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Green next-generation excipients enriched in polyphenols from recovery of grape processing waste black bentonite: Influence of unconventional extraction solvents on antioxidant properties and composition.

Giulia Di Prima [a], Elena Belfiore [b], Giuseppe Angellotti [c], Viviana De Caro [a, d, *]

[a] *Dipartimento di Scienze e Tecnologie Biologiche, Chimiche e Farmaceutiche (STEBICEF), Università degli Studi di Palermo, Via Archirafi 32, 90123, Palermo, Italy*

[b] *Dipartimento di Discipline Chirurgiche, Oncologiche e Stomatologiche (DICHIRONS), Università degli Studi di Palermo, Via L. Giuffrè 5, 90127, Palermo, Italy*

[c] *Istituto per lo Studio dei Materiali Nanostrutturati (ISMN), Consiglio Nazionale delle Ricerche, Via Ugo La Malfa 153, 90146, Palermo, Italy*

[d] *Centro Interdipartimentale di Ricerca Riutilizzo Bio-Based Degli Scarti da Matrici Agroalimentari (RIVIVE), University of Palermo, 90128 Palermo, Italy*

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ABSTRACT

According to the UN Agenda 2030, several actions could be taken to act synergistically towards various areas of critical importance for both the humanity and the planet. Among them, the application of a circular economy model based on the valorisation of the wastes from the local resources could be a virtuous attitude. In this view, this work proposes a dual green approach for recovery of the waste black bentonite from the fining of organic white grape must. Indeed, this virtuous recovery idea is enhanced by the choice of both eco-friendly extraction method (maceration) and solvents. The latter were unconventional solvents selected among the pharmaceutic/cosmetic liquid hydrophilic excipients (waste-to-market approach) characterized by safety and high solvent power toward polyphenols. PEGs (200, 400, 600), Propylene Glycol and Glycerol were tested and the extracted compared by HPLC-DAD analyses as well as the Folin-Ciocalteu, DPPH and Bradford assays. PEG200 emerged as the best one, leading to coloured extract containing great amount of polyphenols (3.123 ± 0.106 mg/g) and specifically Quercetin (60.778 ± 2.307 μg/mL), which confer it interesting scavenger properties, potentially making it directly useful in a wide range of fields from the pharmaceutic to the cosmetic ones.

1. Introduction

Functional ingredients are normally employed excipients characterized by a high added value due to the further presence of active compounds. The latter could determine health benefits, useful in both the cosmetic and pharmaceutical fields, thanks to their own beneficial properties and/or adjuvant/synergistic activity due to the presence of a variegate pool of substances (Cui et al., 2023). Nowadays, toward the main contemplated actives, phytochemicals (e.g., polyphenols, carotenoids, plant sterols and stanols, anthraquinones (Angellotti et al., 2021, 2020; De Caro et al., 2015; Di Prima et al., 2021, 2019)) are under the spotlight as they can exert a wide range of potent biological activities while also being quite safe (Lan et al., 2023; Madore et al., 2023; Oxley and Peart,

* Corresponding author.Via Archirafi 32, 90123, Palermo, Italy.
E-mail addresses: giulia.diprima@unipa.it (G. Di Prima), elena.belfiore@unipa.it (E. Belfiore), giuseppe.angellotti@cnr.it (G. Angellotti), viviana.decaro@unipa.it (V. De Caro).

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2023; Roy et al., 2022; Zhor et al., 2023). Specifically, polyphenols are functional compounds naturally occurring in several fruits and vegetables such as grape, pomegranate, grapefruit and olive (Pagano et al., 2021). They certainly cover a central role in the well-being of plants (Daayf et al., 2012) and are actually focused by researchers working in various fields (e.g., agri-food, pharmaceutical, nutraceutical, cosmetic) due to their wide range of proven biological activities such as antioxidant, antimicrobic, anti-inflammatory, wound-healing, antiaging, anti-hyperpigmentation, photoprotective, chemopreventive, anticancer and immunomodulatory (Pereira and Cotas, 2023). As polyphenols are largely synthetized in the plant kingdom, a conscious way to maximize their "green" use could consist in their extraction from the agri-food wastes and by-products, to which it could be given new life as innovative and precious resources although discarded due to a lack of application. The disposal of the wastes produced by the agri-food sector is currently an expensive process that must be overcome through the application of a circular economy model aimed at extending the life cycle of products through reuse and recycle (Osorio et al., 2021). A further significance could be given by re-using the wastes produced by the main local resources, thus enormously enhancing the circular economy impact. At this regard, Sicily is well known to possess one of the largest areas cultivated with organic grapes in Italy (Sinab, 2023), boasting the production of high-quality autochthonous wines that are internationally recognized. Nevertheless, Sicilian winemaking and grape processing industries produce a huge amount of both organic and inorganic wastes. The organic ones (e.g., pomace, seeds, skins) have been extensively recovered in the last decade to extract several compounds, such as polyphenols and organic acids, in order to produce cosmetics, functionalized animal feed and food products (Hoss et al., 2021; Leal et al., 2020). In contrast, the inorganic wastes have never been valorised yet. Among these, the bentonite is certainly one of the most abundant. The bentonites (mainly consisting of montmorillonite) are classified into natural calcium and natural sodium bentonites as well as activated bentonite, depending on several factors such as the swelling index, the native pH and the type of exchangeable cations (Moreno and Peinado, 2012). They could be used as clarifying agents by themselves (white bentonite, WB) or mixed with active carbon in the 1:1 weight ratio (black bentonite, BB). The latter mixture represents the most used fining agent due to its high deproteinizing power (Morata et al., 2016), but it could also retain other molecules such as polyphenols. Accordingly, the aim of this work was to demonstrate the BB as a valid waste material from which polyphenols can be recovered by extraction with liquid excipients not conventionally used as solvents in order to directly obtain novel secondary raw materials useful for cosmetic/pharmaceutical applications.

