**Characterization of secondary metabolites from green cocoa beans using focusing-
modulated comprehensive two-dimensional liquid chromatography coupled to tandem
mass spectrometry.**

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ABSTRACT

Proanthocyanidins as well as other secondary metabolites present in green cocoa beans were studied thanks to a new method involving the use of on-line comprehensive two-dimensional liquid chromatography coupled to tandem mass spectrometry (LC × LC-MS/MS). In order to enhance the performance of previously developed methodologies, the use of different modulation strategies were explored. Focusing modulation clearly allowed the attainment of higher resolving power and peak capacity compared to non-focusing modulation set-ups. Moreover, the use of active modulation by the addition of a make-up flow efficiently helped to compensate for the solvent strength mismatch produced between dimensions. The optimized method was useful to successfully describe the secondary metabolite composition of green cocoa that was characterized by the presence of 30 main compounds, including 3 xanthines, 2 flavan-3-ols and 24 oligomeric procyanidins with a degree of polymerization up to 12. The obtained results showed that the proanthocyanidins found in the cocoa beans were exclusively B-type procyanidins. The existence of (epi)catechin subunits linked to sugar or galloyl moieties was not observed. The developed method produced a good separation of secondary metabolites allowing an improvement with respect to the available methodologies for the analysis of a complex food sample such as cocoa metabolites in terms of speed of analysis, resolution and peak capacity.

Keywords: Cocoa; Focusing modulation; LC × LC; Phenolic compounds; Proanthocyanidins; Xanthines

1. INTRODUCTION.

Proanthocyanidins (PAs) are mixtures of oligomers and polymers of flavan-3-ol units linked mainly through $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$ bonds [1]. According to the linkage of interflavan bonds, the PAs can be classified into A- or B-type proanthocyanidins. Many foods including cocoa-based products, red wine, coffee, apple and tea exclusively contain B-type procyanidins [2], but occasionally some matrices may present an additional $C_2 \rightarrow O_7$ or $C_2 \rightarrow O_5$ linkage, leading to doubly bonded A-type proanthocyanidins. For example, this kind of bonds can be present in avocados, peanuts and cranberries [3–5].

Flavan-3-ols are the most complex subclass of flavonoids ranging from monomers to oligomeric and polymeric proanthocyanidins [1]. The complexity of the flavan-3-ols is due to their nature as non-planar molecules [6] having two chiral centers at C2 and C3, producing four isomers for each level of B-ring hydroxylation ((+)-epicatechin, (-)-epicatechin, (+)-catechin, (-)-catechin), and for oligomers an additional chiral center at C4. Moreover, these compounds might form more complex structures due to the inclusion of gallic acid and sugar moieties. As can be seen in Figure 1, the main procyanidin monomeric compounds are the (+)-catechin and (-)-epicatechin units.

Some of the main antioxidant-rich food products are those derived from cocoa. Since 2010, cocoa is highlighted as the fourth richest dietary source of polyphenols, just behind cloves, peppermint and star anise [7]. The main polyphenols in cocoa are (epi)catechins, proanthocyanidins, flavanol glycosides, and anthocyanins [8–10]. Procyanidins are, by far, the major flavonoids in cocoa. These compounds are exclusively composed of (epi)catechin units, and are characterized for their high degree of polymerization (DP) [10]. In fact, procyanidins with DP up to 14 have been already described in cocoa [11].

Cocoa procyanidins play an important role in the formation of color, solubility and astringency [12]. For example, the enzymatic browning reaction is responsible for the brown color of cocoa beans [13]. Moreover, the amount of total oligomeric procyanidins directly affects astringency and bitterness [14], whereas the interaction of these polyphenols with proteins forming complexes affects the cocoa aromatic precursors [15]. Many studies have evaluated the biological properties of PAs suggesting that these polyphenols may confer positive effects including antioxidant activity at different levels [16,17], anti-carcinogenic activity reducing the oxidative DNA damage [18], chemopreventive action [19], positive effects on cardiovascular health [20], and anti-inflammatory and immune-modulatory activities [21]. However, the bioavailability, bioactivity and potential nutraceutical properties largely depend on the composition and concentration of the particular procyanidins found [22]. Thereby, better elucidation of the B-type isomers in cocoa as well as the molecular size of the procyanidins is of great interest in order to evaluate the activity, absorption, permeability, the physicochemical properties and the affinity of PAs with proteins during the digestive process [1].

Many strategies such as colorimetric assays, or chromatography coupled to mass spectrometry can be used to determine polyphenols from natural sources. In the particular case of the PAs, colorimetric assays are not specific methods due to severe interferences from phenolic acids, sugars, ascorbic acid or cysteine [23]. Regarding the chromatographic methods, in general, separations from monomers through decamers of A- and B- type procyanidins have been achieved using normal phase liquid separations with diol stationary phases coupled to fluorescence detectors [11]; in this case, compounds can only be separated according to their molecular weight while isomers separation cannot be achieved. On the other hand, smaller compounds with $DP \leq 6$ have been classically elucidated using reverse

93 phase through C18 columns [8,24,25]; through this approach, many of the isomers co-elute
94 and the higher oligomers cannot be separated and elute as an unresolved hump at the end of
95 the chromatogram. For this reason, the application of analytical techniques with higher
96 resolving power is essential.

