Characterization of grape seed procyanidins by comprehensive two-dimensional hydrophilic interaction x reversed phase liquid chromatography coupled to diode array detection and tandem mass spectrometry (HILICxRPLC-DAD-MS/MS).

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ABSTRACT.

In this work, the development and optimization of a new methodology to analyze grape seed procyanidins based on the application of two-dimensional comprehensive LC is presented. This two-dimensional method involves the use of a microbore column containing a diol stationary phase in the first dimension coupled to either a C₁₈ partially porous short column or a C₁₈ monolithic column in the second dimension. The orthogonal HILICxRP-LC system is interfaced through a 10-port 2-positions switching valve. The optimized HILICxRP-LC separation followed by diode array and tandem mass spectrometry detection (HILICxRP-LC-DAD-MS/MS) made possible the direct analysis of a complex grape seed extract and allowed the tentative identification of 46 procyanidins, including monomers and oligomers till a polymerization degree of 7 units with different galloylation degrees. To the best of our knowledge, this is the first time that this powerful analytical technique is employed to characterize complex procyanidins samples. This work successfully demonstrates the great capabilities of the HILICxRP-LC-DAD-MS/MS coupling for the direct analysis of very complex food samples like grape seeds.

Keywords: grape seeds, HILIC, LCxLC, mass spectrometry, procyanidins, two-dimensional comprehensive LC.

INTRODUCTION.

Condensed tannins, more frequently known as proanthocyanidins, are a complex group of phenolic compounds widely distributed in the plant kingdom being the second most abundant group of natural plant phenolic compounds after lignin [1]. Proanthocyanidins are composed of oligomeric and polymeric forms of different flavan-3-ols monomeric units. This wide group of compounds is divided into three smaller categories depending on the monomers included in their composition. The first group procyanidins, are basically composed by catechin and epicatechin units, being the largest class of proanthocyanidins. The other two groups correspond to propelargonidins, formed by afzelechin and epiafzelechin, and prodelphinidins that are made up of gallocatechin and epigallocatechin units [2]. Besides, monomeric forms esterified with gallic acid are also found in nature, such as catechin, epicatechin, gallocatechin and epigallocatechin gallates. This fact makes even more complex the number of possible combinations for the different proanthocyanidins forms, increasing considerably the chemical diversity of these components and, therefore, the complexity of their analysis. Procyanidins are among the most common phenolic compounds in grapes, where can be found both in skins and seeds [3]. In fact, grape seeds are an important natural source of these components, although exist other important food sources, such as cocoa, apples, peanuts or berries [4]. Grape procyanidins have a critical importance during vinification as these compounds have been demonstrated to have a great contribution to the astringency of wines as well as to others organoleptic properties [5]. Besides, it has been suggested that the galloylation degree of the contained procyanidins could increase wine coarseness [6]. The interest on this class of compounds has increased nowadays as procyanidins have been indicated to possess some interesting functional and bioactive

activities, such as antioxidant, antibacterial [7], anti-inflammatory [8] or anti-cancer [9] effects. These important technological and biological characteristics have raised the interest in developing new methods to analyze these compounds.

However, due to their huge chemical complexity and diversity (Figure 1), the analysis of procyanidins is very difficult being typically necessary the use of a separation step followed by mass spectrometry (MS) to identify the multiple compounds present. Different strategies have been explored for their separation, being the most-commonly applied reversed phase LC (RP-LC). In this case, mainly C₁₈ columns are employed, although the complete resolution of all the possible components present on this type of complex profiles is quite difficult to achieve. Besides, the elution order does not correspond to their polymerization degree [4]. Although this RP-LC separation mode could be well suited for monomers and oligomers, the separation of polymers is usually not possible and, generally, polymers of polymerization degree (DP) higher than 3 coelute in a large unresolved peak. In order to partially solve this problem, other separation modes, such as normal phase LC (NP-LC) have been also employed. In this case, procyanidins can be separated according to their polymerization degree [4]. This approach has been also followed to separate procyanidins from grapes [10], among other food samples [11]. The NP approach has been shown to be useful to separate up to DP = 10, however, when the sample is too complex is not possible to have proper resolution between peaks, for instance, when the galloylation degree varies. Besides, all the compounds which present the same DP, coelute. As an evolution of these latter methods, hydrophilic interaction LC (HILIC) has been also employed in order to overcome the problems related to the solvents needed in NP. HILIC popularity is increasing in the last years because it enables to achieve separation similar to those obtained in NP using solvents compatible with RP [12]. The use of water in the mobile

phase allows the formation of a water-enriched layer partially immobilized on the polar stationary phase. Subsequently, the separation is achieved, mainly, by partitioning between the organic rich mobile phase and the water-enriched layer, although other interaction might also occur [13]. However, despite the development of different methods based on the use of these separation methods, the complete analysis of procyanidins still remains unresolved.

