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New advancements in conventional and DES-based extraction of phenolic compounds from Aglianico red grape pomace

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ABSTRACT

Grape pomace is the most abundant winery waste, accounting for about 60 % of solid by-products. Despite being an environmental and economic burden, if properly treated it can be turned into a precious source of bioactive molecules. In this context, we compared the efficiency of extraction protocols based either on deep eutectic solvents (DESs) or conventional ones, with the purpose of proposing convenient procedures to recover residual phenolic compounds from Aglianico red grape pomace. The DES-based extraction protocols comprised choline chloride as a hydrogen bond acceptor and either oxalic or malic acids as hydrogen bond donors; conventional solvents were acetone and methanol. The DES-based protocols outperformed the conventional ones by far, with the mixture composed of a 1:1 molar ratio of choline chloride and oxalic acid with 25 % water (pH 1.65; at 40 °C) exhibiting the highest extraction yields per gram of dry grape pomace. Quali-quantitative LC-MS/MS analyses were conducted to identify specific polyphenols. Additionally, the antioxidant activity of all the extracts was measured by DPPH assay. Again, the DES-based extracts were the most active, the mixture composed of a 1.5:1 ratio of choline chloride and malic acid with 50 % water (pH 2.25; at 40 °C) showing the highest antioxidant properties. Finally, the outcome of the present study, once scaled up to industrial levels, is expected to benefit wineries and other food industries by diversifying their profits within a circular economy.

KEYWORDS: grape pomace, phenolic compounds, Deep Eutectic Solvents (DES), green extraction, antioxidant activity, circular economy, Aglianico

INTRODUCTION

The grape and wine industries are fundamental pillars of global agriculture (Khan *et al.,* 2020), since grapes rank among the main fruit crops cultivated worldwide and play a pivotal role in winemaking. According to the International Organization of Vine and Wine (Annual Assessment of the World Vine and Wine Sector in 2022, 2023), in 2022, European countries led the global grape production, representing over 40 % of the entire world output. It is estimated that approximately half of the harvested grapes are commonly used for wine production. Hence, not surprisingly, winemaking generates bulky quantities of by-products, including grape pomace, lees, stalks, waste waters and filtration residues. Such by-products pose both environmental and economic challenges to winemakers due to stringent disposal requirements. Thus, research on the potential uses of winemaking wastes in accordance with the principles of green chemistry has become critical and urgent (Lavelli *et al.,* 2016).

About 60 % of the total solid waste resulting from winemaking is represented by grape pomace, which consists of erratic quantities of skins, seeds and stems. Traditionally, it has been used for the distillation of spirits, liqueurs and liquors, as well as for the production of animal feed or fertilisers to enrich vineyard soils with nitrogen and minerals (Soceanu *et al.,* 2021; Bamforth & Ward, 2014). However, pomace is rich in fibres, tannins, and anthocyanins that, as a whole, negatively affect animal digestion, leading to severe livestock health problems (Rouches *et al.,* 2016). Additionally, phenolic compounds in grape pomace can inhibit germination on account of their ascertained phytotoxic and antimicrobial properties (Olszewska *et al.,* 2020). Conversely, polyphenols are regarded as valuable molecules thanks to their wide range of health benefits, including antioxidant, vasodilatory, antithrombotic, cardioprotective, anti-inflammatory and anticancer bioactivities (Kato-Schwartz *et al.,* 2020; Zhu *et al.,* 2019; Peixoto *et al.,* 2018). Recent studies have shown that phenolics, particularly in combination with polysaccharide conjugates and pentacyclic triterpenoids, even possess antidiabetic properties (Campos *et al.,* 2021; Errichiello *et al.,* 2023). It follows that, once recovered from residual biomasses, polyphenols can be used across various industrial sectors, including the alimentary, nutraceutical, pharmaceutical and cosmetic ones. In this regard, conventional solid-liquid extraction techniques have been commonly employed, but they have significant drawbacks, such as the use of costly and environmentally unfriendly solvents, high temperatures and prolonged extraction times (Sirohi *et al.,* 2020). In order to overcome these downsides, various innovative and more sustainable methods have been developed, including ultrasound-assisted, microwave-assisted, supercritical fluid, and pressurised liquid extractions (da Rocha & Noreña, 2020; Leyva-Jiménez *et al.,* 2021; Neto *et al.,* 2021); for example, ultrasound-assisted protocols lead to reduced extraction times while enhancing yields and decreasing environmental and economic impacts (Goula *et al.,* 2016). Similarly, pressurised liquid extraction involves shorter extraction times and produces high-quality extracts compared to conventional techniques (Li *et al.,* 2019). Recent studies have focused on Deep Eutectic Solvents (DESs) (Abbott *et al.,* 2003; Smith *et al.,* 2014), which are composed of ionic or molecular compounds that form eutectic mixtures with lower melting points than their components. This would make possible the design of tailored mixtures capable of meeting specific extraction needs by modulating compositions and component ratios (Abbott *et al.,* 2003; Omar & Sadeghi *et al.,* 2022). In this context, Frontini *et al.* (2024) have demonstrated the efficiency of three binary eutectic mixtures based on choline chloride and organic acids, namely lactic and tartaric acid, for extracting polyphenols from grape pomaces derived from either red (Negroamaro, Primitivo, and Susumaniello) or white varieties (Chardonnay, Fiano, and Malvasia bianca). Their results showed that optimised DES formulations can achieve total phenolic yields as high as 120.00 mg/g dry weight, thus significantly outperforming conventional extraction methods.

In a complementary study, Vorobyova *et al.* (2023) investigated several lactic-acid-based DESs for the recovery of phenolic acids from wine production residues. Their work further supported the advantages of DESs in terms of safety, environmental compatibility and extraction efficiency, especially when integrated with ultrasound-assisted procedures.

Despite these advances, there is still room for improvement. Studies usually carry out an overall evaluation of the phenols recovered from the biomass under investigation without any quali-quantitative profiling of specific molecules or classes of compounds. Moreover, some variables, such as temperature or extraction mechanisms, remain poorly explored.

In order to contribute to the advancement of knowledge on DESs aimed at the recovery of polyphenols, we decided to test some protocols based on different mixture of DESs, employing choline chloride as a hydrogen bond acceptor and either oxalic or malic acids as hydrogen bond donors. In addition, different temperatures were tested. These protocols were compared with other extraction procedures based on conventional solvents, and then developed to include some key variables, such as water content, pH, extraction times and agitation techniques. Comparisons were carried out through a combined use of spectrophotometric assays and LC-MS/MS analyses, which allowed us to provide a detailed qualitative and quantitative profile of the extracted compounds.

