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Preparative isolation of polyphenolic compounds from *Vitis vinifera* by centrifugal partition chromatography

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Abstract

This study deals with a centrifugal partition chromatography developed for the separation of phenolic compounds from *Vitis vinifera*. EtOAc grape seed extracts were separated using the solvent system hexane–ethyl acetate–ethanol–water (1:8:2:7; v/v) in two fractions: one containing about 75% of flavanol monomers (catechin and epicatechin) corresponding to 18% of crude extract and another fraction B-type dimers (22% of crude extract). From the stalk extracts, we could separate stilbenoid compounds (resveratrol and its oligomers; 12% of crude extract) which were eluted in less than 30 min from flavanols (which required a few hours of additional elution). Using the same solvent system but in different ratios (4:5:3:3; v/v), we isolated the *trans*-resveratrol (7‰; 90% purity).

Keywords: *Vitis vinifera*; Centrifugal partition chromatography; Preparative chromatography; Polyphenols; Flavonoids; Stilbenoids

1. Introduction

Polyphenols are supposed to exert a beneficial effect on human health and particularly against heart disease [1]. Various studies conducted in vitro on crude extracts of polyphenols showed important biological properties such as: anti-platelet aggregation [2,3], endothelium-dependent vasorelaxation activities [4], inhibition of the low density lipoproteins (LDL) oxidation [5,6], anticarcinogenic properties [7,8].

To continue and improve the current in vitro and to allow the in vivo tests, it is necessary to dispose of large amounts of pure polyphenolic compounds found in grapevine and particularly in grape seed, stalk and stem extracts.

Reversed-phase preparative HPLC, usually used in such a case, did not give the expected results. We observe important sample loss and deterioration of the column with such extracts [9], more than 50% material weight sticks strongly onto the solid support and is never eluted [10]. To circumvent this difficulty, we developed a centrifugal partition chromatography (CPC) method [11], which allowed us to obtain fractions differing by the polyphenolic content and/or the degree of polymerisation in the same family of polyphenols.

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2. Experimental

2.1. Apparatus

The experiments were performed using a centrifugal partition chromatograph, model CPC-LLB-M, from Sanki Engineering Ltd., Kyoto, Japan. The column is a circular partition disk with 2136 channels. The total column capacity is around 215 ml. A 4-way mode switching valve allows to operate in either the ascending or the descending mode. The CPC system was connected to a Bischoff HPLC pump model 2250 (manufactured by Bischoff, Leonberg, Germany) and to a UV–Vis detector from Waters, model Lambda–Max 480 LC spectrophotometer (Waters, Milford, MA, USA). Fractions were collected with a fraction collector model LKB 2111 MultiRac (LKB Instruments, Bromma, Sweden). The sample was injected manually using a Rheodyne valve model 7125 (I.C.S., Belin-Beliet, France) through a 10-ml loop.

2.2. Reagents

All organic solvents were of analytical grade quality and purchased from Merck (Darmstadt, Germany). Water was bi-distilled.

2.3. Preparation of CPC solvents

With CPC, the ternary (or quaternary) systems are usually used and the choice of solvents is made to avoid chlorinated solvents and is the result of sample solubility, and polarity range of compounds to be separated, which can be evaluated from the solvents used for extraction from raw material (ethyl acetate). Taking into account these parameters and the literature survey [12–14], the ternary solvent system ethyl acetate–ethanol–water was selected. To assess our solvent system and to find the appropriate volume ratio of solvents, we used TLC [14] and/or HPLC methods [15]. After numerous series of experiments, the solvent system EtOAc–EtOH–water (8:2:7; v/v) was first chosen. Then, in order to reduce the elution time of weakly polar components such as resveratrol we have increased the hydrophobicity of the mobile phase (organic) by addition of hexane. The quaternary mixture (Q1 mixture) hexane–ethyl acetate–ethanol–water (1:8:2:7; v/v) was used in most cases. Another ratio (Q2 mixture) hexane–ethyl acetate–ethanol–water (4:5:3:3; v/v) was used in one case. CPC solvent systems were prepared by mixing the solvents at room temperature. The resulting two phases were separated just before use. In the standard preparative experiments, 2 g of the crude extract were dissolved in 6 ml of the stationary phase prior to injection.

