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ORIGINAL PAPER

Chemical characterization of Grechetto grape pomace for potential valorization in a biorefinery perspective

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Abstract
The Organisation Internationale de la Vigne et du Vin (OIV) assessed vineyard among the main crops worldwide and the resulting waste represent a critical issue. Winery waste and their content of natural active principles have been extensively explored for changing them into new resources with high added value. The recovery of bioactive phenols and polysaccharides from pomace offers promising opportunities for producing novel ingredients across different sectors, enhancing winery economic sustainability and reducing environmental impact. In this scenario, studies concerning white grape *cv* Grechetto, widely cultivated in Italy, are very limited. The present work studied the possibility of exploiting white Grechetto whole pomace, skins and seeds, in a biorefinery perspective for a large-scale green extraction process to implement in the winery. Grechetto dried pomace were investigated by Fourier Transform Infrared Spectroscopy– Attenuated Total Reflectance (FTIR-ATR) to obtain a qualitative fingerprint. Polyphenols were characterized by High Performance Liquid Chromatography coupled with Diode Array and Mass Detectors (HPLC-DAD-MS) and Folin-Ciocalteu assay; polysaccharides by Dynamic Light Scattering (DLS), chemical hydrolysis and Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectroscopy. Total phenols were 51 ± 1, 25.36 ± 0.08 and 85 ± 2 mg/g GAE on whole dried pomace, skin and seed respectively, with similar trend obtained by HPLC-DAD-MS. Crude polysaccharides, studied for the first time in white Grechetto, were 12%, 13% and 8% on dried whole pomace, skins and seeds. They were present in relatively high quantities, with glucuronic acid, acetyl and methyl groups similar to pectin, suggesting their potential use as a gelling agent.

Keywords *Vitis vinifera* L. · Grechetto · Winery waste · FTIR-ATR · HPLC-DAD-MS analysis · ^{1}H-NMR spectroscopy · Polyphenols · Polysaccharides

Introduction

Background

The Organisation Internationale de la Vigne et du Vin (OIV) reported for 2022 a world wine production of 258mhl, almost stable for the fourth consecutive year and only slightly below its 20-year average. In the same year Italy (49.8mhl), France (45.6mhl), and Spain (35.7mhl) were the main wine producers worldwide, with France recording an increased production (+21% compared to 2021, + 7% with respect to its last five years average) and Italy and Spain substantially stable. Currently, despite the general decline suffered by the wine market due to the Covid-19 pandemic and the war in Ukraine, Italy remains the main exporter accounting for 20% of the global exports in 2022 [1]. Given the large quantities of wine produced, the waste

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of winemaking process represent an important critical issue due to their poor biodegradability and the ability to release volatile organic compounds into the environment and, therefore, the need for specific procedures for disposal [2]. The main waste products of winemaking process are grape pomace, skin, stalks, wastewater, lees, shoots and other filtration residues. Among them pomace, the solid residue obtained after pressing grape and made up of skins, seeds and stalks (these latter if not previously removed), is about 0.16 Kg per liter of wine and it represents the most abundant waste product [3]. The guidelines that regulate the destination of winery waste are continuously updated but, in general, the pomace is delivered to distilleries, converted into biogas or energy, or dispersed on agricultural land. In this latter case, the regulations impose limits on the amounts to spread on the land to avoid environmental impact. In the recent years scientific studies were performed to assess their content of active compounds and possible uses and applications in a wide variety of sectors according to circular economy models [4–6].

Polyphenols and polysaccharides in grape pomace

Red grape pomace may contain anthocyanins such as malvidin 3-O-glucoside, as well as other anthocyanoside compounds such as mono- and di- glucosides of malvidin, cyanidin, delphinidin, petunidin, and peonidin and their esters with acetic, coumaric or caffeic acids. In white winemaking, the vegetal material is removed immediately after pressing the grapes, whereas to produce red wines it is left to ferment with the juice and the anthocyanins are partially or totally transferred from the solid matrix to the wine. Furthermore, given their poor chemical stability, these compounds can largely degrade to uncolored phenolic structures with lower molecular weights during storage or the subsequent transformation processes [7]. Flavanols are among the main compounds in both red and white grape pomace: catechin and epicatechin were found, as well as their oligomers (proanthocyanidins) as such or as galloylated derivatives, in different isomeric forms. Also flavonoids are present such as myricetin, luteolin, quercetin and kaempferol derivatives; hydroxycinnamic derivatives, mainly caffeic, coumaric and ferulic acids esters; gallic acid derivatives; stilbenes (such as resveratrol) and phenolic acids such as p-hydroxyphenylacetic acid, vanillic acid, homovanillic acid, homoprotocatechuic acid, gentisic acid, syringic acid [4, 8–11]. Polyphenolic extracts of pomace demonstrated antioxidant, antimicrobial, anti-inflammatory, antimutagenic/anticarcinogenic, anti-lipogenic properties, and are useful in the prevention of cardiovascular diseases [12, 13]. Grape pomace showed high levels of soluble and insoluble dietary fibre, and crude polysaccharide contents up to 15% weight, these latter based on arabinose, glucose, galactose and mannose with molar ratio 18.4:14.1:10.8:3.0 and prevalent α-pyranose and β-pyranose structures containing also carboxyl groups [14, 15]. Polysaccharide fraction, tested in vivo, showed antioxidant, radical scavenging, antidiabetic, hepatoprotective and anti-inflammatory ability. All these properties can support the use of pomace to extract natural ingredients for the formulation of functional food and nutraceutical products [16]. Studies in the literature reported tests on grape pomace extracts as additives or functional ingredients for beverages, plant origin food, meat, fish, and dairy products, but also in the formulation of cosmetics. Da Rocha et al. (2012) used them to inhibit steel corrosion [9, 13, 17]. Grape seeds are often separated from pomace and used for mechanical extraction of oil, rich of PUFA such as linoleic acid, antioxidant such as tocopherols and bioactive phytosterols. Several analyses demonstrated that the polyphenolic compounds were mostly retained in grape seeds after oil extraction, so the residue could be further exploited as a source of hydro-soluble bioactive compounds [18, 19].

