**PROFILING OF PHENOLIC COMPOUNDS FROM DIFFERENT APPLE
VARIETIES USING COMPREHENSIVE TWO-DIMENSIONAL LIQUID
CHROMATOGRAPHY.**

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ABSTRACT.

An innovative analytical approach based on the use of comprehensive two-dimensional liquid chromatography (LC x LC) is applied to obtain the profiling of phenolic compounds in different apple varieties. The method combines the use of hydrophilic interaction liquid chromatography in the first dimension and a reversed phase separation in the second dimension, as well as the use of diode array and mass spectrometry detection. Using this methodology is possible to obtain in less than 50 min the complete profiling of phenolic compounds in a complex food matrix such as apple. In fact, different flavan-3-ols including procyanidin oligomers with degree of polymerization up to 8, as well as several dihydrochalcones, flavonols and a phenolic acid are tentatively separated and identified in these samples in a single run. Besides, the total phenols and total procyanidins amounts were determined using two in-vitro assays. Reinette apples presented the highest content on total phenols (6.46 mg galic acid equivalents/g dry matter) whereas Granny Smith apples were the richest on total procyanidins (0.73 mg epicatechin equivalents/g dry matter). This work shows the great potential of LC x LC for phenolic compounds profiling in complex food samples.

Keywords: apple; comprehensive LC; flavonoids; LC x LC; phenolic compounds, procyanidins.

1. INTRODUCTION.

Natural phenolic compounds are receiving a lot of attention due to their potential beneficial health properties [1]. As a consequence, a great amount of research is being focused on the determination of this kind of compounds [2]. Although, the links between their consumption and the potential influence on health are still not completely understood [3], these effects can be investigated more in depth, i.e., at molecular level, via the recent foodomic approach, which is expected to provide more sounded evidences on the polyphenols bioactivity [4,5].

The profiling of phenolic compounds of a particular sample is usually a tough task, considering the enormous variability of chemical structures included within this wide group of metabolites [2]. Although LC is mainly used for the profiling of phenolic compounds in food-related samples, depending on the relative complexity of the analyzed sample this technique may lack separation power. In this regard, the use of multidimensional techniques significantly improves the separation capabilities compared to their one-dimensional counterparts [6]. Comprehensive two-dimensional liquid chromatography (LC x LC) takes advantage of the combination of two independent separation mechanisms to effectively improve the available resolving power as well as to produce a dramatic increase on peak capacity. To perform this kind of analyses, two different separation processes have to be coupled on-line, so that fractions of the first dimension (D1) eluate are continuously collected and injected into the second dimension (D2). This coupling is not easy, and different strategies have been already developed and applied to improve the hyphenation [7]. Ideally, the most advantageous approaches are composed by two dimensions in which different separation modes are combined, in order to enhance system orthogonality, maximizing the separation power. Due to its characteristics, LC x LC has potential for being a

powerful tool for profiling studies, in which the complete composition on a particular class of compounds present on a food sample is studied.

Comprehensive LC has been already employed to analyze a variety of food samples [8,9], including the study of some phenolic compounds from juices [10,11] , wines [12,13] and beer [14]. Nevertheless, the complex phenolic compounds pattern of apples has not yet been studied using this technique.

Apples are very rich in phenolic compounds of diverse chemical classes [15] and, up to now, it has not been possible to simultaneously determine the major phenolics present on apple belonging to these different subclasses. In this regard, different methods have been presented focused on the determination of procyanidin oligomers on this sample, using high-speed countercurrent chromatography (HSCCC) [16], size exclusion chromatography (SEC) [17], normal phase LC (NP-LC) [18] or even off-line two-dimensional LC [19]. However, these works were mainly focused on procyanidins, and no other phenolic compounds, also important in apples, were analyzed in the same run. On the other hand, to analyze other phenolic compounds RP-based approaches have been usually employed [20,21] and, although using these methods some procyanidins were also detected, these are just limited to dimers, not being possible the analysis of the whole procyanidin oligomers pattern.

We have recently developed a new hydrophilic interaction liquid chromatography (HILIC) x RP method to characterize grape seed procyanidins [22]; based on that work, in the present manuscript, the method is further optimized and expanded to the detection of several classes of phenolic components, other than procyanidins. The new method was applied to the profiling of phenolic compounds in five different apple varieties. Consequently, the aim of this work was the development of a LC x LC method able to analyze the whole profile of phenolic compounds present in apples, including

procyanidin oligomers as well as other flavonoids and phenolic acids, in a single analytical run.

2. MATERIALS AND METHODS.

2.1. Samples and chemicals.

Five apples (*Malus domestica*) of different varieties (Red Starking, Kanzi, Royal Gala, Reinette and Granny Smith) were purchased in a local supermarket in Madrid, Spain.

