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Development of hybrid elution systems for efficient purification of stilbenoids using centrifugal partition chromatography coupled to mass spectrometry

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ABSTRACT

The phytochemical study of the root extract of the stilbenoid-rich *Vitis riparia* × *Vitis berlandieri* grapevine was carried out by centrifugal partition chromatography (CPC). For this reason, we developed a new elution mode we named back-step, which allowed us to obtain cleaner fractions and a more efficient separation process when used in conjunction with a classical elution approach. Three hydroxystilbenes: (*E*)-resveratrol, (*E*)-ε-viniferin and (*E*)-vitisin C, with greater than 90% purity were thus obtained through such process, with minimal sample handling and purification steps. Online coupling of CPC to ESI mass spectrometry was used for optimization of the separation parameters and to facilitate the characterization of the stilbenoids. This study details the first phytochemical investigation of stilbenoids from the hybrid species together with a new elution mode able to widen the range of ARIZONA biphasic systems.

1. Introduction

Stilbenoids are a group of secondary plant metabolites [1] that have exhibited a number of promising biological activities [2]. Among these, (*E*)-resveratrol, is a widely studied phytoalexin which is biosynthesized in the grape berries and leaves (*Vitis vinifera* L., Vitaceae) in response to fungal infection and other stresses [3,4]. This compound has been proposed to be one of the components in red wine responsible for its health promoting activities [5], including cancer prevention [6], cardiovascular protection [7] and neuroprotection [8]. In addition to resveratrol, recent studies have demonstrated promising *in vitro* biological activities of additional stilbenes found in wine, including, piceid [9], astringin [10] and (*E*)-ε-viniferin [11].

Stilbenoids constitute a group of non-flavonoid polyphenols, which are characterized by two aromatic rings joined by an ethylene bridge (C_6–C_2–C_6). From this relatively simple structure, over a thousand stilbenoid compounds have been characterized, resulting from different chemical substitutions patterns such as methylation, glycosylation or isoprenylation, in addition to oxidative condensations of monomers into dimers and subsequent condensations [12].

In order to undertake further biological evaluation of these promising stilbenoids, large amounts of compounds are required. However, plants extracts are complex biological matrices, and separation of individual compounds is challenging. The currently, existing techniques, such as preparative HPLC, are not ideal for large-scale purification of these compounds due to the volume of solvents needed, time required for multiple purification steps and irreversible adsorptions onto the solid phase material. For these reasons we decided to investigate alternative methods for the purification of these biologically interesting compounds. Centrifugal partition chromatography (CPC) has demonstrated to be a promising tool for this purpose [13]. In particular, isocratic elution CPC has been successfully applied for the purification of phenolic compounds from numerous natural sources [14]. In this method, the determination of the optimal system for one compound, or small class of compounds, requires the determination of the partition coefficient in different solvent systems, with the use of the shake-flask method with HPLC–DAD peak area integration for example [15]. This focus on one compound of interest often leads to co-elution of other compounds that render the separation less effective for these additional compounds. We encountered this problem in applying a traditional CPC elution mode to the separation of stilbenoids with different chemical substituents and different degrees of polymerisation.

In order to efficiently and more precisely monitor the separation of multiple compounds of interest, we coupled semi-preparative CPC to an ESI ion-trap spectrometer [16,17]. This coupled system greatly enhanced the accuracy for monitoring the separation of multiple compounds while optimizing and comparing different solvent systems. This method was used to develop a "hybrid elution

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system", which allowed us to purify compounds more efficiently than with traditional CPC elution modes alone.

The primary goal of this work was to develop an effective method for obtaining pure stilbenoids for further biological and chemical analyses. For this purpose, we chose to investigate the roots of the hybrid *V. riparia* Michx. × *V. berlandieri* Planch. This hybrid species is used in Bordeaux and other wine growing regions as phylloxera resistant rootstock onto which *V. vinifera*, the traditionally used wine grape, is grafted. To the best of our knowledge, no phytochemical evaluation of this economically important plant has been previously reported. Furthermore, stilbenoids are constitutively expressed in roots and have demonstrated to be a good source of diverse stilbenoids [18]. This article will detail the monitoring of multiple ARIZONA systems [19] and the development of a new elution mode we termed "back-step", in order to obtain an effective and efficient separation of the major stilbenes from a *Vitis* root extract.

2. Experimental

2.1. Chemicals and reagents

All organic solvents used for CPC purification were HPLC grade except for the *n*-heptane which was synthesis grade. Ethyl acetate (EtOAc) and *n*-heptane were purchased from Scharlau (Barcelona, Spain) and the methanol (MeOH) from Carlo Erba (Rodano, Italy). Water for the extraction was bi-distilled and acetone was furnished by Xilab (Bruges, France). The extra pure grade methyl *tert*-butyl ether (MTBE), synthesis grade petroleum ether and LC–MS grade acetonitrile were purchased from Scharlau (Barcelona, Spain). H_2O for HPLC–MS analyses was purified using Elga (Bucks, UK) water purification system with a resistivity of not less than 18 MΩ cm^{-1}. HPLC–MS solvents and CPC–MS auxiliary ethanol were acidified with 0.1% formic acid purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC grade ethanol for CPC–MS experiments was purchased from VWR (Fontenay-sous-Bois, France). NMR experiments were performed in acetone-*d6* purchased from Euriso-top (Gif-sur-Yvette, France).

