraction temperature enhanced antiradical activity,

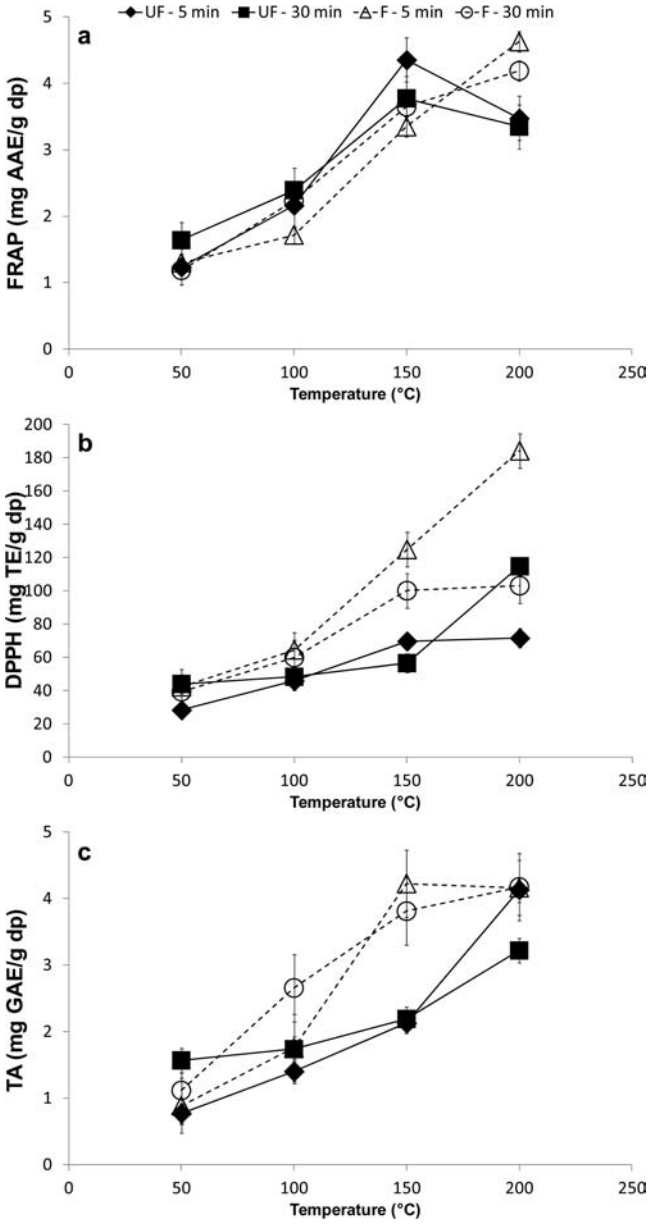

Figure 1. Effects of fermentation and extraction conditions on recovered total antioxidants and antioxidant activity in pressurized hot water extraction (PHWE) grape pomace extracts. (a) Ferric-reducing antioxidant power (FRAP) assay, (b) 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and (c) Folin assay. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

which peaked in the range between 150 and 200 °C (Figure 1b). The temperature effect was more pronounced at temperatures above 100 °C, especially for fermented pomace. The positive effect of temperature on antioxidant PHWE from grape pomace has been reported previously.[20] At 50 and 100 °C, time has no significant effect on extract antiradical activity. At 150 °C, increased extraction time reduced extract antioxidant activity. Additionally, at 200 °C, the antiradical activity of unfermented pomace extracts increased with time while that of fermented pomace extracts decreased. This shows

dx.doi.org/10.1021/jf4010143 | J. Agric. Food Chem. 2013, 61, 6929−6936

that the antioxidant profiles of fermented and nonfermented pomace are different.

Total antioxidant extraction was significantly affected by fermentation and temperature (both p-values <0.001). The maximum TA extraction yield was reached at 150 °C and 5 min for fermented pomace (4.2 mg GAE/g dp) and at 200 °C and 5 min for unfermented pomace (4.1 mg GAE/g dp). In most conditions, the fermented pomace extracts had the highest TA values due to the increased release of phenolic compounds during fermentation. The higher the extraction temperature the higher the TA value, which peaked between 150 and 200 °C (Figure 1c). The positive influence of temperature on the PHWE of polyphenols from grape pomace has been reported previously.[20]

Effect of Fermentation and Extraction Temperature and Time on Anthocyanin Extraction. The anthocyanin extraction yield is affected significantly by all three factors assessed, i.e., fermentation ($p < 0.001$), extraction temperature ($p < 0.001$), and extraction time ($p = 0.001$). Figure 2a shows the effects of these factors, where the highest extraction yield was obtained for unfermented pomace extracted at 100 °C for 5 min.

