# A galloylated dimeric proanthocyanidin from grape seed exhibits dentin biomodification potential

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**A B S T R A C T**

Grape seeds are a rich source of polyphenols, especially proanthocyanidins (PACs), and are also known for the presence of galloylated oligomeric PACs (OPACs). The present study focuses on the phytochemical methodology for grape seed (O)PACs and their potential role as dentin biomodifiers to be used in restorative and reparative dentistry. A new method using centrifugal partition chromatography (CPC) was developed for the preparative separation of the grape seed (O)PACs. Orthogonal phytochemical profiling of the resulting CPC fractions was performed using C18 and diol HPLC, normal phase HPTLC, and IT-TOF MS analysis. A galloylated procyanidin dimer (**1**) was isolated from a CPC fraction in order to evaluate its potential to enhance dentin bio-mechanical properties. Moreover, it helped to evaluate the impact of the galloyl moiety on the observed bioactivity. Structure elucidation was performed using ESI-MS, 1D and 2D NMR analyses. For the first time, $^{1}$H iterative full spin analysis (HiFSA) was performed on this type of molecule, enabling a detailed proton chemical shift and coupling constant assignment. The CPC fractions as well as **1** showed promising results in the dentin stiffness bioassay and indicate that they may be used as dental intervention biomaterial.

*Keywords:*

Centrifugal partition and countercurrent chromatography (CPC/CCC); countercurrent separation (CS), Oligomeric proanthocyanidins (OPACs); dentin stiffness and biomodification; galloylation; HiFSA

*Chemical compounds studied in this article:*

Procyanidin B1 (PubChem CID: 11250133); Procyanidin B3 (PubChem CID: 146798); Epicatechin-(4β→8)-epicatechin-3-*O*-gallate (PubChem CID: 15593124)

## 1. Introduction

Resin-based dental restorations are micromechanically bonded to the tooth. With this purpose, a hybrid structure is built, where the fluid resin infiltrates and is anchored to the dentin by the exposed dentin organic matrix. The greater cosmetic appeal compared to the traditional amalgam based restorations resulted in the popularity of composites for restorative/reparative procedures. However, the relatively short service-life is one of the drawbacks of composite based dental restorations [1, 2]. One of the reasons for the failure of dental adhesive restorations is the weakening of the dentin-resin hybrid layer (Figure 1). Dentin is a calcified tissue that forms the bulk of the tooth and is mainly composed of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals (inorganic content) and type I collagen (organic content), and the latter plays a major role in the dental restorative procedures.

Proanthocyanidins (PACs) can modify the dentin organic matrix by cross-linking collagen, thereby enhancing mechanical strength of the dentin [3-5]. This biomimetic strategy, in addition to other interactions with the non-collagenous components can potentially stabilize the dentin-resin interface and increase the longevity of the adhesive dental restorations.

PACs are naturally occurring polyphenols that belong to the flavonoid class of compounds and are also known as condensed tannins (S1, Supplementary Data). PACs are made up of flavan-3-ol building blocks. Two types of interflavanyl linkages are commonly observed in PACs: in B-type linkages the monomeric flavan-3-ol units are connected via a 4→8 or 4→6 C-C bond between the upper and the lower unit, respectively. Double interflavanyl linkage in the A-type PACs consists of an additional ether (C-O-C) bond between the C-2 of the upper and an oxygen on the C-5 or C-7 position of the lower unit. The majority of the grape seed PACs possess B-type interflavanyl linkages. Although, the basic biosynthetic pathways for PAC precursors has long been known, the exact mechanism of their polymerization is still elusive. There is evidence that potential mechanisms involve enzymatic or non-enzymatic condensation between the electrophilic C-4 position (via quinone methide formation) and the nucleophilic C-8 or C-6 position [6]. PACs are highly abundant in grape seeds and skin. The monomers, (+)–catechin and (-)–epicatechin, are the most commonly occurring building blocks in grapes [6, 7]. The occurrence of galloylated **o**ligomeric **p**ro**a**ntho**c**yanidins (OPACs; see S1, Supplementary Data) is a characteristic feature of the more highly condensed grape seed polyphenols [8-10].

PACs have been evaluated for decades in a number of different bioassays and have been found to be active in-vitro as well as in-vivo [11-13]. Over a thousand reports published in the past four decades have linked the monomeric flavan-3-ols with anti-oxidant activity, with a large number also linking the compounds with anti-inflammatory and cardio-protective activity: in the SciFinder Scholar database, 518 references closely link (O)PACs with anti-oxidant activity, and 1310 references utilize both keywords in the discussion. However, the application of (O)PACs in the field of dentistry as a biomaterial is still relatively unexplored and has only a handful of publications to date.