It is worth underlining that as a biomass for our study we selected the Aglianico (*Vitis vinifera* L. cv) red grape pomace, because i) widely cultivated across Southern Italy, it is one of the most prized vines renowned worldwide for the production of high-quality DOCG (Denominazione di Origine Controllata e Garantita) red wines, such as Taurasi, and ii) it is particularly rich in phenolic compounds, thus is a promising source of phenolic compounds. Despite this, Aglianico has so far received limited attention especially compared to international varieties.

MATERIALS AND METHODS

1. Chemicals and reagents

All reagents were of high-performance liquid chromatography (HPLC) grade. Acetonitrile (99.8 %) was purchased from Panreac Quimica (Barcelona, Spain), while formic acid (99 %) and methanol (99.8 %) were obtained from ChemLab (Zedelgem, Belgium). Folin reagent and sodium hydroxide were purchased from LabChem (ACP Chemicals Inc., Zelienople, PA, USA). Reference standards, containing gallic acid and (+)-catechin, were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetone, choline chloride, malic acid, oxalic acid, sodium carbonate, glacial acetic acid, hydrochloric acid, ethanol, sodium dodecyl sulfate (SDS), triethanolamine, iron chloride, vanillin, 2,3,5-triphenyl-2H-tetrazolium chloride, 22-diphenyl-1-picrylhydrazyl, sulfuric acid, bovine serum albumin (BSA) and metabisulfite were all provided by Fisher Chemical (Geel, Belgium). Water was obtained from a Milli-Q® Direct Water Purification System. Oenin chloride (≥ 95 %) was obtained from Extrasynthese.

2. Grape pomace collection

Aglianico (*Vitis vinifera* L. cv) red grape pomace was collected from the Taurasi area (Avellino, Italy) in November 2022, and was kindly provided by Quintodecimo winery located in Mirabella Ewclano, Italy. The fresh pomace, composed of grape skins, seeds, stalks and residual pulp, was collected after the pressing phase at the end of the alcoholic fermentation. Different samples of grape pomace were collected from a range of stainless-steel tanks to ensure homogeneity.

Immediately after collection, the fresh pomace samples were lyophilised and powdered. The samples obtained were successively extracted, as detailed below.

3. Preparation of Deep Eutectic Solvents (DES)

Prior to the preparation of DES, all solid components (choline chloride and the selected hydrogen bond donors; i.e., malic acid and oxalic acid) were lyophilised for 24 hours using a BenchTop Pro Virtus SP Scientific lyophiliser, in order to remove residual moisture. Subsequently, DESs were prepared by mixing choline chloride with each hydrogen bond donor at predefined molar ratios (Table 1) under continuous stirring at 80 °C until homogeneous and transparent liquids were obtained. The resulting DES mixtures were then diluted with deionised water to obtain final DES solutions containing 25 % or 50 % (*w/w*) water, as detailed in Table 1.

4. Extraction procedures

Conventional or DES-based extraction methodologies were used, as described below and summarised in Table 1.

4.1. Conventional Extractions:

The conventional extractions were conducted using the following mixtures:

- MeOH/H_2O (80:20) *v/v*. (ME); pH 7.0
- MeOH/HCOOH/H_2O (80:1:19) *v/v/v*. (FO); pH 1.5
- MeOH/H_2O(50:50) *v/v* combined with acetone/H_2O(70:30) *v/v* adjusted to pH 2.0 with HCl 6.0 M. (AC)

These three extracting mixtures were used under two different agitation conditions: 1) magnetic stirring (MA) overnight, and 2) T25-ultra-turrax (UT) for six minutes. All the extractions were performed in triplicate.

4.1.1. Extractions by magnetic stirring (MA)

60.0 mL of each extraction mixture was combined with 0.5 g of lyophilised red grape pomace and stirred under a controlled atmosphere using a magnetic stirrer overnight at room temperature. Then, the mixtures were centrifuged at 14000 rpm for 10 minutes at 4 °C using a Hettich Universal 320 Refrigerated Benchtop Centrifuge. The organic solvent was completely removed by evaporation through a Buchi rotavapor R-300 under vacuum at 37 °C. The solid residues were then reconstituted with 1.0 mL of methanol to ensure a standardised final volume was obtained. Then, 3.0 mL of Milli-Q water and 1.0 mL of methanol were added. All the extracts were stored at -20 °C and analysed within a maximum of three days (Figure 1).

4.1.2. Extraction using T25-Ultra-turrax (UT)

A volume of 30.0 mL of each extraction mixture was added to 0.5 g of lyophilised red grape pomace samples. To avoid overheating, the samples were maintained in an ice bath and then homogenised using a T25-Ultra-turrax (IKA-Labortechnik®, Staufen, Germany) for 3 minutes at a rate of 16,000 min^{-1}. The resulting mixture was centrifuged at 14000 rpm for 10 minutes at 4 °C using a Hettich Universal 320 Refrigerated Benchtop Centrifuge. This extraction process was repeated twice with a final extraction mixture volume of 60.0 mL (30.0 mL × 2). Both fractions were combined, and the organic solvent removed by evaporation under vacuum at 37 °C. The solid residues were then reconstituted with 1.0 mL of methanol to ensure a standardised final volume. Then, 3.0 mL of Milli-Q water and 1.0 mL of methanol were added. All the extracts were stored at -20 °C and analysed within a maximum of three days (Figure 1).

4.2. DES-based extraction

The DES-based extractions were carried out using a magnetic stirrer, at both room temperature (RT) and 40 °C (T40) for one hour. 5.0 mL of each DES was added to 0.5 g of lyophilised red grape pomace samples. The resulting mixtures were then centrifuged at 14000 rpm for 10 minutes at 4 °C using a Hettich Universal 320 Refrigerated Benchtop Centrifuge. This extraction process was performed three times for each DES (Figure 1).

