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Chemical characterisation, antioxidant and antimicrobial screening for the revaluation of wine supply chain by-products oriented to circular economy

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ABSTRACT

Aim of the project was the bioassay guided optimisation of extraction methods applied to wine chain by-product to obtain extracts, fractions and biologically active biomolecules with a possible use in the nutraceutical and cosmeceutical industry. Exhausted red and white grape marc were extracted using water:ethanol 50:50 with ultrasound assisted extraction and Naviglio® technology; and also with supercritical fluid extraction (SFE) and steam-distillation obtaining different phytocomplexes. Each extract was characterised by different molecular category: exhausted red grape marc (VCR) by anthocyanins, exhausted white grape marc (VCB) by flavonoids, and grapeseed (VIN) by proanthocyanins. SFE and steam-distillation, instead, highlighted the presence of fatty acids and their ester in every matrix, but terpenoids were not revealed at level major or equal than 0.1%, except for manoyl oxide in VCR (2.89%). VIN was the most abundant matrix in polyphenols (506.24 ± 55.91 mg gallic acid/g dried extract), and it showed the highest antioxidant activity (IC_{50} of 4.30 μg/mL). Regarding the antimicrobial activity, the hydroalcoholic extracts from VCR, VCB and VIN were tested but no noteworthy activities have been recorded.

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Introduction

The constant increase in the anthropic impact on the environment in terms of pollution and exploitation of resources has stimulated governments and research institutions to focus on new production approaches, consolidating the new paradigm of the circular economy, concretized industrially in the concept of bio-refinery. While the concept of circular economy can be defined as a self-sustaining system, the term bio-refinery concretizes the same concept at the level of industrial production processes, in which the output yields are maximized, minimizing or eliminating the production of waste. In this context, what traditionally was considered a production waste becomes a secondary raw material converted into new marketable products using a panel of production strategies with low environmental impact (Lin et al. 2013). This new paradigm of research and production has generated and consolidated the interaction of knowledge and skills to make the reality of bio-refinery more and more concrete. The shift of industry towards greater sustainability, to improve cost-effectiveness, process efficiency and ecological credentials, makes the development of sustainable and innovative waste-re-use strategies economically viable. Waste produced by food processing companies is an example of a type of waste generated on a large scale and globally. This type of waste is becoming increasingly problematic, in some cases it can represent more than 50% of the total waste produced in the countries, of which at least 60–70% is made up by organic substances. In line with this data, the case of the wine industry is exemplificative: in 2016, the International Organisation of Vine and Wine (OIV) indicate that 55% of the global grape production (75.8 million of tons) was wine grape. The volume of the wine industry waste is comprehensibly of the same order of magnitude, it is estimated that for each 6 L of wine, 1 kg of grape pomace is produced (Mendes et al. 2013). The valorization of this secondary raw material – no longer waste – could be an opportunity for the industry to increase its competitiveness by converting its disposal costs into new profit strategies (new products). In general, the state of the art of exploiting agri-food waste essentially consists of two types of approaches (Lin et al. 2013): (a) first generation approach: the waste is sent to composting, used for energy production (anaerobic digestion) or transferred to landfill; (b) second-generation approach: the waste is directed towards processes characterised by high technological level, high sustainability and reduced environmental impact to obtain high value (bio) chemicals. Emerging evidences in the last 20 years have suggested that vinification waste can be a relevant source of useful molecules in the contest of human well-being. Therefore, among the plethora of recycle action implemented by the wine industry, the extraction and the recovery of phenolic compounds also took place (Louli et al.

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2004, Tournour et al. 2015). As mentioned above, one of the main solid by-products produced during the wine-making process is grape pomace, and this, in accordance to the European Council Regulation 1493/1999 on the common organisation of the wine market, must be sent to alcohol distilleries, producing alcohol and tartrates, obtaining another by-product, named exhausted grape marc (EGM). In this contest, the aim of the research was the optimisation of the extraction methods applied to this by-product to obtain extracts and fractions, rich in biologically active biomolecules, with a possible use in the nutraceutical and cosmeceutical industry.

Materials and methods

Plant material

EGM from white (cultivar Trebbiano) and red (cultivar Lambrusco) *Vitis vinifera* L. was provided from Caviro Distillerie (Faenza, Ravenna – Italy) after being harvested in 2016. At the time of delivery, dealcoholized red and white EGM had, respectively, the 51% and the 36% moisture content. They were oven dried at 70 °C for 24 h until constant weight with a high-performance oven (mod. 2100), milled through a 2-mm sieving ring of a Variable Speed Rotor Mill (Fritsch, Germany) and immediately stored at –20 °C until further use. Part of the marc samples, either white (VCB) or red (VCR), was not dried but immediately steam distilled to evaluate the quality and quantity of the volatile component.

