Valorization of wine industry by-products: Characterization of phenolic profile and investigation of potential healthy properties

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Abstract. In the last years, the importance of food waste management and recovery is emphasized by the international guidelines to promote a circular economy approach. Wine industry is one of the sectors with the highest waste production, with a potential negative environmental impact. Winemaking by-products are mainly used to produce distillates, fertilizers and livestock feed, but alternative approaches for their management could be the formulation of healthy products. The aim of this study was the application of *in vitro* methods for a preliminary evaluation of the phenolic pattern and the associated biological properties of winemaking by-products from different red grape varieties. The methods were: 1) Folin-Cocalteau's assay for the assessment of total polyphenol content; 2) the vanillin assay for the quantification of total procyanidin content; 3) the pH differential method for the determination of total anthocyanin content; 4) DPPH and FRAP assays for the measurement of total antioxidant activity; 4) High Performance Thin Layer Chromatography for separation of phenolic substances and assessment of their antioxidant capacity; 5) dipeptidyl peptidase (DPPIV) inhibition assay to evaluate possible effects on glucose homeostasis. The results showed that grape pomace, particularly when including seeds, was a valuable source of polyphenols with significant antioxidant potential and promising activity on DPPIV, supporting its use in formulating healthy foods/food supplements.

1 Introduction

Among the agricultural crops, grape (*Vitis vinifera* L.) is one of the most worldwide cultivated. In 2022, the world area dedicated to vine cultivation for all purposes (wine and juice, table, grapes and raisins) was estimated at 7.3 million hectares (Mha), of which 3.3 Mha are in the European Union [1].

More than 50% of grapes were used for wine production, which was estimated at 258 mhL [2]. The winemaking generates high amounts of by-products: it is estimated that for every 100 L of wine produced, 18-35 kg of grape waste secondary products are accumulated. For this reason, there are difficulties in the management of the grape waste mass with a potential negative impacts on the environment [3]. The practices currently employed to manage grape by-products include their reuse to produce alcohol, distillates, compost and livestock feed. Nevertheless, Directive 2008/98/EC of the European Parliament and the Council of 19 November 2008 established a legal framework for treating waste in the European Union, emphasizing the importance of proper waste management, recovery and recycling approaches to reduce the environmental and human health impact [4].

Also the OIV, in the strategic plan 2020-2024, promotes the circular economy through the reuse of waste and management of by-products, defining and developing guidelines on "green" chemistry.

This situation has encouraged the exploration of novel strategies in utilizing wine by-products, to produce new derivatives, such as enriched foods or "functional" foods.

The development of these approaches has also been driven by consumer demands for both natural sourced ingredients and sustainable practices [3], which include implementing the reuse and recycling of waste products.

The main winery by-products are the grape marc or pomace, which represents between 20 and 25% of the initial grapes' weight.

It is composed of 25% seeds, 25% stalks and 50% skins (left after the crushing and pressing stages of wine production) (Fig. 1) [5]. Grape stalks contain high levels of cations (mainly K and Fe) that can be used for soil amendment [6]. Grape seeds are generally used to produce oil and meal for human and animal consumption, respectively. The seeds and the skins are also rich in soluble fibers, unsaturated lipids, sterols, vitamins, polyphenols and other antioxidants [7]. The potential health-promoting effects of grape pomace are mainly attributed to its polyphenol content. It is estimated that 60-70% of the phenolic compounds of grape remain in the pomace after winemaking, accounting for 4.8-5.4% of pomace dry matter [8]. The content of phenolic compounds, representing about 2-3% of the grape pomace, has raised a great interest since these molecules have been associated with the reduction of risk factors for several chronic diseases [9].

Figure 1. Grape pomace derived from the winemaking process. Adapted from Hoss et al. 2021 [7].

Phenolic compounds of grape marc include several molecules that are further classified into: 1- non-flavonoids, such as hydroxybenzoic acids (i.e. gallic, protocatechuic and vanillic acids), hydroxycinnamic acids (i.e. p-coumaric, caffeic and ferulic acids) stilbenes (i.e. resveratrol, piceatannol and resveratrol dimers), and 2- flavonoids, such as flavanols (i.e. catechins, procyanidins and polymeric procyanidins), anthocyanins (mainly glycosylated form of delphinidin, cyanidin, petunidin, peonidin and malvidin) and flavonols (derivatives of quercetin, myricetin and kaempferol). Grape skins of pomace from red varieties contain mainly anthocyanins. Conversely, grape seeds, skin and stems are an important source of flavanols and procyanidins (PROs), that include oligomers and polymers of flavan-3-ols units [8]. PROs and non-flavonoid compounds are considered responsible for several healthy effects; among them, the improvement of the endothelial function, the increase of the serum antioxidant capacity, the protection of LDLs from oxidation and the reduction of inflammation [9,10]. In addition, there is a growing interest in evaluating the effect of grape pomace on glycaemia modulation, since disorders associated with carbohydrate metabolism (e.g. type 2 diabetes) is one of the major health problems worldwide [8]. For these reasons, the recovery of these compounds after the winemaking could be an interesting alternative approach to reduce the ecological impact of vinification and, in parallel, to formulate food supplements or healthy foods.