2.2. Plant material

The roots of 32 year-old *V. riparia* × *V. berlandieri SO4* (Oppenheim selection no. 4) hybrid species were collected at Château Dubraud, Première Côtes de Blaye appelation, in the Bordeaux region of France in January 2008. The preliminary screening of this plant material demonstrated a high content of (*E*)-vitisin C.

2.3. Extraction of stilbenes

Roots of *V. riparia-berlandieri SO4* rootstock (500 g) were dried in a 40 °C oven for 1 week, finely ground and kept at −20 °C in airtight and light-proof containers. This powder was further extracted two times with 2 L of a mixture of acetone/water (6:4, v:v) under agitation at room temperature for 4 h. After filtration, the aqueous acetone solution was concentrated at 35 °C under reduced pressure. The residual aqueous phase (800 mL) was successively partitioned with petroleum ether (3× 800 mL) and MTBE (6× 800 mL). The MTBE partition was concentrated *in vacuo* at 35 °C, redissolved with a little amount of methanol in water and freeze-dried to afford 22.5 g (4.5%, w/w). Enhancement of stilbene content was done by adsorption onto Amberlite XAD-7 (Sigma–Aldrich, St-Louis, MO, USA), which was washed with water to allow removal of sugars, followed by elution with acetone. This provided a semipurified extract which was then freezed-dried and used for CPC experiments.

2.4. CPC apparatus

The 200 mL CPC used in this experiment, FCPC200®, was provided by Kromaton Technologies (Sainte-Gemmes-sur-Loire, France). The solvents were pumped by a Gilson 321-H1 2-way binary high-pressure gradient pump. The samples were introduced into the CPC column via a high pressure injection valve (3725(i)038 Rheodyne) equipped with a 20 mL sample loop. The effluent was monitored with the aide of our CPC–MS apparatus described below. Fractions were collected directly into 50 mL and 250 mL Schott (St. Gallen, Switzerland) glass bottles according to the extracted ion chromatograms. The experiments were conducted at a regulated temperature of 23 °C.

2.5. HPLC–MS

The chromatography apparatus, Agilent 1200 from Agilent Technologies (Santa Clara, CA, USA), is composed of an autosampler module, a degasser, a binary pump, a column heater/selector and an UV–Visible-DAD from the same provider. The column was a Prontosil C_{18} 250 mm × 4.0 mm, 5 μm, Bischoff (Leonberg, Germany). Fractions and library compounds were eluted at 1 mL/min with a gradient of water-0.1% formic acid (solvent A) and acetonitrile-0.1% formic acid (solvent B) according to the following gradient program (v/v): 0 min 17% B linear, 5 min 17% B, 25 min 30% B, 35 min 38% B, 45 min 100% B linear for 10 min, followed by 10 min for reequilibration. This HPLC was coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker-Daltonics (Billerica, MA, USA). The HPLC output flow of 1 mL/min was split with a passive splitter with an average 1:100 ratio depending on the flow solvent viscosity and rate. Drying gas was set at 5.0 L/min and 320 °C, nebulizer pressure was set to 15 psi. ESI–MS parameters (positive mode): HV capillary 4000 V, end plate offset −500 V, capillary exit 139 V, skimmer 40 V, trap drive 63.7, scan delay 25,000 μs, rolling average 2 and trap averages 5.

2.6. Identification of compounds by HPLC–MS using a compound library

A reference library was developed with compounds previously isolated, purified and identified in our laboratory [13]. ESI–MS parameters were optimized for efficient detection of a large class of hydroxystilbenoids using these compounds. The retention times, ESI–MS in positive mode with parent and fragmentation ions (MS/MS with an isolation width of 2.0 *m*/*z*, 1 V amplitude and 3 spectrum average) of each of these compounds were included in a Bruker LibrarySearch™ database.

2.7. Solvent system selection

2.7.1. Partition coefficient determination using shake-flask method and HPLC–UV

To determine the best suited ARIZONA system, partition coefficient (K_d) of each compound was determined using the shake-flask method [15]. The two-phase solvents systems were prepared separately in 2 mL HPLC glass vials with PTFE-lined stoppers. 1 mL of each phase (upper and lower) of a system was put into vials containing 1 mg of a mixture composed of equal amounts of the following standards: (*E*)-ε-viniferin (1), (*E*)-piceatannol (2), resveratrol trimer mixture (3) and (*E*)-vitisin C (4). The resveratrol trimer mixture is a fraction containing trimers with close partition coefficient that are co-eluting in the systems used in this study. Vials were shaken and allowed to settle for 5 min. A 0.5 mL aliquot of each phase was put into separate vials, which were then dried and reconstituted with 1 mL of acetonitrile:H_2O (1:1) for HPLC–DAD analysis. The K_d values in the biphasic systems were then determined by