2.4. Preparation of the crude phenolic extracts

A 100.0 g dry mass of *Vitis vinifera* grape seeds was extracted with 1 l of water–acetone (3:2, v/v) at room temperature by lixiviation in an open column after a maceration step for 12 h. The extract was concentrated at 40 °C under reduced pressure, and then the residual aqueous phase (300 ml) was washed with ethyl acetate (three times 300 ml). The extraction of dried and finely powdered stalks of *Vitis vinifera* (100.0 g) was carried out in the same fashion, but after evaporation of acetone, the residual aqueous phase (300 ml) was submitted to light petroleum (boiling range 40–65 °C) for a purification step (three times 300 ml), and then extracted with ethyl acetate (three times 300 ml). The EtOAc solutions were concentrated under vacuum at 45 °C and redissolved in water to be freeze dried to yield the ''crude phenolic extract'' in 0.8% from grape seeds and in 1.3% from stalks.

2.5. CPC separation procedure

In the CPC, either the heavier phase (lower phase) or the lighter phase (upper phase) of the biphasic solvent system can be used as the mobile phase. In our experimental conditions, the stationary phase was the lower aqueous phase (ascending mode). The column was first filled with the aqueous stationary phase without rotation. Then, the rotation was brought to 1000 rev./min. The mobile phase was pumped into the column in ascending mode at a flow-rate used for the separation (3 ml/min or 4 ml/min). When the mobile phase emerged from the column, the hydrodynamic equilibrium was reached, the sample dissolved in the stationary phase was injected through the injection valve. The continuous

UV detection of the effluent from the outlet of the column was performed at 280 nm.

2.6. HPLC analysis

The mobile phase was composed of two solvents: A (water with 0.0025% (v/v) TFA) and B (MeOH with 0.0025% (v/v) TFA). Each sample was dissolved in MeOH. All samples were filtered through a 0.45 μm Millipore filter. Analysis was performed with a module system using two Bischoff HPLC analytical model 2250 pumps controlled by computer. Column and guard column were purchased from Bischoff Chromatography. Analysis was performed with an Ultrasep ES 100RP18 column (250 mm×4.0 mm I.D., 6 μm particle size) equipped with a Lichrosorb RP18 precolumn. The linear gradient was 100% solvent A to 100% solvent B in 60 or 100 min. The flow-rate was 1 ml/min. The eluent was monitored at 280 nm by a UV–Vis Spectra-Physics detector model SP8450 (Spectra-Physics, San Jose, CA, USA). Purity of fractions in each characterized component is estimated from the integration of these chromatograms (% of the total area).

2.7. TLC

All the fractions were monitored by thin-layer chromatography (TLC) on an aluminium sheet coated by silica gel $60F_{254}$ (Merck, Darmstadt, Germany) using $CHCl_3$–MeOH–AcOH (85:15:3; v/v) as mobile phase.

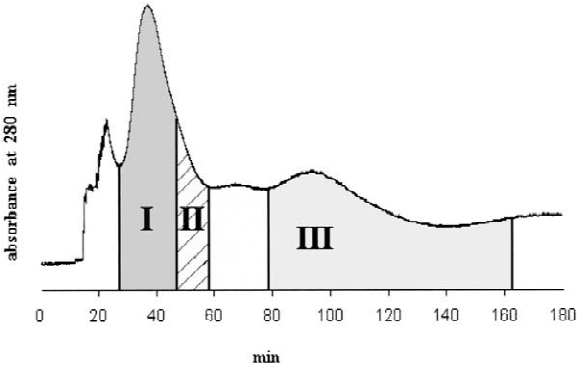

Fig. 1. CPC separation of grape seed procyanidins. Experimental conditions: rotation speed, 1000 rev./min; solvent system, hexane–ethyl acetate–ethanol–water (1:8:2:7, v/v); mobile phase, upper organic phase; flow-rate, 4 ml/min; retention of the stationary phase, 80%; backpressure, 35 bar.

3. Results and discussion

3.1. Separation of grape seed procyanidins

The preparative CPC separation of the grape seed extracts, performed in the Q1 mixture, led to three fractions (Fig. 1). Fraction I contained monomers (75%) catechin and epicatechin; in fraction II we have identified gallic acid and fraction III contains procyanidin dimers (42%) B1, B2, B3, and B4. Fig. 2 shows the HPLC chromatogram of fractions I and III. From 1 g of crude extract, we got 180 mg of monomers I and 220 mg of the dimer fraction III.

3.2. Separation of vine stalk extracts

The preparative CPC separation of the extracts obtained from Merlot stalks was performed in the Q1 mixture. The flow-rate was 3 ml/min. The chromatogram (Fig. 3) shows the four resulting main fractions (A–D).

Fraction A was eluted within 30 min and contains *trans*-resveratrol and its oligomers (55%). Fraction B contains 30% astilbin (dihydroquercetin 3-*O*-rhamnoside) whose interest results from studies with mice showing it could protect liver from damage [16–18]. In fraction C, we found catechin as the main component (about 35%) and fraction D contained all the remaining polar unidentified compounds (40% of crude extract mass).