Chemicals

All the solvents and reagents employed for analyses were chromatographic grade. Standard malvidin-3-O-glucoside was purchased from Extrasynthese (Genay, France). Trolox, DPPH (1,1-diphenyl-2-picrylhydrazil), methanol deuterate, chloroform deuterate, methanol, ethyl acetate, ethanol, formic acid, acetic acid, toluene, natural products-polyethylene glycol reagents (NP/PEG) and gallic acid were purchased from Sigma-Aldrich Italy (Milano, Italy).

Ultrasound-assisted extraction of EGM and grapeseed

Ultrasound-assisted extraction was performed in an ultrasonic cleaning bath (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus) under a working frequency of 48 kHz. Fifteen grams of each sample were placed into a volumetric flask (200 mL), filled with 195 mL of a 50% ethanolic solution as extraction solvent and sonicated for 80 min at room temperature (solvent/solid ratio of 13 mL/g of dried pomace). The extracts were filtered and lyophilized.

Naviglio® extractions of EGM and grapeseed

Naviglio® extractor (Atlas Filtri, Italy) was used to extract solid material with a pressurized solvent extraction method (Naviglio 2003). Briefly, 30 g of each samples were placed in a bag made of 60 μm filtering membrane and transferred into the chamber of the Naviglio extractor, and 400 mL of a 50% ethanolic solution were added. Static phase was set for 5 min, while the dynamic phase was set for 3 min for a total extraction time of 80 min to complete 10 cycles. Each extraction was made in triplicate.

Supercritical fluids extraction of EGM and grapeseed

Samples were subjected to supercritical fluid extraction (SFE) using an Applied Separations (Allentown, PA) model Speed SFE extractor. Extractions were performed on each type of solid matrix (2 g) under the following operating conditions: carbon dioxide flow-rate of 2.5 L/min; oven temperature was set at 42 °C, restrictor temperature at 62 °C, and pressure at 150 atm.

Steam distillation of EGM and grapeseed

Fresh EGM (100 g) was used to obtain essential oils by 4 h steam distillation (DIS) with a Clevenger apparatus according to European Pharmacopoeia methods. The extract yield was determined on a volume to dry weight basis, obtaining the data reported in Table 1. The samples were dried over anhydrous sodium sulphate and stored in glass vials with Teflon-sealed caps at –18 ± 0.5 °C in the absence of light until analysis.

HPTLC analysis of the extracts

Analyses were performed on a high-performance thin layer chromatography (HPTLC) silica gel $60F_{254}$ (10 cm × 20 cm) glass plate (Camag, Swizerland); 50% ethanolic solution of the extracts (20 mg/mL) were applied using Linomat V (Camag, Swizerland). Spots were eluted in two steps with different eluents. First step: ethyl acetate/formic acid/acetic acid/water (100/11/11/20), second step: toluene/ethyl acetate/acetic acid (100/90/10), in two chromatographic chambers (Wagner and Bladt 2009). After development, the chromatogram was derivatized with NP/PEG solution (Wagner and Bladt 2009). The developed plate was dried at room temperature visualized with the Visualizer (Camag, Swizerland).

HPLC analysis of EGM and grape seeds extracts

The analyses of anthocyanins in VCR were performed using a Waters modular HPLC system (MA, model 1525) coupled to a diode array detector (model 2998) linked to a 20-μL sampler loop. The column used was a Luna C18 column (260 mm

Table 1. Procyanidins characterised by ESI-MS^2 in VIN extracts.

Compounds	$[M-H]^-$ (m/z)	MS/MS (m/z)
Epi/catechin	289	
Procyanidin dimer B	577	533, 439, 425, 269
Procyanidin dimer gallate	729	577, 451, 425, 289
Procyanidin trimer	865	739, 695, 577, 407
Procyanidin dimer digallate B	881	729, 577, 559, 407
Procyanidin trimer gallate	1017	865, 729, 695, 577
Procyanidin tetramer	1153	865, 983, 695, 577
Procyanidin trimer digallate	1169	1017, 881, 729, 577
Procyanidin tetramer digallate	1457	1306, 1153, 865, 729

