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Screening of the antioxidant and vasorelaxant activity of wine waste ultrasonic extracts, and HRMS targeted and semi-targeted profiling of glycosylated polyphenols vs free polyphenols

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ABSTRACT

The winemaking process generates a large amount of waste which can be profitably converted into a resource. In this work, extracts from stalks, lees, and pomace of *Magliocco canino* cv (*Vitis Vinifera* L.) were obtained using ultrasounds. A comprehensive metabolomics profile was carried out by high-resolving power mass spectrometry hyphenated with liquid chromatography. Fifty-nine compounds were annotated and successively verified, bearing different chemical classes. Free amino acids were quantified in the different extracts. Distinction of glycosylated polyphenols *vs* their aglycones was accomplished by targeted and semi-targeted LC-MS methods; then selected polyphenolic biomarkers were quantified in the studied extracts. The antioxidant activity was evaluated *in vitro* in a human dermal fibroblasts assay, and the best antioxidant extract was used for the synthesis of an antioxidant DX conjugate by a grafting reaction, to obtain an innovative food ingredient endowed with improved antioxidant activity. The *in-vitro* endothelium-dependent vasorelaxation, recorded for both stalk and lees extracts could be exploited for the development of new antihypertensive nutraceuticals.

1. Introduction

Waste production is a direct consequence of the rising food demand, boosted by rapid population growth, leading to about 9.5 billion people in 2050 (Del Mar Contreraset al., 2022). The wine industry, for example, generates massive quantities of waste and by-products posing severe ecological and economic problems of storage, transformation, or elimination (Soceanu, Dobrinas, Sirbu, Manea, & Popescu, 2021). In the wine sector, about 30% of the processed grapes become waste or by-products, thus representing a severe burden to the environment. Waste includes stems (2.5–7.5 % of the processed grape), pomace (25–45 % of the processed grape), and lees (3.5–8.5 % of the processed grape). As the global 2023 wine grape production was about 30 million tons (International Organisation of Vine and Wine, 2024), last year the winemaking process produced stems, pomace, and lees in the ranges of 0.75–2.25, 7.5–13.5, and 1.05–2.55 million tons, respectively. Moreover, also pruning residues should be considered as they account for about 5 t/ha (Fig. 1).

In this context, it is necessary to develop sustainable methods for waste valorisation (Silva et al., 2021). This strategy fits with the EU goal to reach zero food waste in 2030, stressing the urgency of moving from a linear (make/take/dispose) to a circular (take/make/use) economy approach. Although EC Regulation 479/08 permits distilleries to recover ethanol and tartaric acid from wine by-products, this can be considered

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only a starting point for a more comprehensive and profitable plan of action to support their circular conversion into added-value products.

Several studies have proposed the potential use of winemaking by-products as raw materials for the development of sustainable products, including wastewater treatment absorbents, therapeutics, chemicals, biocomposites, and/or biofuels (Bharathiraja et al., 2020; Niculescu & Ionete, 2023). However, most of the purposes described are based on laboratory-scale prototypes, as industrial applications requiring scale-up, economic analysis and life-cycle assessment are still lacking. (Evtuguin et al., 2024).

Winemaking by-products, including pomace, skins, stalks, leaves, lees, and seeds, are a rich source of bioactive secondary metabolites, especially phenolic compounds (PC). Therefore, the reuse of these compounds represents a valuable strategy to both cut down the costs of waste disposal and to develop functional food formulations (Spigno & De Faveri, 2007) and components in a circular economy approach (Alonso, Guillean, Barroso, Puertas, & Garcìa, 2002). Also, in this case, suitable processing approaches are necessary to make these by-products viable at the industrial level in terms of safety, sustainability, functionality and cost affordability (Evtuguin et al., 2024).

Winemaking lees (WL), accounting for 6% of each grape ton to be processed, are a source of PC including hydroxycinnamic acids, flavan-3-ols, tannins, stilbenes, monomeric and oligomeric flavonols, lignocellulosic compounds (hemicellulose and cellulose), and lignin (Rivas et al., 2021; Sancho-Galán, Amores-Arrocha, Jiménez-Cantizano, & Palacios, 2020; Troilo, Difonzo, Paradiso, Summo, & Caponio, 2020). Grape stalk (GS), an organic waste produced in relevant amounts during vinification (3–6 % of the processed raw matter), contains several PC having high antioxidant and free radical scavenger activities: these are mainly monomeric flavan-3-ols (catechin and epicatechin) and procyanidins (Manca et al., 2019). Grape pomace (GP), the main solid residue (about 8.49 million tons per year worldwide) formed during the maceration and fermentation stages, is a matrix rich in different classes of PC: such as hydroxycinnamic and hydroxybenzoic acids, and flavonoids (Castellanos-Gallo et al., 2022). GP constitutes 60% by weight of the solid side streams of the wine-making process (Perra, Cuena-Lombraña, et al., 2022): this makes GP an excellent raw material for the extraction of compounds with high added value (Carullo et al., 2022; Carullo et al., 2020a; Carullo et al., 2020b; Carullo et al., 2020c; Carullo et al., 2020d; Carullo et al., 2019; Restuccia et al., 2019), or the production of both energy and organic building blocks through strong or mild destruction of the organic matter, respectively (Perra, Cuena-Lombraña, et al., 2022).

The present work focused on stalk, pomace, and lees produced during the processing of a Calabrian-native red grape cultivar, namely *Magliocco Canino*, recorded in the National *Vitis vinifera* variety Register (Schneider et al., 2009). Different MS-based metabolomics approaches, comprehending untargeted, targeted and semi-targeted ones, were applied to acquire a compelling chemical characterisation of the samples. This approach involves the chemical determination of the largest possible amount of (bioactive) compounds in a defined biological sample (cells, tissues, and organs) (Lacalle-Bergeron et al., 2021). The state-of-the-art technique used to characterise polar bioactive compounds is HPLC coupled with high-resolving power mass spectrometry. Here a comprehensive metabolomics characterisation was performed to putatively identify (annotate) or fully identify -through the use of the analytical standard- the largest possible number of potential molecular biomarkers, moreover the quantitative evaluation of a selection of potentially bioactive compounds was also carried out. In particular, polyphenols. the compound class with most interesting antioxidant properties may be identified by analytical standard comparison or annotated through elemental composition study and structural elucidation; the latter can only be obtained using HRMS (High-Resolution Mass Spectrometry), capable to reach a very high accuracy in measuring the mass-to-charge value of an ion with only a few milli-mass units of error with respect to its extact value (Zorzi, Gai, Medana, Aigotti, & Peiretti, 2020). Targeted methods were developed and used to search for known analytes; semi-targeted methods were used in the absence of reference standards to quantify potential biomarkers within, the same organic compounds class. Furthermore, the assessment of the antioxidant activity provided data to be correlated to the content of different chemical species. To enhance the biological availability of the molecules found in the extracts, an innovative, macromolecular, dextran-based conjugate (RAEP), endowed with significant biological properties, was synthesised by an eco-friendly grafting procedure. Dextran (DX) was selected as it is considered a smart starting material for chemical modification reactions and for the design of new functional polymers with promising properties (Heinze, Liebert, Heublein, & Hornig, 2006). DX has a commercial interest because its solubility, viscosity, and thermal and rheological properties allow it to be used in food, pharmaceutical, and research areas, other than showing a prebiotic potential higher than inulin (Díaz-Montes, 2021; Damini, Jagan Tingirikari, & Arun, 2015). In the food industry, DX was proposed as a thickening or gelling agent in applications such as jams, jellies, and confectionery products or as a stabiliser to prevent separation of oil and water phases in products like salad dressings and sauces (Yemenicioğlu, Farris, Turkyilmaz, & Gulec, 2020). Additionally, DX was employed as a sugar substitute and film-forming agent to extend the shelf life of certain food products or to encapsulate flavours, colours, or other active ingredients in food products, helping to protect them from degradation or loss of potency (Hu, Lu, & Luo, 2021). Finally, as the metabolomic profile of the extracts revealed the presence of interesting vasoactive secondary metabolites,