On this basis, the aim of the study was a preliminary characterization of the phenolic pattern and the measure of different *in vitro* biological activities (antioxidant activity, dipeptidyl peptidase IV inhibition) of different winemaking by-products deriving from different *Vitis vinifera* cultivar.

2 Materials and methods

The methods developed for the characterization of the phenolic fraction of pomace samples were based both on spectrophotometric and chromatographic approaches. Spectrophotometric methods included: 1) Folin-Ciocalteau's assay for the quantification of total polyphenol content; 2) the pH differential method for the quantification of total anthocyanin content; 3) vanillin assay for the determination of total flavan-3-ols (proanthocyanidins); 4) DPPH assay for antioxidant activity evaluation; 5) FRAP (Ferric Reducing Antioxidant Power) to evaluate the reduction of ferric-tripyridyltriazine (Fe3-TPTZ) (mmolEFe2+/g). High Performance Thin Layer Chromatography (HPTLC) was used as fast chromatographic approach for the separation and semi-quantitative evaluation of antioxidant properties of pomace active compounds. Finally, dipeptidyl peptidase IV (DPPIV) inhibition assay was used to evaluate the effects of pomace phenol compounds on DPPIV, enzyme involved in glucose homeostasis.

2.1 Samples

The samples included in the study, kindly provided by Dr. Antonella Bosso, CREA (Asti, Italy) were winery by-products from red varieties collected in different winemaking stages (Table 1**)**.

Table 1. Samples included in the study, year of collection, winemaking stage of collection and codes used in the paper.

Samples		**Winemaking stage**	**CODE**
Grignolino, 2015	Seeds	Initial fermentation (2° day)	G-IF-S-15
Grignolino, 2015	Seeds	After fermentation	G-AF-S-15
Grignolino, 2016	Seeds	Initial fermentation (2° day)	G-IF-S-16
Grignolino, 2016	Seeds + skins	After fermentation	G-AF-SS-16
Grignolino, 2018	Seeds + skins	After fermentation	G-AF-SS-18

Uvalino, 2015	Seeds	Initial fermentation (2° day)	U-IF-S-15
Uvalino, 2015	Seeds + skins	After fermentation	U-AF-SS-15
Uvalino, 2016	Seeds + skins	After fermentation	U-AF-SS-16
Barbera, 2017	Seeds + skins	After fermentation	B-AF-SS-17
Barbera, 2018	Seeds + skins	After fermentation	B-AF-SS-18

All samples were maintained at -20 °C till the use.

2.2 Spectrophotometric assays

Four spectrophotometric assays were used in this study.

2.2.1 Folin-Ciocalteu's assay

Total polyphenol content (TPC) was determined according to Singleton and Rossi [11].

About 0.4 g of each blended sample were mixed with 3 mL methanol:water (1:1) mixture, sonicated for 15 minutes using an ultrasonic bath and centrifuged for 15 minutes at 8000 r.c.f. (relative centrifugal force) at 4 °C. The supernatant was collected and filtered on a paper filter. A second extraction was performed on the solid precipitate; the two supernatants were combined and adjusted to volume (5 mL) with methanol:water (1:1) mixture. Aliquots of 300 µL from samples, or water for blank, were mixed in test tubes with: 1.5 mL of Folin-Ciocalteau's reagent (Sigma Aldrich, Germany) diluted 10 times, and 1.2 mL of 7.5% sodium carbonate (Sigma Aldrich, Germany). After 30 minutes, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, California, U.S.A.). Each sample was extracted in triplicate. Results were expressed as mg/g gallic acid (GA) equivalents (dry weight).

