Green and Quick Extraction of Stable Biophenol-Rich Red Extracts from Grape Processing Waste

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ABSTRACT: The extraction of grape processing waste (wine pomace) via microwave hydrodiffusion and gravity (MHG) from three different cultivars grown in Sicily (Syrah, Perricone, and Nero d’Avola) rapidly affords aqueous extracts highly concentrated in valued biophenols including flavonoids, anthocyanins, and phenolic acids. The method does not employ organic solvent, acid, or base and does not require grinding or freeze-drying of the wine pomace nor separation of the grape skins from seeds and stem. All of the extracts have a pronounced stability, as shown by their red-violet color fully retained after storage for more than a year (15 months) in a freezer under air. Concentrations of phenolics up to 2000 ppm were detected in the aged extracts of Sicily’s local cultivar Perricone, which also has the highest content of flavonoids. These findings provide a simple and economically viable extraction route to biophenol-rich red extracts that can be used as food colorants as well as to formulate nutraceutical, cosmetic, and personal care products starting from an agricultural byproduct available in >10 million tonne yearly amount.

KEYWORDS: Grape processing waste, polyphenols, wine pomace, microwave hydrodiffusion and gravity, enocyanin, resveratrol, anthocyanins

1. INTRODUCTION

Available worldwide in over 10 million tonne/year amount (and counting), grape (Vitis vinifera L.) processing waste (wine or grape pomace) has long been identified as a convenient source of valued dietary fiber and phenolic compounds.1 In winemaking after grape pressing, about 60–70% of grape polyphenolic compounds remain in the pomace.2 Red wine pomace, in particular, has become a source of resveratrol used in many cosmetic products, even though many other valued phenolic compounds having health-beneficial properties including anti-inflammatory, antimicrobial, anticancer, and antithrombotic activity can be sourced from this biological resource.3 Alone, the $59 million resveratrol market in 2019 is expected to reach $80 million by 2025, growing at a compound annual growth rate of 7.7%.4

The industrial extraction of grape polyphenols, in general, makes use of conventional solid–liquid extraction via maceration or Soxhlet extraction with polar organic solvents such as ethanol or acetone. In the last two decades (2000–2019), numerous alternative grape polyphenols extraction routes—including acoustic cavitation, microwave-assisted extraction, pressurized solvent, and supercritical fluid extraction—have been demonstrated, though a few have been industrialized.5

Holding large industrial potential,6 microwave-assisted extraction (MAE) of grape polyphenols from grape pomace has been widely explored since the early 2000s. After comprehensive optimization research, scholars in Croatia in 2016 discovered the optimal conditions for the MAE of phenolic compounds and anthocyanins from grape skin pomace: eight consecutive extraction cycles, each lasting 16 min, using 100% methanol added with 1% HCl as solvent at 60 °C for polyphenols; and five consecutive extraction cycles, each lasting 16 min, using 92% methanol solvent added with 0.6% HCl at 45 °C for anthocyanins.7,8

Added as food coloring to several food products including beverages (soft drinks, wine, and liqueurs), jams, candies, ice creams, yogurts, and desserts, enocyanin is the name of the water-soluble grape skin extract widely used as food colorant (labeled E163 in EU countries).9 Its pronounced solubility and high coloring strength allows one to add a very small amount of enocyanin (usually at concentrations between 20 and 60 ppm), causing no change in taste or smell of the treated products.10

Commercially extracted since 1879 (in Italy) first using ethanol, and subsequently for over a century in numerous countries via maceration of wine pomace in a 0.2% sulfur dioxide aqueous solution (to protect the pigment from oxidation and microbial spoilage),11 enocyanin is an important part of the anthocyanin global market currently growing at a fast pace (forecasted to $750 million in 2026).12 Due to its antioxidant and antiproliferative13 activity, which led to the commercialization of numerous cosmetic and...
nutraceutical products using resveratrol as active ingredient, today also this stilbene derivative is widely extracted from red grape berry skin. Now we report the extraction of grape processing waste (wine pomace) via microwave hydrodiffusion and gravity (MHG) from three different grape cultivars grown in Sicily (Syrah, Perricone, and Nero d’Avola). Affording stable aqueous red extracts rich in grape flavonoids, phenolic acids, and anthocyanins, the method does not require grinding or freeze-drying of the wine pomace nor separation from seeds and stem. No organic solvent or acid is used throughout the process, eliminating the need for hazardous or toxic substances to extract the valued biophenols from wine pomace.