Acetonitrile, methanol, and 2-propanol were of HPLC-grade and acquired from Lab-Scan (Dublin, Ireland). Formic acid, sodium carbonate, gallic acid, quercetin, phloridzin dihydrate and 4-dimethylamino cinnamaldehyde (DMAC) were supplied by Sigma-Aldrich (Madrid, Spain), whereas ethanol and acetic acid were purchased from Scharlab (Barcelona, Spain). Folin-Ciocalteu phenol reagent and HCl were acquired from Merck (Darmstadt, Germany). Water employed was Milli-Q grade obtained from a Millipore system (Billerica, MA). (+)-Catechin, (–)-epicatechin, procyanidin B1, quercetin-3-O-rutinoside and quercetin-3-O-galactoside reference standards were acquired from Extrasynthèse (Genay, France).

2.2 Sample preparation.

Apple phenolic compounds were extracted according to a previously published protocol slightly modified [23]. Briefly, fresh whole apples were cut in small pieces before their lyophilization in a freeze-dryer (Labconco Corporation, MO). 19 g of lyophilized apple powder were extracted with 80 mL of acetone/water (70:30, v/v) during 20 min using magnetic stirring and protected from light. The resulting extract was centrifuged for 20 min at 1900 g, the supernatant was decanted and the precipitate was again extracted following the same procedure. Both supernatants were pooled and 50 mL of water were

added before the acetone was removed in a Rotavapor R-210 (Buchi Labortechnik AG, Flawil, Switzerland). Next, phenolic compounds were concentrated using solid phase extraction (SPE). Discovery DSC-18 6 mL SPE cartridges (Supelco, Bellefonte, PA, USA) were conditioned with 3 x 5 mL of methanol and with 3 x 5 mL of water. Then, 10 mL of sample were loaded in the SPE column, rinsed with 10 mL of water, and the polyphenols were extracted with 2 x 5 mL of acetone/water (70:30, v/v). Finally, acetone was evaporated again by rotary evaporation, and the remaining aqueous extract was lyophilized.

2.3. Determination of total phenols content (Folin-Ciocalteu method).

The total phenols content of the different apple samples was measured using the Folin-Ciocalteu assay [24] with some modifications. The total volume of reaction mixture was miniaturized to 1 mL. 600 μ L of water and 10 μ L of each apple sample (1 mg mL⁻¹ of polyphenol extract in methanol) were mixed, to which 50 μ L of undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 150 μ L of 20% (w/v) Na₂CO₃ was added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 μ L of the mixture was transferred into a well of a 96-well microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader Powerwave XS (Bio Tek Instruments, Winooski, VT) and compared to a gallic acid calibration curve (0.032–2 mg mL⁻¹) elaborated in the same manner. Data were presented as the average of triplicate analyses.

2.4. Determination of total procyanidins.

To estimate the total procyanidin content in the apple samples, the p-dimethylaminocinnamaldehyde (DMAC) method was employed according to the work

by Prior et al. [25] with some modifications. In brief, a DMAC solution (0.1% DMAC reagent (w/v) on a mixture of ethanol/water/HCl 75:12.5:12.5, v/v/v) was prepared immediately before use. 70 μ L of each apple sample (0.075 mg mL⁻¹ of polyphenol extract in methanol) were mixed with 210 μ L of the DMAC solution. The mixture was vortexed, transferred into a well of a 96-well microplate and allowed to react at room temperature for 15 min. After this time, the absorbance was read at 640 nm using a microplate spectrophotometer reader Powerwave XS (Bio Tek). Blanks with 70 μ L of methanol instead of sample and a control samples without DMAC solution were also included. Each sample, blank and control was prepared in triplicate. The concentration of total procyanidins was estimated from a calibration curve using epicatechin (0.001-0.02 mg mL⁻¹). Data were presented as the average of duplicate analyses.

2.5. Comprehensive two-dimensional liquid chromatography (LC x LC) analysis of apple phenolic compounds.

2.5.1. Instruments.

LC x 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, hyphenated through an electronic controlled two-position ten-port switching valve. An Agilent 6320 Ion Trap mass spectrometer equipped with an electrospray interface was coupled on-line and operated in negative ionization mode using the following conditions: dry temperature, 350 °C; mass range,

m/z 90–2200 Da; dry gas flow rate, 12 L min⁻¹; and 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).

2.5.2. LC x LC separation conditions.

Samples were prepared by diluting 6 mg of polyphenol extract of each apple variety in 300 µL of methanol and adding 700 µL of acetonitrile to obtain a 6 mg mL⁻¹ solution, which was filtered through 0.45-µm nylon syringe filters (Symta, Madrid, Spain) before injection.