2.2.2 Total Anthocyanin Content

Total anthocyanin content of red pomace samples was determined according to the AOAC method [12]. About 0.4 g of each blended sample were mixed with 3 mL of methanol:HCl 85:15 (v/v), sonicated for 15 minutes using an ultrasonic bath and centrifuged for 15 minutes at 8000 r.c.f. (relative centrifugal force) at 4 °C. A second extraction was performed on the solid precipitate; the two supernatants were combined and adjusted to volume (5 mL) with methanol:HCl 85:15 (v/v). The absorbance of samples, prepared as described in 2.2.1 and suitably diluted with pH 1.0 (0.025M potassium chloride) and pH 4.5 (0.4M sodium acetate) buffers, were measured spectrophotometrically both at 520 and 700 nm, using the last absorbance to correct for haze. Each analysis was performed in triplicate. The content of antocyanin pigments (AP) is expressed as cyd-3-glu equivalents (mg/L), according to (1):

$$AP\ (mg/L) = A \times MW \times DF \times 1000/e \times 1 \qquad (1)$$

where: $A = (A_{520nm}-A_{700nm})_{pH\ 1.0} - (A_{520nm}-A_{700nm})_{pH\ 4.5}$;
MW (molecular weight) = 449.2 g/mol for cyd-3-glu;
DF = dilution factor; l = path length in cm;
e (molar extinction coefficient) = 26,900 for cyd-3-glu;
1000 is the factor for conversion from g to mg.

2.2.3 Vanillin assay

The total content of monomeric and condensed flavanols (proanthocyanidins) was measured by vanillin assay [13]. The reaction involves an aromatic aldehyde, vanillin, that reacts with meta-substituted ring of flavanols to yield a red adduct, with a maximum absorbance at 500 nm. About 0.5 g of blended samples were extracted with 10 mL of methanol and stirred with a magnetic stir for 20 min in the dark. Then, the solutions were centrifuged for 10 minutes at 8000 r.c.f. at 4 °C and filtered with a paper filter. The supernatants were then collected and suitably diluted. The extraction procedure was performed in triplicate. Catechin standard solutions were prepared in methanol using catechin in the range of 50-200 µg/mL. Vanillin reagent was prepared using 1% methanolic solution of vanillin (1%, w/v) mixed with 3% HCl methanolic solution (v/v). Aliquots of 0.5 mL of samples or standard solutions were added with 2.5 mL of vanillin reagent (VR) or 1.5% HCl and maintained at 30 °C form 20 minutes in the dark. Then, the absorbance was measured spectrophotometrically at 500 nm. The Δ absorbance was calculated according (2):

$$\Delta\ absorbance: (A\ sample\ VR - A\ blank\ VR) - (A\ sample\ HCl - A\ blank\ HCl) \qquad (2)$$

A standard curve was obtained by correlating absorbance values with catechin concentrations. Results were expressed as mg catechin (C) equivalents/g of grape by-product.

2.2.4 Antioxidant activity by DPPH assay

The antioxidant activity (AOA) of grape pomace was measured spectrophotometrically, as a measure of radical scavenging activity, using 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) [14,15]. Samples were prepared as described in 2.2.1. Standard solutions of gallic acid (GA) were prepared in methanol:water 1:1 (v/v) in the range of 1-5 µg/mL. Aliquots of 1 mL of DPPH (Sigma Aldrich, Germany) in methanol (5 mg/100 mL) were mixed with 0.5 mL of standard solution or sample suitably diluted. The absorbance was measured after 30 minutes at 517 nm. Results were expressed as equivalents of gallic acid (GA) in mg/g of sample.

2.2.5 Antioxidant activity by FRAP assay

Ferric Reducing Antioxidant Power (FRAP) assay is a method based on the use of antioxidants as reductants in a redox-linked colorimetric reaction, where Fe^{3+} reduced to Fe^{2+} [16] produces a colored ferrous-probe complex from a colorless ferric-probe complex. Samples were prepared as described in paragraph 2.2.1. The antioxidant capacity was calculated using a standard curve of ferrous sulfate heptahydrate (Sigma Aldrich, Germany) ranging from 0.11 to 1.8 mM. Aliquots of 50 µL of standard solution or sample (opportunely diluted), or blank (methanol:water 50:50, v/v) were added with 150 µL of water HPLC grade and 1500 µL of FRAP reactive, mixed, and maintained at 37 °C for 30 min in the dark. The absorbance was measured at 593 nm in a UV–visible spectrophotometer (Varian Cary 50 SCAN, Palo Alto, CA, USA). The FRAP reagent was previously prepared by mixing the 300 mM acetate buffer:10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution:20 mM $FeCl_3*6H_2O$ (iron chloride hexahydrate) solution in a ratio of 10:1:1 (v/v/v). Results were expressed as mmol equivalent of Fe^{2+} (EFe^{2+}) in g of sample.

