Received: 6 November 2018 | Accepted: 22 April 2020 | Published: 6May 2020
DOI:10.20870/oeno-one.2020.54.2.2346

OENO One
VINE AND WINE OPEN ACCESS JOURNAL

Characterisation of polyphenols and antioxidant potential of red and white pomace by-product extracts using subcritical water extraction

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ABSTRACT

A detailed assessment of the content of high added-value compounds in grape pomace varieties was carried out following a subcritical water extraction method. High amounts of anthocyanins and tannins were recovered from fermented grape pomace at differential temperatures with high variability between the by-products observed. Contrary to anthocyanins, high extraction temperatures (about 200 °C) yielded higher amounts of tannins. Overall, we found that grape pomace antioxidant activity and total polyphenols, quantified by the Folin Ciocalteu method, were not directly related to the main polyphenol content in SWE extracts. The data obtained in our study by using laboratory-scale equipment will be useful for developing an industrial-scale SWE process. Finally, it was shown that grape pomace by-products can be considered as an important source of polyphenols. They could therefore potentially provide a basis for the sustainable and integrated exploitation of winemaking by-products, to be used as inexpensive and readily available sources of bioactive compounds for the pharmaceutical, cosmetic and food industries.

KEYWORDS

green process, subcritical water extraction, grape pomace, polyphenols, valorisation

ABBREVIATIONS

AUC Area under curve
DM Dry matter
DS Dry skin
EtOH Ethanol
GA Gallic acid
HCl Hydrochloric acid
HPLC High performance liquid chromatography
PTFE Polytetrafluoroethylene
SWE Subcritical water extraction
T Temperature
ε Dielectric constant of water
Ø Diameter

INTRODUCTION

Grapes are one of the most produced fruit in the world for human consumption, especially for wine production. About 66.4 million tonnes of grapes have been produced annually in recent years (FAOSTAT, 2012). European countries, such as France, Italy, Spain and Germany, account for more than 44 % of the production (OIV, 2013). Such high wine production results in a large amount of grape by-products, in particular grape pomace, which accounts for 17–25 % of the quantity of grape produced (Pinelo *et al.*, 2005; Arvanitoyannis *et al.*, 2006). Grape pomace is a source of polyphenols, oil, alcohol and tartaric acid (Jackson, 2008). The residual alcohol in grape pomace is generally extracted. The seeds found in the pomace are a main source of grape seed oil, while the grape skin - although containing high levels of polyphenols - has not yet been utilised to its full potential. Due to a high polyphenol content, pomace cannot be utilised as feedstock or crop fertiliser (Devesa-rey *et al.*, 2011). For this reason, it is of considerable interest to examine ways of extracting polyphenols from the grape by-product.

Polyphenols are commonly extracted using an organic solvent. The method is simple, because the agents and equipment required for the process are easily obtainable (Spigno and De Faveri, 2007). However, as a result of degradation during solvent regeneration the yield is generally low. In addition, organic solvents substantially increase extraction process costs (Galanakis, 2012). Furthermore, the remaining solvent needs to be regenerated thoroughly from the product, leading to the generation of a large amount of organic solvent wastes (Yammine *et al.*, 2014).

Alternatively, a number of studies have demonstrated the use of water at subcritical (100 °C<T<374.2 °C) conditions (SWE) as an environmentally friendly and effective extraction process (Ju and Howard, 2003). Under these conditions, the dielectric constant of water (ε) changes dramatically with the change in temperature. The value of ε at 25 MPa decreases with temperature from 60 at ambient temperature to 21 at 250 °C, thus the reaction field changes from ionic reaction to radical reaction. In general, several studies have demonstrated that water under subcritical conditions is an effective extraction solvent (Plaza and Turner, 2015), which can be applied for the extraction of several bioactive compounds, such as phenolic compounds from lemon balm (Miron *et al.*, 2013), potato peel (Singh and Saldaña, 2011) and polysaccharides from golden oyster mushroom (Jo *et al.*, 2012).

TABLE 1. Efficiency and operating conditions of SWE-assisted extraction used to extract bioactive compounds from grape by-products

Grape by-product matrix	Operating/extraction conditions	Targeted bio-compounds (Relative increase*)	References
Red grape pomace (A-1575)	10 MPa, 110 °C, 100 % water, 1.4 g/L sodium metabisulfite	Total polyphenols: 6.23 g/100g DM*** Total anthocyanins: 5.93 mg/100g DM	Ju and Howard, 2005
Grape seeds (Tempranillo)	6-7 MPa, 150 °C,	Gallic acid: 232.1 mg/100 g (6.3) DM	García-Marino *et al*., 2006
Red grape pomace (Source n.d.**)	8 MPa, 120 °C, 1:1 (v/v) ethanol, 0.8 % (v/v) HCl	Total polyphenols: 1.26 g/100 g (7) DS Total flavanols: 3.5 mg/100 g (11.6) DS	Luque- Rodríguez *et al.,* 2007
Red grape pomace (Pinot noir)	10 MPa, 150 °C 100 % water	Total polyphenols: 6.070 g/100 g DM Total flavonoids: 1.425g /100g DM	Casazza *et al.*, 2010
Grape skins (Sunbelt grapes)	6.8 MPa, 100 °C 50 % ethanol/water (v/v)	Anthocyanins: 450 mg/100 g (1) DM	Monrad *et al*., 2010a
Red grape pomace (Cortina)	11.6 MPa, 140 °C; 100 % water	Total polyphenols: 3.08 g/100 g DS	Aliakbarian *et al.*, 2012
White grape pomace (Zinfandel)	10 MPa, 140 ° C, 100 % water	Anthocyanins 130 mg/100 g DM Procyanidin 2077 mg/100 g DM	Monrad *et al*., 2012
Red grape pomace (Cabernet-Sauvignon)	10 MPa, 140 °C 70 % ethanol/water (v/v)	Total polyphenols: 16.2 g/100 g DM	Rajha *et al*., 2014
Red grape pomace (Cabernet-Sauvignon)	10 MPa, 100 °C, 100 % water	Total antioxidants 1.06 mg/g Anthocyanins 1.050 g/100 g Condensed tannins 0.52 g/100g	Vergara-Salinas *et al.*, 2015
Red grape pomace (Pinot noir)	10 MPa, 120 °C, 100 % water	Total polyphenols: 7.76 g/100 g	Duba *et al*., 2015a