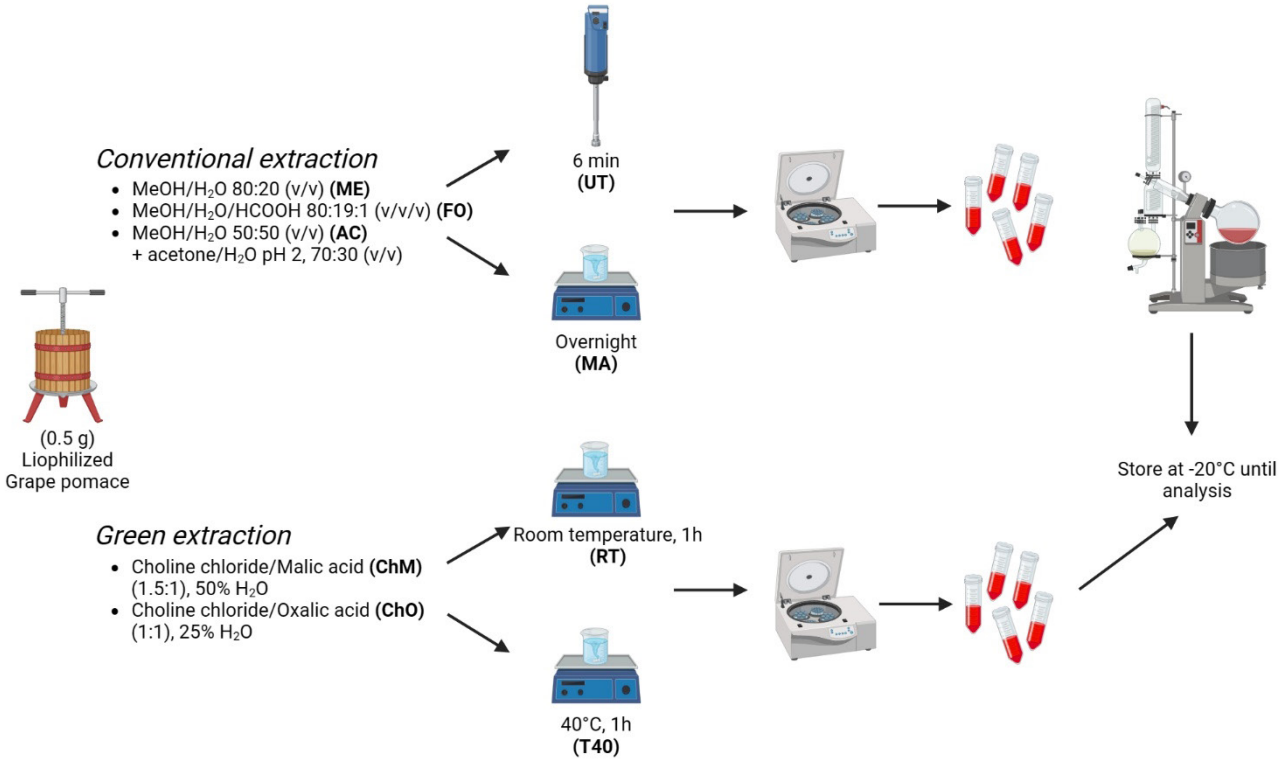


FIGURE 1. The extraction of phenolic compounds from pomace samples involved various processing techniques. In the conventional extraction, both magnetic stirring (MA) and Ultra-Turrax (UT) were utilised. In the green extraction, magnetic stirring was exclusively applied.

TABLE 1. Extraction mixtures composition and abbreviations used.

Conventional extracting mixtures	Abbreviation MA	Abbreviation UT	Ratio
Methanol/water pH 7.0	ME_MA	ME_UT	80:20 *v/v*
Methanol/Formic acid/Water pH 1.50	FO_MA	FO_UT	80:1:19 *v/v/v*
Methanol/Water + Acetone/Water pH 2.0	AC_MA	AC_UT	50:50 + 70:30 *v/v*
DES extracting mixtures	Abbreviation (RT)	Abbreviation (T40)	Ratio
Choline chloride:oxalic acid pH 1.65	RTChO	T40ChO	1:1 + 25 % H_2O
Choline chloride:malic acid pH 2.25	RTChM	T40ChM	1.5:1 + 50 % H_2O

5. Determination of total phenolic content of the extracts

The total phenolic content in each red grape pomace extract was determined using the Folin–Ciocalteu method. Each extract (either conventional or DES-based) was combined with 3.75 µL of Folin–Ciocalteu phenol reagent and 18.75 µL of water in 96-well plates. After 30 seconds of mixing, 75.0 µL of Na_2CO_3 and 152.5 µL of water were added. The mixtures were shaken, incubated at room temperature for 30 minutes in the dark and then analysed.

Absorbance at 750 nm was measured using a plate reader (Biotek, Berthold Technologies, Bad Wildbad, Germany). To quantify the phenolic content, a gallic acid calibration curve ($y = 1.032\times + 0.1041$; $R^2 = 0.9938$) was constructed, with a linear range of 0.0625–3.0 mg/mL. Six replicates of all the analyses were performed (3 experimental replicates × 2 analytical replicates).

6. Determination of Vanillin Reactive Flavans (VRF), Bovine Serum Albumin (BSA) reactive tannins, total anthocyanins and, Polymeric Pigments (PPs)

The VRF assay was used to measure proanthocyanidins, from dimers to tetramers, according to the procedure reported by Gambuti *et al.* (2015). Briefly, one test tube was prepared by diluting (1:10) each extract with pure methanol. Two microcentrifuge tubes were used in the analysis. In the first microcentrifuge tube (1.5 mL) 125.0 µL of diluted extract was mixed with 750.0 µL of a 4 % vanillin solution in methanol. After a 5-minute incubation in water at 4 °C, 375.0 µL of concentrated hydrochloric acid was added. Following a 15-minute incubation at 20 °C, the absorbance was recorded at 500 nm. In a control experiment, the same procedure was applied, but 750.0 µL of pure methanol was used instead of the vanillin solution. The absorbance at 500 nm of this solution was used as a blank. Concentrations were calculated

as (+)-catechin equivalents using a calibration curve with a linear range of 2.0-0.0625 mg/mL. The calibration curve was: y= 0.0246× + 0.00016; R^2= 0.995.

The concentrations of total anthocyanins, BSA reactive tannins, small polymeric pigments (SPPs) and large polymeric pigments (LPPs) were determined using the Harbertson-Adams assay (Harbertson *et al.,* 2003). Concentrations were calculated as (+)-catechin equivalents using a calibration curve with a linear range of 0.057-0.343 mg/mL. The calibration curve was: y= 4.4556× + 0.0589; R^2= 0.995. Chromatic characteristics were assessed using an Agilent Cary 60 UV-Vis spectrophotometer with 10 mm plastic cuvettes. Six replicates of all the analyses were performed (3 experimental replicates × 2 analytical replicates).

7. Radical scavenging assay (DPPH)

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay can assess the free radical scavenging ability of a single compound or extract. The methodology was applied as described in Bondet *et al.* (1997), with some modifications. All extracts were tested at the same concentration (10.0 mg of grape pomace extract per g of methanol) using a 96-well microplate reader (Biotek Powerwave XS with Gen5 software). DPPH (60 µM) was prepared in methanol, with 270.0 µL added to each well, and the reaction was started by adding 30.0 µL of the extract. The antiradical activity was expressed as Trolox equivalents. Six replicates of all the analyses were performed (3 experimental replicates × 2 analytical replicates).