Importantly, the "green soul" of this work is not only related to waste bentonite valorisation as the further aim is to set up "green" extraction procedures by employing both eco-friendly techniques and extraction solvents. Particularly, these will be liquid hydrophilic excipients currently used in pharmaceuticals and cosmetics due to their biocompatibility. This choice relies in a waste-to-market approach aimed at producing novel green biomaterials useful as high value-added functional ingredients to be marketed as such and/or inserted into cosmetics/pharmaceuticals to benefit from the biological activities of polyphenols. It seems obvious that this work perfectly fits with the UN Agenda 2030 purposes, corresponding to 17 Sustainable Development Goals (SDGs) aimed at synergistically acting towards several areas of critical importance for both the humanity and the planet (United Nations, 2015). Specifically, the establishment of a novel production chain focused on waste valorisation and conversion into high value-added products is identifiable as an innovative sustainable development strategy which is strictly in accordance with the SDG 12 (*Ensure sustainable consumption and production patterns*) and particularly with points 12.2 (*Achieve the sustainable management and efficient use of natural resources*), 12.4 (*Achieve the environmentally sound management of chemicals and all wastes throughout their life cycle, in accordance with agreed international frameworks, and significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment*) and 12.5 (*Substantially reduce waste generation through prevention, reduction, recycling and reuse*). Additionally, the further development of the proposed novel biomaterials will also contribute to enhance the regional and national competitivity, as well as the well-being of citizens in terms of employment opportunities and offered products, thus also falling within the SDG 8 (*Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all*; particularly points 8.2 and 8.3) and SDG 3 (*Ensure healthy lives and promote well-being for all at all ages*; in particular point 3.4) (United Nations, 2015).

2. Materials and methods

2.1. Materials

The starting waste material employed in this study was indicated as Black Bentonite (BB) and was provided by Bono & Ditta S.p.A. (Campobello di Mazara, Trapani, Italy) after its use as clarifying agent (to underline the amount of waste produced, please consider that 100 g of BB were used to fine 1 hL of must from white organic grapes). BB is composed by Enobent® Standard (sodium-activated bentonite in powder form) mixed with activated carbon (1:1 w/w). Propylene glycol (PG) and *trans*-resveratrol (RSV) were obtained from A.C.E.F. Spa (Fiorenzuola D'Arda, Italy). Polyethylene glycols 200, 400 and 600 (PEG200, PEG400, PEG600), bovine serum albumin (BSA), glycerol (G) and 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) were purchased from Carlo Erba Reagents (Milan, Italy). Quercetin (QRC) was supplied from Farmalabor (Canosa di Puglia, Italy). Gallic acid (GA), Bradford and Folin–Ciocalteu reagents were purchased by Merck (Darmstadt, Germany). All other chemicals and solvents (analytical grade) were obtained from Carlo Erba Reagents and were used without further purification.

2.2. Recovery and storage of waste BB

The BB appears as a dark and moist mass coming from must filtration and subsequent squeezing. Immediately after its recovery it was kept at −20 °C. An amount (about 10 kg) considered as belonging to the same batch was transported into refrigerated boxes to the laboratories of the University of Palermo where it was subjected to pre-treatment prior to storage.

2.3. Pre-treatment of waste BB

The frozen recovered BB was pulverized in mortar, mixed and sieved to remove any residues from the white grapes processing (e.g., pomace and seeds). Furthermore, it was fractionated (aliquots of about 100 g) and stored at −80 °C (Thermo Forma ultra-freezer −86 °C mod. 902 Thermo Fisher Scientific, Waltham, MA, USA). Additionally, six carefully weighted aliquots of waste BB were freeze-dried (Labconco FreeZone® 2.5 L Freeze Dry System, Kansas City, MO, USA) for 72 h at 0.014 mBar and −50 °C to evaluate the residual water content percentage (W_c%) as follows:

$$W_c\% = \frac{starting\ weight\ (g) - freeze\ dried\ weight\ (g)}{starting\ weight\ (g)} \times 100$$

Results are reported as mean (n = 6) ± standard error (SE).

