Determination of flavanols by liquid chromatography with fluorescence detection. Application to the characterization of cranberry-based pharmaceuticals through profiling and fingerprinting approaches

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ABSTRACT

In this work, a new method based on reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection (FLD) was established for the determination of catechins and related oligomeric proanthocyanidins (PACs) in cranberry-based pharmaceuticals. Compounds were recovered by liquid extraction using methanol/water/hydrochloric acid (60:39:1, *v:v:v*) as the extraction solvent. The chromatographic separation was carried out using a core-shell C18 column under an elution program based on 0.1 % formic acid in water and methanol as the components of the mobile phase. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 µL. Chromatograms were acquired at 280 nm by UV-vis absorption and at λex 280 nm and λem 347 nm by fluorescence spectroscopy. Compared to UV detection, FLD demonstrated both increased sensitivity and selectivity to avoid interfering signals from other phenolic compounds present in the samples. Data resulting from the analysis of cranberry-based products was exploited to tackle an exploratory characterization and classification using principal component analysis. Samples were clustered according to their compositions and those enriched with PACs with antibacterial activity were clearly distinguished from the others.

Keywords: cranberry pharmaceuticals, flavanols, liquid chromatography, profiling characterization, fingerprinting characterization.

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1. Introduction

American red cranberries (*Vaccinium macrocarpon*), typical from the United States and Canada, are increasingly consumed worldwide as fresh fruit or fruit derivatives (e.g. raisins, juices, antioxidant juice cocktails, etc.) as a type of functional food. Besides, cranberries are currently used as raw material to obtain purified extracts to be introduced as the active ingredients of several pharmaceutical products (e.g., capsules, tablets, etc.). Cranberries are rich sources of dietary flavonoids and phenolic acids that may provide a variety of health benefits such as anti-proliferative, antioxidant, anti-inflammatory and antimicrobial properties [1-6]. One of the most appreciated activity is the inhibition of the growth of pathogenic bacteria such as *Escherichia coli* and *Helicobacter pylori* so that cranberry products have traditionally been used to treat and prevent urinary- and digestive-tract infections [3,5-7]. More recently, the antitumor properties of cranberries have made them a popular dietary component to prevent neoplastic diseases [8].

Cranberries contain high concentrations of polyphenols including flavanols, anthocyanins, flavonols and phenolic-acid derivatives [1,4,9]. Regarding flavanols, catechin and epicatechin are the main monomeric components occurring in concentrations of 10 to 100 mg kg^{-1}. Gallic acid derivatives such as gallocatechin, epigallocatechin, gallocatechin gallate and epigallocatechin gallate are found in similar amounts [4,10]. The monomeric flavanol units may be linked in two ways to form oligomers and polymers, the so-called proanthocyanidins (PACs). The most common linkage between the flavan rings is a 4β → 8, which is known as a B-type bond. The A-type linkage is less common and consists of both 4β → 8 and 2β → O → 7 bonds (see Fig. S1 in supplementary material). The presence of A-type linkages provides an additional structural stability to PAC molecules, thus being more resistant to cleavage under harsh conditions such as high temperature or extreme pH values. PACs with A-type linkages and degree of polymerization (DP) up to 10 are abundant in cranberries and have also been found in peanut skin, plum, avocado and curry [11,12].

One of the major protective features of PACs is their ability to bind to and subsequently precipitate proteins, which gives them astringency and makes them unpalatable to potential predators. Recent studies have pointed out the antiadhesive properties of A-type PACs, which seem to confer antimicrobial activity, while B-type counterparts do not exhibit such an activity [3-6].

The characterization and quantification of most of the polyphenolic families in cranberry have been well described elsewhere [1,12-15]. The study of PACs is more challenging due to their oligomeric nature and the lack of commercially available standards so further research strategies are needed [16]. The determination of PACs in matrices of plant origin is a complex issue that requires, in general, an initial extraction step and further determination by liquid chromatography [12-16]. PACs can be recovered from fresh, frozen or dried plant matrices by liquid extraction using acidified hydro-organic mixtures. Solvents such as acetone and methanol, and acids such as acetic, formic, citric or hydrochloric are typically used [13,14]. Plant samples can be subjected to air-drying or freeze-drying processes before grinding, sieving and homogenizing in order to facilitate the extraction and improve the yield. Extract solutions consist of a complex mixture of different classes of phenolic and concomitant substances such as sugars, amino acids, proteins, etc., that may lead to chromatograms with multiple unresolved peaks and overlapping "humps". As a result, further purification steps by adsorption or size-exclusion may be required for interference removal [17,18].

HPLC methods with UV and/or fluorescence detection are often used for PAC quantification [19-23]. However, because of the polymeric structure and the wide molecular mass range of flavanols, their characterization and determination remains as a big analytical challenge. PAC oligomers are hardly separated under reversed-phase mode, thus resulting in broad overlapping bands. Normal-phase mode seems to be more suitable for their separation, leading to peak clusters eluted as a function of DP throughout the chromatogram. Regarding detection, in comparison to UV spectroscopy, fluorescence detection (FLD) provides additional advantages dealing with improved selectivity and sensitivity [19,23]. Interferences from other phenolic compounds can notably be reduced in FLD from an appropriate selection of excitation and emission conditions. The analytical performance can also be enhanced by HPLC-MS [23-28]. The negative ionization in MS is compatible with the anionic nature of analytes although the larger oligomers are poorly detected due to the intense fragmentation under this mode. In contrast, positive ionization with electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) sources seems to be preferred for large PAC molecules. Matrix-assisted laser desorption/ionization coupled with time-of-flight (MALDI-TOF) mass spectrometry has also been introduced for the characterization of flavanols from cranberry, apple and grape [29-30]. In this way, PAC molecules with

high DP can be determined and the structure of monomer building unit and type of inter-flavan linkage (A/B-type) can be differentiated.

This paper is focused on the development of a new HPLC-FLD analytical method for the characterization and quantification of flavan-3-ols in cranberry extracts and pharmaceutical products. It should be pointed out that estimation of PAC amounts is still a controversial issue as values obtained may differ significantly as a function of the method used for the determination. Besides, an overall PAC index may be inefficient to assess the product activity to combat urinary tract infections. Here, a simple and efficient approach for product evaluation according to contents of A-type and B-type molecules was established which seem to be essential from the point of view of the assessment of the antibacterial features. Samples consisting of raw cranberry extracts, antimicrobial pills and capsules and dietary supplements were subjected to an extraction procedure using a methanol/water/hydrochloric acid (60:39:1 *v/v/v*). Analytes (catechin, epicatechin, procyanidin dimers and trimer) from the resulting extracts were separated by reversed-phase HPLC on a core-shell C18 column using an optimized elution gradient based on methanol. The method was applied to the characterization of cranberry-based products under both profiling and fingerprinting approaches using analyte concentrations and FLD chromatograms, respectively. Data was treated chemometrically using principal component analysis and related methods [31]. Here, commercial products can easily be differed according to the flavanol patterns, and samples with high contents of molecules with antimicrobial properties can be identified. The method could be extended to authentication of American cranberry from other types of cranberries or berries that may not contain proper levels of active ingredients.