Both the degree of polymerization (DP) and galloylation of the OPACs have been identified as key structural features that influence the overall dentin-targeting bioactivities [14-16]. The lack of commercially available standards, due to challenges in isolating pure OPACs, is an obstacle that stands in the way of both dental researchers and phytochemists. Notably, a large variety of possible permutations and combinations of flavan-3-ol units give rise to an immense number of structural possibilities for the OPACs. For example, in the case of grapes and considering known monomer substitution patterns and linkage types, more than 16,000 theoretical structural possibilities exist just for tetrameric PACs in grapes, and almost more than double that number in the case of mangosteen [17]. Even if only a fraction of them is in fact biosynthesized by the plants and/or present at abundance levels that enable analysis and isolation, the OPACs contained in the plant metabolome represent thousands of distinct chemical species. This epitomizes why both the analytical separation and preparative fractionation of OPACs has always been a daunting task, typically associated with seemingly poorly resolved chromatograms.

Over time, a large number of chromatographic methods have been developed for separation of proanthocyanidins [18, 19]. This is likely in an attempt to overcome the high level of complexity of OPAC-containing extracts that always leaves room for improvement in the applied separation methodology. The present study offers a new method for the preparative separation of grape seed OPACs using fast centrifugal partition chromatography (FCPC). In addition, the relevance of galloylation in OPACs for increased dentin interaction is highlighted after the evaluation of both the CPC fractions as well as an isolated dimeric gallate, compound **1**.

## 2. Experimental

*2.1 Instrument and materials*

Preparative separation was performed on a Fast Centrifugal Partition Chromatography (FCPC$^R$) type countercurrent separation instrument from Kromaton Technologies (Angers, France). It was equipped with a 1L rotor, a 50 mL sample loop and a Rheodyne injector. An LKB Bromma 2111 MULTIRAC was used for fraction collection. Nano-Sil-20UV$_{254}$ 10 × 20 cm and 0.2 mm thick normal phase HPTLC plates were obtained from Macherey & Nagel. A Camag TLC automatic sampler 4 was used to spot the samples on the plates. Reversed-phase UHPLC profiles were obtained on ACQUITY UPLC column (C18 1.7 µm; 2.1 × 50 mm, Waters Inc.) using a Shimadzu Nexera UHPLC system with a PDA detector. Normal phase HPLC profiles were acquired on a Waters 600 HPLC instrument using a Develosil 5 µm Diol 100A 250 × 4.6 mm column (Phenomenex) with a 2996 PDA detector. Semi-preparative HPLC was carried out on the same Waters HPLC system on YMC-PACK-ODS-AQ 250 × 10 mm with YMC-Guardpack-ODS-AQ 30 × 10 mm guard column at 3 mL/min flow rate. MS analysis was performed in infusion mode on an LCMS IT-TOF hybrid mass spectrometer (Shimadzu, Kyoto, Japan). The 1D and 2D NMR spectra were acquired on an 800 MHz Bruker Avance II spectrometer (Billerica, MA, USA) equipped with a 5 mm triple resonance inverse TXI room temperature probe.

For the dental studies, a universal testing machine, EZ Graph (Shimadzu, Kyoto, Japan) was used to perform the dentin stiffness assay using a 3-point bending method [20].

All the chemicals and solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA). Grape seed extract produced from the seeds of *Vitis vinifera* L. was purchased from Polyphenolic Mega Natural Gold, Madera (California, USA batch no. 206112508-01/122112505-01).

*2.2 Preparation of GSE-UP fraction*

Crude GSE was partitioned with a two phase solvent system composed of methyl acetate/water (v/v) to remove the higher order (DP>7) oligomeric and polymeric PACs from the extract. The lower phase was dried down and repeatedly partitioned (up to 5 times). The yield of the combined upper phase partitions (GSE-UP) was 35% (10 g of GSE yielded 3.5 g of GSE-UP

after partitioning). GSE-UP exhibited higher bioactivity than the crude GSE and hence was used for further studies in this paper.

*2.3 Preparative separation using FCPC*

Hexanes/ethyl acetate/methanol/water (HEMWat) 1/10/1/10 was used as the solvent system for CPC. Separation was performed in the ascending mode, i.e., the upper phase was used as mobile phase and the lower phase was kept stationary. Preparative separation of 3.0 g of GSE-UP was implemented with one injection in a single run. The rotor speed was initially kept at 200 rpm for filling the coil with stationary phase (LP), then increased to 1000 rpm for equilibration with the mobile phase (UP), elution, and extrusion. After equilibration at 20 mL/min flow rate, the stationary phase retention was determined to be 77% with $(V_C - V_M) / V_C * 100$ [21]. Elution was performed in two steps: eluted with 1400 mL of HEMWat UP, then switched to methyl acetate for 1800 mL. Methyl acetate mobile phase was saturated with HEMWat LP in a shake flask prior to use in order to avoid excessive stationary phase bleeding. This was followed by extrusion with 1250 mL of stationary phase. Total 315 fractions were collected with 0.7 min/tube in 3.7 h running time and pooled into 15 fractions on the basis of their TLC behaviors.