In most cases, higher anthocyanin yields were achieved from unfermented pomace. Anthocyanins are water-soluble pigments in the skin of red grapes and are distributed in vacuoles that are covalently associated with pectins.[35] Anthocyanins are extracted mainly in the aqueous phase during maceration prior to fermentation and at the beginning of alcoholic fermentation.[14] Up to 77% of the anthocyanins are released in this process,[36] resulting in a residual pomace with low content of these pigments. Furthermore, within the anthocyanins family, the structural differences between these compounds results in different extractabilities.[36]

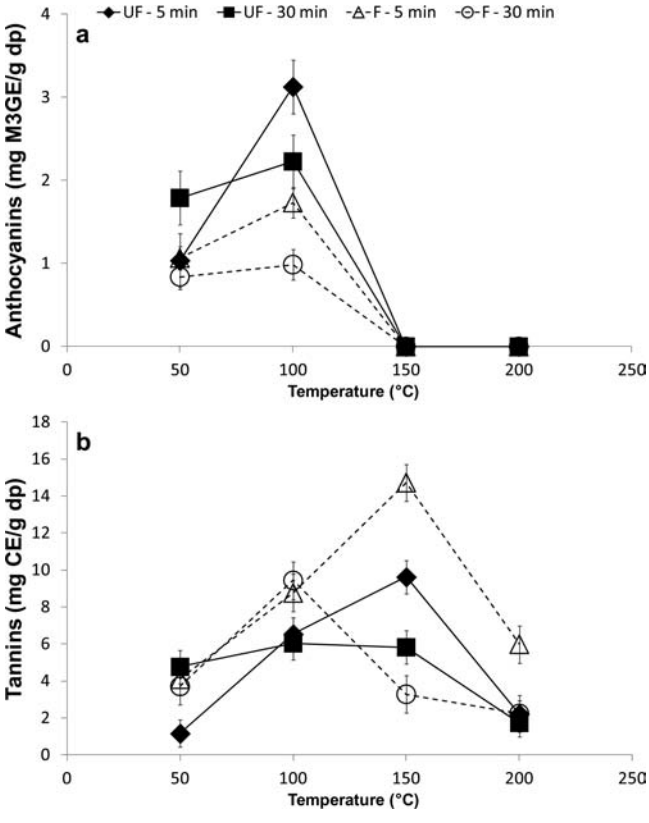

Figure 2. Effects of fermentation and extraction conditions on PHWE recovery of (a) anthocyanins and (b) condensed tannins from grape pomace. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Regarding extraction conditions, an increase from 50 to 100 °C for 5 min increased anthocyanin extraction yield due to increased water solvation power and improved polyphenol solubility.[37] However, at 150 and 200 °C, no anthocyanins were detected in the extracts. Moreover, an increase in extraction time decreased the amount of extracted anthocyanins, which was clearly observable at 100 °C. Both temperature and exposure time have a strong influence on anthocyanin stability. Previous studies have reported that a temperature increase causes a logarithmically increased anthocyanin degradation.[38] Ju et al. also reported that PHWE temperatures above 110 °C decrease individual and total anthocyanins content in dried red grape skin extracts.[39,40] Under excessive heat, grape pomace anthocyanins degrade by opening its pyrilium ring, thereby forming a colorless chalcone equivalent which further degrades to a brown insoluble polyphenolic compound, or by cleaving its sugar moiety to form a more labile anthocyanin aglycon.[41] In our study, these color changes were observed, from red (characteristic of the wine) in the extracts obtained at 50 and 100 °C to brown at 150 and 200 °C. Moreover, the influence of exposure time at high temperatures is very important in anthocyanin degradation. Mishra et al. reported that after 25 min at 126.7 °C grape pomace anthocyanin degradation increased substantially.[41] Additionally, the formation of polymeric pigments (anthocyanins bound to tannins) increases with temperature, especially at temperatures above 100 °C, decreasing the amount of free anthocyanins (monomeric pigments).[40]

Effect of Fermentation and Extraction Temperature and Time on Tannin Extraction. The tannin extraction yield is affected significantly by all three factors assessed: fermentation and extraction temperature and time (all p-values <0.001). The effects of these factors are shown in Figure 2b, where the highest yield was obtained for fermented pomace extracted at 150 °C for 5 min, followed by the unfermented pomace at the same extraction temperature and time.