97 Two-dimensional liquid chromatography (2DLC) is a promising analytical tool since it
98 combines two different separation procedures (dimensions) for the analysis of one sample,
99 allowing the separation of the compounds present in the sample by two different retention
100 mechanisms, greatly increasing peak capacity [26]. 2DLC can be run in heart-cutting (LC–
101 LC) or comprehensive LC (LC × LC) approaches [27], both in off-line, on-line or stop-flow
102 modes [28]. Two-dimensional liquid chromatography has been widely employed for the
103 analysis of phenolic compounds-rich food samples [29] including the analysis of cocoa
104 procyanidins both using off-line 2DLC and stop-flow comprehensive LC [30] while on-line
105 LC × LC methods have been developed for the characterization of proanthocyanidins in
106 grape seeds and apples [31,32], and anthocyanidins in red wine [33].

107 In particular, on-line LC × LC is considered the fastest and more reproducible 2DLC mode,
108 although these separations present several important challenges. The main difficulty of this
109 coupling is to solve the incompatibility of solvents that occurs when two different separation
110 modes are coupled on-line, due to solvent mismatch between dimensions. This issue is
111 responsible for lack of retention in the second dimension (²D) column and results in broad
112 and/or distorted peaks, producing a loss on total peak capacity. Many efforts have been made
113 to solve this problem [34]. Recently, a new strategy named active modulation has been
114 reported [35]. This strategy consists on the use of a make-up flow installed between the
115 output of the ¹D column and the input of the interface useful to adjust solvent strength. The
116 use of this make-up flow rate produce an increment on the focusing effect in the ²D column

allowing a better compatibility between the injection solvent and the ²D stationary phase and therefore, narrower peaks and higher peak capacity [35]. This strategy has also been successfully employed for the separation of complex plant secondary metabolites [36]. Considering the analytical challenges related to the study of the cocoa beans phenolic compounds together with the difficulties that the on-line HILIC × RP coupling involve, the aim of the present work was to optimize a new LC × LC method for the separation and identification of secondary metabolites from unfermented cocoa beans, in order to enhance the performance of the existing methodologies.

2. MATERIALS AND METHODS.

2.1 Samples and reagents.

Cocoa pods (*Theobroma cacao* L., genotype ICS 39) were collected from Villa Santa Monica (San Vicente de Chucurí, Colombia). All chemicals used were of analytical reagent grade with no further purification. (+)-Catechin hydrated (≥ 99%; ASB-000003310), (-)-epigallocatechin gallate (EGCG) (≥ 99%, ASB 00005152), (-)-epicatechin (≥ 99%; ASB-00005127), procyanidin B1 (≥ 90%; ASB-00016230) and procyanidin B2 (≥ 90%; ASB-00016231) were purchased from ChromaDex Inc. (Irvine, CA, USA). Theobromine, theophylline, caffeine, gallic acid, L-cysteine, ascorbic acid, formic acid, trifluoroacetic acid, acetic acid and citric acid were obtained from Sigma Aldrich (St. Louis, MO, USA). All the solvents employed (acetonitrile, ethanol, acetone and methanol) were of HPLC-grade and acquired from VWR Chemicals (Barcelona, Spain). Milli-Q water (Millipore system, Billerica, MA, USA) was used for the preparation of all solutions.

2.2 Preparation of cocoa polyphenols extract

Unfermented green cocoa beans were manually separated from cocoa husk and the mucilage coating around each bean was removed using a home-made mucilage remover. Beans were immediately stored at -20°C until further use. With the aim of protecting polyphenols, the action of polyphenol oxidase enzymes (*i.e.*, POD, and PPO) was controlled by placing the frozen cocoa beans in a solution of 70 mM ascorbic acid/L-cysteine (1:1 v/v) and thermally processing the beans at 96°C for 6.4 min followed by immediate cooling in ice for 30 min. Afterwards, the sample was re-washed with distilled water (4°C) to remove traces of ascorbic acid and L-cysteine. After that, the sample was chopped by hand using a knife and it was directly oven dried (Binder model FD 23, Tuttlingen, Germany) at 70°C for 3 h (final humidity <4% water w/w). The dried sample was milled (Grindomix GM 200, Retsch GmbH & Co., Haan, Germany) at -20°C and sieved through a 120 mesh (W.S., Tyler, OH, USA). The recovery of polyphenol extract consisted on the optimization of a solid-liquid extraction assisted by ultrasounds, as follows: 1 g of sample was mixed with 120 mL of 50% ethanolic solution and submitted to ultrasounds-assisted extraction for 30 min at 20 kHz (Ultrasonic LC 30H, Elma, Singen, Germany), followed by acidification at pH 6 (using 3M citric acid solution) and incubation at 70°C for 45 min under constant stirring. The resulting extract was centrifuged (5000 g, 4°C, 20 min) (Heraeus, Megafuge 16R, Thermo Scientific, Waltham, MA, USA), and the supernatant was collected and filtered with Whatman filter paper N°1. The ethanol was removed by vacuum evaporation (R-100, Büchi, Flawil, Switzerland). The aqueous crude extract was then freeze-dried (Labconco, Kansas City, MO, USA) and the resulting violet-powder crude cocoa extract was stored at -80°C until further use. The studied cocoa extract was characterized by a total phenols content of 112.4 ± 5.7 mg gallic acid equivalents/g dried cocoa beans, measured according to Singleton and Rossi [37], and an

antioxidant capacity value of 1201.5 ± 41.3 $\mu\text{mol Trolox/g}$ dried cocoa beans determined by using the ORAC assay [38].

