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Short communication

Isolation and purification of dimeric and trimeric procyanidins from grape seeds

Baoshan Sun[a], G. Pedro Belchior[a], J.M. Ricardo-da-Silva[b], M. Isabel Spranger[a,*]

[a]*Estação Vitivinícola Nacional, Instituto Nacional de Investigação Agrária, 2560 Dois Portos, Portugal*
[b]*Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1399 Lisbon Codex, Portugal*

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Abstract

An improved method used for the isolation and purification of low-molecular-mass procyanidins (i.e., dimeric B_1, B_2, B_3, B_4, B_1-3-*O*-gallate, B_2-3-*O*-gallate, B_2-3′-*O*-gallate, trimer C_1 and trimer T_2) from grape seeds, is described. Pre-fractionation in oligomers and polymers was performed by chromatography on an open column (LiChroprep RP-18). The oligomer fraction, rich in dimeric and trimeric procyanidins, was then chromatographed on a Toyopearl TSK HW-40 (F) column. The major fractions obtained from Toyopearl TSK HW 40 (F) chromatography were further separated by semi-preparative HPLC, to obtain pure procyanidins. The main advantages of this method are that it is easy to use, less time-consuming and gives very high yield of dimeric and trimeric procyanidins by each manipulation.

Keywords: Grape seed; Procyanidins

1. Introduction

During the last few years, an increasing number of research works have involved high-performance liquid chromatography (HPLC) of dimeric and trimeric procyanidins [1–6] which have been proved to have various biological activities [7–10]. Since these compounds are not commercially obtainable, many authors used catechin to establish calibration curves to quantify them. This is not correct because the response factors of detection (integrator units/μg) for catechin and procyanidin are considerably different [6]. Some methods used for fractionating and isolating proanthocyanidins on a preparative scale have been proposed. These include column chromatography on Sephadex G25 [11], on Sephadex LH-20 [12] and on Toyopearl TSK HW 40 (s) [3]. The best results were obtained using the latter which permitted the separation of grape seed extract into several dimeric and trimeric procyanidin fractions using methanol as mobile phase. The main shortcomings of this method is that the yield of dimeric and trimeric procyanidins is very low since proanthocyanidins in plant tissues exist essentially in highly polymeric forms and dimers and trimers account for only a small percentage [13–15]. The polymeric forms accumulate on the top of the column, and have to be eluted because they block the column. Subsequently lengthy regeneration of the column is required.

To avoid interference by highly polymeric proan-

*Corresponding author. Tel.: +351-61-712-124; fax: +351-61-712-426.
E-mail address: inia.evn@mail.telepac.pt (M.I. Spranger)

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thocyanidins, we developed a pre-fractionation of the crude phenolic extract using open column chromatography with LiChroprep RP-18. The isolated dimeric and trimeric proanthocyanidins are separated on Toyopearl TSK 40 (F), followed by semi-preparative HPLC.

2. Experimental

2.1. Preparation of seeds

Grapes (*Vitis vinifera*, cv. Fernão Pires) were sampled at harvest maturity in September 1996 from vineyards of the INIA-Estação Vitivinícola Nacional (Dois Portos, Portugal). Seeds were removed by hand from the grapes, washed with distilled water, drained, blotted with filter paper, air-dried at 25°C in the dark for one day and stored at −20°C under N_2 until used.

2.2. Preparation of the phenolic extract

The frozen seeds were ground finely under liquid N_2 in a ball grinder. The grape seed powder was immediately used for the extraction of phenolic compounds. Furthermore, a 100-g portion of the powder was extracted using 1.5 l of methanol–water (80:20, v/v) followed by 1.5 l of acetone–water (75:25, v/v). Each solvent extraction was performed by stirring for 3 h under a nitrogen atmosphere at room temperature. The combined supernatants were evaporated at <30°C to remove organic solvents, followed by extraction with hexane (3×300 ml) to eliminate fatty materials, and then filtered through a membrane filter (0.45 μm). The aqueous phenolic solution can be stored at −20°C until used for pre-fractionation on LiChroprep RP-18 column, or lyophilized to obtain a yellow powder, referred as crude phenolic extract, which was also stored at −20°C until needed.