As can be seen in Table 1, almost all cited work has used a subcritical water extraction method on grape by-products. The by-products are different and the type of by-product is not generally comparable, because many studies have simply aimed to analyse the influence of operating parametres on the polyphenolic content release. Only a few studies have tried to optimise specific process parametres, as they relate to the different families of phenolic compounds and the diversity of the by-product. Research has mostly focused on the effects of extraction temperature in terms of yield and selectivity, with optimum extraction temperature dependent on the by-products used and the targeted molecules. When applying the Folin-Coicalteau extraction method, at 100 °C the total polyphenol content generally ranged from 1.26 g/100 g DM (Luque-Rodríguez *et al.*, 2007) to 16.2 g/100 g DM for the Cabernet-Sauvignon by-product (Rajha *et al.*, 2014). Meanwhile, the extraction of anthocyanins from red grape pomace at 110 °C was also variable, ranging from 5.93 mg/100 g DM (Ju and Howard, 2005) to 450 mg/100 g DM (Monrad *et al.*, 2014) for different sources of by-products. The extraction recovery of other families of compounds, such as catechins and proanthocyanidins, showed that selective extractions of compounds also varied. This indicates that extraction temperature, the type of by-product utilised, and the manner in which it was treated before extraction, all have a major influence on extraction.

Thus, the aim of the present work was to characterise the phenolic compounds from the grape pomace by-products of four different cultivars of *Vitis vinifera* (Chardonnay, Cabernet Franc, Merlot, Dunkelfelder), in order to identify any relevant properties they may have that could be used as functional ingredients, and to compare them at different extraction temperatures. This investigation consisted in i) determining the total phenolic and total tannin content of the grape pomace by-products, ii) identifying and quantifying monomeric and oligomeric (dimer and trimer) flavan-3-ol composition using High Pressure Liquid Chromatography (HPLC), and iii) estimating their antioxidant capacity carrying out four different assays (ABTS, CUPRAC, FRAP, and ORAC). The data may contribute to the selection of suitable grape pomace for the development of antioxidant and polyphenolic rich nutraceuticals.

MATERIAL AND METHODS

1. Chemicals

Copper(II) chloride dihydrate, ammonium acetate potassium peroxodisulfate, hydrochloric acid, ethyl alcohol, iron(III) chloride hexahydrate, sodium acetate 3- hydrate, glacial acetic acid, Folin-Ciocalteu reagent, and gallic acid were purchased from Scharlau (Barcelona, Spain). TPTZ (2,4,6-tri-(2-pyridyl)-s-triazine) and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) were obtained from Acros Organics (New Jersey, USA). ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) was obtained from Biochemica (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dodecahydrate, fluorescein, AAPH (2,2′-azobis-(2-methylpropionamidine) dihydrochloride, Neocuproine (2,9-dimethyl-1,10-phenanthroline), phloroglucinol, (+)-catechin, (−)-epicatechin, (−)-epigallocatechin (EGC), (−)-epicatechin- 3-O-gallate (ECG), procyanidin B1 [(−)-epicatechin-(4β-8)-(+)-catechin], and procyanidin B2 [(−)- epicatechin-(4β-8)-(−)-epicatechin] were supplied by Extrasynthèse, Genay, France. Acetonitrile (HPLC grade), formic acid (HPLC grade), methanol (HPLC grade), glacial acetic acid (HPLC grade), L-ascorbic acid, and sodium acetate were purchased from Prolabo-VWR (Fontenay/Bois, France). Trimer C1 [(−)-epicatechin- (4β-8)-(−)-epicatechin-(4β-8)-(−)-epicatechin] was obtained from Phenobio SAS (Martillac, France).

2. Raw material

In this study, grape pomace by-products were used, which were obtained from representative red and white grape varieties (*Vitis vinifera* L.) cultivated in Switzerland: Chardonnay, Cabernet Franc, Merlot and Dunkelfelder. Dunkelfelder is a teinturier grape variety known in Changins, whereas the other three grape varieties are well-known and widely cultivated elsewhere. Samples were provided by the University of Changins winery (Switzerland) during the 2012 and 2013 harvests. To limit the influence of external factors, and to obtain a better comparison among results, all samples were from the same

geographical area (46°23'56.4»N 6°13'58.9»E). The grapes used were harvested at the optimum technological ripeness. While the fourth variety considered, Chardonnay grape pomace was collected the day of grape harvest after destemming and pressing of the grapes under identical conditions.

Once pressed, all the grape pomaces were combined and homogenised to ensure representative sampling of the whole grape pomace. For the three red varieties, pomaces were collected immediately after pressing at 2 105Pa (RPS 50, Bucher Vaslin SA, France) and were treated with 50 mg of SO_2 per kg of raw material (RM). Samples were stored at -20 °C under vacuum until further processing.

In order to avoid complications with repeatability due to the heterogeneity of the raw material, the skins and the seeds were separated with a vibrating sifter (Retsch GmbH, Germany). Small fractions (diameter Ø<2.8 mm) and large fractions (Ø>5.5 mm) were then removed and the two standardised fractions were manually and homogeneously mixed (49 % of seeds and 51 % of skins fresh weight).

3. Process of extraction and parameters

The schematic diagram of apparatus used for the extraction of polyphenolic compounds using subcritical water is shown in Figure 1.

In the extraction system, a HPLC pump (I.C.S. National 1100) was used for deionised water delivery, pressurisation and system pressure control. A pressure transducer (Swagelock NG160) and thermocouple (Eurotherm Automation 90) were installed in the custom-made high-pressure vessel to monitor both pressure and temperature of the system. The extract was collected in an inerted vessel (65 mL volume) once it had passed through an ice bath.

In each run, the pomace (13.00 g) was loaded into the high-pressure vessel, with a capacity of 26 cm3 of material (Figure 1). The liquid-to-solid ratio was maintained at the value of 5, to obtain 65 mL of extract. The vessel was placed in an oven at several temperatures (100 °C, 150 °C, 200 °C). The outlet valve of the extraction vessel was then closed and the system was pressurised to the desired pressure of 25 10^5 Pa at a constant flow rate of 6 mL/min. The pomace and the solution collected in the inerted sampling vessel were then stored at 4 °C for further analysis without any preliminary preparation steps.