8. LC-MS/MS analysis

The identification of each phenolic compound was carried out through Liquid Chromatography-Mass Spectrometry (LC-MS/MS) coupled to a High-Performance Liquid Chromatography (HPLC) system (Vanquish Thermo Fischer Scientific, Waltham, MA, USA). The HPLC system was equipped with an Agilent Poroshell 120 C18 reverse-phase column (250 × 4.6 mm, 2.7 µm particle diameter). The injection volume was set at 5.0 µL, and the mobile phase consisted of two solvents: A) 96 % water, 1 % formic acid and 3 % acetonitrile, and B) 49 % water, 1 % formic acid and 50 % acetonitrile. The gradient profile was initiated with 10 % eluent B, reaching 30 % B after 20 minutes, 35 % B after 37 minutes, 45 % B after 40 minutes, 47 % B after 61 minutes, and ultimately, 100 % B at 65 minutes, which was maintained until 72 minutes. The system was re-equilibrated with 10 % B from 72 to 85 min.

The analysis was conducted by using an Orbitrap™ Exploris 120 mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) and controlled through Orbitrap Exploris 120 Tune Application 2.0.182.35 and Xcalibur 4.4.16.14. The electrospray ionisation source (ESI) was set at a capillary voltage of 3.5 kV, a capillary temperature of 300 °C, and sheath gas and auxiliary gas flow rates of 50 and 10 (measured in arbitrary units as per software settings). The Single Ion Monitoring (SIM) MS scan was performed with a resolution of 30,000, and collision energy settings of 30 V were selected, followed by High-Energy Collision Dissociation (HCD) MS/MS.

Spectra were recorded in both negative- and positive-ion modes within the mass range at *m/z* 100 to 2000. The mass spectrometer was programmed to conduct three scans, including a full mass scan, a zoom scan targeting the most intense ion from the first scan, and an MS/MS scan of the most intense ion, using relative collision energies of 30 V. The entire analysis process was conducted by using Xcalibur version 2.2 software (Thermo Scientific, Waltham, MA, USA). Phenolic compounds identification was achieved by reference standards, comparison of retention times, fragmentation patterns, and exact mass with data available in literature.

9. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (version 29.0.1.0) and R running under Rstudio (Version R-4.3.3, R Foundation for Statistical Computing, Vienna, Austria) (R Core Team, 2019).

One-way ANOVA was used to study the significance of the effect of solvents and mixing techniques on the Folin–Ciocalteu assay, LPPS, SPPS, DPPH, BSA-tannins, Total anthocyanins, VRF and LC-MS/MS. The Tukey post-hoc test ($p \leq 0.05$) was used for mean separation when the data met the assumptions of normality and homogeneity of variance. When these assumptions were not met, the non-parametric Kruskal–Wallis test was applied. To study the differences between the extraction protocols in the measured spectrophotometric parameters, a PCA was performed using as inputs seven variables: LPPs, SPPs, Total anthocyanins, Vanillin-reactive flavans, BSA-reactive tannins, Folin, and DPPH. The PCA and the biplot of the main results were performed using the R package "factoextra". Six replicates of all the analyses were performed (3 experimental replicates × 2 analytical replicates). Mean values and standard deviations were determined.

RESULTS AND DISCUSSION

1. Set-up of extraction protocols and evaluation of polyphenol contents of extracts

In this study, both conventional and DES-based extraction procedures were developed (Table 1) and tested for their efficiency in recovering phenolics from Aglianico grape pomace.

The extraction protocols were developed by referring to available literature reports (Teixeira *et al.,* 2023; Bi *et al.,* 2020) and were designed to include a range of operational variables, such as solvent polarity, water content, pH, agitation procedures, temperature and extraction time. More specifically, both conventional solvents and DESs were chosen on the basis of their documented ability to extract phenolic compounds from plant matrices and wine-making by-products. Among the conventional solvents, methanol and acetone have been widely used in extraction protocols due to their polarity, solubilising capacity and ability to penetrate complex matrices. Specifically, mixtures including

methanol/water (80:20, *v/v*; pH 7), methanol/formic acid/water (80:1:19, *v/v/v*; pH of 1.5), and methanol-acetone aqueous systems adjusted to a pH of 2 are frequently adopted to maximise the solubilisation of phenolics with varying polarity and molecular structures (Teixeira *et al.,* 2023; Spigno *et al.,* 2007). It was crucial to take into account the pH of the extraction media, because of its potential influence on the ionisation state of phenolic hydroxyl groups and, consequently, on the extraction efficiency (Buang *et al.,* 2017). The use of acidic aqueous solutions seems to improve the extraction of acid-stable anthocyanins; likewise, acidified acetone is able to promote the recovery of more hydrophobic compounds including flavonoids and proanthocyanidins (Liu *et al.,* 2024; Tzanova *et al.,* 2020).

Regarding the DES-based systems, our selection was driven by their physicochemical properties and reported efficiency in solubilising phenolic compounds through hydrogen bonding interactions. In particular, choline chloride was used as a hydrogen bond acceptor (HBA), which was paired with either oxalic or malic acid as hydrogen bond donors (HBDs). We decided to use these components on account of their natural origin, low toxicity, and previous successful use in polyphenol extraction from grape-derived matrices (Frontini *et al.,* 2024; Vorobyova *et al.,* 2023). Additionally, water percentage and temperature were varied to modulate the viscosity and polarity of the DESs in order to improve the diffusivity and extraction yield of the phenols without compromising their stability. Therefore, based on prior optimisation studies (Bi *et al.,* 2020; Wu *et al.,* 2020; Neto *et al.,* 2021), we used a water content of 25 % and 50 % (*w/w*). With regard to temperature, we selected two different conditions: room temperature and 40 °C. The pH of the DESs was accurately measured at room temperature immediately after preparation. The choline chloride:oxalic acid DES (ChO) showed a pH of 1.65, while the choline chloride:malic acid DES (ChM) featured a pH of 2.25. These values confirmed the intrinsically acidic nature of both systems, which is expected to promote the solubilisation of acid-stable phenolic compounds such as anthocyanins and hydroxybenzoic acids. Notably, these experimental values are consistent with those reported in the literature for similar formulations (Škulcová *et al.,* 2018).

Once the extraction protocols had been set up to compare their relative recovery efficiency, the total phenolic content of each extract was determined by three different and complementary assays. The colorimetric Folin–Ciocalteau assay was used to estimate the total reducing capacity of the extracts. Given that this method is sensitive to any reducing substances, including ascorbic acid, sugars and certain amino acids, it is worth underlining the possibility that the obtained results do not strictly correlate with the actual phenolic content. We used the Harbertson-Adams assay to identify specific polyphenol classes, namely BSA-reactive tannins, total anthocyanins (TA) and those with both long and short polymeric pigments (LPPs and SPPs). This assay combines protein precipitation with BSA and bisulfite bleaching to differentiate between two types of polymeric pigments: i) LPPs, which precipitate with ii) proteins and SPPs, which do not precipitate. Finally, the Vanillin Reactive Flavans (VRF) assay was employed to measure proanthocyanidins, ranging from dimers to tetramers.