All of the extracts have a pronounced stability, as shown by their red-violet color fully retained after storage for more than a year (15 months) in a freezer under a normal atmosphere. Concentrations of phenolics up to 2000 ppm were detected in the aged extracts of Sicily’s local cultivar Perricone, which also has the highest content of flavonoids.

2. MATERIALS AND METHODS

Wine pomaces from grapes organically harvested in September 2019 originating from Sicily’s vine-growing region of Monreale were kindly provided from Aziende Agricole Biologiche Tamburello (Monreale, Italy). Pomace samples including skins, seeds, residual pulp, and stems were processed as such, with no prior treatment before the MHG extraction. An Ethos X microwave extractor (Milestone, Sorisole, Italy) was used for the extraction of water-soluble compounds from grape pomace samples under the conditions of Table 1.

Table 1. Perricone, Syrah, and Nero d’Avola Wine Pomace Amounts Undergoing MHG Extraction after Soaking in Water

| cultivar         | wine pomace amount (g) | water (mL) |
|------------------|------------------------|------------|
| Syrah            | 550                    | 200        |
| Nero d’Avola     | 560                    | 200        |
| Perricone        | 260                    | 200        |

Each grape processing waste material was soaked in 200 mL of water. The amount of Perricone grape processing waste made available by a winemaking company in Sicily is about half that of the other two cultivars. Future studies will investigate saturation effects (if any) as the two aims of the present study were to assess the feasibility of the MHG extraction of grape processing waste and to identify the key biophenol compounds in the aqueous extracts. Pomace readily adsorbed most of the added water. The resulting hydrated pomace was transferred to the 2 L vessel of the microwave extractor. The aqueous extract generated by microwave irradiation by hydrodiffusion falls under the action of gravity through a spiral condenser outside the microwave cavity (Figure 1). A 1 kW chiller was used to condense the aqueous extract. The extraction was digitally followed via the EasyControl software (Milestone, Sorisole, Italy). Extraction was carried out under the conditions of Table 2 for which a brief step (5 min) using higher power (500 W) was followed by 50 min extraction under relatively low (200 W) microwave irradiation power.

Aqueous extracts deeply colored in red-violet were obtained for each wine pomace. The extracts were poured in 60 mL plastic vials, capped, and stored as such in a freezer for 15 months. No nitrogen or other inert gas was inserted in the vials to remove air. After 15 months, a vial of each extract was left at room temperature for several hours after which the red-violet 60 mL liquid thereby recovered underwent freeze-drying of each aqueous extract using a FreeZone 4.5 (Labconco, Kansas City, MO, USA) freeze-dryer.

Eventually, 111 mg of Nero d’Avola, 110 mg of Syrah, and 107 mg of Perricone powder extracts were recovered. Each powder was added to a 15 mL Falcon conical centrifuge tube. A 3 mL aliquot of HPLC grade MeOH (Sigma-Aldrich, St. Louis, MO, USA) was added to each Falcon.

The ultrasound-assisted extraction of phenolic compounds from each powder was carried out at room temperature using a Transsonic 460 H ultrasonic bath (Elma Hans Schmidbauer, Singen, Germany) operated for 15 min at 35 kHz ultrasonic frequency. After sonication, each tube underwent centrifugation at 5000 rpm for 5 min using a Thermo Fisher Scientific SL 16 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The supernatant was recovered and 3 mL of fresh MeOH were added to the solid residue. This MeOH aliquot was recovered, followed by addition of another 3 mL of MeOH. Overall, the 9 mL methanolic extract was eventually reduced to 1.6 mL via evaporation under reduced pressure.

Only the red-colored Nero d’Avola methanolic extract did not show turbidity. The other two extracts displayed some turbidity and were colored in orange-dark red. The supernatant of each sample was filtered and transferred to glass vials for the high-performance liquid chromatography mass spectrometry (HPLC-MS) analyses conducted using an Alliance e2695 (Waters, Milford, MA, USA) HPLC system equipped with autosampler, degasser, and column heater coupled with a Q-Tof Premier (Waters, Milford, MA, USA) quadrupole time-of-flight mass spectrometer.

Compounds in each sample were separated by a Thermo Fisher Scientific Hypersil GOLD HPLC column (50 × 2.1 mm i.d., Thermo Fisher Scientific, Waltham, MA, USA) kept at 20 °C by injecting each
time a 5 μL sample. All samples were injected in duplicate using a thermostatted autosampler kept at 4 °C. The HPLC eluent was a mixture of 0.1 wt % aqueous formic acid and 0.1 wt % formic acid in MeOH flown at a 0.25 mL/min rate.

