



## In depth study of phenolic profile and PTP-1B inhibitory power of cold-pressed grape seed oils of different varieties



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### ABSTRACT

This paper investigates the phenolic composition of 17 monocultivar commercial cold-pressed grape seed oils. Chromatographic profiles showed the presence of more than 28 molecules, 11 of which were successfully identified by HPLC-DAD-MS-TOF and HPLC-FLD analysis. Pinoreosinol, ethyl caffeate and ethyl gallate were detected for the first time in these oils. The total phenolic content ranged between 0.83 mg/kg for Viognier sample to 15.16 mg/kg for Merlot org sample. The detected ethyl esters can be suggested as markers to evaluate the intensity of fermentation in grape seeds before oil extraction, and to control the sensorial quality of the produced oils. In addition, the inhibitory power of these phenolic extracts against Protein Tyrosine Phosphatase 1B enzyme (PTP-1B), overexpressed in type-two diabetes, was investigated for the first time. Data highlighted a good correlation between total phenolic content and inhibitory power, with pinoreosinol, *p*-coumaric acid and quercetin making the greater contributions.

### 1. Introduction

Grapes are primarily used in winemaking, with several European countries being the main producers together with Argentine and Chile in South America and California in the U.S.A. Grape pomace and grape seeds are the main by-products of the winery industry (Chemat, Li, Tomao, Ginies & Cravotto, 2014). In 2008, Matthäus estimated worldwide grape production at 60 million tons/year, with grape seeds close to 20% of fresh fruit and 40–60% of dried matter. Regarding the oil content, several studies reported values from 13% to a maximum of 19%, but also underlined that the oil is only partially recovered in cold-pressed extraction (Matthäus, 2008; Özcan, Al Juhaimi, Gülcü, Uslu, & Geçgel, 2017; Özcan, Endes, & Er, 2010; Özcan, Al Juhaimi, Uslu, et al., 2017). Other authors (Venkitasamy, The, Atungulu, McHugh & Pan, 2014) reported that 20% of grape production is typically formed by by-products (grape pomace), 47% of which are seeds. Data from the USDA (2013) reported for 2012 an estimated production of 150,000 tons/year of dried seeds, derived from 5.8 million tons of processed grapes. In this case, the reported range of estimated oil production is 10–22% of dry seeds, with values similar to those previously reported (Matthäus, 2008). A not negligible amount of grape seeds as by-products derived

also from juice extraction: in Brazil, approximately 42% of total grape production was marketed as fresh grape fruit (Shinagawa, De Santana, Torres & Mancini-Filho, 2015).

Grape seeds have different morphological aspects, variable content of lipids and minerals (Özcan, 2010), and present a certain difficulty to manage after wine production, mainly due to the risk of fermentation. A large part of these by-products are utilized for oil recovery by solvent extraction; a few preliminary studies were conducted to evaluate the effect of microwave on oil extraction yield (Özcan & Al Juhaimi, 2017), but some aliquots were used for oil extraction by mechanical process only. These latter products are commonly defined as cold-pressed grape seed oils or, in some cases, also virgin grape seed oils. The most critical aspects during production of cold-pressed grape seed oils are linked to seed size and to the drying process applied to strictly control the residual moisture content of the matrix after wine production. At the same time, technological aspects, such as correct screw-press parameters, are recognized as crucial to obtain a good oil with acceptable yields (Venkitasamy et al., 2014; Rombaut et al 2015). ‘Virgin’ grape seed oils are characterized by high levels of poly-unsaturated fatty acids and vitamin E (Bertrand & Özcan, 2011) and a light flavor with fruity notes, even though their organoleptic characteristics are strongly

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affected by the quality of by-products (Zhao, Yagiz, Xu, Fang & Marshall, 2017; Al Juhaimi, Geçgel, Gülcü, Hamurcu & Özcan, 2017; Garavaglia, Markoski, Oliveira & Marcadenti 2016; Shinagawa et al., 2015).

What has been proven for extra virgin olive oil and its properties for health has induced a renewed interest of the market toward cold-pressed grape seed oils. Grape seed oils are often cited for their potential benefits (Shinagawa et al., 2015; Garavaglia et al., 2016) but often it is not specified if the effects have been observed with refined or virgin oils.

Several works are available in literature on phenols in grape seeds extracts, obtained from fresh or dried seeds or from the residual cake after oil extraction (Rustioni & Failla, 2016; Maier, Schieber, Kammerer & Carle, 2009). On the contrary, findings regarding the phenolic profiles of cold-pressed grape seed oils are scarce. Almost all the available works since 2007 (Rombaut et al., 2014; Rombaut et al., 2015; Lutterodt, Slavin, Whent, Turner & Yu, 2011; Matthäus, 2008; Bail, Steuebiger, Krist, Unterweger & Buchbauer, 2008; Baydar, Özcan & Çetin, 2007) report only the evaluation of the total phenolic content, often applying the non-specific Folin Ciocalteu method (Baydar et al 2007; Bail et al 2008; Lutterodt et al., 2011), without identifying the chemical structure of at least the main constituents. Few groups have applied chromatographic analyses to study the composition of this fraction, reporting only the presence of catechin (Assumpção et al., 2014), vanilline and vanillic acid (Rombaut et al, 2014) and, recently, quercetin and rutin together with gallic and chlorogenic acids (Al Juhaimi & Özcan, 2017). Other authors detected epicatechin, epicatechin gallate and pentagalloylglucose in cold-pressed oils obtained from the Muscadine variety (Zhao et al, 2017). Nevertheless, these last works did not report mass spectra or a comparison with pure standards, which are necessary to definitively confirm the structure identification of the detected compounds. Consequently, some doubts remain regarding the presence of very polar phenolic compounds such as glycosylated and galloylated derivatives, which are known to have poor solubility in oil.