4. Conventional extraction experiments

The extraction of polyphenols from grape pomace (100.0 ± 0.1 g) was carried out in a mixture of ethanol and water (50/50, v/v) and maintained at ambiant temperature in a cylindrical extraction cell. The liquid-to-solid

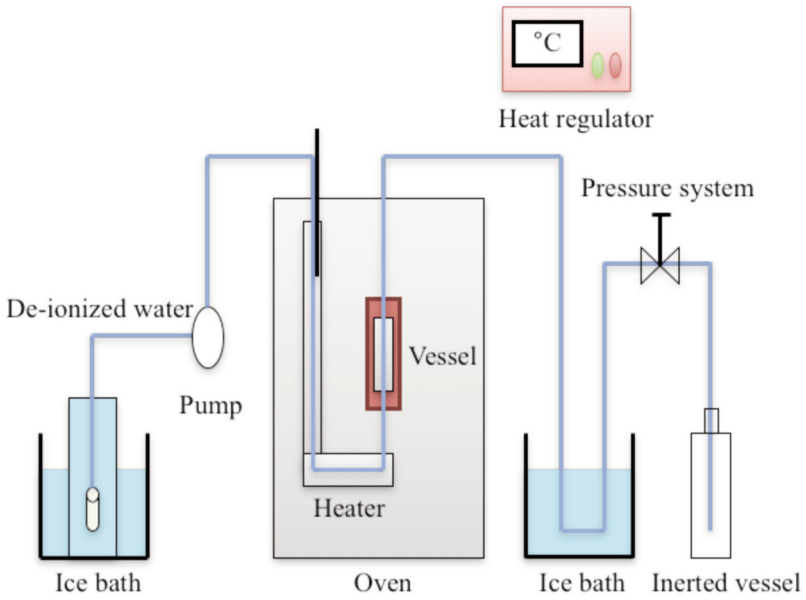

FIGURE 1. Schematic diagram of the pressurised liquid extraction process

ratio was maintained at a value of 5. A gentle agitation at 160 rpm (16.8 rad·s^{-1}) was maintained using a round incubator of 12.5 mm shaking throw (Infors HT Aerotron, Bottmingen, Switzerland). For untreated samples, the same protocol of extraction was used. Regular sampling was carried out during the 420 min of extraction. At the end of the extraction process, the juice was separated from grape pomace by centrifugation (Model 3-16P, Sigma Laborzentrifugen GmbH, Germany) at 3076 *g* for 10 min, and stored at −18 °C for further analysis.

5. Analysis

5.1. Total polyphenol content

The total phenolic content was spectrophotometrically measured according to a modified Folin-Ciocalteu method and applied on 96-well microplates. Stock solutions (10 mg/mL) of the grape pomace extracts were prepared in EtOH/H2O (25:75, v/v), and a microplate spectrophotometer (MultiSkan Spectrum, Thermo Scientific) was used for incubation and measurement. Each well was filled with 184 µL of distilled water and 24 µL of the sample solution, followed by 12 µL of the Folin-Ciocalteu reagent and 30 µL of 20 % (w/v) Na2CO3 solution. Prior to the measurement of the absorbance at 765 nm, the mixture was incubated for 1 h under dark conditions at 25 °C. Gallic acid (0−24 mg/L) was used as a standard for calibration. The results, expressed as milligrams of gallic acid per 100 g of grape pomace sample (on a dry matter basis, DM), were given as the mean of six determinations.

The total proanthocyanidin content of grape pomace by-products were measured using the Bate-Smith reaction (Bate-Smith, 1954).

5.2 Antioxidant activity

Polyphenols extracted from grape are well-known for their antioxidant capacity. This antioxidant activity is not a single reaction, but comprises a wide range of multiple mechanisms. It is usually recommended using several techniques, since no single technique is able to take into consideration all antioxidant mechanisms. Therefore, four different antioxidant capacity assays were used: one fluorometric assay based on hydrogen transfer, (ORAC) and three spectrophotometric assays based on electron transfer (ABTS, CUPRAC and FRAP). FLUOstar Optima (BMG LabTech) was used for the first assay, and an automated microplate reader (MultiSkan Spectrum (Thermo Scientific) for the other three. For the spectrophotometric methods, stem extract solutions (4 mg/10 mL) were prepared in EtOH/H_2O (25:75, v/v). Additional diluted stock solutions of the sample extracts (20 mg/1L) were prepared in 75 mM phosphate buffer (pH 7.4) for the ORAC measurement. The difference in absorbance between the final reading and the reagent blank reading was correlated with Trolox standard curves in all assays. Because the moisture level of each pomace extracted sample was quite different, the antioxidant capacity was reported on a dry weight basis to enhance comparison with the literature. Thus, the results were expressed as milligrams of Trolox per gram of grape sample (DM). Each resulting value was given as a mean of six determinations.

5.3. ABTS Assay

The ABTS radical cation (ABTS•+) was prepared in 96-well microplates by reacting equivalent volumes (1:1) of both aqueous solutions of 7 mM ABTS and 2.45 mM potassium persulfate. This stock solution was left to react for 12–16 h at room temperature in the dark and was then stored in the same thermal and light conditions to be used within two days. At the moment of the analysis 8 mL of the ABTS solution was diluted with EtOH/H_2O (25:75, v/v) in a 100 mL volumetric flask to obtain an absorbance of 1.00 ± 0.02 units at 734 nm. The extract solutions and ABTS reagent (190 µL in each well) were prewarmed in a 96-well microplate at 25 °C for 20 min. Next, a blank reagent reading was taken at a wavelength of 734 nm. The reaction was carried out by adding 10 µL of the pomace extract solution to each well. After 3 min of shaking, the mixture was incubated at the same temperature for a 30 min period, and then the absorbance decrease was measured at the same wavelength.

Trolox standard solutions were prepared at a concentration ranging from 0 to 0.8 mM (R2 = 0.995), by using EtOH/H_2O (25:75, v/v) as a solvent.

5.4. CUPRAC Assay

The cupric reducing antioxidant capacity analysis of the sample extracts was carried

out in 96-well microplates. The CUPRAC reagent was prepared just before the analysis by reacting equal volumes (1:1:1) of 10 mM Cu(II) aqueous solution, 7.5 mM neocuproine (in 96 % freshly prepared EtOH), and ammonium acetate buffer (1 M, pH 7). Pomace extract solution and 190 µL of CUPRAC reagent for each determination were incubated in a 96-well microplate under the same conditions as the ABTS assay. Once the initial absorbance had been read at 450 nm, 10 µL of the pomace extract solution was added to each well. After 3 min of shaking, the mixture was incubated at 25 °C for 30 min, and then the absorbance increase was measured at the same wavelength. The Trolox standard curve was linear between 0 and 1.3 mM (R2=0.996).

5.5. FRAP Assay

The ferric reducing antioxidant power assay was carried out in 96-well microplates. The fresh working FRAP reagent was prepared by mixing a 0.01 M TPTZ solution in 0.04 M HCl, a 0.02 M FeCl3·6H2O aqueous solution, and an acetate buffer (pH 3.6, 3.1 g of sodium acetate and 16 mL of acetic acid glacial per liter of buffer solution) at a ratio of 1:1:10. All of these solutions were prepared on the day of analysis, except for the buffer and hydrochloric solutions. For the measurement of the antioxidant activity by the FRAP method, the protocol and experimental conditions were exactly the same as those reported for the ABTS and CUPRAC assays. However, the increase in absorbance was measured at 593 nm and the Trolox calibration curve was obtained using concentrations of 0 to 1.6 mM (R2=0.996).