In the first dimension, a Lichrospher diol-5 (150×1.0 mm, 5 µm, HiChrom, Reading, UK) column was employed with a precolumn with the same stationary phase. The optimized flow rate employed was 21 µL min⁻¹, from minute 0 to 24, and 15 µL min⁻¹ from minute 24 to the end of the analysis. 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; 2 min, 0 % B; 5 min, 20 % B; 30 min, 20 % B; 40 min, 30 % B; 50 min, 30 % B. The injection volume was 20 µL.

In the second dimension, an Ascentis Express C₁₈ (50×4.6 mm, 2.7 µm d.p., Supelco, Bellefonte, CA) partially porous column was employed together with a C₁₈ precolumn. During the whole LC×LC separation, 78 s-repetitive second dimension gradients were employed, being also 78 s the modulation time programmed in the switching valve. Two different gradient profiles were employed throughout the analysis. During the first 25.4 min of two-dimensional analysis, the mobile phase employed in D2 consisted of water (0.1 % formic acid, A) and acetonitrile (B) eluted according to the following gradient: 0 min, 0 % B; 0.1 min, 15 % B; 0.8 min, 50 % B; 1.0 min, 70 % B; 1.01 min, 0 % B. From minute 25.4 till the end of the analysis, the mobile phase composition was

changed to water (0.1 % formic acid, A) and acetonitrile/methanol (50:50, v/v) (B) using the following program: 0 min, 0 % B; 0.1 min, 15 % B; 0.3 min, 25 % B; 1.0 min, 45 % B; 1.01 min, 0 % B. The flow rate was 3 mL min⁻¹.

The wavelength used to monitor the separations was 280 nm, although UV–Vis spectra were collected from 190–550 nm during the whole analysis 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, so that the flow rate entering the MS detector was approximately 600 µL min⁻¹.

3. RESULTS AND DISCUSSION.

3.1. Optimization of sample preparation for the analysis of phenolic compounds from apple.

The first part of this study consisted on the search of optimum extraction conditions to obtain a representative sample of apple phenolic compounds. Apples are very well-known for possessing high amount of procyanidins as well as other phenolic compounds [26]. Although different advanced extraction techniques have been already employed to obtain particular apple polyphenols, such as supercritical fluid extraction [27], microwave-assisted extraction [28] or pressurized liquid extraction [29], for the aim of the present study, a relatively fast and easy extraction method capable of providing with a wide mixture of the entire pattern of phenolic compounds present on these samples was sought. To do this, different extraction methodologies were initially tested, including extraction with ultrasounds as well as magnetic stirring using acetone:water 70:30 (v/v) as extraction solvent. The optimization of the extraction procedure was monitored using a HILIC-based method, which was the basis of the first

dimension of a previous LC x LC method involving the separation of procyanidins polymers on grape seeds depending on their degree of polymerization (DP) [22]. The use of magnetic stirring followed by a SPE clean-up and concentration step provided the best results. Once selected the initial methodology for the extraction, the employment of different SPE cartridges with diverse stationary phases was tested in order to find the best conditions to clean-up and concentrate the phenolic compounds-rich extract from apples. Namely, amino, HLB and C₁₈ stationary phases were studied. Using the C₁₈ cartridges, extracts that produced a better distribution in the HILIC separation were attained (Figure 1A). Thus, this stationary phase was finally selected to carry out the extraction procedure. Once all the steps of the extraction protocol were optimized, the phenolic compounds-rich extracts from apples were obtained by extracting 19 g of lyophilized apple with 80 ml acetone:water 70:30 (v/v) twice, and the resulting extracts were further concentrated using SPE as detailed above.

3.2. HILIC x RP-DAD-ESI-MS analysis of phenolic compounds from apple.

The next step consisted on the optimization of the separation method. Although, as it has been already mentioned, apple procyanidins have been previously analyzed using NP-LC [18], SEC [17] and HSCCC [16], all these methods have several drawbacks, mainly involving lengthy analysis times and the impossibility to separate the different procyanidins having the same DP. Since our goal was to separate apple procyanidins together with other phenolic compounds by using an LC x LC approach, a set-up comprising a HILIC separation in the first dimension (D1) coupled to a fast RP-LC separation in the second dimension (D2), previously developed for the separation of grape seeds proanthocyanidins, was initially tested [22]. However, since evident differences were expected between the compositions of these samples, mainly

considering that procyanidins pattern in apples is simpler than in grape seeds whereas apples contain greater amounts of other flavonoids and phenolic acids than grape seeds, no complete separations could be achieved with the original method and further re-optimization of the HILIC x RP method was necessary to obtain a clear profile of the entire phenolic compounds composition from apples. As the optimization of a two-dimensional separation is not an easy task, each dimension was studied separately, although LC x LC analyses during optimization were also necessary, not only to confirm the efficacy of the changes performed but also to suggest further modifications for the fine tuning of the employed conditions.

