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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

Table 1
Values of K_d determined by HPLC–DAD.

	(*E*)-ε-viniferin (1)	(*E*)-piceatannol (2)	Mix of trimers (3)	(*E*)-vitisin C (4)
ARIZONA-K (descending) Upper [1.5/58.8/36.2/3.5] Lower [60.8/11.3/0.15/27.8]	3.33 (2.70)	1.15 (1.18)	1.28 (1.47)	2.94 (2.78)
ARIZONA-L (ascending) Upper [0.9/49.9/46.1/3.0] Lower [52.8/14.1/0.03/33.1]	2.27 (1.52)	3.70 (2.86)	6.67 (4.55)	5.26 (4.00)

Between parentheses are the K_d values determined experimentally on a CPC chromatogram. Percent composition of the system phases are given in the format [H_2O/EtOAc/Heptane/MeOH].

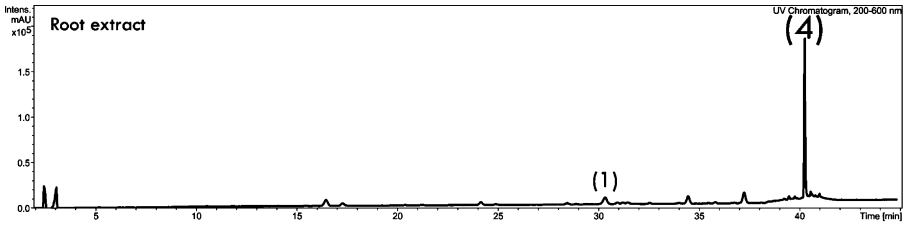

Fig. 1. HPLC–DAD chromatogram (200–600 nm) of the root extract.

the ratio of the compound concentration in each phase. These values were calculated using the HPLC-UV integration at 280 nm of the chromatographic peaks with the same retention time in each phase, avoiding the need of calibration curves for each compound.

2.7.2. Two-phase solvent system preparation and analysis

For the CPC experiments, the ARIZONA-K and L solvent systems were individually prepared according to the data shown in Table 1 which were published by Berthod and Carda-Broch [15]. Validity of our phase compositions was assessed by gas phase chromatography and is described in the Supplementary material.

2.7.3. Partition coefficient determination using CPC chromatogram

In order to facilitate the selection between different solvent systems, a simulation was done with the ScicosLab 4.4b7 free software, detailed in Supplementary data, using the shake-flask determined K_d. The results obtained from this simulation are shown in Figs. 2 and 3.

Due to differences observed between the preliminary CPC trials and the simulated elution results using the shake-flask determined partition coefficients, we determined the experimental partition coefficient from the CPC experiments using the following equation:

$$K_d = \left(\frac{t_{peak} * flowrate - V_{stat}}{V_{mobile}} \right)$$

where t_{peak} is the compound elution time (corrected by the injection time if not zero). V_{mobile} is the volume of mobile phase in the column determined by the time at which the first drop of mobile phase is eluted. V_{stat} is the column stationary phase volume, corresponding to $V_{column} - V_{mobile}$.

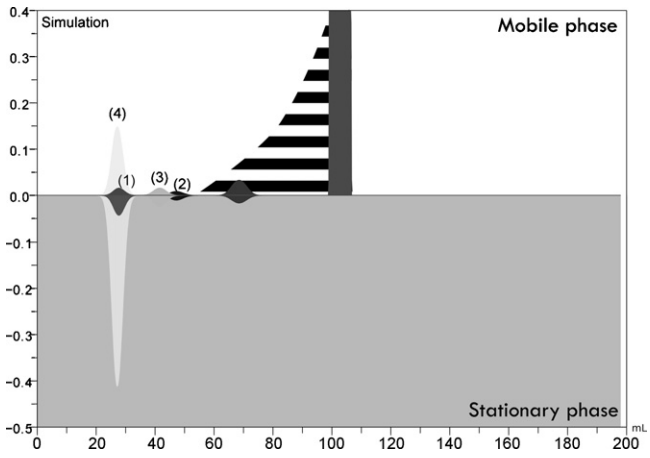

Fig. 2. Simulation of the column content using HPLC-UV determined K_d of some stilbenoids of the extract for ARIZONA-K solvent system in descending mode. The highly polar compound area is traced over the graph with horizontal stripping.