×4.6mm, 5 μm; Phenomenex) at a flow rate of 1.0 mL/min. The mobile phase consisted of solvent solution B (methanol, water and formic acid, 50:40:10 v/v/v) and A (water and formic acid 90:10). The gradient system adopted was a suitably modified version of the one described by Favretto and Flamini (2000). Briefly (1) B raised progressively from 15 to 45% in 25 min; (2) B then raised to 70% at 45 min; (3) B achieved 90% at 55 min and 99% at 60 min. Individual stock solutions of standards of anthocyanins were prepared in methanol acidified with 0.5% HCl. Six calibration solutions were prepared within the range: 10–100 μg/mL. Each calibration solution was injected into HPLC in triplicate. The calibration graphs were provided by the regression analysis of peak area of the analytes versus the related concentrations (Table 2). The characterisation of flavonoids in VCB was performed using an Eclipse-PLUS-C18 (250 mm × 4.6mm, 5 μm; Phenomenex) column, at a flow rate of 1.0 mL/min. Conditions are reported in Tacchini et al. (2015). Following chromatogram recording, sample peaks identification was carried out by comparison of UV spectra and retention time with those of pure standards. The analyses of the phytocomplexes (10 mg/mL) were performed under the same experimental conditions. The obtained calibration graphs allowed the determination of the concentration of the three components. Three batches of extractions were tested.

GC-MS analysis

The samples obtained from steam-distillation were checked with NMR (data not shown) that evidenced a relevant amount of free fatty acids. For this reason, an aliquot of few milligrams of the extracts have been mixed with 200 μL of BSTFA (1% TMCS) (bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane) (Sigma-Aldrich) for 45 min at 80 °C with the purpose of obtaining the trimethylsilylethers (TMS) of free fatty acids. Then 1 μL of solution was directly injected in gas chromatography (GC). GC analysis was performed by a Varian GC-3800 gas chromatograph equipped with a Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 μm), a Varian MS-4000 mass spectrometer using electron impact (EI) and hooked to NIST library. TMS were identified by comparing their GC retention time and the MS fragmentation pattern with those of pure components (Sigma-Aldrich), the methylesters of fatty acids with 37 pure component FAME mix (Supelco, Sigma-Aldrich), and others molecules matching the retention indices (AI) with those in the literature (Adams 2007). Operating conditions are reported in Radice et al. 2014. Briefly: injector temperature, 300 °C; carrier (helium) flow rate, 1,2 mL/min and split ratio, 1:50. Oven temperature was initially increased from 130 °C to 200 °C at a rate of 1 °C/min, then from 200 °C to 250 °C at a rate of 5 °C/min and from 250 °C to 320 °C at a rate of 10 °C/min, finally for 3 min the temperature was maintained at 320 °C. The MS conditions were: ionization voltage, 70 eV; emission current, 10 mAmp; scan rate, 1 scan/s; mass range, 29–600 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. One microliter of each sample was injected. Samples were analysed in GC-FID (GC-flame ionization detector) for quantitative determination through the normalization method, without using correction factors: the relative peak areas for individual constituents were averaged on three different chromatograms of three independent reactions. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector maintained at 300 °C; all the others GC conditions were the same of GC-MS method.

Table 2. Statistical data of the evaluation of linearity regression, LOD and LOQ in the anthocyanins quantification by RP-HPLC-DAD.

Compound	R^2	Regression equation Linearity range 10-100 μg/mL	LOD μg/mL	LOQ μg/mL
Cyanidin-3-O-glucoside	0.9981	y = 63632x + 185474	0.28949	0.87726
Delphinidin-3-O-glucoside	0.9990	y = 29673x-16816	0.53933	1.63434
Malvidin-3-O-glucoside	0.9928	y = 65789x-126525	0.40976	1.24171

Mass spectroscopy

Lyophilized extracts were dissolved in a 50% ethanol–water solution and directly infused in a Mass Spectrometer Thermo Finningan (Ringoes) ESI Q-Duo linear trap set as follow: spray voltage 4,5 KV, sheath gas flow rate 20, capillary voltage 10 V, capillary temperature 160 °C and ion negative and positive modes. The samples were opportunely diluted and filtered before infusion. Opportune collision energies were used for the fragmentation and the simultaneous monitoring of the parent ions. The same conditions were applied for the mass analysis after chromatographic separation. Three batches of extractions were tested.

Total polyphenols quantification

The determination of the total polyphenolic contents in active extracts were performed using a ThermoSpectronic Helios-γ spectrophotometer and performed according to previously described methods (Rossi et al. 2012; Tacchini et al. 2015). The former's results are expressed as milligram of gallic acid per gram of crude. Each experiment was made in triplicate.