All the results were expressed as mg of extracted analyte per gram of pomace (dry weight). Specific and detailed results are reported in Figure 2 and in Tables 2–3. A careful comparison of the obtained data allowed us to draw some insightful conclusions. Firstly, the results of all of the employed assays clearly indicated that the DES-based protocols were by far more efficient than the conventional ones. More specifically, among the DESs, on the basis of the Folin–Ciocalteu assay, T40ChO showed the highest value (43.00 mg GAeq/g), which was approximately double those obtained in the three remaining DES-based extraction conditions. This highlights T40ChO's capacity to recover the greatest content of redox-active compounds, among which polyphenols could be reasonably considered as the most abundant (Figure 2). When compared with ChO, the positive role of temperature defined the final extraction yield. However, the same effect was not recorded in the case of ChM-based protocols. According to previous reports (Wu *et al.,* 2020) both temperature and water seem to play a pivotal role in driving the phenolic compounds recovery from by-products, but with no ascertained interactive effects. Under our experimental conditions, different water contents (25 % for ChO and 50 % for ChM) had no impact on the phenolic compounds' extraction yields, in contrast to temperature, which remarkably increased the extraction efficiency of ChO (T40ChO). Considering that temperature had no impact on the extraction when using ChM-based protocols, it can be concluded that an optimal combination of water content (25 %) and high temperature (40 °C) was responsible for the significantly high yield obtained by the T40ChO protocol. Moreover, the higher extraction yields obtained with DES-based systems, particularly T40ChO, can be rationalised by considering both their physicochemical properties and their interaction mechanisms with phenolic compounds. The acidity of ChO (pH ~1.65) likely favoured protonation of phenolics to a greater extent, thus improving their solubility in the slightly polar eutectic matrix (Rente *et al.,* 2021). This is consistent with the known behaviour of anthocyanins, which are more stable and soluble in acidic conditions (Frontini *et al.,* 2024; Errichiello *et al.,* 2023). In conclusion, the presence of oxalic acid, a stronger acid than malic acid, may have reinforced the solubilisation of phenols (Chitra *et al.,* 2004). It follows that the acidity of the DES formulations may have contributed to their high extraction efficiency; it could thus be considered a relevant parameter for future optimisation studies in order to provide additional insights for optimising extraction conditions and improving selectivity toward specific phenolic subclasses.

Among the conventional solvents, AC_MA, FO_MA, and ME_MA did not statistically differ from each other in terms of phenolic compounds extraction yields, ranging from 1.6 (FO_MA and Me_MA) to 2.3 mg/g DW (AC_MA). Likewise, the extraction mixtures combined with UT did

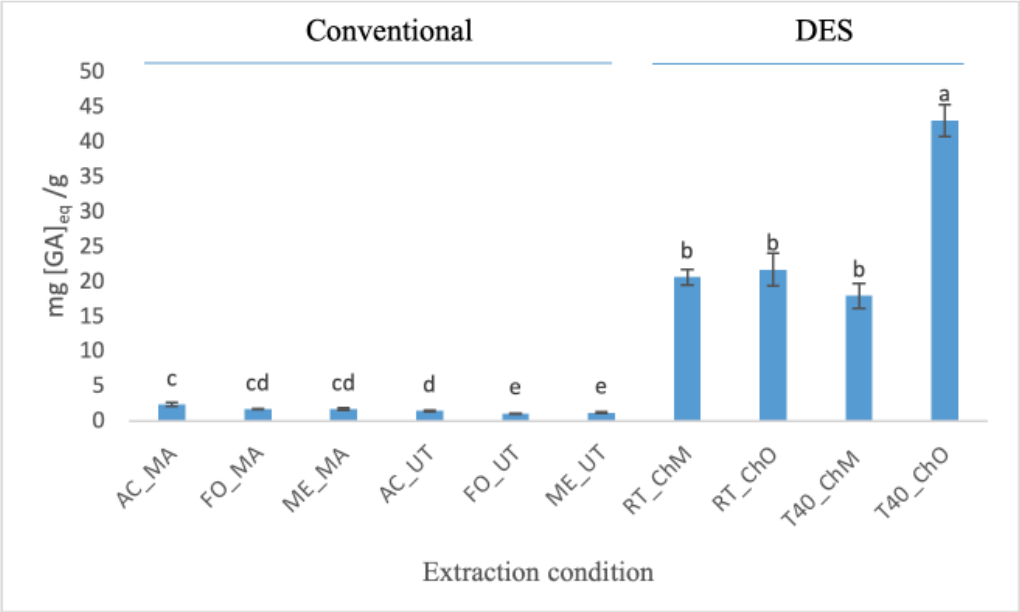


FIGURE 2. Total reducing compounds determined by the Folin–Ciocalteu assay, expressed as gallic acid equivalents (mg GAeq/g of dry pomace). Data are presented as mean ± standard deviation (SD) across 3 experimental replicates × 2 analytical replicates. Different letters indicate statistically significant differences between extraction conditions at $p < 0.05$.

not show significant differences between each other, except for AC_UT. Finally, each solvent mixture used with MA was more efficient than the corresponding mixture used in combination with UT. However, it should be emphasised that the extractions carried out with solvents combined with UT, even if performed for only 6 minutes, allowed 60 % -70 % of the total polyphenols to be recovered. Therefore, while MA might provide gentler and more continuous contact between the solvent and the pomace compared to UT, it still needs longer extraction times (Spigno *et al.,* 2007). In this regard, it is worth underlining that extraction times longer than 6 minutes were not advisable when using UT due to a significant temperature increase.

As reported above, we further analysed our extracts by means of the Harbertson-Adam method to identify specific polyphenol classes, namely LLPs, SPPs, BSA-reactive tannins and anthocyanins. In addition, proanthocyanidins ranging from dimers to tetramers were investigated by the vanillin reactive flavans assay.

Table 2. Long polymeric pigments (LPPs), short polymeric pigments (SPPs), BSA reactive tannins and total anthocyanins from Aglianico grape pomace extracted using conventional and DES-based mixtures and determined by the Harbertson-Adams assay. LPPs and SPPs are expressed as absorbance (Abs), BSA reactive tannins are expressed as equivalents of catechin (mg CAEeq/g of DW), and total anthocyanins are expressed as equivalents of malvidin-3-*O*-glucoside (mg MAEeq/g of DW).