Valorisation of grape pomace: biorefinery approaches towards circular economy

According to these results and considerations winery waste, grape pomace in particular, could be transformed from environmental and economic burdens into new resources with high added value according to innovative models of circular economy, by integrating the different technologies in a biorefinery process to obtain a wide range of bio-based products [2, 20]. The implementation of sustainable models of circular economy in the agrifood sector acquires increasing importance as it directly meets the goals of 2030 Agenda for Sustainable Development in terms of food security, sustainable consumption and production, sustainable agriculture and causes positive repercussions on climate change, well-being, economic growth. Events such as Covid-19 pandemic, and epidemics that in recent years spread also due to the increase in population density, especially in particular areas, have highlighted the fragility of the interactions among elements of the food supply chain (production, transformation, distribution, consumption, disposal), and the need for innovative models for food systems [21]. Circular economy could improve these systems making them more robust and resilient.

Scientific studies are available in the literature concerning laboratory scale and industrial processes to recover and exploit grape pomace. Solid-liquid extraction is traditionally among the main extraction techniques for polyphenols and polysaccharides; selectivity depends on the used solvents and yields are dependent from time and temperature of extraction [22]. The large use of organic solvents and

long times of extraction made this technique not always a sustainable solution, so more eco-friendly methods were studied, aimed at enhancing solvent extraction or based on different principles. Enzymes, ultrasound and microwave-assisted extraction, the use of deep eutectic solvents, high-pressure applied to solid-liquid extraction, sub-critical or super-critical fluid extraction are among the green technologies that also allow for industrial scalability [22–27]. Concerning bioresources of antioxidant compounds in general, and grape pomace in particular, it is crucial to evaluate the effects of extraction and transformation technologies on qualitative and quantitative yields, especially for obtaining functional food or nutraceutical ingredients [28]. Emerging technologies such as high pressure processing and pulsed electric field extraction showed positive effects on polyphenols extraction yields from fresh grape and grape pomace, also taking into account the need to optimize the parameters according to the raw material and the target compounds to avoid the latter from degrading [29, 30]. The high content of organic matter makes grape pomace also suitable to produce energy by thermal conversion or by converting the biodegradable fraction into biogas through anaerobic digestion; bio-fuels such as bio-ethanol, bio-oil, bio-diesel can be obtained by fermentation. Aerobic fermentation allows the conversion of grape pomace into compost, to obtain eco-friendly fertilizers and soil amendments [31]. The integration of two or more processes in the same plant could allow to fully exploit grape pomace, although there are few studies concerning practical applications. As an example, Almeida et al. [32, 33] assessed the influence of polyphenol extraction on subsequent pyrolysis or methane production by anaerobic digestion, finding that the potential yields remain statistically similar. Lucarini et al., 2018 [18] explored the possibility of recovering phenolic compounds after oil extraction from grape seeds, demonstrating that these processes could be integrated in a biorefinery plant. Technical-economic assessments were also carried out, concluding that the economic sustainability of a biorefinery plant mainly depends on the possibility of obtaining more than one bio-based products as well as on their prices, and the variety of involved market sectors [31]. Hence, obtaining a range of differentiated products acquires increasing importance, and makes it crucial their characterization and the study of suitable technologies for the exploitation of the different classes of compounds in distinct fractions. In-depth studies are present in the literature on pomace from numerous white grape cultivars, but in most cases the characterization of only one class of compounds is reported, and studies that specifically concern white pomace *cv* Grechetto are very limited. Furthermore, the differences between pomace obtained from white and red winemaking still need to be explored in more detail, in terms of both their chemical composition and possible applications [23, 34, 35].

Object and purpose of the study

The present study focused on white grape pomace *cv* Grechetto, little investigated so far. This cultivar, probably originating from Greece as evidenced by the toponymic root present in its name, was anciently imported to Italy through Magna Graecia and spread mainly in the regions of Umbria, Lazio, Tuscany and Marche. The clones currently present in the National Register of Varieties are considered native to central Italy. The research concerned the characterization of polyphenol content and polysaccharide fraction of dried grape pomace resulting after the production of wine in Cortona, a city located in Tuscany, to explore the possibility of exploiting them in a biorefinery context. Both the whole grape pomace (G_PO) and the skins (G_SK) and seeds (G_SE) after separation were investigated, to compare their compositions in active principles and allow the assessment, in a future valorization perspective, of using the whole pomace as such or introduce a step of separation of skins from seeds into the process [36]. A preliminary characterization was carried out by Fourier Transform Infrared with Attenuated Total Reflectance (FTIR-ATR) for determining the fingerprint of the different powdered samples based on the qualitative analysis of the main functional groups.

The techniques used for studying the profile of main components were Liquid Chromatography coupled with Diode Array Detector and Mass Spectrometry (HPLC-DAD-MS), Dynamic Light Scattering (DLS) and proton Nuclear Magnetic Resonance (^{1}H-NMR), with the purpose of obtaining, respectively, a qualitative and quantitative analysis of the polyphenolic compounds and a detailed characterization of the polysaccharide content. These results are the basis for testing scalable extraction processes that could be integrated into a biorefinery process, aimed at the recovery and exploitation of Grechetto pomace, to obtain products differentiated by chemical composition for use in different production sectors.

Materials and methods

Samples and reagents

All solvents for ^{1}H-NMR and HPLC-DAD-MS analyses (HPLC grade), formic acid, gallic acid, catechin hydrate, epicatechin, p-coumaric acid, caffeic acid (analytical grade) were purchased from Sigma Aldrich Chemical Company Inc. (Milwaukee, WI, USA). Procyanidin B1, B2, B3 and C1, quercetin 3-*O*-glucoside and kaempferol 7-*O*-glucoside

were supplied by Extrasynthèse S.A. (Lyon, Nord-Genay, France). HPLC-grade water was obtained via distillation and purification with a Labconco Water Pro PS polishing station (Labconco Corporation, Kansas City, MO, USA). The grape pomace was obtained after wine-making at Consulente Enologica Srl (Cortona, AR, Italy) from white grapes *cv* Grechetto harvested in September, 2023. A portion of pomace was dried as such (G_PO), whereas a second portion was separated in skins (G_SK) and seeds (G_SE) by using an inox vibrating sieve before the drying process. The drying process was performed at E.LAB Srl (Pistoia, Italy) in a dryer cell with bottom-up hot air input at 28 °C, for 48 h, with 80% yield of dried material as previously reported [37]. A rotor-knife mill (IKA M20 universal mill, IKA, Staufen im Breisgau, Germany) was used to obtain from each of the dried samples a homogeneous powder that was used to prepare the hydro-alcoholic and the aqueous extracts.