DPPH assay, evaluation of the antioxidant properties of ethanolic extracts

The DPPH assay was performed following the method by Cheng et al. (2006). After 30 min of incubation in the dark at room temperature, the microplates were analysed with a microplate reader (Biorad, 680 XL) and the absorbance was read in triplicate against a blank at 515 nm. The DPPH inhibition in percentage was determined by the following formula: IDPPH% = [1 − (A1/A2)] × 100; where A1 was the DPPH absorbance with the extracts and A2 without extracts. Eight different concentrations (range 20–0.16 μg/mL) of Trolox were prepared and used as positive control. Antioxidant activity of the extract was expressed as IC_{50}, concentration providing 50% inhibition of the radical, and calculated as described in Nostro et al. (2016). All experiments were assessed in triplicate and values were reported as mean ± standard deviation.

Antimicrobial activity

The antimicrobial activity has been evaluated against phytopathogenic fungi and bacteria to preliminarily verify phytoiatric properties. *Pseudomonas syringae* pv. *syringae* van Hall ATCC 19310 was used to evaluate the antibacterial activity. The experiments (minimum inhibitory concentration [MIC] and minimal bactericidal concentration [MCB]) were set up following the indications given by the National Committee for Clinical Laboratory Standards (NCCLS, standard M7-A6). For the evaluation of the antifungal activity, *Sclerotinia minor* and *Sclerotinia sclerotiorum* were used. The preparation of the filamentous fungi cultures and the experiments of antifungal activity were carried out following the indications reported in Guerrini et al. (2009). The MIC of fungal growth and minimum fungicide concentration were determined following the indications reported in Cavaleiro et al. (2006). Finally, the effective concentration able to give a 50% maximal effect (EC_{50}) was calculated. Each experiment was made in triplicate.

Results

Extraction yield and total polyphenols

The first target of the study was the isolation and quantitation of polyphenols, and the solvent system that showed the best compromise between water-soluble and alcohol-soluble molecules, obtaining the richest fingerprinting, resulted to be water:ethanol 50:50 (Figure 1). As reported in Table 3, ultrasound assisted extraction (UAE) showed the highest yields among the extraction processes: 25.13 ± 3.71% against 10.42 ± 2.38% of NAV for VCR and 27.59 ± 1.68% against 13.42 ± 6.25% for VCB.

On the other side, the extraction yields achieved by SFE and DIS were much lower than the previous, with the highest value obtained by VIN for SFE (8.36%) and VCR for DIS (0.05%). The UAE extraction was more performing than NAV regarding the total extraction yield and the total polyphenol content (Table 3), except for VIN, in which NAV method showed the same efficiency. The polyphenols richest samples were the extracts of grapeseed performed with both methods. Moreover, Student *t* test indicated significant differences in polyphenols content between the grape pomace extracts and grape seeds extract ($p = .0006$), and this was also valid for the antioxidant activity evaluation ($p = .0339$).

HPLC/MS analysis of UAE and NAV extracts

The mass spectrometry (MS) analysis, in positive-ion mode, and the reversed phase liquid chromatography (RP-HPLC-DAD) performed on VCR extractions highlighted the presence of anthocyanins, in particular: malvidin-3-O-glucoside (*m/z* 493), the most abundant; peonidin (*m/z* 301), malvidin (*m/z* 331), cyanidin-3-O-glucoside (*m/z* 449), peonidin-3-O-glucoside (*m/z* 463), delphinidin-3-O-glucoside (*m/z* 465), malvidin-3-O-(6-O-acetyl)glucoside (*m/z* 535) and malvidin-3-O-(6-O-p-coumaroyl)glucoside (*m/z* 639). Being the malvidin-3-O-glucoside the most abundant molecule (it had a relative abundance of 12,68% and 12,05% respectively in UAE and NAV), we expressed the quantification of the whole unknown anthocyanins fraction in grams of malvidin-3-O-glucoside/100 g of crude extract (Table 4).

As regards VCB extracts (both UAE and NAV), the most abundant molecular categories were represented by flavonols. The RP-HPLC-DAD analysis, confirmed by MS, highlighted the presence of seven main flavonoids: three glycosylate derivatives of quercetin (quercetin-3-O-rutinoside, quercetin-3-O-glucuronide and quercetin-3-O-galactoside), two glycosylate derivatives of kaempferol (kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside) and the two free aglycons (Figure 2). The MS and the HPTLC analyses permitted to discriminate the co-elution of quercetin-3-O-glucuronide and quercetin-3-O-galactoside (data not shown).

The VIN extracts were mainly characterised by procyanidins (PAs). The PAs were identified by direct infusion of the extracts in electrospray ionization-MS, negative-ion mode without performing chromatographic separation. The analysis highlighted the presence of epi/catechin monomer and oligomers (Table 1). Each molecule was confirmed by MS/MS analyses.