Firstly, the HILIC separation was optimized modifying the gradient employed in order to get a better distribution of the sample through the available separation space. In this regard, it is important to keep in mind that the chromatographic conditions employed in the D1 will significantly influence the D2. A maximum injection volume in the D2 of 30 μL was considered, limited by the maximum capacity of the injection loops. Nevertheless, as we formerly showed, the transfer of smaller volumes to the injection loops installed in the switching valve used as interface between injections ($< 20 \mu\text{L}$), allowed the dilution of the D1 eluate with D2 initial mobile phase, producing better peak shapes and significantly less peak distortion in this latter separation [22]. Consequently, $15 \mu\text{L min}^{-1}$ was selected as flow rate, meaning that the transfer of $20 \mu\text{L}$ into the injection loop would last 78 s, which would be the modulation time as well as the time available to carry out each single D2 separation. Such slow flow rates imply the use of microbore columns so that the needed analytical performance can be attained. After several modifications in the composition and characteristics of the mobile phases and gradients employed, it was decided to maintain the same initial mobile phases using a less steep gradient. Figure 1B shows the chromatogram obtained under the optimum

D1 separation conditions. Final gradient conditions are detailed in section 2.5.2. As it can be observed comparing with Figure 1A, the peaks were more evenly distributed during the analysis.

For the D2 conditions optimization, 78 s was fixed as target analysis time. Preliminary LC x LC analyses using the gradients from the original method clearly showed that in the last part of the 2D plot peaks were clearly and sufficiently separated. However, some peaks were not completely resolved in the first part of the 2D plot. To solve this problem, dynamic gradients were employed in the D2 analyses maintaining a flow rate of 3 ml min⁻¹. The LC x LC analysis was divided into two well differentiated zones: from 0 to 25.4 min and from 25.4 to the end of the analysis. The original conditions involving the use of water plus 0.1 % formic acid (A) and acetonitrile/methanol 50:50 v/v (B) as mobile phases were maintained in the final part, while the separation conditions of the initial part were completely re-optimized. Different mobile phases and gradients were employed, finally achieving as optimum conditions the use of water plus 0.1 % formic acid (A) and acetonitrile (B) reaching higher proportions of the organic modifier during the separation. The final separation conditions involved in the D2 are specified in section 2.5.2. In Figure 2A and 2B, a comparison between the separations obtained in the first part of the two-dimensional analysis before and after optimization, respectively, is shown. As it can be appreciated, the peaks coeluting around 60-65 s (Figure 2A) were more clearly resolved after optimization (Figure 2B). It is also possible to observe the separation obtained in the second part of the analysis under the optimum conditions. Moreover, as the relative complexity in this first part in terms of number of different compounds was lower, it was decided to increase the D1 flow rate in order to speed-up the analysis in a certain extent, taking advantage of the volume of the injection loops installed in the switching valve. Thus, the D1 flow rate for the first

part of the analysis (Figure 2B) was established at 21 $\mu\text{l min}^{-1}$, whereas in the second part (Figure 2C), D1 flow rate was maintained at 15 $\mu\text{l min}^{-1}$ to take advantage of the dilution effect produced by the partial use of the injection loop volume available.

3.3. Profiling of phenolic compounds in different apple varieties.

Once the analytical method was optimized, the two-dimensional LC procedure was coupled to MS, including the use of an ESI negative ionization mode in order to assist in the characterization of the apple samples. Five apple varieties, namely Red Starking, Kanzi, Royal Gala, Reinette and Granny Smith, were studied to further demonstrate the applicability of the developed procedure. Table 1 summarizes the main phenolic compounds detected and identified in the five apple varieties studied using the optimized HILIC x RP-DAD-MS/MS methodology. Besides, in Figure 3 a comparison among the typical profiles obtained for all the samples is shown. As can be observed, the phenolic composition of all samples was dominated by the presence of a high number of different flavan-3-ols, mainly catechin and epicatechin as well as procyanidin oligomers up to a DP = 8. Besides, it was also possible to find several dihydrochalcones (phloretin-glucoside, phloretin-xilosyl-glucoside and hydroxyphloretin-diglycoside), flavonols (quercetin-related compounds) and a hydroxycinnamic acid (dicaffeoylquinic acid). The identification of the compounds present on the samples was performed thanks to the information provided by the two detectors coupled in series, DAD and MS, as well as the information collected from MS/MS experiments and the use of commercial standards when available. Figure 4 shows some examples of how the identification was carried out. Among the flavan-3-ols, catechin and epicatechin were correctly identified due to the detection of characteristic ions at m/z 289.7 and 289.5 ($[\text{M}-\text{H}]^-$), respectively. These compounds were differentiated by comparing their