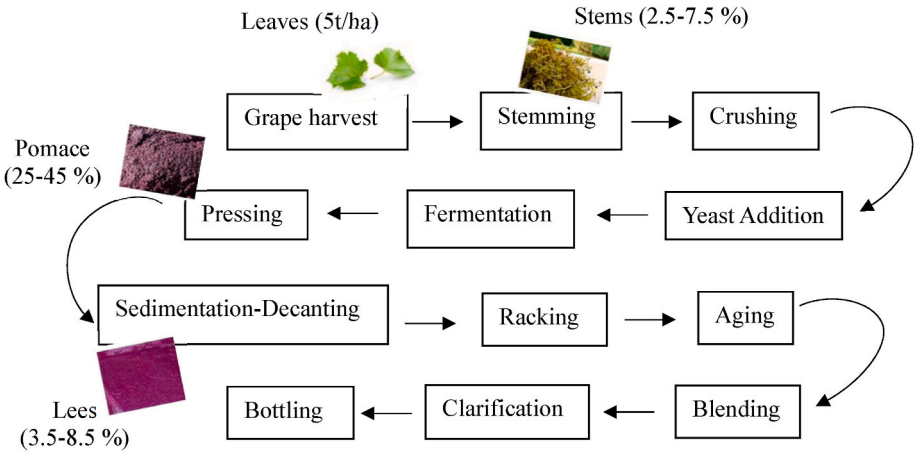

Fig. 1. Red wine production process and related by-products. Percentages are intended as % w/w of the processed grapes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

their *in vitro* vascular activity was investigated. Results point to these winemaking by-products as raw materials worth considering for a potential, future nutraceutical and/or food supplement development.

2. Materials and methods

2.1. Standards, solvents, and materials

Ethanol (absolute), HCl, CH_3OD-d4, and D_2O, were purchased by VWR International (Italy). Acetonitrile (ACN) and methanol (MeOH) LC-MS grade were purchased from VWR (Milan, Italy), formic acid 98–100% from Merck (Darmstadt, Germany), ultrapure water was produced through a VWR apparatus (Milan, Italy). All the standard compounds were from Sigma-Aldrich (Milan, Italy) and were used without further purification. DX from *Leuconostoc* spp. was purchased from Merck-Sigma-Aldrich. The chemicals used include acetylcholine, apigenin-7-glucoside, kaempferol, nifedipine, phenylephrine, quercetin (Sigma Merck, Milan, Italy), and sodium nitroprusside (Riedel-De Haën AG, Seelze-Hannover, Germany). All other substances were of analytical grade and used without further purification. Phenylephrine was solubilised in 0.1 M HCl. Nifedipine was dissolved directly in ethanol and diluted at least 1000 times before use. Wine lees, grape stalk, and pomace of *Magliocco canino* cv were kindly provided in September 2021 by Azienda Agricola Donna Fidelia (Belvedere Marittimo, Italy).

2.2. Extracts preparation

Lees (V), stalk (F), and pomace (R) were stored at −20 °C until extraction. One g of F, V, and R was extracted in an ultrasound bath at 40 kHz, 30 °C for 15 min, using 200 mL of the following solvents: water pH 2 (A), to get extracts VA, FA, and RA; ethanol (E) to get extracts FE, VE, and RE; water/ethanol (AE) 50:50, v/v to get extracts FAE, VAE, and RAE. The crude extracts were centrifuged for 15 min at 9500 rpm, filtered using a syringe filter PTFE 0.2 μm, either concentrated to dryness, (FAE, VAE, and RAE) or freeze-dried (FA, VA, RA, FE, VE, and RE), and stored in the dark at −20 °C until use. Ten mL of MeOH were added to about 20 mg of samples obtained by hydrolysis from wine lees, stalks, and pomaces, in a polypropylene tube, to perform the analytical extraction. The solution was vortexed for 10 s and put in an ultrasonic bath for 10 min. After the extraction, a centrifugation step (9000 rpm for 10 min) was necessary to remove the insoluble vegetal matrix. A 0.2 mL volume of supernatant was then diluted 50-fold in MeOH in a 1.5 mL vial.

2.3. HPLC-HRMS untargeted and precursor ion scan analysis and polyphenol quantitative analysis

To investigate the molecular composition of the samples, an HPLC-HRMS analysis, through an Ultimate 3000 HPLC system hyphenated with an Orbitrap Fusion Tribrid Series HRMS (ThermoFisher Scientific, Milan, Italy), was performed. The HPLC was equipped with a Luna C18 column (2.1 × 150 mm, 3 μm) (Phenomenex, Bologna, Italy) and the system was operated in binary gradient mode. Water at 0.1 % of formic acid was used as solvent A and ACN as solvent B. The chromatographic gradient was set as follows: flow 0.2 mL/min; t: 0 min B: 5 %; t: 34 min B: 100%; t: 37 min B: 100 %; column reconditioning 10 min at B: 5 %. The HRMS -operated both in positive and negative ion mode- was coupled to the HPLC system with a H-ESI source with the following parameters: source voltage 4 kV (ESI+) and 3.2 kV (ESI-); sheath gas and aux gas flow rate 35 and 20, respectively, capillary temperature was set to 270 °C. The mass spectrometer was operated in the full-scan mode in the range of 100–1000 *m/z*, with a resolution of 30 000 in FTMS. DDA tandem mass experiments were performed with a surveyor scan ra range of 100–1000 *m/z* for both polarities. Collision energy was set at 22 (arbitrary units) for all signals exceeding a treshold of 1.0E4. All spectra were acquired in profile mode. Xcalibur 4.0 software (Thermo Scientific, Bremen, Germany) was used for acquisition, data evaluation, elaboration and calculation. Semi-targeted quantitation of the annotated compounds was obtained in the same analytical conditions of the untargeted analysis, based on the calibration curves built using the following standard compounds: apigenin-7-glucoside, kaempferol and quercetin.

Raw data, acquired in profile mode, were converted in centroid using MSconvert software by ProteoWizard (ProteoWizard, Palo Alto, Santa Clara County, USA) using the CWT peak peaking algorithm with 0.1 SNR for both mass levels. Centroided raw data were processed by MZ Mine 3, which was used to perform the feature detection and alignment along with the automatic annotation using the MONA and KEGG databases. After this step, the annotations were manually verified by using Xcalibur software.

2.4. Samples preparation and LC-MS quantitative analysis of amino acids

10mL of ACN (acidified with 20 μl of HCl 1M) were added to 10 mg of hydrolytic-extracted samples in a polypropylene tube to perform an organic extraction. The solution was vortexed for 10 s and put in an ultrasound bath for 10 min. After the extraction, a centrifugation step (9000 rpm for 10 min) was necessary to remove the insoluble vegetal matrix. A 0.2 mL volume of supernatant was then diluted 50-fold in H_2O: ACN (0.01 M HCl) in a 1.5 mL vial.

The LC-MS quantitative analysis of amino acids was performed with an LCMS-8045 Shimadzu liquid chromatograph – ESI MS triple quadrupole analyser equipped with a Raptor Polar X (100 × 2.1 mm, 2.7 μm particle size) (Restek, Milan, Italy) column. Solvent A was acqueous 0.5 % formic acid; solvent B was 7:3 ACN: H_2O, in which the water fraction was a solution of 20 mM of ammonium formiate adjusted with formic acid at pH 3. Chromatographic conditions were set as follows: flow: 0.5 mL/min; t: 0 min B: 88 %; t: 3.5 min B: 88 %; t: 8 min B: 30 %; t: 8.01 min B: 88 %; t: 10 min B: 88 %; t: 10 min B: 88 %. The ESI ionization source was working under the following conditions heating gas flow: 30 (L/min); interface temperature: 300 °C; desolvation temperature: 526 °C; DL temperature: 250 °C; heating block temperature: 400 °C; drying gas flow: 10 L/min. Two MRM (multiple reaction monitoring) events were used to identify each analyte (described in Table 3).