The aim of this work was to investigate the phenolic composition of cold-pressed grape seed oils from both qualitative and quantitative points of view. Furthermore, until now no data have been available regarding cold-pressed grape seed oils and the interaction with the enzyme Protein Tyrosine Phosphatase 1B (PTP-1B), a typical target of investigations on type-two diabetes. A recent review discussed the numerous approaches applied to find selective inhibitors of PTP-1B enzyme (Verma, Ji Gupta, Chaudhary & Garg, 2017), which acts as a negative regulator of insulin and leptin receptor signaling pathways. Pharmacological inhibition of PTP-1B enhances insulin sensitivity, improves glycemic control, and favors loss of body weight (Qian, Zhang, He, Wang & Liu, 2016).

Our investigation focuses on studying, by HPLC-DAD-MS-TOF and HPLC-FLD, the phenolic profile of several cold-pressed grape seed oils from qualitative and quantitative points of view, and to examine the *in vitro* power of their phenolic fractions to inhibit the PTP-1B enzyme, overexpressed in type-two diabetes. It is worth noting that the study is based on 17 commercial oils, most of them monocultivar, obtained not at laboratory scale but at industrial scale and derived from the same producer.

## 2. Material and methods

### 2.1. Chemicals

Ethanol, hexane and formic acid of analytical reagent grade were from Sigma-Aldrich (Steinheim, Germany). Acetonitrile of HPLC and HPLC-MS grade were purchased from Panreac (Barcelona, Spain). The Milli-Q-system (Millipore SA, Molsheim, France) was used to produce deionized water. Pinoresinol ( $\geq 95\%$ ) and *p*-coumaric acid ( $\geq 98\%$ ) from Sigma-Aldrich (Steinheim, Germany) were used as standard

**Table 1**

List of the analyzed grape seed oil samples.

n°	Sample name	Best before date
1	Viognier	Jan-16
2	Sangiovese	Jan-16
3	Cabernet Sauvignon	Oct-16
4	French Colombard	Oct-16
5	Sauvignon blanc	Oct-16
6	Riesling	Oct-16
7	Chenin blanc	Nov-16
8	Pinot noir	Dec-16
9	Merlot	Dec-16
10	Petite Sirah organic	Mar-17
11	Merlot organic	Mar-17
12	Cabernet Sauvignon organic	Mar-17
13	Zinfandel	Mar-17
14	Chardonnay	Sep-17
15	Sirah	Nov-17
16	Sample A organic*	Sep-16
17	Sample B organic*	Sep-16

\* From Italian market.

compounds. Other standards as vanillic, ferulic and syringic acids, *E*-resveratrol, tyrosol, and kaempferol were purchased from Extrasynthèse (France).

Human recombinant PTP-1B was expressed in *Escherichia coli* TB1 strain, and purified as previously described (Paoli et al., 2013). 4-nitrophenyl phosphate disodium salt hexahydrate were from Santa Cruz Biotechnology.

### 2.2. Samples

#### 2.2.1. 'Virgin' grape seed oils

All the selected mono-varietal cold pressed grape seed oils are listed in Table 1, with the best-before-date and the corresponding grape variety. The 15 Californian samples, three of which being organic samples, were from SaluteSantè, Napa, California, USA, while the further two organic samples of unknown variety were purchased from Italian market. All samples from SaluteSantè were from sun-dried grape seeds, then cold pressed with a screw press applying a pressure of 35–55 MPa. One ton of grape seeds was pressed in each extraction cycle; the oil yields were about 10% w/w. No filtration was applied, but the oils were clarified by sedimentation.

#### 2.2.2. Grape seeds

A sample of sun-dried grape seeds of Sangiovese variety was purchased from a Tuscan farm, then milled at laboratory scale in order to obtain a homogenous powder which was used for the successive extraction with the sunflower oil.

### 2.3. Extraction of phenolic compounds

#### 2.3.1. Extraction of oil samples

The extraction conditions to recover the phenolic fraction were the same already applied to olive oil samples (Cecchi et al., 2017). Briefly, 20 g of sample were added to a flask, extracted in 60 mL of ethanol/acidic water (pH 3.2 by formic acid) 7:3 v/v and stirred for 30 min. The obtained mixture was defatted three times with *n*-hexane (20 mL each time); when the separation of the two phases was incomplete due to formation of emulsion, few mL of ethanol were added to broke this emulsion. The hydroalcoholic phase was recovered, evaporated under vacuum at room temperature, and the residue redissolved in 1.5 mL of ethanol/acidic water (pH 3.2 by formic acid) 7:3 v/v. The obtained solution was centrifuged at 14,000 rpm and the supernatant was used for the chromatographic analysis.

### 2.3.2. Extraction of grape seed sample

The seeds from Sangiovese were extracted in commercial sunflower oil; the oil was previously analyzed to rule out the presence of native phenols. 10 g of powdered grape seeds were added to a flask together with 100 g of sunflower oil and extracted for 25 min in an ultrasound bath at 30 °C, and then stirred for 60 min at room temperature. The mixture was filtered and the phenolic fraction was extracted from the obtained oil as previously described for the cold pressed oils.