To avoid the lack of resolution provided by the mentioned monodimensional separation strategies, bidimensional approaches might be utilized. Comprehensive LC (LCxLC) is characterized by an enhanced resolving power, which can be very useful for complex samples analysis [14]. This technique has been already applied for the separation and characterization of different food-related complex matrices [15,16]. In LCxLC the sample is subjected to two independent separation processes, so that different fractions from the first dimension are continuously transferred to the second dimension for further separation. This approach is far from being straightforward because multiple parameters have to be optimized [17,18,19]. For instance, very fast analyses are needed in the second dimension to be able to handle all the fractions coming from the first one, and consequently, very high flow rates should be employed. In order to keep the system backpressure under attainable conditions, two types of columns are typically employed in the second dimension in comprehensive LC systems: monolithic columns and partially-porous columns. Monolithic columns are formed by a porous continuous gel with a porosity typically 15 % higher than a conventional packed columns. On the other hand, partially porous columns are packed with particles with 2.7 μm of diameter containing a solid core of 1.7 μm and a superficial porous section of 0.5 μm . These particles provide a decrease on analyte diffusivity into the column compared to conventional fully porous particles allowing higher mass transfer rates and higher flow

rates without compromising the column efficiency and generating significantly less backpressure. Two-dimensional LC in off-line mode has already been applied to the separation of procyanidins from apple and cocoa [20]. In that work, a HILIC separation was coupled to a RP-based separation in the second dimension. However, in the mentioned work, fractions eluting from the first dimension were collected and, later on, injected in a second dimension making the procedure lengthy and laborious.

The aim of the present work is to develop a new LCxLC method to automatically analyze grape seed procyanidins without any previous pretreatment. To do that, the performance of different HILIC and RP columns is tested and compared in both dimensions, and all the parameters involved in this challenging coupling closely studied. By using this novel LCxLC-DAD-MS/MS approach, it is possible to separate and identify 46 different procyanidins in the studied grape sample.

EXPERIMENTAL

Samples and chemicals

Grape seeds corresponded to *Vitis vinifera*, variety Malvar, that was provided by IMIDRA from El Encín research fields (IMIDRA, Madrid, Spain).

All the solvents employed (acetonitrile, methanol and 2-propanol) were of HPLC-grade and acquired from Lab-Scan (Dublin, Ireland). Formic acid was supplied by Sigma-Aldrich (Madrid, Spain) whereas acetic acid was purchased from Scharlab (Barcelona, Spain). The water employed was Milli-Q Water obtained from a Millipore system (Billerica, MA). Catechin and epicatechin standards were acquired from Extrasynthèse (Genay, France) and Fluka Analytical (Madrid, Spain), respectively.

Sample preparation

A fast and simple extraction protocol was followed for the extraction of proanthocyanidins from grape seeds: briefly, 80 g of finely grounded grape seeds were weighted and added to 90 mL of extraction solvent (methanol/water 80:20, v/v). The solution was sonicated during 15 min. Afterwards, the solution was left to stand protected from light for 2 h and then, it was again sonicated during 15 min. The extract was centrifuged at 8000 rpm for 20 min. The supernatant was recovered and filtered through 0.45 μ m nylon syringe filters (Symta, Madrid, Spain). Lastly, after evaporation of methanol in a Rotavapor R-210 (Buchii Labortechnik AG, Flawil, Switzerland), the extract was lyophilized using a freeze-dryer (Labconco Corporation, MO).

For the LCxLC analysis of procyanidins, a 50 mg/mL solution of the dried extract was prepared in methanol and filtered (0.45 μ m). Then, 300 mL of this solution were added to 700 mL ACN to obtain a 15 mg/mL solution that was again filtered (0.20 μ m, Symta) and finally injected.

