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# Revalorization of winery by-products as source of natural preservatives obtained by means of green extraction techniques

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ABSTRACT

Winemaking industry produces huge amounts of by-products such as grape seeds, pomace and stems with important remaining polyphenol content after vinification process. Extracts obtained from two green extraction techniques, accelerated solvent extraction (ASE) and ultrasound assisted extraction (UAE), were evaluated to inhibit the growth of bacteria implicated in alimentary pathologies and to avoid the oxidation reactions. Hydroalcoholic extracts obtained by UAE resulted to have higher antibacterial and antioxidant properties compared to aqueous extracts from ASE. Results confirmed differences in bacteria susceptibility to extracts. Among Gram-negative bacteria, *E. coli* were especially sensitive to the inactivation of grape seeds and pomace extracts ($IC_{50}$ ranged between 0.33–0.12 mg $mL^{-1}$), meanwhile stem extracts were particularly effective against *S. aureus* growth ($IC_{50}$ = 0.09 mg $mL^{-1}$). No significant correlation was found between antibacterial activity and the phenolic composition, suggesting that structure-function of phenolic extracts and the interspecific relationship among different bacteria strains have a great influence. On the other hand, strong correlations were found among antioxidant capacity of extracts and phenolic composition (DPPH, r = 0.778, $P < 0.05$; ABTS, r = 0.879, $P < 0.01$). Due to the high content of hidroxycinamic acid derivatives, flavonols, tannins, catechins and anthocyanins, winery by-products are postulated as a good source of natural preservatives whose antibacterial and antioxidant properties can be customize to satisfy the requirements of the diverse food industries.

## 1. Introduction

It is well known that food processing industries generate large amounts of wastes and residues which threaten the environment. This fact represents one of the main social challenges, which must be addressed to minimize the environmental impact. A well management of these residues can help offset growing environmental problems. On the other hand, in most cases, agric-food residues can turn into new available natural resources of valuable compounds whose extraction can entail a sustainable strategy. In this sense, valorization and integral use of by-products seem to offer also a profitable activity improving the economic feasibility of the main process by the production of secondary streams of value-added compounds (Vardanega et al., 2015).

*Vitis vinifera* is one of the fruit crops most widely cultivated throughout the world (Winkler et al., 1997). Wine making is the main industrial activity whose production generates high amounts of by-products such as grape seeds, pomace and stems, representing a waste manage issue both ecologically and economically (FAO, 2012). Alternative uses have been applied to winery by-products as soil conditioner, source of fibers and energy (Kamel et al., 1985), for oil extraction, as source of protein for animal feed, enzyme production (Masutti et al., 2015), tocopherol and tocotrienol recovery (Gornas et al., 2015) or for improving sensorial characteristics of beverages (De Torres et al., 2015; Villegas et al., 2016). However, in the last years, a rising attention has been paid on the revalorization of the winery by-products based on the recovery of bioactive compounds (Teixeira et al., 2014). This relevance relies on the polyphenolic compounds remaining which are recognized as important phytochemicals due to their antioxidant and antimicrobial activity (García-Ruiz et al., 2009).

Currently, there is a growing demand by consumers for clean label food products where artificial additives are replaced by natural ones to ensure food safety. Consequently, vegetal sources rich on polyphenols from agro-industrial by-products and the screening of raw materials for identifying new natural preservatives are in the spotlight (Moure et al., 2001).

In general, red grape pomace, skins and seeds are characterized by high content of flavonoids: such as flavan-3-ols, flavonols and anthocyanins (Garrido and Borges, 2013). All these substances have

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demonstrated their antioxidant and antimicrobial properties in grape pomace, skins and seeds (Delgado Adámez et al., 2012; Furiga et al., 2009; Jayaprakasha et al., 2003; Kataliníc et al., 2010; Mattos et al., 2017). On the other hand, phenolic compounds can be also found in other parts of the grape cluster, such as grape stems, which are usually discarded at the first stage of winemaking process. Consequently, grape stems preserve their phytochemical composition almost completely. However, the bioactive properties of grape stems have been received less attention in comparison with grape pomace and seeds (Anastasiadi et al., 2009; Dias et al., 2015; Ruiz-Moreno et al., 2015).

In the last years, sustainable green alternative technologies for the extraction of bioactive compounds from winery wastes and by-products have been established (Barba et al., 2016). Green technologies such as sub- and supercritical fluid extraction, ultrasounds or high pressure processing among others, often entail the use of GRAS (Generally Recognized As Safe) solvents under temperature and pressure controlled to improve the solvation power and extraction efficiency of these solvents. These facts point out to green extraction techniques as an excellent tool to extract target compounds preserving their functional properties (Barba et al., 2016). However, the use of the different techniques under diverse conditions modifies the extraction process and the subsequent chemical composition of the extracts which entails different spectra of functional properties.

Therefore, winery by-products are postulated as natural source of bioactive compounds such as polyphenols, whose antioxidant and antimicrobial activities can be useful as natural preservatives. However, despite the high number of studies concerning the use of grape products as antimicrobial agents, there is still a lack of literature information involving similar combination of raw material type and preparation, extraction method and microbiological assays methodology applied to determine the antimicrobial and antioxidant activity, in order to compare the results. With this aim, the antimicrobial and antioxidant properties of extracts from different by-products (grape pomace, seeds and stems) obtained by two different green extraction methods: pressurized liquid extraction and ultrasound extraction, have been evaluated. Furthermore antimicrobial and antioxidant capacities were related to the different families of polyphenols in order to point out the best extract from winery by-products capable to be used as natural preservative mainly in food industry.