2. Experimental

2.1. Reagents and solutions

The reagents and solvents used for the preparation of HPLC mobile phase and sample analysis were formic acid (>96%, Sigma-Aldrich, St Louis, USA), methanol (99.9%, UHPLC PAI-ACS SuperGradient, Panreac, Castellar de Valles, Barcelona, Spain) and Mili-Q Water, purified using an Elix 3 coupled to a Milli-Q system (Bedford, USA) and filtered through a 0.22 µm nylon filter integrated into Milli-Q system.

The polyphenolic standards used in this work were epicatechin and catechin from Sigma-Aldrich, procyanidin A2 and procyanidin C1 from PhytoLab (Vestenbergsgreuth, Germany) and procyanidin B2 (>98%) from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Stock standard solutions of polyphenols were prepared at ~1000 mg L^{-1} in methanol using amber glass vials. Intermediate working solutions for method optimization and calibration were prepared by proper dilution in 50% methanol at 1, 5, 10, 20, 40, 60, 80 and 100 mg L^{-1}. All stock and standard solutions were stored at 4 °C. Apart from flavanols, other common polyphenolic compounds were also assayed to assess chromatographic and spectral selectivity issues including gallic acid, 3,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 4-hydroxybenzoic acid, homovanillic acid, vanillic acid, syringic acid, salicylic acid, piceid, resveratrol, chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, quercetin, fisetin and kaempherol, all of them from Sigma-Aldrich.

2.2. Samples and sample treatment

A total of 2 raw cranberry extracts and 17 anti-cystitis products and dietary supplements were analyzed in this work. Raw cranberry extracts referred to as E01 and E02 were kindly provided by Deiters S.L. (Barcelona, Spain). These products were used as the main materials to prepare some pharmaceuticals. Dietary supplements and parapharmaceuticals were purchased from pharmacies and specialized shops in Gdansk (Poland) and Barcelona (Spain). They were coded as follows: C01 and C02 were tablets containing ca. 250 mg of cranberry extracts (equivalent to 130 mg PACs) together with other natural plant extracts; C3 to C14 consisted of gelatin capsules filled with amounts of cranberry extracts ranging from 175 to 425 mg and, depending on the cases, with some co-adjuvants such as ascorbic acid, grape, heather and salvia extracts; Sa01 and Sa02 were sachets containing 240 mg PAC; Sy01 was a syrup with 12 mg mL^{-1} PAC.

The sample extraction procedure was as follows: 0.1 g of sample (weighed with a PB1502-L analytical balance, Mettler-Toledo, Columbus, OH, USA) were dispersed in 10 mL of methanol/water/hydrochloric acid (60:39:1 *v/v/v*) solution. Samples were shaken (Genius 3 Vortex mixer IKA, Staufen, Germany), sonicated for 30 min at room temperature (Branson 5510, Richmond VA) and centrifuged for 15 min at 3600 *g* (Rotanta 460 RS, Hettich, Tuttlingen, Germany). Afterwards, the supernatant extracts were separated from the solid and filtered through 0.45 μm nylon filters (Whatman,

Clifton, NJ, USA). In order to avoid an initial concentration decay due to analyte adsorption in the membrane, 1 mL of sample was kept in an amber vial after rejecting the first 2 mL of filtrate. Solutions were stable for at least 1 month when stored in the refrigerator at 4ºC. Sample extraction was carried out in triplicate to obtain independent extract solutions. A quality control (QC) was prepared by mixing 100 µL of all sample extracts.

2.3. Chromatographic method

The chromatographic system consisted of an Agilent Series 1100 HPLC chromatograph (Agilent Technologies, Palo Alto, California, USA) with binary pump (G1312A), degasser (G1379A), automatic injection system (G1329B), diode array detector (G1315B) and fluorescence detector (G1321A). All these components were from the 1100 series except the automatic injector that belonged to the 1200 series. An Agilent ChemStation for LC (Rev. A. 10.02) software was used for instrument control and processing.

The chromatographic separation was carried out using a Kinetex C18 reversed-phase (100 mm x 4.6 mm I.D., 2.6 µm particle size) column with a Gemini C18 pre-column (4.0 mm x 6.0 mm I.D.) from Phenomenex (Torrance, California, USA). The elution program was generated with 0.1 % formic acid in water (*v/v*) (solvent A) and methanol (solvent B) as follows: 0 to 7 min, B(%) 12→35 (linear increase); 23 to 33 min, B(%) 35→95 (linear increase). Subsequently, the column was cleaned for 2 min at 95% B and conditioned for 3 min at 12% B. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 µL. Chromatograms were acquired at 280 nm by absorption, and at λ_{ex} 280 nm / λ_{em} 347 nm by fluorescence detection (FLD) spectroscopy.

Sample extracts were injected randomly to minimize any possible influence of chromatographic drifts over time on the results. The QC solution was injected throughout the series of runs, at the beginning, at the end and every 5 extract injections.

2.4. Data analysis

Data was treated by Principal Component Analysis (PCA) and other chemometric methods using the PLS-Toolbox (version 3.5, www.eigenvector.com) for MATLAB. The study was performed under the following approaches:

(i) Profiling using flavanol concentrations. Data was autoscaled to equalize influence of major and minor components on the model.

(ii) Fingerprinting using FLD chromatograms recorded at λ_{ex} 280 nm / λ_{em} 347 nm within the time window 5- 28 min. Data was normalized to minimize the effect of overall component concentration. In order to improve the quality of the model, a precision filter based on the reproducibility of QC data was applied to detect and remove noisy variables with RSD values higher than 20 %.

In the two approaches, the plot of scores showed the distribution of the samples on the principal components (PCs) to reveal the main patterns and the influence of raw extracts on the pharmaceuticals. The variability of the chromatographic runs throughout the data set was assessed from the dispersion of QCs, which should be located in a compact cluster in the center of the graph. The plot of loadings showed the distribution of variables and their influence on the sample properties. The most descriptive variables could be also deduced.

3. Results and discussion

3.1. Optimization of the experimental conditions

The separation of analytes relied on chromatographic methods previously reported by Aznar, Raja and coworkers [32,33] for the determination of phenolic acids and flavonoids in wines and fruit samples. Compounds were separated using an elution gradient based on increasing the percentage of methanol. Chromatograms were preliminarily recorded by UV-vis spectroscopy at 280, 310, 370 and 550 nm to gain information on the sample complexity regarding other polyphenols such as cinnamic acids, flavonoids and anthocyanins. Although this paper is mainly focused on flavanols, it should be pointed out that compositional data from other families of polyphenols could also be exploited for additional descriptive purposes.

The elution program was first optimized to tackle the resolution of A2, B2 and C1 PACs using the corresponding pure standards. Fig. 1 shows that all the analytes were reasonably resolved (in the most critical case, the resolution between epicatechin and procyanidin C1 $Rs_{epicatechin/procyanidinC1}$ was 1.3). For A2, which was the active antimicrobial compound, appeared fully separated from the other components. The chromatogram of a cranberry fruit extract (Fig. 2) showed multiple peaks from other polyphenols, some of them overlapping with those of flavanols, such as in the case of epicatechin partly resolved from caffeic acid (t_R 17.1). Other compounds such as chlorogenic or coumaric acids (t_R 15.5 and 18.3 min, respectively) also displayed intense absorbing peaks at 280 nm. It was thus encountered that UV detection was not specific for catechins and multiple components were detected at 280 nm.