2.3. Pre-fractionation of proanthocyanidins using LiChroprep RP-18

A 100-ml volume of the aqueous phenolic solution (20 mg dry matter/ml) or an equivalent aqueous solution prepared from the *crude phenolic extract* was loaded onto an open column (200×25 mm I.D.) packed with LiChroprep RP-18 (25–40 μm particle size, Merck) already pre-conditioned with distilled water adjusted to pH 7.0. The fractionation procedures were similar to those already described using C_{18} Sep-Pak cartridges [14]. Elution began with 100 ml of distilled water adjusted to pH 7.0 to eliminate phenolic acids, followed by 150 ml ethyl acetate to elute catechins and oligomeric proanthocyanidins ($F_{cat+olig}$). The polymeric proanthocyanidins adsorbed on the top of the bed were eluted with 100 ml methanol. For further separation of $F_{cat+olig}$ into catechin fraction (F_{cat}) and oligomeric proanthocyanidin fraction (F_{olig}), the $F_{cat+olig}$ was evaporated to dryness under vacuum at 25°C, dissolved in distilled water and then redeposited onto the same column pre-conditioned with distilled water. Elution was performed with 150 ml diethyl ether (F_{cat}) and then with 100 ml methanol (F_{olig}).

2.4. Fractionation of proanthocyanidins using Toyopearl TSK HW-40 (F)

The *crude phenolic extracts*, $F_{cat+olig}$ and F_{olig} were dissolved in methanol, each of which was made to give a concentration of 100 mg/ml. A 3-ml volume of each solution was separately fractionated on a Toyopearl TSK HW-40 (F) column (32–63 μm particle size, 230×25 mm I.D.), using methanol as mobile phase as previously reported [3].

2.5. Analytical HPLC

Catechin and procyanidin compositions in each fraction isolated from Toyopearl TSK HW-40 (F) column were controlled by HPLC analysis as reported in our previous work [14].

2.6. Semi-preparative HPLC

Isolation and purification of procyanidins from the fractions isolated from the Toyopearl column were performed by semi-preparative HPLC using a μBondapark C_{18} column (10 μm particle size, 300×7.8 mm). The elution conditions were the same as used for analytical HPLC, but the flow-rate was 2 ml/min and the injection volume was 100 μl. Each procyanidin peak was collected in several runs,

pooled, gently evaporated at <30°C with addition of water for several times to eliminate acetic acid. The aqueous solution was lyophilized and the corresponding purified procyanidin powder was obtained.

3. Results and discussion

Direct application of *crude phenolic extract* on the Toyopearl column permitted separation of phenolic compounds into nine fractions (Fig. 1). HPLC analysis (Fig. 2) revealed catechin and procyanidin compositions in these fractions as follows: fraction 3, (+)-catechin and (−)-epicatechin; fraction 4, procyanidin B_1, B_2, B_3 and B_4; fraction 5, procyanidin B_1, B_2, B_3, B_4, B_2-3-*O*-gallate and procyanidin T_2; fraction 6, procyanidin T_2, procyanidin B_2-3-*O*-gallate and procyanidin C_1; fraction 7, procyanidin B_2-3′-*O*-gallate and procyanidin B_1-3-*O*-gallate. Each of the procyanidins was tentatively identified by microthiolysis [16] and confirmed by HPLC with co-injection of pure procyanidins isolated in our laboratory as already described [3].

On the other hand, although phenolic compositions of fractions 8 and 9 were not identified, both of these two fractions gave positive reaction with vanillin–H_2SO_4, indicating that each of them contained flavan-3-ols, probably other types of dimeric and/or trimeric procyanidins or even their higher condensed forms (degree of polymerization>3). By comparison, neither fraction 1 nor fraction 2 gave coloration with vanillin–H_2SO_4, indicating that none of them contained flavan-3-ols. Furthermore, fraction 2 strongly absorbed 313 nm, suggesting that this fraction contained some phenolic acids.