For the two-dimensional LC analyses, 100 mg of extract were dissolved in 1 mL of acetone/water/acetic acid (78:20:2 v/v/v), and then centrifuged at 5000 g for 5 min. Solutions were filtered through hydrophilic Durapore PVDF membrane 0.22 μm (Millipore, USA) prior injection.

2.3 LC $\times$ LC Instrumentation

Comprehensive two-dimensional liquid chromatography instrumentation consisted on a first dimension (^1D) composed by an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a diode array detector which was connected at the exit of the second dimension. Additionally, a Protecol flow-splitter (SGE Analytical Science, Milton Keynes, UK) was placed between the ^1D pump and the autosampler with the aim of obtaining reproducible low flow rates. The second dimension (^2D) was carried out using an additional LC pump (Agilent 1290 Infinity). An electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) was used to connect both dimensions. Sampling loops (50 μL) or C_{18} trap columns (10 x 3 mm, 2.6 μm , Accucore, Thermo Scientific, Waltham, MA, USA) were connected to the switching valve to collect the fractions from the ^1D and to inject them into the ^2D . Separations were recorded at 280, 320 and 370 nm although, in addition, UV-Vis spectra were collected from 190 to 550 nm at a sampling rate of 20 Hz in the DAD.

For the active modulation configuration, an additional make-up flow was provided by a third LC pump (Agilent 1200 series) connected through a T-piece between the outlet of ^1D and the

switching valve. LC image software (Zoex Corp., Houston, TX, USA) was used to plot the results as 2D and 3D images.

An Agilent 6320 ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used for the identification of the separated compounds interfaced by an ESI ion source. MS/MS analyses were carried out to obtain the structural information of the separated compounds. The ion trap mass analyzer was controlled by the 6300 series trap control software (Bruker Daltonik GmbH, V. 6.2). The flow eluting from second dimension was splitted via a T-piece in order to allow the introduction of a flow of *ca.* 700 $\mu\text{L min}^{-1}$ in the MS. The instrument was operated under negative and positive ESI modes using the following conditions: mass spectra recorded from m/z 50 to 2200; nebulizer pressure, 40 psi; dry gas, 12 L min^{-1} , and; dry temperature, 350 °C.

2.4 LC $\times$ LC separation conditions

A HILIC separation in the ^1D was coupled to a reversed phase separation in the ^2D . After optimization (see Sections 3.1 and 3.2), the following conditions were employed:

^1D separation: a Lichrospher Diol-5 (150 $\times$ 1.0 mm, 5 μm d.p., HiChrom, Reading, UK) column was employed protected with a security guard column containing the same stationary phase. Acidified acetonitrile (2% acetic acid, v/v , A) and methanol/water/acetic acid (95:3:2 v/v , B) were used as mobile phases. Under optimum conditions, the gradient was: 0-1 min, 0% B; 5 min, 20% B; 18 min, 35% B; 50 min, 42% B; 75 min, 100% B; 77 min, 100% B, 80 min, 0%B. The flow rate was set at 20 $\mu\text{L min}^{-1}$, the column temperature at 30°C and the injection volume was 3 μL .

^2D separation: two partially-porous columns with different length (Ascentis Express C₁₈ 30 $\times$ 4.6 mm, 2.7 μm , and 50 $\times$ 4.6 mm, 2.7 μm , Supelco, Bellefonte, CA, USA) were tested.

Gradient, flow rate, temperature, type of acid and solvents used were optimized separately. The optimal conditions were achieved using the 30 mm column with acidified H₂O (0.05 % TFA, solvent A) and ACN (0.05 % TFA, solvent B) as mobile phases. The optimum gradient was as follow: 0 min, 2% B; 0.05 min, 8% B; 0.5 min, 20% B; 0.9 min, 25% B; 0.91 min, 2% B and re-equilibrium at starting conditions until 1 min. Unless stated otherwise, the flow rate was set at 3.5 mL min⁻¹ and the column temperature was 30 °C maintained using a temperature control module (Cecil Instrument Ltd, Cambridge, England). Once these parameters were optimized, the coupling between both dimensions was achieved employing 1 min repetitive second dimension separations; thus, the modulation time was 1 min. The third pump used for achieving the active modulation was set at 100 µL min⁻¹, delivering solvent with the same composition than the initial ²D gradient conditions (2% B).

3. RESULTS

Plant extracts are well known for the complexity of their chemical composition. In general, this is due to the diversity of different families of compounds that can form their metabolic profile. That is the case of the cocoa extract, mainly composed by phenolic compounds, in particular proanthocyanidins. Due to the importance of these compounds as potential beneficial health ingredients, the knowledge about its chemical composition is essential. As mentioned, the chemical complexity of these compounds makes their elucidation a big challenge and the use of conventional separation techniques does not provide enough separation power to obtain the comprehensive map of compounds. For this reason, in this work a LC × LC method has been developed for the exhaustive characterization of this complex extract. An initial individual optimization for both, ¹D and ²D was carried out. Once the preliminary conditions were set, their coupling was also studied and fine-tuned.