The detection of flavanols was also established by FLD. 3-dimensional excitation and emission spectra of epicatechin, catechin, A2, B2 and C1 standards were recorded as follows: excitation range, 260-300 nm in steps of 5 nm; emission range, 270-400 nm in steps of 1 nm. The maximum fluorescence intensity of procyanidin A2 was at λ_{exc} 280 and λ_{em} 347 nm (see Fig. S2 in supplementary material). Spectra of the other catechins were highly similar so these values were selected for FLD. It was found that other common sample components such as hydroxybenzoic acids also produced a residual fluorescence under these conditions while hydroxycinnamic acids and flavonoids were undetectable. Apart from selectivity, the sensitivity was also enhanced. In the example of Fig. 2 compounds such as catechin, hardly detectable in UV mode, produced intense peaks in FLD. In this regard, detection limits of the HPLC-DAD-FLD method were about 10-fold better for FLD than for UV (see "Figures of merit" section). In conclusion, FLD at λ_{exc} 280 and λ_{em} 347 nm seemed to be more appropriate than UV spectroscopy for the determination of analytes so this detection technique was chosen in further studies.

Extraction conditions were thoroughly optimized via experimental design to gain information on the principal factors influencing on the yield and selectivity of extracts. Previous extraction studies recommended acidified water/acetone solutions for the recovery of PACs from cranberry and other fruits [16]. Initial conditions such as sample amount, solvent volume and time to start the optimization were adopted from previous studies [9,33]. Here, a grid design consisting of three solvent percentages (60, 70 and 80% *v/v*) at four hydrochloric acid levels (0, 0.5, 1 and 2% *v/v*) was planned with a total of 12 experiments to be run. Other conditions were as follows: sample weight ~

0.1 g, solvent volume 10 mL, extraction method by sonication, extraction time 30 min. Results in Fig. 3a indicated that, in general, the overall PAC extraction slightly increased with the acidity of the extraction solvent from 0 to 1% *v/v* hydrochloric acid and decreased at 2%. Regarding acetone content, it was found that the PAC recovery decreased with increasing the solvent percentage. These general patterns were also observed when focusing on individual compounds such as A2 and epicatechin as representative major components of this fruit extract. As a result, the best recoveries were attained at 60% *v/v* acetone and 1% *v/v* HCl. Unfortunately, direct injections of acetone extracts were not recommendable as they led to poor separation performance with double and unresolved peaks for most of the components. This drawback was solved by solvent evaporation and re-dissolution of the residue in the mobile phase.

In order to try to overcome the main disadvantages of acetone-based extraction, methanol was investigated as an alternative solvent. 12 experiments were run at solvent percentages of 0 (water), 50 and 60% and acid levels of 0, 0.5, 1 and 2% *v/v*. Results obtained indicated that overall PAC recovery and individual recoveries of epicatechin and A2 increased in the range 0 to 1% *v/v* HCl and slightly decreased up to 2%. The effect of methanol on the extraction yield showed a progressive increase with increasing the solvent content reaching the maximum at 60% *v/v*. This behavior was similar to the acetone system although, from a quantitative point of view, the overall recovery was 35% higher with MeOH. In addition, MeOH extracts could be directly injected into the HPLC without affecting the separation performance and, thus, pre-chromatographic solvent evaporation was not required. The composition of the extraction solvent finally selected was methanol/water/hydrochloric acid 60:39:1 *v/v/v*.

3.2. Figures of merit

Analytical parameters of the proposed method established with analytes standards are summarized in Table 1. Retention times of catechin, epicatechin, procyanidin B2, procyanidin C1, and procyanidin A2 were 10.8, 16.3, 13.3, 16.8, and 23.3 min, respectively. Repeatabilities estimated as the relative standard deviation (RSD%) from 6 replicate injections at 10 mg L^{-1} each flavanol were about 0.1% in terms of retention time and 0.3% in terms of peak area. For fluorescence detection at λ_{exc} 280 and λ_{em} 347 nm, calibration models were linear in the range 0.3 to 100 mg L^{-1} for most of the analytes (0.5 to 100 mg L^{-1} for procyanidin C1), with correlation

coefficients better that 0.999. Limits of detection (LODs) of FLD estimated for a signal-to-noise ratio of 3 were below 0.11 mg L^{-1}, except for procyanidin C1 which was 0.14 mg L^{-1}. Comparatively, LODs calculated in UV detection at 280 nm were one order of magnitude poorer: catechin, 0.7 mg L^{-1}; epicatechin, 0.8 mg L^{-1}; procyanidin B2, 0.5 mg L^{-1}; procyanidin A2, 0.7 mg L^{-1}; procyanidin C1, 0.7 mg L^{-1}.

Analytical parameters were also assessed from independent analyses of sample extracts. The RSD values of retention time (n=10) of compounds were below 0.05%. In the case of peak area repeatability, RSD values were 3.3% for catechin, 4.8% for epicatechin, 3.6% for procyanidin B2 and 2.9% for procyanidin A2 (procyanidin C1 was not detected). The occurrence of matrix effects was previously investigated and results indicated that ratios (in percentage) of sensitivities in pure synthetic and extracted matrices were close to 100%, thus suggesting the absence of such a kind of deviations [9,32,33].

3.3. Determination of PACs in extracts and pharmaceutical samples

PAC contents in cranberry extracts and pharmaceutical samples were determined using the HPLC-FLD method developed here. Three independent extractions of each sample were carried out as described in the experimental section using 10 mL of methanol/water/hydrochloric acid 60:39:1 *v/v/v* solution.

Results from various representative examples are depicted in Fig. 4. The composition of raw cranberry extracts referred to as samples E1 and E2 were remarkably different, being E1 rich in A2 (and B2) while E2 mainly contained monomers. The low concentration of A2 in E2 could be attributed to the use of cranberry varieties poor in A2, inefficient extract purification or even adulteration issues. Indeed, a previous publication by Navarro *et al.* suggested that the composition of this extract was more similar to grapes than to American cranberry (16). The analysis of the pharmaceutical samples revealed that some capsules and pills displayed a pattern similar to E1 (e.g., C2 with high levels of A2) while others resembled E2 (e.g., C10 with poor amount of A2). This finding suggested that these pharmaceutical products were manufactured from one or another kind of raw material. A third compositional pattern was also encountered which corresponded to samples such as C12 with high levels of B2 and monomers and undetectable concentrations of A2.

Overall results showed that A2 content ranged between not detected (*n.d.*) and 7.4 mg g^{-1} depending on the samples, with a mean value of 1.6 mg g^{-1} (see Fig. S3 in supplementary material). In the case of B2, concentrations were in the range of *n.d.* to 12.6 mg g^{-1}, being the richest samples those with negligible amounts of A2. Regarding monomers, epicatechin was, in general, more abundant, but samples similar to E2 raw material contained high levels of catechin.