corresponding retention times with those of their available commercial standards. In the case of procyanidin dimers, trimers and tetramers, their typical molecular ions were detected as $[M-H]^-$ at m/z 577, 865 and 1153, respectively. In each case, different fragment ions were produced in the MS/MS experiments, which confirmed the assignments; for example, procyanidin dimers presented fragments at m/z 425, corresponding to a retro-Diels-Alder mechanism ($[M-H-152]^-$), as well as ions of the monomer (m/z 289). Procyanidin trimers presented fragment ions corresponding to the loss of a phloroglucinol unit (m/z 739) as well as to the loss of one or two (epi)catechin molecules, m/z 577 and 289, respectively. On the other hand, procyanidin tetramers were mainly characterized by the presence of ions related to shorter oligomers that would be formed after collision induced dissociation, such as m/z 865 and 577. Longer procyanidin oligomers could not be detected as monocharged ions. Indeed, procyanidin pentamers, hexamers and heptamers were detected as doubly-charged ions, as can be observed in Table 1. Procyanidin pentamers were detected as m/z 720 ($[M-2H]^{2-}$) which fragmentations gave rise to ions related to the losses of a phloroglucinol unit, and one, two or three (epi)catechin moieties (m/z 1315, 1151, 863, 577, respectively), as can be observed in Figure 4A. Similar fragmentation patterns allowed the identification of ions at m/z 864 and 1008 ($[M-2H]^{2-}$) as procyanidin hexamers and heptamers, respectively. Lastly in this group, two procyanidin octamers were detected in several samples as ($[M-3H]^{3-}$) with m/z 768.

Regarding the rest of identified compounds, two phloretin-glycosides were tentatively identified (peaks 4 and 6) in agreement with the main molecular ion detected at m/z 435.6 ($[M-H]^-$) together with the presence of a fragment corresponding to the loss of an hexoside ($[M-H-162]^-$) matching with phloretin aglycone (m/z 273). Another fragment at m/z 167 derived from phloretin has been also previously detected [30]. Moreover, the

UV-Vis spectra of these peaks confirmed the identification showing the maximum of absorbance of phloretin (285 nm) as well as the comparison with its corresponding commercial standard. Other dihydrochalcones identified on the samples were phloretin-xylosyl-glucoside (peaks 7, 11, 16 and 18) and hydroxyphloretin-xylosyl-glucoside (peak 32). The identification of these compounds was performed similarly as for phloretin-glucoside, thanks to the detection of their corresponding molecular ions and fragments as well as from the UV-Vis spectra obtained (see Figure 4B). On the other hand, six different quercetin-related flavonols were also tentatively identified. Three of them corresponded to quercetin-glucoside, namely peaks 15, 22 and 23, although peak 15 was tentatively assigned to quercetin-galactoside, according to their elution order [31]. These three compounds presented similar molecular ion at m/z 463 ($[M-H]^-$) and produced a fragment after MS/MS corresponding to quercetin (m/z 301) as well as similar UV-Vis absorption maximum at 354 nm. Besides, it was also possible to tentatively assign peak 3 to dihydroquercetin-rhamnoside thanks to the detection of an ion at m/z 549 which produced a fragment at m/z 303. Likewise, quercetin-rhamnoside (peak 17) and quercetin-rutinoside (peak 24) were also identified. All these compounds have been already found in several apple-derived materials [31,32].

The basic phenolic profiles of the five apple varieties were quite similar (see Figure 3), formed by groups of peaks belonging to the above-mentioned chemical classes. Nevertheless, the relationships among them were not the same; interestingly, Reinette apples contained higher relative abundance of dihydrochalcones (peaks 4, 6, 7, 11, 16, 18 and 32) than procyanidins. On the other side, Granny Smith apples presented a high number of different procyanidin peaks with significantly higher intensity than other flavonoids. Besides, some characteristic peaks of just one variety could also be detected. In Figure 5, a reconstructed 2D plot is presented in which it is possible to observe the

compounds that were present in all the studied samples, as well as those that were markers for only one sample. For instance, dihydroquercetin-rhamnoside (peak 3) and quercetin-rhamnoside (peak 17) were only present in Kanzi apples. Royal Gala apples possessed two differential procyanidin peaks, a tetramer (peak 40) and a heptamer (peak 62), while Red Starking presented a procyanidin tetramer (peak 33), a pentamer (peak 41), two hexamers (peaks 49 and 55) as well as quercetin–rutinoside (peak 24). Granny Smith, possessed three procyanidin oligomers (peaks 52, 59 and 60) that were not present in any other sample. Thus, the only apple variety which did not present at least a differential phenolic compound was Reinette.