Elution started with 95% aqueous formic acid and 5% methanol formic acid, isocratic for 1 min. In the subsequent 14 min, the solvent became 100% MeOH, and remained isocratic for the subsequent 5 min (from min 15 to min 20). After 30 s, the eluting solvent mixture was reverted to 95% aqueous formic acid and 5% methanolic formic acid and kept as such for another 30 s. The whole run lasted 21 min. Every sample was injected twice. The concentration values reported in Table 4 are the arithmetic mean of the values measured in each run.

Quantification of quercetin, resveratrol, and gallic acid used as standards authentic samples of HPLC purity purchased from a chemical supplier (Sigma-Aldrich, St. Louis, MO, USA). For the remaining compounds, we used the calibration curve of quercetin for the detection of flavonoids and anthocyanins and the calibration curve of resveratrol for the assessment of other biophenols. The former calibration curve was prepared using a standard (1000 ppm) solution dissolving 10 mg of quercetin in 10 mL of methanol. Similarly, the other calibration curve was prepared starting from a standard (1000 ppm) solution of trans-resveratrol in 10 mL of methanol. Both calibration curves were obtained using the peak areas from 0.5, 1, 5, 10, and 20 ppm solutions.

The MS experiments were performed on the aforementioned Q-ToF Premier mass spectrometer using dynamic range enhancement (DRE) as the acquisition mode that avoids MCP saturation while keeping a fairly good sensitivity. This allows one to correctly quantify abundant as well as trace level compounds, providing results suitable for statistical analysis. Electrospray ionization (ESI) in negative mode was used under the following conditions: capillary, 2.0 kV; extraction cone, 2.0 V; ion guide, 2.0 V; source temperature, 80 °C, cone gas, N₂, flow 35 L h⁻¹; desolvation gas, N₂, flow 300 L h⁻¹.

The following compounds were researched in each wine pomace analyzed: quercetin, naringin, sinapinic acid, rutin, quercetin-3-glucuronide, naringenin, hesperidin, hesperetin, eriodictyol, eriocitrin, diosmin, casticaric acid, petunidin-3-O-(6′-acetylglucoside), cyanidin-3-O-glucoside, malvidin-3-O-pentoside, peonidin-3-O-glucoside, resveratrol, resveratrol dimer, resveratrol trimer, resveratrol tetramer, resveratrol glucoside, kaempferol, kaempferol-7-O-hexuronide, quercetin-3-O-(6′-O-malonylglucoside), quercetin-3-O-hexuronide, kaempferol-3-O-glucoside, quercetin-3-O-galactoside, myricetin-3-O-glucoside, myricetin, epicatechin-3-O-coumarate, epicatechin-3-O-vanillate, epigallocatechin, procyanidin B3, catechin, procyanidin B2, procyanidin C1, procyanidin dimer B type, galloctachin, ferulic acid, ellagic acid, prodelphinidin B, epicatechin-3-glucoside, caffeic acid methyl ester, 3-O-(cafeoyl-5′-O-p-coumaroylquinic acid, p-coumaric acid, gallic acid ethyl ester, vanillic acid, caffeic acid, p-hydroxybenzoic acid, and gallic acid.

3. RESULTS AND DISCUSSION

The system was able to reliably follow the extraction heating program with a full traceability of time, power, and temperature. Under the conditions applied (Table 2), the temperature in the extraction vessel rapidly reached 78 °C and subsequently remained constant. The overall extraction lasted each time 55 min, after which an aqueous extract deeply colored in red (Figure 2, top) was readily isolated for all three cultivar wine pomaces, leaving the extracted wine pomace (Figure 2, bottom) ready for composting.

Results in Table 3 show an exceptionally high content of polyphenols for the Perricone wine pomace and a very high amount of the same compounds in the Syrah pomace. Only the pomace residual of Nero d’Avola winemaking process had a polyphenol content <1000 mg/kg. For simplicity, Table 3 and Table 4 list anthocyanins alone (i.e., not amid flavonoids), although anthocyanins are flavonoids with a positive charge at the oxygen atom of the C-ring of basic flavonoid structure.
Table 4. Flavonoids, Phenolic Acids, and Anthocyanins in the MHG Aqueous Extracts of Perricone, Syrah, and Nero d’Avola Red Wine Pomaces