3.4. Classification of PAC samples

First sample characterization was attempted using flavanol concentrations determined in the previous section as the analytical data. The PCA model working with autoscaled data retained 48.1, 33.6 and 17.1% of data variance on PC1, PC2 and PC3, respectively. The map of scores (Fig. 5a) showed that QCs appeared together in a compact cluster in the center of the graph which demonstrated that data was highly reproducible. The reproducibility of the extraction process could be also evidenced from the analysis of independent sample triplicates, which were always located in close positions. Samples were mainly distributed according to three principal patterns, being E1, E2 and C12 representative examples of each trend. The information gained from the loadings (Fig. 5b) denoted that sample patterns corresponded to (i) extracts rich in A2, (ii) extracts rich in monomeric species and (iii) extracts rich in B2, respectively. From the point of view of the biological effects, it could be expected that samples with compositional profiles similar to E1 reasonably exhibited antimicrobial activity while the other would be less effective against urinary tract infections [3-5].

The fingerprinting approach relied on FLD chromatograms recorded at λex 280 nm / λem 347 nm within the working time window 5 - 28 min. Chromatogram segments from 0 to 5 and from 28 to 38 min were excluded from the analysis as they corresponded to signals of sample front and cleaning and conditioning steps which might distort the sample description. Apart from flavanols, chromatographic fingerprints contained other relevant features related to benzoic acids and stilbenes. Various peaks were identified in the chromatograms of QCs (Fig. 6c) including gallic, 3,4-dihydroxybenzoic, 2,5-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, and syringic acids, and epigallocatechin, piceid and resveratrol. Data was normalized and filtered as explained in the experimental section prior to PCA. Under these conditions, PC1 captured 52.2%, PC2 18.0% and PC3 6.6% of data variance. As shown in the plot of

scores (Fig. 6b), QCs were compactly grouped thus suggesting the absence of noticeable chromatographic drifts during the measurements. The distribution of samples on PC1, PC2 and PC3 was in agreement with that from the profiling approach (see above), with three patterns corresponding to representative samples such as E1, E2 and C12. PC1 partly described the overall response intensities and its contribution to class discrimination was limited. In contrast, PC2 and PC3 were highly useful to distinguish samples according to phenolic contents. The relevance of flavanols and other polyphenols as class descriptors was deduced from the plot of loadings (Fig. 6c). Apart from flavanols, gallic, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, and syringic acids were other noticeable markers that contributed to sample discrimination.

4. Conclusions

The method developed here based on liquid chromatography with fluorescence detection (HPLC-FLD) opened up new opportunities to tackle the determination of flavanols in cranberry extract samples. HPLC-FLD resulted in an excellent combination to improve both sensitivity and selectivity in comparison with other existing methods based on UV detection. Owing to the great stability of resulting chromatographic data, HPLC-FLD was highly recommendable for processing large sets of samples such as in the course of routine control or characterization studies. The application of experimental design to the extraction process facilitated the optimization of solvent and acidity conditions. Sample characterization by Principal Component Analysis relied on profiling and fingerprinting approaches using flavanol concentrations and FLD chromatograms, respectively. Sample behavior was analogous in the two models and products rich in the active ingredient against urinary tract infections were easily recognized. Concentration data was certainly simpler in terms of matrix dimensions and interpretation of loadings but involved a more expensive and time-consuming quantification step using standards. The use of fingerprints required high reproducibility in the chromatographic domain throughout the entire series of runs. In the present case, this requirement was successfully achieved, as deduced form the behavior of QCs, but it could be a serious drawback when dealing with data of higher variability. As a potential advantage, chromatographic fingerprints contained additional features related to phenolic acids that could be of great interest as complementary descriptors for sample characterization, classification and authentication.

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Table 1. Figures of merit of the proposed method.

compound	retention time (min)	retention time repeatability RSD%	peak area repeatability RSD%	Linear range (mg L^{-1})	Sensitivity (A.U. min L mg^{-1})	r^2	LOD (mg L^{-1})
Catechin	10.8	1.10	0.29	0.2 – 100*	10.67	0.999	0.06
B2	13.3	0.37	0.30	0.4 – 100*	6.46	0.999	0.09
Epicatechin	16.3	0.12	0.27	0.2 – 100*	9.22	0.999	0.06
C1	16.9	0.88	0.35	0.5 – 100*	4.59	0.999	0.15
A2	23.3	0.31	0.23	0.4 – 100*	4.67	0.999	0.1

* maximum assayed

Figure captions

Figure 1. Chromatogram of a standard mixture of flavanols obtained by HPLC-FLD at λex 280 nm / λem 347 nm. Analyte concentration 10 mg L^{-1} each. Peak assignation: 1 = catechin, 2 = procyanidin B2, 3 = epicatechin, 4 = procyanidin C1, 5 = procyanidin A2.

Figure 2. Chromatogram of a cranberry extract obtained by HPLC-DAD-FLD. Acquisition conditions: (a) DAD, λ 280 nm; (b) FLD, λex 280 nm / λem 347 nm. Peak assignation: see Fig. 1.

Figure 3. Experimental design for the evaluation of the influence of acidity and organic solvent percentage on the flavanol extraction. (a) Acetone; (b) Methanol.

Figure 4. Composition of selected samples. Analyte assignation: 1 = catechin, 2 = epicatechin, 3 = procyanidin B2, 4 = procyanidin A2.

Figure 5. Characterization of cranberry pharmaceutical samples by principal component analysis using concentrations of flavanols as the data. (a) Plot of scores; (b) Plot of loadings.

Figure 6. Characterization of cranberry pharmaceutical samples by principal component analysis using chromatographic fingerprints as the data. (a) Chromatograms of representative samples C12, E1, E2 and QC; (b) Plot of scores; (c) Plot of loadings. Analyte assignation: 1 = catechin, 2 = epicatechin, 3 = procyanidin B2, 4 = procyanidin A2; a = gallic acid; b = 3,4-dihydroxybenzoic acid; c = 2,5-dihydroxybenzoic acid; 4-hydroxybenzoic acid; e = homovanillic acid; f = vanillic acid; g = syringic acid; h = salicylic acid; i = piceid; j = resveratrol.