In order to quantitatively assess these differences on the phenolic profiles, two in-vitro assays were carried out. Firstly, the total phenols amount present on the different samples was determined following the Folin-Ciocalteu method. The obtained results are summarized in Table 2. As it can be observed, the apple variety with the higher phenols content was Reinette (6.46 mg GAE g⁻¹ d.m.) followed by Granny Smith and Red Starking, whereas Kanzi presented by far the lowest amount of total phenols (1.21 mg GAE g⁻¹ d.m.). A second in-vitro assay, based on the reaction with p-dimethylaminocinnamaldehyde (DMAC), was employed to determine the total procyanidin content of the studied samples. DMAC has been shown to specifically react with flavanols, enhancing the sensitivity and accuracy for the determination of procyanidins compared to other procedures [33]. As it can be appreciated in Table 2, Granny Smith was the richest apple variety on this class of compounds, reaching 0.73 mg ECE g⁻¹ d.m., followed by Reinette (0.56 mg ECE g⁻¹ d.m.). Again, Kanzi was the sample with the lowest amounts of flavan-3-ols. Interestingly, as was previously pointed out from the analysis shown in Figure 3, the relationships between procyanidins and other compounds were not the same among samples. In fact, the apple variety with the

richest content on procyanidins was Royal Gala, with more than 19% of total phenols corresponding to procyanidins; Granny Smith also contained high amounts of procyanidins (13%) whereas Reinette was the sample with the lowest relative abundance of procyanidins (8.7 %), in agreement with its corresponding LC x LC analysis. These results corroborate the applicability of the developed methodology based on HILIC x RP-DAD-MS/MS, to characterize complex samples involving a great number of compounds belonging to different chemical classes. It is also worth to mention that the total analysis time needed to obtain the complete phenolic compounds' profile of each sample was less than 50 min, which is a rather fast analysis for a comprehensive two-dimensional method. In fact, these analysis times are directly comparable to other one-dimensional methods aimed to the separation of different phenolic compounds in apples which provided significantly less separation power and information [21,32,34-36]. Besides, the optimized HILIC x RP methodology allowed the separation of procyanidin oligomers in the first dimension according to their DP, and their subsequent differentiation using the second dimension, as can be clearly seen in Figure 5.

4. CONCLUSIONS.

In this work, the phenolics profiling of different apple varieties using comprehensive two-dimensional LC is shown for the first time. The developed method, based on a HILIC x RP-DAD-MS/MS coupling was capable to provide the 2D plot of each sample in less than 50 min, allowing the tentative identification of ca. 65 compounds on each studied sample, including flavan-3-ol oligomers up to a DP = 8, dihydrochalcones, flavonols and phenolic acids. By attaining these 2D profiles, a fast visual comparison was possible enabling to distinguish among different phenolic compound classes in a

single run, depending on the elution zone of the 2D plane in which they appear. Among the studied samples, Reinette and Granny Smith were the richer cultivars in terms of total phenolic compounds and procyanidins, respectively. Thus, the applicability of 2D LC for the profiling of complex food samples has been demonstrated, opening new possibilities for the application of procedures based on this technique for other target and non-target metabolomics-related studies.

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FIGURE LEGENDS.

Figure 1. HILIC chromatograms (280 nm) corresponding to the separation of phenolic compounds from Red Starking apple depending on the nature of the SPE cartridges employed during their extraction (A) and under the optimized conditions used for the comprehensive two-dimensional LC analyses (B).

Figure 2. Two-dimensional plots (280 nm) corresponding to the separation of the first eluting apple phenolic compounds before (A) and after (B) optimization, as well as to the second part of the separation in which a different gradient program is employed in the optimized HILIC x RP method (C).

Figure 3. Comparison of the 2D profiles (280 nm) obtained for the five apple varieties studied using the optimized HILIC x RP-DAD-MS/MS methodology. A, Red Starking; B, Kanzi; C, Royal Gala; D, Reinette; E, Granny Smith. For peak identification see Table 1.

Figure 4. UV-Vis and MS/MS spectra of phloretin-xylosyl-glucoside (A) and a procyanidin pentamer (B).

Figure 5. Reconstructed 2D plot of the phenolic compounds identified in the different apple varieties studied showing the compounds present in all the samples (pink spots), in several apples (grey spots) and those differentially found in just one sample. Kanzi, yellow spots; Royal Gala, green spots; Red Starking, red spots; Granny Smith, orange spots. Circles represent the separation of procyanidins according to their degree of polymerization (DP) in the first dimension.