FTIR-ATR analysis

The FTIR spectra were recorded on a Nicolet iS10 FT-IR spectrometer (Thermo-Fisher Scientific, Waltam, MA, USA) equipped with a diamond crystal cell for attenuated total reflection (ATR) operation. The spectra were acquired (64 scans per sample or background) in the range of 4000–600 cm^{-1} at a nominal resolution of 4 cm^{-1}. The spectra were corrected using the background spectrum of air. The analysis was carried out at room temperature. For a measurement, a dried powdered sample was placed on the surface of the ATR crystal. Before acquiring a spectrum, the ATR crystal was carefully cleaned with wet cellulose tissue and dried using a flow of nitrogen gas. The cleaned crystal was checked spectrally to ensure that no residue was retained from the previous sample and, as a first step, spectra were analyzed with respect to the band positions in order to identify the signatures of the major functional groups. An assignment of the main bands was carried out by analyzing the acquired spectra and by comparing them with the literature.

Extraction and characterization polyphenols

Extraction of polyphenols

The extraction was carried out by dispersing 5.0 g of each of the three different matrices in 30.0 mL of 70:30 EtOH: H_2O (pH 2.5 by addition of formic acid). The mixture was kept under mechanical stirring, at room temperature, for 24 h, then centrifuged at 5000 rpm for 10 min to separate the solid from the extract. The plant matrix was recovered and kept further 24 h under stirring with 30.0 mL of the same solvent, under the same conditions, then again separated by centrifugation. The two extracts were combined to determine total phenolic content and analyzed by HPLC-DAD-MS.

Total phenolic content and qualitative/quantitative HPLC-DAD-MS analysis

Total phenolic content was evaluated using the spectrophotometric test with Folin-Ciocalteu reagent. The latter is a mixture of phosphotungstic and phosphomolybdic acids which is reduced by reaction with phenols, producing a blue coloration proportional to the concentration of phenolic compounds. Phenol concentration in the sample was determined by measuring the absorbance at 725 nm, by a UV-Visible spectrophotometer (Agilent 8453, Agilent technologies, CA, USA), of a solution of sample and Folin-Ciocalteu reagent, after adding 20% Na_2CO_3 and incubating for 40 min., using a calibration curve built by measuring the absorbance of five reaction solutions containing gallic acid at different concentrations from 0.018 mg/mL up to 0.560 mg/mL. The phenol content of each sample is expressed as mg GAE (Gallic Acid Equivalents) per gram of dried vegetal material.

The HPLC-DAD-MS analyses were performed with a HP-1260 liquid chromatograph equipped with a DAD detector and an MSD API-electrospray (Agilent Technologies, Santa Clara, CA, USA) operating in negative and positive ionization mode. Mass spectrometer operating conditions were the following: gas temperature 350 °C at a flow rate of 10.0 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C, and capillary voltage 3500 V. The fragmentor was set at 120 eV. For the chromatographic separation a Lichrosorb RP18 250 × 4.60 mm i.d, 5 µm column was used (Merck Darmstadt, Germany). The eluents were H_2O, adjusted to pH 3.2 with formic acid, and CH_3CN. A four-step linear solvent gradient was used, starting from 100% H_2O up to 100% CH_3CN, for 117 min at a flow rate of 0.8 mL/min [38]. The phenolic compounds were identified by using data from HPLC-DAD-MS analyses, by comparing their retention times, UV-VIS and mass spectra with those of the available specific commercial standards and according to the available literature data.

The quantitative analysis was performed by HPLC-DAD using five-point regression curves built with the available standards; calibration curves with an $r^2 > 0.9998$ were considered. Calibration was performed at the wavelength of the maximum UV-VIS absorbance, by applying the correction of molecular weights. In particular gallic acid, catechin, epicatechin, proanthocyanidins were calibrated at 280 nm with gallic acid and catechin hydrate respectively. Coutaric acid was calibrated at 308 nm with p-coumaric acid; caftaric acid was calibrated at 330 nm with caffeic acid. Quercetin,

kaempferol and their derivatives were calibrated at 350 nm with quercetin 3-*O*-glucoside and kaempferol 7-*O*-glucoside respectively. The determinations of the polyphenol contents were carried out in triplicate; the results are given as means with the respective standard deviations.

Extraction and characterization of polysaccharides

Extraction of polysaccharides

For polysaccharides extraction, 5.0 g of each of the powdered samples (whole pomace, skin and seed) were suspended in 200.0 mL hot water and kept boiling for 1 h under magnetic stirring. Once at room temperature, each mixture was kept under maceration for 12 h, then centrifuged at 5000 rpm, at 15 °C, for 15 min. The obtained aqueous extracts were freeze-dried. For the isolation of crude polysaccharides, the freeze-dried extracts were suspended in 80% EtOH in proportion 1 g:50mL, treated with ultrasounds in ultrasonic processor (DU-32 S ARGO Lab, Carpi, MO - Italy) at 40 kHz, 120 W and 35 °C for 2 min to homogeneously disperse the powders and maintained at 0 °C in ice bath for 1 h. For each of the samples, the mixture was filtered under vacuum, then the insoluble fraction of polysaccharides was washed with 20 mL of 96% EtOH. The filtered solution was recovered and the process repeated, then the insoluble fraction was freeze-dried and weighed to obtain the yield in crude polysaccharides.

Dynamic light scattering

Dynamic Light Scattering (DLS, Zetasizer NanoSeries ZS90, Malvern Instruments, Worcestershire, UK) was employed for the following measurements: Z-average (Z-Ave, nm), size distribution by intensity (polydispersity index, PdI), and average molecular weight of the polysaccharide. The measurements were conducted at 25 °C in ultra-pure water (3 mg/mL) with a scattering angle of 90°, using the ALV-60 × 0 software package (Malvern, ver. 7.2). Each sample (G_PO, G_SK, G_SE) was analyzed in three separate experiments, with three readings for each sample. As for DLS, each replicate was an average of 12–16 readings made by the instrument.