### 2.4. HPLC-DAD-MS-TOF and HPLC-DAD-FLD analysis of phenolic extracts

The analyses of phenolic compounds were carried out with an HP 1100 Liquid Chromatograph coupled with DAD and TOF Mass Spectrometer detector equipped with electrospray interface (ESI), all from Agilent Technologies (Palo Alto, CA, USA). The column was a Poroshell 120, EC-C18 (150 mm × 3 mm i.d., 2.7 μm) equipped with a precolumn of the same phase (Agilent Technologies); oven temperature, 26 °C. Solvents for elution were (A) 0.1% formic acid/water and (B) acetonitrile. The multi-step linear solvent gradient varied as follows: 0–5 min 10–15% B; 5–15 min 15–30% B; 15–20 min 30–35% B; 20–23 min 35–40% B; 23–26 min 40–45% B; 26–32 min 45–100% B; 32–37 min 100% B; 37–42 min 100–10% B; equilibration time 10 min; flow rate 0.4 mL/min. We acquired chromatograms at 240 nm, 280 nm, 330 nm, 350 nm and 540 nm, and UV spectra in the wavelength range of 200–600 nm. Mass spectra were acquired in negative ion mode in a mass range of 80–1200 *m/z*. The ESI source was set as follows: drying gas (N<sub>2</sub>), temperature 350 °C, drying gas flow rate 6 L/min, nebulizer 20 psi, capillary voltage 3800 V, fragmentation 150 V, skimmer 60 V. The acquisition data was done by the Agilent MassHunter Qualitative Analysis Software, version B.06.00 (Agilent Technologies). The TOF mass spectrometer was calibrated immediately before the analyses and no internal reference was used. The accurate mass of the molecules related to the main peaks was measured and the elemental compositions were calculated, considering a maximum difference of 10 ppm between the mass of the calculated and measured formulas.

To better investigate the lignan content, some analysis were repeated using the same chromatographic conditions and the same apparatus, but equipped with a fluorimetric detector (FLD). Regarding the FLD, the excitation wavelength was 280 nm, and the emission wavelength was set at 339 nm, according to Servili et al. (2007).

Quantification of phenolic compounds was carried out by the external standard method, using the following standards: *p*-coumaric acid was used to build a five-point calibration curve at 280 nm; vanillic acid, *p*-hydroxybenzoic acid, syringic acid, *p*-coumaric acid, ferulic acid, ethyl gallate, ethyl caffeate, *E*-resveratrol, quercetin and kaempferol were expressed as mg of *p*-coumaric acid per kg of oil (mg<sub>*p*-cum</sub>/kg). Total phenolic content (TPC) was evaluated on the total area of peaks in the range 4–33 min of the chromatograms at 280 nm and was expressed as mg<sub>*p*-cum</sub>/kg. Pinoreosin was used to build a five-point calibration curve at 280 nm; pinoreosin was expressed as mg of pinoreosin per kg of oil (mg<sub>pin</sub>/kg). All the analytes in Table 2 were evaluated at 280 nm with the only exception of *p*-coumaric acid, for which a calibration curve at 330 nm was built because this molecule was partially co-eluted with ethyl gallate, which does not shown absorbtion at 330 nm.

Limit of quantifications (LOQ) were estimated according to the Eurachem Guide (Magnusson & Ormemark, 2014) using the standards pinoreosin (LOQ, 0.053 mg/kg) and *p*-coumaric acid (LOQ, 0.015 mg/kg).

### 2.5. Inhibition's assays of enzyme PTP-1B by the phenolic grape seed oil extracts

Inhibition assays of the enzyme PTP-1B was carried out using *p*-nitrophenylphosphate (pNPP) 2.5 mM as substrate. The assay buffer contained sodium β,β-dimethylglutarate buffer (75 mM, pH 7.0), EDTA (1 mM), and dithiothreitol (1 mM) in addition to pNPP. Solutions of

**Table 2**  
Phenolic compounds identified in the cold pressed oils. Results are expressed as mg<sub>*p*-cum</sub>/kg (mean ± SD); LOQ, 0.015 mg/kg.

