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# Why proanthocyanidins elute at increasing order of molecular masses when analysed by normal phase high performance liquid chromatography? Considerations of use

Esperanza Guerrero-Hurtado[a], Alba Gutiérrez-Docio[a], Rebeca Fiedorowicz[a], Esperanza Mollá[b], Guillermo Reglero[a], Marin Prodanov[a,*]

[a] Departamento de Producción y Caracterización de Nuevos Alientos, Instituto de Investigación en Ciencia de la Alimentación (CIAL), (CEI, CSIC-UAM), Universidad Autónoma de Madrid, calle Nicolás Cabrera, 9; campus Cantoblanco, Madrid 28049, Spain
[b] Departamento de Química Agrícola y Bromatología, Universidad Autónoma de Madrid, Madrid 28049, Spain

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ABSTRACT

Although it is widely known that proanthocyanidins elute at an increasing order of molecular masses when analysed by normal phase high performance liquid chromatography (NP-HPLC), there is no a consistent explanation of the mechanisms of their separation until now. Therefore, the aim of the present study was to give a reliable response to this question, using a complex procyanidin-rich grape seed extract. For this, an off-column static simulation of extract injection and a fragmented-column dynamic procyanidin location tests were studied to show their precipitation in an aprotic solvent, besides another off-column static simulation and multiple contact dynamic solubilisation tests to confirm procyanidin redissolution in an aprotic/protic solvent system. The results showed that separation of procyanidins in the aprotic/protic solvent system of Diol-NP-HPLC was governed by precipitation/redissolution mechanism, that could be extended to all known plant proanthocyanidin homopolymers, including hydrolysable tannins, if they are able to accomplish this condition. However, separation of monomer species, namely catechins and some hydroxybenzoic acids, was based on classic adsorption/partition mechanism. Other factors, such as analyte solubility, chromatographic conditions and sample preparation, that affect the viability of proanthocyanidin analysis by NP-HPLC were stand out and guidelines for its durable and reproducible use were defined.



## 1. Introduction

Proanthocyanidins (flavan-3-ol polymers or condensed tannins) are the most abundant end products of the flavonoid biosynthetic pathway of plants. They are synthesized in response to predators [1] and multiple stress conditions [2] and are accumulated mostly into the solid parts of vegetables, leaves, fruits, fruit skins, barks, seeds, seed coats and roots. They impart bitterness and astringency to products obtained from proanthocianidins-rich plants, as well as a large number of health-promoting biological activities, such as antioxidant, anti-inflammatory, cardioprotective, anticarcinogenic, antiaging, etc. [3–7].

Proanthocyanidins present practically unlimited diversity of structures based on the condensation of only few flavanol elemental units, mainly (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatchin, among others less frequently found flavan-3-ols [1,8,9]. This diversity is due basically to the stereochemistry and hydroxylation patterns of the flavan-3-ol elemental units, the position of interflavan bond, the length of the polymer chain (degree of polymerization), the presence of an additional (second) ether linkage between an upper and an extension elemental unit, the number, position and type of esterification or glycosylation of hydroxyl groups of the flavan skeleton and the position of esterification or glycosylation in the polymer. These vast structural diversities pose also, a real challenge when quantitative determination of these compounds is required.

Global quantification of proanthocyanidins is based on diverse methods that include protein precipitation, acid hydrolysis in the presence of butanol or condensation with aldehydes [9,10]. These methods are simple and fast, but they are also unspecific and show low reproducibility's.

* Corresponding author.
*E-mail address:* marin.prodanov@uam.es (M. Prodanov).

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The most used technique for individual assessment of proanthocyanidins is the reverse-phase mode of high performance liquid chromatography (RP-HPLC), using bonded stationary phases, such as C18 and gradient elution [11]. In this way, proanthocyanidins elute at a decreasing order of polarities, allowing fine separation and quantification of up to pentamers, but not all, if not only some of them [6]. Nevertheless, if identification of proanthocyanidins from one and the same homologous series can be achieved, it becomes obvious that they elute at increasing order of molecular masses [12]. A good example of such a homologous series are cocoa procyanidins, which are formed mainly by the only elemental unit of epicatechin and can be easily identified (note that monomeric catechin and epicatechin, which are not procyanidins, don't accomplish this order and elute at a decreasing order of polarities). In the case of cocoa procyanidins, the increase of the order of molecular masses agree also with the increase of the hydrophobic part of the polymer chains (decrease in polarity), maintaining thus the established adsorption/partition mechanism of RP-HPLC separation. Nevertheless, differentiation of simple proanthocyanidin homologous series on the background of the huge complexity of most plant secondary metabolite constituents, that include all other proanthocyanidin homologous series present in the same extract is very difficult and can be achieved after multistep purification [12,13], and does not imply any analytical advantage from practical point of view.

Other HPLC alternative for proanthocyanidin analysis is the normal phase (NP) HPLC modality with silica or diol bonded phases [5,14]. In these cases, they elute at increasing order of molecular masses and their separation is not so precise, but allows quantification of oligomeric procyanidins with degree of polymerisation of up to 7 for some relatively simple polymers, such as those of cocoa procyanidins [14,15]. For more complex procyanidins, as those from grape seed extracts, for which differentiation between non-galloylated and galloylated forms is impossible because of their large overlapping, only small polymers (oligomers) have been quantified successfully [16]. Although these limitations, as far as we are aware, NP-HPLC is the only high resolution analytical technique that allows separation of oligomeric proanthocyanidins in one set. Furthermore, the most important advantage of NP-HPLC is that it allows separation of the polymer (chromatographically non-separable) proanthocyanidins in a relatively singular peak at the end of the chromatogram, that has allowed some researchers to quantify the whole polymer fraction individually [16,17].

Nevertheless, this chromatographic modality has some drawbacks that have to be taken in consideration, such as not very high reproducibility, peak movements, pressure rise, irreversible adsorption of some solutes in the stationary phase or election of the proper solvent for sample solubilisation. Other shortcoming of this method is that in spite of its worldwide acceptance and use since 1979 [18], to the best of our knowledge, there is no a consistent explanation of the question, why proanthocyanidins elute at increasing order of molecular masses? Therefore, the aim of the present study was to give a reliable response to this question, providing a better understanding of the separation mechanism of proanthocyanidins when analysed in NP-HPLC, and defining main guidelines for getting better chromatographic stability and reproducibility. For this, a complex procyanidin-rich grape seed extract was used as experimental material.

## 2. Experimental section

### 2.1. Materials

Industrially obtained crude procyanidin-rich grape seed extract was used in this study as those described by Gutiérrez-Docio et al. [5]. The extract was freeze-dried and stored until use in plastic bags under vacuum at 4 °C.

HPLC grade methanol and acetonitrile were purchased from Scharlab (Barcelona, Spain) and glacial acetic acid (AA) (p.a.) was obtained from Sigma-Aldrich (Madrid, Spain). Milli-Q grade water was produced in-house by a Milli-Q® Integral 3 purification system (Merck Millipore, Burlington, MA, USA). For peak identification, HPLC grade (+)-catechin (C), (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), procyanidin dimers $B_1$ [EC-(4–8)-C] and $B_2$ [EC-(4–8)-EC] were purchased from Extrasynthèse (Genay, France). Gallic acid and ethyl gallate were acquired from Sigma-Aldrich (Madrid, Spain). Purified oligomeric procyanidin (OPC) extract from cocoa (Breko GmbH, Bremen, Germany) was used as a complex reference sample for the identification of non-galloylated oligomer procyanidins.