534 **Table 1.** Main phenolic compounds tentatively identified in the five different apple varieties studied using the optimized HILIC×RPLC-DAD-
535 MS/MS profiling methodology.

ID	Identification	Total t _R (min)	D2 t _R (s) ± sd	[M-H] ⁻	Main MS/MS fragments	UV-Vis maxima (nm)
1	Catechin *	6.90	24.17 ± 0.09	289.7	245	280
2	Epicatechin*	6.98	28.83 ± 0.16	289.5	245	280
3	Dihydroquercetin rhamnoside	7.57	64.22 ± 0.12	549.3	303	340
4	Phloretin-glucoside *	11.07	40.23 ± 0.12	435.6	273, 167	285
5	Dicaffeoylquinic acid	12.18	28.94 ± 0.11	515.4	353, 191	278
6	Phloretin-glycoside	18.82	37.12 ± 0.27	435.8	273	285
7	Phloretin-xylosyl-glucoside	27.91	36.80 ± 0.11	567.4	273	285
8	Procyanidin dimer	29.03	25.79 ± 0.08	577.5	425, 289, 559	280
9	Procyanidin dimer	29.05	27.03 ± 0.15	577.2	425, 289, 559	280
10	Procyanidin dimer	29.16	33.40 ± 0.16	577.2	425, 289, 559	280
11	Phloretin-xylosyl-glucoside	29.23	38.01 ± 0.17	567.3	273, 167	285
12	Procyanidin dimer	30.41	30.40 ± 0.17	577.8	425, 289, 559	280
13	Procyanidin dimer	30.44	32.21 ± 0.20	577.5	425, 289, 559	280
14	Procyanidin dimer	30.66	45.49 ± 0.21	577.5	425, 289, 559	280
15	Quercetin-galactoside *	30.73	49.86 ± 0.22	463.3	301	354
16	Phloretin-xylosyl-glucoside	30.80	54.33 ± 0.29	567.4	273	285
17	Quercetin rhamnoside	30.81	54.68 ± 0.04	447.3	301	350
18	Phloretin-xylosyl-glucoside	30.84	56.32 ± 0.33	567.4	273	285
19	Procyanidin dimer	31.72	31.12 ± 0.05	577.3	425, 289, 559	280
20	Procyanidin dimer	31.75	32.75 ± 0.07	577.2	425, 289, 559	280
21	Not identified	31.97	46.02 ± 0.19	599.1	447, 295	280
22	Quercetin-glucoside	32.05	50.84 ± 0.28	463.5	301	356
23	Quercetin glucoside	31.12	55.01 ± 0.09	463.8	301	350
24	Quercetin-rutinoside *	33.29	47.58 ± 0.58	609.7	301	350
25	Procyanidin trimer	34.30	29.70 ± 0.23	865.5	739, 577, 289	280
26	Procyanidin trimer	34.32	31.28 ± 0.22	865.6	739, 577	280
27	Procyanidin trimer	34.33	31.47 ± 0.19	865.3	739, 577, 425, 289	280
28	Procyanidin trimer	34.37	34.39 ± 0.23	865.4	739, 577	280