	LPPs (Abs)			SPPs (Abs)			BSA-reactive tannins (CAE_{eq} mg/g DW)			Total anthocyanins (MAE_{eq} mg/g DW)		
AC_MA	1.82 ± 0.01	e		0.97 ± 0.01	f		1.78 ± 0.01	e		0.25 ± 0.00	e	
FO_MA	1.44 ± 0.03	f		1.13 ± 0.02	e		1.26 ± 0.02	f		0.20 ± 0.00	g	
ME_MA	0.54 ± 0.01	h		1.27 ± 0.00	d		1.05 ± 0.01	g		0.24 ± 0.00	f	
AC_UT	0.74 ± 0.02	g		0.80 ± 0.02	h		1.06 ± 0.00	g		0.17 ± 0.00	h	
FO_UT	0.49 ± 0.04	i		0.96 ± 0.02	f		0.55 ± 0.01	h		0.14 ± 0.00	i	
ME_UT	0.20 ± 0.03	l		0.85 ± 0.01	g		0.56 ± 0.01	h		0.11 ± 0.00	l	
RT_ChM	26.37 ± 1.18	c		14.80 ± 0.31	a		7.40 ± 0.28	c		2.35 ± 0.03	c	
RT_ChO	30.65 ± 0.72	b		10.37 ± 0.70	b		11.23 ± 0.11	b		2.57 ± 0.01	b	
T40_ChM	22.67 ± 0.36	d		7.04 ± 0.26	c		6.38 ± 0.05	d		1.80 ± 0.01	d	
T40_ChO	42.60 ± 0.58	a		9.92 ± 0.54	b		19.41 ± 0.04	a		3.29 ± 0.02	a	

Data are presented as the mean ± standard deviation (SD) across 3 experimental replicates × 2 analytical. Letters (a, b, c, d, e, f, g, h, i, l) indicate statistically significant differences between the solvents at $p < 0.05$.

TABLE 3. Vanillin reactive flavans (VRF) from Aglianico grape pomace extracted by conventional and DES-based mixtures. Vanillin reactive flavans (VRF) are expressed as equivalents of (+)-catechin (mg CAEeq/g of DW).

	VRF (CAEeq mg/g DW)		
AC_MA	1.39	±	0.01 e
FO_MA	0.84	±	0.01 h
ME_MA	1.01	±	0.02 f
AC_UT	0.98	±	0.02 g
FO_UT	0.49	±	0.00 l
ME_UT	0.65	±	0.00 i
RT_ChM	117.83	±	0.76 c
RT_ChO	129.97	±	5.17 b
T40_ChM	103.45	±	1.35 d
T40_ChO	197.52	±	0.82 a

Data are presented as the mean ± standard deviation (SD) across 3 experimental replicates × 2 analytical. Letters (a, b, c, d, e, f, g, h) indicate statistically significant differences between the solvents at $p < 0.05$.

TABLE 4. Sum of the quantified (mg/g of DW) phenolic classes analysed by LC-MS/MS.

	Anthocyanins				Flavan-3-ols				Phenolic acids				Stilbens				Flavonols			
AC_MA	9.032	±	0.002	e	0.092	±	0.000	d	0.033	±	0.001	de	0.051	±	0.000	e	0.158	±	0.001	f
FO_MA	4.516	±	0.001	i	0.056	±	0.001	h	0.038	±	0.000	cd	0.048	±	0.000	f	0.150	±	0.000	g
ME_MA	2.164	±	0.001	l	0.029	±	0.001	i	0.008	±	0.001	e	0.009	±	0.000	l	0.041	±	0.000	l
AC_UT	8.149	±	0.001	f	0.077	±	0.002	f	0.033	±	0.001	de	0.041	±	0.000	g	0.148	±	0.001	h
FO_UT	6.706	±	0.001	h	0.062	±	0.002	g	0.033	±	0.001	de	0.035	±	0.000	h	0.136	±	0.001	i
ME_UT	7.201	±	0.004	g	0.083	±	0.002	e	0.033	±	0.000	de	0.030	±	0.000	i	0.191	±	0.000	e
RT_ChM	144.898	±	0.028	b	0.773	±	0.015	b	0.253	±	0.006	bc	0.238	±	0.007	a	1.276	±	0.009	b
RT_ChO	147.473	±	0.021	a	0.847	±	0.027	a	0.292	±	0.009	a	0.156	±	0.005	b	1.429	±	0.006	a
T40_ChM	75.218	±	0.021	d	0.686	±	0.013	c	0.257	±	0.002	abc	0.092	±	0.005	c	0.854	±	0.012	d
T40_ChO	102.852	±	0.032	c	0.661	±	0.021	c	0.287	±	0.004	ab	0.082	±	0.002	d	1.127	±	0.007	c

Abbreviations: AC = Methanol/Water + Acetone/Water pH 2 (50:50 + 70:30 *v/v*); FO = Methanol/Formic acid/Water (80:1:19 *v/v/v*); ME = Methanol/water (80:20 *v/v*); ChO = Choline chloride:oxalic acid (1:1 + 25 % H_2O); ChM = Choline chloride:malic acid (1.5:1 + 50 % H_2O); RT = room temperature; T40 = 40 °C; MA = magnetic stirrer agitation; UT = ultra-turrax agitation. Different letters indicate a statistically significant difference between the solvents (a, b, c, d, e, f, g, h, i, l) (*e.g.*, AC_MA *vs.* AC_FO *vs.* RTChM *vs.* RTChO.). The data are expressed as means ± standard deviation, ($p < 0.05$) over four replications.

As shown in Tables 2 and 3, DES-based mixtures extracted all of the polyphenol classes more efficiently than the conventional solvents. Once again, among the DES-based solvents, T40ChO was the one that showed the highest extraction yield out of all the evaluated polyphenol classes (Tables 2 and 3), except the SPPs, for which the best extracting conditions were obtained by RTChM. The higher water content of ChM (50 %) could be responsible for the latter case (Table 2), which can be easily explained by the fact that SPPs tend to be more hydrophilic than LPPs and are therefore more readily extracted in a medium with a higher water content (Vilková *et al.,* 2020; Obluchinskaya *et al.,* 2021).

With regards to the conventional protocols, no dramatic differences were detected between the different tested procedures in terms of polyphenol yields. As in the case of the Folin–Ciocalteu assay, magnetic agitation turned out to be more efficient in extracting all of the investigated polyphenol classes than Ultra-Turrax. However, this latter methodology might be preferred since it allows much lower extraction times than MA (6 minutes versus overnight), thus conveniently counterbalancing the slightly lower yields.

2. Phenolic compound profile by LC-MS/MS targeted analysis

All of the obtained extracts were finally analysed by LC-MS/MS to identify and quantify the major phenolic compounds (Table 4; Tables S1 and S2). From a qualitative perspective, the metabolites identified in all of the extracts (conventional and DES-based solvents) were basically the same. However, from a quantitative standpoint, and consistent with the results obtained by the Folin–Ciocalteu and Harbertson-Adams assays, DES-based extraction outperformed the conventional solvent mixtures (Table 4; Table S2).