LCxLC instrumentation

Comprehensive two-dimensional LC analyses were carried out on an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector and an autosampler. In order to have robust and reproducible low flow rates and gradients in the first dimension, a Protecol flow splitter (SGE Analytical Science, Milton Keynes, UK) was placed between the first dimension pumps and the autosampler. Besides, an additional LC pump (Agilent 1290 Infinity) was coupled to this instrument to perform the second dimension separation through an electronic controlled 2-position 10-port switching valve. In addition, an Agilent 6320 Ion Trap mass spectrometer equipped with an electrospray interface operating in negative

ionization mode was coupled on-line. The mass spectrometer operated using the following parameters: dry temperature, 350 °C; mass range, m/z 90-2200 Da; dry gas flow rate, 12 L/min; nebulization pressure, 40 psi. The LC data were elaborated and visualized in two and three dimensions using LC Image software (version 1.0, Zoex Corp., Houston, TX).

LCxLC separation conditions

Different columns and conditions were tested for the optimization of the HILICxRP separation of grape seeds procyanidins. In the first dimension, a Synchronis HILIC column (250 x 2.1 mm, 5 μ m d.p., Thermo Scientific, Waltham, MA) as well as a Lichrospher diol-5 (150 x 1.0 mm, 5 μ m d.p., HiChrom, Reading, UK) column were tested. Under the optimum conditions, the Lichrospher diol column with a precolumn with the same stationary phase was employed using 15 μ L/min as flow rate. The mobile phases employed were (A) acetonitrile/acetic acid (98:2, v/v) and (B) methanol/water/acetic acid (95:3:2, v/v/v) eluted according to the following gradient: 0 min, 0 %B; 5 min 20 %B; 10 min, 30 %B; 30 min, 50 %B; 40 min, 50 %B; 50 min, 80 %B; 75 min, 100 %B. The injection volume was 20 μ L.

In the second dimension separation, two different columns were tested. The optimum separation conditions were separately optimized. In first place, a partially porous column Ascentis Express C₁₈ (50 x 4.6. mm, 2.7 μ m d.p., Supelco, Bellefonte, CA) was employed. During the whole LCxLC separation 1.3 min repetitive second dimension gradients were employed, being also 1.3 min the modulation time programmed in the switching valve. Water (0.1 % formic acid, A) and acetonitrile/methanol (B) were the mobile phases, using a repetitive gradient consisting of: 0 min, 0 %B; 0.1 min, 15 %B; 0.3 min, 25 %B; 1 min, 45 %B; 1.01 min, 0 %B. The flow rate was 3 mL/min.

The second column tested in the second dimension was a C₁₈ monolithic column (Onyx C₁₈, Phenomenex, Torrance, CA) was also eluted using 1.3 min repetitive gradients. Two different gradient profiles were employed throughout the analysis. During the first 52 min, the mobile phases employed consisted of water (0.1 % formic acid, A) and methanol (B) eluted according to the following gradient: 0 min, 0 %B; 0.1 min, 15 %B; 0.3 min, 25 %B; 1.0 min, 45 %B, 1.01 min, 0 %B. For the rest of the analysis, the mobile phase composition was changed to water (0.1 % Formic acid, A) and acetonitrile/methanol (B) using the following program: 0 min, 0 %B; 0.1, 15 %B; 0.3, 25 %B; 0.8 min, 45 %B; 0.9, 90 %B; 1.0 min, 0 %B. In this case, the flow rate employed was 4 mL/min.

In any case, 280 nm was the wavelength used to monitor the separations although UV-Vis spectra were collected from 190-550 nm using a sampling rate of 20 Hz in the diode array detector. The MS was operated under negative ESI mode. The flow eluting from the second dimension column was splitted before the MS instrument, introducing approximately 600 µL/min into the MS detector.

RESULTS AND DISCUSSION

The coupling of a HILIC column in the first dimension to a RP-LC column in the second dimension to separate grape procyanidins by LCxLC is, theoretically, a promising alternative. However, this coupling is far from being straightforward due to numerous technical difficulties that arise during the method optimization [21]. For this reason, an initial optimization of the two intended dimensions separately was carried out. Once the preliminary conditions were obtained for both columns, their coupling

was set-up and fine-tuned, as well as the electrospray conditions prior to MS analysis. Data from DAD and MS were combined to chemically characterize the grape seed sample studied.