Sample	<i>p</i> -hydroxybenzoic acid	vanillic acid	syringic acid	<i>p</i> -coumaric acid	ethyl gallate	ferulic acid	<i>E</i> -resveratrol	quercetin	ethyl caffeate	kaempferol	TPC
Vignier	< LOQ	< LOQ	0.079 ± 0.004	0.161 ± 0.008	< LOQ	< LOQ	< LOQ	0.082 ± 0.018	< LOQ	< LOQ	0.827 ± 0.099
Sangiovese	< LOQ	0.095 ± 0.005	0.104 ± 0.005	0.381 ± 0.020	0.017 ± 0.002	0.029 ± 0.003	< LOQ	< LOQ	< LOQ	< LOQ	2.713 ± 0.326
Cabernet Sauvignon	0.043 ± 0.003	0.127 ± 0.007	0.260 ± 0.013	0.191 ± 0.010	< LOQ	0.038 ± 0.004	0.096 ± 0.010	0.207 ± 0.046	< LOQ	0.043 ± 0.007	4.629 ± 0.556
French Colombard	0.036 ± 0.003	0.122 ± 0.007	< LOQ	0.573 ± 0.030	< LOQ	0.048 ± 0.004	< LOQ	0.343 ± 0.076	< LOQ	0.031 ± 0.005	4.973 ± 0.597
Sauvignon Blanc	0.032 ± 0.003	0.124 ± 0.007	< LOQ	0.712 ± 0.037	< LOQ	0.051 ± 0.005	< LOQ	0.379 ± 0.084	< LOQ	0.025 ± 0.004	5.563 ± 0.668
Riesling	0.040 ± 0.003	0.130 ± 0.007	< LOQ	0.891 ± 0.047	< LOQ	0.068 ± 0.006	< LOQ	< LOQ	< LOQ	0.029 ± 0.005	6.776 ± 0.813
Chenin Blanc	0.036 ± 0.003	< LOQ	< LOQ	0.717 ± 0.038	< LOQ	0.052 ± 0.005	< LOQ	0.493 ± 0.109	< LOQ	0.025 ± 0.004	5.896 ± 0.708
Pinot Noir	0.060 ± 0.005	0.437 ± 0.025	0.398 ± 0.020	0.257 ± 0.014	0.590 ± 0.051	0.131 ± 0.012	0.252 ± 0.026	0.241 ± 0.053	0.341 ± 0.027	0.349 ± 0.057	9.172 ± 1.101
Merlot	0.025 ± 0.002	0.101 ± 0.006	0.114 ± 0.006	0.552 ± 0.029	< LOQ	0.031 ± 0.003	0.030 ± 0.003	0.129 ± 0.029	0.062 ± 0.005	0.036 ± 0.006	2.944 ± 0.353
Petit Syrah Org.	0.023 ± 0.002	0.190 ± 0.011	0.420 ± 0.021	0.249 ± 0.013	0.061 ± 0.005	0.064 ± 0.006	0.055 ± 0.006	0.458 ± 0.101	0.048 ± 0.004	0.131 ± 0.022	6.253 ± 0.750
Merlot Org.	0.080 ± 0.006	0.537 ± 0.030	0.874 ± 0.044	0.528 ± 0.028	0.170 ± 0.015	0.092 ± 0.008	0.094 ± 0.010	1.094 ± 0.242	< LOQ	0.218 ± 0.037	15.158 ± 1.819
Cabernet Sauvignon Org.	0.049 ± 0.004	0.317 ± 0.018	0.831 ± 0.042	0.459 ± 0.024	0.085 ± 0.007	0.061 ± 0.006	0.060 ± 0.006	< LOQ	0.100 ± 0.008	0.227 ± 0.036	9.962 ± 1.196
Zinfandel	< LOQ	0.066 ± 0.004	< LOQ	0.500 ± 0.026	< LOQ	0.031 ± 0.003	< LOQ	< LOQ	< LOQ	0.016 ± 0.003	2.553 ± 0.306
Chardonnay	0.045 ± 0.004	< LOQ	< LOQ	0.218 ± 0.011	0.072 ± 0.006	0.042 ± 0.004	0.041 ± 0.004	0.132 ± 0.029	< LOQ	0.051 ± 0.008	3.835 ± 0.460
Italian SampleA Org.	0.050 ± 0.004	0.289 ± 0.016	0.518 ± 0.026	0.615 ± 0.032	0.083 ± 0.007	0.089 ± 0.008	< LOQ	< LOQ	< LOQ	0.036 ± 0.006	8.173 ± 0.981
Italian SampleB Org.	0.037 ± 0.003	0.314 ± 0.018	0.579 ± 0.030	0.417 ± 0.022	0.078 ± 0.007	0.068 ± 0.006	0.048 ± 0.005	< LOQ	0.040 ± 0.003	0.148 ± 0.024	7.861 ± 0.943
Italian SampleB Org.	0.047 ± 0.004	0.328 ± 0.018	0.629 ± 0.032	0.434 ± 0.023	0.091 ± 0.008	0.080 ± 0.007	0.049 ± 0.005	< LOQ	0.049 ± 0.004	0.179 ± 0.029	9.202 ± 1.104

grape seed oil extracts (0.66 g<sub>oil</sub>/mL) were used as putative inhibitor of PBP-1B.

Inhibitory assays were carried out at 37 °C on a solution of inhibitor (10 µL) and substrate (990 µL). Reactions started by addition of aliquots of the enzyme preparation (Paoli et al., 2013) and stopped with KOH 0.2 M (2 mL). The released *p*-nitrophenolate was quantified by reading the absorbance of the final solution at 400 nm ( $\epsilon = 18,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). Percentage of inhibition of each extract was calculated by comparing the absorbance of the assays with that of a control test, carried out in the same condition but in absence of the inhibitor solutions. The results of all the assays were reported as a mean of three experiments.

IC<sub>50</sub> values for some inhibitors were calculated. To this aim, 12 different dilutions of the phenolic extracts (range of concentration 0.001333–13.33 g<sub>oil</sub>/mL) obtained from grape seed oils as described in paragraph 2.3, were used for the inhibitory assays, carried out as described above. The IC<sub>50</sub> values for the PTP-1B inhibitors were determined by fitting experimental data using the following equation (Paoli et al., 2013):

$$y = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x}{\text{IC}_{50}}\right)^{\text{slope}}} + \text{Min}$$

where  $y = v_i/v_o$ , is the ratio between the activity measured in the presence of the inhibitor ( $v_i$ ) and the activity of the control without the inhibitor ( $v_o$ ). The parameter “ $x$ ” is the inhibitor concentration.

## 2.6. Precision parameters

To evaluate the precision of the procedure for the quantitation of each phenolic compound, we prepared a blend of all the oil samples, weighting and mixing aliquots of each of them until reaching a homogeneous oil solution. This solution was used as reference sample. The extraction and analysis of the phenolic compounds were repeated six times starting from different aliquots of the reference sample and the obtained results expressed in terms of CV% (Supplementary Table S1).

## 2.7. Statistical analysis

All computations related to the Pearson Correlation Coefficient reported in Fig. 4 were carried out by EXCEL software (version 2013) in-house routines

## 3. Results and discussion

### 3.1. Phenolic characterization

Our aim was to investigate the phenolic composition of ‘virgin’ grape seed oils. To this aim, we selected and analyzed 17 oils by HPLC-DAD-MS and HPLC-DAD-FLD, in order to compare their phenolic profiles and to estimate the total phenolic content using suitable external standards. The applied liquid/liquid extraction was similar to that previously used to recover the minor polar compounds of olive oil (Cecchi et al., 2017) and the separation was obtained without the need to add Tween 20, as previously suggested for these oils (Maier, et al., 2009). Fig. 1 shows the chromatographic profiles at 280 nm for the Pinot Noir sample: 11 compounds were successfully assigned to specific phenols on the basis of their retention time, UV-Vis and mass spectral data, and the comparison with a pool of pure standards (Supplementary material, Table S1 and Fig. S1). Other peaks were not identified despite the acquisition of their spectral data (Supplementary material, Table S2) and the consultation of a specific data bank for phenolic compounds (<http://phenol-explorer.eu/>). Further analytical efforts will be required for their structural identification. With the help of pure standards and use of the extract ion technique, it was possible to exclude the presence of detectable amounts of epicatechin and catechin, previously cited as main components of the phenolic fraction of these oils (Assumpção et al., 2014; Zhao et al., 2017), and of epicatechin gallate and pentagalloyl glucose recently reported in oils from Muscadine variety (Zhao et al., 2017). Analogously, gallic and chlorogenic acids, previously found in ‘virgin’ grape seed oils from Muscadine variety, were not detected in our samples (Zhao et al., 2017). Furthermore, the total amounts reported by these latter authors appears really too high, with values up to 697 mg/kg; these values are largely higher even than the amounts detected in high quality extra virgin olive oils. The amount of identified phenolic compounds in each of the analyzed grape seed oil samples is summarized in Table 2.