| phenolic class          | Perricone (mg/kg) | Syrah (mg/kg) | Nero d’Avola (mg/kg) |
|-------------------------|-------------------|---------------|----------------------|
| flavonoids              |                   |               |                      |
| quercetin               | 178.60            | 170.23        | 51.34                |
| quercetin-3-O-glucuronide | 76.36            | 33.83         | 63.89                |
| quercetin-3-O-galactoside | 50.04           | 39.35         | 31.08                |
| eriodictyol             | 22.60             | 21.01         |                      |
| kaempferol              | 43.54             | 34.04         | 22.05                |
| kaempferol-7-O-glucuronide | 23.01           | 10.40         | 21.90                |
| kaempferol-3-O-galactoside | 29.27           | 26.26         | 25.11                |
| myricetin               | 22.89             | 21.13         | 22.42                |
| myricetin-3-O-glucoside | 31.50             | 26.14         | 24.90                |
| naringenin              | 30.51             | 30.65         | 30.48                |
| hesperidin              | 22.87             | 23.06         | 20.34                |
| procyanidin B2          | 52.48             | 34.14         | 25.01                |
| epicatechin-3-O-vanillate | 23.53            | 11.17         | 10.55                |
| phenols                 |                   |               |                      |
| resveratrol             | 32.65             | 8.80          | 9.22                 |
| resveratrol dimer       | 13.34             |               | 9.56                 |
| resveratrol trimer      | 11.30             | 9.87          | 13.05                |
| resveratrol tetramer    | 16.20             |               | 10.22                |
| p-hydroxybenzoic acid   | 9.78              | 9.57          | 15.56                |
| p-coumaric acid         | 13.63             | 18.46         | 12.87                |
| gallic acid ethyl ester | 434.95            | 344.22        | 155.61               |
| caffeic acid            | 15.78             | 25.30         | 12.87                |
| gallic acid             | 518.47            | 277.84        | 151.58               |
| syringic acid           | 350.32            | 270.85        | 122.49               |
| anthocyanins            |                   |               |                      |
| cyanidin-3-O-glucoside  | 24.49             | 23.11         | 28.63                |

Results in Table 4 show that cyanidin-3-O-glucoside was the dominant anthocyanin found in the aqueous extract under the applied MHG extraction conditions, in an amount (~25 mg/kg) almost independent of the cultivar.

This outcome is in agreement with literature data for which the dominant anthocyanin in the Perricone wine and grape is the malvidin-3-O-glucoside, followed by substituted anthocyanins and particularly by petunidin, peonidin, and delphinidin 3-O-glucosides, because winemaking techniques limit the amount of anthocyanin that can be extracted from the berry skins to less than 7% of cyanidin-3-O-glucoside.

Table 4 lists the flavonoid and other phenolic compounds found using the highly sensitive HPLC-MS technique employed for the analysis.

Amid phenolic acids, gallic acid, which is known to be along with catechin, and epicatechin particularly abundant in the seeds of red and white grape varieties, was found in very high amount (519 mg/kg) in the Perricone wine pomace extract, along with gallic acid ethyl ester (435 mg/kg) and syringic acid (350 mg/kg), another hydroxybenzoic acid typical (with p-hydroxybenzoic acid) of wines and grapes.

While gallic acid is found in the grape itself, it is also released following the hydrolysis of the gallic acid esters of flavan-3-ols, whereas the large amounts of gallic acid ethyl esters likely formed thanks to the acid-catalyzed esterification reaction of gallic acid and ethanol formed during sugar fermentation occurring also in the wine pomace. These results are partly in agreement with the analyses of the red wine pomace of Cabernet Sauvignon pomace and Feteasca Neagra cultivars also finding abundant amounts of gallic and syringic acid.

Resveratrol isomers were found in all three cultivar wine pomaces. Again, resveratrol, present in all the natural forms, was particularly abundant in the Perricone cultivar wine pomace (free, dimer, trimer, and tetramer together >73 mg/kg) and also in the Nero d’Avola wine pomace (free, dimer, trimer, and tetramer together >42 mg/kg). The Syrah processing waste extract had the lowest resveratrol content (<20 mg/kg). For comparison, the amount of resveratrol extracted by researchers in Italy with MeOH/EOH from freeze-dried grape skins and grape pomace of Nero d’Avola was 27.5 and 6.00 mg/kg, respectively.

In agreement with previous findings concerning the wine pomace of another Sicilian red grape cultivar (Nerello Mascalese), quercetin (and its glycoside derivatives quercetin-3-O-glucuronide and quercetin-3-O-galactoside) was the dominant flavonoid detected in the three grape cultivar lyophilized aqueous extracts, with the Perricone extract containing almost 180 mg/kg of this dietary flavonol known for its powerful antioxidant, antihypertensive, anti-inflammatory, antiobesity, and antiatherosclerotic activities.