GC-MS analysis of SFE and DIS extracts

To complete the overview of these exhausted matrix content, the lipophilic part of the biomass was investigated by SFE and DIS followed by GC-MS analyses. Results of the chromatographic analysis performed on SFEs extracts (data not shown) highlighted the presence of the characteristics fatty acids of grape seeds: linoleic, elaidic, oleic and stearic acid were the most abundant; and myristic, palmitoleic, 11-

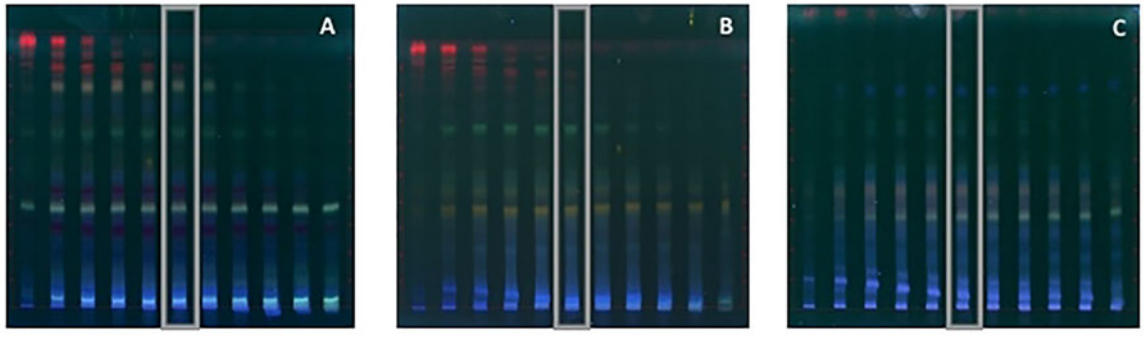

Figure 1. HPTLC analyses of the extractions of red grape (A), white grape (B) and grapeseed (C) performed with different percentages of ethanol–water (from left to right, form 100% ethanol to 100% water in step of 10%, for each matrix).

eicosanoic and arachidonic acids were detected in smaller quantities.

GC-MS analysis performed on the DIS extracts confirmed the data obtained by SFE, highlighting samples predominantly characterized by fatty acids (Table 5). All presented samples showed a substantially similar qualitative profile, while from a relative quantification point of view some differences emerged: in VCR, the most abundant compounds (expressed per area%) were palmitic acid (53.69%), myristic acid (7.60%), lauric acid (6.13%) and oleic acid (5.21%); while VCB exhibited the highest quantity of palmitic acid (43.04%), palmitoleic acid (7.01%), oleic acid (5.72%) and myristic acid (5.03%). The distillate of VIN exhibited a chemical profile mainly characterized by palmitic acid (29.60%), linoleic acid ethyl ester (19.4%), palmitic acid ethyl ester (11.1%), linoleic acid (10.48%) and oleic acid (6.86%).

Table 3. Yields % of the extraction processes, spectrophotometric quantitation of the total phenolic content and evaluation of antioxidant activity through DPPH test (expressed as half maximal inhibition concentration, IC_{50}).

Sample	Extraction methods	Extraction yield (%)	Total phenolic content (milligram gallic acid per gram of dried extract)	Antioxidant activity –DPPH – IC50 (μg/mL)
VCR	UAE	25.13 ± 3.71	189.11 ± 5.95	10.99 ± 1.74
	NAV	10.42 ± 2.38	159.58 ± 1.57	15.31 ± 4.95
VCB	UAE	27.59 ± 1.68	116.44 ± 3.49	22.44 ± 2.30
	NAV	13.41 ± 6.25	106.11 ± 5.46	20.82 ± 3.72
VIN	UAE	11.61 ± 4.39	446.72 ± 22.16	5.44 ± 0.40
	NAV	9.52 ± 0.76	506.24 ± 55.91	4.30 ± 0.31

Table 4. Anthocyanins quantification by RP-HPLC-DAD, expressed as milligram of standards per gram of dried extract.

	Red grape (mg/100 g dried extract)	
	VCR NAV	VCR UAE
Cyanidin-3-O-glucoside	14.50 ± 0.64	15.88 ± 1.03
Delphinidin-3-O-glucoside	108.60 ± 4.80	102.10 ± 9.50
Malvidin-3-O-glucoside	259.40 ± 14.62	276.80 ± 12.10
Unknown anthocyanins	1770.07 ± 115.03	1788.08 ± 109.11
Total	2152.67	2182.86

The *unknown anthocyanins* are quantified as malvidin-3-O-glucoside.