5.6. ORAC Assay

The oxygen radical absorbance capacity analysis was carried out using 96-well fluorescence microplates. The reaction was carried out in a phosphate buffer (75 mM, pH 7.4). In this order, 30 µL of the pomace extract solution, 180 µL of fluorescein (117 nM final concentration), and 90 µL of AAPH (40 mM) were added to each well. The mixture was shaken and left to stand for 1.5 h at 37 °C. Fluorescence was recorded every minute during this period at excitation and emission wavelengths of 485 and 530 nm respectively. A blank sample (phosphate buffer replaced the sample) and Trolox calibration solutions (1–40 µM) were also performed (R2=0.983) simultaneously on the same microplate. The area under the curve (AUC) was calculated for each extract sample by integrating its relative fluorescence curve. By subtracting the AUC of the blank, the net AUC of the pomace extracts was calculated and correlated with Trolox concentrations.

5.7. Anthocyanin analysis

Quantitative and qualitative analyses of anthocyanins were performed on the extracts with high performance liquid chromatography (HPLC) after 420 min of extraction. Samples of extracts were diluted (ratio 1/10) in acidified water (0.1 % formic acid) and then filtered through Polyamide filters (pores diameter Ø = 0.45 µm). The system used for the anthocyanin analysis was an Agilent 1200 HPLC Series (Agilent Technologies) equipped with a diode array detector. The separation was carried out with a Prontosil C18AQ column (4.6 × 250 mm, 5 µm, Bischoff Chroma-tography, Germany) operated at 25 °C in reverse phase.

UV/VIS spectra were recorded in the range of 200–600 nm. Two mobile phases, (A) water/acetonitrile/formic acid (87/3/10, v/v/v), and (B) water/acetonitrile/formic acid (40/50/10, v/v/v) were used for the separation of phenolic compounds. The elution gradient had the following profile: *t*0 min B (6 %), *t*15 min B (30 %), *t*30 min B (50 %), *t*35 min B (60 %), *t*41 min B (6 %), *t*45 min B (6 %). The injection volume was 30 µL and the flow rate was set to 1.0 mL·min^{-1}. Anthocyanins were detected at 518 nm. Individual anthocyanins were quantified using a calibration curve of the corresponding standard compounds. Results were expressed as weight (g) of individual anthocyanin extracted/100 g DM.

5.8 Flavan-3-ols and gallic acid analyses

The HPLC system used for flavan-3-ols and gallic acid analysis was an Agilent 1200 HPLC Series (Agilent Technologies, Germany) equipped with a diode array detector. The samples were diluted (ratio 1/10) in water and then filtered through PTFE filters (Ø = 0.45 µm). A volume of 60 µL was injected into a Prontosil C18AQ column (4.6 × 250 mm, 5 µm, Bischoff Chromatography, Germany), operated at 25 °C in reversed phase. Solvent A, 0.1 % trifluoroacetic acid (TFA) in water and solvent B, 0.1 % TFA in acetonitrile, were used for elution at a flow rate of 1 mL·min− 1.

The elution gradient had the following profile: *t*0 min B (7 %), *t*2 min B (7 %), *t*10 min B (16 %), *t*40 min B (31 %), *t*45 min B (50 %), *t*48 min B (100 %), *t*53 min B (100 %), *t*54 min B (7 %), and *t*59 min B (7 %). The detection wavelength of 280 nm was used for gallic acid, while a fluorimetric detector was set at 280 nm for excitation and 320 nm for emission wavelengths. Individual flavan-3-ols and gallic acid were identified using the corresponding standard compounds. Results were expressed as g of catechin equivalent/100 g RM for monomers and g of procyanidin B1 equivalent/100 g RM for oligomers.

6. Statistics

The Variance analysis was used for data analysis. The statistical significance of the differences in the data was obtained using the Tukey's test (α = 5 %). Data processing was carried out using XLSTAT (Addinsoft SARL, France) software.

RESULTS AND DISCUSSION

1. Total polyphenol content

The total phenolic content of subcritical water extracts of grape pomace of four different grape varieties according to extraction temperature is shown in Table 2. The total phenolic content ranged from 0.59 ± 0.05 to 3.66 ± 0.19 g of Gallic acid GA/100 g Dry Matter (DM) for the Cabernet Franc extraction at 100 °C and the Dunkelfelder extraction at 200 °C respectively. The Dunkelfelder variety harvested in 2012 yielded the highest values followed by Chardonnay and Dunkelfelder 2013, then Merlot and Cabernet Franc, in that order. Since the red pomace samples were collected for vineyards in close proximity to each other and underwent the vinification and pressing procedure, the differences observed in total phenolics are mainly due to the year of harvest and the inherent characteristics of each grape variety that was studied. Meanwhile, the difference between the white Chardonnay and the other red pomace can be linked to grape variety and vinification practices. As shown in Table 2, significant differences ($p<0.05$) in total phenolic content was found among all varieties (except between Dunkelfelder 2012 and Dunkelfelder 2013 extracted at 100 °C).

Temperature had a high influence on the extracted total polyphenols. For example, for Merlot pomace an increase of 1.08 ± 0.2 g to 2.29 ± 0.18 g of GA/100g DM in polyphenol content was observed with a 100 °C to 200 °C temperature increase, and the polyphenol content was above that of conventional solvent extraction at temperatures higher than 150 °C.

Another important factor was the influence of harvest year on the Dunkelfleder variety, with significant differences for total polyphenol content being linked to all three extraction temperatures; for example, the extraction of the 2012 harvest and 2013 harvest at 200 °C resulted in 3,66 ± 0,19 and 2,72 ± 0,09 g of GA/100 g DM respectively.

In other studies using subcritical extraction and conventional solvent extraction, polyphenol content has been found to largely vary from 1.06 to 6.23 g of GA/100 g depending on the grape cultivar, geographical origin and vintage winemaking process (Ju and Howard, 2003; García-Marino *et al.*, 2006; Luque-Rodríguez *et al.*, 2007; Casazza *et al.*, 2012; Vergara-Salinas *et al.*, 2013; Duba *et al.*, 2015a; Vergara-Salinas *et al.*, 2015). The results obtained in the present study are in agreement with the aforementioned range. Specifically, total phenolic content of weight (g) of GA/100 g was given by Aliakbarian *et al.* (2012) for grape red Cortina pomace. These results are also comparable with those in studies which utilised organic solvent for the extraction (González-centeno *et al.*, 2012; Ky *et al.*, 2014).