235

236 **3.1 Optimization of the HILIC × RP-DAD-ESI-MS/MS method for the chemical** 237 **characterization of proanthocyanidins of cocoa beans**

238 The use of 2DLC is only justified when the complexity of the sample is complex enough that
239 1DLC is not able to guarantee the complete resolution of the analytes. In other words,
240 samples that are analyzed by 2DLC present more than one chemical variable defined as the
241 sample dimensionality [39]. This fact means that a new sample that is considered for a 2DLC
242 analysis is different and presents its own particular characteristics. Some of the characteristics
243 that greatly affect the separation are the matrix effect of the sample and the composition of
244 the analytes of interest. Therefore, a complete evaluation of the method developed for its 2D
245 analysis is required. Although proanthocyanidins have been previously analyzed using
246 different 2DLC approaches [25,30-32], the analysis of a new sample, such as cocoa green
247 beans extracts, always involves an in-depth study of all the parameters that affect the 2D
248 separation.

249

250 *3.1.1 Operational conditions for HILIC-based ¹D separation*

251 HILIC has been successfully employed for the separation of procyanidins before, involving
252 conventional LC. In particular, Robbins et al. [11] achieved the separation of
253 proanthocyanidins from a cocoa extract according to their degree of polymerization, getting
254 the separation of proanthocyanidins up to 14 units of (epi)catechin with a Develosil Diol
255 column. In the present work, HILIC has also been used as ¹D separation mechanism in order
256 to obtain the separation of phenolic acids, alkaloids as well as monomeric and polymeric
257 procyanidins eluted as a function of their DP. Separation optimization is challenging as LC
258 × LC conditions imply to run ¹D under suboptimal chromatographic conditions [40–42]. The

injection solvent composition is a critical parameter in a microbore column; solvents similar to the initial mobile phase should be used to promote the sorption of the analytes into the column in a narrow band. For this reason, the solvent used to inject the sample in the microbore ¹D column was carefully studied and optimized; three different solvents were tested to dissolve the crude cocoa extract: methanol, acetonitrile/methanol (70:30 v/v), acetone/water/acetic acid (78:20:2 v/v/v). Considering the initial conditions of the analysis, acetonitrile should be selected as solvent to dissolve the sample since it is the weak organic solvent for the ¹D. However, proanthocyanidins are not well dissolved in acetonitrile and precipitated. Instead, the most favorable conditions included the use of acetone/water/acetic acid (78:20:2 v/v/v) as sample solvent.

Figure 2 shows the ¹D separation of the crude cocoa extract at optimum conditions. As can be observed, with a run time of ca. 75 min and a slow gradient the elution of all the components present in the sample using a low flow rate was achieved. The first chromatographic peaks in the chromatogram correspond to alkaloids, mainly theobromine and caffeine, immediately followed by monomeric (epi)catechin. Between 30-50 min the oligomeric proanthocyanidins up to DP 8 were separated and at the end of the analysis (~65 min) the higher proanthocyanidin polymers were eluted.

3.1.2 Operational conditions for ²D separation

The appropriate optimization of individual ²D separation conditions is critical to achieve a successful LC × LC separation. In LC × LC, the ²D available analysis time and re-equilibration time is equal to the modulation time, and this time has to be as short as possible in order to get the maximum number of ²D analyses per ¹D peak. However, this short time implies the need to carry out very fast ²D analyses. Besides, in the ²D is where the solvent

mismatch between dimensions becomes apparent, hampering the retention and proper elution of peaks, and having a critical influence on the total peak capacity attainable.

With the aim to optimize the ²D separation in the shortest possible analysis time, two C18 partially porous columns of different length (30 and 50 mm) were tested. In this regard, different total ²D separation available times (60 and 90 s), gradients, flow rates (from 2 to 4 mL min⁻¹), mobile phases (water, acetonitrile, methanol and their combinations) and acidic modifiers (formic acid and trifluoroacetic acid) were studied. Slightly better peak shapes were obtained using TFA, probably due to its pK_a value and interaction with ionizable groups of flavonoids, whereas the presence of methanol was always detrimental for the elution of the extract components. In Figure S1, the 2D chromatograms of the analysis employing acetonitrile (Figure S1A) and methanol (Figure S1B) as organic mobile phase under preliminary gradient conditions are showed. Comparing both analysis, a great fraction of the compounds eluted in the void volume without any retention when methanol was employed, whereas using acetonitrile the retention of the compounds is improved and, besides, a better peak resolution is obtained. Once the mentioned separation conditions were studied, the influence of the separation temperature was also tested. It is well known that high separation temperatures may favor resolution and reduce analysis times due to a decrease on the solvent viscosity and to an improvement in mass transfer kinetics [43]. Separation temperatures between 30-55 °C were evaluated. However, a significant difference in the separation of oligomeric compounds was not observed and, thus, the minimum temperature (30 °C) was maintained.