2.5. LC-MS qualitative analysis of glycosylated polyphenols

The analytical method of Tian, Giusti, Stoner, and Schwartz (2005) was adapted to the condition of this work. Analyses were performed through an LCMS-8045 Shimadzu instrument; a Luna C18 (150 × 3 mm, 2.7 μm particle size) (Phenomenex, Bologna. Italy) was used in the chromatographic method. The system was operated in binary mode: solvent A was H_2O with 0.1 % of formic acid and solvent B was ACN with 0.1 % of formic acid. Chromatographic conditions were set as flow: 0.2 mL/min; t: 0 min B: 5 %; t: 6 min B: 5 %; t: 34 min B: 100 %: t: 37 min B: 100 %; t: 40 min B: 5 %; t: 46 min end run. The ESI ionization source, used to couple LC and MS instruments, was working under the following conditions: heating gas flow: 30 (L/min); interface temperature: 300 °C; desolvation temperature: 525 °C; DL temperature: 250 °C; heating block temperature: 400 °C; drying gas flow: 10 L/min. The mass analyzer was working in precursor ion scan mode for 11 chosen polyphenolic compounds (summarized in Table S1).

2.6. Synthesis of the antioxidant dextran conjugate by grafting reaction

In a reaction flask, 500 mg of DX were dissolved in 30 mL of purified water. Then, 12.5 mL of H_2O_2 (120 vol) and 250 mg of ascorbic acid were added. The solution was kept under stirring and after 2 h an amount of RAE (previously solubilised in 7.5 mL of purified water), equivalent to 70 mg of CT, was added into the reaction flask. After 24 h, the polymer solution was purified by dialysis (MWCO: 3500 Da) in purified water at 20 °C for 72 h. The solution obtained was subsequently frozen at −18 °C and freeze-dried to obtain a vaporous solid (labelled

RAEP). Using the same conditions, a polymer of DX alone was prepared and used as a control (Restuccia et al., 2019).

2.7. Antioxidant power measurements

Total phenolic content (TPC), total content of phenolic acids (PAC), and total flavonoid content (FC) of the extracts and RAEP were assessed following the procedures described by Carullo et al. (2020) with slight modifications (SI section). TPC was expressed as the amount of GA per gram of sample (mg GA g^{-1}), while PAC and FC values were expressed as the amount of CT per gram of sample (mg CT g^{-1}). The scavenging activity of the extracts and RAEP in both organic (against DPPH radicals) and aqueous (against ABTS radicals) environments was assessed using the procedure of Carullo et al. (2022) with slight modifications (SI section). The scavenging activity of RAE and RAEP on H_2O_2-induced reactive oxygen species production by human dermal fibroblasts was expressed as IC_{50}.

2.8. Cell cultures and reactive oxygen species detection

Primary human dermal fibroblasts were a kind gift from Dr. D. Franci (Dept. of Medicine, Surgery and Neuroscience, Siena, Italy). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose, supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cell cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Intracellular reactive oxygen species (ROS) formation was assessed using 2′,7′-dichlorofluorescein diacetate (DCF-DA). Human dermal fibroblasts were seeded (1,5 × 104 cells/well) into a 96-well plate, grown for 24 h under standard conditions, washed with phosphate-buffered saline (PBS) and loaded with 10 μM DCF-DA for 30 min at 37 °C. The cells, rinsed twice with PBS, were treated with either RAE (10, 100, and 300 μg mL^{-1}) or RAEP (0.1, 1, and 10 μg mL^{-1}), and 0.2 mM H_2O_2; the intracellular fluorescence was read for 60 min with the Thermo Labsystems Synergy HTX reader (BioTek, Winooski, VT, USA) (485 nm excitation, 528 nm emission). The Area Under the Curve (0–60 min) describing changes in fluorescence monitored every 5 min was calculated (De Luca et al., 2023).

2.9. Animals

All the study procedures were in strict accordance with the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and approved by the Animal Care and Ethics Committee of the University of Siena and Italian Department of Health (7DF19.N.TBT). Male Wistar rats (250–350 g) were purchased from Charles River Italia (Calco, Italy) and maintained in an animal house facility at 25 ± 1 °C and 12:12 h dark-light cycle with access to standard chow diet and water ad libitum. Animals were anaesthetised with an isoflurane (4%) and O2 gas mixture using Fluovac (Harvard Apparatus, Holliston, Massachusetts, USA), decapitated and exsanguinated. The thoracic aorta was immediately isolated and placed in a modified Krebs-Henseleit solution (KHS) and prepared as detailed below.

2.10. Aorta ring preparation and effect of extracts on phenylephrine- and high KCl-induced contractions

The thoracic aorta was gently cleaned of adipose and connective tissues and cut into 3-4-mm wide rings. These were mounted in organ baths between two parallel, L-shaped, stainless-steel hooks, one fixed in place and the other connected to an isometric transducer (Cuong et al., 2014). Rings were allowed to equilibrate for 60 min in KHS (composition in mM: 118 NaCl, 4.75 KCl, 1.19 KH_2PO_4, 1.19 $MgSO_4$, 25 $NaHCO_3$, 11.5 glucose, 2.5 $CaCl_2$, gassed with a 95% O_2–5% CO_2 gas mixture to create a pH of 7.4) under a passive tension of 1 g. During this equilibration period, the solution was changed every 15 min. Isometric tension was recorded using a digital PowerLab data acquisition system (PowerLab 8/30; ADInstruments). Ring viability was assessed by recording the response to 0.3 μM phenylephrine and 60 mM KCl. Where needed, the endothelium was removed by gently rubbing the lumen of the ring with a forceps tip. This procedure was validated by adding 10 μM acetylcholine at the plateau of phenylephrine-induced contraction: a relaxation ≥75% or less than 15% denoted the presence or absence of functional endothelium, respectively (Carullo et al., 2020e). Aorta rings were pre-contracted pharmaco-mechanically with 0.3 μM phenylephrine (endothelium-intact or denuded) or electro-mechanically with 25–35 mM or 60 mM KCl (endothelium-denuded) (Carullo et al., 2021). Once the contraction reached a plateau, extracts were added cumulatively into the organ bath to assess their vasorelaxant activity. At the end of the concentration-response curve, 100 μM sodium nitroprusside alone (phenylephrine-induced contraction) or 1 μM nifedipine followed by sodium nitroprusside (KCl-induced contraction) were added to test the functional integrity of smooth muscle. Vasodilation was calculated as a percentage of the contraction induced by either phenylephrine or KCl (taken as 100%).

2.11. Statistical analysis

Analysis of data was accomplished using GraphPad Prism version 5.04 (GraphPad Software Inc.). Data are reported as mean ± SD; n is the number of rings analysed (indicated in parentheses), isolated from at least three animals. Statistical analyses and significance as measured by Student's *t*-test for unpaired samples (two-tailed) or ANOVA and Bonferroni post-test were obtained using GraphPad Prism version 5.04. In all comparisons, $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Winemaking by-products extraction

The choice of a specific extractive method and its features (e.g., solvent type, sample/solvent ratio, time of extraction, and temperature) are crucial for the isolation of targeted metabolites from agrochemical wastes (Casquete et al., 2022). As compared to conventional methodologies (e.g., maceration), the Ultrasound-Assisted Method (UAM) can increase extraction yields through enhanced mass transfer and heat (because of the ultrasonic cavitation phenomenon and the acoustic impact; (Tao & Sun, 2015), has a mild impact on structure integrity (as sonication usually avoids oxidative side reactions), and requires less time and low temperatures (Casquete et al., 2022). Therefore, UAM was applied to the waste matrix, at 30 °C to preserve their chemical integrity, using green solvents such as water (A), ethanol (E), and hydroalcoholic mixtures (AE) to reduce the environmental impact. (Galviz-Quezada, Ochoa-Aristizábal, Zabala, Ochoa, & Osorio-Tobón, 2019). Table 1 summarises the yields of wine lees, stalk, and pomace. Lees and stalk displayed the highest values in water extraction (81.3 % and 68.7 %, respectively), while pomace in the hydroalcoholic mixture and pure ethanol. However, the choice of the best extraction conditions should also consider the amount of total phenolic content. The hydroalcoholic mixture provided the highest values for all by-products (V, F, and R) in terms of both yield and concentration of molecules with potential biological activity. RAE displayed the highest TPC value (132.32 mg CT g^{-1}), which was almost two-fold that of VAE, and one order of magnitude higher than that of FAE.Table.2

The choice of only three solvents and one matrix/solvent ratio may be a potential limitation of this approach. However, this environmentally friendly method proved valuable for subsequent analysis and testing.Table. 3

Table 1
Extraction conditions and Total Phenolic Amount of the wine by-product extracts.