Kaempferol, another powerful bioactive flavonoid with a broad range of physiological (anticancer, antioxidant, and anti-inflammatory) activities, was also abundant in all three cultivar aqueous MHG extracts. The three forms found, including kaempferol-7-O-glucuronide and kaempferol-3-O-glucoside, approach 100 mg/kg in the case of the Perricone extract, followed by ~70 mg/kg for both the Syrah and the Nero d’Avola extracts.

Myricetin (and its glycoside myricetin-3-O-glucoside) was also found in approximately 50 mg/kg amount in each cultivar extract. Again, the flavonoid exhibits an ample spectrum of physiological (anticancer, anticancer, anti-diabetic, anti-inflammatory, and neuroprotective) activities. Numerous studies have suggested that the compound may be beneficial to protect against diseases such as Parkinson’s and Alzheimer’s.

In brief, likewise to red wine, polyphenols are a complex mixture of flavonoids (mainly anthocyanins and flavan-3-ols) and nonflavonoids (such as resveratrol and gallic acid) with oligomeric and polymeric procyanidins often representing 25–50% of the total phenolic constituents. Contrary to red wine, however, in the wine pomace aqueous extracts sustainably sourced via microwave hydrodiffusion and gravity, flavanols and procyanidins are not the most abundant biophenols.

Procyanidin B2, the most widely distributed natural procyanidin, is abundant (52 mg/kg) in the Perricone extract and still substantially present (25 mg/kg) in the Nero d’Avola extract (24 mg/kg in the Syrah extract). The molecule was recently found to mitigate glucose-induced reticulum stress in endothelial implicated in the pathophysiology of diabetes and its vascular complications.

Finally, naringenin, at approximately 30 mg/kg, and hesperidin, at about 21 mg/kg, are present in almost equal amounts in all three cultivar extracts. Both known amid red wine polyphenols, the former flavonone and the latter flavanone glycoside have wide-scope physiological activities, including broad-scope antiviral activity.

Originally introduced by Chemat, Visinoni, and co-workers in 2008 for the extraction of essential oils without adding any solvent or water, microwave hydrodiffusion and gravity was successfully applied to extract valued bioproducts to the pomace of numerous fruits, including bilberry, citrus, and...
apple. The first application to the solvent-free extraction of grape-pressing byproducts was reported by Chemat and co-workers in 2013. The team added the MHG extract to the grape juice obtained in the laboratory along with the grape pomace. The resulting fortified grape juice had significantly higher total phenolics content and was redder and less sweet in comparison to the natural grape juice.

In this work, MHG has been applied to different industrial wine pomaces after soaking the pomace in water in order to study the (i) phenolic profile from different red grape cultivars grown in Sicily and (ii) the stability of the red biophenol-rich extracts thereby obtained. The lack of chemical stability of anthocyanins in the presence of air’s oxygen and thus during food processing, storage, and commercialization is the main issue that limits the widespread uptake of these colorant molecules in the food industry. Quick oxidation occurs, and the red color is converted to an undesirable brown, a color particularly disliked by food and beverage consumers. Several stabilization methods have been developed, including additions of co-pigments, phenolic compounds, metals, and exclusion of O₂ during processing and storage.

Now, we show evidence that the MHG aqueous extract of red wine pomace is so stable that it can be safely handled in an open atmosphere, stored in a freezer for over 1 year (15 months), lyophilized for several days, and eventually analyzed without alteration of its deep red-violet color, while retaining exceptionally high content of phenolic compounds. Likewise to what was observed with Opuntia-ficus indica red betalains obtained from the fruit peel via MHG, stabilization against oxidative degradation is due to the concomitant extraction of numerous strongly antioxidant water-soluble biophenols.

These findings provide a simple and economically viable extraction route to anthocyanin-based red extracts that can be used as food colorants as well as to formulate nutraceutical, cosmetic, and personal care products starting from an agricultural byproduct annually produced in >10 million tonne amount.

Finally, it is also of relevance to this report that in scaled-up MHG extraction of phenolics from fruit, the cost of manufacturing largely depends on the cost of raw material (81.09%), followed by the cost of utilities (13.09%). As the cost of wine pomace is extremely low, MHG is particularly well suited for the valorization of red wine pomace from a cost of wine pomace is extremely low, MHG is particularly well suited for the valorization of red wine pomace also from a practical application viewpoint.

Given the large amounts of bioactive compounds and in agreement with recent studies on grape pomace using different extraction methods, it is anticipated that these red wine pomace extracts obtained using water as the only extraction solvent may have significant physiological activity, especially those originating from the Perricone and Syrah cultivars. The outcomes of biological experiments carried out using these extracts will be reported in due course.

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