**Table 1**
Mobile phase elution conditions of the modified NP-HPLC method.

| Time (min) | component A (%) | component B (%) | component C (%) |
|---|---|---|---|
| 0 | 95 | 5 | 0 |
| 32 | 64 | 36 | 0 |
| 37 | 55 | 45 | 0 |
| 49 | 0 | 100 | 0 |
| 65 | 0 | 100 | 0 |
| 70 | 0 | 0 | 100 |
| 74 | 0 | 0 | 100 |
| 76 | 95 | 5 | 0 |
| 86 | 95 | 5 | 0 |

### 2.2. Sample preparation and HPLC analysis

For HPLC analysis, solutions of 5 mg of dry crude grape seed extract $mL^{-1}$ of components B/C (60/40) were prepared and analysed by a Prominent modular HPLC (Shimadzu Co., Japan), according to the methodology described by Kelm et al. [19] for analysis of cacao extracts and adapted for grape seed extracts in our laboratory as follows. The HPLC equipment was composed of SIL-$20A_{HT}$ autosampler, a DGU-$20A_{5R}$ on-line degassing unit, a quaternary solvent delivery unit LC-20AD, a CTO-20A column thermostat and a photodiode array detector (PAD), model SPD-M20A. The stationary phase was a Kromasil 60 Diol adsorbent (5 µm particle size, 6 nm pore size) packed in a 250 × 4.6 mm i.d. column (part number TR-012,309) from Teknokroma (Sant Cugat del Vallés, Barcelona, Spain), provided with a guard column (10 × 4.6 mm i.d.) (part number TR-C-160–15) from the same adsorbent material and thermostated at 40±0.1 °C. The mobile phase consisted of 3 components, A (acetonitrile/AA (98/2, v/v)), B (methanol/water/AA (95/3/2, v/v/v)) and C (water/AA (98/2, v/v)). Elution was carried out at a flow rate of 0.8 mL $min^{-1}$ as shown in Table 1. Chromatograms were registered at 280 nm.

Sample preparation and HPLC analytical methodology were divided in two areas:

#### 2.2.1. Procedures that aimed to reveal precipitation of procyanidins when injected in aprotic solvent systems

For studying procyanidin precipitation at the moment of their injection two types of tests were carried out: 1) an off-column static simulation of sample injection and 2) a fragmented-column dynamic location of solutes. The off-column static simulation test of sample injection was carried out by 10 mL volumetric series that were suitable for correct instrumental turbidity measurements. Amounts of 100, 30, 10, 3 and 1 mg of grape seed extract powder were dissolved in 1 mL of mobile phase components B/C (60/40) and were shaken until they became completely dissolved. For the simulated injection, an amount of 0.5 mL of these solutions were

mixed with 10 mL of mixture of 95% mobile phase component A and 5% of component B (proportion that corresponds to the moment of sample injection) to obtain final concentrations of approximately 4.8, 1.4, 0.5, 0.15 and 0.05 mg $mL^{-1}$. After short manual shaking, turbidity of the thus obtained suspensions was measured in 10 mL glass cuvettes by a model D-112 turbidimeter in the interval of 0 to 800 NTU (Dinko Instruments, Barcelona, Spain).

Next, the five suspensions were filtered through 0.45 µm pore size polytetrafluoroethylene (PTFE or Teflon) filters from Symta SLL (Madrid, Spain). However, this pore size filters were not able to separate completely the finest solids of the suspensions of the most abundant precipitations (4.8, 1.4 and 0.5 mg $mL^{-1}$), so 0.22 µm Nylon 25 mm disk filters from Pall España SAU (Madrid, Spain) were used for their clarification. Nevertheless, it should be pointed out that 7 and 2 filters were necessary for complete clarification of the suspensions with the most abundant precipitations (4.8 and 1.4 mg $mL^{-1}$, respectively), while for the rest of suspensions (0.5–0.05 mg $mL^{-1}$) 1 filter was enough. The filtered supernatants (soluble grape seed fractions) were submitted to NP-HPLC analysis at normal gradient conditions of elution (Table 1). The precipitates retained on only 1 filter were washed with 1.5 mL of solvent with composition corresponding to those of the mobile phase at the moment of injection (components A/B, 95/5) in order to remove rests of the filtered liquid phase and redissolved afterwards by passing consecutively 5.25 mL of mobile phase component B and 5.25 mL of mobile phase component C through the filter (both components B and C were used because one part of the precipitates was soluble only in component B and other, in component C). These solutions were the redissolved insoluble fractions of the extract and were analyzed by NP-HPLC as described in Table 1. The washing effluents were discarded. The only exception was done with the two most concentrated suspensions (4.8 and 1.4 mg $mL^{-1}$) which precipitates were retained on 7 and 2 filters, respectively. In these cases, the precipitates were redissolved by passing consecutively 0.75 mL (for the 4.8 mg $mL^{-1}$ suspension) and 2.6 mL (for the 1.4 mg $mL^{-1}$ suspension) of mobile phase component B and the same amounts of mobile phase component C through each filter to obtain final volumes of 10.5 mL.

Solutions of purified reference procyanidin dimer B1 and epicatechin gallate were also submitted to the off-column simulated injection test. For this 0.1 mL of 1 mg $mL^{-1}$ solutions of both compounds were injected in 1 mL of the aprotic component A of the mobile phase (non-solvent) to a final concentration of 0.1 mg $mL^{-1}$. The thus obtained suspensions were filtered through 0.22 µm Nylon filters, the precipitates were redissolved with 1.1 mL of mobile phase component B and both, soluble and insoluble fractions of each reference solution were analysed by NP-HPLC as described in the previous paragraph for the grape seed extract.

Fragmented-column dynamic location test of solutes was carried out by injection of aliquots of 10 µL of grape seed extract at 5 mg $mL^{-1}$ of concentration into the Diol HPLC column at normal gradient conditions of elution (Table 1). For this, the possibility of opening and replacement the guard cartridge as a part of the whole chromatographic column was taken in advantage. In this way, the chromatographic run was stopped for short time after sample injection, just for replacement of the guard column with other new or clean one and restarted again at the condition at which it was disrupted. After finishing the elution time, the new guard column cartridge was replaced with the initial one and the chromatograph was run again at normal gradient conditions of elution (Table 1). The run was started with injection of methanol instead of grape seed extract, chromatographing in this way rather the grape seed material retained in the initial guard column. As the mobile phase front took 3.8 min to cross the whole column, the front corresponding to the guard column was calculated to require 1/26 times shorter time, that corresponds to 0.15 min. This means that only longer intervals of time should be considered for stopping the chromatographic run. Taking in account these concerns, intervals of 6, 2.1 and 0.25 min after sample injection were established for testing the distribution of procyanidins along the chromatographic column.

### 2.2.2. Procedures that aimed to reveal redissolution of procyanidins in protic solvents after precipitation in aprotic/protic solvent systems

For solubility behavior of procyanidins in low to medium polarity solvent systems, 2 types of tests were carried out also, 1) an off-column static simulation of grape seed extract solubilisation and 2) a multiple contact dynamic test of procyanidin redissolution in an aprotic/protic NP-HPLC solvent system at real chromatographic conditions of solubilisation. The first one was also divided in 2 sub-stages with 1 and 4 contact extraction tests, respectively. The 1 contact extraction test consisted in a simple extraction by only 1 contact of the freeze-dried grape seed extract and the mobile phase components A (acetonitrile/AA, 98/2 (v/v)) and B (methanol/water/AA, 95/3/2 (v/v/v)). For this, an amount of 5 mg of extract powder were mixed with 1 mL of mobile phase component A in a 2 mL centrifugal Eppendorf tube; the mixture was sonicated for 5 min in ultrasonic bath (model 3,000,513, from J.P. Selecta, SA, Abrera, Barcelona, Spain) with 1.5 L of water at 40 kHz of agitation frequency; the temperature of the mixture increased from 23 to 28 °C; after sonication, the added grape seed extract maintained at the bottom of the tube almost intact and the liquid phase became turbid; after centrifugation by MiniSpin® Plus centrifuge (Eppendorf) at 14,000 g, the supernatant was recovered and 10 µL of the clarified solution were assessed by NP-HPLC-PAD at the conditions described in Table 1; other 5 mg of extract powder were mixed with 1 mL of mobile phase component B; after sonication for 5 min at the already described conditions, the extract was dissolved almost completely and formed slightly cloudy solution; after centrifugation at 14,000 g, the supernatant was recovered and 10 µL of the clarified solution were assessed by NP-HPLC-PAD; the insoluble part recovered from the mobile phase component B suspension was dried by a $N_2$ stream, redissolved in 1 mL of a mixture of water/AA (98/2, v/v) to clear solution and assessed by NP-HPLC.

The 4 contacts extraction test was an extension of the 1 contact test of the insoluble part of the grape seed extract, left after centrifugation of the mobile phase component B suspension and repeating the previous procedure for three times more. Finally, the insoluble part of the grape seed extract recovered from the $4^{-th}$ mobile phase component B suspension was dried by a $N_2$ stream, redissolved in 1 mL of a mixture of water/AA (98/2, v/v) to clear solution and assessed by NP-HPLC.