2.8. CPC/ESI–MS hyphenation

Performing CPC experiments and then analyzing the collected fractions is a time consuming process. To shorten experimentation times, the output of the CPC was coupled to a mass-spectrometer. Since the high flow-rate of the CPC output (4–10 mL/min) is highly concentrated (mg/mL scale) and that ESI mass-spectrometers require a highly diluted low flow-rate (μL/min) input, an active-splitter was used. This allowed a small amount of the CPC output to be mixed with the HPLC–MS solvent flow. Solvent addition to the ActiveSplitter-MS chain is crucial for an effective phase mixing and MS detection. For this purpose, ethanol with 0.1% formic acid was used at a flow-rate of 1 mL/min. This ActiveSplitter-MS system configuration allowed the use of the splitting power of both the active and the passive splitter, thus achieving a 50,000:1 split ratio in terms of compound concentration to the mass spectrometer (10 μL/min of a 500 times diluted flow, achieved by the active splitter). The active-splitter, a Rheodyne MRA100-000, was provided by Kromaton technologies (Sainte-Gemmes-sur-Loire, France). It was set at a 500:1 splitting ratio (setting 6 for CPC flow-rate of 4 mL/min) to provide the appropriate analytical quantity from the CPC flow into the DAD-MS analytical flow.

ESI Source parameters: drying gas was set at a flow-rate of 9.0 L/min and a temperature of 320 °C, nebulizer pressure was set to 35 psi. ESI–MS parameters in negative mode: HV capillary 4300 V, end plate offset −500 V, capillary exit −176.4 V, skimmer −29.5 V, trap drive 75, scan delay 25,000 μs, rolling average 10, trap averages 10.

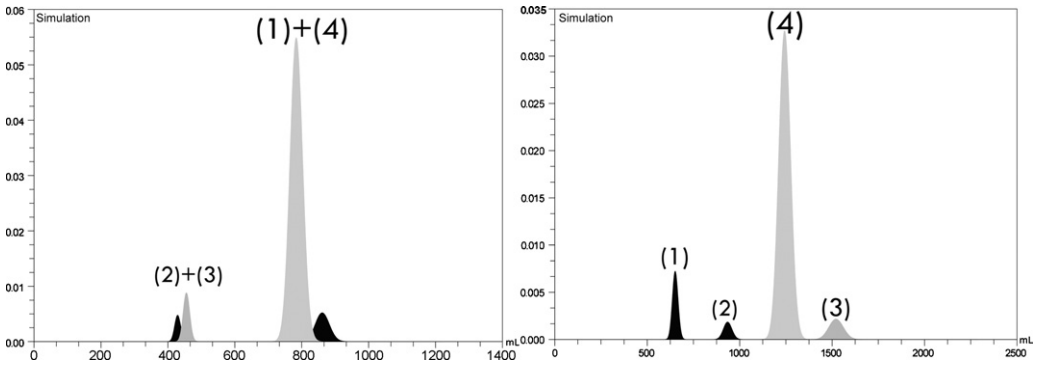

Fig. 3. Left: simulation of ARIZONA-K in descending mode with shake-flask determined K_d. Right: simulation of ARIZONA-L in ascending mode with shake-flask determined K_d.

Extracted ion and DAD chromatograms (200–600 nm and 280 nm) were obtained with Bruker Data Analysis 3.2 software and further treated with Inkscape 0.48 and GNU Image Manipulation Program (Gimp 2.7.2 Nightly). Chromatograms were not smoothed or filtered for the figures.

2.9. CPC/ESI–MS procedures

2.9.1. General procedure for CPC experiments

For all CPC experiments, the column was filled at a flow-rate of 10 mL/min with 600 mL of the stationary phase in the specified experiment elution mode. Equilibration with mobile phase was done at 1300 rpm with a 4 mL/min flow-rate. Changes to other mobile phases, were made using the purged first solvent pump line. For each experiment, the samples were injected in 10 mL of a 50:50 (v:v) stationary and mobile phase mixture. Washing was executed with 600 mL of 1:1 (v:v) acetone and H_2O at 10 mL/min.