Sample	Extraction Conditions				Yield (%)	TPC (*mg GA g−1 sample*)
	Solvent	°C	minutes	mL		
FA	Water	30	15	200	81.3 ± 3.1[a]	4.61 ± 0.18[g]
VA	Water	30	15	200	28.6 ± 1.1[d]	33.18 ± 2.14[c]
RA	Water	30	15	200	68.7 ± 2.8[a]	104.78 ± 5.21[b]
FE	Ethanol	30	15	200	4.3 ± 0.2[f]	49.56 ± 2.30[e]
VE	Ethanol	30	15	200	40.7 ± 1.4[c]	52.75 ± 1.94[de]
RE	Ethanol	30	15	200	14.2 ± 0.5[e]	78.74 ± 3.40[c]
FAE	Water/ethanol (50:50 v/v)	30	15	200	13.7 ± 0.6[e]	13.72 ± 0.48[f]
VAE	Water/ethanol (50:50 v/v)	30	15	200	57.9 ± 1.9[b]	56.39 ± 2.01[d]
RAE	Water/ethanol (50:50 v/v)	30	15	200	74.9 ± 1.5[a]	132.32 ± 5.74[a]

TPC = Total Phenolic Content; FA = Wine lees of *Magliocco canino cv* in water; RA = Grape stalk of *Magliocco canino cv* in water; VA = Pomace of *Magliocco canino cv* in Water; FE = Wine lees of *Magliocco canino cv* in Ethanol; RE = Grape stalk of *Magliocco canino cv* in Ethanol; VE = Pomace of *Magliocco canino cv* in Water/Ethanol; FAE = Wine lees Pod of *Magliocco canino cv* in water/Ethanol; RAE = Grape stalk of *Magliocco canino cv* in Water/Ethanol; VAE = Pomace of *Magliocco canino cv* in Water/Ethanol; GA = Gallic Acid; CT = Catechin. Different letters are significantly different (Tukey HSD test) at $p < 0.05$.

3.2. HPLC-HRMS analysis

Untargeted analysis, through the DDA experiment, represents a powerful strategy to try to identify unknown molecules contained by complex samples and matrices. Indeed, each sample revealed a large number of molecules: small organic acids, simple and glycosylated polyphenolic compounds, monomeric and polymeric sugars, amino acids and also sphingosines and betaines. Table 3 summarises the molecules and parameters functional to the annotation process. Fifty-nine compounds were annotated by the software and manually verified. Seventeen were classified as sphingosines and betaines, 8 as mono- and polysaccharides, and 13 as simple and glycosylated polyphenols. Twenty-four molecules were found in pomaces. These were mono- and polysaccharides as well as flavonoids such as kaempferol, catechin and quercetin. These plant secondary metabolites are usually found in the form of glucosides as in the case of quercetin 3-*O*-glucuronide and aurantio-obtusin beta-D-glucoside. Thirty-one sphingosine, such as phytosphingosine and eicosasphinganine, were detected in lees samples. Lastly, 35 analytes belonging to different molecular classes were identified in stalks. These included quercetin (polyphenols), myristamidopropyl (betaines), and arginine (amino acids) Table. 4. A potential limitation of the untargeted hypothesis process is represented by the need to confirm all of the annotated structures with certified standards to assign the maximum identification level according to Schymansky rules (Schymanski et al., 2014). This is the reason to use the term annotation instead of identification. In any case, barring unlikely isomers, a large number of known plant metabolites have been recognised. To complete our metabolomics characterisation, a semi-quantitative determination using some selected polyphenol analytical standards was performed to better define the profile of the potential biomarkers present in the extracts. The results of the quantitation are reported in Table 5. The quantified polyphenols are representative of only a fraction of the TPC previously obtained because not all compounds belonging to this class that are present could be detected in the untargeted process. However, a significant presence of glycosylated flavonoids can be observed: about 7.5 mg/g in FAE, 241.6 mg/g in VAE and 43 mg/g in RAE.

Table 2
Quantitative analysis of amino acids; the following parameters are shown: MRM transitions (from molecular ions to fragment ions); Rt: retention time; concentration of each compound in samples of lees, pomaces, and stalks ± SD: standard deviation (n.f. stands for not found).

Compound	MRM (*m/z*)	Rt (min)	FAE (g/kg ± SD)	RAE (g/kg ± SD)	VAE (g/kg ± SD)
Aspartic acid	134 > 74 134 > 88	7.6	0.333 ±0.042	0.093 ±0.007	0.079 ±0.006
Cystine	241 > 152 241 > 122	6.5	n.f	n.f	n.f
Glutamic acid	148 > 84 148 > 130	6.1	1.517 ± 0.072	0.113 ± 0.009	0.087 ±0.019
Lysine	147 > 110 147 > 90	3.6	0.271 ± 0.006	0.014 ±0.004	0.010 ±0.005
Histidine	156 > 110 156 > 96	3.1	0.317 ±0.036	0.107 ±0.008	0.120 ±0.019
Arginine	175 > 70 175 > 129	2.7	1.625 ±0.151	0.374 ± 0.019	0.379 ±0.178
Phenylalanine	166 > 149 166 > 120	1.2	0.305 ±0.029	0.144 ±0.003	0.153 ±0.036
Serine	106 > 60	2.9	0.119 ±0.019	0.048 ±0.007	0.011 ±0.005
Glycine	76 > 30 76 > 59	2.7	0.090 ±0.021	n.f.	n.f.
Threonine	120 > 74	2.4	0.219 ±0.025	0.141 ±0.002	0.147 ±0.024
Alanine	90 > 73 90 > 44	2.3	2.157 ±0.017	0.120 ±0.014	0.018 ±0.0005
Proline	116 > 70	2.0	5.122 ±0.198	2.224 ±0.059	2.264 ±0.237
Valine	118 > 72	1.7	0.146 ±0.005	0.092 ±0.002	0.106 ±0.009
Tyrosine	182 > 136 182 > 149	1.5	0.218 ±0.029	0.119 ±0.003	0.137 ±0.028
Methionine	150 > 104 150 > 133	1.4	0.151 ±0.011	0.069 ±0.002	0.082 ±0.019
Isoleucine	132 > 86	1.3	1.096 ±0.043	0.177 ±0.004	0.345 ±0.054
Leucine	132 > 86	1.4	n.f	0.12 ± 0.003	0.17 ±0.040
Total amino acids concentration			**13.686**	**3.959**	**4.103**

3.2.1. Quantitative analysis of amino acids

Amino acids are the main components conferring taste to wine and, generally, to all fermented food. A targeted approach was developed, allowing easy retention and separation of underivatised amino acids with nonpolar, polar, positively charged, and negatively charged side chains in a single analytical run. The LOQ level was 200 μg/kg. A good linear correlation of all the analytes was obtained ($R^2 > 0.999$) within a

Table 3
Annotated molecules in positive and negative analytical mode. Each column describes the parameters used for molecular annotation: molecular ion mass, retention time (Rt), chemical formula, mass error value (Δppm), some representative fragment ions, hypothetical molecular losses due to fragmentation and the presence of each molecule in a different sample of winemaking waste products.