Fig. 4 displays the HPLC chromatogram of fraction A. The three first major peaks in the order of increasing retention time could be identified, respectively, to pallidol, piceatannol and *trans*-resveratrol. The fourth one revealed to be a mixture of compounds whose structures are not thoroughly identified. This fraction was concentrated under vacuum and the residue was re-injected in ascending mode on the CPC column in the same conditions as above but with Q2 mixture. The CPC chromatogram is shown in Fig. 5. Fraction 3 contained 90% pure resveratrol which is supposed to possess important biological properties: recent studies have shown cardiopro-

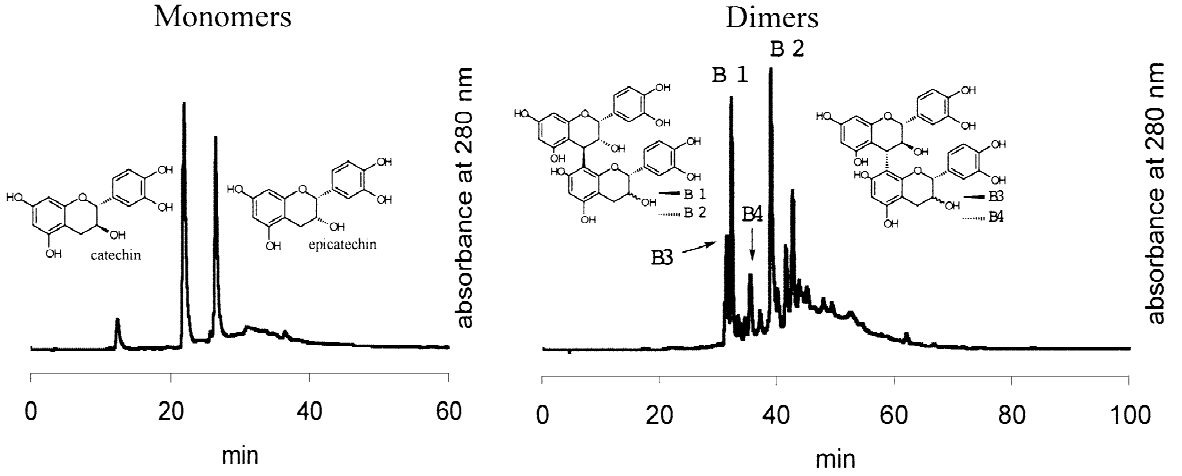

Fig. 2. HPLC chromatogram of procyanidin fractions. Experimental conditions: column, Ultrasep ES 100RP18 (250 mm×4.0 mm I.D.); column temperature, 23 °C; mobile phase with 0.0025% (v/v) TFA: water–methanol (linear gradient, 100:0 to 0:100:0 v/v in 60 or 100 min); flow-rate, 1 ml/min; detection, 280 nm.

tective effects [3,5,19] and it has been reported to have cancer chemopreventive properties [7,20].

Fraction 4, collected between 90 and 150 min of elution, contains piceatannol (30%) and other components which are assumed to be resveratrol oligomers from their TLC peculiar behaviour and from their crude proton NMR spectra. Piceatannol is an inhibitor of several protein-tyrosine kinases which play an important role in regulation of cell proliferation [21,22]. Piceatannol inhibits selectively the tyrosine phosphorylation of signal transducers and activators of transcription STAT3 and STAT5 [23].

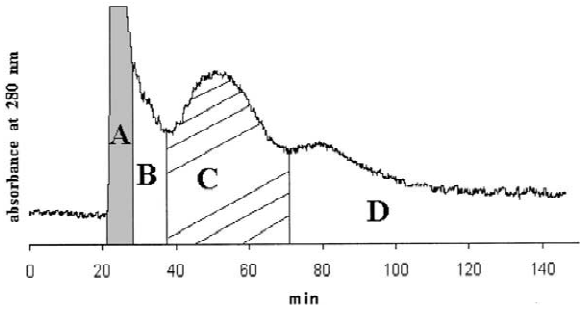

Fig. 3. CPC chromatogram of the stalk extracts. Experimental conditions: rotation speed, 1000 rev./min; solvent system, hexane–ethyl acetate–ethanol–water (1:8:2:7, v/v); mobile phase, upper organic phase; flow-rate, 3 ml/min; retention of the stationary phase, 80%; backpressure, 35 bar.