In most of the extraction conditions tested (except 150 °C and 30 min), the fermented pomace showed higher tannin yields than unfermented pomace. Only a small amount of tannins are released during fermentation, resulting in a fermented pomace with high tannin content and increased tannin extractability. Fournand et al. reported that tannin extraction efficiency from unfermented grape skins in a hydroalcoholic solution similar to wine was lower than 38%.[36] Because tannin−cell wall interactions (hydrogen bonding and hydrophobic interactions) are determined by tannin and cell wall sugar structure and content,[42] the cell wall degradation during fermentation and the PHWE operating conditions enhance tannin recovery.

Higher tannin yields were achieved at 150 °C and 5 min, while at 200 °C the extraction yield greatly decreased from both unfermented and fermented pomace. In PHWE of tannins from grape seeds, where grape tannins are most concentrated,[43] increasing the extraction temperature increases tannin extraction yield, peaking at 150 °C.[9] Likewise, Monrad et al. found

Table 1. Proanthocyanidins in Grape Pomace Extracts Obtained with Different Pressurized Hot Water Extraction (PHWE) Conditions, As Analyzed and Identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry[a]

				50 °C		100 °C		150 °C	
proanthocyanidin subclass	compd	Na adduct (obsd)	Na adduct (calcd)	5 min	30 min	5 min	30 min	5 min	30 min
procyanidins	dimer (B)[48]	601.1	601.0	F		F		F	
	trimer (B)[48]	889.3	889.1	F	F	F	F		
	dimer (B-1 GE)[48]	753.2	753.0	U/F	U/F	U/F	U/F	F	F
	trimer (B-1 GE)[48]	1041.3	1041.1	U/F	U/F	U/F	U/F		
	tetramer (B-1 GE)[48]	1329.4	1329.2	U/F	U/F	U/F	U		
	pentamer (B-1 GE)[48]	1617.4	1617.2		F	F			
prodelfinidins	dimer (A-1 EGC)	615.1	615.0			U			
	dimer (A-2 EGC)	631.1	631.0			U			
	dimer (B-1 EGC)	617.1	617.0	U/F	U	U			
	trimer (B-1 EGC)[47,48]	905.3	905.1	U/F	U/F	U/F	U/F		
	tetramer (B-1 EGC)[47,48]	1193.3	1193.1	U/F	U/F	U/F	U/F		
	pentamer (B-1 EGC)[47,48]	1481.4	1481.2	U/F	U/F	U/F	U		
	hexamer (B-1 EGC)[47,48]	1769.3	1769.3		F	F			
	dimer (B-1 EGC-1 GE)	769.1	769.0	F	F	F			
	trimer (B-1 EGC-1 GE)	1057.2	1057.1	U/F	U/F	U/F			
	tetramer (B-1 EGC-1 GE)[48]	1345.2	1345.2	U/F	U/F	U/F			
	pentamer (B-1 EGC-1 GE)[48]	1633.2	1633.2	U	U/F	U/F			
	dimer (B-2 EGC)	633.1	633.0	U/F	U/F	U	U	U	

[a]obsd, observed; calcd, calculated; A, type-A bonds; B, type-B bonds; GE, galloyl ester; EGC, (epi)gallocatechin; EA, (epi)afzelechin; U, identified in unfermented pomace; F, identified in fermented pomace; U/F, identified in unfermented and fermented pomace. Superscripts indicate the reference in which this compound has been reported in grapes.

that the optimum temperature in the semicontinuous PHWE of grape pomace tannins is 140 °C.[44] To our knowledge, there are few studies about tannin stability at temperatures above 100 °C. However, it has been reported that the onset temperature of degradation of these polyphenols is approximately 150 °C and is dependent on factors such as acetylation and the amount of carbohydrates in the extract.[45]