2.4. Extraction procedure

The extractions were carried out as previously reported by maceration (Di Prima et al., 2022a). Briefly, 3 g of BB were mixed with 12 g of the extraction solvents (PEG200, PEG400, PEG600, PG, G) and kept for 1 h in the dark at 25.0 ± 0.5 °C under vigorous magnetic stirring (Heidolph MR3001K Hotplate Stirrer with Heidolph EXT3001 Temperature Probe, Heidolph Instruments, Schwabach, Germany). The obtained suspensions were transferred into 15 mL PE falcon and centrifuged for 1 h at 5000 rpm and room temperature. The supernatants were then filtered through a 0.22 μm nylon syringe filter, collected into amber glass vials, weighed, and stored at 4 °C in the dark. The yield % of the whole procedure, in term of amount of liquid recovered respect the starting used one, was calculated as follows:

$$Yield\ \% = \frac{amount\ of\ recovered\ extract\ (g)}{starting\ amount\ of\ solvent\ (g)}\ x\ 100$$

Each extraction was repeated six times (n = 6) and results are reported as means ± SE.

2.5. Density evaluation

500 μl of each extract carefully collected using a calibrated micropipette (Ultra High-Performance single-channel Pipettor VWR®) were accurately weighted by using an analytical balance to 5 decimal figures (Mettler, Columbus, OH, USA, Mod. AE 240) to mathematically calculate the density, reported as g/mL. Each measurement was repeated two times for each sample (n = 12) and results are reported as means ± SE.

2.6. pH measurement after water dilution

100 mg/mL stock solutions of each extract in ultrapure water were prepared and their pH was measured by using a pH meter HI 2211 pH/ORP Meter, Hanna Instrument (Woonsocket, RI, USA). Each experiment was performed in duplicate for each extract (n = 12) and results are reported as means ± SE.

2.7. Total phenolic content by Folin–Ciocalteu assay

100 mg/mL stock solutions of each extract in ultrapure water were prepared and then 50 μL of the latter solutions were diluted with 2 mL of ultrapure water previously inserted into a 15 mL plastic tube and well mixed. Subsequently, 130 μL of Folin-Ciocalteu reagent were added and the resulting solutions were vigorously mixed and then left to settle for 5 min in the dark. Afterwards, 370 μL of Na_2CO_3 solution (0.2 g/mL in ultrapure water) were added and vortexed. Samples were maintained at room temperature in the dark for 2 h and then subjected to UV-VIS analysis by using a Shimadzu 1700 instrument (Kyoto, Japan). Each experiment was performed in duplicate on each prepared extract (n = 12) and results are expressed as mg equivalent of GA per gram of extract (means ± SE). To construct the standard GA calibration curve six standard solutions (50–500 μg/mL in ultrapure water) were prepared and analysed as follows:

- linearity range (considering sample operative dilution): 0.98–9.80 μg/mL; λ_{max} = 760 nm; regression equation: Abs = 0.039 + 65.08 × [mg/mL] (R = 0.999).

Intraday and interday variations were lower than the sensitivity.

2.8. HPLC-DAD analysis and quantification of three representative compounds

200 μl of each extract were diluted 1:1 (v/v) with methanol and then subjected to HPLC-DAD analysis to quantify GA, RSV and QRC as representative compounds. The chromatographic separation was achieved by using a HPLC Agilent 1260 Infinity Instrument equipped with a G7129C automatic vial sampler (injected volume: 20 μL; column temperature: 25 °C) a Quaternary Pump G1311B, a Diode Array Detector 1260 Infinity II and a computer integrating apparatus (OpenLAB CDS ChemStationWorkstation, Stockholm, Sweden). The stationary phase consisted in an Ace® Excel Super C18 reversed-phase column (length and inner diameter: 125 × 4.60 mm; pore size: 100 Å; particle size: 5 μm) while the mobile phase was a mixture of 0.1% (v/v) TFA water solution (solvent A) and acetonitrile (solvent B) at 1 mL/min in gradient conditions as follows: 0–2 min isocratic conditions A:B = 90:10; gradient from 2 min A:B = 90:10–22 min A:B = 5:95; 22–23 min isocratic conditions A:B = 5:95, gradient from 23 min A:B = 5:95 to 25 min A:B = 90:10 and finally 25–27 min isocratic conditions A:B = 90:10. The DAD spectrum covered the range between 190 and

800 nm. In these conditions, the retention time of GA, RSV and QRC were 2.77, 11.25 and 12 min respectively. For each compound the appropriate calibration curve was constructed by analysing 6 standard solutions:

- GA → linearity range: 0.5–100 μg/mL; λ_{max} = 271 nm; regression equation: Area = −4.38 + 32113.53 × [mg/mL] (R = 0.999).
- RSV → linearity range: 0.1–50 μg/mL; λ_{max} = 305 nm; regression equation: Area = 49.47 + 142283.71 × [mg/mL] (R = 0.999).
- QRC → linearity range: 1–100 μg/mL; λ_{max} = 370 nm; regression equation: Area = −14.63 + 77606.83 × [mg/mL] (R = 0.999).