From 1 g of crude extract from stalks, we get 120 mg of fraction A. The obtained enrichment is of the order of 8. Fraction 3, collected after 90 min in the second CPC conditions contains about 7 mg of pure *trans*-resveratrol. After the outlet of fraction 4, if we switch to the descending mode, we obtain after a 20-min delay, a fraction principally containing pallidol (32%).

It is worthy of note that we can obtain pure *trans*-resveratrol, by injecting directly the crude extract of *Vitis vinifera* in the ascending mode when using the Q2 mixture. We get the same type of separation as the one presented in Fig. 5 with the same retention time but the recovery of the other products (as for example pallidol, astilbin) needs a greater elution time [11].

4. Conclusion

We have performed a CPC method which appears to be the most convenient way to obtain almost pure stilbenoids (monomers and oligomers) in a short time. Using stalk extracts, it is possible to separate stilbenoid compounds (major constituents in the extracts) from flavanols (minor compounds).

The most salient results, here obtained, are: (i) the possibility to isolate directly from 1 g of crude stalk

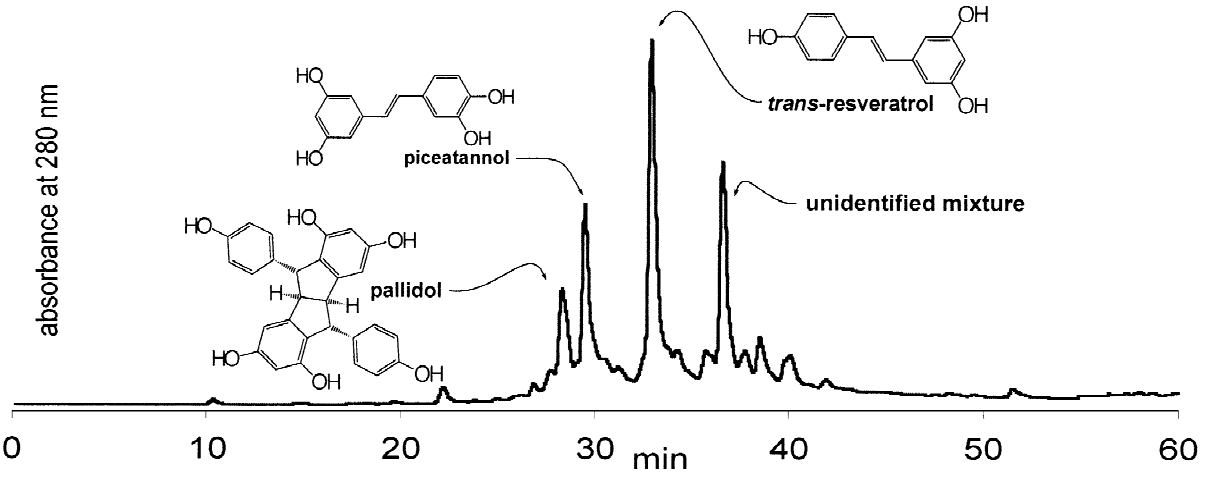

Fig. 4. HPLC chromatogram of the stalk fraction A. Experimental conditions: column, Ultrasep ES 100RP18 (250 mm×4.0 mm I.D.); column temperature, 23 °C; mobile phase with 0.0025% (v/v) TFA: water–methanol (linear gradient, 100:0 to 0:100 v/v in 60 min); flow-rate, 1 ml/min; detection, 280 nm.