2.3 Fast chromatographic methods: High Performance Thin Layer Chromatography (HPTLC)

Thin Layer Chromatography (HPTLC) is a fast and suitable method for screening different classes of molecules, allowing the fingerprint characterization of several botanical products [17]. Furthermore, HPTLC technique can be used for the assessment of biological properties such as the semi-quantitative measure of antioxidant activity.

In this study, HPTLC technique was used to perform a screening of the most representative polyphenol classes (phenolic acids, flavonols, flavanols and anthocyanins) in grape by-products and evaluate in parallel the associated antioxidant activity.

2.3.2 Polyphenol profile and antioxidant activity of pomace samples

Aliquots of 10 µL of standard solutions (200 µg/mL) of the main pomace polyphenols (kaemferol-3-glu, hyperoside, caftaric acid, quercetin-3-O-glu, epicatechin) were applied on silica-gel plates. Sample volumes of 5 µL samples, prepared as described in 2.2.1, were loaded onto the plate. At the end of the chromatographic run, performed using 10 mL of mobile phase (toluene:acetone:formic acid 4.5:4.5:1, v/v/v) the plate was sprayed with a DPPH (Sigma Aldrich, Germany) methanolic solution (0.05%, w/v) and dried for 1 min at room temperature in an extractor hood. The dried plate was wrapped with aluminium foil for 30 minutes and exposed at UV (366 nm) or at visible light.

2.3.3 Anthocyanin profile of pomace samples

Aliquots of 10 µL of standard solutions (cyaniding 3-O-glucoside, delphinidin 3-O-glucoside, pelargonidin 3-O-glucoside, malvidin 3-O-glucoside, peonidin 3-O-glucoside) at the concentration of 200 µg/mL and 15 µL of sample (prepared as described in paragraph 2.2.2) were applied on silica-gel plate using a semi-automatic applicator, Linomat 5 (Camag, Muttenz, Switzerland). At the end of the chromatographic run, performed using 10 mL of mobile phase (1-butanol:acetic acid:water 4:1:5), the dried plate was exposed at visible light.

2.4 Dipeptidyl peptidase IV inhibition assay

The DPP-IV inhibitory activity assay was performed using a DPP- IV Inhibitor Screening Kit (MAK203, Sigma-Aldrich, Milan, Italy), suitable for the screening of potential DPP4 inhibitors. DPP4 activity is measured by cleaving the substrate to yield an amount of fluorescent product, proportional to the enzymatic activity. The effectiveness of the test inhibitors may be compared with the DPP4 inhibitor (sitagliptin) provided as control. Inhibitors of DPP4 reduce the degradation of glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 by DPP4 and have emerged as oral anti-diabetic agents [18, 19]. The DPP-IV inhibitory activity assay was performed according to the method proposed by Su et al., 2019 [20]: briefly, 50 µL of enzyme solution and 25 µL of sample solution were premixed and incubated at 37 °C for 10 min; then 25 µL of DPP-IV substrate was added. Fluorescence (λex=360 nm, λem=460 nm) was measured once every min up to 60 min, using a VICTOR X3 Multilabel Plate Reader (Perkin Elmer, Milano, Italy). The efficacy of the test inhibitors was compared to the DPP4 inhibitor (sitagliptin) provided as a control, and to remove background interference, the buffer was added instead of enzyme solution.

Relative Inhibition (%), was calculated according to (3):

$$\text{DDP-IV Relative Inhibition} = (\text{SlopeEC} - \text{SlopeSM})/\ \text{SlopeEC}) \times 100\% \quad (3)$$

where:
SlopeSM = the slope of the Sample
SlopeEC = the slope of the Enzyme Control (Blank).

3 Results and Discussion

3.1 Spectrophotometric assays

Figure 2 reports total polyphenol content (TPC) and total proanthocyanidin content (Tpro) of samples included in the study.

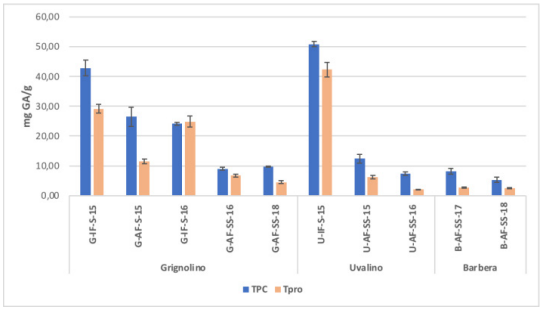

Figure 2. Concentrations of total polyphenol (TPC) and procyanidin (Tpro) in the samples, mean±SD, $n = 3$.