Figure 1

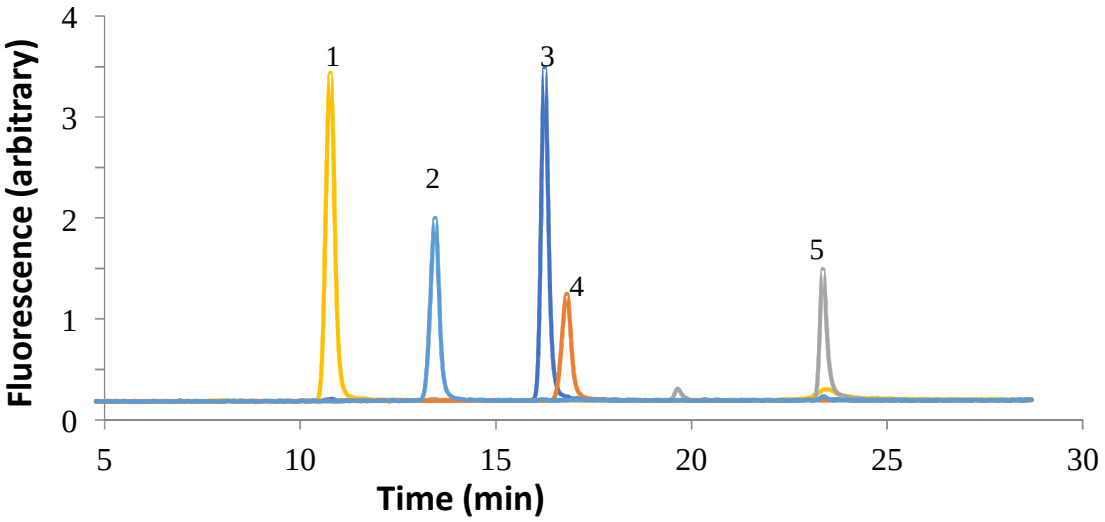


Figure 2

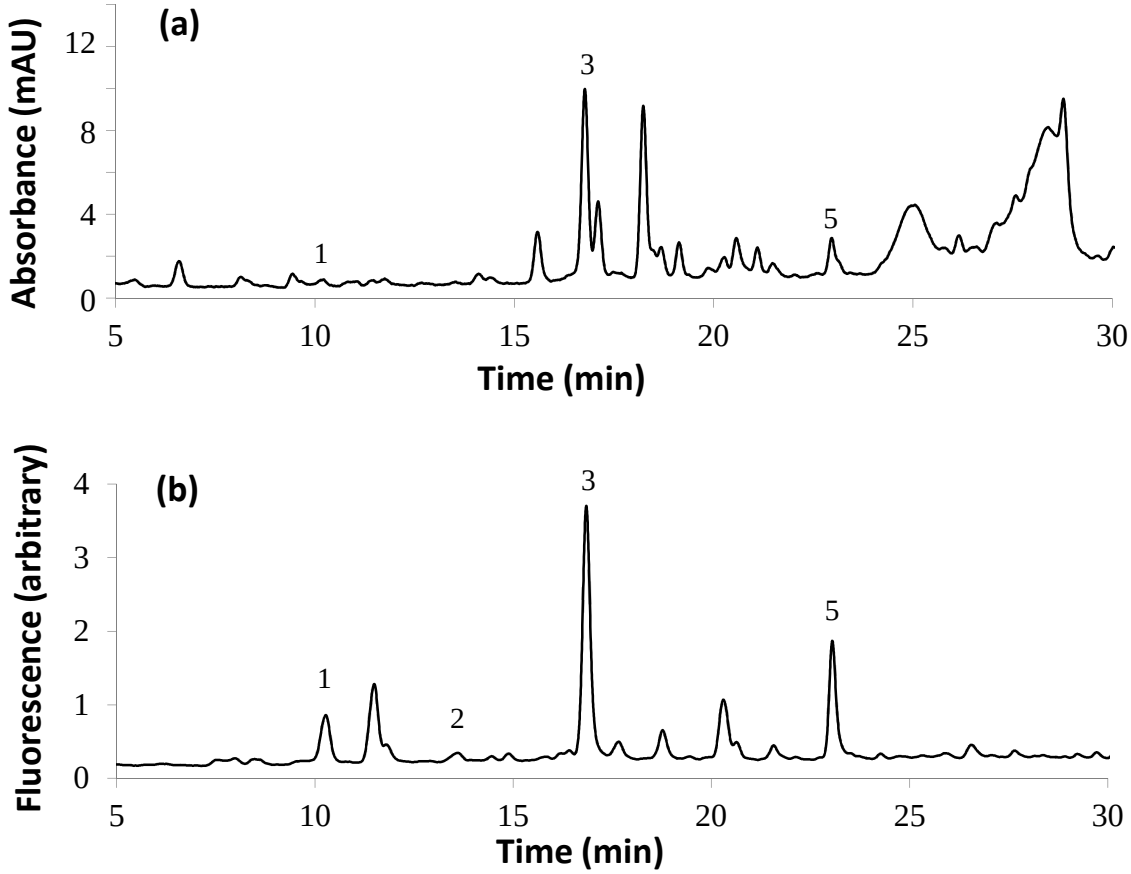

(a)
Absorbance (mAU)
12
8
4
0
1
3
5
5
10
15
20
25
30
Time (min)
(b)
Fluorescence (arbitrary)
4
3
2
1
0
1
2
3
5
5
10
15
20
25
30
Time (min)