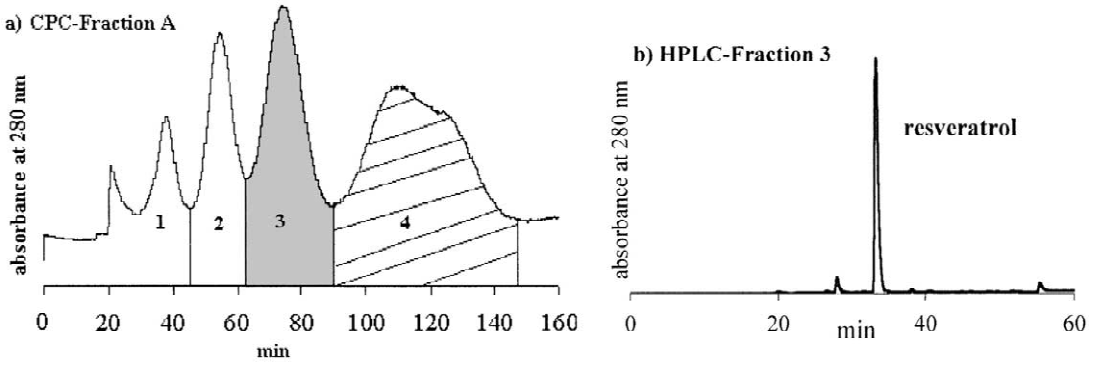

Fig. 5. CPC fractionation of fraction A from stalks. Experimental conditions: (a) CPC: rotation speed, 1000 rev./min; solvent system, hexane–ethyl acetate–ethanol–water (4:5:3:3, v/v); mobile phase, upper organic phase; flow-rate, 3 ml/min; retention of the stationary phase, 72%; backpressure, 45 bar. (b) HPLC: column, Ultrasep ES 100RP18 (250 mm×4.0 mm I.D.); column temperature, 23 °C; mobile phase with 0.0025% (v/v) TFA: water–methanol (linear gradient, 100:0 to 0:100 in 60 min, v/v); flow-rate, 1 ml/min; detection, 280 nm.

extract, within 30 min, a fraction eightfold enriched in resveratrol and its oligomers, and (ii) the possibility to obtain *trans*-resveratrol (90% pure) in one step of 90 min (7 mg from 1 g of crude stalk extract).

With the same experimental CPC conditions, from crude phenolic grape seed extracts, we isolated a fraction containing only flavanol monomers (catechin and epicatechin) and another one with B-type dimers.

References

[1] S. Renaud, M. De Lorgeril, Lancet 39 (1992) 1523.
[2] A.A.E. Bertelli, L. Giovannini, D. Giannessi, M. Migliori, W. Bernini, M. Fregoni, A. Bertelli, Int. J. Tissue React. 17 (1995) 1.
[3] C.R. Pace-Asciak, O. Rounova, S.E. Hahn, E.P. Diamandis, D.M. Goldberg, Clin. Chim. Acta 246 (1996) 163.
[4] U. Jager, H. Nguyen-Duong, Arzneimittelforschung 49 (1999) 207.

[5] E.N. Frankel, J. Kanner, J. German, E. Parks, J. Kinsella, Lancet 341 (1993) 454.
[6] L. Fremont, L. Belguendouz, S. Delpal, Life Sci. 64 (1999) 2511.
[7] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W.W. Beecher, H.H.S. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto, Science 275 (1997) 218.
[8] K. Subbaramaiah, W.J. Chung, P. Michaluart, N. Telang, T. Tanabe, H. Inoue, M. Jang, J.M. Pezzuto, A.J. Dannenberg, J. Biol. Chem. 273 (1998) 21875.
[9] T. Yoshida, T. Hatano, T. Okuda, J. Chromatogr. 467 (1989) 139.
[10] L. Balas, Ph.D. Thesis, University of Bordeaux 2, Bordeaux, France, 1992.
[11] J. Vercauteren, C. Castagnino, J.C. Delaunay, Patent EP 0998445 (WO 9903816), 1999.
[12] A.P. Foucault, Centrifugal partition chromatography, in: Chromatographic Science Series, Vol. 68, M. Dekker, New York, 1995.
[13] N.B. Mandava, Y. Ito, Countercurrent chromatography, in: Chromatographic Science Series, Vol. 44, M. Dekker, New York, 1988.
[14] A. Marston, K. Hostettmann, J. Chromatogr. A 658 (1994) 315.
[15] R. Margraff, in: A.P. Foucault (Ed.), Centrifugal Partition Chromatography, Chromatographic Science Series, Vol. 68, Marcel Dekker, New York, 1995, p. 331.
[16] D. Closa, M. Torres, G. Hotter, G. Bioque, O.S. Leon, E. Gelpi, J. Rosello-Catafau, Prostaglandins Leukot. Essent. Fatty Acids 56 (1997) 331.
[17] Q. Xu, F. Wu, J. Cao, T. Chen, J. Jiang, I. Saiki, A. Koda, Eur. J. Pharmacol. 377 (1999) 93.
[18] T. Chen, J. Li, Q. Xu, K. Komatsu, T. Namba, Planta Med. 65 (1999) 56.
[19] L.M. Hung, J.K. Chen, S.S. Huang, R.S. Lee, M.J. Su, Cardiovasc. Res. 47 (2000) 549.
[20] M.V. Clément, J.L. Hirpara, S.A. Chawdhury, S. Pervaiz, Blood 92 (1998) 996.
[21] J.M. Oliver, D.L. Burg, B.S. Wilson, J.L. McLaughlin, R.L. Geahlen, J. Biol. Chem. 269 (1994) 29697.
[22] J.D. Peters, M.T. Furlong, D.J. Asai, M.L. Harrison, R.L. Geahlen, J. Biol. Chem. 271 (1996) 4755.
[23] L. Su, M. David, J. Biol. Chem. 275 (2000) 12661.