2.9.2. Descending mode back-step ARIZONA-K, water, ARIZONA-K

Stepwise and gradient elution modes in CPC are typical transitions from one mobile phase to another phase with an increase in elution strength [20,21]. The innovation in this study is that we used gradients or steps going from higher to lower elution strength. Therefore, we call these elution modes "back-steps" or "back-gradients". The most successful experiment of this kind was an ARIZONA-K experiment with a water back-step performed using the following protocol: after an elution corresponding to $V_{mobile}/2$, a switch to water equilibrated with stationary phase (1:1 volume ratio with the ARIZONA-K upper phase) was made. After another elution of $V_{mobile}/2$, the mobile phase was switched back to the ARIZONA-K lower phase.

3. Results and discussion

The main purpose of this study was to find an effective and efficient way to obtain pure stilbenoids from a crude stilbenoid-rich extract (HPLC–DAD chromatogram in Fig. 1) with minimal sample manipulation since stilbenoids have demonstrated to be unstable. To achieve this goal we evaluated several ARIZONA systems and optimized the removal of unwanted molecules from the compounds of interest by adding a water back-step.

3.1. Visualizations of back-step conditions for the removal of unwanted molecules

Fig. 2 represents the simulated column content at a time corresponding to an elution of $V_{mobile}/2$ using the K_d values of pure stilbenoids determined via HPLC-UV for ARIZONA-K in the descending mode. The area in grey represents the stationary phase while the mobile phase is in white. The superimposed horizontal striped area is representative of the area occupied by the first elution peak. Fig. 3 Fig. displays the simulations of elution using the HPLC-UV determined K_d of compounds for ARIZONA-K in descending mode and ARIZONA-L in ascending mode.

3.2. Comparison of K_d determined by shake-flask method and experimental CPC experiment data

The K_d values obtained via the shake-flask method described above, when compared to the measurements from the CPC chromatograms, are substantially different. One explanation for these differences may be due to the interactions between compounds during co-elution. In the case of the ARIZONA-L experiment, the faster elution and subsequent co-elution were assumed to be related to the lower phase retention as well. The K_d values of compounds obtained from the different systems and by using the two different methods are given in Table 1.

3.3. Solvent systems stabilities and capacities

According to the simulation results, ARIZONA-L was the only ARIZONA system that afforded the separation of each of the major compounds. However, the predicted results were not in agreement with the experimental results. This may have been caused by the substantial loss of stationary phase during the experiment together with interactions between compounds. When more than 500 mg of sample was injected, we observed a complete loss of stationary phase, most likely due to a destabilization of the phase equilibrium by the extract (stable emulsion, gelification, etc.). Injection before the column equilibration, sandwich mode, did not provide any improvement over the phase retention. Modifications to relative phase proportions and reduction of injection volume did not provide any significant improvement. Moreover, when injecting 300 mg with ARIZONA-L in ascending mode (V_{mobile} = 80 mL), compound 1 co-eluted with 2 and compound 4 with 3. Therefore, we found that the ARIZONA-L system was not able to provide any benefit over other systems. We then tried using the ARIZONA-K system, which supported 1 g injection without significant separation quality deterioration (V_{mobile} = 73 mL). The ARIZONA-K experiment showed equivalent separation capabilities as ARIZONA-L, in that the compounds also co-eluted by pairs, but the capacity of injection and stability of this system was greater.

Other variations of different steps and gradients were tested, including an ARIZONA-L ascending mode with an ARIZONA-M back-gradient, an ascending ARIZONA-L with an ARIZONA-K forward-gradient. These systems did not provide any significant improvement over the traditional ARIZONA-L mode and are presented in the supplementary data.

3.4. Application for stilbenoids purification

3.4.1. Purification of the MTBE partition using ARIZONA-K with a water back-step

The ARIZONA-K was effective for the initial step of separation in descending mode, however unwanted low K_d molecules overlapped several compounds of interest in the beginning of the run. In order to remove these unwanted compounds, we used a lower elution strength mobile phase during the experiment, allowing the elution of these compounds without the compounds of interest. To achieve this we initiated the separation of the compounds in the column by pumping a $V_{mobile}/2$ volume of ARIZONA-K mobile phase. As shown in Fig. 2, the unwanted compounds were initially eluted to near the middle of the column. Using a water back-step, we were able to remove the unwanted compounds from the column, without removing our compounds of interest. After a $V_{mobile}/2$ elution with water, we stepped back to the ARIZONA-K mobile phase in order to separate the stilbenoids. The appeal of water as a mobile phase is due to its high polarity, yet relatively low interaction with the compounds of interest, this mobile phase is unable to elute them, since this allowed for a separation comparable to ARIZONA-K, without the interfering compounds, as shown in Fig. 4. Results of ARIZONA-K with and without the back-step are compared in Fig. 4, showing the DAD at 200–600 nm chromatogram and extracted ion chromatograms of the corresponding mass of the compounds of interest. Major differences between the isocratic and the back-step experiments include a more concentrated F2 fraction with the back-step experiment and the F1 fraction was more pure compared to F3 as shown in Fig. 5. The ARIZONA-