Optimization of the first dimension separation

To achieve a proper LCxLC separation of procyanidins, it was decided to employ a HILIC column in the first dimension in order to obtain a first distribution of the components present in the sample according to their increasing DP. This novel approach would allow the coupling to a RP-based second dimension avoiding problems related to solvent immiscibility. Nevertheless, as in every comprehensive multidimensional system, some requirements should be met in order to have a good coupling depending on the configuration chosen. One of the most successful multidimensional approaches is to use a switching valve as interface between dimensions equipped with two identical sample loops to transfer the fractions collected from the first dimension to the second dimension [16]. This set-up was the one chosen in this work. In this kind of systems, the first dimension separation has to be carried out at very low flow rates so that enough time is allowed to fill one of the loops while the fraction previously collected in the other loop is being analyzed in a fast second dimension. For this reason, the optimization of the first dimension separation implies great difficulty, mainly related to the proper distribution of wide peaks throughout the chromatogram and the effective translation of the gradient program to the column at those very low flow rates. In this sense, obviously, the nature of the column employed is of great importance as it will influence how the components of the sample will interact with the whole chromatography system during the analysis. To achieve the separation of the procyanidins present on the grape seed sample studied, two different columns were

studied from the wide group of stationary phases available to carry out HILIC separations [22], a silica column and a bonded diol column. Different mobile phases and gradients were studied, always keeping a small proportion of water during the separation. In any case, the retention obtained in the silica column was stronger making very difficult the correct separation of the procyanidins under the desired conditions. The diol column offered better capabilities in this application, allowing a better separation and distribution of the different procyanidin oligomers during the first dimension analysis. The particular conditions were optimized by changing the mobile phases employed as well as the gradients and flow rates and also studying the influence of the sample solvent. This factor was shown to have a critical importance on the separation. In fact, at the beginning the sample was injected dissolved in methanol, which is a good solvent to dissolve the procyanidins oligomers and polymers. However, considering the narrow dimensions of the column employed (150 x 1.0 mm), the introduction of the sample in a solvent as methanol with a higher eluotropic power than the acetonitrile-rich initial mobile phase (under HILIC conditions) prevented the separation as the components of the sample did not have enough time to interact with the stationary phase and eluted practically unretained. For this reason, the sample was prepared more concentrated in methanol and then diluted in acetonitrile (to maintain the same concentration). This change modified completely the behavior of the chromatographic process allowing the separation of the different components. In Figure 2, a typical first dimension chromatogram obtained under optimum conditions is shown. As it can be observed, procyanidins were grouped and distributed along the analysis according to their different DP, as it was expected. The flow rate employed was 15 $\mu\text{l}/\text{min}$. This flow rate was selected as it was low enough to permit the transfer of a relatively low volume of first dimension eluate to the second dimension separation.

Optimization of the second dimension separation

The second dimension separation conditions should allow the fast separation of the components present on each fraction of the first dimension transferred. The length of the second dimension analysis directly influence the volume being transferred in each fraction as each first dimension fraction cannot be transferred until the second dimension analysis is not finished and the second dimension column is prepared. For this reason, 20 μ l was the fraction volume targeted from the beginning of the second dimension optimization, to keep a reasonable injection volume in these analyses. This fact is interesting because even if the solvents employed in both dimensions of a HILICxRP coupling are compatible, the strength of the solvents relative to the two different stationary phases brings about an important problem. Indeed, the stronger solvent in the first dimension will be the weaker one in the second dimension and *vice versa*. Considering the flow rate employed in the first dimension (15 μ l/min), ca. 1.3 min were available for the separation and re-equilibration of the second dimension column after injecting the mentioned 20 μ l from the first dimension. Thus, very fast analyses are needed, and consequently, very high flow rates should be employed. In order to keep the system backpressure under attainable conditions, two types of columns have been mainly employed in the second dimension in comprehensive LC systems: monolithic columns and partially-porous columns. In this work, both types of columns with C₁₈ stationary phases were studied carrying out a preliminary optimization of the separation conditions for each column injecting 20 μ l of the whole sample. Different mobile phases (acetonitrile, methanol, acetonitrile/methanol 80:20, v/v, and acetonitrile/2-propanol 80:20, v/v), flow rates (2-4 ml/min), gradients and temperatures (30-55 °C) were tested. According to the results obtained, mobile phases composed by