It is worth pointing out that none of the previous works mentioned the presence of lignans in the phenolic fraction of cold-pressed grape seed oils. In the present work, the presence of these molecules was confirmed by HPLC-MS-TOF analyses in negative ionization mode, the extract ion chromatogram at 357.13 Th, and the comparison with pinoresinol standard (retention time, UV and mass spectra). A second

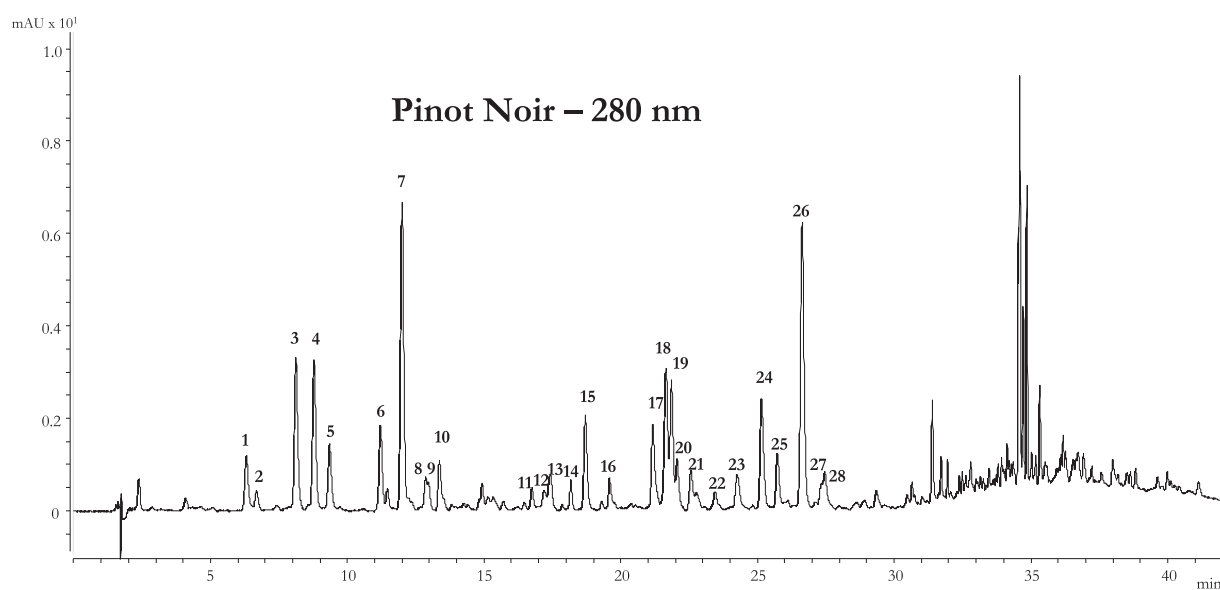


Fig. 1. Chromatographic profile at 280 nm for the Pinot Noir sample. The identified molecules: 2, *p*-hydroxybenzoic acid; 3, vanillic acid; 4, syringic acid; 7, ethyl gallate + *p*-coumaric acid; 10, ferulic acid; 15, *E*-resveratrol; 17, quercetin; 18, pinoresinol; 19, ethyl caffeate; 24, kaempferol. (Spectral data of both identified and unidentified compounds are reported in Supplementary material, Tables 1S and 2S).