Varying the extraction time had different effects on tannin recovery, depending on the type of pomace (fermented or unfermented) and the extraction temperature. With unfermented pomace extracted at 50 °C, increasing the extraction time from 5 to 30 min increased the tannin extraction yield. Because at low temperatures the mass transfer rate of tannins is slow, increasing the extraction time results in higher yields. However, in the case of fermented pomace extracted at 50 °C, the time increment produced no change in tannin extraction yield. Pomace degradation during fermentation facilitates the release of compounds reaching solubility equilibrium at short extraction times. In contrast, at 150 and 200 °C, increasing extraction time decreased tannin extraction efficiency for both unfermented and fermented pomace because long exposure times and high temperatures favors polyphenol degradation.[45]

Proanthocyanidin Profiles Observed by MALDI-TOF Analysis. *Compound Identification.* Tentative proanthocyanidin identification was performed by comparing the masses observed on mass spectra with the calculated mass for each compound. For identification, differences less than 0.3 Da between observed and calculated masses were considered acceptable. Proanthocyanidin results are summarized in Table 1.

The proanthocyanidins identified are mainly procyanidins and prodelphinidins with polymerization degrees up to 5 and 6, respectively. These findings are consistent with similar studies performed with positive ion reflectron mode where the polymerization degree ranged between 2 and 6.[32] It should be considered that in grape seeds, highly polymerized procyanidins are generally more abundant than oligomers.[46] The detection limits of the MS and the poor extractability of these large polymers may hamper their identification.

Grape seeds possess significant amounts of procyanidins only, while grape skins and stems also contain (epi)-gallocatechins units and therefore contain both B-type procyanidins and prodelphinidins.[47] Six procyanidins (B-type bond) were identified: a dimer and a trimer of (epi)catechin, and a dimer, trimer, tetramer, and pentamer of (epi)catechin with one gallate. These compounds have been found in grapes previously.[48] Twelve compounds were identified from the prodelphinidin family: two dimers (A-type bond) with one and two (epi)gallocatechins, one dimer (B-type bond) with two (epi)gallocatechins, oligomers from dimer to hexamer (B-type bond) with one (epi)gallocatechin, and oligomers (B-type bond) from dimer to hexamer with one (epi)gallocatechin and one gallate. Prodelphinidins trimers up to hexamers has been previously reported in grapes.[47]

Effect of Extraction Temperature and Time on Proanthocyanidins Profile. Fermentation, extraction temperature, and extraction time caused striking changes in the grape pomace extract proanthocyanidin profiles (Table 1). Procyanidin dimers and trimers were found only in fermented pomace extracts. In fermented extracts, prodelphinidins and procyanidins with one gallate present higher polymerization degrees than those found in unfermented extracts. Additionally, more procyanidins and prodelphinidins with one gallate were identified in the extracts from fermented versus unfermented grape pomace. During fermentation, lower proanthocyanidin extraction yields, especially those with a high degree of polymerization, are observed.[36] These differences in extractabilities result in fermented pomace extracts with different proanthocyanidins profile than unfermented extracts. Moreover, degradation of the cell wall during fermentation[14] facilitates extraction of a greater variety of proanthocyanidins.

Most of the proanthocyanidins detected by MALDI-TOF were recovered at 50 and 100 °C. Additionally, the highest proanthocyanidin yields and polymerization degrees, from both fermented and unfermented pomace extracts, were found after 100 °C and 5 min extractions. Higher extraction temperatures and times dramatically reduced the number of proanthocyanidins detected. At 150 °C, only a few proanthocyanidins were detected, and at 200 °C no proanthocyanidins were recovered.

Effect of Fermentation, Temperature, and Time on Polymeric Pigments. Polymeric pigments consist of anthocyanins (monomeric pigments) bound to tannins or flavan-3-ols such as catechin or epicatechin that are formed in wine after fermentation.[49] Fermentation and extraction temperature and time significantly affected recovery of SPP, LPP, and TPP (all p-values <0.001). The highest SPP extraction yield was obtained in unfermented pomace extracted at 150 °C for 30 min, whereas the highest LPP and TPP extraction yields were obtained in fermented pomace extracted at 150 °C for 5 min.

In most cases, unfermented pomace showed higher SPP values than fermented pomace, especially with 30 min extraction times (Figure 3a). In contrast, higher LPP and TPP yields were obtained with fermented pomace (Figure 3b,c). LPP and TPP showed similar extraction yield patterns because the LPP group is the largest contributor to TPP. In the Harbertson–Adams assay, LPPs represent the colored fraction of the condensed tannins, hence these two values are directly related.[49] Fermented pomace gives higher tannin extraction yields that are representative of higher LPP values.