The amount of *crude phenolic extract* applied onto the Toyopearl column should not be over 0.3 g dry matter to avoid too high operating pressure. Since the concentration of small oligomeric procyanidins in grape seeds is much lower than that of larger-molecular-mass procyanidins [13–15], only very small amounts of dimeric and trimeric procyanidins are obtained. In addition, after each chromatography,

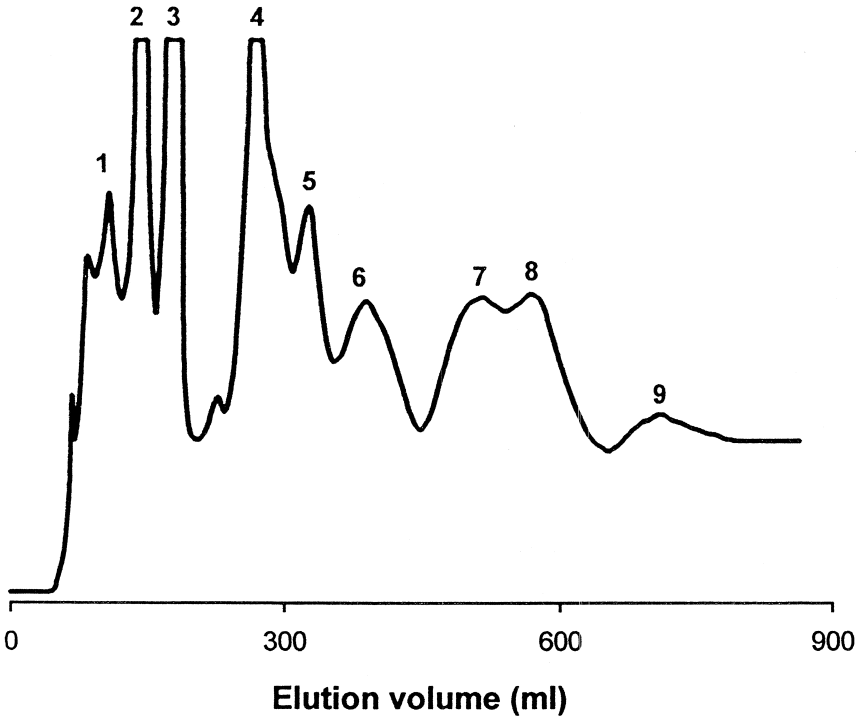

Fig. 1. Elution diagram of *crude phenolic extract* on Toyopearl TSK HW 40 (F) using methanol as eluent; flow-rate: 1.8 ml/min, detection wavelength: 280 nm.