Annotation	Molecular Ion	Rt	Chemical Formula	Δppm	Fragments	Hypothetical Loss	RAE	FAE	VAE
1,2,3-Trihydroxybenzene	127.0392	1.9	$C_6H_7O_3^+$	1.81	109.0284 81.0335	H_2O CH_2O	x		x
2′-Amino-2′-deoxyadenosine	267.1198	21.02	$C_{10}H_{15}N_6O_3^+$	−1.1	136.0618 250.0935	$C_5H_9NO_3$ NH_3	x		
3,7-Di-O-methylquercetin	331.0809	15.25	$C_{17}H_{15}O_7^+$	−1.36	316.0578 301.0707	$CH_3^·$ CH_2O			x
3-Buten-2-one, 4-(2,6-dimethoxy-4-pentylphenyl)	277.1794	31.62	$C_{17}H_{25}O_3^+$	−0.9	235.1693	C_2H_2O		x	x
5-Aminopentanamide	117.1022	2.04	$C_5H_{13}N_2O^+$	−0.34	100.0757 70.0651	NH_3 CH_3NO	x		
5-Cyclohexylsalicylic acid	221.117	28.69	$C_{13}H_{17}O_3^+$	−0.99	203.1067 175.1117	H_2O CH_2O_2	x	x	
8-Hydroxy-5-O-beta-D-glucopyranosylpsoralen	381.0801	41.73	$C_{17}H_{17}O_{10}^+$	−1.08	219.0288 291.0499	$C_6H_{10}O_5$ $C_3H_6O_3$	x	x	
Adenine	136.0619	1.73	$C_5H_6N_5^+$	−1.04	95.0478	$CHN_2^·$		x	
Arginine ethyl ester	203.1506	1.43	$C_8H_{19}N_4O_2^+$	1.71	186.1237 144.1019	NH_3 CH_5N_3			x
Aurantio-obtusin beta-D-glucoside	493.1338	2.21	$C_{23}H_{25}O_{12}^+$	−0.51	331.0821	$C_6H_{12}O_5$			x
Cocamidolpropil betaine	343.2957	24.27	$C_{19}H_{39}N_2O_3^+$	0.53	325.285 240.2322	H_2O $C_4H_7NO^{·+}$	x	x	
Eicosasphinganine	330.3366	25.14	$C_{20}H_{44}NO^+$	−0.17	312.3261 296.3104	H_2O C_3H_6		x	
Erucamide	338.3414	39.93	$C_{22}H_{44}NO^+$	−1.01	321.3152 291.3208	NH_3 CH_3NO		x	x
3-Buten-2-one, 4-(2,6-dimethoxy-4-pentylphenyl)	277.1794	25.28	$C_{17}H_{25}O_3^+$	−1.27	235.1693	C_2H_2O	x		
Hexadecasphinganine	274.2736	20.52	$C_{16}H_{36}NO_2^+$	−0.93	256.2635 213.2213	H_2O C_2H_3N	x		
Hispidulin 4′-O-|A-D-glucopyranoside	463.1221	13.38	$C_{22}H_{23}O_{11}^+$	−0.09	301.0707	$C_6H_{10}O_5$	x		
L-Arginine	175.1192	1.54	$C_6H_{15}N_4O_2^+$	1.41	158.1162 117.0784	H_2O CH_4N_3			x
Myristamidopropyl betaine	371.3271	34.75	$C_{21}H_{43}N_2O_3^+$	0.53	268.2635	$C_4H_9NO_3$	x	x	
N-(2-Hydroxyethyl) octadecanamide	328.3212	2.53	$C_{20}H_{42}NO_2^+$	2.51	310.3104 298.3104	H_2O CH_2O		x	
N-Acetyl-D-glucosamine	222.0971	1.75	$C_8H_{16}NO_6^+$	−0.51	204.0866 130.0499	H_2O $C_3H_8O_3$		x	
N-Lauryldiethanolamine	274.2737	39.83	$C_{16}H_{36}NO_2^+$	2.29	256.2635 213.2213	H_2O C_2H_3N		x	x
Octadecanamide	284.2944	25.21	$C_{18}H_{38}NO^+$	−1.82	267.2682 211.2482	NH_3 C_3H_5NO		x	
Octylamine	130.1591	41.36	$C_8H_{20}N^+$	−0.66	74.0964 113.1325	C_4H_8 NH_3		x	
Oleamide	282.1464	36.59	$C_{18}H_{36}NO^+$	−1.39	265.2526	NH_3			x
Oleamidopropyl betaine	425.2146	37.46	$C_{25}H_{49}N_2O_3^+$	0.68	322.3104	$C_4H_9NO_3$	x		
Phytosphingosine	318.3007	1.47	$C_{18}H_{40}NO_3^+$	−3.91	300.2897 196.2186	H_2O $C_4H_7NO_2$		x	
Prenylcaffeic acid	249.1121	1.94	$C_{14}H_{17}O_4^+$	−0.14	231.1016 204.1145	H_2O $CHO_2^·$	x		
Quercetin	303.0498	37.42	$C_{15}H_{11}O_7^+$	−0.43	194.021	$C_6H_5O_2^·$	x	x	
Shikimate	175.0601	1.58	$C_7H_{11}O_5^+$	0	157.0495 130.0624	H_2O $CHO_2^·$		x	
Sphinganine	300.2893	24.05	$C_{18}H_{38}NO_2^+$	−1.35	282.2791 270.2791	H_2O CH_2O	x	x	
Tetradecanamide	228.2324	32.66	$C_{14}H_{30}NO^+$	−0.57	211.2056	NH_3	x		
1,5-Anhydro-D-fructose	161.0436	1.81	$C_6H_9O_5^-$	−0.29	131.035 73.0295	CH_2O $C_2H_2O_2$	x		x
2-(1,2-Dihydroxyethyl)-6-(hydroxymethyl) oxane-2,3,4,5-tetrol	239.0744	1.84	$C_8H_{15}O_8^-$	−0.179	192.0639	CH_4O_2			x
4-Hydroxy-5-methyl-3-furanone	113.023	1.78	$C_5H_5O_3^-$	−1.038	85.0295	CO	x		
Catechin	289.0684	14.74	$C_{15}H_{13}O_6^-$	0.134	245.0819	$C_{14}H_{13}O_4$	x		
Cellobiosan	323.095	1.85	$C_{12}H_{19}O_{10}^-$	1.331	179.0561 143.035 161.0455	$C_6H_6O_4$ $C_6H_{10}O_6$ $C_6H_{10}O_5$			x
Citric acid	191.0175	2.82	$C_6H_7O_7^-$	0.171	173.0092 111.0088	H_2O CH_2O_3	x	x	
Dodecyl 2-deoxy-beta-D-arabino-hexopyranoside	331.2453	26.41	$C_{17}H_{15}O_7+$	−0.301	316.0578 301.0707 163.0612	$CH_3^·$ CH_2O $C_{12}H_{25}^·$	x	x	x
Gingerol	293.1725	27.15	$C_{17}H_{25}O_4^-$	0.23	236.1054 221.0819	$C_4H_9^·$ $CH_3^·$	x	x	x
Gluconolactone	177.0382	4.17	$C_6H_9O_6^-$	−0.911	103.0037	$C_3H_6O_2$			x

(*continued on next page*)

Table 3 (*continued*)

Annotation	Molecular Ion	Rt	Chemical Formula	Δppm	Fragments	Hypothetical Loss	RAE	FAE	VAE
Glucose acetate acetate	295.0639	2.14	$C_{10}H_{15}O_{10}^-$	0.78	276.0565 179.0561	H_2O $C_4H_6O_8$	x		x
Glutammic acid	146.0441	1.69	$C_5H_8NO_4^-$	0.316	103.0037 128.0353	CO_2 H_2O		x	
Kaempferol	285.0378	23.18	$C_{15}H_9O_6^-$	2.241	132.0217	$C_7H_6O_4$	x		
Maleic acid	279.157	27.16	$C_{16}H_{23}O_4^-$	−0.086	251.1289 125.0608	$C_2H_4O_2$ C_7H_{10}	x	x	x
Malic acid	133.0125	2.63	$C_4H_5O_3^-$	−1.854	115.0037 89.0244	H_2O CO_2	x		
Methyl 5-acetoxyhexanoate	187.0953	19.49	$C_9H_{15}O_4^-$	−0.44	143.0414 125.0608	C_2H_4O H_2O			
Monoglyceride citrate	265.0536	2.82	$C_9H_{13}O_9^-$	−0.008	173.0092	$C_3H_8O_3$		x	
Piceatannol	243.1573	25.71	$C_{14}H_{11}O_4^-$	−4.863	225.0557	H_2O	x		
Quercetin	301.0319	21.61	$C_{15}H_9O_7^-$	0.08	149.0244	$C_7H_6O_4$	x		
Quercetin 3-O-glucuronide	477.0622	18.22	$C_{21}H_{17}O_{13}$.	0.705	301.0354	$C_6H_8O_6$	x		
Sucrose	341.1051	1.83	$C_{12}H_{21}O_{11}^-$	0.485	161.0455 179.0561	$C_6H_{12}O_6$ $C_6H_{10}O_5$	x	x	x
Tartaric acid	149.0074	2.41	$C_4H_5O_6^-$	−0.41	103.0037 87.0088	CO_2 H_2O	x	x	x
Undecanedioic acid	215.1264	22.84	$C_{11}H_{19}O_4^-$	0.082	197.1183 153.1285	H_2O CO_2	x	x	x

Table 4
Phenolic acid, flavonoid content, and scavenger activity of the extracts from wine lees, grape stalk, and pomace of *Magliocco canino cv*.

