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Assessment of alcoholic distillates for the extraction of bioactive polyphenols from grapevine canes

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ABSTRACT

The suitability of alcoholic distillates, from wine production wastes, as green solvent for the extraction of polyphenolic compounds from grapevine canes is evaluated. Two flavanols (catechin and epicatechin) and four stilbenes (*trans*-resveratrol, *trans*-piceatannol, *trans*-piceid and *trans*-ε-viniferin) are chosen as model compounds in order to investigate the effect of (1) the technique (maceration, Soxhlet and pressurized liquid extraction, PLE), and (2) the solvent (ethanol, ethanol:water, methanol, acetone and alcoholic distillates) in the yield of the extraction. Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) was used as determination technique. Concentrations measured for above compounds, relative extraction efficiencies obtained for additional polyphenols (flavanols and stilbenes), and determination of the total polyphenol content of samples proved that alcoholic distillates achieve equivalent extraction yields to purified solvents, and/or ethanol:water mixtures. Levels of polyphenols in canes from local varieties of *Vitis vinifera* are provided. The potential contamination of polyphenol extracts with fungicides sprayed on vineyards is also discussed.

1. Introduction

Stilbene and flavanol polyphenols have attracted the interest of the scientific community due to their healthy properties and commercial applications. Within the first group, resveratrol is employed by the food supplements industry. Resveratrol is also included in active packing films to improve the stability of both, the packaging and the food (Agustin-Salazar et al., 2014; Barbosa-Pereira et al., 2014). Preliminary studies reporting the use of resveratrol for (1) wine stabilization, as alternative to sulphites (Pastor et al., 2015); and (2) for the post-harvest surface treatment of fruits and vegetables, instead of synthetic fungicides (Jiménez et al., 2005), have been also published. The resveratrol dimer *trans*-ε-viniferin is employed in the formulation of cosmetics (Mora-Pale et al., 2015). Moreover, *in-vitro* studies have suggested the potential of this viniferin for the treatment of some types of hepatic cancer (Ozdemir et al., 2014). The flavanol catechin and its derivatives are also regarded as valuable compounds due to their antimicrobial and antioxidant properties (Friedman, 2014). The above applications have led to a demand for sustainable, cost-effective and safe sources of both kinds of polyphenols, as well as for extraction approaches to recover these compounds from natural sources.

The wine production industry generates different sub-products which are rich in polyphenols. Attending to waste amounts, grapevine canes and grape pomace are the most relevant ones. The former matrix contains higher concentrations of viniferins and other resveratrol oligomers than the former one (Rayne et al., 2008; Guerrero et al., 2016; Wei, 2016); moreover, canes are easier to handle, to store and to preserve from microbiological contamination than grape pomace. Nowadays, pruned canes are usually burned at vineyards, a practice which introduces large amounts of carbon dioxide in the atmosphere, destroying a valuable sub-product.

The extraction of polar polyphenols from vegetal waste materials involves the use of organic solvents which must be compatible with the further uses of these bioactive compounds as food supplements, in food packing and/or in cosmetics. Most methods employ acetone, ethanol, and less often methanol (Rayne et al., 2008; Soural et al., 2015). Whatever the extraction technique, the use of purified solvents increases the cost of the polyphenols enriched extract. A further question to address during extraction of polyphenols from grapevine canes is to evaluate the potential transfer of unwanted chemicals (e.g. pesticides) from the wooden stuff to the extracts. Under conventional vineyards management practices, the use of systemic pesticides (mainly fungicides) is a globalized trend (Cabras and Angioni, 2000; Rodríguez-Cabo et al., 2016). These compounds are able to penetrate and to travel inside the wooden parts of vines; thus, they might persist in canes until pruning. Nonetheless, to the best of our knowledge, this topic has not

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been evaluated in previous studies reporting the extraction of polyphenols from vine canes.

The current study investigates, at laboratory scale, the feasibility of developing a green, sustainable method to recover two different classes of polyphenols (flavanols and stilbenoids) from grapevine canes using alcoholic distillates as alternative to purified solvents. Destillates are also obtained from wastes (fermented grape pomace and low quality wine) generated during wine elaboration. A circular economy extraction process, without external inputs to wine production, is therefore proposed. The extraction efficiencies for six polyphenolic species (catechin, epicatechin, *trans*-resveratrol, *trans*-piceatannol, *trans*-piceid and *trans*-ε-viniferin) obtained under different conditions (techniques and solvents) are quantified and compared. Concentrations of above compounds are determined by liquid chromatography (LC) with quadrupole time-of-flight (QTOF) mass spectrometry (MS). Relative extraction yields obtained for additional polyphenolic compounds, belonging to flavanol and stilbene classes and identified from their accurate MS and MS/MS spectra, are also provided. In addition, the total polyphenolic content of conventional extracts (corresponding to purified solvents) and those obtained using alcoholic distillates are compared. The presence of fungicides residues in the extracts from grapevine canes is investigated by LC-QTOF-MS, using previously reported determination conditions (Fontana et al., 2011).

2. Material and methods

2.1. Standards and solvents

Standards of *trans*-resveratrol, *trans*-piceatannol, *trans*-piceid, catechin, epicatechin and *trans*-ε-viniferin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of these polyphenols were prepared in methanol. Further dilutions were made in the same solvent. Standards of the following fungicides: cyprodinil, pyrimethanil, iprovalicarb, metalaxyl, benalaxyl, tebuconazole, azoxystrobin, dimethomorph and fenhexamid were also purchased from Sigma-Aldrich. The selection of fungicides was made taking into account their occurrence in wines and grape pomace produced in the same geographic area (Northwest Spain) where grapevine canes have been obtained (Rodríguez-Cabo et al., 2016; Celeiro et al., 2014).