^{1}H-NMR analysis

^{1}H-NMR data were obtained by a Bruker Avance 400 instrument (Bruker, Bremen, Germany). The proton spectra were used to determine the percentage of galacturonic acid, degree of acylation (DA), and degree of methylation (DM). The polysaccharides were analyzed after dialysis at concentrations ranging from 4.8 to 6 mg/mL. The percentages of DM and DA were determined according to Müller-Maatsch et al. [39] with slight modifications. Briefly, the initial hydrolysis process was conducted directly in a ^{1}H-NMR tube. The samples, with concentrations ranging from 2.1 to 3.8 mg/mL, were dissolved in 0.4 M NaOH in D_2O. After an incubation period of 120 min at room temperature, 25 µL of an internal standard solution (5.44 mg/mL maleic acid in D_2O) was added. Then, the samples were subjected to analysis using ^{1}H-NMR. To determine the concentrations of acetic acid and methanol after basic hydrolysis, we integrated the C-methyl groups at 1.80 ppm and 3.22 ppm, respectively. Additionally, the proton signal of maleic acid (6.24 ppm) was used as a reference for quantitative evaluation, applying a formula previously used for this purpose [40, 41]. The content of acetic acid and methanol in the polysaccharide sample was determined as follows:

$$\mathrm{C}\,(\%) = \frac{I_{CH3}}{I_{istd}} \times \frac{N_{istd}}{Nx} \times \frac{MW_x}{MW_{istd}} \times \frac{W_{istd}}{W_x} \times P_{istd}$$

C (%), the concentration of methanol or of acetic acid.
I_{istd}, the integral of the two protons of the ISTD, maleic acid.
I_{CH3}, the integral area of proton signal of CH_3 groups of methanol or acetic acid.
Nx, the number of protons for the CH_3 groups of methanol or acetic acid (3 protons) N_{istd}, the number of protons for ISTD (2 protons).
MW_x, the molecular weight of methanol (32 g/mol) and acetic acid (60 g/mol) MW_{istd}, the molecular weight of ISTD (116.1 g/mol).
W_x, weight (mg) of dry polysaccharide W_{istd}, weight of maleic acid.
P_{istd}, purity degree of maleic acid.

The content of galacturonic acid in each sample was determined after acid hydrolysis, in a Pyrex tube, at 100 °C for 120 min, according to Giner et al. [42]. The amount of galacturonic acid (Gal-ac) was obtained by the integration of the anomeric protons of the α-Gal-ac at 4.559–4.569 ppm and β-Gal-ac at 3.865–3.884 ppm, by using the same internal standard. The formula previously reported was applied considering: C%, concentration of Gal-ac; Nx, the number of anomeric protons (1) for α and β forms respectively; MW_x, molecular weight of galacturonic acid: 194 g/mol.

Through the moles of methanol, acetic acid and those of galacturonic acid, the degree of methylation (DM) and acetylation (DA) were calculated, according to the following formulas [39]:

$$\mathrm{DM}\,(\%) = (\%\ \text{moles of methanol/moles of galacturonic acid})$$

Statistical analysis

Data were processed with DSAASTAT software and Microsoft Excel. A one-way analysis of variance (ANOVA) was performed with significance level set at $p=0.01$. Means separation was carried out using Tukey's HSD test.

Results and discussion

The chemical characterization of Grechetto winery waste was below described and discussed. G_PO, G_SK and G_SE samples were explored as follows:

- Qualitative Analysis by FTIR-ATR;
- HPLC-DAD-MS analysis of polyphenols;
- Dynamic Light Scattering analysis of polysaccharides;
- ^{1}H-NMR analysis of polysaccharides.

Qualitative analysis by FTIR-ATR

A preliminary rapid characterization was carried out by FTIR-ATR. This analytical technique provides a qualitative fingerprint of the different samples based on their main functional groups. FTIR-ATR Infrared spectroscopy represents an interesting alternative to the conventional analytical methods as it does not require pretreatment of the samples. In addition, this technique is considerably more environmentally friendly since no solvents or carrier gases are used. FTIR-ATR spectra of G_PO, G_SE and G_SK samples are shown in Fig. 1. FTIR-ATR spectra (Fig. 1) represent a molecular fingerprint of the samples, by providing characteristic signatures of the substances present in the samples by featuring their molecular vibrations (stretching, bending, and torsions of the chemical bonds) [43].

Different peaks, which correspond to functional groups and modes of vibration of the individual components, can be discerned. The description of the main features of G_PO, also characteristic of all three samples, is reported below.

The broad band extending at around 3306–3265 cm^{-1} corresponds to the OH stretching modes (hydroxyl group), that can be related to the polysaccharide fraction and/or lignin, cellulose, and hemicellulose [44–46]. The peak at 3009 cm^{-1} is due to the C-H stretching vibration of the cis-double bond (=CH) groups. Asymmetric and symmetric stretching vibrations of CH_2 and CH_3 groups at 2962, 2921, 2873 and 2852 cm^{-1} are found [47]. The peak at 1743 cm^{-1} and the shoulder one at 1710 cm^{-1} is attributed to the absorption generated by stretching vibration of the–COOR groups [48] and it could be related to the presence of the fatty acids and their glycerides, as well as pectin and lignins [18, 49]. The broad bands at 1607 cm^{-1} and 1532 cm^{-1} associated to amide I could be related to the proteins in the extracellular matrix or in cell walls on the skin external surface as reported by Fasoli et al. (2016) [49]. Also, the bands 1607–1532 cm^{-1} can be associated to vibrations of aromatic C=C groups in pectin and phenolic compounds or cutan as reported by Spinei et al. [50].

As previously marked by Lucarini et al. [36], the region from 1500 to 800 cm^{-1} represents the fingerprint region rich in peaks produced by different vibrations - stretching, bending, rocking, scissoring, and torsional modes. At the same time, it represents a region rich in information on organic compounds (i.e. sugars, alcohols, organic acids, and so on) in the samples [36]. The stretching of aromatic C-C at ~1532 cm^{-1} and ~1440 cm^{-1} is related to phenolic molecules [44]. The out of plane bending of the CH_3 at 1377 cm^{-1}, the vibration at 1241 cm^{-1}, and the stretching of C-O at ~1032 cm^{-1} are related to polysaccharide structures [44, 48, 51]. The bands at 1134 cm^{-1}, 1097 cm^{-1}, 1032 cm^{-1} are related to cellulose and hemicellulose [49]; the peak around 904 cm^{-1} is related to the absorption peak of β-glycosidic linkage in pectin [52–54]. The peak at 1134 cm^{-1} is related to the stretching of aromatic C-H and the band at 763 cm^{-1} is related to the rocking vibration of CH_2, typically of phenolic compounds [45].