The multiple contact extraction test of procyanidin solubility at real (dynamic) aprotic/protic NP-HPLC conditions was carried out using the proper Diol HPLC column as an extraction unit, the mobile phase as an extraction solvent and the PAD for monitoring the effect of extraction on different grape seed constituents at 280 nm. For this, subsequent extractions (elution's) were carried out with solvents that were mixtures of components A/B at the following proportions of A, 100, 95, 90, 80, 70, 60, 50, 40, 30, 20 and 0%, maintaining always component C at 0% and temperature at 40±0.1 °C. The thus obtained mixtures were pumped isocratically through the column during the first 37 min. From this point onward, the gradient changes were those already described in Table 1, with the only exception that conditioning of the column for each next analysis (min 76–86) was carried out with the corresponding proportion of the component B for the next isocratic elution. As an example, the chromatographic elution conditions at isocratic methods for the first two elution's (100/0 and 95/5, A/B) are described in Table 2. For each isocratic elution, new sample of crude grape seed extract was used.

**Table 2**
Mobile phase elution conditions used in the multiple contact extraction test for the first two isocratic elution's of 100/0 and 95/5 of mobile phase components A/B.

| Retention time | Isocratic elution at 100/0% of components A/B | | | Isocratic elution at 95/5% of components A/B | | |
|---|---|---|---|---|---|---|
| | A | B | C | A | B | C |
| (min) | (%) | (%) | (%) | (%) | (%) | (%) |
| 0 | 100 | 0 | 0 | 95 | 5 | 0 |
| 37 | 100 | 0 | 0 | 95 | 5 | 0 |
| 49 | 0 | 100 | 0 | 0 | 100 | 0 |
| 65 | 0 | 100 | 0 | 0 | 100 | 0 |
| 70 | 0 | 0 | 100 | 0 | 0 | 100 |
| 74 | 0 | 0 | 100 | 0 | 0 | 100 |
| 76 | 95 | 5 | 0 | 90 | 10 | 0 |
| 86 | 95 | 5 | 0 | 90 | 10 | 0 |

*2.2.3. Procedures that aimed to reveal solubility behavior of procyanidins in high to medium polarity solvent systems*

On the other hand, for studying the solubility behavior of procyanidins in high to medium polarity solvent systems, 5 mg of dry crude grape seed extract were contacted with 1 mL of mixtures between the mobile phase components B and C at the following proportions of B: 0, 10, 20, 30, 40, 50, 60, 70, 80 and 100%. After sonication during 5 min at temperatures from 23 to 28 °C, dissolved extracts and suspensions were centrifuged at 14,000 g and 10 µL of the clarified extracts were assessed by NP-HPLC at the gradient conditions already described in Table 1.

For sample preparation, 0.45 µm pore size hydrophilic polytetrafluoroethylene (PTFEH or hydrophilic Teflon), polyvinylidene fluoride (PVDF), polypropylene (PP) and polyamide (PA or Nylon) and hydrophobic PTFE filters were obtained from Symta SLL Madrid, Spain. Hydrophilic filters of mixed cellulose esters (MCE) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and hydrophobic polyether sulfone (PES) filters, from Pall España SAU (Madrid, Spain). The hydrophobic PTFE filters were hydrophilised by flushing them with 3–5 mL of acetonitrile and were tested for their retention of polymeric procyanidins.

Peaks were identified according to Gutiérrez-Docio et al. [5] by the use of the already mentioned reference substances in Section 2.1. We take the opportunity here to point out that gallic acid elutes always overlapped or immediately after catechin in NP-HPLC and that this was erroneously indicated in the chromatograms shown in Gutiérrez-Docio et al. [5].

*2.3. Statistical analysis*

All instrumental analyses were completed in duplicate and expressed as a mean value and standard deviation. Statistical analysis of analytical data was evaluated by analysis of variance (ANOVA) and significant differences between mean values ($p < 0.05$) were evaluated using Tukey's test with the statistical software IBM SPSS Statistics for Windows, Version 26.0 (IBM Co., Armonk, NY, USA).

## 3. Results and discussion

*3.1. Solubility behavior of procyanidins in low to medium polarity or aprotic to protic solvent systems*

Since proanthocyanidins are highly to moderate polar species, they are usually analysed in reverse-phase mode HPLC, using weakly polar C18 bonded stationary phase and gradient elution with highly to moderate polarity solvents, such as water/acetonitrile or water/methanol, where analytes are separated by adsorption and partitioning between the stationary and mobile phase and elute at decreasing order of polarities. On the contrary, NP-HPLC mode is used commonly for assessment of low to moderate polar dipole molecules, using high to moderate polarity Silica or Diol bonded stationary phases and gradient elution with low to moderate polarity solvents (dichloromethane to methanol) or aprotic to protic solvents (acetonitrile to methanol), where the low to moderate polarity species are completely dissolved in the initial solvent of the mobile phase and elute at increasing order of polarities [20]. Nevertheless, the huge experience accumulated until now with Silica or Diol NP-HPLC assessment of oligomers of proanthocyanidin undoubtedly sustains that they elute at increasing order of molar masses [14,15], but due to the huge diversity of their structures, most of them overlap [5].

In the case of the studied here grape seed extract, singular peaks for only non-galloylated (min 15.3) and monogalloylated procyanidin dimers (min 19.6) were found (Fig. 1). Maximums of the non-galloylated procyanidin trimers to hexamers were also matched, as they are mayor species among the grape seed procyanidins [6,11], but they were rather overlapped with oligomers with different degree of galloylation. As such, the peak corresponding to procyanidin non-galloylated trimers included also the digalloylated procyanidin dimer. Procyanidins with higher degree of polymerization eluted at the end of the chromatogram as a singular broad peak (PPC). For better understanding of this common PPC peak, it is noteworthy to mention that it includes all other non-galloylated and galloylated procyanidin species with degree of polymerization higher than hexamers and that they elute at increasing order of molecular masses within the same peak, i.e. smaller PPC elute at the beginning of the peak and PPC with higher molecular masses elute later along the first half of the peak [21].

Regardless of this overlapping, oligomeric procyanidins eluted not at increasing order of polarities, but rather at increasing order of molecular masses, which is a clear contradiction to the widely accepted adsorption mechanism of separation in NP-HPLC. In such a relationship, it becomes obvious that the mechanism of separation when highly to moderate polar dipoles are injected in a NP-HPLC elution system is different and most probably related to their poor solubility in a such a mobile phase, thus suggesting that sample/solvent interaction phenomena should be studied first.

*3.1.1. Precipitation of procyanidins when injected in aprotic solvent systems*

For studying physical behavior of soluble grape seed procyanidins when injected in an aprotic solvent system, such as those of the used here Diol-NP-HPLC, two types of tests were carried out, one off-column simulation of grape seed extract injection and another, fragmented-column dynamic location of solutes as described in experimental Section 2.2.1.