Code	PAC (*mg CT g−1 polymer*)	FC (*mg CT g−1 polymer*)	IC_{50} (*mg mL^{-1}*)	
			DPPH Radical	ABTS Radical
FAE	2.67 ± 0.10^c	–	0.6976 ± 0.0148^a	0.6966 ± 0.0341^a
RAE	89.64 ± 3.51^a	21.81 ± 0.94^a	0.0322 ± 0.0010^c	0.0144 ± 0.0003^c
VAE	24.68 ± 1.02^b	7.87 ± 0.21^b	0.3605 ± 0.0180^b	0.3920 ± 0.0141^b

FAE = Wine lees Pod of *Magliocco canino cv* in water/Ethanol; RAE = Grape stalk of *Magliocco canino cv* in Water/Ethanol; VAE = Pomace of *Magliocco canino cv* in Water/Ethanol; PAC = Phenolic Acid Content; FC = Flavonoid Content; CT = Catechin; DPPH = (2,2-diphenyl-1-picrylhydrazyl); ABTS = (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)). Different letters are significantly different (Tukey HSD test) at $p < 0.05$.

specific concentration range. SD was calculated on three replicates of the same sample. In total, 17 amino acids were quantified (Table 2). The total amino acid concentration for stalks, pomaces, and lees was quantified at 13.69, 3.96 and 4.10 g/kg respectively. Proline was the most abundant in all samples, with concentration values of 5.12, 2.22, and 2.26 g/kg. In conclusion, the analytical comparison between the three winemaking waste products highlighted significant differences in amino acid concentration but not in selective composition; almost all the searched analytes were found in each sample.

3.2.2. Qualitative and semiquantitative analysis of glycosylated polyphenols

A semitargeted search of the glycosylated adducts of polyphenols was carried out to better characterise the potential antioxidant properties of the extracts. The glycosides of catechin, pelargonidin, cyanidin, delphinidin, peonidin, petunidin, malvidin, kaempferol, quercetin, myricetin, isorhamnetin, syringetin, and laricitrin were detected by a precursor ion MS/MS approach and the results are reported in Table 5. The precursor ion study allows the detection of all the species that are able to generate the selected product ion (in this case the aglycone portion of the molecule, identified by the corresponding protonated ion). As described in Table 3 (Annotation of glycosylated polyphenols) a large number of glycosylated compounds was highlighted with this method; most of these compounds were not detected by the untargeted analysis. As described in paragraph 2.8, 11 aglycons were recognised as product ions, but for some of them, no conjugated compound was found. In total 22 molecules were annotated, as precursor ions of 10 polyphenols. Kaempferol, pelargonidin, and myricetin displayed the largest number of glycosylated adducts. Most of the analytes, 16, were found in stalks samples, some in pomaces but no remarkable peaks were evidenced in lees chromatograms analysis. The initial untargeted approach defined the most important molecular classes present in the three winemaking waste products. The data obtained in precursor ion scan mode were used to realise a semitargeted quantitation. The flavonoid content of the extracts was quantified using standard polyphenol compound calibration curves obtained with LC-HRMS. Overall values of 7.54, 241.6 and 42.97 mg/g of extract were found for FAE, RAE and VAE respectively. RAE fraction displays the highest total quantity of phenolics, with the prevalence of two molecules only, malvidin and petunidin. VAE fraction was shown to be rich in the same compounds, but also in quercetin derivatives. In addition to simple and conjugated flavonoids, untargeted analyses revealed that lees and pomaces were characterised by the presence of organic acids, amines, amides, and sugars; in fact these plant matrices are derived from the peel, seeds, and pulp of the fruit, generally rich in these types of components. On the other hand, stalks, representing the bunch wooden part, are particularly rich in amino acids, sphingosines and betaines that, however, are particularly concentrated also in the peel, the most abundant component of lees winemaking waste product. A large variety of amino acids, typical components of red fruits such as grapes, was found in lees and, to a lesser extent, in pomaces and stalks. Their presence shapes the organoleptic characteristic of wine, as demonstrated by several experimental evidence (Ozcan & Senyuva, 2006). The semi-targeted analysis performed on glycosylate compounds found a variety of analytes not detected previously by the untargeted approach. Likewise, stalks, composed essentially of cellulose and lignin that render its analysis very difficult, contained a great variety of glycosylated polyphenols. The comparison between untargeted and targeted analysis provided interesting insights into the composition of the extracts. However, only targeted methods identified additional secondary metabolites, thus improving the sample metabolomic profiling. This is not surprising, as untargeted analysis detects above all highly concentrated molecules due to its high selectivity but low sensitivity. For example, more polyphenolic compounds were annotated in pomace, and stalks than in lees. Similarly, only a few amino acids were detected by the untargeted analysis, whereas the targeted and quantitative analysis allowed a complete evaluation of this class. Finally, the precursor ion scan method expanded the glycosylated

Table 5
Annotation of glycosylated polyphenols.

Polyphenol	Transitions *(m/z)*	Precursor ion mass	Rt	Annotation	FAE	RAE	VAE
					HRMS quantitation (mg/g extract)		
Syringetin	200-800 > 346	509.4	2.9	Syringetin 3-O-galactoside	0.16 ± 0.05	8.97 ± 2.61	2.76 ± 0.80
Petunidin	200-800 > 331	479.3	3.1	Petunidin 3-galactoside	0.23 ± 0.07	8.02 ± 2.33	0.99 ± 0.29
Isorhamnetin	200-800 > 317	479.3	3.1	Isorhamnetin 4′-galactoside			
Malvidin	200-800 > 317	493.3	3.2	Malvidin-3-galactoside	0.39 ± 0.11	110.4 ± 32.1	11.4 ± 3.33
Cyanidin	200-800 > 287	449.3	3.1	Cyanidin 3-O-galactoside	0.11 ± 0.03	2.72 ± 0.79	0.30 ± 0.09
Myricetin	200-800 > 319	495.5	3.2	Myricomplanoside	0.24 ± 0.07	0.42 ± 0.12	1.04 ± 0.30
		481.5	2.5	Myricetin 3-O-glucoside	1.59 ± 0.46	0.56 ± 0.16	1.05 ± 0.31
		548.5	3.1	Myricetin 3-(3″,4″-diacetylrhamnoside)	n.d.	0.30 ± 0.09	1.95 ± 0.57
		388.5	36.6	5-Hydroxy-3,3′,4′,5′,7-pentamethoxyflavone	0.12 ± 0.03	0.19 ± 0.06	0.04 ± 0.01
Peonidin	200-800 > 301	463	3.2	Peonidin-3-glucoside	0.17 ± 0.05	39.0 ± 11.3	6.63 ± 1.93
		463.2	3.2	Peonidin 3-O-beta-D-glucopyranoside			
Quercetin	200-800 > 303	383	25.5	Quercetin 3-sulfate	n.d.	0.04 ± 0.01	0.09 ± 0.03
		479.1	21.6	Quercetin 3-O-glucuronide	n.d.	0.08 ± 0.02	3.96 ± 1.15
		435.2	3.1	Quercetin 3-O-beta-D-arabinofuranoside	0.29 ± 0.09	2.67 ± 0.78	0.44 ± 0.13
		465.3	3.1	Isoquercetin			
Pelargonidin	200-800 > 271	595.2	3.1	Pelargonidin 3-O-(6-caffeoyl-beta-D-glucoside)	n.d.	0.33 ± 0.09	0.24 ± 0.07
		403.7	3.1	Pelargonidin 3-arabinoside	0.08 ± 0.02	2.86 ± 0.83	0.21 ± 0.06
Laricitrin	200-800 > 332	509	3.0	Laricitrin 3-glucuronide	0.05 ± 0.01	0.54 ± 0.16	n.d.
Kaempferol	200-800 > 287	449.3	3.1	Kaempferol 3-O-D-galactoside	0.11 ± 0.03	2.72 ± 0.79	0.30 ± 0.09
		491.6	34.5	Kaempferol 3-O-beta-(2″-acetyl) galactopyranoside	n.d.	2.04 ± 0.59	n.d.
		559.4	35.2	CHEMBL519761	2.86 ± 0.84	11.1 ± 3.22	3.45 ± 1.00
		355.5	35.2	8-Isopentenyl-kaempferol	n.d.	n.d.	n.d.
				Sum	7.54	241.6	43.0

Transitions used for the precursor ion scan experiments; the precursor ions mass/charge ratio found, retention time (Rt), molecular annotations, and the relative presence in different samples are also shown.

polyphenols annotation, detecting more compounds in stalks and pomaces. Taken together, these observations indicate that though untargeted analysis allows a fairly complete description of the principal metabolomics classes, the development and improvement of targeted and quantitative methods are necessary to provide a complete metabolomics profile of this type of plant samples.