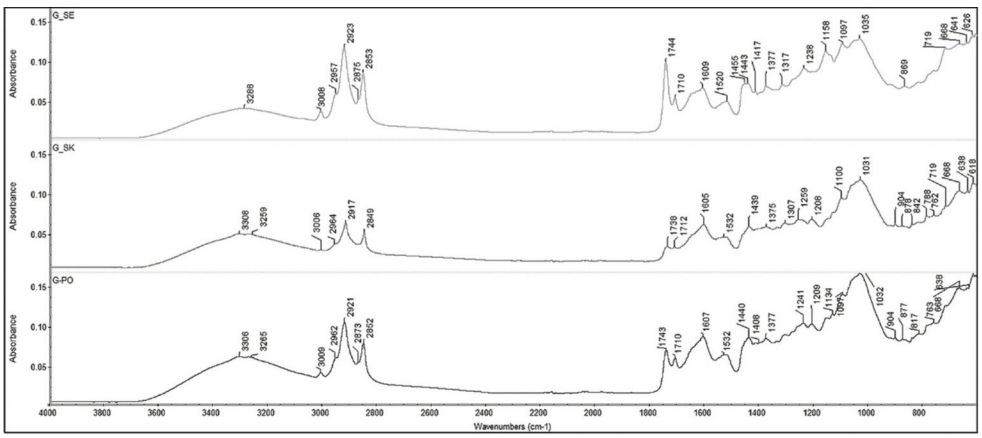

Fig. 1 FTIR-ATR spectra of G_PO, G_SE and G_SK samples

Characterization of polyphenols

Total phenol content was evaluated by in vitro spectrophotometric test with Folin-Ciocalteu reagent, an electron transfer-based assay widely used for screenings of total phenols and polyphenols in plant-derived extracts, foods and drinks, and also reported by OIV among the official international methods of analysis for red and white wines. A direct correlation was demonstrated between phenolic content measured by Folin-Ciocalteu assay and the antioxidant activity evaluated with other antioxidant assays such as ORAC, ABTS, and DPPH [55, 56]. The hydro-alcoholic extracts of the three samples under examination were tested, showing total phenolic contents of 51 ± 1 mg/g GAE (G_PO), 25.36 ± 0.08 mg/g GAE (G_SK) and 85 ± 2 mg/g GAE (G_SE), as reported in Table 1. HPLC-DAD-MS analysis was performed for the qualitative and quantitative characterization of the polyphenolic fraction. This analytical technique is widely used, mostly using reverse stationary phases, for the characterization of polyphenols in general due to their polar nature, and in particular those in grape pomace. The analysis of chromatographic, spectrophotometric and spectrometric data, the comparison with specific analytical standards and previous literature results allow to identify and quantify the individual compounds belonging to the different phenolic subclasses, also distinguishing the different proanthocyanidin isomers and oligomers which often remains challenging [36, 37, 57]. The HPLC-DAD-MS analysis of the polyphenolic extracts revealed the presence of 34 ± 1 mg/g polyphenols for G_PO, 8.5 ± 0.3 mg/g for G_SK and 50 ± 2 mg/g for G_SE. These results follow the same trend as the Folin-Ciocalteu data: seeds show the highest polyphenol content, whereas skins contain the lowest amount. This is not only because seeds have the highest concentration of proanthocyanidins, which contribute significantly to the weight of total polyphenols due to their high molecular weights, as the molar concentrations also reflect the same trend: 62 ± 2 µmol/g, 15.9 ± 0.5 µmol/g and 91 ± 3 µmol/g total polyphenols for G_PO, G_SK and G_SE respectively (data not reported in Tables). It should be noted, however, that several previous works in the literature reported higher polyphenol levels for grape seed extracts than for skin extracts [58, 59].

Proanthocyanidins are the main compounds in all of the three samples, accounting for 94.9% weight of total polyphenols in G_PO, 88.7% in G_SK and 94.6% in G_SE as shown in Table 2. Among them, procyanidin dimers B1, B2, B3 and trimer C1 were identified by comparison with the specific commercial standards according to their chromatographic, spectrometric and spectrophotometric data and to the literature [58–60]. Moreover, they were tentatively identified 6 different dimers and two trimers of catechin or epicatechin, two epigallocatechin dimers and two isomers of epicatechin gallate according to their retention times, UV-VIS absorption profiles and $[M+H]^+$ molecular ions, also taking into account our previous results concerning the characterization of grape and its by-products [18, 36, 37]. Among the proanthocyanidins, dimers are by far the most abundant compounds accounting for over 90% of the total proanthocyanidins in all three samples.

No catechin or epicatechin tetramers were detected in the samples. This should be better investigated, because flavanol tetramers are generally reported for grape and its by-products, mainly seeds. Previously reported results assessed their presence in polyphenolic extracts of grape pomace, in particular white grape pomace var. Zalema [61]. Moreover, the controlled soft drying process used for G_PO. G_SK and G_SE was previously validated by this research group for winery waste from different grape cultivars, also detecting the presence of preserved high-molecular weight oligomers in the dried products [37]. On the other hand, in recent studies about white grapes, the analyses of phenolic compounds are performed on extracts obtained with specific methodologies for polysaccharides, making it difficult a comparison with hydro-alcoholic extracts specific for polyphenols. As an example, Curiel-Fernàndez et al. found only flavanolic monomers and procyanidin dimers in nine white grape pomace samples of varieties from the Castilla y León region in northern Spain, after extraction in 2.5 g/L tartaric acid in water [65]. Among the flavan-3-ols catechin and epicatechin, the basic monomers of the detected procyanidins, catechin is significantly more abundant than its epimer epicatechin in all three samples and in particular in the skins (catechin: epicatechin ratios: 1.5, 2.8 and 1.3 for G_PO, G_SK and G_SE), according to the available literature about grape pomace and also in agreement with what previously reported for fresh grape berries [28, 31–33]. They account together for 3.9%, 3.2% and 5.0% weight on total polyphenols in G_PO, G_SK and G_SE respectively. The other identified polyphenolic compounds are gallic acid and monogalloyl-glucose, present in low percentages in all of the samples, whereas flavonols (quercetin and kaempferol glycosides) and hydroxycinnamic derivatives (trans-caftaric and trans-coutaric acids) were detected only in skins and pomace. Flavonols, among which quercetin glucuronide is the most represented, are particularly abundant in skins (6.12% of total polyphenols vs. 0.82% in pomace), in accordance with literature data [61–65].