2. Total proanthocyanidins content

The total proanthocyanidin content of grape pomace by-products, obtained with the Bate-Smith reaction, are shown in Table 2. Similar to phenolic content quantification using the Folin Ciocalteu method, Dunkelfelder subcritical water extraction at 200 °C gave the highest value of 72.52 ± 2.43 mg/g DM, whereas Cabernet Franc pomace had the lowest value of 11.67 ±1.67 mg/g DM. Significant differences ($p<0.05$) were observed among the grape varieties, years of harvest and extraction temperatures.

Extraction temperature had a high influence on total extracted proanthocyanidins. In our case, for example, polyphenols extracted from Chardonnay pomace showed an increase of 54.20±1.33 to 68.37±4.17 mg/g DM in content of total proanthocyanidins by increasing the

TABLE 2. Total phenolic, proanthocyanidin and flavan-3-ol content of grape pomace samples (SWE: 100 % water, P= 25 10^5 Pa; Control: 50 % ethanol/water 20 °C)

By product	Extraction parameters	Total phenolics mg of GA/100 g DM		Total pro-anthocyanin mg of tannins/g DM		Gallic acid mg/100 gr DM		Phenolic content mg of GA/100 g DM		Flavan-3-ol concentration (mg/100 gr DM)						Total
										Catechin		Epicatechin		Procyanidin dimers	Procyanidin trimers	
Dunkelfelder 2012	SWE 100 °C	2.53	± 0.11	62.23	± 3.79	11.85	± 0.41	55.54	± 3.01	25.65	± 1.95	18.83	± 0.44	4.96	± 0.77	104.97
	SWE 150 °C	2.92	± 0.17	68.76	± 2.55	20.46	± 0.96	65.08	± 2.11	35.75	± 1.09	18.65	± 0.31	7.27	± 0.13	126.75
	SWE 200 °C	3.66	± 0.19	72.52	± 2.43	38.83	± 1.39	94.78	± 0.49	65.84	± 2.74	28.49	± 0.70	9.75	± 0.31	198.86
	Control	3.07	± 0.67	67.25	± 5.29	12.52	± 1.82	47.21	± 0.89	30.21	± 2.06	19.83	± 1.40	4.23	± 0.94	101.47
Dunkelfelder 2013	SWE 100 °C	2.11	± 0.14	44.26	± 1.43	8.58	± 1.01	46.87	± 2.21	19.49	± 2.30	13.12	± 0.53	4.27	± 0.39	83.75
	SWE 150 °C	2.44	± 0.06	52.31	± 0.59	18.37	± 0.73	60.42	± 4.95	31.14	± 1.13	17.55	± 1.21	6.65	± 0.79	115.76
	SWE 200 °C	2.72	± 0.09	67.19	± 1.63	30.86	± 0.33	78.73	± 4.98	59.37	± 2.79	24.69	± 1.24	7.23	± 0.68	170.02
	Control	2.38	± 0.08	49.11	± 2.93	13.10	± 1.74	49.66	± 3.12	29.50	± 1.92	16.07	± 0.90	4.67	± 0.71	99.89
Cabernet Franc	SWE 100 °C	0.59	± 0.05	11.67	± 1.67	15.08	± 1.58	18.58	± 1.73	15.17	± 1.01	13.87	± 1.06	2.91	± 0.17	50.54
	SWE 150 °C	0.82	± 0.10	20.26	± 0.90	26.20	± 1.19	34.79	± 1.13	18.29	± 1.50	15.13	± 0.47	2.89	± 0.64	71.10
	SWE 200 °C	1.42	± 0.12	34.17	± 2.76	31.41	± 3.36	55.87	± 0.36	21.29	± 0.32	17.54	± 0.90	2.29	± 0.40	97.00
	Control	0.49	± 0.01	16.07	± 0.42	24.66	± 2.20	23.41	± 1.68	18.28	± 1.40	15.50	± 0.79	2.08	± 0.74	59.27
Merlot	SWE 100 °C	1.08	± 0.20	21.63	± 2.02	0.63	± 0.04	13.29	± 2.53	7.32	± 0.89	5.20	± 0.47	2.08	± 0.18	27.90
	SWE 150 °C	1.56	± 0.06	38.51	± 2.03	1.75	± 0.11	16.78	± 1.02	11.65	± 0.67	6.86	± 1.09	2.82	± 0.33	38.11
	SWE 200 °C	2.29	± 0.18	44.31	± 1.48	1.86	± 0.06	19.69	± 2.09	15.48	± 0.74	3.03	± 1.56	0.49	± 0.35	38.70
	Control	1.26	± 0.19	36.17	± 3.96	1.52	± 0.07	13.75	± 0.10	11.77	± 0.38	5.11	± 0.70	2.19	± 0.24	32.81
Chardonnay	SWE 100 °C	2.11	± 0.08	54.20	± 1.33	1.78	± 0.33	30.62	± 2.74	26.13	± 2.40	8.24	± 0.80	5.58	± 0.08	70.58
	SWE 150 °C	2.82	± 0.12	63.48	± 2.29	2.11	± 0.37	37.30	± 3.69	28.48	± 1.08	12.03	± 0.17	6.04	± 0.46	83.85
	SWE 200 °C	3.06	± 0.09	68.37	± 4.17	3.59	± 1.85	45.03	± 1.23	31.91	± 0.97	14.53	± 0.48	5.88	± 0.22	97.35
	Control	2.91	± 0.76	57.17	± 3.69	1.97	± 0.41	38.36	± 0.15	26.78	± 4.06	13.10	± 1.08	5.62	± 0.42	83.86

temperature from 100 °C to 200 °C, and the polyphenol content was above conventional solvent extraction at temperatures higher than 100 °C.

The year of harvest also had an important influence on proanthocyanidin content. For the Dunkelfleder variety, there were significant differences in proanthocyanidin content depending on the harvest year for each of the three extraction temperatures; for example, at 150 °C the extractions of the 2012 harvest and 2013 harvest resulted in 68.76 ± 2.55 and 52.31 ± 0.59 g of tannins/100g DM respectively.