Figure 3

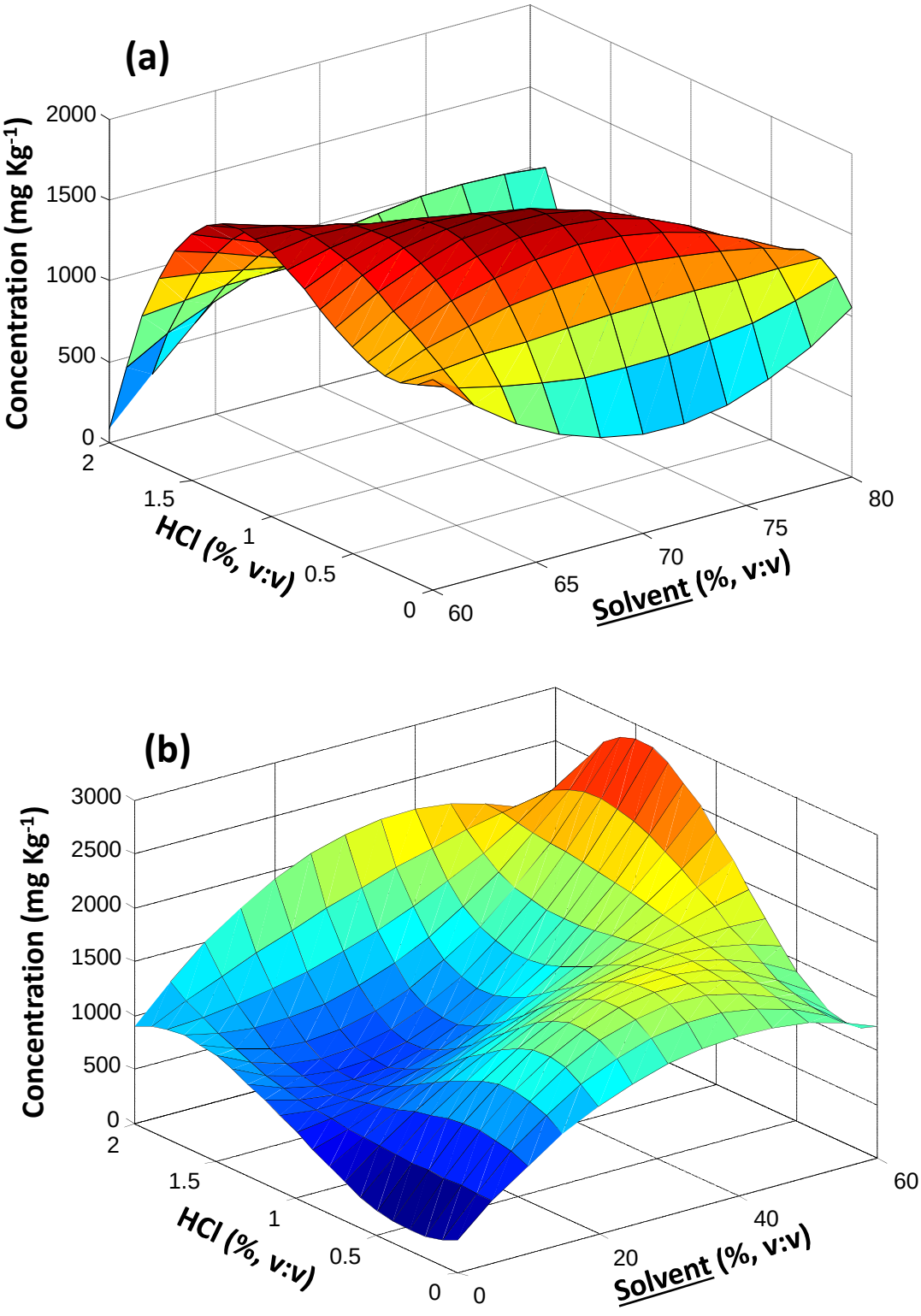

(a)
Concentration (mg Kg-1)
2000
1500
1000
500
0
2
1.5
1
0.5
0
HCl (%, v:v)
60
65
70
75
80
Solvent (%, v:v)
(b)
Concentration (mg Kg-1)
3000
2500
2000
1500
1000
500
0
2
1.5
1
0.5
0
HCl (%, v:v)
0
20
40
60
Solvent (%, v:v)