Moreover, the gradient in the ²D was deeply studied to enhance the separation of dimer, trimer and tetramer isomers and to achieve a good peak resolution for oligomeric and polymeric proanthocyanidins previously separated in the ¹D. In this sense, the finally

selected gradient consisted on a progressive increment of the organic phase up to a 25 %. With this optimum gradient, the oligomeric proanthocyanidins (DP 2-6) and polymers eluted in the middle and at the end of the ²D analysis, respectively, as can be observed in Figure 3A, where peaks in the ²D separation occur between 25 and 45 s.

In spite of this optimization of the separation conditions, the complexity of cocoa proanthocyanidins, implying a mixture of very similar chemical structures, meant that the separation of the different isomers was not completely achieved. For this reason, other modulation strategies including a focusing effect were studied in a second step.

3.1.3 Focusing modulation to improve the ²D separation of the proanthocyanidins isomers.

3.1.3.1 Focusing configuration using trapping columns

The use of trapping columns instead of loops in the interface has been reported as a good alternative to produce a focusing effect for other food samples [29,36,44]. These trapping columns must possess the same or similar selectivity than the ²D stationary phase in order to produce a pre-concentration and retention step of the analytes eluted from ¹D in the trapping columns during the collection position, which will be then eluted from the interface with the ²D solvents once the valve is actuated to the injection position. Therefore, to produce this trapping effect, two C18 trapping columns (to match the selectivity of the ²D column) with a void volume of 42 µL were installed in the switching valve on a forward elution mode using the same separation conditions previously optimized for the LC × LC method without focusing. In this work, forward elution mode from the traps was selected in opposite to backflush elution based on previous experience in which a slight but noticeable improvement in separation was observed when forward elution was employed [36]; since the trapping column is not actually filled with ¹D effluent between modulations, longer interaction is

allowed in forward elution mode. Comparing loops vs trapping columns, using the latter a slightly better retention of the compounds was achieved (Figure 3B) since in each ²D separation, peaks appear more defined than in the non-focusing configuration (Figure 3A). Indeed, as can be observed, when trapping columns are used peaks are slightly more separated as well as more intense, probably due to the minimization of the solvent strength mismatch produced and an improvement of ²D peak shapes. As a result of the better separation, the use of trapping columns allowed the separation of several isomers from each degree of polymerization that were previously not resolved using the non-focusing approach (Figures 3A and B).

3.1.3.2 Focusing configuration using active modulation

Active modulation consists on the use of the same trapping columns configuration to which an additional make-up flow composed by a weak solvent for the ²D (ideally the same composition of the initial ²D gradient) is added between the ¹D exit and the interface [35]. The role of this additional flow is to reduce or completely remove the ¹D solvent by the dilution of the transferred fraction with a ²D compatible solvent combined with the use of trapping columns. This fact produces an increment of the retention of the analytes in the interface and, therefore, an effective pre-concentration of the analytes in the trapping columns [36]. Thereby, in this work, an additional make-up flow pump was connected through a 3-way PEEK connection in which one way was connected to the ¹D effluent, whereas the others corresponded to the flow from the additional pump and to the exit to the switching valve. The composition of the make-up flow employed was 98% acidified H₂O (0.05% TFA) and 2% ACN (0.05% TFA) to match the initial conditions found at the start of the ²D optimum gradient. The influence of the make-up flow rate on the separation of the

cocoa extract was also evaluated; flow rates ranging from 50–160 $\mu\text{L min}^{-1}$ were studied. As can be seen in Figure 4, the lowest flow rate did not improve the separation of compounds (procyanidins window), as this additional flow would not be enough to completely eliminate the solvent strength mismatch between ^1D and ^2D solvents, as can be from Figure 4A. On the other hand, flow rates higher than 100 $\mu\text{L min}^{-1}$ positively affected the retention of the sample components, achieving better separation and resolution. From a comparison between Figures 4B and 4C and Figure S2, it can be inferred that no significant differences on separation were obtained. Thus, the lowest make-up flow rate (100 $\mu\text{L min}^{-1}$) was retained as optimum conditions.

Moreover, the performance of the different LC $\times$ LC studied set ups was evaluated by determination of the attainable peak capacity. Using the optimized method for both ^1D and ^2D separations, the peak capacity was calculated according to Davis et al. [45], as follows:

$$n_c = 1 + \frac{t_g}{\left(\frac{1}{n}\right) \sum_1^n w}$$

where, n_c represents the peak capacity, n is the number of compounds, t_g is the gradient time and, w is the average peak width. The total theoretical peak capacity of two-dimensional LC is the product of the peak capacities of the first and second dimension, $^{2D}n_c = {}^1n_c \times {}^2n_c$. More recently, Li et al. [46] defined the effective 2D peak capacity taking into account the modulation time, the influence of the under-sampling of first dimension and peak broadening, as follows:

$$^{2D}n'_{c,} = \frac{{}^1n_c \times {}^2n_c}{\sqrt{1 + 3.35 \times \left(\frac{{}^2t_c {}^1n_c}{{}^1t_G}\right)^2}}$$

where $^{2D}n_c$ is the effective 2D peak capacity, 2t_c is the second dimension cycle time and