3.3. Biomolecule class profile definition and antioxidant activity of the water-ethanol extracts

The synergic cooperation between solvents at different polarities makes hydroalcoholic mixtures an effective extraction medium. Colorimetric assays were performed to define the biomolecule class profile of the hydroalcoholic extracts as well as their antioxidant features. As shown in Table 4, the highest concentration of phenolic acid was found in RAE, representing 67.8 % of total phenolic content, while lower amounts were recorded in the other samples. Flavonoids showed a similar trend, representing about 16.1 % of the total phenolic measured in RAE. Semi-quantitative analysis of glycosylated polyphenols showed a profile in partial agreement with total flavonoids, indicating VAE and RAE as the richest extracts.

The antioxidant properties of the hydroalcoholic extracts were investigated by probing their scavenger activity both in aqueous and organic environments against specific radical species. In line with the above results, RAE was the best-performing extract, with IC_{50} values against DPPH and ABTS radicals more than one order of magnitude lower than those of the other matrices. The highest levels of quercetin-related compounds found in RAE are consistent with these findings.

3.4. Synthesis and antioxidant properties of RAEP

Chemical modification of the DX mainly exploits functional hydroxyl groups, providing an easy plug for chemical conjugation with other molecules. In this context, the selection of the extract able to guarantee better performance plays a decisive role. A detailed evaluation of the antioxidant performances of the extracts returned RAE as the best valuable extract in terms of both PAC and scavenger activity. The synthesis of DX conjugate RAEP involved ascorbic acid/H_2O_2 redox pair as radical initiators. Specifically, the reaction between hydroxyl radicals and DX chains activated the polysaccharide towards radical reactions, promoting the insertion of antioxidant molecules in the extract. To settle the antioxidant features of RAEP, a control polymer was also synthesised in the same reaction conditions, but without any extract. Antioxidant properties of the conjugate and the control were studied operating the same tests previously described for the extracts. Specifically, the Folin-Ciocolteau test returned a TPC value equal to 64,7 mg GA g^{-1} of the sample, while PAC and FC displayed lower amounts, equal to 25.8 and 12.5 mg CT g^{-1} of the sample. Similarly, scavenger activity highlighted the best performances of the conjugate in the aqueous environment against ABTS radical species (IC_{50} equal to 0.06 mg mL^{-1}), compared to the organic one (IC_{50} of the DPPH test equal to 0.20 mg mL^{-1}). Finally, the control polymer did not display any interference, providing negative results to all the performed tests.

3.5. Effect of RAE and RAEP on H_2O_2-Induced ROS production in human dermal fibroblasts

In human dermal fibroblasts, treatment with H_2O_2 markedly increased the intracellular level of ROS (Fig. 2). Pre-incubation with either RAE or RAEP significantly prevented ROS production in a concentration-dependent manner, suggesting that the extract and the polymer protected cells against oxidative stress damage. Furthermore, RAE polymerisation with DX improved the antioxidant activity of the extract.

3.6. Extracts relax phenylephrine-induced contraction

A series of experiments was performed to investigate the effect of extracts on pharmaco-mechanical coupling. In endothelium-intact preparations, RAE and VAE caused concentration-dependent relaxation of the α_1 adrenergic receptor agonist phenylephrine-induced contraction (Fig. 3A–C); this was characterized by a hormetic profile. FAE was effective only at the highest concentration assessed (Fig. 3B). Endothelium removal significantly reduced the efficacy of the three extracts.

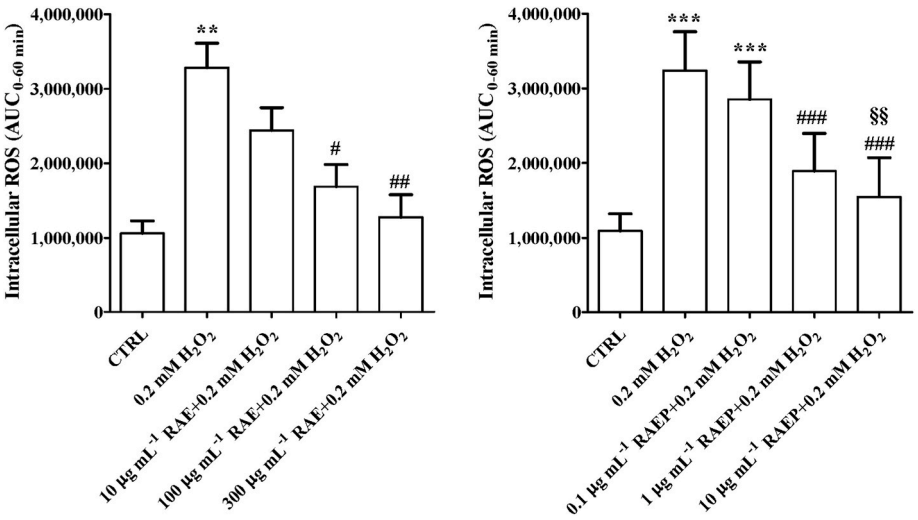

Fig. 2. Total amount of intracellular reactive oxygen species in the control condition (CTRL), in the presence of 0.2 mM H_2O_2, of either RAE (10, 100 and 300 µg mL^{-1}) or RAEP (0.1, 1 and 10 µg mL^{-1}) plus 0.2 mM H_2O_2 during 60 min, expressed as Area Under $Curve_{(0–60\ min)}$. Data are reported as the mean ± SD of at least 3 independent experiments. **p < 0.01, ***p < 0.001 vs. CTRL, #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. 0.2 mM H_2O_2, §§p < 0.01 vs 0.1 µg mL^{-1} RAEP+ 0.2 mM H_2O_2, ANOVA followed by Bonferroni post-test.

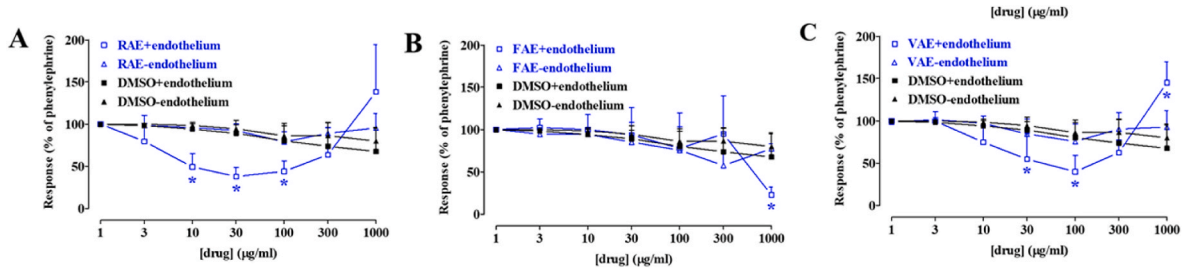

Fig. 3. Effect of the extracts on phenylephrine-induced contraction of rat aorta rings. Concentration-response curves of (A) RAE, (B) FAE, and (C) VAE on endothelium-denuded or endothelium-intact preparations pre-contracted by 0.3 µM phenylephrine. The effect of vehicle (DMSO) is also shown. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine, taken as 100%. Data are mean ± SD (n = 3–9). *P < 0.05 vs –endothelium, Student's *t*-test for unpaired samples.