*3.1.1.1. Off-column static simulation of grape seed procyanidin injection in an aprotic solvent.* As it was not possible to know the exact proportion of the sample to mobile phase volumes during the entry of the analytes in the column, amounts of 100, 50, 40, 30, 20, 10, 5, 3 and 1 mg of grape seed extract powder were dissolved in 1 mL mobile phase components B/C (60/40) and 0.5 mL of each of these solutions were mixed with 10 mL of mobile phase with com-

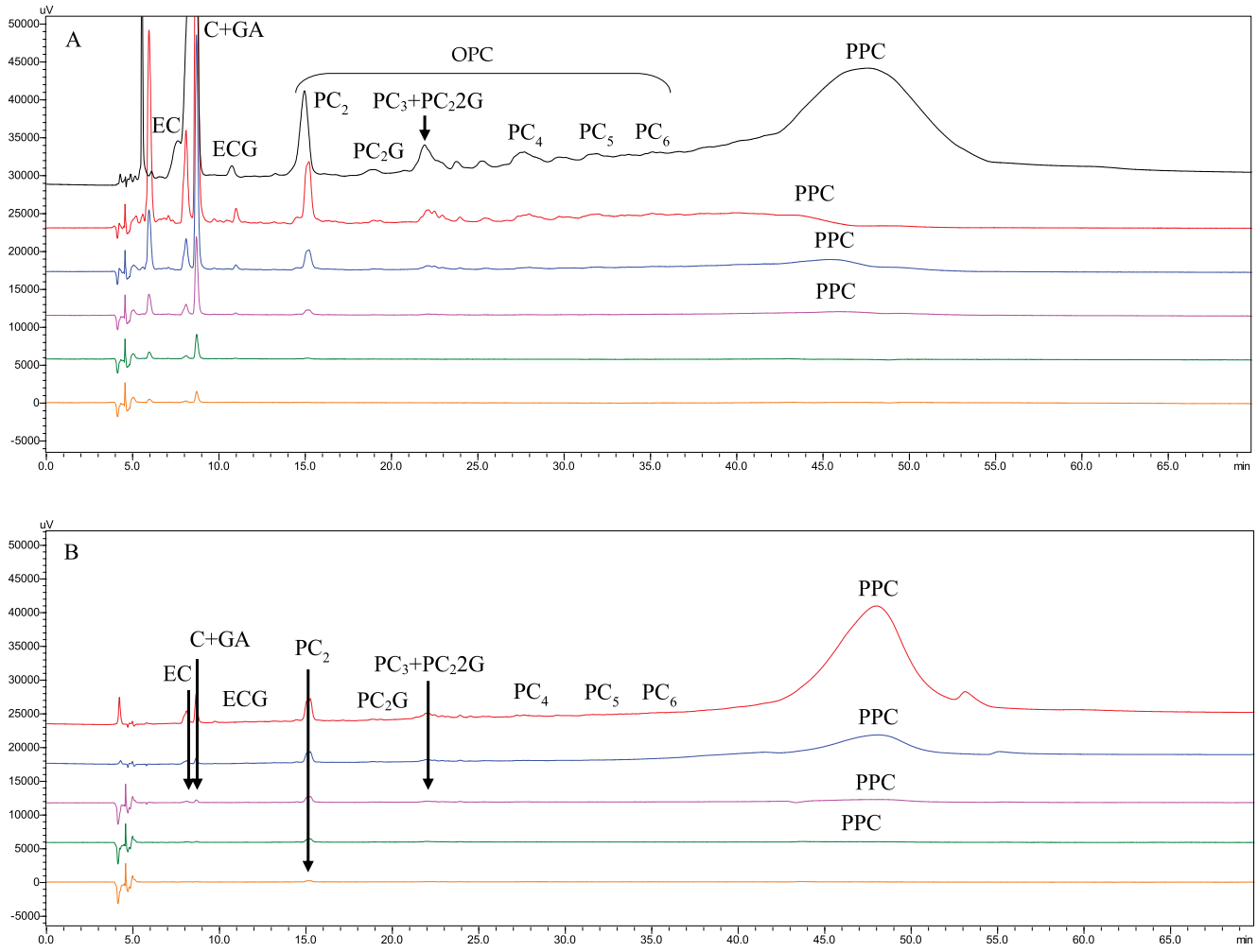


**Fig. 1.** Diol-NP-HPLC chromatogram traces (280 nm) of the 5 mg $mL^{-1}$ whole grape seed extract (black) and the soluble (A) and insoluble (B) fractions of the studied grape seed extracts after off-column simulated injection test at final concentrations of 4.8 (red), 1.4 (blue), 0.5 (violet), 0.15 (green) and 0.05 (orange) mg $mL^{-1}$. EC -epicatechin, C - catechin, GA – gallic acid, ECG – epicatechin gallate, $PC_x$ – non-galloylated procyanidin oligomers, $x$ = 2–6, $PC_2G$ – galloylated procyanidin dimers, $PC_2 2G$ – digalloylated procyanidin dimer, OPC – oligomeric procyanidins, PPC – polymer procyanidins.

position corresponding to the moment of sample injection (aprotic component of the mobile phase). In this way, suspensions with final concentrations of 4.8, 2.4, 1.9, 1.4, 1.0, 0.5, 0.2, 0.15 and 0.05 mg $mL^{-1}$ were obtained. Contacting the extracts with concentrations from 100 to 10 mg $mL^{-1}$ with the aprotic mobile phase produced immediate spontaneous cloudiness (precipitation) of the solutions, visible to a naked eye. Measurement of turbidity of the suspensions showed that as higher were the concentrations of the final suspensions, higher were also their cloudiness and that their dependence could be adjusted to a power function with very high correlation coefficient ($R^2$>0.99) (Fig. S1 from the supplementary material) (suspensions with final concentrations of 4.8 and 2.4, and 0.15 and 0.05 mg $mL^{-1}$ were excluded from the graphic, because the first two showed values of turbidity out of the calibration range >800 NTU and the last two showed values <0.5 NTU).

These data showed clearly that contacting grape seed extracts with the aprotic mobile phase produced phase separation (precipitation) of some solutes and that this precipitation was concentration dependant. These findings also mean that the aprotic component of the mobile phase acted as a non-solvent with respect to the precipitated solutes. However, these data were not sufficient to explain the nature of the precipitated solutes.

To give an answer to this question, the above mentioned suspensions of 4.8, 1.4, 0.5, 0.15 and 0.05 mg $mL^{-1}$ were filtered through 0.22 µm pore size Nylon filters and the clarified solutions (soluble grape seed fractions) were analyzed by Diol-NP-HPLC as described in experimental Section 2.2.1. The fine solids retained on the filters (the insoluble fractions of the extract) were redissolved by passing successively 2 mL of mobile phase component B and C through the filters, as described in experimental Section 2.2.1. The results of NP-HPLC analysis of the soluble and insoluble fractions at final concentrations of 4.8, 1.4, 0.5, 0.15 and 0.05 mg $mL^{-1}$ are shown in Fig. 1. For better understanding of the distribution of oligomeric and polymeric procyanidins among the soluble and insoluble fractions at each concentration of the studied suspensions they are shown also in Fig. S2 from the supplementary material.

The results of Diol-NP-HPLC analysis (Fig. 1) showed that the precipitated solid phases obtained from extracts with higher concentrations were composed mostly by highly polymerized procyanidins (PPC), whereas oligomeric procyanidins and small part of the PPC maintained mainly in the supernatants. Decreasing the concentration of the studied extracts produced gradual decrease of all dissolved procyanidin species in the supernatant liquid phase, as well as their progressive appearance in the solid phase as precipitates. This finding suggests that at higher concentrations, polymeric and oligomeric procyanidins needed higher volumes of non-solvent to precipitate, while at low concentrations, all procyanidins (oligomeric and polymeric) had enough amount of non-

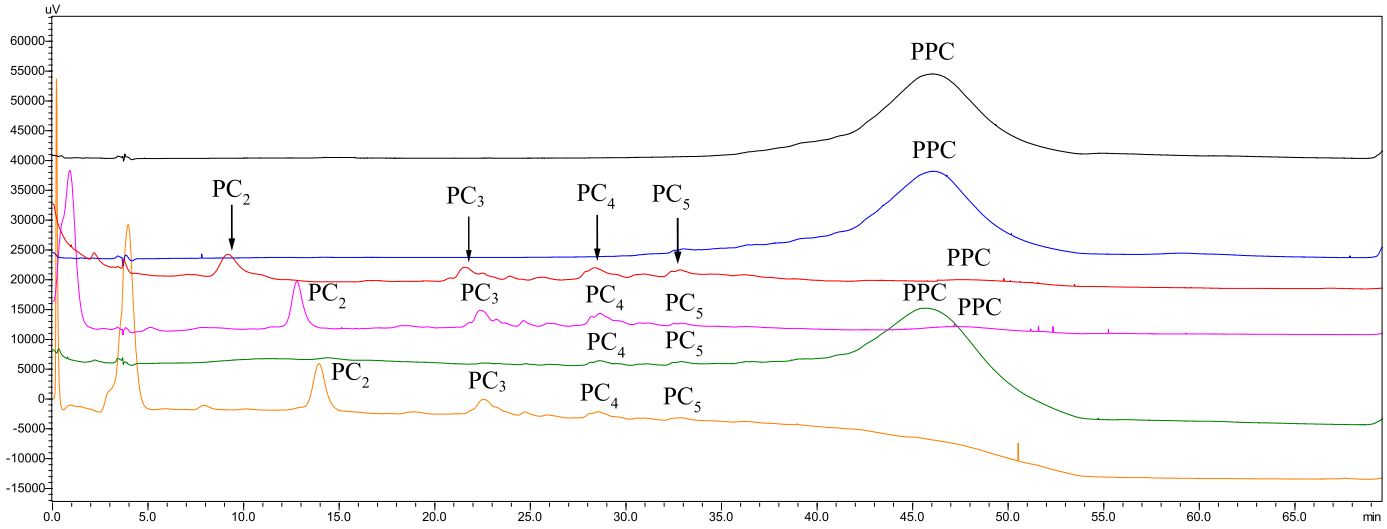


**Fig. 2.** Diol-NP-HPLC chromatogram traces (280 nm) of the analytes retained 6, 2 and 0.25 min in the column before disrupting the chromatographic run (red, violet and orange, respectively) and those retained in the guard column (black, blue and green, respectively) in the fragmented-column dynamic test for procyanidin location. $PC_x$ – non-galloylated procyanidin oligomers, $x$ = 2–6, PPC – polymeric procyanidins.