Antioxidant and antimicrobial evaluation

Extracts of EGM (VCR, VCB) and grape seeds (VIN) have been preliminarily evaluated for the *in vitro* antioxidant (DPPH assay), and antimicrobial properties. Regarding the antioxidant properties (property of sure interest for future possible

Table 5. Chemical analyses of VCB, VCR and VIN steam distillation by GC-MS and GC-FID, expressed in area % of the total.

Compound[a]	VCR %	VCB %	VIN %	ID method[b]
Caprylic acid ethyl ester	–	0.15	–	GC-MS, AI
3-Octenoic acid	–	0.11	–	GC-MS
Caprylic acid	0.28	0.93	–	GC-MS, Co-GC
Nonanoic acid	–	0.16	–	GC-MS, Co-GC
Capric acid	0.21	1.29	0.14	GC-MS, Co-GC
9-Oxo-nonanoic acid ethyl ester	0.41	1.54	–	GC-MS
Lauric acid, ethyl ester	0.47	0.29	–	GC-MS
Lauric acid	6.13	2.12	2.23	GC-MS, Co-GC
Octanedioic acid	–	0.22	–	GC-MS
Myristic acid ethyl ester	0.74	0.43	0.30	GC-MS, AI
Azelaic acid	0.66	2.78	–	GC-MS
6,10,14-trimethyl-2-pentadecanone	0.18	0.30	0.13	GC-MS, AI
Myristic acid methyl ester	–	–	3.93	GC-MS, Co-GC
Myristic acid	7.60	5.03	–	GC-MS, Co-GC
Cis-10-pentadecenoic acid	0.13	0.76	0.14	GC-MS, Co-GC
n-Pentadecanoic acid	0.86	1.23	0.33	GC-MS, Co-GC
Palmitoleic acid ethyl ester	0.28	2.74	0.67	GC-MS
Manoyl oxide	2.89	1.85	–	GC-MS, AI
Palmitic acid ethyl ester	7.78	5.42	11.10	GC-MS, AI
Palmitoleic acid	0.58	7.01	1.44	GC-MS, Co-GC
Palmitic acid	53.69	43.04	29.60	GC-MS, Co-GC
Linoleic acid methyl ester	1.25	0.38	0.27	GC-MS, Co-GC
Oleic acid methyl ester	1.94	1.88	0.21	GC-MS, Co-GC
Linoleic acid ethyl ester	–	–	19.40	GC-MS
Linolenic acid ethyl ester	–	–	2.60	GC-MS
Oleic acid ethyl ester	–	–	7.24	GC-MS
Elaidic acid ethyl ester	–	0.42	0.35	GC-MS
Stearic acid ethyl ester	0.43	0.80	1.25	GC-MS, AI
Linoleic acid	2.42	0.99	10.48	GC-MS, Co-GC
Oleic acid	5.21	5.72	6.86	GC-MS, Co-GC
Elaidic acid	0.36	0.94	0.19	GC-MS, Co-GC
Stearic acid	0.90	1.64	0.33	GC-MS, Co-GC

[a]Free fatty acids were determined as trimethylsilylethers.
[b]GC-MS: gas-chromatography-mass spectrum; Co-GC: co-injection with authentic compound; AI: experimental retention indices compared with literature.

