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Analytical Methods

Preparative separation of grape skin polyphenols by high-speed counter-current chromatography

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ABSTRACT

To develop an efficient method for large preparation of various individual polyphenols from white grape skins (Fernão Pires; *Vitis vinifera*) by preparative high-speed counter-current chromatography (HSCCC) and preparative-HPLC, an optimized preparative HSCCC condition with two-phase solvent system composed of Hex-EtOAc-H_2O (1:50:50, v/v) was used to separate grape skin polyphenols into various fractions. Both the tail-head and head-tail elution modes were used with a flow rate of 3.0 ml/min and a rotary speed of 950 rpm. Afterwards, a preparative-HPLC separation was applied to isolate individual polyphenols in each of the fractions from HSCCC. Total of 7 fractions (Fraction A to G) were obtained from grape skin extract by HSCCC. After preparative-HPLC isolation, fifteen individual compounds were obtained, most of which presented high yields and purity (all over 90%). The HSCCC method followed with preparative-HPLC appeared to be convenient and economical, constituting an efficient strategy for the isolation of grape skin polyphenols.

1. Introduction

Plant polyphenols constitute one of the most numerous and widely distributed groups of natural products (Antoniolli, Fontana, Piccoli, & Bottini, 2015). From chemical point of view, polyphenols are characterized by a benzene ring with one or more hydroxyl group and can in general be classified into two main groups: non-flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, stilbenes and phenolic alcohols) and flavonoids (anthocyanins, flavanols, flavonols and dihydroflavonols) (Fanzone et al., 2012). Recently, there has been much interest in these phenolic compounds because of their wide range of bioactivities as antioxidants (Jara-Palacios, Hernanz, Escudero-Gilete, & Heredia, 2014), antimicrobials (Kemperman et al., 2013), *anti*-inflammatory agents (Mossalayi, Rambert, Renouf,

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Micouleau, & Mérillon, 2014), and *anti*-cancer drugs (Sun, Chen, Wu, Yao, & Sun, 2012).

Grapes and red wines are among the richest sources of phenolic compounds and polyphenols presented in grapes are essentially in their solid parts, i.e., seed, skin and stem (Sun, Ribes, Leandro, Belchior, & Spranger, 2006). As a consequence, grape pomaces, particularly those from white wine-making technology, provide a rich source of these compounds. Today, various polyphenols products can be found on the market but most of them are originated from grape seeds, i.e., OPCs (oligomeric procyanidins) and few of them from grape skins. Grape skins contain not only procyanidins, as grape seeds, but also other phenolic compounds such as flavonols, flavanonols, flavones, derivatives of cinnamic acids and tartaric acid (Cheynier, Moutounet, & Sarni-Manchado, 1998); for grape skins from red varieties, they contain also large amount of anthocyanins (Ribéreau-Gayon, 1982). Enological tannins from grape skins have in general better quality than those from grape seeds and grape stems, due essentially to their polyphenols composition (Vivas et al., 2004). From quantitative point of view, grape pomaces are mainly composed of grape skins and the number of polyphenols in grape skins is higher than in grape seeds. Because the disposal of grape skins is one of environmental problems during wine-making process, the recovery of phenolic compounds from these waste materials is of undoubtedly economical and social significance.

The selection of suitable and effective methods for large separation of bioactive phenolic compounds from plant tissues is always a critical problem in the scientific community. Traditional separation methods for polyphenols such as liquid-liquid extraction (Sun, Ferrão, & Spranger, 2003; Sun et al., 2006), thin-layer chromatography (Jesionek, Majer-Dziedzic, & Choma, 2015; Sun, Leandro, Ricardo-da-Silva, & Spranger, 1998), column chromatography (Lu & Foo, 1999; Sun, Belchior, Ricardo-da-Silva, & Spranger, 1999), semi-preparative and preparative HPLC (Makhotkina & Kilmartin, 2012; Sun, Belchior, et al., 1999) have disadvantages like time-consuming, secondary-pollution, complex-process, low-yield and high-cost. High-speed counter-current chromatography (HSCCC), a supporting free liquid-liquid partition system where solutes are partitioned between two immiscible phases, offers various advantages such as high solute loading capability, high recovery, high repeatability and low solvent consumption (Zhang et al., 2015). Furthermore, this technique has a great advantage over the traditional methods by eliminating the complications resulting from the solid support matrix, such as irreversible adsorptive sample loss and deactivation, tailing of solute peaks and contamination (Wang et al., 2015).