Figure 4

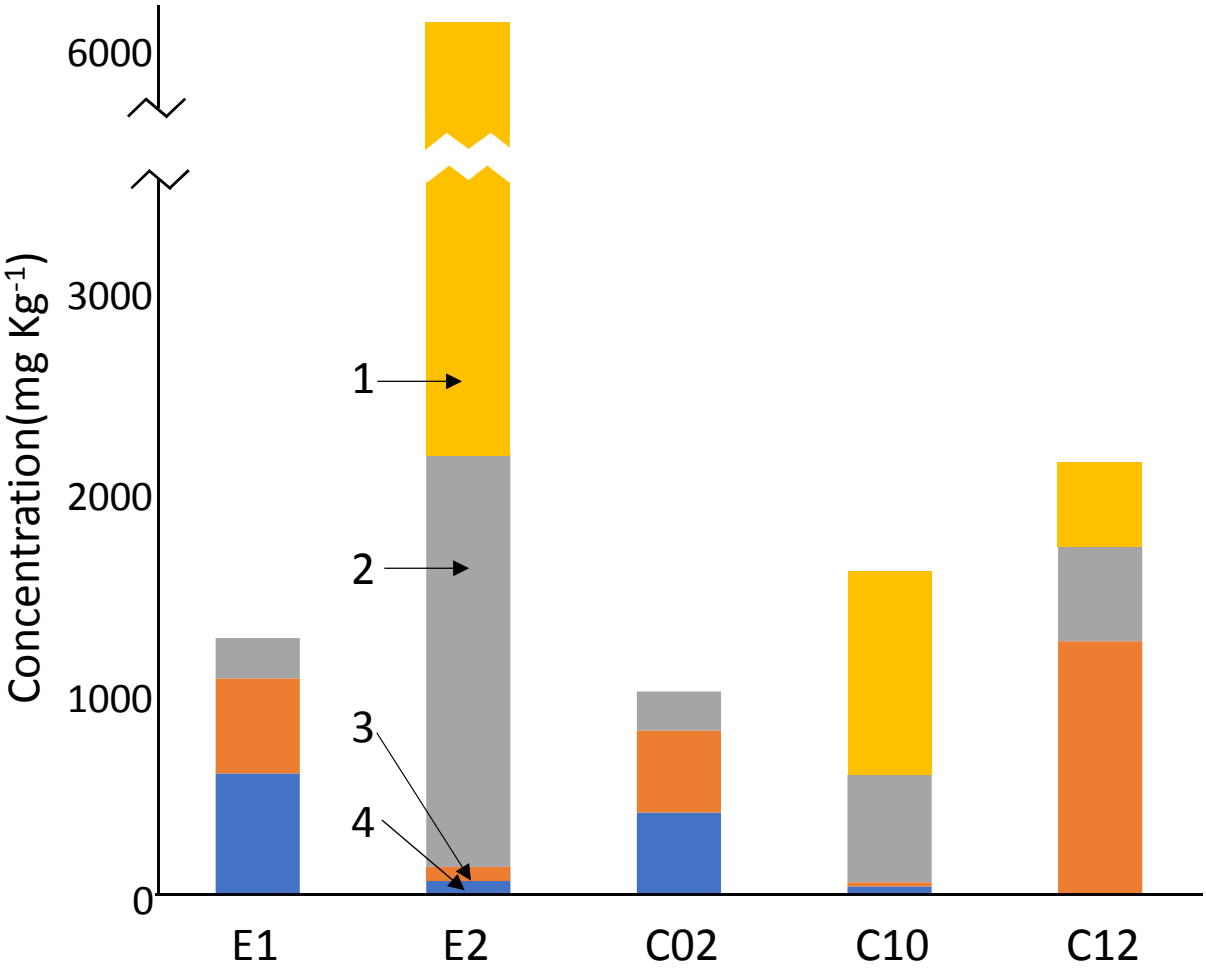

6000
3000
2000
1000
0
Concentration(mg Kg-1)
1
2
3
4
E1
E2
C02
C10
C12

Figure 5

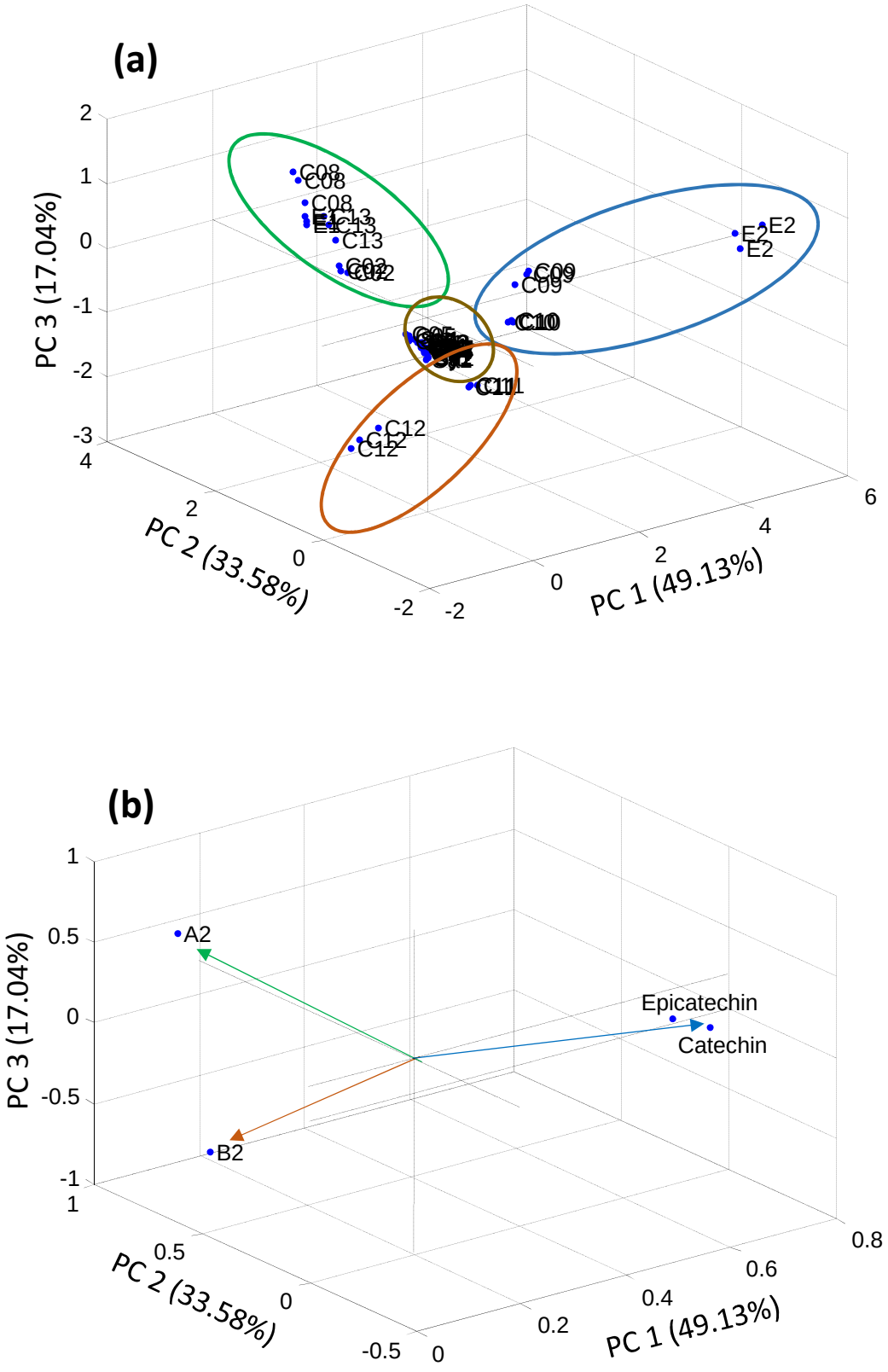
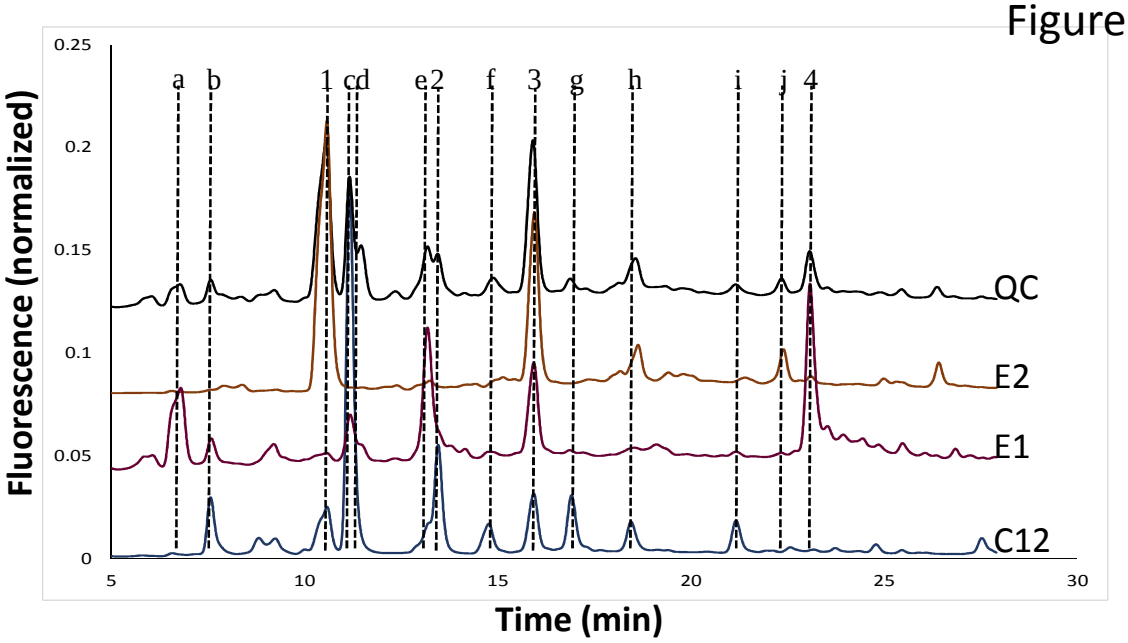
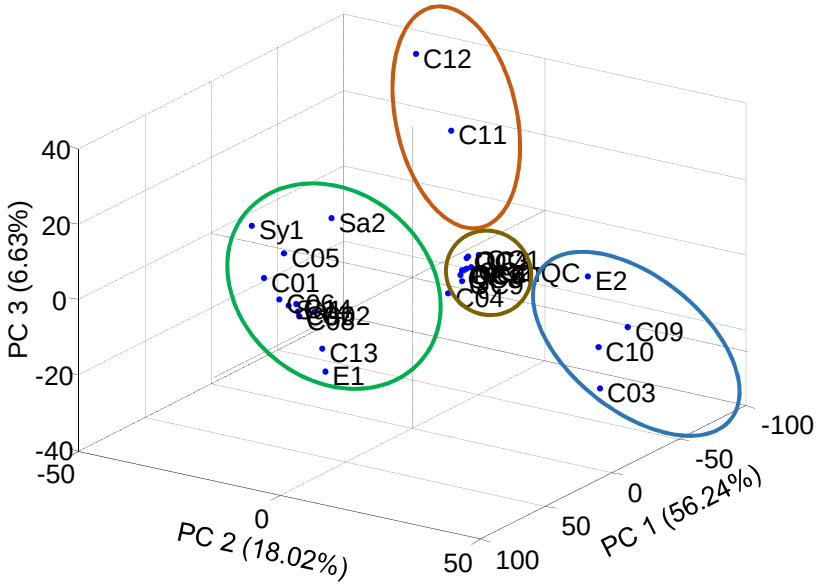
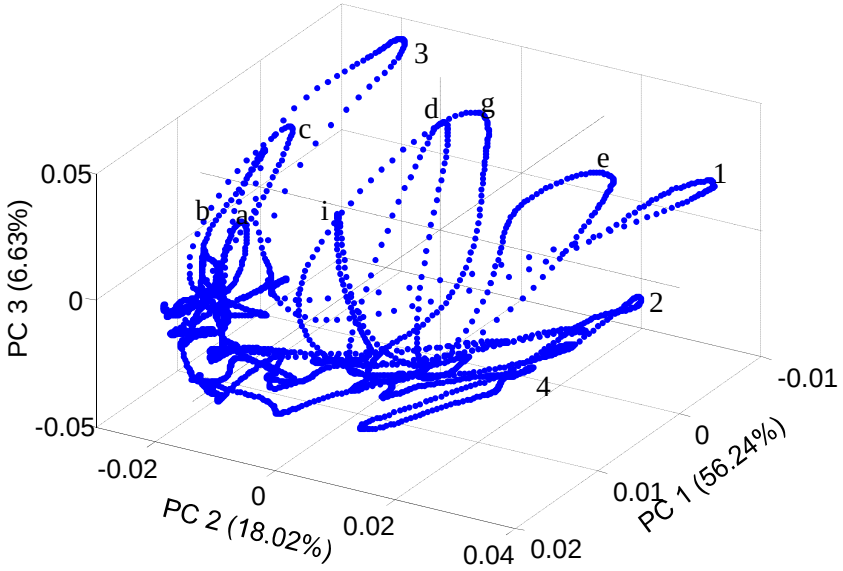