Among the identified anthocyanins and in accordance with previous studies, the least hydrophilic coumaroyl derivatives turned out to be the most abundant, since they were not massively extracted during the grape maceration

(Errichiello *et al.,* 2023; Oliveira *et al.,* 2015). Accordingly, malvidin-3-*O*-*trans*-*p*-coumaroylglucoside was the most abundant in all of the extracts, reaching concentrations of 77.81 mg/g in RTChM and 82.55 mg/g in RTChO. Regarding specific anthocyanin derivatives, vitisins (A- and B-types) were recovered in notable concentrations: vitisin B derived from malvidin-3-*O*-glucoside reached 6.21 mg/g DW (RTChM), while vitisin A derived from malvidin-3-*O*-coumaroylglucosides reached 2.09 mg/g DW in the same solvent. For ethyl-(epi)catechin-anthocyanin derivatives, the isomers of malvidin-3-(6-*O*-*p*-coumaroylglucoside)-8-ethyl-C(E) were quantified at significant levels, with the first isomer reaching 3.21 mg/g in RTChM. As for flavan-3-ols, ChM exhibited the best performance, with (+)-catechin and (-)-epicatechin detected at 0.23 mg/g and 0.20 mg/g, respectively in RTChM. Among the phenolic acids, gallic acid hexoside was the only one to be identified, with a maximum concentration of 0.29 mg/g in RTChO. Likewise, RTChM was the best mixture for stilbenes, among which resveratrol tetramer reached 0.24 mg/g. Finally, among the identified flavonols, quercetin-3-*O*-hexuronide and quercetin aglycone reached concentrations of 0.27 mg/g in RTChM and 0.62 mg/g in RTChO, respectively.

3. Antioxidant activity of the extracts

The antioxidant activity of the grape pomace extracts obtained by both conventional and green methodologies was assessed by the DPPH assay, which is a quick, widely-accepted and straightforward means of assessing the free radical scavenging ability of a compound or even an extract.

All of the DES-based extracts (RTChM, RTChO, T40ChM, and T40ChO) demonstrated significantly higher antioxidant activity compared to the extracts obtained through conventional solvents (AC_MA, FO_MA, ME_MA, AC_UT, FO_UT, and ME_UT) (Figure 3). This suggested that DESs, composed of choline chloride with either malic acid or oxalic acid, are more effective in extracting bioactive compounds with strong antioxidant properties (Gómez-Urios *et al.,* 2023; Airouyuwa *et al.,* 2023). Within the DES group, T40ChM and T40ChO exhibited higher antioxidant activity than their counterparts RTChM and RTChO. In particular, T40ChM showed the highest radical-scavenging activity, even if its overall yield in phenolic compounds was lower than that of T40ChO according to the Harbertson-Adams assay. Different reasons could explain this datum. It could be due to specific phenolic compounds showing stronger antioxidant activities than the others (Xu & Chang, 2009; Li *et al.,* 2024). Furthermore, the presence of undetected antioxidant components not identified by our targeted LC-MS/MS analysis but that might significantly contribute to the observed radical-scavenging activity should not be ruled out. Further investigation aimed at identifying these potential components could provide a more comprehensive understanding of the antioxidant properties of T40ChM.

In the case of conventional extraction methods, samples subjected to MA generally showed higher DPPH activity

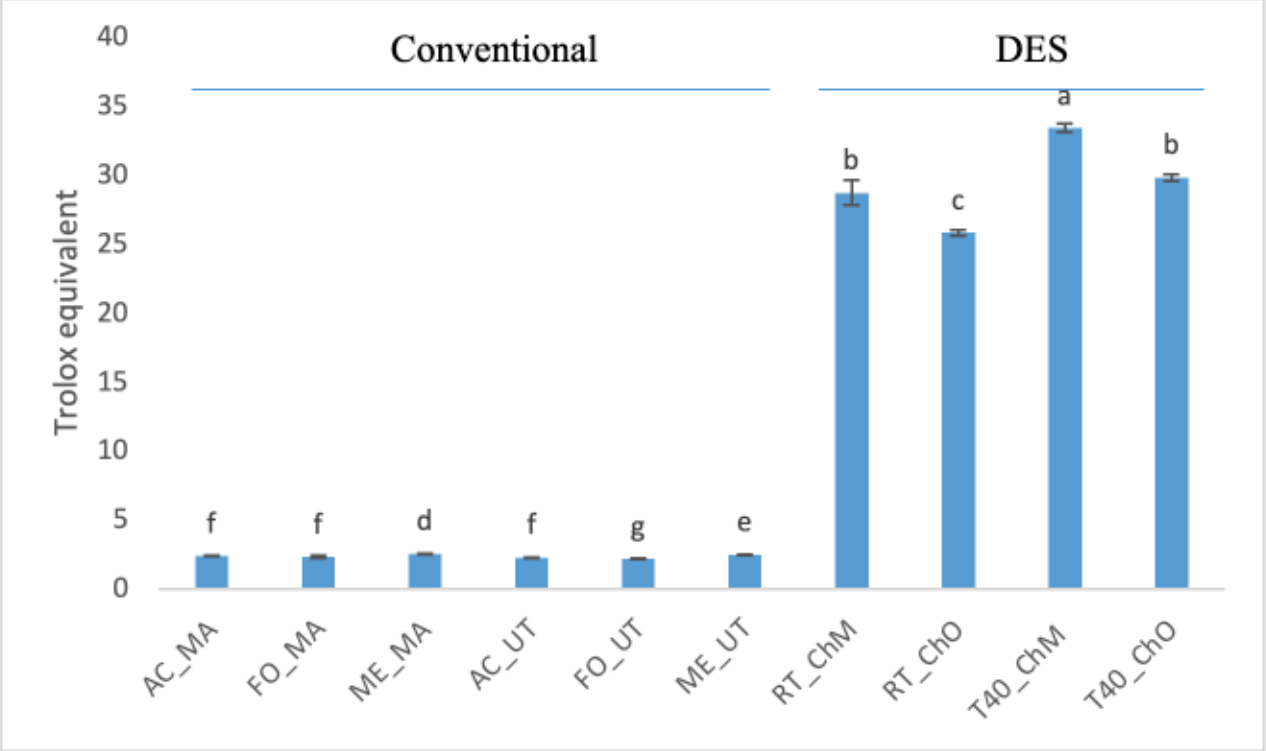


FIGURE 3. DPPH radical scavenging activity of the obtained extracts. Data are express in Trolox equivalents as the mean ± standard deviation (SD) across four replicates. Letters (a, b, c, d, e, f, g) indicate statistically significant differences between the solvents at $p < 0.05$.

compared with the UT extracts, except ME_UT (Figure 3). In particular, ME_MA exhibited a greater antiradical activity compared to AC_MA and FO_MA. Regarding the UT samples, it was interesting to note that, despite exhibiting lower total phenol recovery by the Folin assay, they demonstrated DPPH activity comparable to the MA extracts, which possessed significantly higher levels of phenolic compounds. As discussed above, this datum seems once again to support the hypothesis that antioxidant activity is mainly due to the type of phenolics rather than to their overall quantity (Sassi *et al.,* 2022; Ioannou *et al.,* 2023).