*2.4 CPC fraction profiling*

*2.4.1 HPTLC profiling*

The recombined fractions 1 to 15 were analyzed using HPTLC. Chloroform/methanol/acetic acid/water in a 60/40/2/2 ratio was used as mobile phase for developing the plates. The plates were observed under UV 254 nm and later sprayed with acidified vanillin solution and heated until color development. Pictures were taken at UV 254 nm and in white light after the vanillin treatment (Figure 2).

*2.4.2 UHPLC profiling*

Each sample was prepared at a concentration of 5 mg/mL with methanol. Solvent A was 0.1 % formic acid and solvent B was acetonitrile. The gradient used was 2-3% B in 1 min; 4% B at 3 min; 11% B at 20 min; 22% B at 21 min; 90% B from 22-23 min and 5 min of re-equilibration time with flow rate at 0.5 mL/min. The PDA chromatograms were extracted at 280 nm for data analysis. These data were exported as ASCII format and imported into Origin Pro 9.1 for plotting the 3D stacked chromatograms using waterfall Z color mapping (Figure 3).

*2.4.3 Diol normal phase HPLC profiling*

Each sample was prepared at a concentration of 10 mg/mL in methanol for normal phase HPLC analysis. Acetonitrile/acetic acid (98/2) was solvent A, and methanol/water/acetic acid (95/3/2) was solvent B. HPLC analysis was performed according to a method previously reported in the literature [22]. The PDA chromatograms were extracted at 280 nm, stacked in Origin Pro 9.1 using a protocol similar to that used for the UHPLC profiles. On diol stationary phase, OPACs are predominantly separated according to their degree of polymerization.

*2.4.4 IT-TOF MS analysis*

IT-TOF MS analysis was performed on fractions 1-15 using the flow injection method. The analyses were done in full scan mode. Mass spectra were acquired in both positive and negative modes over a mass range of *m/z* 250 to *m/z* 1600 Da. ESI type of ion source was used with the following parameters: capillary voltage of 1.5 kV, CDL temperature 200 °C, and a nebulizing gas flow of 1.5 L/min.

*2.5 Isolation and structure elucidation using 1D and 2D NMR*

Epicatechin-(4β→8)-epicatechin-3-*O*-gallate (**1,** 9.0 mg from fraction F5), procyanidin B1 (**2**, 10 mg from F7), and procyanidin B3 (**3**, 2.0 mg from F7) were purified by semi-preparative HPLC on a YMC ODS AQ column. Solvent B was acetonitrile and solvent A was 0.1% aqueous formic acid. A gradient was initiated at 20% B, linearly increased to 100% B in 20 min, and kept at 100% B for 3 min before going back to the initial 20% B in 0.5 min to re-equilibrate the column for 10 min.

NMR data was acquired on a Bruker 800 MHz Avance II spectrometer at low temperature (255 K). The sample was dissolved in methanol-$d_4$ and its residual proton signal was used as an internal reference. NMR data was processed with MestReNova v9 software. NMR simulation and $^1$H iterative full spin analysis (HiFSA) was performed with PERCH NMR software package version 2013.1 SA (PERCH Solutions Ltd. Kuopio, Finland). The chemical shifts and coupling constants reported here are generated by PERCH software.

*2.6 Dentin mechanical bioassay*

Recently extracted human sound molars were selected after approval from the Institutional Review Board Committee of the University of Illinois at Chicago (protocol no. 2011-0312). Teeth

were cut to obtain dentin beams with 0.5 × 1.7 × 6.0 mm (H × W × L). The beams were demineralized using 10% phosphoric acid as previously described [23]. The fractions 1-15 were prepared at 0.65% w/v concentration dissolved in 0.02 M HEPES buffer (pH 7.2). Each dentin beam was immersed in 100 µL treatment solution for 1 h in a 96 well plate, protected from light. The modulus of elasticity (in MPa) was obtained at baseline and post treatment using a universal testing machine in a three-point flexural assay [5, 16].

## 3. Results and Discussion

Our recent research has shown that (O)PACs from certain plant sources can greatly improve the strength of demineralized dentin [3, 5, 16]. Accordingly, OPACs have the potential to be a viable resource of dental biomaterials [17]. This was demonstrated by screening a panel of various commercially obtained polyphenol rich plant extracts including cocoa extract, pine bark extract, green tea extract, and grape seed extract (GSE), and a homemade 70% methanolic extract of cinnamon bark. Grape seed extract was found to be the most active amongst all the screened extracts [16] and was also distinguished by the presence of galloylated OPACs. Hence GSE was chosen for further study. On partitioning GSE between methyl acetate and water, the upper phase of the two- phase system (GSE-UP) was found to be more active in the dentin stiffness assay than both the lower phase and the crude GSE, suggesting that the intermediate order OPACs with a degree of polymerization (DP) of *ca*. 2 to 5 possess better dentin biomodification potential than the higher order oligomeric (DP > 7) or the very high order polymeric PACs (data not shown).