The total proanthocyanidin Bate-Smith test is a coloration method used to detect the presence of condensed tannins, which comprise an important fraction of the extract that is usually overlooked when quantifying subcritical water pomace extracts. For this reason, it was difficult to compare results to other subcritical water extracts. Nonetheless, the results obtained in the present study were similar to those previously reported in the literature for pomace by-products from white and red grape varieties extracted using an organic solvent (Rockenbach *et al.*, 2011; Mandic *et al.*, 2008; Obreque-Slier *et al.*, 2010; Travaglia *et al.*, González-centeno *et al.*, 2012). Nonetheless, the observed total tannins values of 68 mg/g DM for Chardonnay pomace was 2.2-fold higher than those obtained by González-centeno *et al.* (2012) using a solvent of MeOH/water (60:40, v/v) for extraction. These differences can be attributed to the different vintage and viticulture conditions of the samples.

As previously observed in several studies (Mandic *et al.*, 2008; Lorrain *et al.*, 2013; Ky *et al.*, 2014) a highly significant correlation was found between the total phenolic and total proanthocyanidin contents of the grape pomace extracts ($r=0.94$, $p<0.05$).

3. HPLC analysis of monomeric and oligomeric flavan-3-ols

The monomeric and oligomeric flavan-3-ol composition of grape pomace by-products from four grape varieties extracted via subcritical water at different temperatures was investigated and is given in Table 2. All the extracts were analysed using HPLC to identify and quantify the flavan-3-ols procyanidin B1, (+)- catechin, (−)-epicatechin, and the trimer C1, in that order of elution.

The combined amount of the above flavan-3-ols in grape pomace by-products ranged from 27.90 to 198.86 mg/100 g DM, for the Merlot (SWE 100 °C) and Dunkelfelder (2012, SWE 200 °C) varieties. These results are in accordance with those published by Luque-Rodríguez *et al.* (2007) with a total flavan-3-ol range of 29 to 199 mg/100 g DM for a red grape pomace by-product (8 MPa, 120 °C, 1:1 (v/v) ethanol, 0.8 % (v/v) HCl). Significant differences were found among the four varieties, year of harvest, and extraction temperature ($p<0.05$), with both Dunkelfelder and Chardonnay exhibiting the highest total flavan-3-ol content of 198 mg/100 g DM and 97 mg/100 g DM respectively at 200 °C.

The amount of extracted Flavan-3-ols can be linked to the temperature of the subcritical water extraction ; for example, increasing the temperature from 100 °C to 200 °C led to a 1.37- to 1.91-fold increase in Flavan-3-ol extracts. Temperature had a differential influence on individual compounds; (+)-catechin and (-)-epicatechin were optimally extracted at 200 °C for all grape pomaces, while Proanthocyanidins B1 and C1 were optimally extracted at 150 °C for Cabernet Franc, Merlot and Chardonnay.

The content ratio of both monomers, (+)-catechin and (-)-epicatechin, accounted for 65 to 81 % of the total flavan-3-ol quantified content of grape pomaces, depending on the grape variety, extraction temperature and year of harvest. Apart from the Chardonnay variety, the monomeric fraction was generally greater than the dimeric and trimeric ones at high temperatures. This observation agrees with that reported by Monrad *et al.* (2014) for red grape pomace (*V. labrusca L.*) , as well as with the results described by different authors for red grape pomace (*V. vinifera* L.) (Vergara-Salinas *et al.*, 2015; Duba *et al.*, 2015b). The optimal extraction conditions are shown in Table 1.

A similar ranking of the individual flavan-3-ol compounds was detected throughout all the investigated by-products. (+)-catechin was the major flavan-3-ol component, representing 46 to 74 % of the monomeric fraction and 44 to 55 % of the quantified flavan-3-ol content. (-)-epicatechin was the second main component quantified in all extracted by-products, except for Merlot, which had a higher of (−)-

TABLE 3. Total anthocyanin content of the grape pomace samples (SWE: 100 % water, P= 25 10^5 Pa; Control: 50 % ethanol/water 20 °C)

By product	Extraction parameters	Anthocyanins (mg/g of DM)										
		Cyanidine-3-O- Glucoside		Delphinidine-3- O-Glucoside		Malvidine-3-O- Glucoside		Petunidine-3-O- Glucoside		Peonidine-3-O- Glucoside		Total
Dunkelfelder 2012	SWE 100 °C	0.21	± 0.01	1.45	± 0.08	34.79	± 1.20	4.78	± 0.55	6.72	± 1.02	47.94
	SWE 150 °C	0.08	± 0.00	0.30	± 0.04	19.18	± 1.75	2.71	± 0.10	7.80	± 0.45	30.07
	SWE 200 °C	0.05	± 0.02	0.13	± 0.02	8.46	± 0.41	1.08	± 0.05	3.64	± 0.17	13.36
	Control	0.15	± 0.02	0.97	± 0.08	16.42	± 1.27	4.83	± 0.07	4.51	± 0.07	26.89
Dunkelfelder 2013	SWE 100 °C	0.19	± 0.03	1.33	± 0.02	31.49	± 0.15	4.54	± 0.02	2.49	± 0.27	40.04
	SWE 150 °C	0.08	± 0.01	0.19	± 0.08	18.17	± 1.35	2.60	± 0.05	7.77	± 0.97	28.81
	SWE 200 °C	0.04	± 0.00	0.11	± 0.07	6.96	± 0.09	0.91	± 0.07	2.99	± 0.41	11.02
	Control	0.13	± 0.02	0.83	± 0.06	15.14	± 0.05	0.49	± 0.04	3.89	± 0.08	20.48
Cabernet Franc	SWE 100 °C	0.51	± 0.03	2.15	± 0.06	5.99	± 0.08	1.87	± 0.04	1.69	± 0.02	12.21
	SWE 150 °C	0.21	± 0.02	1.33	± 0.02	4.31	± 0.01	0.92	± 0.02	0.15	± 0.08	6.93
	SWE 200 °C	0.19	± 0.01	1.47	± 0.10	1.36	± 0.04	0.54	± 0.06	0.12	± 0.00	3.67
	Control	0.43	± 0.07	0.17	± 0.03	0.43	± 0.03	0.18	± 0.03	0.12	± 0.07	1.32
Merlot	SWE 100 °C	0.14	± 0.01	1.59	± 0.06	2.18	± 0.08	4.93	± 0.07	1.33	± 0.04	10.17
	SWE 150 °C	0.10	± 0.00	1.42	± 0.05	1.67	± 0.04	3.59	± 0.03	1.09	± 0.14	7.86
	SWE 200 °C	0.03	± 0.00	0.54	± 0.02	0.97	± 0.22	1.54	± 0.02	0.17	± 0.00	3.25
	Control	0.13	± 0.03	1.39	± 0.03	1.89	± 0.06	3.84	± 0.05	0.99	± 0.07	8.23

In units of mg/g DM pomace, Data are expressed as the mean of triplicate ± standard deviation,
TPC, total phenol content; total anthocyanins; Dp, delphinidin-3-O-monoglucoside; Cy, Cyanidin-3-O-monoglucoside; Pt, petunidin-3-O-monoglucoside (eq, malvidin-3-O-glucoside) ; Pn, peonidin-3-O-monoglucoside (eq, malvidin-3-O-glucoside); Mv, malvidin-3-O-monoglucoside;vi)

epicatechin to (+)-catechin when extracted at 200 °C.