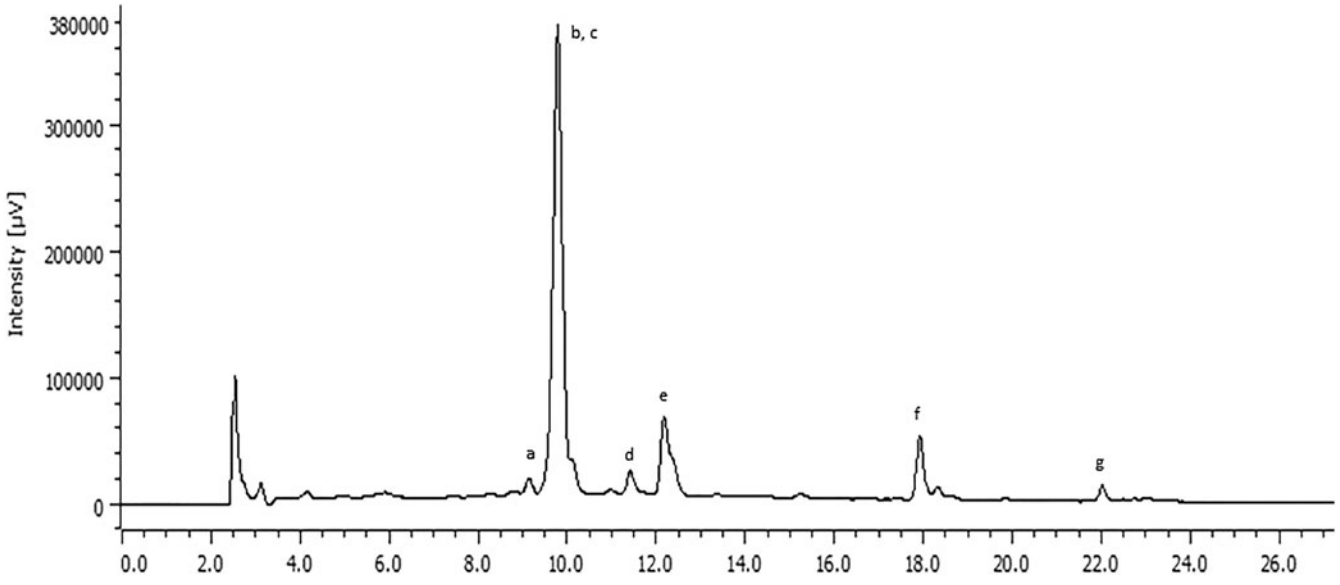

Figure 2. RP-HPLC-DAD chromatogram of the white grape extract, indicating the presence of quercetin-3-O-rutinoside (a), quercetin-3-O-gucoronide (b), quercetin-3-O-galactoside (c), kaempferol-3-O-rutinoside (d), kaempferol-3-O-glcoside (e), quercetin (f) and kaempferol (g).

Table 6. Antimicrobial activity of hydroalcoholic extracts of pomace from red and white grapes (VCR, VCB) and grape seeds (VIN).

	Antibacterial (MIC μg/mL)	Antifungal (EC_{50} μg/mL)	
	Pseudomonas syringae pv. *syringae*	*Sclerotinia minor*	*Sclerotinia sclerotiorum*
VCR UAE	>1000	n.d.	n.d.
VCR NAV	>1000	n.d.	n.d.
VCB UAE	>1000	n.d.	n.d.
VCB NAV	>1000	n.d.	n.d.
VIN UAE	>1000	>15	>15
VIN NAV	>1000	>15	>15
Positive Control	125	<0.5	<0.5

The results are expressed as MIC growth (μg/mL) for antibacterial activity and as an effective concentration able to give 50% maximal effect (EC_{50}) as regards the antifungal activity. n.d.: not defined; the strain showed a strong increase in growth instead of an inhibition, probably due to the sugary component of the extracts. Positive control: Delan 70 WG, per *Pseudomonas syringae pv. syringae*); Heliocuivre S, per *Sclerotinia* sp.

nutraceutical and cosmetic projections), the hydroalcoholic extracts obtained with the NAV and UAE method were considered because they are characterized by the most abundant polyphenolic component and normally taken as reference for expressing antioxidant activity (Table 3).

As far as the antimicrobial activity is concerned, the hydroalcoholic extracts from VCR, VCB and VIN matrices were tested (Table 6). As a fact, no noteworthy activities have been recorded. On the contrary, with reference to antifungal activity, an increase in growth was observed.

Discussion

The selection and optimization of the extraction processes are key factors for the valorization of secondary raw materials of the agro-food supply chains with respect to a bio-based industrial profile and circular economy. For these reasons, the choice of the extraction methods was driven by efficiency criteria (high yield), sustainability (low or no use of organic solvents) and technological transfer opportunities (scale up from laboratory towards industry), but they were also selected considering the type of vegetable matrix, its condition and size (powdered or not), and the type of target biomolecules that would characterise the new finished products. In relation to these aspects, it was decided to glean in the ample and modern context of the *green chemistry* the UAE, the pressurised fluid extraction with Naviglio® technology (NAV), the supercritical CO_2 extraction (SFE) and the steam distillation (DIS) (Baiano 2014).