### *3.1 Preparative separation of grape seed OPACs by CPC and characterization of the fractions*

A new method using CPC, a hydrostatic form of countercurrent separation (CS), and a solvent system specifically optimized for the grape seed OPAC mixtures was developed for preparative separation of polyphenols. While it has been shown that the hydrodynamic countercurrent columns afford better chromatographic resolution than the hydrostatic systems [21, 24], another crucial factor affecting chromatographic resolution in CS is the stationary phase retention (S*f*). As OPACs require highly polar solvent systems such as HEMWat +7 or +8 [25] to match their polarities and achieve favorable K values, the relatively lower retention of these polar solvent systems on hydrodynamic instruments compromises their separation performance. Better stationary phase retention of polar solvent systems in hydrostatic columns makes CPC actually

more suitable for such separations [21, 24]. In addition, CPC offers the advantage of a high sample loading capacity on bench top instruments, which enables the production of relatively large amounts of fractionated material suitable for the biomechanical bioassay in a research laboratory.

Compared to published CS methods, the present method features a two-step elution protocol. Step 1 followed a standard procedure where the aqueous stationary phase was eluted with the organic mobile phase of the HEMWat 1/10/1/10 solvent system, operating in ascending mode. This enabled the separation of the monomeric flavan-3-ols and dimers from the other OPACs. Continued elution with the mobile phase was not effective for the separation of trimers and higher oligomers, and resulted in an extensive overlap of compounds. Hence, a second elution step with a saturated methyl acetate was introduced in order to improve the resolution. This step markedly improved the chromatographic resolution between the galloylated dimers from their non-galloylated counterparts, and also gave a relatively better distribution of trimers and higher order oligomers in the fractions. As the separation involved elution with methyl acetate, the K values of compounds eluting from step 2 could not be determined directly due to the non-equilibrated system resulting from the newly introduced solvent.

The CPC fractions could be classified into different OPAC groups based on their DP, using IT-TOF MS analysis (Table 2) as well as diol normal phase HPLC profiles (Figure 3). Mass-to-charge ratio (*m/z*) values obtained in the negative mode of the MS analysis were used. Fractions F1-F4 (tubes 5-27) represented galloylated and non-galloylated monomeric flavan-3-ols. A salient feature of the CPC method is that the galloylated dimers could be well separated from their non-galloylated counterparts with almost no overlap. F5 (tubes 94-128) was enriched in mono-galloylated dimers (S2), whereas F7 (tubes 143-159) was enriched in non-galloylated dimeric OPACs. F8 (tubes 160-175) was mainly composed of non-galloylated OPAC dimers and trimers. There was a considerable overlap in F9 and F10 (tubes 176-212), consisting of prodelphinidin type of dimers and OPAC trimers. Mono-galloylated OPAC trimers and di-galloylated dimers were also detected in F9 and F10. F11-F15 were characterized by the presence mono- and di-galloylated PAC trimers, tetramers, and pentamers. While orthogonal separation steps will have to be developed in order to characterize the trimeric and tetrameric PACs, the present study yielded a mono-galloylated dimer (**1**) and two non-galloylated dimers procyanidin B1 (**2**, epicatechin-(4β→8)-catechin) and B3 (**3**, catechin-(4α→8)-catechin), the former was purified on semi-preparative ODS HPLC column from fractions F5, and the latter were

from F7, respectively. Furthermore, the purity of **1** in fraction F5 was determined to be 68.57% w/w by the 100% qHNMR method [26, 27] (S3), a value consistent with the typically observed residual complexity of OPAC isolates. Overall, the CPC method was efficient in separating the mono-galloylated dimeric OPACs from both the monomers and the rest of the oligomers and yielded a fraction highly enriched in **1**.

The concurrent use of HPTLC and HPLC for the profiling of the fractions was capable of assessing the overall quality of the separation and complemented the mass spectrometric evidence (Figures 2 and 3). These phytochemical profiles can act as OPAC fingerprints of the materials and help categorizing the fractions used for the evaluation of dentin biomechanical properties. These phytochemical profiling methods could be applied in general to obtain fingerprints of any polyphenol rich plant extracts for future studies. The diol NP-HPLC profiles show the distribution of oligomers in the different fractions based on their retention behavior (Figure 3); this was not achieved with RP-18 sorbents. As the DP increases in the later CPC fractions (F9-F14), the complexities of the OPACs also increase as indicated by the large hump peaks around 35-65 min. As galloylated OPACs are more polar than their non-galloylated counterparts, they are retained longer on the diol stationary phase. Accordingly, galloylated dimers are eluted closer to the trimeric PACs, and galloylated trimers overlap with the tetrameric OPACs. Consequently, in grape seed extracts, the oligomer distribution pattern in diol-based HPLC is complicated further by the presence of galloylated OPACs. In contrast, the lack of galloyl OPACs in, for example, cocoa extract leads to their elution in an orderly fashion, directly based on their DP [28].