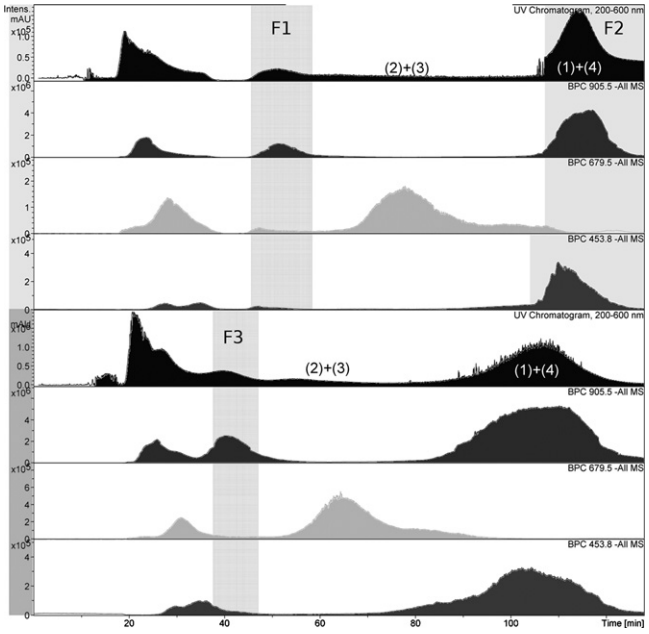

Fig. 4. Experimental CPC-DAD and extracted ions chromatograms showing the results of the water back-step. Series are delimited by grey blocks in the ordinate axis. Upper: ARIZONA-K with water backstep (300 mg injected). Lower: ARIZONA-K (500 mg). F1 and F3 are the fractions containing (*E*)-Vitisin A. F2 is the fraction containing compounds 1 and 4.

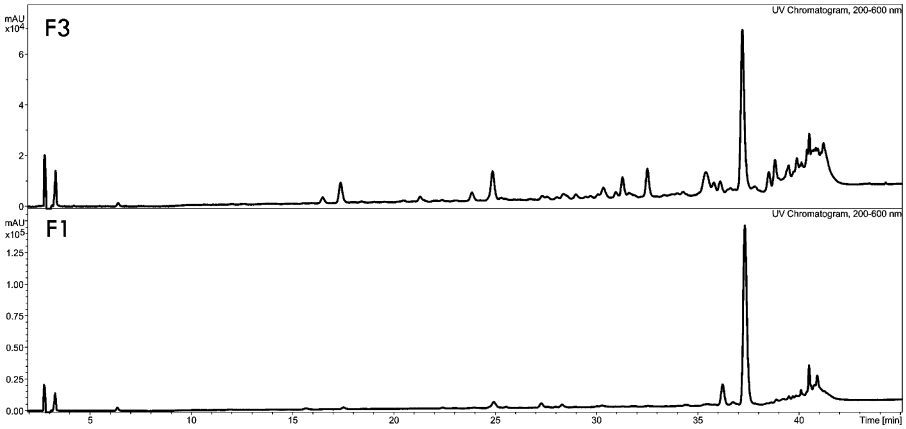

Fig. 5. HPLC–DAD chromatograms (200–600 nm) of vitisin A fraction F3 from the ARIZONA-K solvent system in descending mode and vitisin A fraction F1 from the ARIZONA-K system in descending mode with a water back-step.