(a)
PC 3 (17.04%)
PC 2 (33.58%)
PC 1 (49.13%)
C08
C13
C12
E2
C09
C10
C11
(b)
PC 3 (17.04%)
PC 2 (33.58%)
PC 1 (49.13%)
A2
B2
Epicatechin
Catechin

Figure 6


Fluorescence (normalized)
0.25
0.2
0.15
0.1
0.05
0
a b 1 c d e 2 f 3 g h i j 4
QC
E2
E1
C12
5
10
15
20
25
30
Time (min)


C12
C11
Sy1
Sa2
C05
C01
QC
E2
C04
C09
C10
C03
C13
E1
PC 3 (6.63%)
PC 2 (18.02%)
PC 1 (56.24%)


3
d
g
c
e
1
b
a
i
2
4
PC 3 (6.63%)
PC 2 (18.02%)
PC 1 (56.24%)

Supplementary material

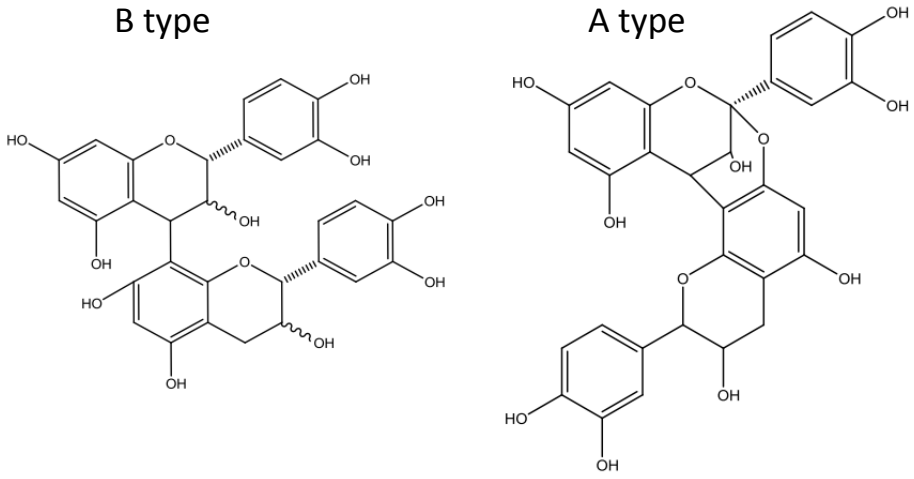


Figure S1. Chemical structure of B- and A-type interflavan links of proanthocyanidins.

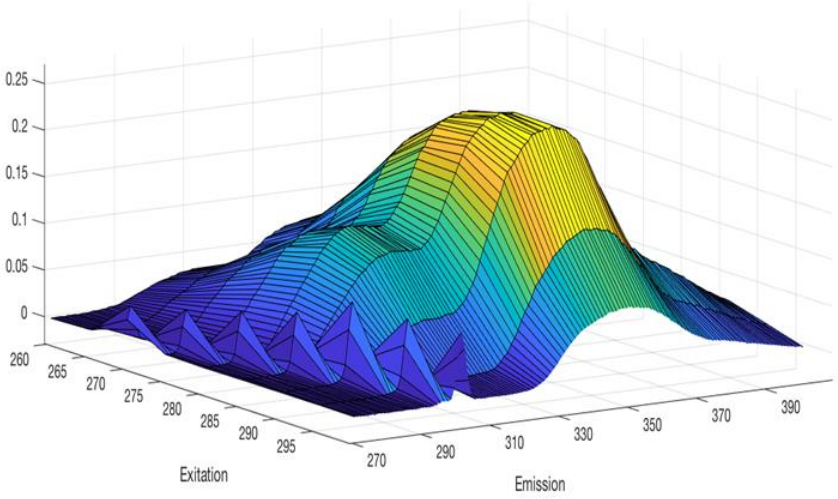


Figure S2. Excitation-emission spectra of 10 mg L^{-1} procyanidin A2. Excitation: 260 – 300 nm in steps of 5 nm. Emission: 270 – 400 nm in steps of 1 nm.

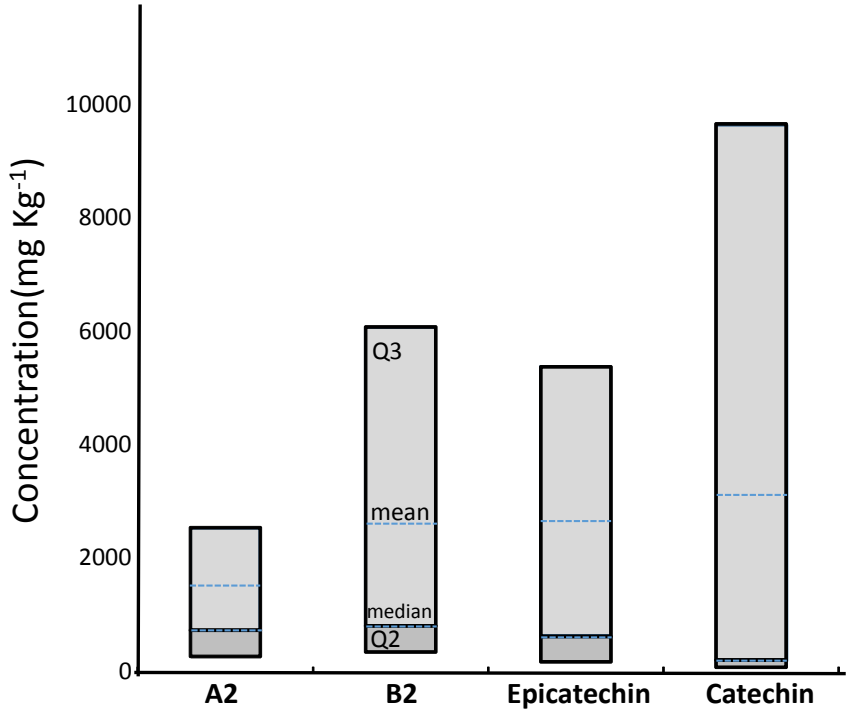


Figure S3. Box plot corresponding to the determination of flavanols in the set of samples with mean, median and quartile 2 (Q2) and quartile 3 (Q3) ranges.