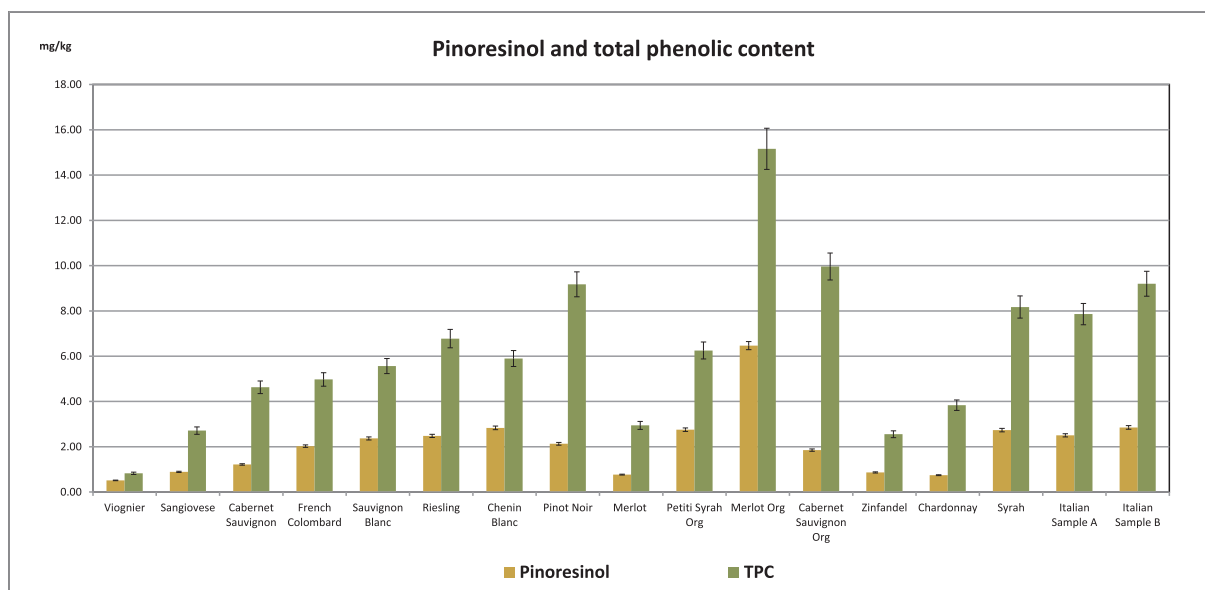


Fig. 2. Pinoresinol and total phenolic content (TPC) of the 17 oil samples. Pinoresinol content is expressed as mg<sub>pin</sub>/kg; total phenolic content is expressed as mg<sub>coum</sub>/kg.

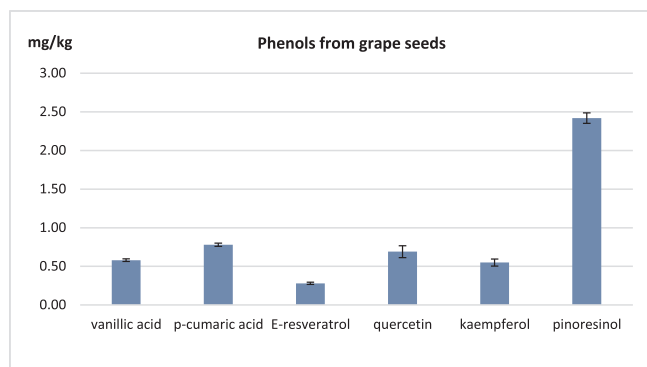


Fig. 3. Phenols extracted by refined oil from dried powder of Sangiovese grape seeds. All phenolic compounds are expressed as mg<sub>p-coum</sub>/kg, with the only exception for pinoresinol, which is in mg<sub>pin</sub>/kg.

peak with the same molecular ion of pinoresinol (confirmed by the adduct with formic acid) and retention time higher than pinoresinol was detected in a few samples. In order to better investigate the structure of this molecule, the same chromatographic analyses were repeated using a fluorimetric detector (FLD). This detector, selective for the lignans pinoresinol and 1-acetoxypinoresinol in olive oil (Servili et al., 2007), allowed exclusion of this molecule as an isobaric derivative of pinoresinol. As shown in Fig. 2, the presence of pinoresinol was confirmed in all the analyzed samples, with values in the range between 0.513 mg/kg (Viognier sample) and 6.468 mg/kg (Merlot Org sample), with a mean concentration close to 2–3 mg/kg in the other oils.

The presence of the ethyl esters of caffeic and gallic acids in the phenolic fraction of several oils was revealed; ethyl gallate was detected in nine of the 17 samples in concentrations up to 0.590 mg/kg, and ethyl caffeate was detected in seven oils in concentrations up to 0.341 mg/kg. The highest content of the two esters was detected in the Pinot Noir sample (Table 2), suggesting these grape seeds had undergone to a stronger fermentation of the residual sugars, presumably developed during the drying process (Ovcharova, Zlatanov & Dimitrova, 2016). It is well known that sugar fermentation leads to ethanol formation (Angerosa, Lanza & Marsilio, 1996) which is needed for the synthesis of ethyl esters. According to the literature, these esters can be suggested as possible markers to evaluate the intensity of the

fermentation process in grape seeds before oil extraction, but also to control the sensorial quality of the final pressed oil (Di Serio et al., 2017).