*3.2 Isolation and structural analysis of PAC dimers*

One prerequisite of evaluating isolated OPACs in the dentin stiffness bioassay is that the phytochemical protocol, including structure elucidation, needs to be performed with underivatized material. This requirement not only limits the usefulness of NMR data available in the literature, which predominately stems from derivatized compounds, but also increases the complexity of the spectroscopic task due the presence of rotamers. At the same time, considering the stereochemical complexity of the PACs, in order to establish solid links between PAC structure and dentin-enhancing activity, it was important to unambiguously assign the structures of all isolates, including presumably known compounds.

Isolated **1-3** were dried and re-dissolved in methanol-$d_4$ for NMR analysis. The NMR data were recorded at low temperature on a Bruker 800 MHz spectrometer. The use of low temperature is essential for the NMR analysis of many OPACs with B-type interflavanyl linkage, in order to obtain interpretable spectra and achieve complete NMR structural assignments [29-31]. The reason for low-temperature measurements is the presence of dynamic equilibria caused by atropisomerism, which is commonly observed in OPACs due to restricted rotation along the interflavanyl bond [29-31]. At room temperature, steric hindrance near this linkage leads to slow rotation on the NMR timescale. This variation of the magnetic environment results in the observation of a large number of rotameric forms on the NMR timescale, thus causing broad signal or "humps" in the NMR spectrum (Figure 4). The interflavanyl rotation was further restricted in the low temperature to lead to the observation of typically one or two major rotamers in the minimized energy. At the same time, signal line shape and overall resolution are improved, thus enabling a more detailed interpretation of the NMR spectra. In the present study, the assignment of **1-2** was achieved through interpretation of their low temperature NMR data, respectively. Interestingly, **3** representing a dimeric PAC of similar constitution yielded clean and interpretable spectra at room temperature. Accordingly, it is likely that the structure is symmetric, i.e., the two monomer units in **3** are (+)-catechin.

Out of the three isolated dimers, only **2** and **3** are commercially available, while **1** is not, in spite of being isolated and/or synthesized previously. Of the three existing reports on the NMR structural assignments of **1** [32-34], the most comprehensive data set had been acquired in a $D_2O$/acetone-$d_6$ mixture at 243 K on a 400 MHz instrument using a synthetic sample of both derivatized and non-derivatized material [32]. However, definitive structural dereplication by NMR was still hampered by the lack of conclusive H, H-coupling information (*J* values and signal multiplicity) for proton H-3 of the lower ECG moiety, which has always been reported as a multiplet. Moreover, no assignments have been reported previously for the B ring protons of both the upper and the lower unit. Herein, the complete $^1$H chemical shift and coupling constant assignments are reported for the underivatized form of **1** (major rotamer), using a solution of **1** in methanol-$d_4$ and an 800 MHz instrument at 255 K (Table 1, Figure 4). Signals from the minor rotamer are also assigned unambiguously, except for position H-3 of the lower unit as the signal is ~~completely~~ overlapped with the HDO resonance. This coincidence was confirmed by the observation of an HSQC cross-peak centered at $\delta_C$ 69.804 ppm, belonging to C/H-3. The presence

of the gallate functional group was readily confirmed by the singlet observed at 7.009 ppm, integrating for two chemically equivalent protons. The 4→8 interflavanyl linkage was confirmed via the observed HMBC correlation of H-2 of the lower unit (L-2) and, H-4 of the upper unit (U-4) with C8a of the lower unit. The 4β→8 absolute configuration was confirmed from the circular dichroism (CD) data, by the positive Cotton effect observed between 210-240 nm (S7). The relative configurations at the positions L-2/L-3 and U-2/U-3 could also be determined as *cis* from the smaller coupling constants of 1.47 Hz and 1.04 Hz between the respective protons of the lower and upper unit, respectively. This readily explained the observation of broad singlets of slightly different shape. Considering the literature, as well as the higher natural abundance of *epi*-catechin relative to *ent-epi*-catechin, **1** is tentatively determined to possess *epi*-catechin as the upper unit and *epi*-catechin gallate as the lower unit (Fig 5).

The $^{1}$H iterative full spin iterative (HiFSA) analysis [35, 36] of **1** was performed with the PERCH NMR software tools and is shown in Figure 6. PERCH uses quantum mechanical calculations in order to simulate $^{1}$H NMR spectra from the spin parameters ($J$, ν) of the molecule and utilizes iterative calculations to fit both the integrals as well as the total line shape of the experimental signals. Achievement of a perfect match between the calculated and the observed spectrum is visualized and also indicated by the RMS value of the residual spectrum. Through this process, a digital spectrum (HiFSA profile) was generated and saved as a text file (PMS file). It contains all coupling constant and chemical shift values of **1**. This information can be used to re-generate the spectrum at any field strength (HiFSA fingerprint) for verification and dereplication as well as quantitation of **1** in a less complicated mixture. The structures of **2** and **3** were also confirmed by NMR analysis, literature reports [31, 37, 38], and via HiFSA fingerprinting (S6, S7, S9, S10, Supplementary Data). The purity (%, w/w) of **1**, **2**, and **3** was determined to be 88.5%, 74% and 89% respectively by qHNMR using the 100% method [26, 27] (S4, S8, S11, Supplementary Data).