Table 1 Folin-Ciocalteu assay and HPLC-DAD-MS analysis of G_PO, G_SK and G_SE polyphenolic extracts

Folin-Ciocalteu assay						
			Total phenolics (mg/g GAE)			
			G_PO	G_SK	G_SE	
			51 ± 1[a]	25.36 ± 0.08[b]	85 ± 2[c]	
HPLC-DAD-MS analysis						
Compound	RT (min)	λ_{max} (nm)	$[M+H]^+$ (m/z)	Concentration (mg/g sample)		
				G_PO	G_SK	G_SE
Monogalloyl glucose	11.7	274	331[1]	0.030 ± 0.001[a]	ND	0.052 ± 0.002[b]
Gallic acid	13.3	272	169[1]	0.088 ± 0.001[a]	0.048 ± 0.001[b]	0.147 ± 0.003[c]
Trans-caftaric acid	25	326	311[1]	0.0162 ± 0.0002[a]	0.092 ± 0.002[b]	ND
Procyanidin B1	30	280	579	0.36 ± 0.01[a]	0.139 ± 0.004[b]	0.47 ± 0.01[c]
Trans-coutaric acid	30.5	314	295[1]	0.0097 ± 0.0002[a]	0.027 ± 0.001[b]	ND
Procyanidin B3	32	280	579	0.250 ± 0.008[a]	ND	0.48 ± 0.01[b]
Catechin	34.16	280	291	0.800 ± 0.009[a]	0.202 ± 0.002[b]	1.42 ± 0.03[c]
Catechin/epicatechin trimer 1	36.8	280	867	ND	ND	0.325 ± 0.009
Catechin/epicatechin dimer 1	39.2	280	579	ND	ND	0.51 ± 0.01
Procyanidin B2	42.5	280	579	0.290 ± 0.002[a]	ND	0.75 ± 0.03[b]
Epicatechin	46.2	278	291	0.52 ± 0.01[a]	0.073 ± 0.001[b]	1.06 ± 0.02[c]
Procyanidin C1	54.6	280	867	0.134 ± 0.002[a]	ND	0.338 ± 0.005[b]
Epigallocatechin dimer 1	55.9	280	731	0.59 ± 0.02[a]	0.152 ± 0.004[b]	1.11 ± 0.03[c]
Epigallocatechin dimer 2	56.9	280	731	0.045 ± 0.001[a]	ND	0.40 ± 0.01[b]
Catechin/epicatechin trimer 2	59.2	280	867	0.248 ± 0.005[a]	0.053 ± 0.002[b]	0.142 ± 0.003[c]
Catechin/epicatechin dimer 2	61.1	280	579	0.067 ± 0.003[a]	0.33 ± 0.01[b]	0.259 ± 0.008[c]
Epicatechin gallate isomer 1	62	280	443	0.79 ± 0.02[a]	0.143 ± 0.004[b]	1.43 ± 0.04
Catechin/epicatechin dimer 3	64.2	280	579	0.27 ± 0.01[a]	ND	0.53 ± 0.02[b]
Catechin/epicatechin dimer 4	75.7	280	579	7.5 ± 0.2[a]	1.29 ± 0.04[b]	11.2 ± 0.4[c]
Epicatechin gallate isomer 2	79.2	280	443	0.124 ± 0.004[a]	0.027 ± 0.001[b]	0.271 ± 0.008[c]
Catechin/epicatechin dimer 5	85.8	280	579	20.5 ± 0.8[a]	4.2 ± 0.1[b]	27.8 ± 0.8[c]
Quercetin glucuronide	87	256, 354	477[1]	0.110 ± 0.002[a]	0.218 ± 0.004[b]	ND
Quercetin galactoside	89	254, 354	463[1]	0.0160 ± 0.0003[a]	0.0185 ± 0.0004[b]	ND
Quercetin glucoside	89	254, 354	463[1]	0.048 ± 0.001[a]	0.102 ± 0.001[b]	ND
Kaempferol galactoside	93	254, 342	447[1]	0.031 ± 0.001[a]	0.046 ± 0.001[b]	ND
Kaempferol glucoside	95	256, 342	447[1]	0.075 ± 0.002[a]	0.138 ± 0.003[b]	ND
Catechin/epicatechin dimer 6	95.6	280	579	1.27 ± 0.05[a]	1.27 ± 0.05[b]	1.03 ± 0.04[b]
Total polyphenols				34 ± 1[a]	8.5 ± 0.3[b]	50 ± 2[c]

Results in mg GAE (Gallic Acid Equivalents) per gram of dried sample and mg individual compounds per gram of dried sample. Data reported as the means of 3 measures ± standard deviations. For each line, data with different letters are significantly different according to Tukey's HSD test ($p < 0.01$)

[1]$[M - H]^-$ (*m/z*); *ND* Not Detected

Table 2 Amounts of individual polyphenolic subclasses with respect to total polyphenols (% w/w) in the analyzed samples

Subclass	% G_PO	% G_SK	% G_SE
Pro.	94.9	88.7	94.6
Cat.	2.34	2.37	2.87
Epi.	1.53	0.86	2.14
Flav.	0.82	6.12	ND
Gal.	0.35	0.56	0.40
Hydr.	0.08	1.39	ND

Pro. Proanthocyanidins, *Cat.* Catechin, *Epi.* Epicatechin, *Flav.* Flavonols, *Gal.* Gallic acid derivatives, *Hydr.* Hydroxycinnamic derivatives

Characterization of polysaccharides

Yields of crude polysaccharides

The yields of crude polysaccharides were calculated according to the weight of crude polysaccharides with respect to the freeze-dried decoction or the dried vegetal material. The percentage yields were: 43%, 46% and 40% w/w on freeze-dried decoction for G_PO, G_SK and G_SE respectively. Analogously, the yields expressed on the dried vegetal material were 12%, 13% and 8% for G_PO, G_SK and G_SE respectively. It is important to note that this green extraction process to prepare an extract rich in polysaccharides is simple and easily scalable.