Intraday and interday variations were lower than the sensitivity. Each experiment was performed in duplicate on each prepared extract (n = 12) and results are expressed as means ± SE.

2.9. Antioxidant power by DPPH assay

The DPPH assay was performed as previously described (Angellotti et al., 2023b). Briefly, 100 mg of each extract were accurately weighted in a 5 mL volumetric amber flask and dissolved in methanol (concentration 20 mg/mL). 100 μl of the latter solutions were inserted into a quartz cuvette containing 2 mL of DPPH stock solution (40 μg/mL in methanol), well-mixed and immediately subjected to UV-Vis measurements every 5 min for 1 h at room temperature by using a Shimadzu 1700 instrument (Kyoto, Japan). Blank control samples were prepared analogously by using the fresh extraction solvents at the same dilution. Each experiment was performed in duplicate on each extract (n = 12) and results are expressed as percentage amount of residual DPPH (means ± SE) as a function of time. To quantify the residual DPPH free radical (referred to the starting amount intended as 100%) the appropriate calibration curve was constructed as follows by preparing 5 standard DPPH free radical solutions:

- linearity range: 4–40 μg/mL; λ_{max} = 515 nm; regression equation: Abs = 0.018 + 28.59 × [mg/mL] (R = 0.999).

Additionally, to further elaborate the experimental data and calculate the antioxidant power of the extract by referencing to a standard antioxidant molecule, standard DPPH curves were obtained by similarly analysing 5 GA standard solutions (concentration range: 15–50 μg/mL in ultrapure water) in triplicate (n = 3). The residual DPPH % values at 3 selected time points (10, 30 and 60 min) were used to construct 3 calibration curves useful to quantify the antioxidant power of samples in terms of mg equivalents of GA per gram of extract (means ± SE; n = 12). The obtained standard curves were the following:

- 10 min: residual DPPH % = 90.65 – 1153.55 × [mg/mL] (R = 0.996);
- 30 min: residual DPPH % = 91.38 – 1293.95 × [mg/mL] (R = 0.999);
- 60 min: residual DPPH % = 90.65 – 1338.35 × [mg/mL] (R = 0.999).

Intraday and interday variations were lower than the sensitivity.

2.10. Total protein content by Bradford assay

100 mg/mL stock solutions of each extract in ultrapure water were prepared and then 200 μL of the latter solutions were added to 600 μL of ultrapure water previously loaded into a 2-mL plastic Eppendorf. Subsequently, 200 μL of Bradford reagent were added and the solutions were vortexed, kept for 30 min in the dark at room temperature and then subjected to UV-Vis analysis (Shimadzu 1700 instrument, Kyoto, Japan). Each experiment was performed in duplicate on each prepared extract (n = 12) and results are expressed as mg equivalent of BSA per gram of extract (means ± SE). To construct the standard BSA calibration curve five standard solutions in ultrapure water were prepared and analysed as follows:

- linearity range 2–7 μg/mL; λ_{max} = 595 nm; regression equation: Abs = 0.1700 + 0.0332 × [μg/mL] (R = 0.998).

Intraday and interday variations were lower than the sensitivity.

2.11. Control groups

All the experiments were also conducted by using fresh BB (not used to clarify the must) with the purpose of detecting any possible interference. No compounds were extracted from the control BB and the obtained control samples did not possess any antioxidant power, phenolic and protein contents nor HPLC-DAD peaks, so results are not reported.

2.12. Data analysis

The data are expressed as mean ± standard error (SE). All differences were statistically evaluated with the Student's *t*-test or the one-way analysis of variance (ANOVA or F-test). Data were considered statistically significant when $p < 0.05$.

3. Results and discussion

The bentonite from the fining of white musts and wines could be highlighted as not just an abundant waste but as an interesting material deserving a special attention as a novel, valuable source of precious polyphenols. When manipulating a discarded product, it is crucial to define the best extraction method to be employed. The latter should be chosen according to several factors such as scalability, costs, and environmental impact. To stay in a green context the extraction method and solvent should be carefully chosen in order to be completely eco-friendly. Over the decades, many extraction techniques have been proposed specifically to recover polyphe-