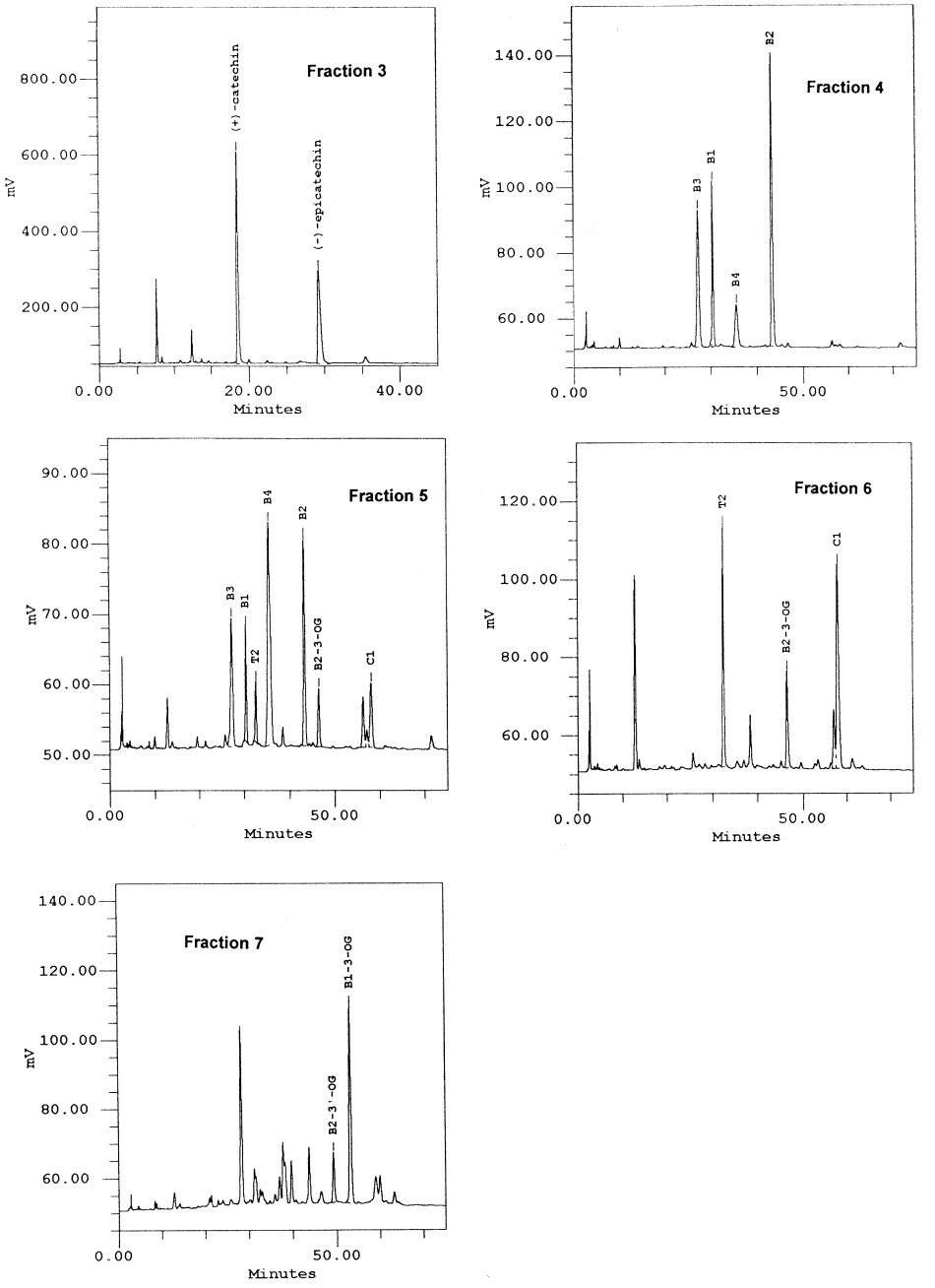

Fig. 2. HPLC chromatograms recorded at 280 nm of the major fractions in Fig. 1.

polymeric forms bound in the top of the column have to be eluted by acetone (75% in water) which requires regeneration by methanol before re-chromatography.

Fig. 3 presents the chromatogram issue from the Toyopearl chromatography of the fraction $F_{cat+olig}$ under the same conditions as those for *crude phenolic extract.*