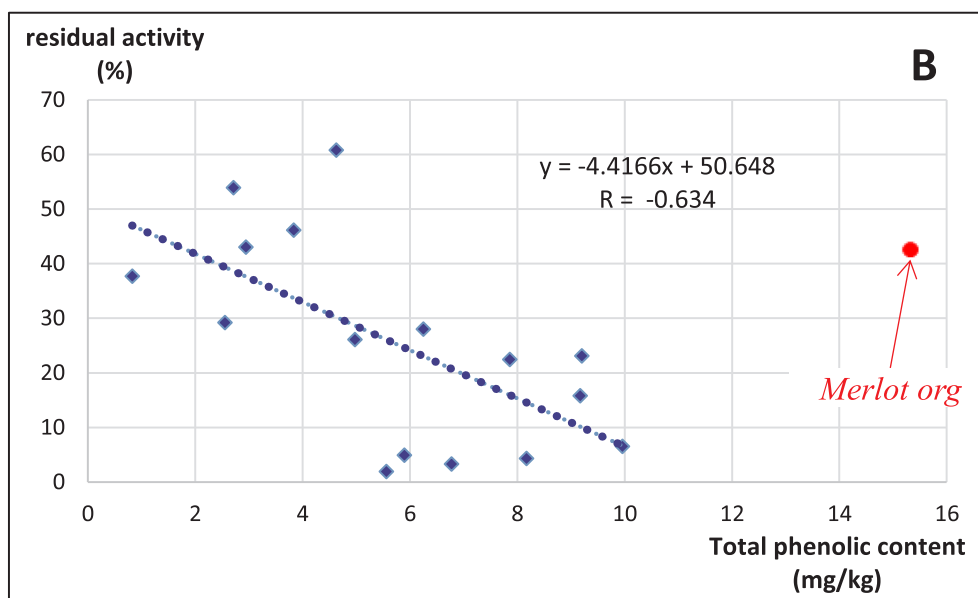
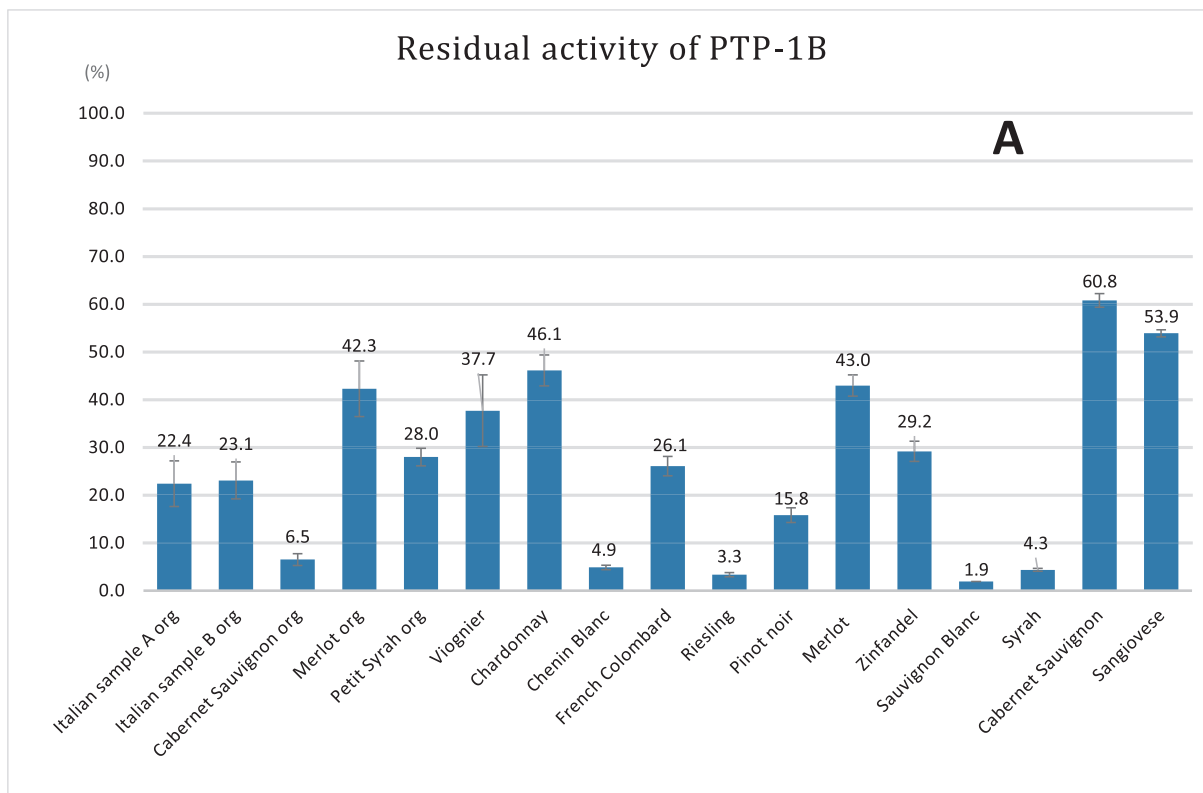
Overall, the quantitative data for the total phenolic compounds content are in agreement with those obtained with a similar analytical approach by Maier et al. (2009), who reported 2.9 mg/kg as maximum amount. At the same time, our results strongly disagree with other authors who recently reported concentrations of total phenols over 600 mg/kg (Zhao et al., 2017); a clearly described quantitative procedure was not applied in this work. However, bearing in mind that the total phenolic compounds content in extra virgin olive oils exceeds only in a few cases 500–600 mg/kg, the values indicated for the grape seeds oils by these latter authors seem to be largely overestimated.

### 3.2. Research of pinoresinol in grape seeds

To the best of our knowledge, this is the first time the presence of pinoresinol is reported in cold-pressed grape seed oils; we presume it is also present in grape seeds. In another oleaginous matrix, namely olives (*Olea europaea* L.), the presence of lignans (pinoresinol and 1-acetoxypinoresinol) before the milling process has not yet been confirmed, despite their presence in the corresponding virgin olive oils (Cecchi et al., 2013; Cecchi, Migliorini, Cherubini, Innocenti & Mulinacci, 2015).

In this study, we analyzed a set of oils purchased from a production plant that works with about one ton of seeds per time and, consequently, it is not possible to completely exclude the co-presence of vine shoots as a possible source of lignans. To clarify this, we investigated the presence of the lignan pinoresinol at laboratory scale in fresh grape seeds of a widespread wine variety, namely Sangiovese. In order to simulate the seed contact with the extracted oil during the productive process, seeds were ground and extracted with a commercial refined sunflower oil, previously analyzed to exclude the presence of detectable amounts of phenolic compounds.

The histogram in Fig. 3 clearly shows that the typical components (catechins, procyanidins, gallicolates) of either aqueous or hydroalcoholic extracts of grape seeds are absent in the oil extract. On the other hand, the presence of pinoresinol as the principal extractable compound from the seeds was confirmed. Even though the test was carried out on a raw material different from that used to produce the analyzed oils, it demonstrated that this lignan can be considered a



**Fig. 4.** Residual activity of PTP-1B in presence of hydroalcoholic extracts of the analyzed samples evaluated at the same concentration (6.67 mg<sub>oil</sub>/mL) (A). Correlation between residual activity of PTP-1B and total phenolic content of the analyzed extracts (B); the red point indicates the outlier. Correlations between the amount of each phenolic compound and residual activity of PTP-1B for the seventeen 'virgin' grape seed oils (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

component of grape seeds. To date, pinoresinol has never been detected in this matrix, presumably because the extraction procedures were not suitable to recover this lipophilic phenol and/or because the molecule is present at very low concentrations in seeds.

### 3.3. *In vitro* inhibition of PTP-1B enzyme

To evaluate the potential contribution of consuming cold-pressed grape seed oils to reduce the risk of type-two diabetes, a study on a specific enzymatic target, PTP-1B, was initially carried out working with the phenolic extracts of the selected oils.