nols from grape by-products. These are enzyme-assisted extraction, employ of supercritical fluids, ultrasound- and/or microwaves-assisted extractions, percolation, conventional solid liquid extraction and maceration (Hoss et al., 2021). The latter is a simple technique which still remains the most commonly used as it is fully accessible, easily scalable and, by accurately choosing the extraction solvents, completely green. For the first time at the end of 2022, our research team demonstrated the feasibility to recover polyphenols from the waste white bentonite (WB) by maceration with PEG200 and PG as innovative, unconventional and green extraction solvents (Di Prima et al., 2022a). On the other hand, in this work, the waste BB was exploited as further and better extraction matrix to obtain polyphenols-enriched secondary raw materials characterized by a high value-added and thus potentially marketable and/or useful in several fields. The BB can be ideally considered as the best bentonite to be recovered because it is actually the most commonly employed clarifying agent (and consequently the most representative inorganic waste) of musts and wines as it possesses higher proteins entrapment efficacy than the WB. This certainly results in higher deproteinizing power and maybe also in higher starting content of polyphenols to be recovered. To the aim of comparing the extracts previously obtained by the WB with the newest from the BB, the recovered waste was pre-treated as already reported (pulverization, sieving, aliquoting and low-temperature storage) to minimize its intrinsic inhomogeneity as well as eliminate any residual of grape skins and seeds. Subsequently, to maintain the bentonite:solvent ratio used in the previous work carried out on the WB so that a comparison can be made, the residual W_c% was firstly calculated by freeze drying. It resulted significantly high and equal to 55.32 ± 1.27%, that is really close to the experimental value previously obtained for the WB (54.15 ± 1.56%). As a consequence, the extraction parameters were maintained as previously reported: maceration for 1 h at 25 ± 0.5 °C under vigorous stirring in the dark and employing a bentonite:solvent ratio equal to 1:4 (w/w). These extraction parameters (ratio, time and temperature) had been previously optimized following numerous attempts (Di Prima et al., 2022a) and therefore have not been modified, again with a view of comparing the effects of the extraction process on the two different wastes.

Concerning the extraction solvents, in this context five different unconventional solvents were tested to perform the polyphenols extraction. They were chosen among the well-known liquid hydrophilic excipients already approved for pharmaceutical and cosmetic use due to their safety and solvents/cosolvents properties against polyphenols. The choice of well-known safe liquid excipients is in accordance which a general waste-to-market idea. Indeed, as both the solvents and the extracted molecules by themselves are non-toxic, the whole resulting product should be safe and potentially directly marketable as novel enriched and value-added raw material and/or useful to be inserted in cosmetic and pharmaceutical formulations useful for several applications. Particularly, the tested excipients were PEGs (200, 400 and 600), PG and G. The obtained coloured extracts are depicted in Fig. 1.

Table 1 reports the yields % of the extraction procedures as well as the firstly evaluable characteristics of each obtained extract: density and pH after water dilution. The yield % refers to the recovered amount of extract referred to the starting amount of employed extraction solvent. In view of a scalable and cost-effective procedure this is a crucial parameter to be evaluated. Yet, it should be considered that the here reported procedure in only at a lab-scale and thus it might determine higher loss than a semi-industrial or industrial procedure.

As noticeable, the use of G resulted in the lowest yield %. This is probably due to the high viscosity of the obtained fine suspension, leading to difficulties and relevant losses during the final filtration phase. The density of each extract was close to the starting density of the corresponding pure solvent, while the pH after water dilution resulted drastically changed. In particular, when dissolving each pure solvent in distilled water its pH was not affected and resulted about 6.7–7. In contrast, when diluting each extract with distilled water its pH significantly decreased reaching the range 3.4–4.5. This could be due to the presence of acidic recovered substances, such

Fig. 1. Photograph of the freshly prepared extracts obtained from the waste BB by using the five selected unconventional green solvents. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Characteristics of the extracts in terms of solvent yield% (n = 6), density and pH after water dilution (n = 12). Means ± SE.

Sample	Yield%	Density (g/mL)	pH after water dilution
PEG200	65.7 ± 2.4	1.140 ± 0.005	4.47 ± 0.14
PEG400	63.3 ± 2.0	1.122 ± 0.005	3.54 ± 0.06
PEG600	49.0 ± 10.0	1.111 ± 0.009	3.57 ± 0.02
PG	71.8 ± 1.9	1.039 ± 0.004	4.30 ± 0.21
G	35.3 ± 6.0	1.226 ± 0.005	3.45 ± 0.01

as polyphenols (able to determine acidic pH (Smith and March 2007) and organic acids (e.g., tartaric and malic acids which are widely present in must and wine (Todorov et al., 2023). To assess any differences between the 5 obtained extracts, they were then subjected to quali-quantitative analyses aimed to i) determine the total phenolic content (Folin-Ciocalteu assay); ii) evaluate the chromatographic profile while quantifying 3 representative molecules (HPLC-DAD analyses); iii) estimate their scavenger activity (DDPH assay); iv) verify the presence and amounts of proteins (Bradford assay). Clearly, the below reported and collected knowledge about the prepared extracts is not completed. For instance, the variegate pool of extracted molecules was not completely assessed. Based on the nature of the starting waste material, the extracts could contain polyphenols, proteins, acids, sugars, etc. However, the complete characterization of the extract will need to be performed using other analytical techniques (e.g., HPLC-MS) but this is not the aim of this work which has the purpose of evaluating i) if the BB is a better source of polyphenols than the WB and ii) among the tested extraction solvents, which is the best one in terms of polyphenols content and scavenger activity.