solvent that let to their precipitation. Furthermore, the results from Fig. 1 showed also that the highly polymerised non-separable procyanidins precipitated first, followed by the oligomeric species at decreasing order of molecular masses, that could suggest that procyanidins should be ordered from bigger to smaller in the stationary phase just after extract injection. An example of precipitation of purified procyanidin dimer $B_1$ as a representative molecule of the smallest procyanidins and epicatechin gallate at final concentrations of 0.1 mg $mL^{-1}$ is shown also, in Fig. S3 from the supplementary material.

*3.1.1.2. Fragmented-column dynamic test for procyanidin location in an aprotic/protic NP-HPLC solvent system.* To confirm the results from the off-column static simulation of grape seed procyanidin injection in an aprotic solvent, a fragmented-column dynamic test for procyanidin location was carried out with the Diol column used for all analysis of this study, that was coupled to a detachable guard column as described in experimental Section 2.2.1. For this, several chromatographic runs were disrupted for an instant after sample injection, the guard column (1) was replaced with other clean one (2) and the run was restarted again at the condition at which it was stopped. After finishing each elution time, the guard column cartridge (2) was replaced with the initial one (1) and the chromatograph was run again by injection of methanol instead of grape seed extract. Two chromatograms were registered in this way for one injected sample, one with the part of analytes that have entered in the column before disrupting the chromatographic run (2) and a second, with the rest of the solutes that were retained in the guard column (1). In this way, chromatographic runs were disrupted at 6, 2.1 and 0.25 min after sample injection (the interval of 0.25 min was very close to the time at which the front of elution could go through the guard column (0.15 min). The obtained results are shown in Fig. 2.

These results (Fig. 2) revealed that after 6 min of run, all oligomer procyanidins and a small part of the polymer procyanidins were located in the part of the HPLC column, while the bigger part of polymer procyanidins were found in the guard column, most probably at the head of the column. Approaching the time of disruption (min 0.25) to the time at which the front of elution leaved the guard column (min 0.15) besides the polymer procyanidins, some part of dimers and higher oligomer procyanidins were retained in the guard column. This means that most oligomers precipitated also together or a little bit after the polymers on the head of the column or very close after it and confirms that precipitation could contribute to the establishment of one partial primary distribution of the procyanidin oligomers at decreasing order of molecular masses along the initial part of the HPLC column.

The obtained here results for the studied aprotic solvent system are directly transferable to systems of low to medium polarity solvents, such as those used in Silica NP-HPLC, as shown in other studies [9,14,17].

*3.1.2. Redissolution of procyanidins in protic solvents after precipitation in aprotic/protic solvent systems*

*3.1.2.1. Off-column static simulation of procyanidin redissolution in a protic solvent from an aprotic/protic solvent system.* For studying procyanidin behavior when contacted with the protic solvent from an aprotic/protic solvent system, an off-column static simulation test of procyanidin solubilisation was carried out. For this, amounts of 5 mg $mL^{-1}$ of freeze-dried grape seed extract were contacted with the mobile phase components A (acetonitrile/AA, 98/2 (v/v)) and B (methanol/water/AA, 95/3/2 (v/v/v)), as described for 1 contact extraction test in experimental Section 2.2.2. and aliquots of 10 µL of each of them were assessed by NP-HPLC. The insoluble part of the extracts from the mobile phase component B suspensions were contacted with a mixture of water/AA (98/2, v/v) and were assessed also by NP-HPLC. The results are shown in Fig. 3.

These results (Fig. 3) showed that only a small part of the grape seed constituents (monomeric species eluting from min 3.5 to 11.0, that include ethyl gallate, epicatechin, catechin, gallic acid and epicatechin gallate) (second chromatogram from the top of Fig. 3) dissolved in the aprotic and less polar (weaker) mobile phase component A and with the exception of epicatechin gallate eluted at an increasing order of their polarities, as it is usual in NP-HPLC analysis (for order of polarities see [6] and [11]). In opposite, the biggest part of the grape seed extract constituents dissolved in the protic and most polar (stronger) mobile phase component B and peaks appeared all over the chromatogram (first chromatogram from Fig. 3).

Analysis of the insoluble part of the grape seed extract in the mobile phase component B, that was re-dissolved with a mixture of water/AA, 98/2 (v/v), showed the presence of peaks, corresponding to its most characteristic components, catechins and oligomeric and polymeric procyanidins, but with smaller heights

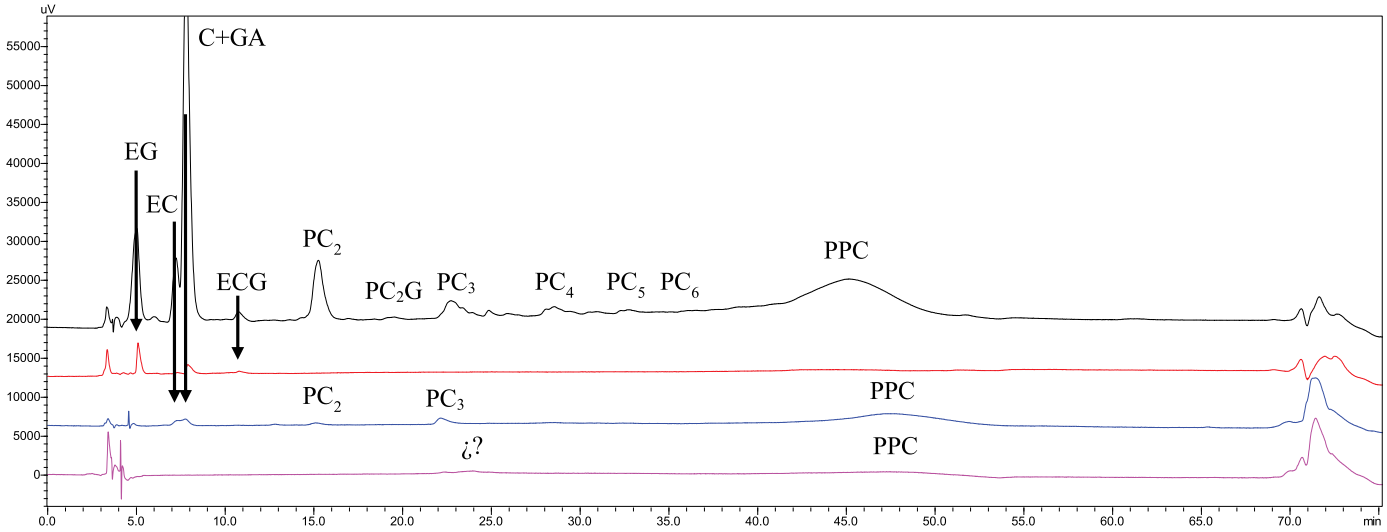


**Fig. 3.** NP-HPLC chromatogram traces (280 nm) of grape seed extract dissolved in a mobile phase component B (black), mobile phase component A (red), the insoluble part of the grape seed extract recovered from the mobile phase component B suspension and dissolved in a mixture of water/AA, 98/2 (v/v) (blue) and the insoluble part of the grape seed extract recovered from the 4$^{-th}$ mobile phase component B suspension and dissolved in a mixture of water/AA, 98/2 (v/v) (rose-violet), EG – ethyl gallate, EC - epicatechin, C - catechin, GA – gallic acid, ECG – epicatechin gallate, $PC_x$ – non-galloylated procyanidin oligomers, $x$ = 2–6, $PC_2G$ – galloylated procyanidin dimers, PPC – polymer procyanidins.