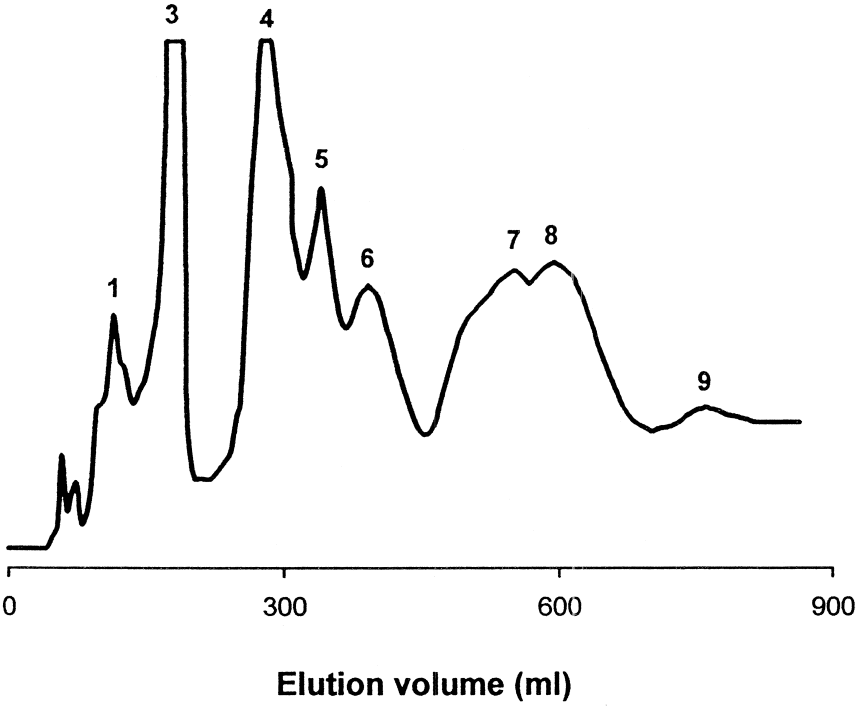

Fig. 3. Elution diagram of $F_{cat+olig}$ on Toyopearl TSK HW 40 (F) using methanol as eluent; flow-rate: 1.8 ml/min, detection wavelength: 280 nm.

Except for lack of peak 2 (phenolic acid fraction), the chromatogram presented in Fig. 3 is nearly identical to that presented in Fig. 1. HPLC analysis revealed that the procyanidin composition of each fraction in Fig. 3 was identical to those in Fig. 1.

In other words, the pre-fractionation of *crude phenolic extract* using LiChroprep RP-18 permitted isolation of catechins and oligomeric procyanidins and allowed one to discard unwanted phenolic acids and polymeric procyanidins. This pre-fractionation technique is simple and easy to operate. An amount of the *crude phenolic extract* as high as 2 g dry matter can be applied onto the column, which yields 0.4 to 0.5 g dry matter of $F_{cat+olig}$.

The application of $F_{cat+olig}$ instead of *crude phenolic extract* on the Toyopearl TSK 40 (F) column presented some advantages as follows: (1) after fractionation of procyanidins, no regeneration of the bed is required (at least six runs); (2) under the same chromatographic conditions, using $F_{cat+olig}$ gives rise to less column pressure than using *crude phenolic extract*, which makes manipulation easier, reduces the risk of leak and the high compression of the gel bed, and permits increasing the sample size (up to 0.5 g dry matter of $F_{cat+olig}$); (3) the yields of procyanidins by using $F_{cat+olig}$ were much higher than those by using *crude phenolic extract* (Table 1).

It should be noted that increasing $F_{cat+olig}$ sample size increased the overlap between peaks 4, 5 and 6. However, this did not affect the further isolation of each dimeric or trimeric procyanidins in these fractions by semi-preparative HPLC because the elution program of HPLC permits good separation of all these procyanidins.

Moreover, $F_{cat+olig}$ can be further separated into catechin fraction F_{cat} and oligomeric procyanidin fraction F_{olig} by the LiChroprep RP-18 column as described in Section 2.3. HPLC analysis revealed that F_{cat} contained (+)-catechin and (−)-epicatechin, whereas F_{olig} contained all dimeric and trimeric procyanidins detected in $F_{cat+olig}$. As expected, the Toyopearl TSK 40 (F) chromatogram of F_{olig} (Fig. 4) is similar to that of $F_{cat+olig}$ except the lack of peak 3 (catechin fraction). HPLC analysis showed

Table 1
Yield of procyanidins by the original method and by the proposed method (n=3; mean value±SD)