The first two extraction methods (UAE and NAV) were performed using water and ethanol in different proportions, in accordance to the principle of the green chemistry. Figure 1 highlighted how a 50:50 mixture had the highest affinity for the extraction of polyphenols, obtaining extracts fingerprinting that showed the best compromise between water-soluble and alcohol-soluble molecules for all production waste of the wine production chain. Regarding the extraction yield, the UAE method was the most performing for all matrixes, except for VIN extracts, were the results of UAE was in line with the data obtained by the NAV extraction (9.74 ± 1.47% of UAE compared with 9.52 ± 0.76% of NAV). Going more in detail, VIN NAV extract obtained the lowest extraction yield, but, in comparison between extraction yield and quantity of total polyphenols where VCR and VCB showed a direct correspondence between extraction yield and polyphenol content, VIN NAV extract differs from this trend, showing the highest polyphenol content. The high abundance of PAs that characterise these extracts (Table 1) could explain these results. This molecular category is well known for its use in nutritional supplements, functional foods, in the cosmetic and pharmaceutical products (Nagasako-Akazome 2014; Xia et al. 2014; Panickar 2015; Martinez et al. 2017) and these extraction strategies could implement the revaluation of this by-product, making it a new profitable resource. VCR extracts, in turn, showed higher polyphenolic content than VCB samples, with an average difference of 39%, most probably because of the presence of anthocyanins. This is by far the most characterising molecular category of VCR extraction, and both extraction strategies considered (UAE and NAV) showed the same specificity towards these compounds (Table 4), in the range of the standard deviation. VCB extracts, instead, exhibited the presence of flavonols, most probably because white grape varieties do not undergo maceration during winemaking, leaving part of these molecules in the vinification wastes. Although the literature reports numerous examples of polyphenolic quantification of extracts obtained by grape wastes compatible with those considered in this article (Tournour et al. 2015; Ferri et al. 2016; Trikas et al. 2016), it is not possible to make a constructive comparison because of the grape cultivar chosen for the research, and because it is difficult to identify the point of the supply chain where they come from.

Considering the extractions of the lipophilic part of the vinification by-products achieved by SFE and DIS, they showed extractions yields much lower than the previous obtained by UAE and NAV (e.g. 0.05% was the highest yield among all the waste matrixes), making the hypothesis of industrial scale-up of these processes substantially impractical. With regard to the steam distillation of the considered by-products, there are no literature data that allowed us to compare these results; however, it is possible to make some considerations with respect to the predetermined target. The expected phytocomplex would be mainly characterised by terpenoids, whereas these compounds were present in small quantities just in VCR with a relative presence of 2.89%. This aspect could probably be explained by the fact that the fresh pomace, before being supplied for the analyses, had undergone conditions for which most of the volatile components had disappeared. Instead, the fatty acid composition was both qualitatively and quantitatively richer than the extracts obtained by SFE. Since the samples extracted with SFE had been dried, while the same distilled samples had been extracted fresh (hoping to extract the volatile terpene component), it is possible to hypothesize that the pre-treatment in stove at 70 °C degraded most of the fatty acid component, which remained in the fresh matrices instead.

In the preliminary evaluation of the antioxidant activity with DPPH assay, VIN NAV extract showed the highest antioxidant capacity and it showed a directly correlation with the polyphenols content, since the richest samples in

polyphenols showed the lowest IC_{50}, therefore, the highest activity. The antioxidant activity of grape seeds extracts is well known in literature and the results are in line with the those evaluated from our tests (Li et al., 2008; De Sá et al., 2014). The direct correlation between total polyphenols quantity and antioxidant activity is evincible for the VCR extracts, but these cases differs from the previous because UAE exhibited higher activity than NAV. The main contribution to the antioxidant capacity of VCR extracts is probably due to anthocyanins and their synergistic effect, already reported in literature (Sun et al. 2015). VCB extracts, instead, are the only samples in which no direct correspondence was observed between the content of polyphenols and the antioxidant activity, in effect, the NAV extract showed a lower polyphenol content than UAE extract, but slightly higher antioxidant activity (20.82 ± 3.72 μg/mL of NAV vs. 22.44 ± 2.30 μg/mL of UAE).

The preliminary *in vitro* tests of antimicrobial activity have been set up with the precise aim of identifying promising extracts for further investigations with respect to the phytoiatric activity, or for the sustainable defence of the crops as a further applicative impact. As mentioned in the result paragraph, no noteworthy activities have been recorded, most probably because of the sugary content of the extracts.

The obtained extracts have shown that the waste of the wine production chain can be an excellent source of biomolecules useful for different application contexts, to realize the exploitation of resources in a circular economy perspective. In addition to these considerations, preliminary bioactivity assessments related to chemical evidence impose the need to deepen the chemical characterization of the extracts, to better identify any molecules or fractions responsible for the bioactivity, although weak, with nutraceutical, cosmetics and/or phytoiatrics projections. This would lead, by means of bioguided strategy, to modify the extractive conditions by identifying those parameters that determine the achievement of enriched extracts with the desired biological activities making the process of exploitation of secondary raw materials realistically and concretely operative also for an industrial scale up.

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