The total phenolic content (TPC) was assessed by the well-known Folin-Ciocalteu assay which is based on a colorimetric reaction occurring between the Folin-Ciocalteu reagent and phenols due to reduction in alkaline environment (Shanaida et al., 2021). Due to the presence of a variegate pool of phenol-based molecules in the proposed extracts, it is necessary to quantify the TPC by using a standard reference molecule. The latter was GA as usual, and thus results (Table 2) are expressed as equivalent mg of GA per 1 g of each extract.

As noticeable, PEGs showed a TPC inversely proportional to their molecular weight (MW), and G displayed the lowest TPC value. This trend was also confirmed by the quantitative HPLC-DAD evaluations, aimed at determining the amount of GA, RSV and QRC in each extract. As reported in Fig. 2, the PEG200-based extract showed the highest amounts of the three representative compounds. In contrast, PEG400, PEG600 and PG did not give significant differences. Again, the G-based extract appeared as the worse one as it did not contain any amount of GA and both RSV and QRC amounts were the lowest.

Clearly, the quantification of just three polyphenols limited further deeper considerations. As a consequence, to better compare the extracts, it is relevant to observe the whole chromatographic profiles. Indeed, numerous peaks attributable to a complex pool of extracted molecules (polyphenols, proteins and maybe other undefined compounds are observable. For this purpose, the 3D-plot chromatograms are reported in the same scale (Fig. 3). As noticeable, the PEG200-based extract resulted in higher and more numerous peaks than the others. On the other hand, again, the use of G as extraction solvent seemed to be ineffective as less compounds as well as lower amounts of them were extracted. It is relevant to underline that despite the DAD detector was used and thus the UV-Vis spectrum of each peak is available, a tentative of identification by using just this information is not possible as different polyphenols belonging to the same sub-class and having a common basic structure exhibit similar UV-Vis spectra, making their identification fail in absence of reference standards.

The rich and variegate pool of polyphenols already highlighted might confer to the extracts some relevant properties which can make them directly useful for several purposes in accordance with the waste-to-market approach. Generally, polyphenols are well-

Table 2
Total phenolic content of the extracts calculated by the Folin-Ciocalteu assay and expressed as equivalent mg of GA per gram of extract and per gram of waste treated. Means ± SE (n = 12).

Sample	TPC (mg/g of extract)	TPC (mg/g of waste)
PEG200	3.1 ± 0.1	12.4 ± 0.2
PEG400	2.1 ± 0.1	8.4 ± 0.2
PEG600	1.7 ± 0.2	6.8 ± 0.5
PG	1.8 ± 0.1	7.2 ± 0.2
G	1.6 ± 0.1	6.4 ± 0.2

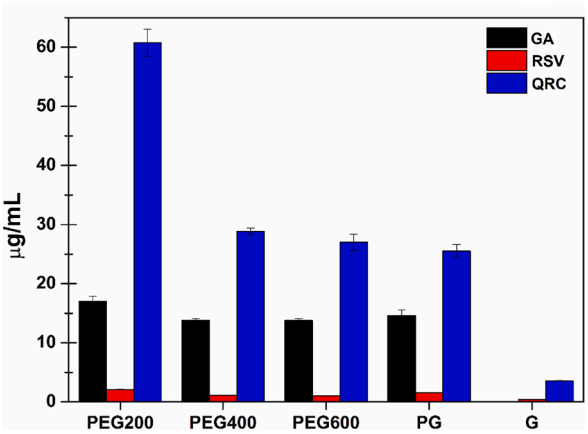

Fig. 2. HPLC-DAD analyses: concentration (μg/mL) of Gallic Acid (GA; black), Resveratrol (RSV; red) and Quercetin (QRC; blue) detected in each extract. Means ± SE (n = 12). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