3.7. Extracts are ineffective on high KCl-induced contraction

The vasorelaxant activity of the extracts was assessed on rings pre-contracted by either moderate (25–35 mM) or high (60 mM) KCl concentrations, which cause membrane depolarisation and $Ca_V1.2$ channel opening. In aorta preparations depolarised by 60 mM KCl, FAE was ineffective whereas the maximal concentrations of VAE and RAE caused a weak vasocontraction (12 % and 28 % of control, respectively; Fig. 4A–C).

In rings stimulated by 25–35 mM KCl, FAE was still ineffective whereas VAE and RAE caused a marked vasocontraction. The experimental evidence here presented is consistent with the hypothesis that winemaking by-products represent a valuable source of vasorelaxant agents. Whether the vascular activity described here is due to a single component of the extracts or rather to the additive or synergistic effect of several molecules is difficult to determine. Several compounds herein detected, such as the flavonoids quercetin (Trezza et al., 2022), luteolin and genistein (Ahmed et al., 2022), catechin (Menendez et al., 2011), naringenin (Saponara et al., 2006), and quercitrin (Calderone et al., 2004), the fatty acid derivative oleamide (Hernández-Díaz et al., 2020), the lignan (+)-pinoresinol (Lapi et al., 2015), and the anthraquinone aurantio-obtusin beta-D-glucoside (Li et al., 2015) are effective vasodilators. However, other flavonoids, such as myricetin and kaempferol (Fusi, Spiga, Trezza, Sgaragli, & Saponara, 2017), are capable of

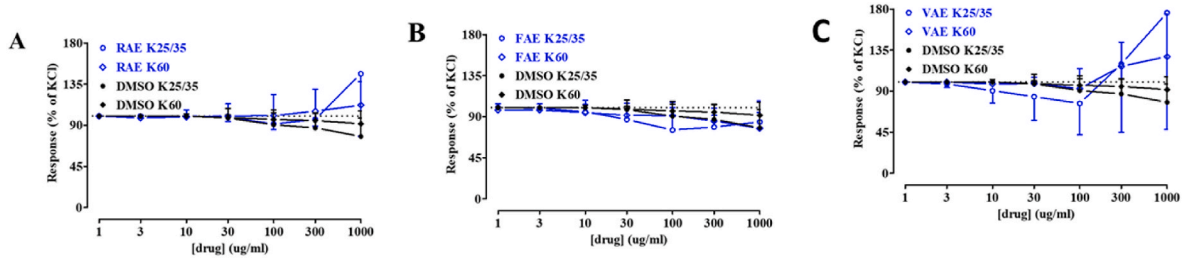

Fig. 4. Effect of the extracts on KCl-induced contraction of rat aorta rings. Concentration-response curves of (A) RAE, (B) FAE, and (C) VAE on endothelium-denuded preparations pre-contracted by either 25–35 mM (K25/35) or 60 mM KCl (K60). The effect of vehicle (DMSO) is also shown. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by KCl, taken as 100%. Dotted lines represent 100% response. Data are mean ± SD (n = 3–9).

stimulating $Ca_V1.2$ channels and, along with the alkaloid ecgonine, to evoke vascular smooth muscle contraction (Kurth et al., 1993; Fusi et al., 2003). Therefore, it is reasonable to hypothesise that the vascular activity of the extracts depends on the combined action of more than one component.

The experimental protocols used to investigate the vascular activity of the extracts provided interesting insights into the mechanism underpinning the myorelaxant effect observed. It is clear that RAE and VAE evoked an endothelium-dependent vasorelaxation, evident within a reasonable range of concentrations, which was characterised by a hormetic effect, already showed by another variety of *Magliocco*, i.e., *Magliocco dolce* cv (Carullo et al., 2020b). FAE also evoked an endothelium-dependent relaxation, but this property occurred only at very high concentrations. Arginine ethyl ester, found in VAE, enters the endothelial cell by passive diffusion and releases free arginine via the action of cellular esterases. However, arginine that does not enter the cell via the cationic transport system only weakly stimulates eNOS activity (Shin et al., 2011) and, therefore, arginine ethyl ester can only in part contribute to the endothelial-dependent relaxation of VAE. On the other hand, naringenin (Tan et al., 2021), oleamide (Hernández-Díaz et al., 2020), and aurantio-obtusin beta-D-glucoside (Li et al., 2015), all found in RAE, and the lignan (+)-pinoresinol found in RAE, VAE, and FAE (Lapi et al., 2015), might contribute to this phenomenon as their vascular activity is indeed characterised by the release of endothelium-derived molecules. Limitations in the availability of the extracts, however, did not allow an in-depth analysis of the modulators (e.g., NO, prostacyclins, endothelium-derived hyperpolarising factor, etc.) or pathways (e.g., eNOS, TRPV channels, etc.) involved in this phenomenon. Further experiments will help clarify the mechanisms implicated.

The effects of the extracts on preparations depolarised with high KCl indicated that stimulation of K^+ channels and/or inhibition of $Ca_V1.2$ channels are not involved in their vascular activity. In fact, on one hand, RAE, FAE, and VAE did not relax vessel tone evoked by moderate concentrations of KCl, an experimental setting where K^+ channel openers can repolarise the cell membrane, thus causing the closure of $Ca_V1.2$ channels and myorelaxation (Gurney, 1994). Rather, RAE and VAE increased vessel tone, though this effect was observed only at very high concentrations. On the other, they did not relax the contraction evoked by high KCl concentrations, an experimental setting where binding of Ca^{2+} antagonist to $Ca_V1.2$ channels and block of the Ca^{2+} current is promoted by membrane depolarisation, which stabilises the channel in its inactivated state (Gurney, 1994).

The existence of an inverse correlation between the amount of amino acids found in each extract and its vasoactivity provided some indications of the role played by these components. In fact, the higher the quantity of amino acids (as in FAE), the lower the vasoactivity, either in terms of pharmaco-mechanical coupling antagonism or electro-mechanical coupling enhancement. Extracts characterised by a one-order of magnitude lower concentration of amino acids, i.e., VAE and RAE, conversely, displayed comparable vasoactivity in all the experimental settings assessed. This evidence is consistent with the hypothesis that amino acids can limit in some way the efficacy of the vasoactive components of the extracts, impeding their interaction with the targets located at the endothelium and smooth muscle level and/or affecting the pathways associated with target activation.

4. Conclusions

The main results of the present study may represent a step forward in the valorisation of winemaking by-products: 1) targeted and semi-targeted analyses revealed interesting secondary metabolites, sometimes hidden due to the use of inappropriate analytical techniques or hard extractive methods. The combined use of high resolving power MS and of selective precursor ion tandem MS has proven to be a powerful molecular identification tool in plant metabolomics analysis; 2) the best extract, RAE from GP waste, showed a promising *in vitro* antioxidant activity; 3) the functional DX synthesised with RAE showed improved antioxidant power, exploitable in functional food or new antioxidant ingredients; 4) the vasorelaxant activity, shown by both RAE and FAE, suggests that these extracts may provide novel nutraceuticals. Taken together, the present data, along with others previously published, strongly recommend the recycling of these wastes for the development of nutraceuticals and/or food antioxidants/supplements beneficial to human cardiovascular health.

Notes The authors declare no competing financial interest.

CRediT authorship contribution statement

Claudio Medana: Writing – review & editing, Supervision, Software, Resources, Methodology, Investigation. **Umile Gianfranco Spizzirri:** Methodology, Formal analysis, Data curation. **Valentina Schiavo:** Formal analysis, Data curation. **Fabio Fusi:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Alice Panti:** Formal analysis, Data curation. **Simona Saponara:** Writing – original draft, Validation, Methodology, Investigation. **Paola Marcolongo:** Validation, Resources, Methodology, Investigation, Formal analysis. **Alex Affricano:** Formal analysis, Data curation. **Alberto Asteggiano:** Formal analysis, Data curation. **Francesca Aiello:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Donatella Restuccia:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: FRANCESCA AIELLO Alex Affricano CRediT: Data curation NO CONFLICT OF INTEREST If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116666.

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