*3.3 Changes in dentin biomechanical properties by the CPC fractions and isolated compounds*

In the dentin mechanical bioassay, fraction F1 containing galloylated flavan-3-ol monomers was more than twice as active as F2 which is mainly composed of non-galloylated monomers. Also, fractions F5 and F6 consisting of mono-galloylated PAC dimers exhibited a higher potential to enhance dentin mechanical properties than fractions F7 and F8, which are mainly composed of

non-galloylated OPAC dimers (Figures 2 and 3). These observations suggest that galloylation plays a role in the dentin biomodification potential of the OPACs. Such a differentiation is hard to make in the rest of the OPAC fractions with DP more than 3, due a greater overlap between the galloylated and non-galloylated trimeric and tetrameric OPACs. Among the tested pure isolates, **2** showed only a two-fold increase in activity from the baseline control, whereas **1** (a dimer, similar to **2** except for a gallate moiety attached at L-C-3) exhibited a 5.7 fold increase in the bioassay (Figure 7). The observation that the galloylated dimer, **1**, is more than twice as active as non-galloylated compound **2** reinforces the importance of galloylation in interaction of PACs with detin matrix. This data is in agreement with our previous studies on the evaluation of galloylated and non-galloylated monomeric flavanols as dentin biomodifying agents [16]. Fraction F5 shows a 7.14 fold increase in the dentin stiffening bioactivity which is significantly higher than that of **1** (5.7 fold) isolated from this fraction. This suggests that other structural analogues and/or isomeric forms of compound **1** might play an additive role in the observed bioactivity of the fraction. It remains to be shown if the galloylated and non-galloylated trimers and tetramers follow an analogous trend in terms of their bioactivity. However, considering the analytical challenge, further method development is needed in order to isolate galloylated higher order OPACs from grape seeds.

Studies performed on the interaction between collagen and plant derived polyphenols suggest that mostly non-covalent mechanisms such as hydrogen bonding and hydrophobic interactions [35, 36]. Presence of a galloyl moiety offers more hydroxyl groups for cross-linking and might be a reason for higher dentin stiffness observed on treatment with gallates. Some studies have also speculated on the possible covalent interactions of polyphenols with collagen [39, 40].

Interpreting the bioactivity profile of the fractions, it appears that all the fractions are active to a certain extent. This is due to the fact that the phytochemical components in all the fractions have a similar skeletal structure. Also, it is important to highlight that in the context of dentin biomodification, the OPACs, unlike drugs or other small molecules, do not target a single receptor or an enzyme but mediate collagen cross-linking and also interact with other non-collagenous components in the dentin [41]. This is a rich micro-environment with abundant sites available for cross-linking. For this reason, a significantly high concentration of the active material (0.65 to 6.5% w/v) is needed to produce a mechanically observable effect when compared to other in-vitro bioassays. Reflecting back on our earlier work, it is also important to recall that only certain

plant-specific OPAC mixtures act as dentin bio-modifiers, with grape seed extract being one of the strongest crude agents tested in our laboratory to date. Isolating major compounds is a prerequisite for determining the most active components of the extract or an enriched fraction and will further help with the characterization and production of a standardized intervention material. The intervention material itself will most likely be a mixture of potent active OPACs that promote dentin substrate strengthening and biostability over time.

## 4. Conclusions

The new CPC-based preparative method developed for the separation of grape seed OPACs offers a good blend of chromatographic resolution and high throughput for large scale sample production. In spite of CPC being a primary fractionation step, a fraction highly enriched in dimeric PAC gallates was obtained and enabled the isolation of **1**. One key feature of the CPC based 2-step elution method is the achievement of clean separation between galloylated and non-galloylated dimeric proanthocyanidins. The combined bioassay and phytochemical data suggest that the presence of galloylated OPACs makes GSE a promising source for dental biomaterial development.

Inevitably, the galloylation of OPACs in GSE complicates the chromatographic separation and subsequent spectroscopic analysis. The use of low temperature 1D and 2D NMR studies was vital for obtaining interpretable NMR spectra for **1** and **2**. Accompanied by HiFSA, advanced $^{1}$H NMR analysis of OPACs enables the complete assignment of proton chemical shifts and coupling constants for the underivatized form of these molecules, which are available for biological evaluation. The HiFSA fingerprints are both highly specific for the compounds and field independent, and therefore can be used for structural dereplication and/or mixture analysis [36].