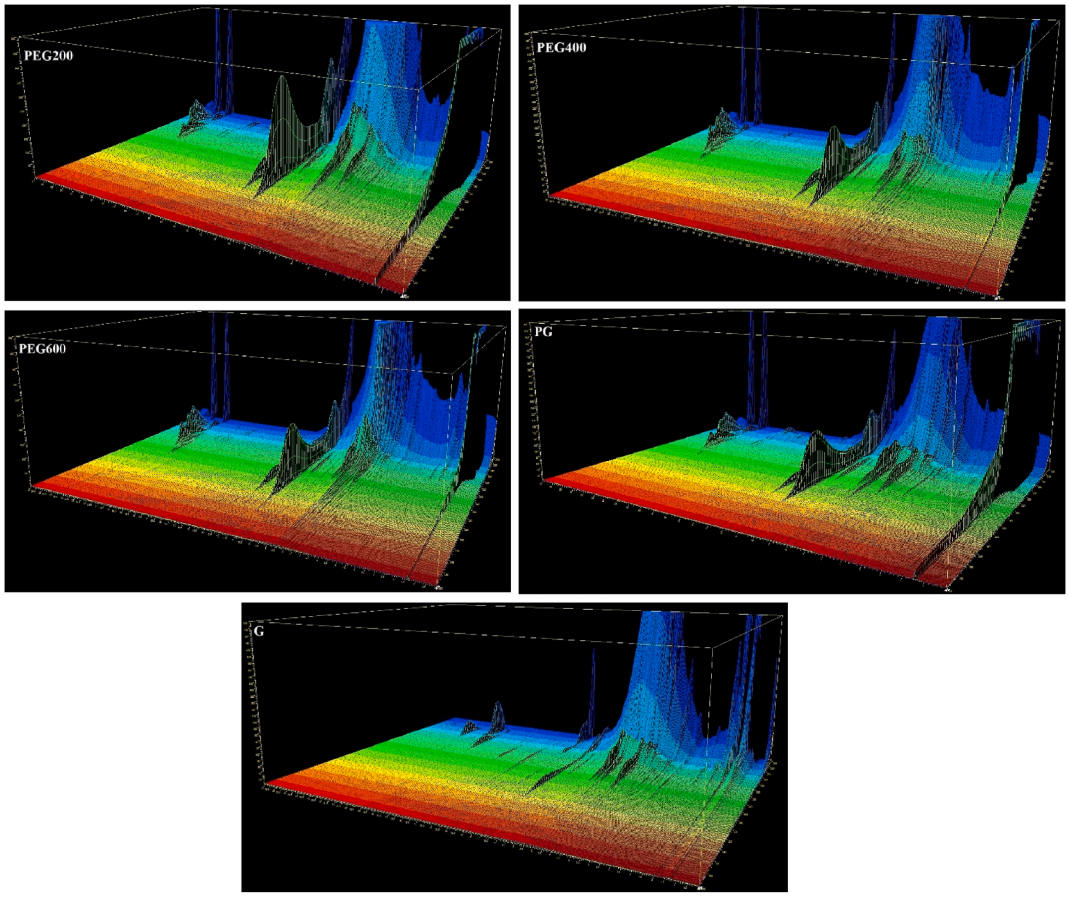

Fig. 3. 3D-plot chromatograms of the extracts reported in the same scale.

known for their excellent scavenger and antioxidant properties resulting useful in a wide variety of fields such as the pharmaceutical (e.g., treatment, prevention and adjuvant therapy against inflammatory disease (Angellotti et al., 2023a; Bruno and Ghiadoni, 2018; Lomartire and Gonçalves, 2023) and cosmetic (e.g., antiaging purposes (de Lima Cherubim et al., 2020; Nichols and Katiyar, 2010) ones. The DPPH assay is a well-known, simple and widely employed method to assess the scavenger properties of substances or mixtures as it is based on the reaction between the stable DPPH radical (DPPH$^{\bullet}$) and the anti-radical compounds (De Caro et al., 2019; Munteanu and Apetrei, 2021). In accordance with the previously set up protocol (Di Prima et al., 2022a), the antioxidant assays were performed at a fixed DPPH:extract ratio as a function of time until 1 h. In Fig. 4 the residual amount % of unreacted DPPH radical is

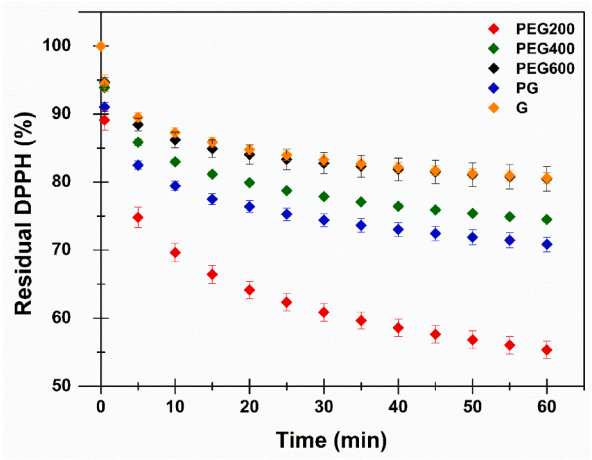

Fig. 4. DPPH antioxidant assay: residual DPPH (%) as a function of incubation time when evaluating all the prepared extracts. Means ± SE (n = 12).

shown over time. Unlike most DPPH assay protocols reported in the literature, here it was additionally evaluated the kinetic of DPPH consumption instead of a simple one-point evaluation. This choice is related to the presence of a mixture of several antioxidants which might display different rate of reaction with the DPPH free radical thus determining specific patterns of its consumption.