(A) water (0.1 % formic acid) and (B) acetonitrile/methanol eluted at 3 ml/min were selected for the partially porous column whereas (A) water (0.1 % formic acid) and (B) methanol eluted at 4 ml/min were chosen for the monolithic column. The gradients employed have been described in the Experimental section. The separation temperature did not influence significantly the separation and only slight decreases on the system backpressure were observed. For this reason, 30 °C was selected as the separation temperature. At the optimum analysis conditions, the backpressures observed were under the instrument capabilities ranging from 150 bar to 320 bar for the monolithic and partially porous columns, respectively. Using both sets of conditions it was possible to have the separation finished and the column reconditioned in 1.3 min. Therefore, this time could be selected as modulation time. To this time corresponds the transfer of a volume of 19.5 μ l of eluate from the first dimension. In this regard, it is important to note that although the sample solvent has great importance on the analysis obtained in the second dimension, as it can be observed in Figure 3A, in LCxLC the second dimension injection solvent cannot be selected as it is imposed by the fractions eluting from the first dimension. Consequently, in the present HILICxRP approach, 19.5 μ l of a strong solvent are injected in the second dimension, which deteriorates the separation obtained. To partially solve this problem, the use of loops in the switching valve with different internal diameter was studied. The idea was to take advantage of the miscibility of the solvents employed in HILIC and RP modes to dissolve the first dimension fraction in second dimension mobile phase in order to avoid the problems generated by the solvents incompatibility. As it is shown in Figure 3B, loops with internal volume of 20, 30 and 50 μ l were tested. It was observed that using a 30 μ l loop the separation was significantly improved at the same time that the volume of injection in the second dimension was kept as low as possible.

Overall HILICxRPLC-DAD-MS optimization

Under the conditions optimized for each dimension, the negative effect of the differential HILIC and RP solvent strength was successfully solved. Thus, HILICxRPLC separations of grape seed procyanidins were carried out using the best conditions for the combinations diol x partially porous columns and diol x monolithic columns. In both cases, the modulation time was 1.3 min corresponding as mentioned to 19.5 μ l of first dimension eluate being transferred each time and diluted in the second dimension mobile phase up to 30 μ l, that corresponds to the internal volume of the loop installed on the switching valve. As it can be observed in Figures 4 and 5, good separations of most of the compounds included in this complex sample were attained. In order to try to improve the separation and ionization yield in the electrospray of the compounds (mainly focusing on those eluting at the end of the first dimension separation, i.e., procyanidins with higher DP) different gradients and mobile phases were tested. In the case of the partially porous column, no significant improvement was observed when the gradient was modified from minute 52. On the other hand, a slight improvement was attained for the monolithic column changing the composition of the mobile phases in this last part, above all, regarding the ionization of these components. Thus, after this optimization, when using the monolithic column in the second dimension, water (+0.1% formic acid) and methanol were employed as mobile phases during the first 52 min, while water (0.1% formic acid) and acetonitrile/methanol were used for the rest of the analysis. Besides, the gradient was also slightly modified, as can be observed in Figure 4.

Concerning the MS detection of these components, negative ESI ionization was employed, as this method is widely considered as the most suitable to obtain a high

response for this kind of compounds [4]. The particular ionization parameters, namely, dry temperature, dry gas flow rate and nebulization pressure, were adapted to the second dimension flow rate splitted to the MS.

It is important to note that the procyanidin polymers with higher DP were not completely resolved. In this sense, although those polymers were eluted at the end of the first dimension analysis, the extremely fast second dimension analysis needed to perform LCxLC did not provide the conditions required to have these compounds properly separated. Nevertheless, it has to be also remarked, that procyanidin polymers with $DP > 4$ have not been fully resolved by monodimensional RP [23]. Thus, the application of the methodology developed in this work implies a clear a significant improvement over the previously published analytical methods.

To proceed with the exhaustive characterization of the natural food sample, the use of the partially porous column in the second dimension was selected as this set of columns allowed using less amount of solvents compared to the set of columns including the monolithic column. Using the optimized method, the peak capacity of the system was studied according to Neue [24]. Theoretical peak capacity of the separation, assuming that the total peak capacity of the two-dimensional system will be the product of the two independent peak capacities values, was 1435. As usual, it is assumed that the entire two-dimensional plane might be occupied by peaks, which is not really the case. For this reason, other approaches have been developed to obtain more realistic peak capacity values. Following the equation developed by Li et al. [25] a value of effective peak capacity equal to 875 is obtained. In any case, this value clearly shows the extremely high potential and enhanced separation power of the developed method, and its application to complex real samples. Besides, it is important to remark the low

correlation obtained between the retention mechanisms employed in both dimensions, as it can be clearly appreciated in Figures 4 and 5, thus, assuring high orthogonality.

Characterization of grape seed procyanidins by HILICxRP-LC-DAD-MS/MS

The development of this HILICxRP method allowed the comprehensive two-dimensional analysis of procyanidins for the first time. The tentative identification of the separated compounds was carried out combining the information from the two detectors employed, DAD and MS detectors. In Table 1, the data corresponding to the separated compounds as well as their tentative identification is presented. In Figure 5, the two-dimensional chromatogram obtained for the grape seed procyanidins analyzed under optimum conditions is also shown. As it can be observed, the compounds were mainly grouped according to their size, firstly eluting the monomers and then eluting the different oligomers according to their increasing DP and increasing galoylation degree (for the same DP) with respect to the first dimension separation.