Increasing extraction temperature increased the recovered SPP and LPP, and therefore the TPP values, peaking at 150 °C and markedly decreasing at 200 °C (except for SPP extracted for only 5 min). Increased extract polymeric color with increasing extraction temperature, especially at temperatures above 100 °C, has been reported previously.[40] The contribution of polymers to extract color indicates that extensive "degradation" of anthocyanins at high extraction temperatures occurred either by thermal degradation or polymeric pigment formation.[40] Extraction time showed no clear impact on polymeric pigment recovery from grape pomace. The highest SPP values were found at extractions times of 30 min in most conditions tested. Longer extraction times, hence, high anthocyanin-tannin reaction times, could favor the formation of SPPs at low temperatures and the breakdown of LPPs at high temperatures, increasing the SPP content in both cases. In contrast, longer extraction times have negative (especially at high temperatures) or no effect on LPP and TPP yields.

Correlation between Polyphenol Subclasses, Total Antioxidants, and Antioxidant Activity. Several different assays were statistically correlated using the Pearson correlation coefficient (Table 2). We calculated correlations after separating the data according to treatment (unfermented or fermented) and extraction time (5 or 30 min). Analysis of antioxidant activity and TA showed a strong positive correlation that was more pronounced with fermented pomace. This observation agrees with previous findings in polyphenol PHWE.[50] However, both antioxidant activity and TA showed strong negative correlations with total recovered anthocyanins, especially for fermented pomace. This is expected because anthocyanins are degraded at temperatures above 100 °C, while antioxidant activity as well as TA increased with temperature and peaked at 150 to 200 °C. At extraction times of 5 min, total tannins showed weak positive correlations with antioxidant activity and TA, while at 30 min these correlations were negative. Increased extraction time decreases tannin extraction efficiency at high temperatures due to thermal degradation,[45] while in most cases the extraction time has little or no effect on antioxidant activity and TAs.