1t_g is the first dimension gradient time. To more realistically describe the method performance, orthogonality (A_0) was also estimated in agreement with the asterisk equations method [47]. This value was subsequently employed to correct the effective peak capacity values as follows:

$${}^{2D}n_{c,corrected} = {}^{2D}n'_c \times A_0$$

Moreover, dilution factor (DF) produced during the analysis was also calculated. In agreement with the equations proposed by Vivó-Truyols et al. [48], considering that dilution factor produced in each dimension is

$$DF = \sqrt{2\pi} \frac{\sigma}{V_i} F$$

Where F and V_i are the flow rate and injection volume, respectively. The dilution of the complete 2D process will be:

$${}^{2D}DF = {}^1DF \times {}^2DF$$

As summarized in Table 1, compared to the effective peak capacity of the non-focusing configuration of this work (712) the peak capacity was higher when active modulation was used, reaching a maximum effective peak capacity equal to 879. This value was also higher than the data provided in other works (551 and 771) [30], although in this case it is important to remark that the comparison is not completely fair as the samples analyzed are also different. These data confirmed that the additional make-up flow allows improving the separation and the resolution of complex compounds as procyanidin isomers. An inspection of the theoretical (1184) and effective (879) peak capacities obtained for the optimized method demonstrates that a good modulation performance was obtained, as the two values are very close. In any case, the orthogonality values obtained for all three set-ups were

relatively low, reducing the values of corrected peak capacity (Table 1). Nevertheless, as can be deduced from the values shown in Table 1, the improvement that active modulation produces in the 2D separation was evident; 40% higher orthogonality values were obtained using active modulation compared to non-focusing modulation. Besides, the positive effect of focusing due to active modulation is directly related to the sensitivity that is related to the dilution factor (DF). This value was reduced by 20% using the active modulation approach, and therefore a gain in sensitivity was potentially achieved.

In summary, after studying and optimizing all possible configurations and conditions, the final selected conditions to carry out the in depth characterization of cocoa extracts were as follows (Figure 3C): ¹D separation was carried out on Lichrospher Diol-5 column, using ACN/AcOH (98:2 v/v) and MeOH/H₂O/AcOH (95:3:2 v/v/v) as mobile phases at a flow rate of 20 µL min⁻¹. In the ²D, the short C18 partially porous column (30 × 4.6 mm, 2.7 µm) H₂O (0.05% TFA, solvent A) and ACN (0.05% TFA, solvent B) were used as mobile phases using a 1 min gradients, whereas 98% H₂O (0.05% TFA) and 2% ACN (0.05% TFA) was used as make-up flow for active modulation at 100 µL min⁻¹.

3.2 Characterization of the cocoa extract

As can be seen in Table 2, the optimized focusing modulated LC × LC method was able to separate 30 compounds as a function of two different mechanisms; in the ¹D the proanthocyanidins were separated as function of their DP while in the ²D the separation of different isomers within the same DP was possible. Table 2 summarizes the tentative identifications of each separated peak as well as their main MS and MS/MS features. In addition of flavan-3-ols, three xanthines also present in the metabolic profile of cacao were

422 similarly assigned. The identification of these three alkaloids was carried out in positive
423 ionization mode. On the other hand, procyanidins were detected under negative ionization
424 due to the acidity of the phenolic protons which mean that these are better detected under
425 that mode. Besides, there are more available charges as the chain length increases [49].
426 Figure 5 shows the 3D plot of the studied crude cocoa extract separated under the optimized
427 LC $\times$ LC conditions. As can be observed, good separation in the complex mixture of flavan-
428 3-ols units and their isomers was attained. As expected, the compounds can be grouped in
429 two main areas, corresponding to xanthine family and monomeric (epi)catechin at the
430 beginning of the analysis, and, on the other hand, oligomers of (epi)catechin units according
431 to their increasing degree of polymerization (up to DP 12). Oligomers higher than $DP \geq 12$
432 were not completely resolved mainly due to their low concentration in the studied extract.
433 The first eluted compound corresponds to caffeine that presents a molecular ion ($[M+H]^+$) at
434 m/z 195.1 (peak 1), followed by theophylline and theobromine (peaks 2 and 3) with m/z 181.3
435 and 181.1 ($[M+H]^+$), respectively, which were assigned by comparison with commercial
436 standards. The presence of others common xanthines in cocoa such as paraxanthine or 7-
437 methylxanthine was not detected.

438 Regarding flava-3-ols, the cocoa extract sample contained (+)-catechin and (-)-epicatechin,
439 in agreement with other reports [8,10]. Indeed, (+)-catechin and its isomer (-)-epicatechin
440 eluted at 17.3 min and 17.6 min, respectively (peaks 4 and 5) and were precisely assigned by
441 comparison with commercial standards. No evidence of galloylated catechin was found.
442 Among the observed peak signals detected through the rest of the analysis, these show
443 molecular ion species consistent with those of procyanidins oligomers [49]. For example, by
444 comparing with commercial standards, the identification of procyanidin dimers B2 [EC-
445 $4\beta \rightarrow 8$ -EC] at 35.5 min (peak 8) and B1 [EC- $4\beta \rightarrow 8$ -C] at 35.7 (peak 9) min was confirmed;

both showed ions at m/z 577.8 and 577.4, respectively, whose fragmentation generated ions at m/z 425, 289 and 245. Thanks to the combination of HILIC and RP separations, the present development allowed the separation of different isomers for a given polymerization degree, as can be observed from the information collected in Table 2.