29	Procyanidin trimer	34.40	35.71 ± 0.21	865.3	739, 577, 289	280
30	Procyanidin trimer	34.42	36.96 ± 0.22	865.3	739, 577	280
31	Procyanidin trimer	34.55	44.81 ± 0.18	865.2	739, 577, 289, 245	280
32	Hydroxyphloretin-xylosyl-glucoside	34.62	49.08 ± 0.30	583.2	289, 167	285
33	Procyanidin tetramer	35.66	33.50 ± 0.40	1153.5	1027, 983, 865, 739	280
34	Procyanidin tetramer	36.85	27.29 ± 0.45	1153.3	1027, 983, 865, 739, 575	280
35	Procyanidin tetramer	36.89	29.68 ± 0.37	1153.6	1135, 983, 865, 739, 575	280
36	Procyanidin tetramer	36.91	30.66 ± 0.05	1153.5	1135, 983, 865, 739, 577	280
37	Procyanidin tetramer	36.93	31.79 ± 0.23	1153.5	1135, 983, 865, 739, 575	280
38	Procyanidin tetramer	36.99	35.26 ± 0.08	1153.6	1135, 983, 865, 739, 575	280
39	Procyanidin tetramer	37.01	36.71 ± 0.22	1153.3	1135, 1027, 983, 865, 739, 575	280
40	Procyanidin tetramer	37.23	49.77 ± 0.84	1153.3	1135, 1027, 983, 865, 739, 575	280
41	Procyanidin pentamer	37.28	49.52 ± 0.63	719.8 ^a	1315, 1151, 1025, 863, 575, 287	280
42	Procyanidin pentamer	39.46	27.52 ± 0.33	720.4 ^a	1315, 1151, 1027, 863, 577, 287, 245	280
43	Procyanidin pentamer	39.49	29.42 ± 0.19	720.6 ^a	1315, 1151, 1027, 863, 739, 577, 289, 245	280
44	Procyanidin pentamer	39.50	29.83 ± 1.16	720.5 ^a	1315, 1151, 1027, 863, 577, 289	280
45	Procyanidin pentamer	39.53	31.57 ± 0.42	720.6 ^a	1315, 1151, 1027, 863, 577, 289	280
46	Procyanidin pentamer	39.54	32.64 ± 0.31	720.4 ^a	1315, 1151, 1027, 863, 577, 289	280
47	Procyanidin pentamer	39.61	36.72 ± 0.13	720.5 ^a	1315, 1151, 863, 577, 289	280
48	Procyanidin pentamer	39.62	37.49 ± 0.57	720.4 ^a	1315, 1151, 1027, 863, 577	280
49	Procyanidin hexamer	40.89	35.15 ± 0.46	864.1 ^a	1603, 1315, 1153, 719, 575, 287	280
50	Procyanidin hexamer	42.12	30.94 ± 0.42	864.6 ^a	1603, 1441, 1151, 1027, 719, 577, 287	280
51	Procyanidin hexamer	42.14	32.48 ± 0.16	864.5 ^a	1603, 1441, 1315, 1153, 719, 575, 289	280
52	Procyanidin hexamer	42.20	36.12 ± 0.08	864.6 ^a	1605, 1441, 1315, 1151, 719, 575, 287	280
53	Procyanidin hexamer	42.23	37.73 ± 0.43	864.5 ^a	1604, 1441, 1315, 1151, 719, 577, 289	280
54	Procyanidin hexamer	42.25	38.73 ± 0.24	864.6 ^a	1603, 1151, 719, 577, 289	280
55	Procyanidin hexamer	43.51	36.62 ± 0.39	864.4 ^a	1603, 1441, 1153, 719, 577, 287	280
56	Procyanidin heptamer	44.73	31.98 ± 0.25	1008.5 ^a	1729, 1603, 1441, 1316, 1151, 863, 577	280
57	Procyanidin heptamer	44.83	37.56 ± 0.05	1008.7 ^a	1727, 1605, 1153, 1027, 865, 739, 577, 287	280
58	Procyanidin heptamer	44.85	39.04 ± 0.28	1008.5 ^a	1605, 1439, 1314, 1151, 865, 577, 287	280
59	Procyanidin heptamer	44.70	29.88 ± 0.13	1008.6 ^a	1605, 1441, 1153, 863, 575	280
60	Procyanidin heptamer	44.73	31.80 ± 0.17	1008.5 ^a	1441, 1153, 863, 575, 287	280
61	Procyanidin heptamer	46.06	33.80 ± 0.36	1008.6 ^a	1605, 1441, 1153, 863, 575	280
62	Procyanidin heptamer	46.10	33.77 ± 0.12	1008.6 ^a	1605, 1441, 1153, 863, 739, 577	280
63	Procyanidin heptamer	46.15	38.89 ± 0.20	1008.7 ^a	1605, 1441, 1315, 1151, 863, 577	280

64	Procyanidin octamer	48.75	39.21 ± 0.62	768.1 ^b	1727, 1153, 865, 739, 577, 289	280
65	Procyanidin octamer	49.94	32.69 ± 0.58	768.0 ^b	1316, 1153, 863, 575, 287	280

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536 ^aIons detected as [M-2H]²⁻; ^bIons detected as [M-3H]³⁻; * indicates identification confirmed using commercial standards.

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Table 2. Total phenols amount (given as mg GAE (galic acid equivalents)/g dry matter) and total procyanidins amount (given as mg ECE (epicatechin equivalents)/g dry matter) present in the five studied apple varieties calculated according to the Folin and DMAC methods, respectively. Values provided as mean $\pm$ sd of three independent assays.

Apple variety	Total phenols (mg GAE/g dry matter)	Total procyanidins (mg ECE/g dry matter)	% procyanidins
Red Starking	3.57 $\pm$ 0.07	0.38 $\pm$ 0.00	10.62
Kanzi	1.21 $\pm$ 0.13	0.14 $\pm$ 0.01	11.19
Royal Gala	1.77 $\pm$ 0.07	0.34 $\pm$ 0.00	19.41
Reinette	6.46 $\pm$ 0.22	0.56 $\pm$ 0.01	8.71
Granny Smith	5.45 $\pm$ 0.23	0.73 $\pm$ 0.00	13.37

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552 **Figure 2**

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Figure 3.

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560 **Figure 4.**

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Figure 5.