TPC ranged between 50.83±0.89 mg GAE/g (Uvalino seeds, 2015) and 5.30±0.89 mg GAE/g (Barbera skins+seeds, 2018). Generally speaking, procyanidins represented about 50% of the total polyphenol content, both in samples containing only seeds and in those including also the skins. These data are in line with literature data [21]. Interestingly, TPC of Grignolino samples containing only seeds collected before the winemaking was mainly represented by procyanidins. As conformation, TPC and TPro were highly correlated (R^2>0.91).

According to Rodriguez-Perez et al. (2019), grape seed procyanidins include oligomers and polymers of flavan-3-ol units, composed mainly by catechin and epicatechin monomers, followed by their galloylated forms [21].

These compounds are raising great interest among researchers and food industry due to their antioxidant potential, in fact, oxidative stress is recognized as one of the key factors for the progression of several chronic disease. On these bases, several authors investigated new functional foods containing different source of proanthocyanidins, where methods aimed at improving their bioavailability were tested. Tang et al. (2018) reported that the total flavonoid content in grape seeds is up to ten times higher than grape peel, reinforcing the importance of this grape-product [22].

Anthocyanins mainly in their glycosylated forms, are another group of phenolic substances present in red grape pomace having antioxidant and anti-inflammatory activity. In Fig. 3 total anthocyanin content (TAC) of samples is reported.

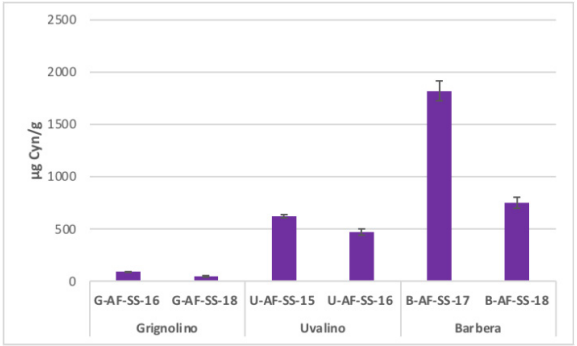

Figure 3. Total anthocyanin content (TAC) (mg/g of cyanidin equivalents, mean±SD; $n = 3$) of winemaking by-products containing skins. Data with different letters are significantly different ($p < 0.001$). For abbreviations see Table 1.

Total anthocyanin content (TAC) range between 49.31±6.83 µg/g (Grignolino 2018, G-AF-SS-18) and 1822±91.31 µg/g (Barbera 2017, B-AF-SS-17). Anthocyanin concentration and profile are influenced by several factors such as varietal diversity, soil composition, environmental conditions, vineyard management and grape ripening. In addition, some grape varieties can present high anthocyanin concentration but a low extractability index, resulting in pomaces still rich in these compounds [23]. In our study, Barbera by-products collected in 2017 showed the highest anthocyanin content (1822±91.31 µg/g /g cyanidin equivalents), which was from two to twenty times higher than the other cultivar by-products. On the other hand, Barbera samples collected in 2018 showed an anthocyanin concentration significantly lower than the by-products from the same variety collected in 2017, indicating that climatic conditions could lead to a great variability in phenol compound content. However, our data are consistent with literature where Barbera variety shows anthocyanins in the range of 4.00 and 12.00 mg/g of skin berry [24]. On this basis, Barbera pomace can be considered a valuable source of anthocyanin compounds.

As regards the other samples, after fermentation, no significant differences were found between TAC of Grignolino samples collected in different years, while TAC of Uvalino 2015 showed a little, but significant difference respect to the same variety collected in 2016. In particular, Uvalino variety, a Pedimont indigenous cultivar shows an interesting anthocyanin content [25]; this aspect should be taken into account as a part of biodiversity protection concept.

As procyanidins and anthocyanins are the most interesting and abundant compounds in pomace, the antioxidant potential of the samples included in the study was measured. Table 2 reports total antioxidant activity (AOA, measured by DPPH and FRAP assays) of the samples analyzed.

Table 2. –AOA of samples measured by DPPH and FRAP assay, mean± SD ($n = 3$).