Regarding higher procyanidins, these compounds are more difficult to assign due to the lack of commercial standards. In this regard, their tentative identification was based on mass spectra data, correlation of retention time for both 1D and 2D , UV-Vis spectra and careful correlation of LC $\times$ LC analysis (see Figure S3 as example). Moreover, the detected molecular ions followed a particular pattern described by the following formula: $[290 + 288(n-1) + 152g + 162h]^+$, where 290 represents the molecular weight of terminal catechin/epicatechin unit, n is the degree of polymerization, g is the number of galloyl esters and h the number of hexosides. This formula may indicate the presence of glycosidic and galloylated procyanidin structures [3,49].

The results showed that different series of ions (peaks 13-23) separated by 288 Da from m/z 865 to 1730, were consistent with procyanidins with DP 3-6. For example, ions at m/z 865, 1153, 1441, and 1729 correspond to molecular species of mono-charged trimers, tetramers, pentamers and hexamers, respectively. The fragmentation pattern also followed a retro-Diels-Alder fission mechanism (RDA) as well as quinone methide mechanism (QM) which is in agreement with previous reports [50]. For example, the ion at m/z 577 presents a loss of 288 Da (final m/z 289) and corresponds to QM cleavage whereas the fragments with m/z 425 can be assigned to RDA ($[M-H-152]^+$) re-arrangements.

As mentioned before, each DP of proanthocyanidins present numerous isomers, and the composition and number of isomer depends on several factors such as the plant variety or the geographical area where the plant is harvested. Thanks to the separation obtained by the

developed LC × LC method, the identification of the particular isomer composition of these oligomers present in cocoa beans was possible. In particular, three dimeric-, three trimeric-, four tetrameric-, three pentameric- and three hexameric procyanidins isomers that could not be separated employing 1DLC were identified.

Doubly-charged ions ($[M-2H]^{2-}$) species with fragmentation patterns consistent with procyanidins oligomers were detected for higher oligomers starting from DP 7. Indeed, ions with m/z 1008, 1153, 1297, 1442, 1599, and 1733 (peaks 24-30) were tentatively attributed to procyanidin heptamers, octamer, nonamer, decamer, undecamer, and dodecameric respectively. The formation of multiply charged species beyond the doubly-charged ions was not detected. The identification of larger tannins was difficult due to the lower polymer concentration, peak signal dispersion, formation of multiple ions, and limitations of the ion trap MS analyzer. In addition, presence of prodelphinidins, A-type procyanidins and galloylated or glycosylated (epi)catechin were not detect.

The obtained results are in agreement with previous reports by Kalili and De Villiers [25,30] In those works, off-line and on-line comprehensive two-dimensional LC methods were employed for the analysis of procyanidins from cocoa. The off-line method was able to separate A- and B- type procyanidins among other compounds, at the cost of very long analysis times. On the other hand, using the on-line method, authors obtained completely resolved peaks of procyanidins up to DP = 8 using a most sensible fluoresce detector in 100 min. In the present work, the application of a focusing modulation approach through an active modulation strategy coupled to a DAD allowed obtaining an effective separation of different procyanidin isomers up to DP = 12, reducing analysis time to 75 min. Moreover, although comparatively, off-line two-dimensional LC analyses offer less restriction on ²D analysis time compared to on-line LC × LC, the on-line method developed in the present contribution

provides advantages such as full automation, faster analyses and less risk of analyte loss.

In summary, the application of the developed on-line LC $\times$ LC-DAD-MS/MS method for the analysis of procyanidins from green cocoa allowed a good separation of main monomeric and oligomeric (epi)catechins. Thirty compounds including 3 xanthines, 2 monomeric catechins and 24 oligomeric procyanidins with a degree of polymerization up to 12 were separated and tentatively identified. It was possible to separate not only procyanidins in agreement to their increasing DP, but also their major isomers. Consequently, the present developed method implies a step forward the existing methodologies for the analysis of procyanidins in cocoa.

4. CONCLUSIONS

The use of a new LC $\times$ LC coupled to ESI-MS/MS allowed the attainment of significant gains for the separation and elucidation of secondary metabolites present in cocoa, mainly flavan-3-ol monomers and procyanidin oligomers. After careful optimization of the separation conditions, including flow rate, temperature, type of solvent and gradients employed in both dimensions, the implementation of focusing modulation approaches based on the use of trapping columns and active modulation was also studied. The results obtained showed that the combination of trapping columns and active modulation allowed better separation of proanthocyanidins and their isomers with a DP up to 12. This is the first time that active modulation is used for the separation of proanthocyanidins. Using this approach, solvent mismatch effects between dimensions are minimized producing better retention, peak shapes and sensibility of procyanidins. This novel method reinforced the applicability of two-dimensional LC $\times$ LC for the analysis of food complex samples, such as procyanidins from cocoa.

518

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FIGURE LEGENDS

Figure 1. Chemical structure of methylxanthines and procyanidins. Skeleton of: A) monomeric catechins, B) xanthines, and C) condensed procyanidins.