As expected from the already collected data, the use of PEG200 as unconventional extraction solvent produced the secondary raw material with the highest antioxidant power as it is able to consume a greater amount of DPPH radical than the other extracts. According to the calculated TPCs, PEGs still exhibited a trend which is inversely proportional to their MW. However, it is noticeable that the TPC values by themselves are not enough to predict the potentiality of each extract. Indeed, the PG-based extract showed a TPC lower than that of the PEG400 one, while it resulted a stronger anti-radical agent. This is probably due to the different ratios of recovered biomolecules in the extracts, having each proper and characteristic reaction kinetics and stoichiometry of DPPH consumption. To better compare the obtained results, the antioxidant power of each extract was quantified at 3 different time points by constructing standard DPPH consumption curves by using again GA as reference molecule. The construction of standard curves of DPPH consumption by reaction between fixed doses of DPPH and 5 different standard concentrations of GA was already previously proposed (Di Prima et al., 2022a, Di Prima et al., 2022b) and gave interesting results in term of kinetic behaviour. Indeed, this approach permits to highlight important differences in the kinetic trend of DPPH consumption when it reacts with a pure substance (GA) compared to a complex mixture of antioxidants such as that contained in the extracts. In particular, the DPPH consumption curves for the GA reached a plateau after almost 10 min of incubation, suggesting a complete reaction between the GA and the free DPPH radical (data are not shown as already reported in the reference papers). Consequently, when the quantification of the antioxidant power of the extracts (in terms of mg GA equivalent per gram of extract, Table 3) was performed at 3 different time point, a time-dependant trend of DPPH consumption was observed, without a plateau, suggesting that the mixture of antioxidants in the extracts continue to react over time, indicating a different kinetic of reaction (Di Prima et al., 2022a,b). Results are reported in Table 3 as mg GA equivalent per gram of extract.

Clearly, the data fully confirm the previous considerations while also greatly highlighting the worse results which were those obtained for the PEG600 and G-based extracts.

Finally, as the waste bentonite is conceptually rich in proteins due to its use to eliminate protein haze from musts and wines, the total protein content (TPtC) of each extract was assessed by the Bradford assay and expressed as mg BSA equivalent (used as standard protein reference) per gram of extract (Kielkopf et al., 2020). Results were reported in Table 4.

The experienced TPtC values in all extracts always resulted very low (about one order of magnitude lower than the TPC) and again demonstrated that the extraction capability of PEGs was inversely proportional to their MW: the PEG having the lowest MW possessed the highest TPtC value. However, G resulted in high TPtC values, which were very close to those of PEG200 (highest ones). This could be attributable to its great hygroscopicity (Verdier-Sévrain and Bonté, 2007) as high amount of water could determine an increased solvent power toward proteins as well as to be responsible for the limited polyphenols extraction previously experienced.

4. Conclusions

The proposed work is fully inserted into a green frame of circular economy and waste valorisation. Here for the first time the waste BB from white organic grape must fining has been proven as a valuable source of precious polyphenols. Additionally, together with a waste recovery approach, the green soul of this work was exploited by mean of eco-friendly extractions based on the maceration of the chosen discarded product using unconventional extraction solvents. PEGs, PG and G were chosen to apply a waste-to-market approach and the resulting extracts were compared. Particularly, PEG200 emerged as the most effective extraction solvent, leading to coloured extract enriched in polyphenols and characterized by relevant scavenger properties. The data collected further valorised the choice of the waste BB instead of the WB as the latter is less polyphenols-rich. Indeed, the BB-based extractions could be compared

Table 3
Antioxidant power of the extracts expressed as mg GA equivalent per gram of extract, after 10, 30 and 60 min of experiment. Means ± SE (n = 12).

Sample	mg GA equivalent per gram of extract		
	t = 10 min	t = 30 min	t = 60 min
PEG200	0.90 ± 0.06	1.17 ± 0.05	1.30 ± 0.05
PEG400	0.33 ± 0.02	0.52 ± 0.01	0.60 ± 0.01
PEG600	0.19 ± 0.05	0.33 ± 0.06	0.38 ± 0.07
PG	0.48 ± 0.03	0.65 ± 0.03	0.73 ± 0.04
G	0.14 ± 0.03	0.31 ± 0.03	0.37 ± 0.03

Table 4
Total protein content of the extracts by the Bradford assay, expressed as mg BSA equivalent per gram of extract and per gram of waste. Means ± SE (n = 12).

Sample	TPtC (mg/g of extract)	TPtC (mg/g of waste)
PEG200	0.25 ± 0.02	1.00 ± 0.08
PEG400	0.19 ± 0.01	0.76 ± 0.02
PEG600	0.17 ± 0.01	0.68 ± 0.02
PG	0.16 ± 0.01	0.64 ± 0.02
G	0.21 ± 0.01	0.84 ± 0.02

with the previously reported pilot WB-based ones. Considering specifically the results obtained when employing PEG200 as solvent, it appears evident that the BB possesses higher amounts of polyphenols to be extracted: the RSV, GA and QRC concentrations into the recovered products are significantly higher when using the BB and particularly resulted doubled for RSV and almost 5-times increased for QRC. Also, the TPC is enhanced coming from 2.58 mg/g to 3.12 mg/g from the WB-based and BB-based extracts respectively, while the TPtC remained almost unchanged probably due to proteins' limited solubility in PEG200. Ultimately, this work is completely in agreement with the sustainable development goals of the UN Agenda 2030.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

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