Although two different detectors were employed, it is important to remark that the UV-Vis spectra of the different grape seeds procyanidins were quite similar, thus, not allowing the differentiation among them. Therefore, the information from the MS detector was critical in order to provide a tentative identification of each separated compound. The first identified compounds were the monomers catechin and epicatechin. Both compounds provided molecular ions with m/z 289 ($[M-H]^-$). The fragmentation of those ions generated ions at m/z 245 as a result of loss of CO_2 . By comparing with commercial standards, peaks 2 and 3 could be identified as catechin and epicatechin, respectively. Other compounds were detected in the area in which these monomers eluted; peak 1 produced an ion at m/z 418.2 ($[M-H]^-$) whose fragmentation

generated ions at m/z 373, 289 and 127. Peak 4 was tentatively identified as gallic acid ethyl ester, according to the molecular ion detected (m/z 197.0, $[M-H]^-$). Different ions at m/z 577 ($[M-H]^-$) were detected corresponding to peaks 6, 7 and 8. The fragmentation pattern of these three compounds was the same (see Table 1) giving prominent fragments at m/z 469 and 425. These latter fragments would be theoretically formed through a retro-Diels-Alder mechanism ($[M-H-152]^-$) [23].

Ions with m/z of 865 (peaks 9, 15, 16 and 18) were assigned to procyanidins trimers. The fragments derived from these ions consisted on m/z 739 and 577, corresponding probably, to the loss of a phloroglucinol unit and to the loss of a (epi)catechin molecule, respectively. These fragments have been previously observed in procyanidins trimers [25,4]. On the other hand, compounds with m/z 729 ($[M-H]^-$) generated fragments at m/z 577, 559, 441, 407 and 289. Consequently, these components (peaks 10-13) were tentatively identified as procyanidin dimers monogallates. The fragments with m/z 577 corresponded to the loss of the galloyl group, whereas the fragments at m/z 559 would be formed as a result of the subsequent loss of a water molecule. The fragments with m/z 441 and 289 were detected as a result of the loss of a (epi)catechin unit and its detection, respectively. The ion at m/z 407 would correspond to the loss of water from a fragment formed through a retro-Diels-Alder mechanism from the m/z 577 ion. Besides, eluting in the second dimension between these components, a single peak with m/z 881.2 ($[M-H]^-$) was detected. This compound was assigned to a procyanidin dimer digallate according to the fragment at m/z 729 detected after its MS/MS analysis.

Peaks 17, 19 and 22 were tentatively assigned to procyanidin trimers monogallates. The detection of molecular ions ($[M-H]^-$) at m/z 1017 together with the presence of fragments at m/z 729 (loss of a (epi)catechin unit), 865 (loss of galloyl group), 577 (loss of a (epi)catechin unit with a galloyl group) and 891 allowed the identification. These

fragment ions have been described as common fragments resulting from these compounds [4]. In Figure 6, a typical fragmentation pattern for these compounds is shown. Besides, a doubly-charged ion ($[M-2H]^{2-}$) with m/z 584.5 was also detected. The MS/MS analysis of this compound (peak 21) allowed the detection of fragment ions closely related to losses of galloyl moieties (m/z 1017 and 999), (epi)catechin units (m/z 880) and both (m/z 729). Consequently, peak 21 was assigned to a procyanidin trimer digallate.

Several procyanidin tetramers and procyanidin tetramers monogallates were found. Procyanidin tetramers were detected either as single-charged ions ($[M-H]^-$) m/z 1153 or doubly-charged ions (m/z 576.7). In any case, the fragmentation pattern was quite similar producing fragments at m/z 1027 (loss of phloroglucinol unit), 865 (loss of (epi)catechin), 575 (loss of two (epi)catechin units) as well as other typical fragments (m/z 739, 403). Likewise, procyanidin tetramers monogallates (peaks 27-31) were mostly detected as doubly-charged ions ($[M-2H]^{2-}$) at m/z 652, although the detection of singly-charged ions was also possible with m/z 1305.4 ($[M-H]^-$). Moreover, a procyanidin tetramer digallate was also detected as $[M-2H]^{2-}$ with m/z 728.5 (peak 36). The fragments found corresponding to losses similar to those already described confirmed the identification of all these DP = 4 procyanidins.