Compound **1** shows prominent activity in the dentin stiffness assay and is significantly more active than **2**. Also, some commercially available PACs such as procyanidin B2, A1, A2, and the trimer, procyanidin C1, were tested in the dentin stiffness assay in our previous experiments [41]. The OPAC **1** was found to possess a superior dentin stiffness enhancing potential compared to all the aforementioned commercially obtained OPACs. The prominent dentin activity of **1** can be attributed to the presence of a galloyl functional group. Although, it must be noted that gallic acid on its own does not have a significant dentin biomodification potential and needs to be attached to a flavanol core in order to enhance the dentin stiffness [41].

The data obtained in the present study suggest that grape seed OPACs have a strong dentin biomodification potential and are favorable candidates for developing a clinical intervention material. The increase in the dentin mechanical properties promoted by monomer and dimers is dependent on the degree of polymerization and galloylation. The focus of future studies will be to compare the bioactivities of the galloylated and non-galloylated trimers and tetramers.

## Acknowledgements

The authors are particularly grateful to Dr. Benjamin Ramirez from the Center for Structural Biology at UIC for his general NMR support and his invaluable assistance in performing the low temperature NMR experiments. We also thank an anonymous reviewer for helpful suggestions regarding the nomenclature of oligomeric proanthocyanidins. This research was funded by grant R01 DE021040 from NIDCR/NIH.

## Appendix A. Supplementary Data

Supplementary data to this article (PDF files with Appendix; ZIP file with raw and processed qHNMR data) can be found online at http://dx.doi.org/10.1016/j.fitote.2015.xx.xxx.

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## Figures and tables

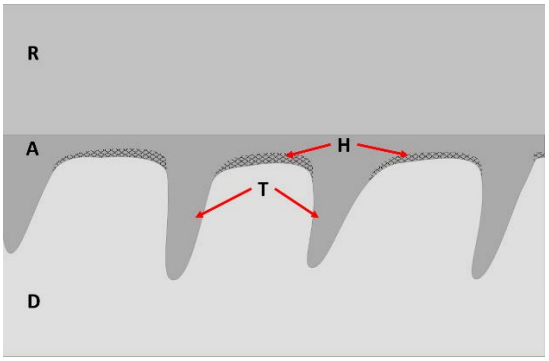


**Fig. 1.** Scheme representing the main components of dentin-resin interface. D – Dentin; A – resin-based dental adhesive; T – resin tags (infiltration of the adhesive into the dentin tubules); H – hybrid layer (resin infiltrated dentin organic matrix); R- resin composite.

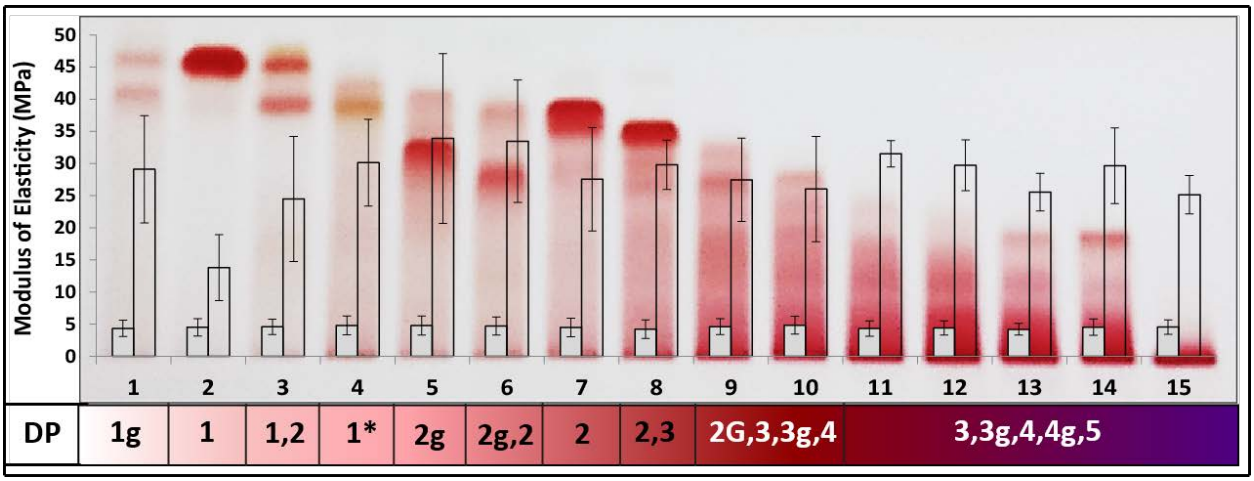


**Fig. 2.** Classification of CPC fractions F1-F15 according to the DP with the dentin modulus of elasticity (MPa) at baseline (white bar) and post treatment (no fill bar) overlaid on HPTLC profile. The figure shows the relationship between the bioactivities of fractions and their DP. Gradation of the *Rf* values on HPTLC plate highlight the overall quality of separation achieved and show that the separation is according to the DP. The exact DP of PACs contained in each individual fractions have been obtained from IT-TOF MS analysis; Number: DP, g – degree of galloylation, G – "gallo" or 3,4,5-trihydroxy substitution pattern on a B ring, * - flavonoids other than proanthocyanidins.