4. Principal Component Analysis and Correlation analysis

To better visualise the differences between conventional and green extraction methods, Principal Component Analysis (PCA) was applied, enabling the examination of how different extraction methods influence the composition of phenolic compounds and the antioxidant activity of the analysed extracts. A biplot highlighted the principal components responsible for the variability in the data, clearly separating the extraction methods used.

The biplot of the first two principal components (Dim1 and Dim2) effectively distinguished the different extraction methods based on the six spectrophotometric parameters measured simultaneously (Figure 4). Dim1 and Dim2 collectively accounted for 98.3 % of the total variance in the six original variables included in the PCA. Extraction methods employing green solvents were predominantly located in the two right quadrants (associated with positive Dim1 values) and were characterised by higher extraction efficiencies for all of the phenolic classes, as well as relatively elevated DPPH values. Dim1 distinctly separated green extraction methods from conventional ones.

Dim2, which explained 4.7 % of the variance, further differentiated T40ChO from the other green solvents (associated with positive Dim2 values), thus highlighting its comparatively higher extraction of BSA-reactive tannins, VRF and LPPs. The lower-right quadrant (negative Dim2, positive Dim1) included methods associated with relatively higher extractions of short polymeric pigments (SPPs) and higher DPPH values.

The PCA results underscored a greater affinity of green solvents for the analysed compounds with respect to conventional solvents. Conversely, PCA analysis did not reveal any notable differences between the conventional solvents.

CONCLUSIONS

This study demonstrated that deep eutectic solvents (DESs), particularly the choline chloride:oxalic acid system at 40 °C

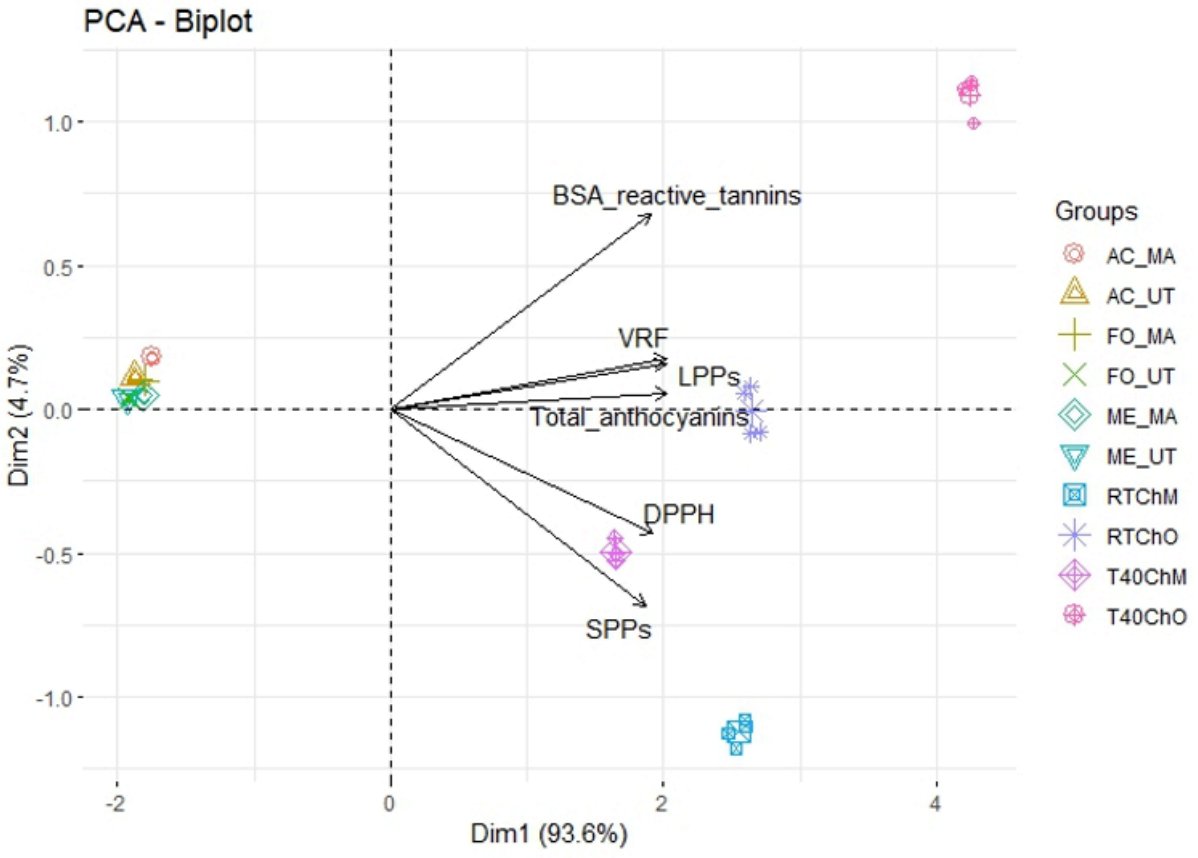


FIGURE 4. Biplot of ten extractions methods for the first two principal components (Dim1 and Dim2) extracted by the PCA. The vectors indicate the loadings of the original variables.

(T40ChO), significantly outperformed conventional solvents in extracting phenolic compounds from Aglianico red grape pomace. Of the variables tested (extraction time, temperature, agitation techniques, acidity and water percentages), both temperature and acidity were found to be the key ones. T40ChO yielded the highest concentrations of anthocyanins, flavan-3-ols, phenolic acids, stilbenes and flavonols, while also exhibiting a superior performance across multiple phenolic classes based on the multivariate analysis. Although T40ChM showed the highest antioxidant activity, this effect appeared to be related more to phenolic composition than to total yield. Overall, the T40ChO protocol emerges as a promising and green alternative for the valorisation of grape pomace. Further research is needed to assess its scalability and potential applications in the food, nutraceutical and cosmetic industries within a circular economy.

ABBREVIATIONS

DES	Deep eutectic solvent
ME	MeOH/H_2O (80:20) *v/v*
FO	MeOH/HCOOH/H_2O (80:1:19) *v/v/v*
AC	MeOH/H_2O (50:50) *v/v* combined with Acetone/H_2O (70:30) *v/v* adjusted to pH 2 with HCl 6M
MA	Magnetic stirring agitation
UT	Ultra-turrax agitation
RT	Room temperature
T40	40°C
ChO	Choline chloride:oxalic acid 1:1 25 % H_2O
ChM	Coline chloride:malic acid 1.5:1 50 % H_2O

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AUTHOR CONTRIBUTION

Errichiello F.: Experimental work: extraction, sample preparation, Mass Spectrometry Analysis and writing original draft. Azevedo J.: Antioxidant studies; Basile B. and Mataffo A.: Statistical analyses. De Freitas V.: Data curation, Supervision and reviewing Forino M.: Data analyses, Supervision and Writing. Oliveira J. and Soares S.: Data analyses, Reviewing and Editing.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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