These ratios of higher quantity of monomers with respect to dimer and trimer have been previously observed in the literature for skins and/or seeds of different grape pomaces extracted by subcritical water (Srinivas *et al.*, 2011; García-Marino *et al.*, 2006; Vergara-Salinas *et al.*, 2013; Bucić-Kojić *et al.*, 2011; Monrad *et al.*, 2014).

4. HPLC Analysis of anthocyanins for red grape by-products

The anthocyanin content of skin extracts was analysed using HPLC and the concentrations of the obtained compounds are shown in Table 3.

For total anthocyanins, Dunkelfelder 2012 and 2013 extracted at 100 °C (47.94 mg/g DM and 40.04 mg/g DM respectively), and Cabernet Franc (12.1 mg/g DM) superior amounts to lower temperatures. Grape variety had a stronginfluence on quantity of total anthocyanins and the ratios of individual anthocyanins.

Undeniably, "teinturier" cultivars (i.e., Dunkelfelder) had higher anthocyanin content than "non-teinturier" grapes (i.e., Merlot and Cabernet Franc). As can be seen in Table 3, the Dunkelfelder pomace mainly contained malvidin-3-O-glucoside (72–78 %) out of the total anthocyanins found in the extract in contrast to the other two cultivars used for the extraction. It also contained unusually higher amounts of peonidin- 3-O-glucoside (6.7.1 mg/g DM at SWE 100 °C) than Merlot and Cabernet Franc under the same extraction conditions. These results are in accordance with several studies that have been carried out on teinturier grape varieties (Hermosín-Gutiérrez and García-Romero, 2004; Ky *et al.*, 2014), in which higher ratios of peonidin-3-O-glucoside were found in teinturier grape varieties than in other varieties.

In the 2012 and 2013 vintages, pomace from Dunkelfelder contained variable levels of anthocyanins the ratio of the anthocyanins stayed the same. For both Dunkelfelder vintages, the major anthocyanin was malvidin-3-O-monoglucoside, accounting for 72 % total anthocyanins, while for the other varieties it ranged from 21 to 65 %. In the 2013 by-products, less anthocyanins were observed. Values ranged from 1.4 mg/g DM to 10.6 mg/g DM for glycosylated anthocyanins,

The temperature of subcritical water extraction was the most important factor to contribute to a varied amount of extracted anthocyanins; for example, increasing the temperature from 100 °C to 200 °C led to a 1.37- to 1.91-fold decrease. Temperature had a differential influence on individual compounds; (+)-catechin and (-)-epicatechin were optimally extracted at 200 °C for all grape pomaces.

The optimum extraction temperature depended on the molecules; with average optimum temperatures (0.47 mg/100 mg) of around 100 °C. These molecules were optimally extracted at a lower temperature than flavonoid and phenolic acids. The presence of a sugar molecule in glycoside anthocyanins tends to make them more soluble in polar substances, and they can therefore be extracted better at a lower temperature than less-polar flavonoid and phenolic acids (Monrad *et al.*, 2010b). Furthermore, anthocyanins are highly thermolabile compounds, due to the presence of the glucoside function that leads degradation at high temperatures during extraction (Ko *et al.*, 2014). Proanthocyanidins B1 and C1 were optimally extracted at 150 °C for Cabernet Franc, Merlot and Chardonnay.

5. Antioxidant capacity

The antioxidant potential of each sample was determined in order to select the most active grape pomace among studied varieties. The antioxidant capacity of each extract cannot be assessed by applying a single method. Indeed, antioxidant measurements can be related to the capacity of extracts to either directly transfer hydrogen to a radical (ABTS, FRAP) or to act as competitors for the peroxy radicals (ORAC, CUPRAC) (Roginsky and Lissi, 2005). Hence, more than one type of antioxidant measurement needs to be performed to take into account the various mode of action of antioxidants (Huang *et al.*, 2005). In that context, the free radical scavenging capacities of seed and skin extracts were evaluated using the following four tests: the FRAP, ABTS decolorisation tests and the CUPRAC and ORAC assays.

The results of these tests are shown in Table 4. Similar behavior patterns were observed in all four tests, regardless of their action

mechanism. The Dunkelfelder variety produced the highest antioxidant capacities extracted at 200 °C.

Meanwhile, the Merlot variety had the lowest values at the same temperature, with an antioxidant potential 1.6 times lower than that observed for the Dunkelfelder variety. Furthermore, in the CUPRAC assay, significant differences ($p<0.05$) were found among the antioxidant capacity values of all four grape pomaces. In the ORAC assay, however, no significant difference was found between the Merlot and Cabernet Franc varieties, which had the lowest antioxidant potential ($p>0.05$). A considerable rise in antioxidant activity was observed for all the examined grape pomace extracts when the temperature was increased from 100 °C to 200 °C. Out of the the four varieties studied, total phenolic content was particulary high in the Dunkelfelder variety.

The comparison of literature on the antioxidant capacity of winemaking by-products is quite challenging, due to the fact that we used different analytical methods (such as CUPRAC, ABTS and FRAP, etc.), a variety of standards and reference units, and importantly, differing grape materials of reference. The antioxidant capacity is also affected by other factors, such as the winemaking procedure, geographical origin of the samples and the extraction methodology.

Nonetheless, in our study, the same orders of magnitude as those previously described in literature for the antioxidant capacity of grape pomace extracts were observed, irrespective of the analytical method applied or grape variety used. It was difficult to find an antioxidant assay on extracts from grape pomace to compare with. Ju and Howard (2005) reported antioxidant capacity ranges measured by the ORAC assay to be higher than those extracted at 160 °C in the present research (1105 mg of Trolox/g DM). A larger scope of comparison to grape extracted solvent gave lower antioxidant values: Sánchez-Alonso *et al.* (2007) reported the antioxidant capacity of Airén white grape pomace, measured by ABTS (71.1 mg of Trolox/g DM) and FRAP assays (116.6 mg of Trolox/g DM) as being similar to the extracts obtained at 100 °C.