## 2. Materials and methods

### 2.1. Plant material

Different winemaking by-products, grape seeds, pomace and stems from *Vitis vinifera* L. cv. Tempranillo, and seeds from *Vitis vinifera* L. cv. Cabernet Sauvignon, were obtained from the Institute of Vine and Wine of Castilla-La Mancha (IVICAM, Tomelloso, Ciudad Real, Spain). Samples were ground with addition of dry ice and cooling jacket at 0 °C, using a crusher Stephan UMC5 (Stephan Food Service Equipment GMBH). Then, they were frozen at −20 °C until their processing.

### 2.2. Accelerated solvent extraction (ASE)

Aqueous extracts were obtained according to the method previously described by Alañón et al. (2017) using an accelerated solvent extractor ASE 200 (Dionex Corp. Sunnyvale, CA). Eight grams of each winemaking by-product were mixed with 2 g of a dispersing agent, diatomaceous earth, in order to reduce the dead volume of an 22 mL stainless steel cell. The extractions were carried out at a temperature of 120 °C and a pressure of 1500 psi, performed two extraction cycles of 10 min. The extraction system was rinsed between samples to avoid possible contaminations. The extracts done by duplicate were frozen at −20 °C and then freeze-dried under vacuum ($1.1 \times 10^{-2}$ mB) using a Cryodos −50 lyophilizer (Telstar, Barcelona, Spain). Condenser temperature was −53.2 °C and power supply was 230 V (50 Hz).

### 2.3. Ultrasound assisted extraction (UAE)

Hydroalcoholic extracts were obtained according to the method previously optimized by Marchante et al. (study submitted for publication), using a QSONICA sonicator (53 CHURCG HILL RD. Newtown, CT USA). Five grams of each winemaking by-product were placed in a beaker together with 20 mL of hydroalcoholic solvent (44% of ethanol). The operation conditions were as follows: frequency of 20 KHz at 500 W; duty cycle of 15 s turn on and 5 s off; and extraction time of 3 min. The extraction temperature was never higher than 50 °C, for which a bath at 4 °C was used. After two extraction cycles, extracts were mixed and centrifuged at 7000 rpm for 5 min. The supernatants were filtered under vacuum. The UAE extracts done by duplicate were frozen at −80 °C and then freeze-dried under vacuum ($1.1 \times 10^{-2}$ mB). Condenser temperature was −53.2 °C.

### 2.4. Strains and culture conditions

The antimicrobial activity of the extracts obtained by different extraction methods was evaluated against Gram-positive bacteria *Staphylococcus aureus* CECT 86 (ATCC 12600) and Gram-negative bacteria *Escherichia coli* CECT 45 (ATCC 4157), *Pseudomonas aeruginosa* CECT 111 (ATCC 9027), *Salmonella enterica* serovar. Typhimurium CECT 443(ATCC 13311).

An overnight culture of each strain grown in Mueller Hinton broth (MHB) was diluted in fresh medium to achieve a concentration of approximately 1 to $2 \times 10^8$ colony-forming units (CFU) $mL^{-1}$.

### 2.5. Antimicrobial activity assay

The antimicrobial activity assays were performed using the microdilution method as reported by García-Ruiz et al. (2011). Briefly, a volume of 300 µL of plant extracts (30 $mg_{lyophilized}\,mL^{-1}$) prepared in MHB were transferred into the wells of the first column of the 96 well microplates. Two-fold serial dilutions were performed on the rest of wells containing 150 µL of MHB, achieving a range of concentrations from 22.5 to 0.05 mg $mL^{-1}$. Fifty microliters of bacterial suspension was added to each well to obtain $5 \times 10^5$ CFU $mL^{-1}$. The following controls were included in each plate: a positive control with ciprofloxacine (from 200 µg $mL^{-1}$ to 0.1 µg $mL^{-1}$), a sterility control (extract in MHB without bacterial suspension), a growth control (inoculated MHB without extract) and a blank control (uninoculated MHB only). Plates were incubated at 37 °C for 24 h and bacterial growth was determined by reading the optical density (OD) at 630 nm. Growth inhibitory activity was expressed as a mean percentage (%) of growth inhibition with respect to a control without antimicrobial extract. Assays were conducted in triplicate. The inhibition percentage was calculated as:

$$\%inhibition = \frac{(\text{ODfSample} - \text{ODiSample}) - (\text{ODfBlank} - \text{ODiBlank})}{(\text{ODfGrowth} - \text{ODiGrowth}) - (\text{ODfBlank} - \text{ODiBlank})}$$

where $ODi_{Sample}$ and $ODf_{Sample}$, corresponded to the $OD_{630}$ of the strain growth in the presence of the extracts before and after incubation, respectively; $ODi_{Blank}$ and $ODf_{Blank}$ corresponded to the broth medium with extracts before and after incubation, respectively; and $ODi_{Growth}$ and $ODf_{Growth}$ corresponded to the strain grown in the absence of the extract solution before and after incubation, respectively. The survival parameter $IC_{50}$ and $IC_{90}$ values, defined as the concentration required to obtain a 50% and 90% inhibition of growth respectively, were estimated by a logarithmic regression by the IBM SPSS statistics 22.0 for Windows statistical package (SPSS Inc., Chicago, IL, USA).

### 2.6. Antioxidant activity

#### 2.6.1. DPPH method

Antioxidant activity was determined by DPPH method based on the