The procyanidins with DP = 5 detected included pentamers (peaks 32-35, 37 and 39), procyanidin pentamers monogallates (peaks 31 and 38) and procyanidin pentamers digallates (peaks 40-42). All these components were detected as doubly-charged ions, with the exception of two procyanidin pentamers that were detected as $[M-H]^-$ with m/z 1141.5. Concerning the procyanidin pentamers fragmentation pattern, several fragment ions which confirmed the identification were detected; among them, fragments with m/z 1315 corresponding to a phloroglucinol unit loss, m/z 1153 matching with a

(epi)catechin loss, m/z 1027 equivalent to a (epi)catechin with a phoroglucinol unit loss, m/z 863 corresponding to the loss of two (epi)catechin units and m/z 577 matching the loss of three (epi)catechin units. Ions with m/z 796.4 and 872.5 were assigned to procyanidin pentamers monogallates and digallates ($[M-2H]^{2-}$) and presented typical procyanidin oligomers fragments that confirmed the identification.

Lastly, the highest DP compounds identified corresponded to procyanidin hexamer (peak 44, m/z 864.7), procyanidin hexamer monogallates (peaks 43 and 45, m/z 940.8, see Figure 6) and procyanidin heptamer (peak 46, m/z 1008.4) which were detected as doubly-charged ions. Their fragmentation patterns were consistent with those typical of procyanidin oligomers.

Thus, the application of this new two-dimensional comprehensive HILICxRP-LC-DAD-MS method to the analysis of procyanidins from grape seeds allowed the separation and tentative identification of 46 compounds, including monomers and oligomers up to $DP = 7$.

CONCLUSIONS

In this work, the development of a new two-dimensional comprehensive HILICxRP-LC-DAD-MS/MS method to analyze grape seed procyanidins is presented. This application included the use of a microbore column with diol stationary phase for the HILIC separation in the first dimension and, either, a C_{18} partially porous column or a C_{18} monolithic column to carry out the RP-LC separations in the second dimension. These combinations provided high orthogonality to the multidimensional system. The optimization of this methodology allowed the separation and tentative identification of 46 procyanidins including monomers (catechin and epicatechin) and procyanidin

oligomers up to $DP = 7$. To the best of our knowledge, this is the first time that on-line two-dimensional comprehensive LC is applied to the separation of procyanidins in such a complex natural sample, like grape seeds. This work also opens new possibilities to the analysis of such complex compounds as proanthocyanidins in other food and natural complex sources.

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FIGURE LEGENDS.

Figure 1. Chemical structure of the most common flavan-3-ols forming part of procyanidins.

Figure 2. HILIC chromatogram (280 nm) of a grape seed procyanidins extract obtained under the optimum first dimension separation conditions using a microbore column (diol stationary phase, 150 x 1.0 mm, 5 μ m). DP, polymerization degree.

Figure 3. A) Effect of the injection solvent on a 2D separation using the partially porous column, and B) effect of the use of loops with different internal volume installed in the switching valve maintaining 19.5 μ l as transfer volume.

Figure 4. Two-dimensional HILIC x RP plot (280 nm) of the separation of grape seed procyanidins using a monolithic column in the second dimension. Scheme of the gradients employed during the analysis in both dimensions. D1, (A) acetonitrile/acetic acid (98:2, v/v) and (B) methanol/water/acetic acid (95:3:2, v/v/v); D2 purple line, (A) water (0.1 % formic acid) and (B) methanol; D2 red line, (A) water (0.1 % formic acid) and (B) acetonitrile/methanol.

Figure 5. Two-dimensional HILIC x RP plot (280 nm) of grape seed procyanidins separated using a partially porous column in the second dimension. For peak identification see Table 1.

Figure 6. UV-Vis and MS spectra of a procyanidin hexamer monogallate (A) and a procyanidin trimer monogallate (B). Main detected fragments of m/z 940.7 ($[M-2H]^{2-}$):

m/z 1593 ([M-H-(epi)catechin]⁻), m/z 1305 ([M-H-2(epicatechin)]⁻), m/z 1153 ([M-H-2·(epicatechin)-phloroglucinol]⁻), m/z 577 (dimer), m/z 289 ((epi)catechin). Main detected fragments of m/z 1017.2 ([M-H]⁻): m/z 865 ([M-H-phloroglucinol]⁻), m/z 729 ([M-H-(epi)catechin]⁻), m/z 577 (dimer).