HSCCC is now recognized as an efficient preparative technique, and widely used for separation and purification of catechins (Wang et al., 2008; Xia, Hong, & Liu, 2014), oligomeric proanthocyanidins (Esatbeyoglu & Winterhalter, 2010) and other phenolic compounds from natural plants (Shu et al., 2014). Degenhardt, Hofmann, Knapp, and Winterhalter (2000) used firstly HSCCC technique for preparative isolation of anthocyanins from grape skins of red varieties.

In practice, grape skins can be largely obtained from pomaces of vinification, but only grape skins from white winemaking preserve nearly all their polyphenols while those from red winemaking are less interesting because they suffered maceration process during alcoholic fermentation and thus their important part of polyphenols are transferred into wine (Sun, Pinto, Leandro, Ricardo-Da-Silva, & Spranger, 1999).

The purpose of this work was to develop an efficient method using preparative HSCCC combined with preparative HPLC for large separation of the main polyphenols in grape skins from white winemaking pomace.

2. Experimental

2.1. Apparatus

The preparative HSCCC employed in study was a model TBE-300B HSCCC (Tauto Biotechnique Company, Shanghai, China). The apparatus with the maximum rotational speed of 1000 rpm was equipped with three polytetrafluoroethylene preparative coils (ID 1.9 mm, total volume is 300 ml) and a 20 ml sample loop. The HSCCC system consists of a TBP-5002 pump, a UV2000D detector (Shanghai Sanotac Scientific Instrument Co., Ltd., Shanghai, China) and a DC-0506 low constant temperature bath (Tauto Biotechnique Company, Shanghai, China) which was used to control the separation temperature. EasyChrom-1000 chromatography workstation (Shanghai Sanotac Scientific Instrument Co., Ltd., Shanghai, China) was employed to record the chromatograms.

2.2. Reagents and materials

Sufficient amount of grape skins were manually isolated from non-macerated white grape pomace (Fernão Pires; Vitis vinifera, cv.), air-dried at <40 °C dark for one day, sealed with N_2 and stored at −20 °C until used. Oligomer proanthocyanidin fraction (OPC) and polymer proanthocyanidin fraction (PPC) used for determination of *K* values of HSCCC were prepared from grape skins by column chromatography on Lichroprep RP-18 column according to Sun et al. (1998); both OPC and PPC presented high purity (92% and 93%, respectively) (Spranger, Sun, Mateus, Freitas, & Ricardo-da-Silva, 2008). (−)-Epicatechin was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). Phloroglucinol was purchased from Aladdin reagent (Shanghai, China). All organic solvents used for prep-HSCCC (analytical grade) and for prep-HPLC separation and HPLC/UPLC analysis (chromatographic grade) were purchased from Chemical Branch of Shandong Yuwang industrial Co., Ltd. (Shandong, China).

2.3. Preparation of crude grape skin phenolic extract

The skins isolated from the pomace will be first immersed in liquid nitrogen and then ground finely by a miller. Each powder obtained will be immediately used for the extraction of total phenolic compounds using the method described previously (Sun, Belchior, et al., 1999). Furthermore, a 200 g portion of the powder is extracted using 3.0 L of methanol-water (80/20; v/v) followed by 3.0 L of acetone-water (75/25; v/v). Each solvent extraction is performed by stirring for 3 h under a nitrogen atmosphere at room temperature. The combined supernatants will be evaporated at <30 °C to remove organic solvents, followed by extraction with hexane (3 × 600 ml) to eliminate fatty materials, and then filtered through a membrane filter (0.45 μm). The aqueous phenolic solution will be lyophilized. The powder thus obtained as crude grape skin phenolic extract (GSE), was stored at −20 °C until used.

2.4. Selection of the two-phase solvent systems

The following six solvent systems were selected based on the partition coefficient values (*K*) of (−)-epicatechin, OPC, PPC and GSE: (1) n-butanol: ethyl acetate: water (1:20:20); (2) methyl alcohol: ethyl acetate: water (1:25:25); (3) ethyl acetate: water (1:1); (4) n-hexane: ethyl acetate: water (1:20:20); (5) n-hexane: ethyl acetate: water (1:50:50); (6) n-hexane: ethyl acetate: methyl alcohol: water (1:50:1:50). Each solvent mixture was thoroughly equilibrated in a separator funnel at room temperature and the two phases were separated shortly before use.