Sample	DPPH mg GA/g	FRAP mmolFe^{2+}E/g
G-IF-S-15	35.17±0.81	0.57±0.04
G-AF-S-15	32.27±3.13	0.40±0.04
G-IF-S-16	18.58±2.10	0.37±0.02
G-AF-SS-16	5.34±0.24	0.14±0.01
G-AF-SS-18	3.51±0.43	0.15±0.01
U-IF-S-15	42.15±3.83	0.72±0.08
U-AF-SS-15	7.59±0.25	0.18±0.01
U-AF-SS-16	4.31±0.25	0.12±0.01
B-AF-SS-17	5.25±0.32	0.14±0.02
B-AF-SS-18	2.83±0.03	0.07±0.004

Samples containing only seeds show the highest antioxidant activity (AOA), in particular Uvalino (2015, U-IF-S-15) and Grignolino (2015, G-IF-S-15). The presence of the skins, representing 50-52% (w/w) of grape pomace, significantly decreased AOA by 30% in all the samples. This could be due to the higher solubility of anthocyanins in ethanolic-water solution and to the surface exposed to the solvent during the fermentation process. On the other hand, procyanindins in the seeds, together with minor compounds such as flavonols and phenolic acids, show high radical scavenger capacity.

Results obtained using DPPH and FRAP assays showed a similar trend as shown by the high linear regression between results from the two tests (R^2 = 0.95) (Fig. 4).

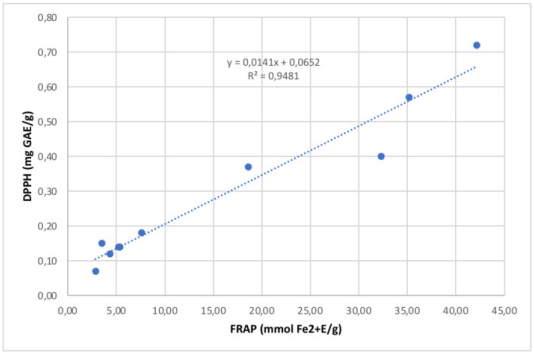

Figure 4. Linear regression between DPPH and FRAP assays. Results are expressed as mg of gallic acid equivalent (GA)/g (DPPH) and mmol Fe^{2+} equivalents/g.

In addition, TPC was well correlated also with AOA, measured by both DPPH and TRAP assays, TPC, as shown by the good linear correlation coefficients (R^2=0.99 and 0.93, respectively).

In order to understand the contribution of the different phenol compounds to AOA, the content of procyanidins and anthocyanins was considered showing that only procyanidins had a good correlation with AOA.

The correlation between total polyphenol content, total anthocyanins and procyanidins and AOA was evaluated using Pearson's correlation coefficients (threshold for statistical significance: p<0.01) (Table 3).

Table 3. Correlation between parameters measured by spectrophotometric assays in the by-products containing anthocyanins (n = 6 samples).

Parameter	Pearson correlation coefficient (r)	statistical significance (p)	Strength
Total polyphenols Vs. Antioxidant capacity	r = 0.734	p<0.01	Positive strong correlation
Total anthocyanins vs Antioxidant capacity	r = 0.246	ns	Positive weak correlation
Total flavan-3-ols vs Antioxidant capacity	r = 0.520	p<0.05	Positive moderate correlation

The data reported in Table 3 suggest that flavan-3-ols, compared to anthocyanins, contribute more to the antioxidant activity, even if the role of other molecules contained in the seeds, such as flavonols (e.g. kaempferol-3-glucoside and quercetin-3-glucoside) and phenolic acids cannot be excluded. Further studies will clarify these aspects.

3.2 Thin layer chromatography (HPTLC)

The HPTLC technique allowed a parallel evaluation of phenol compound distribution and antioxidant activity in pomace samples.

3.2.1 Phenolic pattern and antioxidant activity of grape samples

The innovative approach of HPTLC technique allowed to obtain: 1- the separation and the relative abundance of anthocyanins in samples containing skins (n = 6); 2- the correlation of polyphenol pattern with the relative antioxidant activity.

Figure 5 shows anthocyanin pattern obtained after the exposure of the plates at visible light.

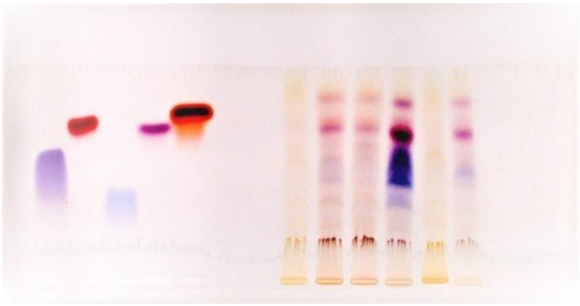

Figure 5. HPTLC anthocyanin patterns of samples after exposure of the plate at visible light. Standard anthocyanins are run in parallel.