Table 1. Main procyanidins tentatively identified on grape seed using the optimized HILICxRPLC-DAD-MS/MS optimized methodology. Peaks as shown in Figure 5.

Peak	Identification	Total t_R (min)	D2 t_R (s)	[M-H] ⁻	[M-2H] ²⁻	Main fragments
1	<i>Not identified</i>	10.77	22.2	418.2		373, 289, 127
2	Catechin	10.92	31.7	289.7		245
3	Epicatechin	11.02	36.9	289.3		245
4	Gallic acid ethyl ester	9.81	42.6	197.0		
5	<i>Not identified</i>	9.87	46.3	573.3		525, 377, 329, 195
6	Procyanidin dimer	35.55	26.9	577.3		469, 425
7	Procyanidin dimer	35.58	28.7	577.8		469, 425
8	Procyanidin dimer	35.65	32.4	577.3		469, 425
9	Procyanidin trimer	38.26	33.8	865.3		739, 577
10	Procyanidin dimer monogallate	38.29	35.3	729.7		577, 559, 407
11	Procyanidin dimer monogallate	38.31	36.7	729.7		577, 559
12	Procyanidin dimer monogallate	38.58	52.5	729.2		577, 559, 441
13	Procyanidin dimer monogallate	38.61	54.7	729.2		577, 559, 441, 407, 289
14	Procyanidin dimer digallate	39.67	39.8	881.2		729
15	Procyanidin trimer	40.78	28.9	865.5		739
16	Procyanidin trimer	40.89	35.3	865.5		739
17	Procyanidin trimer monogallate	41.02	43.0	1017.3		891, 847, 729, 577, 441
18	Procyanidin trimer	41.96	21.4	865.3		739, 695, 577
19	Procyanidin trimer monogallate	42.10	29.9	1017.4		729, 865, 999, 577
20	Procyanidin tetramer	42.12	30.9	1153		
21	Procyanidin trimer digallate	42.18	35.0		584.5	999, 880, 1017, 865, 729
22	Procyanidin trimer monogallate	42.39	47.5	1017		
23	Procyanidin tetramer	43.35	26.9	1153.5		1027, 983, 865, 739, 575, 403
24	Procyanidin tetramer	43.38	29.1		576.7	1027, 863, 739, 577
25	Procyanidin tetramer	43.48	34.7	1153.4		1027, 983, 865, 739, 575
26	Procyanidin tetramer	43.50	36.2	1153.4		1027, 983, 865, 739, 575
27	Procyanidin tetramer monogallate	43.33	38.5		652.3	1179, 1017, 863, 729, 575
28	Procyanidin tetramer monogallate	43.63	43.7		652.4	1179, 1017, 863, 729, 575
29	Procyanidin tetramer monogallate	43.71	48.4		652.4	1179, 1017, 863, 729, 575
30	Procyanidin tetramer monogallate	44.67	28.0	1305.4		
31	Procyanidin tetramer monogallate	44.72	31.4	1305.4		
32	Procyanidin pentamer	44.76	33.3		720.7	1316, 1153, 1027, 863, 729, 577
33	Procyanidin pentamer	44.86	39.4		720.9	1316, 1153, 1027, 865, 577
34	Procyanidin pentamer	46.01	30.3		720.8	1316, 1153, 1027, 863, 577
35	Procyanidin pentamer	46.10	35.7	1441.5		
36	Procyanidin tetramer digallate	46.13	37.5		728.5	1331, 1169, 1017, 879, 719, 575
37	Procyanidin pentamer	46.22	43.1		720.6	1315, 1153, 1027, 863, 577, 441
38	Procyanidin pentamer monogallate	46.24	44.6		796.4	1468, 1304, 1179, 1017, 863, 575
39	Procyanidin pentamer	47.31	30.8	1141.4		
40	Procyanidin pentamer digallate	47.41	36.6		872.5	1575, 1458, 1303, 575
41	Procyanidin pentamer digallate	48.65	33.1		872.6	1457, 1327, 1169, 1017, 575
42	Procyanidin pentamer digallate	48.72	37.4		872.7	1576, 1457, 1169, 1017, 729
43	Procyanidin hexamer monogallate	48.86	45.7		940.8	
44	Procyanidin hexamer	49.93	31.9		864.7	1441, 1314, 1151, 575
45	Procyanidin hexamer monogallate	50.01	36.5		940.8	1593, 1441, 1303, 1152, 864, 739
46	Procyanidin heptamer	51.32	37.2		1008.4

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.