Figure 2. ¹D Chromatogram (280 nm) for cocoa extract under the optimum conditions using a Lichrospher Diol-5 column. The mobile phase consisted of ACN/AcOH (98:2 v/v) and MeOH/H₂O/AcOH (95:3:2 v/v/v) at flow rate of 20 μL min⁻¹. For more details, see text.

Figure 3. Two-dimensional plots (280 nm) of cocoa secondary metabolites using optimum separation conditions for each modulation set-up: A) sampling loops, B) C18 trapping columns and, C) C18 trapping columns + active modulation. ¹D separation was carried out on Lichrospher Diol-5 column, and mobile phase consisted of ACN/AcOH (98:2 v/v) and MeOH/H₂O/AcOH (95:3:2 v/v/v) at flow rate of 20 μL min⁻¹. ²D separation was carried out Ascentis Express C₁₈ partially-porous column, using H₂O (0.05% TFA) and ACN (0.05% TFA) as mobile phases at flow rate of 3.5 mL min⁻¹. For the rest of separation conditions, see text.

Figure 4. Two-dimensional plots (280 nm) of cocoa secondary metabolites obtained under optimum separation conditions using active modulation with different make-up flow rates: A) 50 μL/min, B) 100 μL/min, C) 160 μL/min.

Figure 5. Three-dimensional chromatogram of cocoa secondary metabolites obtained under the optimized conditions using the novel LC × LC method developed. For detailed separation conditions, see text. Peak identification as in Table 2.

708 **Table 1.** Peak capacity values calculated for the LC $\times$ LC focusing modulation set-ups
709 studied.

Parameter	Non-focusing modulation	Focusing Modulation	
	Loops	Trapping Columns	Trapping columns + active modulation
¹ D peak capacity, ¹ n_c	37	37	37
² D Peak capacity, ² n_c	26	28	32
Theoretical peak capacity, ^{2D} n_c	962	1036	1184
Effective peak capacity, ^{2D} n'_c	712	767	879
Orthogonality, A_0	47%	56%	66%
Corrected peak capacity, ^{2D} $n_{c,corr}$	335	430	580
Dilution Factor, ^{2D} DF	36.7	33.9	29.3

710 A_0 , orthogonality; ^{2D} $n_c = {}^1n_c \times {}^2n_c$; ^{2D} n'_c : calculated according to [46]; ^{2D} $n_{c,corr}$: ^{2D} $n_c \times A_0$;
711 ^{2D}DF = ¹DF $\times$ ²DF
712

Table 2. Characterization of cocoa secondary metabolites separated using the optimized LC × LC-ESI-MS/MS method developed.

Peak	Compound	Retention Time (min)	m/z	Main Fragments
Xanthines ([M+H]⁺)				
1	Caffeine	15.3	195.1	175; 158; 121
2	Theophylline	15.5	181.3	137; 117; 99
3	Theobromine	15.6	181.1	158; 138; 104
Monomeric flavan-3-ols ([M-H]⁻)				
4	Catechin	17.3	289.3	245; 195
5	Epicatechin	17.6	289.4	245; 195
6	Unknown compound	27.6	589.0	589; 441; 295; 113
Oligomeric proanthocyanidins ([M-H]⁻)				
7	Procyanidin Dimer	35.4	578.1	425; 290; 245; 205
8	Procyanidin Dimer	35.5	577.8	577; 425; 245; 289
9	Procyanidin Dimer	35.7	577.4	577; 425; 245; 289
10	Procyanidin Trimer	40.6	865.6	577; 455; 384; 289
11	Procyanidin Trimer	41.5	865.4	578; 441; 384; 289
12	Procyanidin Trimer	41.8	865.7	739; 577; 425; 289
13	Procyanidin Trimer	43.5	865.4	664; 580; 440; 113
14	Procyanidin Tetramer	44.7	1153.3	865; 531; 384; 289
15	Procyanidin Tetramer	45.5	1153.5	865; 577; 455; 289
16	Procyanidin Tetramer	45.6	1153.8	866; 575; 395; 289
17	Procyanidin Tetramer	45.8	1153.7	865; 575; 395; 289
18	Procyanidin Pentamer	47.8	1441.3	1153; 865; 384; 289
19	Procyanidin Pentamer	48.8	1441.5	1153; 865; 775; 384
20	Procyanidin Pentamer	49.6	1441.5	1154; 863; 720; 384
21	Procyanidin Hexamer	51.6	1730.2	1146; 865; 384; 289
22	Procyanidin Hexamer	51.7	1729.2	1151; 865; 384; 289
23	Procyanidin Hexamer	53.6	1729.3	1151; 865; 384; 289
Doubly-charged proanthocyanidins ([M-2H]²⁻)				
24	Procyanidin Heptamer	55.7	1008.6	865; 515; 384; 289
25	Procyanidin Heptamer	57.7	1008.7	779; 531; 384; 289
26	Procyanidin Octamer	59.7	1153.3	779; 515; 384; 289
27	Procyanidin Nonamer	63.8	1297.4	1175; 865; 739; 515
28	Procyanidin Decamer	65.9	1442.5	1152; 865; 384; 289
29	Procyanidin Undecamer	68.0	1599.2	865; 515; 384; 289
30	Procyanidin Dodecamer	69.8	1733.9	1440; 865; 383; 290