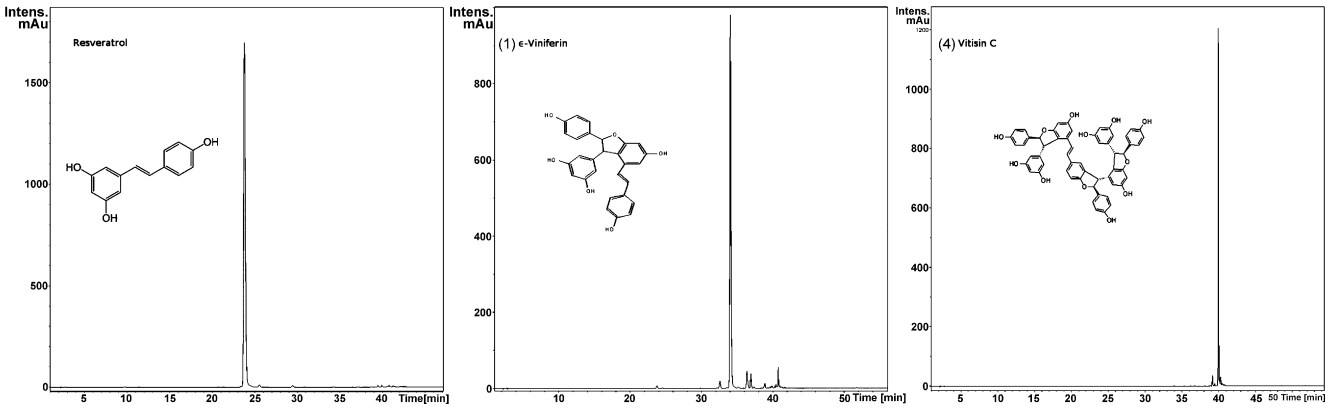

Fig. 6. HPLC–DAD (200–600 nm) of compounds 1, 4 and trans-resveratrol obtained during the second step of the extract separation using ARIZONA-L.

K system with the back-step part afforded a fraction F0 (470 mg) of low K_d compounds in addition to a greatly enriched (*E*)-vitisin A fraction, F1 (14 mg), immediately afterwards. The isocratic part of the experiment afforded the F2 fraction (not weighed) containing compounds 1 and 4 a fraction (42 mg) that contains 2 and 3 compounds.

3.4.2. Purification of the F2 fraction

The F2 fraction was then separated using the ARIZONA-L system in ascending mode. This gave us a V_{mobile} = 80 mL, and a significant loss of stationary phase during the experiment. However, 3 compounds (*E*)-resveratrol (20 mg), (*E*)-ε-viniferin (1, 39 mg) and (*E*)-vitisin C (4, 255 mg), with a ≥90% purity assessed by NMR and HPLC–DAD, were obtained (Fig. 6).

4. Conclusions

This study shows that using back-step and back-gradient experiments may be an efficient way to optimize difficult separations using CPC when traditional ARIZONA isocratic systems have shown stability or separation ability insufficiencies. While a change to a forward-gradient did not manage to bring any sufficient improvement upon the separation, the water back-step has shown promising and effective enhancements. Furthermore, we have demonstrated that the mobile phase change of the water back-step and different gradients of ARIZONA mobile phases did not necessarily alter the stability of the systems, as no significant stationary phase loss was observed within our experiments. Using a mobile phase with a lower elution force for a back-step experiment, we were able to elute the less retained compounds without any overlap of our compounds of interest. Starting with a stronger solvent elution strength allowed the compounds of interest to separate from the earlier eluting compounds. This reduced the viscosity of the sample zone in addition to co-elution phenomena and allowed the unretained compounds to elute faster which reduced the experiment time.

The CPC–MS hyphenation technique used in these experiments provided a fast and efficient method to check the different elution programs without having to individually treat and analyze every fraction obtained. Using one back-step CPC experiment in conjunction with a classical experiment, we were able to obtain 3 compounds with a ≥90% purity, (*E*)-resveratrol, (*E*)-ε-viniferin (1) and (*E*)-vitisin C (4), along with a greatly enriched (*E*)-vitisin A fraction. We were able to obtain compounds of a higher purity than with previous methods in just two step [13], in addition to several highly enriched fractions available for further work.

Capabilities of mobile phase changes during ARIZONA experiments have been shown in this study, and were successfully applied for the first phytochemical study of this plant material. However, methods of execution and analysis of the stationary phase composition changes, especially with gradients between mobile phases, would be needed to improve the effectiveness and versatility of this technique. Similarly, further work on the simulation program, such as inclusion of a gradient and step feature, in combination with molecular modelling [22] would provide more ease when working with such elution modes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.03.020.

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