Dynamic light scattering

DLS has been used in previous studies to analyze the particle size of different polysaccharides extracted from olive pomace, pomegranate peel and date palm [66, 67]. The by-products of grape pomace, skin and seeds were analyzed using DLS to acquire information on the shape and hydrodynamic volume of the polysaccharides in water solution because DLS is a simple and powerful technique for detecting variations in the size of polymeric structures, such as polysaccharides. A single peak in the DLS profile indicates a highly homogeneous sample with polysaccharides of similar size, in contrast multiple peaks suggest the presence of a heterogeneous group of polysaccharides. The polydispersity index (PdI) of the polysaccharides extracted from grape pomace ranged from 0.18 for G_SE, to 0.23 for G_SK indicating in both the cases a homogeneous distribution of polysaccharide sizes. Furthermore, the PdI values are correlated to the area percentage values of the main peak (Pk1), which in almost all the samples was 100%, suggesting the presence of only one main population. Moreover, the Z-average size is the primary and most stable parameter in DLS and provides a reliable measure of the average size of a particle distribution. It is often preferred in DLS for evaluating the size diameter of the principal component in solution. The Z-average values of the main peak in the polysaccharide samples show an average diameter (Z-Pk1) ranges from 166.1 nm (G_SE) to 305.3 nm (G_SK). Moreover, the molecular weight estimated (Est MW) in grapes polysaccharides ranged from $6.4*10^4$ to $3.2*10^5$ KDa for the dominant peak (Pk1). This is consistent with the expected size range for polysaccharide molecules (Table 3). Overall, the DLS analysis provides valuable insights into the size distribution and estimated molecular weight of the polysaccharides in aqueous solution. Samples G_PO, G_SK, and G_SE, exhibit moderately uniform size distributions with diameters ranging from 166 to 305 nm.

Recently, alternative techniques have been employed to investigate polysaccharide, in particular pectin molecular size, including high-performance size-exclusion chromatography coupled with multi-angle light scattering and refractive index detection (HPSEC-MALS-RI), which revealed average molecular weights (Mw) of 478 ± 6 kDa and 449 ± 3 kDa for pectin extracted from grape pomace [68]. Similarly, Spinei and Oroian [69] reported molecular weight ranges of 4.19×10^4 to 5.59×10^4 g/mol using high-performance size-exclusion chromatography (HPSEC), which align with our findings.

Table 3 DLS analyses of samples G_PO, G_SK, G_SE. PdI, polydispersity index; D, diameter size Nm; Z-Pk1, Z-average particle size of the main peak; Pk1, the detected peak in each sample; KDa, Kilodalton; est MW, estimated molecular weight

Samples	PdI	Z-Pk1 D (nm)	Pk 1 D (nm)	Pk 1 Area %	Est MW Pk 1 (KDa)
G_SK	0.23 ± 0.05	249.1 ± 2.5	305.3 ± 10.6	100	1.9*105
G_SE	0.18 ± 0.02	166.1 ± 2.9	199.6 ± 7.9	100	6.4*104
G_PO	0.21 ± 0.01	300.1 ± 3.8	334 ± 15.8	100	3.2*105

^{1}H-NMR analyses

The ^{1}H-NMR spectra of the polysaccharides extracted from Grechetto pomace revealed a high similarity (Fig. 2). All the polysaccharide samples exhibited an intense singlet at 3.76 ppm, indicating the presence of numerous CH_3O- groups bonded to galacturonic acid units, a characteristic feature observed in various natural pectin structures, also consistent with the spectra of two commercially available pectin with degree of esterification ≥ 85% and 55–70% (Fig. S1). Among the samples, the proton spectra of G_SE displayed a slight variation in the chemical shift (δ) of the acetyl (CH_3CO-) group at 2.1 and 2.14 ppm (Fig. 2c). Additionally, a less intense signal was evident at 1.20 ppm, attributable to the methyl (CH_3-) group of the rhamnose unit. The intensity of these signals (CH_3O-; CH_3CO-) can be correlated with the degree of methylation (DM) and the degree of acetylation (DA) of the pectic polysaccharides [70]. These data can be used as indicators of the water solubility and gel-forming ability of the polysaccharides. To determine the DM and DA of the recovered polysaccharides, ^{1}H-NMR analysis was performed on samples after acidic and basic hydrolysis. This allows for the quantification of galacturonic acid content and the relative abundance of methylated and acetylated groups by integration of the signals area at 1.82 ppm and 3.21 ppm respectively. To confirm the presence of pectin in grape pomace, the content of galacturonic acid was measured in all the samples by performing acidic hydrolysis at 100 °C as described by Giner et al. [42]. The concentration of galacturonic acid was then determined by ^{1}H-qMR, with maleic acid as internal standard. The signals in the anomeric region of the spectrum of the polysaccharide after hydrolysis were attributable to the anomeric proton as α (4.559–4.569 ppm) and β (4.559–4.569 ppm) forms of galacturonic acid (Fig. 2). The attribution was confirmed by a comparison with the spectrum of the pure standard in the same condition (Fig. S2), and the constant ratio of α/β forms (which ranged between 0.82 and 0.88 for all the samples) indicated the same balanced presence of both anomeric forms in water. The galacturonic acid content (Gal-ac) ranged from 26.7% (G_SE) to 34.2% (G_SK). To avoid a partial loss of the produced methanol and acetic acid generated from the *O*-methyl and acetyl groups of the polysaccharides, the degree of methylation (DM) and acylation (DA) was determined after alkaline hydrolysis at

Table 4 Content of galacturonic acid (Gal ac), degree of methylation (DM) and degree of acetylation (DA) expressed as % on dry sample

Samples	% α Gal ac	% β Gal ac	Tot % Gal-ac	α/β	% MeOH	% CH_3COOH	DM	DA
G_SK	15.4 ± 0.3	18.7 ± 0.4	34.2 ± 0.6	0.82	1.85 ± 0.2	0.57 ± 0.3	32.9	5.4
G_SE	12.93 ± 0.4	15.75 ± 0.3	28.7 ± 0.6	0.82	1.97 ± 0.4	1.27 ± 0.2	41.6	14.3
G_PO	15.11 ± 0.5	17.1 ± 0.4	32.2 ± 0.7	0.88	1.69 ± 0.5	0.63 ± 0.4	31.8	6.35