Cy=Cyanidin-3-O-glu
Peo=Peonidin-3-O-glu
Del=Delphinidin-3-O-glu
Mal=Malvidin-3-O-glu
Pel=Pelargondin-3-O-glu

S4= G-AF-SS-16
S6=U-AF-SS-15
S7=U-AF-SS-16
S8=B-AF-SS-17
S9=G-AF-SS-18
S10=B-AF-SS-18

Cy Peo Del Mal Pel **S4 S6 S7 S8 S9 S10**

Results obtained from HPTLC confirmed the spectrophotometric data: Barbera samples S8 and S10 (SB-AF-SS-17 and B-AF-SS-18, respectively) showed the highest abundance of anthocyanins, followed by Uvalino S6 (U-AF-SS-15) and S7 (U-AF-SS-16) and Grignolino S4 (G-AF-SS-16) and S9 (G-AF-SS-18). Malvidin-3-O-Glucoside was the most abundant compound, especially in Barbera by-products, that showed in parallel significant amount of cyanidin-3-O-glucoside and delphinidin-3-O-glucoside, as already reported by other authors [26]. Besides malvidin-3-O-glucoside, Uvalino samples were characterized by peonidin-3-glucoside as well, confirming data by Borsa et al. (2010) [25]. In Grignolino samples anthocyanins were not detectable, in line with the low concentration

measured by spectrophotometric assays (90.87±3.96 µg/g in G-AF-SS-16 and 49.31±6.83 µg/g in G-AF-SS-18).

To test the antioxidant activity, the plates were exposed at 366 nm (not shown) and visible light after derivatization with the DPPH solution. Figure 6 shows the phenol distribution and the associated antioxidant activity of samples, when plated were exposed to at visible light after the derivatization step.

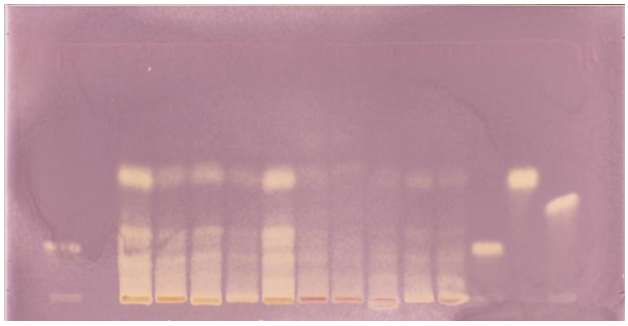

Figure 6. HPTLC patterns of samples after exposure of the plate at visible light and derivatization with DPPH solution. Standard flavonoids are run in parallel.

Q1 = Quercetin-3-O-glu
K = Kaempferol-3-O-glu
S1= G-IF-S-15
S2= G-AF-S-15
S7=U-AF-SS-16
S8=B-AF-SS-17
S9=G-AF-SS-18
S10=B-AF-SS-18
S3=G-IF-S-16
S4= G-AF-SS-16
S5=U-IF-S-15
S6=U-AF-SS-15
Hy= Hyperoside
EC = Epicatechin
CA= Caftaric acid

Samples S1 (Grignolino seeds 2015 before fermentation), S2 (Grignolino seeds 2015 after fermentation), S3 (Grignolino seeds 2016 before fermentation) and S5 (Uvalino seeds 2015 before fermentation) showed the highest flavonoid abundance and antioxidant capacity, as shown by the strong discoloration of the bands, proportional to polyphenol radical DPPH scavenger activity. Samples S1 (G-IF-S-15) and S5 (U-IF-S-15) showed the highest relative abundance of epicatechin, being one of the most characterizing compounds of seed procyanidins.

Data from HPTLC were in agreement with spectrophotometric results (Fig. 2), reporting that Grignolino and Uvalino samples were the richest in polyphenols and proanthocyanidins. Samples including the skins showed a reduced content of polyphenols with antioxidant activity, since these compounds are highly extracted during winemaking.

Caftaric acid, quercetin-3-O-glucoside and hyperoside were not detectable in the sample analyzed.

3.3 Dipeptidyl peptidase IV inhibition assay

In the last years, dipeptidyl peptidase IV (DPP-IV) has been recognized as a novel target for managing and positively modulate glucose homeostasis in both healthy people with little alteration of basal glycaemia and patients affected by type 2 diabetes. The formulation of functional foods enriched with compounds able to inhibit DPP-IV is the focus of new studies aimed at reducing risk factor for chronic metabolic diseases or at integrating pharmacological treatments [27].

In literature, very few data have been produced on the bioactivity of grape polyphenols on DPPIV; in fact, most studies are focused on food peptides deriving from protein hydrolysis [27].