(third chromatogram from Fig. 3), that was indicative for an incomplete extraction of these solutes and was due to the only 1 extraction contact between the substrate and the mobile phase. On the other hand, this analysis showed also that the mobile phase component B dissolved preferentially smaller polymer procyanidins, as the peak corresponding to them eluted earlier (min 45.4) than the peak corresponding to polymer procyanidins dissolved in water/AA (min 47.8) and indicates that procyanidins with higher molecular masses were prone to retain more time in the column.

In an effort to overcome the drawback of the 1 contact test, extraction of up to 4 sequential contacts of the grape seed extract with the protic mobile phase component B was carried out. The results showed that even the increased number of extraction contacts, they were not enough to dissolve the extract completely (data not shown). For this, the insoluble part of the fourth extraction was contacted with a mixture of water/AA, 98/2 (v/v), dissolved and analysed by NP-HPLC. In this case, an aliquot of 100 µL was injected to make the analytical response appreciable. The results of this analysis (last chromatogram of Fig. 3) showed the presence of only few unknown and unresolved peaks, as well as rests of the peak of polymeric procyanidins and indicated that there were some species that could be retained into the column if they were not pulled out completely from it. This retention could cause a continuous increase of the pressure and therefore, subsequent shrinking of the analytical resolution and reproducibility of the method and was one of the main reasons for extending the chromatographic elution proposed by Kelm et al. [19] from up to 40 to 100% of component B and introduction of a third, more polar component (C) (water/acetic acid (98/2, v/v) to the mobile phase at the end of the elution, for assuring complete column washing.

*3.1.2.2. Multiple contact dynamic test of procyanidin redissolution in an aprotic/protic NP-HPLC solvent system.* Hence, a multiple contact dynamic extraction test was designed, using the proper NP-HPLC column as an extraction unit and sequential isocratic extractions (elution's) with solvents of increasing proportions of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 100% of the component B (vs. component A, at 0% component C), as described in experimental Section 2.2.2.

The next Fig. 4 picks-up an overlay of the NP-HPLC chromatograms corresponding to the whole grape seed extract analysed at isocratic conditions of elution of 0, 10, 30, 50, 70% of the mobile phase component B and at normal NP-HPLC gradient elution.

Analysis of the grape seed extract at isocratic elution with 100% of mobile phase component A (first chromatogram from the top of Fig. 4), followed by sequential washing of the column with 100% of mobile phase component B and then C, permitted attaining of a complete view of the most species absorbing at 280 nm. At the isocratic part of the chromatogram (min 0–37), only the monomers ethyl gallate, epicatechin, catechin, gallic acid and epicatechin gallate were identified. They eluted later (min 5–18, instead of min 5–11) and were better resolved when analyzed at normal gradient elution conditions (the last chromatogram of Fig. 4). With the exception of epicatechin gallate, elution of these compounds followed an increasing order of polarities, that is consistent with a whatever elution of low to moderately polar molecules in NP-HPLC and means that separation of these compounds has followed the classic adsorption/partition phenomena of NP-HPLC. On the other hand, none of the identified procyanidin oligomers (OPC) or non-separable polymers (PPC) eluted at these isocratic conditions, that means they were not soluble in the weaker aprotic mobile phase component A (non-solvent). Nevertheless, they eluted later (min 44–55), in the interval that corresponds to the fast increase of the component B in the mobile phase of up to 100% (min 37–49, Table 2) (washing part of the chromatogram), which was stronger solvent for procyanidins (Fig. 4) and produced their joint elution from the column.

By increasing the proportion of component B in the next isocratic elution's (Fig. 4), procyanidin oligomers were mobilized (solubilized), showing a clear tendency of elution with a shorter retention time. Proportions of up to 50% of the component B, moved all oligomers to the beginning of the chromatogram and left the peak of the polymers completely separate from oligomers, but also with diminished area ($p \leq 0.05$) (Fig. 5), because of the elution of some of the non-separable polymers (probably the smallest by size). Proportions of component B higher than 70% redissolved completely the non-separable procyanidin polymers and all together eluted in a singular peak at the beginning of the chromatogram.

All these findings suggest that the peaks eluted at gradient conditions during the first 9 min corresponded to molecules that

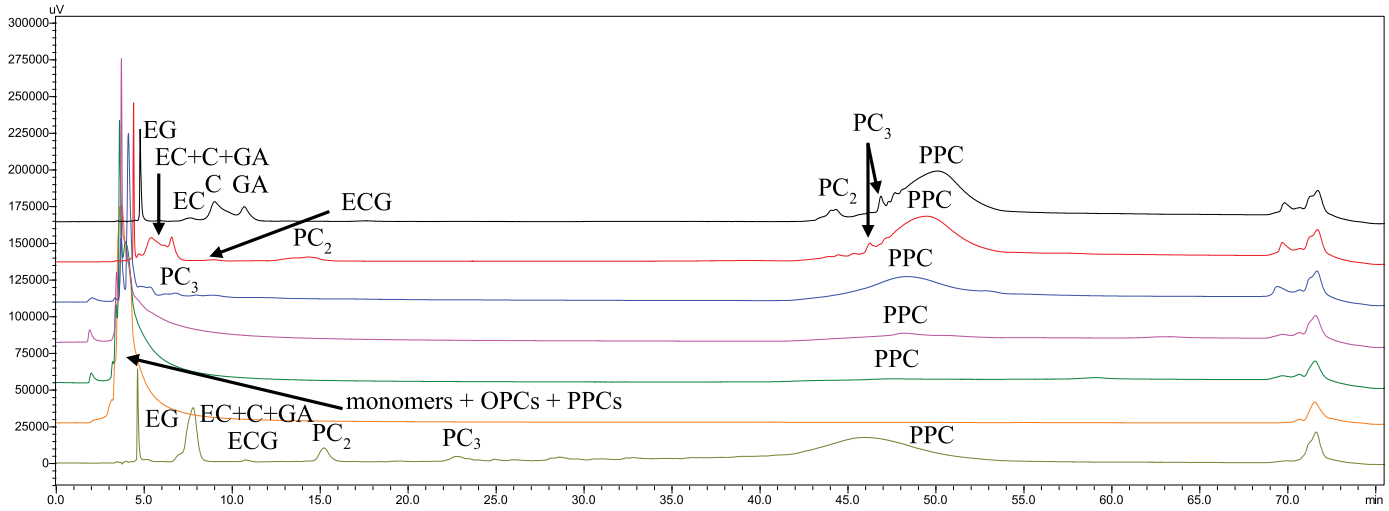


**Fig. 4.** NP-HPLC chromatogram traces (280 nm) of the whole grape seed extract analysed at isocratic conditions of elution, 100% of A/0% of B (black trace), 90% of A/10% of B (red trace), 70% of A/30% of B (blue trace), 50% of A/50% of B (rose-violet trace), 40% of A/60% of B (green trace), 30% of A/70% of B (orange trace) and at normal gradient elution (greenish blue), EG – ethyl gallate, EC - epicatechin, C - catechin, GA – gallic acid, ECG – epicatechin gallate, $PC_x$ – non-galloylated procyanidin oligomers, $x = 2–3$, PPC – polymer procyanidins.