Gal-ac was determined after acid hydrolysis, while DM and DA were evaluated after basic hydrolysis; all values were evaluated by ^{1}H-qNMR and were expressed as mean of triplicates for each sample

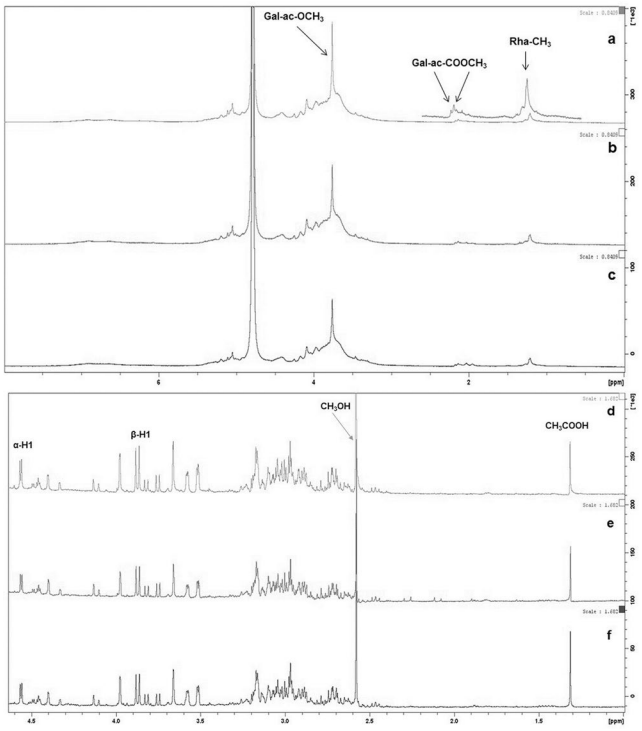

Fig. 2 ^{1}H-NMR spectra of polysaccharide of **a** G_PO in D_2O; **b** G_SK in D_2O; **c** G_SE in D_2O; **d** ^{1}H-NMR spectra of hydrolysed polysaccharides from **d** G_PO; **e** G_SK; **f** G_SE. For each sample, approx. 5–6 mg/mL in 2 M H_2SO_4 at 100 °C were used

room temperature. The results obtained for all the samples are summarized in Table 4. The DM % consistently varied, from 31.8% for G_PO to a maximum of 41.6% for G_SE. Concerning DA%, the sample G_SE resulted highly acetylated with value 14.3 compared with 5.4 in G_SK sample.

Overall, the structural properties of these polysaccharides suggest their potential use for various applications depending on the specific requirements for gelling, thickening, and stabilizing in food. Accurate quantification and characterization of polysaccharides by ^{1}H-qNMR are essential for better understanding their functional properties. The degree of methylation (DM) and acetylation (DA) are crucial factors for determining the functional properties of polysaccharides and how a polysaccharide could be used in food applications.

Few studies have comprehensively examined the extraction of pectin from grape pomace. Minjares-Fuentes et al. [71] reported that microwave extraction produced pectin containing 64.7–65.5% galacturonic acid (GalA) with 55.2% degree of methylation (DM). Similarly, González-Centeno et al. [72], using ultrasound-assisted extraction, found that the GalA content ranged from 40.9 to 64.3%, while DM varied between 38.6 and 52.7%. In contrast, Spinei and Oroian in another study [73] demonstrated that extraction conditions significantly influenced pectin composition, with GalA content varying from 33.1 to 54.9 g/100 g, as determined by UV-Vis-NIR spectroscopy, and DM values ranging between 73 and 75%. Notably, Hayrapetyan et al. [68], utilizing supercritical fluid extraction with water, found a substantially lower GalA content of 6.8% (dry weight) with a correspondingly low DM (~23%). Alternatively, conventional extraction, as reported by Spinei and Oroian [69], produced 9.96–11.1% of pectin, with GalA content of 79.91–80.9 g/100 g and DM of 81%. Comparing these data with our results (Table 4), the GalA content in our samples (28.7–34.2%) was considerably lower than those reported by Minjares-Fuentes et al. [71] and González-Centeno et al. [72]. However, direct comparisons are challenging, as UV-VIS methods generally yield higher galacturonic acid values than our ^{1}H-NMR approach. Additionally, our samples showed varying degrees of acetylation (DA), with G_SE displaying the highest value (14.3%), a parameter not extensively reported in previous grape pomace studies.

Conclusion

To the best of authors knowledge, no in-depth studies on the composition of *cv* Grechetto pomace are reported so far. The present study aimed to chemically characterize the whole pomace and their fractions (skin and seeds) obtained from this cultivar. Two of the most important chemical classes were analyzed: polyphenols and polysaccharides, which play a fundamental role both during fermentation and in the maturation and refining of wine, but are also retained in considerable amounts in pomace, giving it great potential for their recovery to obtain different functional ingredients for different applications. The present study furnished the basis to design and test an integrated process of pre-treatment, extraction, purification, and concentration steps to valorize this by-product as a whole but also for their subfractions for obtaining several bio-based products for application in different sectors. The separation of pomace into skins and

seeds could also allow for exploiting these latter in a biorefinery perspective, to obtain oil by pressing before the extraction of polysaccharides and polyphenols, also given the recent results that assessed the economical convenience of fully exploiting this material in integrated industrial plants. According to the regulations applied to ensure the safety of food ingredients, the marketing of the produced extracts will require chemical and microbiological analyses by accredited laboratories. These include: multi-residue test for pesticides, determination of ochratoxin, SO_2 and metals in accordance with the European Commission regulations and using analytical techniques such as chromatography coupled with UV-VIS or MS detectors and atomic absorption. Routine microbiological controls on the different batches of the final products will complete the safety assessment. Besides an increased environmental sustainability, the application of circular economy models to wine industry for obtaining products applicable in multiple sectors will expand the opportunities for collaboration between companies. It will also help create new links between different supply chains and their stakeholders, promoting a process of industrial symbiosis capable of giving companies greater efficiency and stability.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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