Procyanidin	Yield (mg/ml Toyopearl gel)	
	The original method[a] [3]	The proposed method[b]
B_1	0.018±0.003	0.090±0.014
B_2	0.044±0.007	0.221±0.026
B_3	0.012±0.002	0.059±0.007
B_4	0.031±0.005	0.157±0.020
B_1-3-*O*-gallate	0.022±0.005	0.109±0.013
B_2-3-*O*-gallate	0.013±0.004	0.066±0.016
B_2-3′-*O*-gallate	0.003±0.001	0.013±0.004
Trimer C_1	0.026±0.006	0.128±0.018
Trimer T_2	0.023±0.006	0.117±0.020

[a] Application of 0.3 g dry matter of the crude phenolic extract on Toyopearl TSK 40 (F) column.
[b] Application of 0.3 g dry matter of $F_{cat+olig}$ on Toyopearl TSK 40 (F) column.

that procyanidin composition in each fraction in Fig. 4 was identical to that in corresponding fraction in Fig. 3.

However, since the amount of catechins in $F_{cat+olig}$ was relatively small and that Toyopearl TSK 40 (F) fractionation permitted well separating

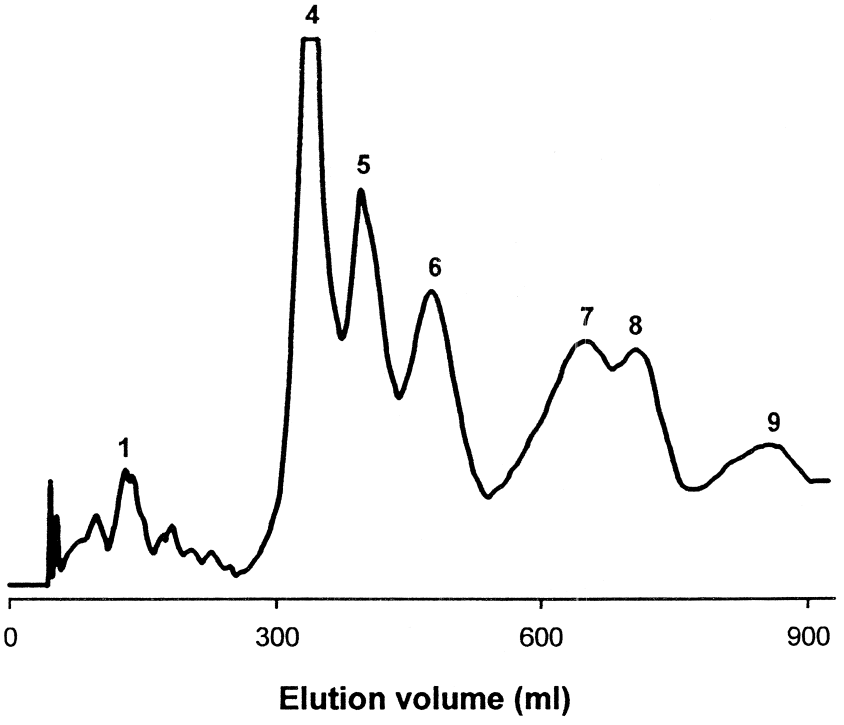

Fig. 4. Elution diagram of F_{olig} on Toyopearl TSK HW 40 (F) using methanol as eluent; flow-rate: 1.8 ml/min, detection wavelength: 280 nm.

catechins from procyanidins, the use of $F_{cat+olig}$ instead of F_{olig} as Toyopearl TSK 40 (F) fractionation sample is more appropriate because the isolation procedure of $F_{cat+olig}$ is much simpler than that of F_{olig}.

Finally, each procyanidin isolated by semi-preparative HPLC should be re-chromatographed using the same HPLC elution program in order to obtain chemically pure product. The level of purity of each procyanidin was higher than 96%, which was confirmed by HPLC with diode array detection.

It is concluded that pre-fractionation of the *crude phenolic extract* using a LiChroprep RP-18 column to eliminate polymeric procyanidins, improves fractionation on Toyopearl TSK 40 (F). The new fractionation and isolation procedure is easy to operate and each manipulation give very high yields of dimeric and trimeric procyanidin products. The proposed method is suitable for laboratory research and potentially applicable in industry for a large-scale production.

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