Pearson's correlation coefficients were calculated to evaluate the agreement of the expression of the grape pomace antioxidant capacity among the four assays applied. Regardless of the pair of methods considered, a high, significant and positive correlation was observed ($r\geq0.84$, $p<0.05$), suggesting that ABTS, CUPRAC, FRAP, and ORAC assays give comparable and interchangeable antioxidant capacity values for grape pomaces. Correlation coefficients among antioxidant capacities based on ORAC and FRAP assays were the highest ($r\geq0.96$), whereas ABTS data exhibited little to low correlation values ranging from 0.62 to 0.89. The different degrees of correlation among these four assays may be due to the different chemical information provided, depending on the electron or hydrogen transfer mechanism on which they are based.

A correlation with total phenolic content was exhibited by the ORAC, ABTS, FRAP and CUPRAC assays ($r=0.35$ 0.53, 0.22 and 0.15 respectively at $p<0.05$); this low correlation has not previously been observed in the literature for skins, seeds, and grape pomaces (Ju and Howard, 2005; Lafka *et al.*, 2007; Aliakbarian *et al.*, 2012). When comparing the total tannin content and the antioxidant capacity of the grape pomace extracts , a lower correlation was observed ($0.12\geq r\geq0.51$, $p<0.05$). Similar results were obtained when correlating total anthocyanin content and the antioxidant capacity of the grape pomace extracts : a lower correlation was observed ($0.05\geq r\geq0.16$, $p<0.05$). This is unexpected, because the anthocyanins were degraded at temperatures above 100 °C, while the antioxidants increased with temperature and peaked at 150 to 200 °C.

CONCLUSION

The present research carried out a detailed evaluation of the phenolic composition (total phenolic and total proanthocyanidin content, monomeric and oligomeric flavan-3-ol composition, and proanthocyanidin profile, anthocyanins) and antioxidant potential of white grape pomace by-products derived from the vinification process. To the best of our knowledge, no studies addressing this variability of the by-product in such a detailed way for multiple grape varieties have been previously published.

In conclusion, by using the subcritical water extraction method, high amounts of anthocya-

TABLE 4. Antioxidant capacity determined by ABTS, CUPRAC, FRAP, and ORAC assays for grape pomace samplesa

By products	Extractions parameters	ABTS	CUPRAC	FRAP	Orac
Dunkelfelder 2012	100 % water/100 °C/50 10^5Pa	124.74 ± 9.55	163.04 ± 3.92	99.90 ± 5.81	123.00 ± 1.46
	100 % water/150 °C/50 10^5Pa	239.95 ± 15.05	213.64 ± 1.20	192.17 ± 6.99	236.62 ± 5.37
	100 % water/200 °C/50 10^5Pa	290.39 ± 14.02	379.57 ± 8.00	232.57 ± 5.17	286.36 ± 20.03
	50 % ethanol/water 20 °C	144.08 ± 13.82	181.01 ± 7.95	162.85 ± 6.25	136.93 ± 2.47
Dunkelfelder 2013	100 % water/100 °C/50 10^5Pa	70.60 ± 7.01	159.17 ± 6.24	73.37 ± 2.87	87.00 ± 8.79
	100 % water/150 °C/50 10^5Pa	149.12 ± 12.60	336.23 ± 2.21	154.99 ± 5.73	183.77 ± 8.96
	100 % water/200 °C/50 10^5Pa	219.00 ± 13.49	493.78 ± 1.34	227.61 ± 6.32	269.89 ± 5.63
	50 % ethanol/water 20 °C	87.40 ± 9.85	194.34 ± 1.23	78.79 ± 2.34	123.53 ± 4.53
Cabernet Franc	100 % water/100 °C/50 10^5Pa	60.56 ± 4.58	113.63 ± 2.28	67.82 ± 2.26	66.40 ± 6.76
	100 % water/150 °C/50 10^5Pa	107.97 ± 7.90	202.59 ± 8.21	120.92 ± 3.93	118.40 ± 2.37
	100 % water/200 °C/50 10^5Pa	218.60 ± 4.75	410.17 ± 7.11	244.81 ± 1.21	239.71 ± 5.54
	50 % ethanol/water 20 °C	72.34 ± 5.25	132.80 ± 9.80	98.88 ± 7.02	73.47 ± 4.25
Merlot	100 % water/100 °C/50 10^5Pa	28.76 ± 1.16	97.72 ± 5.15	47.78 ± 5.65	43.44 ± 4.14
	100 % water/150 °C/50 10^5Pa	49.13 ± 2.59	166.95 ± 6.42	81.62 ± 3.81	74.21 ± 3.09
	100 % water/200 °C/50 10^5Pa	97.50 ± 3.20	331.31 ± 6.34	161.98 ± 7.75	147.27 ± 8.34
	50 % ethanol/water 20 °C	32.49 ± 1.62	111.77 ± 7.29	53.86 ± 4.41	55.46 ± 5.41
Chardonnay	100 % water/100 °C/50 10^5Pa	97.90 ± 4.17	71.38 ± 2.51	55.95 ± 6.60	62.30 ± 1.51
	100 % water/150 °C/50 10^5Pa	150.01 ± 6.01	109.38 ± 1.92	85.73 ± 5.86	95.46 ± 5.60
	100 % water/200 °C/50 10^5Pa	271.90 ± 7.46	198.25 ± 9.59	155.40 ± 4.21	173.02 ± 8.34
	50 % ethanol/water 20 °C	123.80 ± 3.95	81.15 ± 3.07	70.43 ± 3.23	82.34 ± 2.53

[a]Antioxidant capacities expressed as equivalents of mg of Trolox/g DM. Letters following the values in each column show the significant differences among grape varieties ($p<0,05$).

nins and Flavan-3-ols were recovered from fermented grape pomace at different temperatures with a high variability between by-products. Contrary to anthocyanins, high extraction temperatures (about 200 °C) yielded higher amounts of tannins. Overall, we found that grape pomace antioxidant activity and total polyphenols, quantified by applying the Folin Ciocalteau method, were not directly related to the main polyphenol content in SWE extracts; this critical point needs to be investigated further. The data obtained here from laboratory-scale equipment could be useful for developing industrial scale SWE processes.

Finally, it has been shown that grape pomace by-products can be considered as an important source of polyphenols. They could therefore potentially provide a basis for the sustainable and integrated exploitation of winemaking by-products, and be used as inexpensive and readily available sources of bioactive compounds for the pharmaceutical, cosmetic and food industries.

Acknowledgements: This study has received financial support from the Agence Nationale de Recherche under the 1st transnational Call of ECO-INNOVERA (ERA-NET, ANR-12-INOV-0001- 04).

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