Figure 7 shows the preliminary results on the ability of pomace samples (10 mg/mL) to inhibit DPPIV activity.

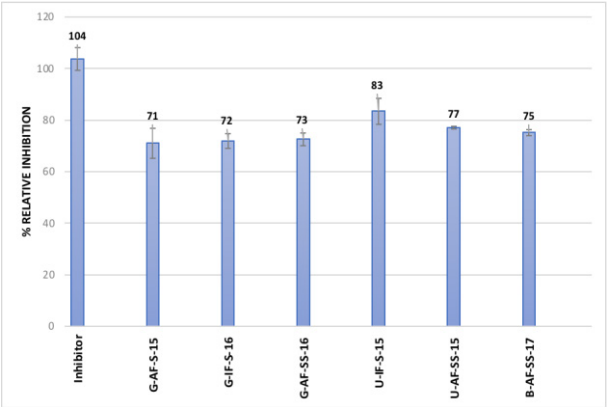

Figure 7. Percentage of DPPIV inhibition obtained after the incubation of pomace samples for 60 min.

The relative percentage inhibition of DPPIV ranged between 71.0±5.8% and 83.0±5.0%, indicating that all the by-products assayed were active on this target. No significant differences were observed among samples at the concentration of 10 mg/mL. Moreover, the inhibition of DPPIV was obtained after only 30 min, as showed by the enzyme slope (Fig. 8).

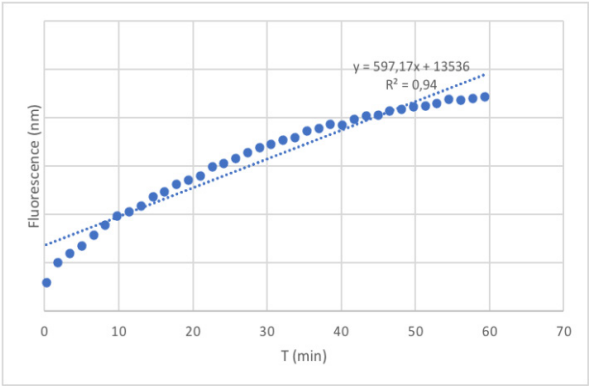

Figure 8. DPPIV slope measured at T0-T60. After 30 min, the enzyme activity, proportional to fluorescence, reached the plateau.

Further studies will be performed to define the concentration of samples able to inhibit 50% of DPPIV (IC_{50}) and identify the phenolic molecules mostly involved in this bioactivity.

4 Conclusions

The present study is focused on the preliminary characterization of the phenolic fraction of grape pomace samples and the evaluation of their *in vitro* biological properties using different analytical approaches. Spectrophotometric methods included a preliminary assessment of total polyphenol content in order to identify the most promising ones. In parallel, total procyanidins and anthocyanins were quantified to estimate their contribution to both TPC and antioxidant activity, the latter performed using two different assays. Generally speaking, procyanidins were the most abundant compounds in all the samples assayed and were less affected by the fermentation process. In addition, these compounds were also significantly correlated with antioxidant activity, measured by both DPPH and FRAP assays (p<0.05). The content of anthocyanins, present in the samples containing the skins, was weakly correlated with AOA (R^2<0.5), suggesting that these molecules are less involved in this biological activity. HPTLC confirmed the spectrophotometric data and showed interesting results not only for the screening of active compounds, but also for the evaluation of the antioxidant activity associated with each molecule. Malvidin 3-O-glucoside was the most abundant anthocyanin in all the by-products assayed, followed by cyanidin-3-O-glucoside and peonidin-3-O-glucoside. Grignolino and Uvalino samples were the richest in antioxidant molecules, represented mainly by epicatechin, belonging to procyanidins compounds with the highest antioxidant activity. Quantitative analysis is currently in progress to identify the most bioactive compounds. Finally, preliminary results on DPPIV showed that all samples were active in inhibiting the enzyme activity. Further studies will be conducted to confirm the results obtained. In conclusion, our data confirm that winemaking by-products could be used as a source of healthy compounds, according to the great scientific attention paid to winery by-products, especially to grape skins [7,8]. Our data show that also the seeds can be recovered and used for different purposes (dietary supplement and cosmetic formulations, food preservatives) implementing the circular economy. Grape skins from Barbera variety showed an unexpected content of anthocyanins; since in our previous studies [28] the same variety showed significant *in vitro* anti-inflammatory properties at gastric level, further studies will be performed to identify if other compounds, other than anthocyanins, could be involved in this biological activity.

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