Methanol, acetone and ethanol, trace analysis solvents, were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was also from Merck. Formic acid was provided by Sigma-Aldrich. Ultrapure water was obtained from a Milli-Q Gradient A-10 system (Millipore, Bedford, MA, USA).

2.2. Cane samples and alcoholic distillates

Grapevine canes were pruned during last week of January 2016. After reception, they were stored in the dark, at room temperature (19 ± 1 °C). Pooled mixtures of powdered (sieved to 2 mm) canes, corresponding to three different grape vine varieties (Mencía, Albariño and Grenache), were prepared when needed, within the next two months, and employed to investigate the effect of sample preparation conditions in the responses measured for different polyphenols. Additionally to the pooled samples used during method development, individual powdered samples, from six different *Vitis vinifera* varieties (36 canes from 12 different plants of each variety were sampled at vineyards to prevent variations between plants), were prepared after storing the pruned material for two months under identical conditions. Controlling the storage time is required to investigate changes in the concentrations of polyphenols among grapevine canes from different varieties. At least in the case of *trans*-resveratrol and *trans*-piceatannol, it is known that their concentrations change during the storage of pruned canes (Gorena et al., 2014; Houillé et al., 2015).

A sample of *Polygonum cuspidatum* root was purchased in a local herbalism. This plant is usually employed as a source of resveratrol in many food supplements (Wang et al., 2013).

Alcoholic distillates (obtained from grape pomace and commercialized as spirit beverages for human consumption, *grappa*) were purchased in local markets. A pooled solution of several spirits (ethanol content 42%, v:v) was used for extraction purposes as alternative to purified solvents.

2.3. Extraction procedures

Pressurized liquid extraction (PLE) assays were carried out using an ASE 200 instrument (Sunnyvale, CA, USA) equipped with 11 mL volume stainless cells. The amount of sample (powdered grapevine canes) was 1 g. It was mixed with 3 g of sand and loaded into the PLE cell containing 5 g of sand at the bottom. The empty volume in the cell was also filled with sand. Extractions were carried out at 100 °C, in 3 static cycles (5 min each), with extraction cells maintained at 1500 psi. The above conditions are similar to those reported for the extraction of *trans*-resveratrol from freeze-dried grape samples (Piñeiro et al., 2006). PLE extracts were collected in glass vials and made up to a final volume of 30 mL with the same solvent employed in the extraction step.

Soxhlet extractions were performed with 5 g of powdered grapevine cane samples and 100 mL of solvent. The total extraction time was 3 h, with a Soxhlet cycle every 4 min. The final extract was adjusted to 100 mL. Solid-liquid extractions were carried out overnight, using 1 g of sample and 25 mL of solvent, under magnetic stirring. Thereafter, the mixture was centrifuged and the extract made up to 25 mL.

Before LC-QTOF-MS determination of polyphenols, extracts were diluted 10 times using a 1:1 mixture of acetonitrile:water. Undiluted extracts were employed to investigate the presence of fungicide residues in grapevine canes.

2.4. LC-QTOF-MS determination conditions

Target polyphenols were quantified using a LC-ESI-QTOF-MS system acquired from Agilent Technologies (Wilmington, DE, USA). It consisted on an Agilent 1200 Series LC system, an autosampler and an oven for the LC column. The QTOF was an Agilent 6520 model, equipped with a Dual-Spray ESI source.

LC separations were developed in a Ultisil XB-C_{18} column (100 mm × 2.1 mm, 3 μm) provided by Welch Materials (Zhejiang, China). The column was connected to a C_{18} guard cartridge (4 mm x 2 mm) from Phenomenex (Torrance, CA, USA). Column and pre-column were maintained at 30 °C and operated at a constant flow of 0.2 mL min^{-1}. The mobile phases were ultrapure water (A) and acetonitrile (B) both 0.1% in formic acid. The composition of the mobile phase was programmed as follows: 0–1 min, 5% B; 24 min, 55% B; 25–28 min, 100% B; 29–38 min, 5% B. The injection volume was 5 μL.

The QTOF system was operated at 2 GHz and used in the single MS mode for quantification purposes. The pseudo-molecular ions ($[M-H]^-$) of selected polyphenols were extracted with a mass window of 20 ppm and the corresponding peak areas compared to those obtained for calibration standards prepared in acetonitrile: water (1:1). Under above conditions, the LC–MS system provided linear responses within the range of concentrations between 25 and 10,000 ng mL^{-1} for the six compounds quantified in this study.

The *Find by Molecular Feature* function, incorporated in the *Mass Hunter* software used to control the LC-QTOF-MS instrument, was used to detect the most intense features in the LC-ESI(−)-MS chromatograms of canes extracts. In a further injection, these compounds were submitted to cell-induced collision and their accurate product ion spectra obtained. Their identities are proposed from comparison of the experimental spectra with those existing in spectral databases (https://metlin.scripps.edu/) and/or with those previously published for wine extracts (Moss et al., 2013; Flamini et al., 2016).

Fungicide residues in the extracts from grapevine canes were also investigated by LC-QTOF-MS, operating the ESI source in the positive