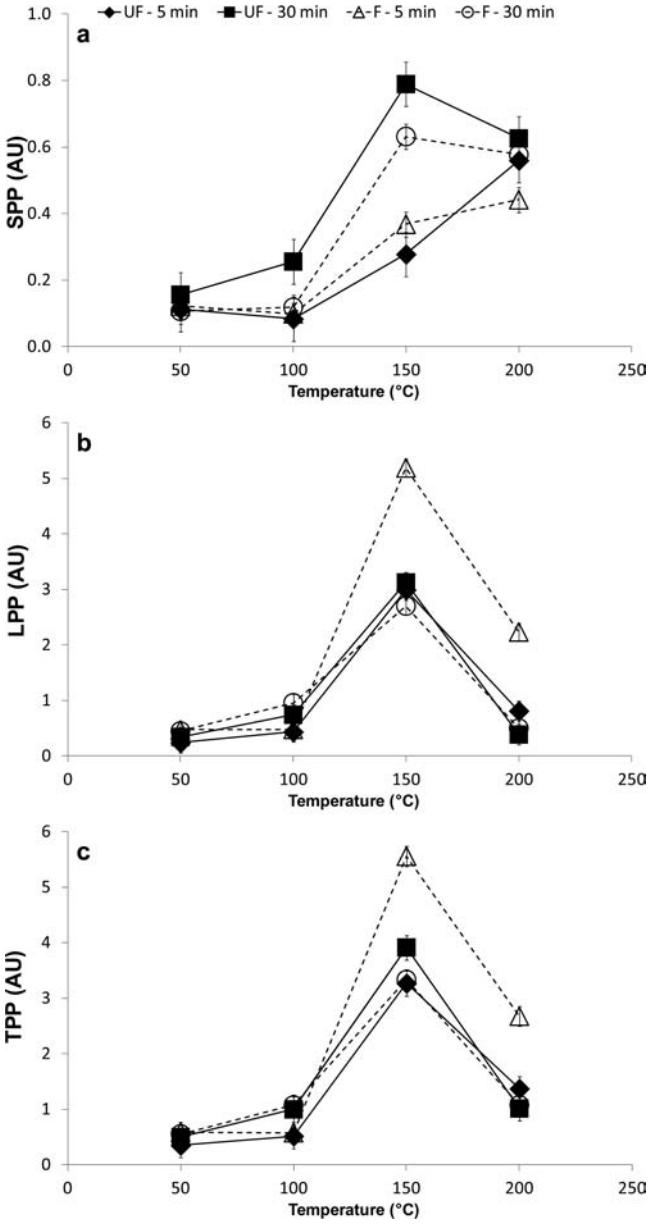

Figure 3. Effects of fermentation and extraction conditions on PWHE recovery of (a) small polymeric pigment (SPP), (b) large polymeric pigment (LPP), and (c) total polymeric pigment (TPP) from grape pomace. Curve symbols ◆ and ■ correspond to unfermented pomace extracted for 5 and 30 min, respectively. Curve symbols △ and ○ correspond to fermented pomace extracted for 5 and 30 min, respectively. UF and F correspond to unfermented and fermented conditions, respectively. Bars represent the upper and lower limits of the 95% confidence interval.

Increasing extraction temperature above 100 °C decreased polyphenol content but increased antioxidant activity and TA. It has been reported that high temperatures favor the formation of derived antioxidant compounds from polyphenols[9,39,51,52] as well as antioxidant Maillard reaction products such as melanoidins.[23]

dx.doi.org/10.1021/jf4010143 | J. Agric. Food Chem. 2013, 61, 6929–6936

Table 2. Pearson Product−Moment Correlation Coefficient between the Different Assays of Antioxidants Recovered during Grape Pomace PHWE[a]

	U		F	
assays	5 min	30 min	5 min	30 min
FRAP−DPPH	0.953	0.529	0.999	0.991
FRAP−folin	0.651	0.689	0.931	0.987
DPPH−folin	0.840	0.979	0.918	0.977
FRAP−anthocyanins	−0.596	−0.870	−0.849	−0.894
DPPH−anthocyanins	−0.563	−0.675	−0.821	−0.926
folin−anthocyanins	−0.557	−0.795	−0.859	−0.825
FRAP−tannins	0.637	−0.202	0.240	−0.430
DPPH−tannins	0.451	−0.919	0.213	−0.465
folin−tannins	−0.088	−0.839	0.576	−0.284

[a]Correlation coefficients were calculated with the values of all extraction temperatures separated by treatment (unfermented or fermented) and extraction time (5 or 30 min). U, unfermented pomace; F, fermented pomace; FRAP, ferric-reducing antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay.

In conclusion, the PHWE of antioxidants from fermented grape pomace, in most of the extraction conditions tested, allows recovery of a greater amount of TAs and antioxidant activity equivalent than from unfermented pomace. In both fermented and unfermented pomace, the highest antioxidant recoveries were obtained at temperatures above 150 °C. Although the majority of anthocyanins were removed during fermentation, high amounts of anthocyanins were recovered from fermented grape pomace using moderate temperatures (100 °C) and short extraction times. Contrary to anthocyanins, high extraction temperatures (about 150 °C) and short times yielded higher amounts of tannins. Extraction temperature determined the proanthocyanidin profile, different in fermented and unfermented pomace, where the greatest amount of these compounds was recovered at lower temperatures (50 and 100 °C). Overall, we found that grape pomace antioxidant activity and TA were not directly related to the main polyphenol content in PHWE extracts. The data obtained here in a laboratory-scale equipment will be useful to develop an industrial scale PHWE processes.

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Funding

We thank project 07CT9PDT-47 from INNOVA-Chile and grant Anillo en Ciencia y Tecnología ACT1105 from Conicyt for financial support. J.R.V. thanks CONICYT and DIPEI-PUC for a Ph.D. grant and VRAI-PUC for a visiting scholarship to CSIC-Barcelona. J.P.-J. thanks the ISCIII for a *Sara Borrell* postdoctoral contract (CD09/00068).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Viña Carmen and Felipe Ibañez for their support during this research. Editorial assistance from BioScience Writers LLC is greatly appreciated.

ABBREVIATIONS USED

TA, total antioxidant; PHWE, pressurized hot water extraction; DPPH, 2,2-diphenyl-1-picrylhydrazyl; TPTZ, tripyridyl triazine; Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid; IC_{50}, half-maximal inhibitory concentration; TEAC, Trolox equivalent antioxidant capacity; TE, Trolox equivalent; FRAP, ferric-reducing antioxidant power; AAE, ascorbic acid equivalent; GAE, gallic acid equivalent; TPP, total polymeric pigment; SPP, small polymeric pigment; LPP, large polymeric pigment; CE, catechin equivalent; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; MS, mass spectrometry; DHB, 2,5-dihydroxybenzoic acid

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