After some preliminary tests (necessary to select the suitable

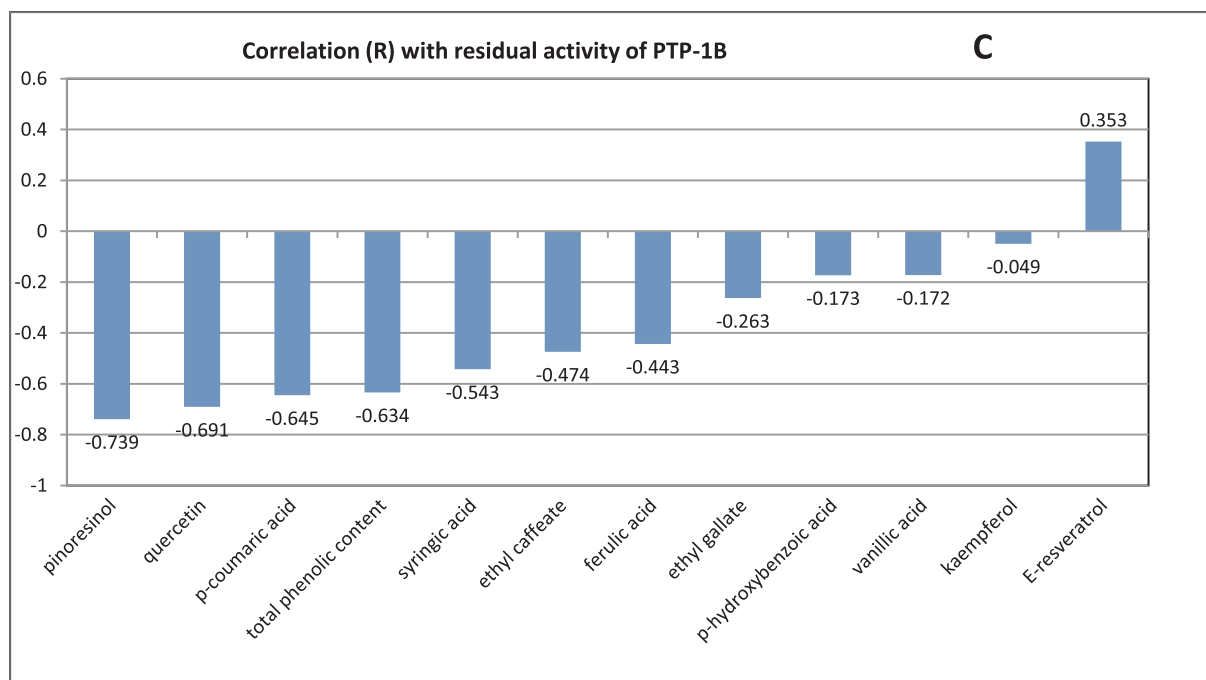


Fig. 4. (continued)

concentration), the inhibitory power of the extracts was evaluated testing all the samples at a concentration of 6.67 mg<sub>oil</sub>/mL. As summarized in Fig. 4A, different potencies were found for the 17 samples. Maximum inhibition was close to 93–98% for a group of five oils, while minimum inhibition power was shown by Caberbet Sauvignon (close to 40%).

In order to verify a correlation between the inhibitory activity and the total phenolic content (TPC), we estimated the IC<sub>50</sub> value of the samples at the lowest (Viognier) and highest (Merlot org) TPC. The curves in Figures A and B in Supplementary Fig. S2 show a very similar power for both extracts (corresponding to 5.33 mg<sub>oil</sub>/mL) in spite of the consistently different phenolic content (Fig. 2), suggesting no correlation between TPC and inhibitory power. Nevertheless, Fig. 4B shows a good correlation between TPC and the inhibitory power and points out that Merlot Org is clearly an outlier. This result can partially explain the similar IC<sub>50</sub> values obtained for Viognier and Merlot Org samples.

Fig. 4C reports the Pearson correlation coefficients between the residual activity of PTP-1B and the amount of each identified phenolic compound for the 17 ‘virgin’ grape seed oils. Pinoresinol showed the highest negative correlation value among the identified phenols (R, -0.739); a slightly lower correlation was observed for *p*-coumaric acid and quercetin. The latter molecule is a flavonol already known as an inhibitor of PTP-1B, with an IC<sub>50</sub> value of 0.98 μM as pure molecule (data not shown). All the other phenolic compounds identified in the extracts showed R values close to or below -0.5.

These preliminary results showed that the inhibitory power is partially correlated to the phenolic content of these ‘virgin’ grape seed oils and that pinoresinol and quercetin seem to give the highest contribution within this group of molecules. However, further studies are needed to complete identification of the other minor constituents of the seed oils and of their inhibition power of PTB-1B enzyme.

#### 4. Conclusions

A detailed study on the phenolic content of a large pool of commercial cold-pressed grape seed oils determined by HPLC-DAD-MS-TOF and HPLC-FLD analysis is reported in this work. The presence of pinoresinol was confirmed for the first time in all these oils, together with some main flavonols such as quercetin and kaempferol, while catechin

and its gallate forms were not detected, a result in disagreement with some previous works. Ethyl caffeate and ethyl gallate, detected in many of these oils, can be suggested as markers to evaluate the intensity of fermentation in grape seeds before oil extraction, but also to control the sensorial quality of the final oils.

Lastly, an inhibitory activity exerted by the phenolic fraction isolated from these oils against PTP-1B, an enzyme overexpressed in type-two diabetes, was demonstrated. This interesting data begs for further studies to confirm this action with other cold-pressed grape seed oils and to understand the mechanism behind this action.

Overall, these results highlight the greater health properties of the cold-pressed grape seed oils with respect to refined oils, which do not contain the pool of the phenolic molecules.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.07.140>.

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