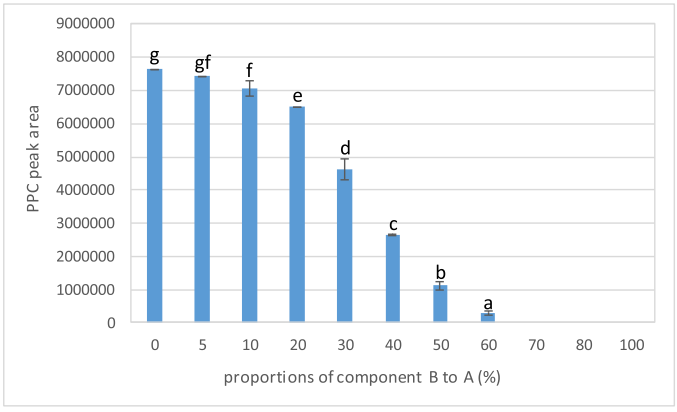


**Fig. 5.** Areas of the peak of polymer procyanidins at each isocratic elution at increasing proportion of component B (to A). Data marked with different letters indicate significant difference between values ($p \leq 0.05$).

dissolved in the component A of the mobile phase at the moment of injection and were separated according to the classic adsorption/partition phenomena of NP-HPLC. The rest of molecules, namely procyanidins, were not soluble in the component A of the mobile phase (non-solvent) and consequently precipitated at the moment of injection of the extract and retained in the stationary phase until the proportion of the more polar (stronger solvent) component B was increased at amounts to dissolve these molecules into the mobile phase. In this case and according to the thermodynamic theory of polymer solubility of Flory–Huggins [22], solvation starts first with the smallest oligomers and continue with polymers with higher degree of polymerization, that results in their elution of increasing order of molar masses. Epicatechin gallate seems elute at a mixed mechanism, as it appeared in the mobile phase component A, but not according to the position corresponding to its lowest polarity before the other identified peaks, rather at the end of them. This order of elution suggests that initially epicatechin gallate probably underwent precipitation and then redissolved for a time that delayed its appearance in the chromatogram. Applying the off-column simulation test for solubility of epicatechin gallate in the aprotic component A of the mobile phase of the used here Diol-NP-HPLC system showed its complete precipitation at concentrations of 0.1 mg $mL^{-1}$ (Fig. 3S) that confirms the same precipitation/redissolution mechanism of its separation, as those already established for the oligomeric procyanidin species.

In light of the foregoing, it becomes clear that separation of catechin, epicatechin and some hydroxybenzoic acids by NP-HPLC was governed by adsorption/partition, while separation of epicatechin gallate and oligomeric procyanidin polymers was based exclusively on their insolubility in the first aprotic (non-solvent) and solubility in the second protic (strong) mobile phase component (solvent) and accomplish a precipitation/redissolution mechanism. As most other known plant proanthocyanidins have very similar structures and differ among them mainly by the position and degree of hydroxylation of their flavan skeleton, the number of elemental units of their polymer chain and the presence of ether linkages in the case of A-type procyanidins [8], these finding should be extended to them, including hydrolysable tannins [23,24] and whatever natural or synthetic homologous series of compounds (e.g. acylcarnitines [25]) or polymers able to precipitate in the first component (non-solvent) of the mobile phase and to redissolve in the second component (strong solvent). This is especially true for the field of synthetic chemistry, where the need for characterization of polymers during their study and production from the last 3–4 decades has promoted the creation and use of huge diversity of chromatographic methods known under the terms of high-performance precipitation liquid chromatography (HPPLC), precipitation-redissolution liquid chromatography (PRLC), gradient polymer elution chromatography (GPEC) or interaction chromatography (IC) [26–28]. The common of these methods is that they are based on the tandem non-solvent/solvent system and according to the polarity of the studied polymers, can take place in both modalities, NP- or RP-HPLC [26,28,29]. With this respect and having in mind the term that better describes the separation mechanism of procyanidin assessment by NP-HPLC is precipitation/redissolution, the most proper definition should be PR-HPLC.

### *3.2. Solubility behavior of procyanidins in high to medium polarity solvent systems*

Since procyanidins were insoluble in the aprotic component A at the moment of their injection, it was essential to study also their solubility behavior in a binomial system of high to medium polarity solvents, such as components C and B of the mobile

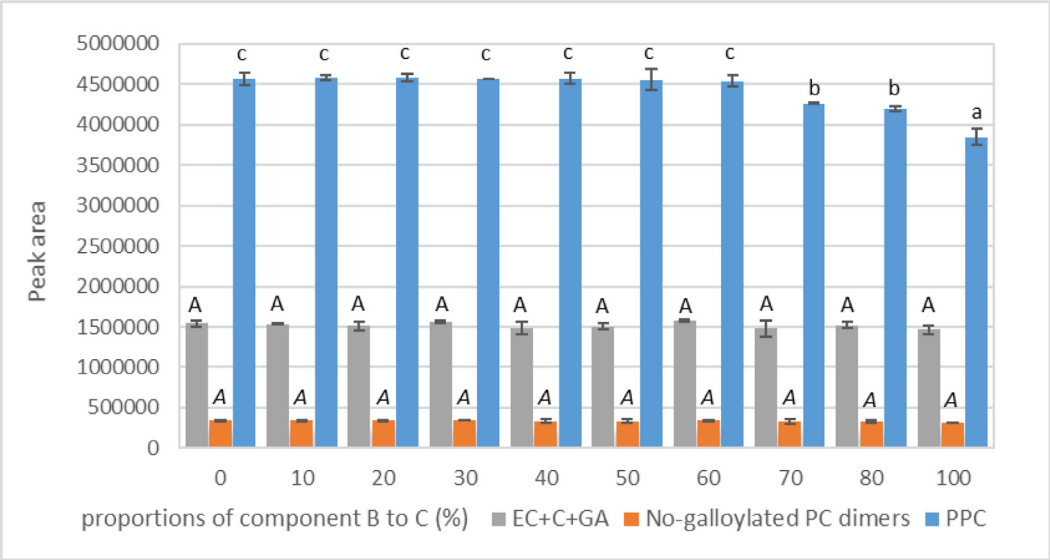


**Fig. 6.** Areas of the peaks corresponding to the sum of epicatechin, catechin and gallic acid (EC+C+GA), non-galloylated procyanidin dimers and procyanidin polymers of the whole grape seed extract dissolved in mixtures of the mobile phase components B (methanol/water/AA, 95/3/2 (v/v/v)) and C (water/AA, 98/2 (v/v)) at proportions of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 100% of B. Data marked with the same letters within the same series of bars indicate no significant difference between values ($p \leq 0.05$).

phase are, as an important step for accurate introduction of the grape seed extract into the chromatographic system. For this, 5 mg of freeze-dried grape seed extract were contacted with mixtures of the mobile phase components B (methanol/water/AA, 95/3/2 (v/v/v)) and C (water/AA, 98/2 (v/v)) at proportions of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 100% of B, sonicated for 5 min and centrifuged at 14,000 g for separation of suspended solids, where they appeared (visual turbidity was found in the samples with proportions of 100, 80 and 70% of B) (experimental section 2.2.3). Peaks corresponding to the sum of epicatechin, catechin and gallic acid (EC+C+GA), non-galloylated procyanidin dimers ($PC_2$) and procyanidin polymers were integrated and their areas were plotted as a function of the corresponding proportions of component B (vs. component C) (Fig. 6).

As it can be seen from Fig. 6, significant differences in solubility ($p \leq 0.05$) were observed only for procyanidin polymers at proportions of B to C from 70 to 100%. This means that the solubility of these procyanidins decreased substantially with the decrease of the solvent polarity and shows that mixtures with up to 60% of component B/C were the solvents of choice for solubilisation of highly polymerized procyanidins. This effect was already established earlier [30]. Within this interval, proportions of at least 20% of component B always are preferable (but not exclusive), because of the antimicrobial effect of methanol that allows better conservation of samples. Whatever deviation of this interval of proportions or solvent system should be tested carefully before use, because important changes could take place. Among them, the most tentative is the substitution of both components B and C of the mobile phase with their precursors, methanol and water. In this case, the solubility of polymeric procyanidins were affected from 60/40 to 100/0% of methanol/water (data not shown) and means that only mixtures with up to 50% of methanol/water were proper.

### *3.3. Other factors that affect the usefulness of NP-HPLC for proanthocyanidin analysis*

Regardless of that NP-HPLC was very good established in many laboratories working with plant proanthocyanidins, this technique has some important limitations that should be taken in consideration for avoiding technical problems, as well as, achieving better chromatographic reproducibility's and result interpretations. Therefore, the main problem derives directly from the already defined mechanism of separation of this chromatographic modality, the precipitation of proanthocyanidins in the non-solvent component of the mobile phase at the moment of their injection. This leads instantly to a certain pressure rise that decrease gradually with the increase of the strong solvent component of the mobile phase. If the sample is prepared correctly, there is no noticeable problems on the chromatographic run, but usually they appear at medium and long terms of time with slow and progressive increase of the pressure that leads subsequently to peak movement and loss of reproducibility. To avoid this inconvenience several cautions should be considered, that should be divided in 2 groups:

#### *3.3.1. Adjustment of the chromatographic conditions in a way to prevent or minimize the long term effects of irreversible retention of some plant metabolites in the stationary phase*

- The methodology proposed by Kelm et al. [19] was based on a gradient elution of 5 to 40% of the component B (methanol/water/AA, 95/3/2 (v/v/v)) to the component A (acetonitrile/AA, 98/2 (v/v)) of the mobile phase. The results of our study showed that for complete solubilisation of the polymer procyanidins from the column, proportions of at least 70% of B/A were required (Fig. 5). For this reason, a gradient that includes an isocratic elution with 100% of component B during 6 min was introduced.
- The present study also showed that even an extension of 100% of mobile phase component B wasn't enough to avoid the irreversibly retention of some compounds in the column. This was the reason for the introduction of the third component C (water/AA (98/2, v/v)) to the mobile phase at the end of the elution for additional 11 min. This means that for binary solvent HPLC systems, a final post-analytical elution/cleaning of the column with water/AA (98/2, v/v) is highly recommended.
- Column/solutes interactions depend highly on heat of the system; therefore, temperature control is one of the conditions with mayor influence on the analytical reproducibility of polymer procyanidins. Therefore, the use of high precision column thermostat for proanthocyanidin analysis by NP-HPLC is highly recommended, as well. In order to minimize the effect of pressure fluctuation after polymer precip-

itation in the column, maintaining of the column temperature in the interval of 35 to 45 °C is preferable.
- Polymer precipitations take place mostly at the head of the column; then, saving of the column for longer time requires the use of guard-columns.
- Monitoring of the pressure recovery from the moment of injection to the end of analysis, should be established as a routine activity for proanthocyanidin assessment by NP-HPLC. This is still more important when unknown vegetable material is explored.

*3.3.2. Sample preparation*
- Not all kinds of hydrophilic vegetable extracts could be submitted directly to NP-HPLC. It is recommended to test their solubility in each of the 2 last components of the mobile phase. If the extract is not soluble in the second component (B) and the insoluble part of B is not soluble in the third component (C) or in mixtures of both, this extract is potentially hazard for the stationary phase and should not be injected into the system. Only the clear soluble part of solutions of B, C or mixtures of them could be used for analysis, as the insoluble phase is of non-proanthocyanidin nature and its luck in the solution does not affect the proanthocyanidin chromatographic profile. Concerning this test is especially important for extracts with high concentrations of native or added proteins that are usually insoluble in the component B of the mobile phase (for example proteolytic enzymes from digestive experiments).
- Not all membrane filters available in the market are adequate for grape seed extract filtration. In general, all of them are designed to retain suspended particles and leave small analytes to pass freely through them. With regard to this, they accomplish in most of the cases this objective. The problem comes when polymers with high molecular masses (macromolecules), that have colloidal behavior when they are dissolved, become object of analysis. In these cases, for filtration of protein solutions most recommended are low protein-binding cellulose-derived filters, but this is not the case of tannin macromolecules, for which there is no specific filter media. This means that for adequate filtration of tannins previous testing of commercially available filters is essential. For this, a viable reference PPC peak should be established. The only way to do it is injection of a samples with minimal clarification treatment, that could include as strong as possible centrifugation, followed by soft filtration with low binding capacities filters, such as those from glass microfibers (0.7 µm pore size). The main problem with these filters is that they are not membranes and their pore size is rather nominal, that means that particle retention is not thorough and that one and same sample should be filtered 2–3 times with new glass filters in an effort to separate maximum amount of insoluble particles. Taking these requirements into account, several of the most used hydrophilic (PTFEH, PTFE(ACN), MCE, PP, PVDF and PA) and hydrophobic (PTFE and PES) membrane filters with a pore size of 0.45 µm were tested. The obtained results (Fig. S4) showed that, with the exception of the PA and PVDF filters, all tested hydrophilic filters, PTFE(ACN), PTFEH, PP, MCE and PA, including the hydrophobic PTFE filter, produced 0 binding of polymeric procyanidins (tannins). The main surprise was that even the highly hydrophobic PTFE filter didn't retain polymeric procyanidins, despite its considerable resistance to the extract flow. But probably, the main drawback of the use of this filter and especially those with the bigger diameter (25 mm) was that it repelled water extracts so strongly that only up to 400 µL of 1 mL of sample could be recovered. The rest of the sample was retained in the proper filter housing without any possibility of recovery. In contrast, the use of the smaller 13 mm diameter filters allowed recovery of the whole sample volume, even they also required considerable physical effort to pass the sample through the filter. Nevertheless, this problem should be partially overcome by hydrophilisation of the PTFE filter by passing through it 3–5 mL of acetonitrile. The problem of this conditioning is that the filter has to be dried after the passage of the acetonitrile or some part of the initial sample flow has to be discarded to avoid dilution.

Other surprise of the filter test was the considerable retention of polymeric procyanidins from the hydrophilic PA and PVDF filters, which are usually used for filtration of grape seed extracts when analyzed for monomeric and oligomeric flavan-3-ols in RP-HPLC. In the case of PVDF filters (and not PA), when the grape seed extract was dissolved in mixtures of 30 to 100% of components B/C of the mobile phase, spontaneous precipitation in the filtered extracts was produced and this precipitation was more abundant as higher was the proportion of component B (data not shown). The fact that these filters were stable when filtering only the same mixtures of components B and C (without dissolved extract) excludes the possibility of disintegration of the filter material. Therefore, the only explanation of this effect was that PVDF filter media provoked the spontaneous precipitation of part of the colloidal polymeric procyanidins that were solubilized by sonication (Section 2.2)(sections 2.2.2 and 2.2.3) that then have led these solutions to the limits of their solubility (saturation) and that they became unstable in these solvent systems (Fig. 6). Nevertheless, when only low molecular mass species should be analyzed in RP-HPLC and the extracts are dissolved in mixtures with proportions of components B/C $<$ 60%, PVDF filters offer excellent clarification of the samples.

Precautions for using 0.22 µm membrane filters should be still higher.

- Minimizing the amount of injected sample: as a rule, as small is the sample load (volume and concentration), lower is the risk of pressure increase. This requirement was stated also in previous studies for the analysis of other polymers [31].

## Conclusions

The results from the off-column static injection simulation test and the fragmented-column dynamic location of procyanidins, as well as the off-column static simulation solubilisación and the multiple contact dynamic tests used in this study led to the conclusion that separation of grape seed procyanidins and epicatechin gallate in aprotic/protic Diol-NP-HPLC was governed by precipitation/redissolution phenomena, while separation of monomeric species, namely catechins and some hydroxybenzoic acids was based on the classic adsorption/partition mechanism. The wide use of other chromatographic methodologies, known under the term of precipitation/redissolution HPLC in the assessment of synthetic homologous series that are based on the same mechanism of separation, allowed to extend this conclusion to all known plant proanthocyanidin homopolymers, including hydrolysable tannins, if they are able to accomplish this condition. Even this normal phase (precipitation/redissolution) HPLC technique was the only one that allowed a global view of all oligomeric and polymeric procyanidins, it also presents some limitations and particularities, such as the need of adaptation of specific chromatographic conditions and sample preparation, that should be taken in consideration for achievement of better chromatographic stability and reproducibility.

## CRediT author statement

**Esperanza Guerrero-Hurtado:** data curation, formal analysis, investigation, resources, visualization, review & editing, **Alba Gutiérrez-Docio:** data curation, formal analysis, investigation, project administration, resources, visualization, review & editing, Rebeca Fiedorowicz: data curation, resources, review & editing, **Esperanza Mollá:** conceptualization, investigation, project administration, supervision, validation, writing - review & editing, **Guillermo Reglero:** conceptualization, supervision, validation, writing - review & editing, **Marin Prodanov:** conceptualization, data curation, funding acquisition, investigation, methodology; project administration, resources, supervision, validation, visualization, writing - original draft, writing - review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

No data was used for the research described in the article.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.463957.

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