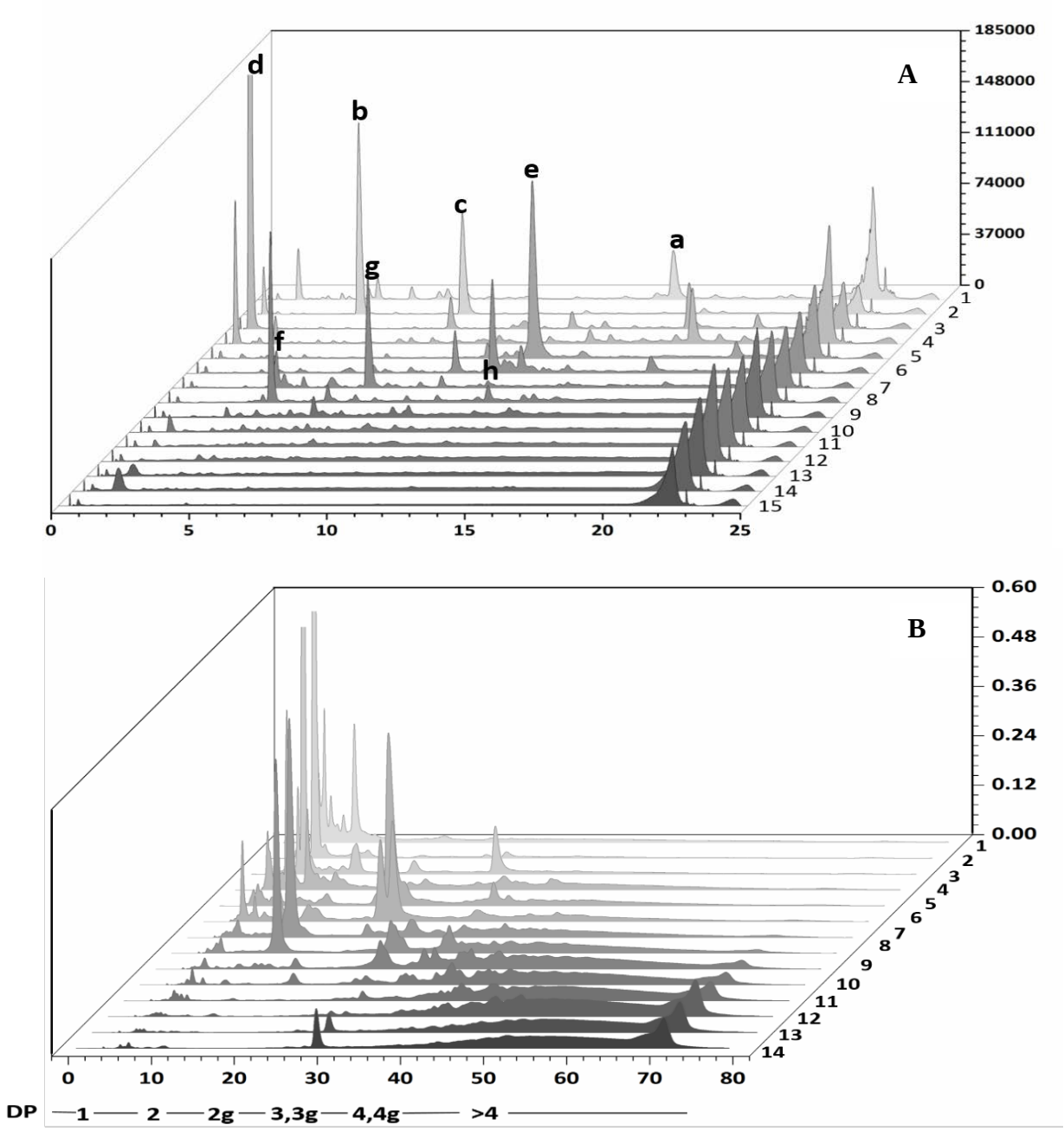


**Fig. 3.** The $C_{18}$-UHPLC profiles of fractions F1-F15 (A) and diol normal phase HPLC profiles of fractions F1-F14 (B). The $C_{18}$-UHPLC profiles of the fractions in panel A help in the identification of some monomers and PAC dimers in the earlier CPC fractions (F1-F8); a: epicatechin gallate, b: catechin, c: epicatechin, d: gallic acid, e: compound **1**, f: procyanidin B1, g: procyanidin B2, h: procyanidin C1. The diol normal phase HPLC profiles in panel B help in classification of fractions according to their DP based on the retention times and also provide complementary evidence to the mass spectrometric data. (X-axis – retention time in minutes; Y-axis – UV absorption intensity; z-axis – fraction numbers). The chromatograms were extracted at UV 280 nm.

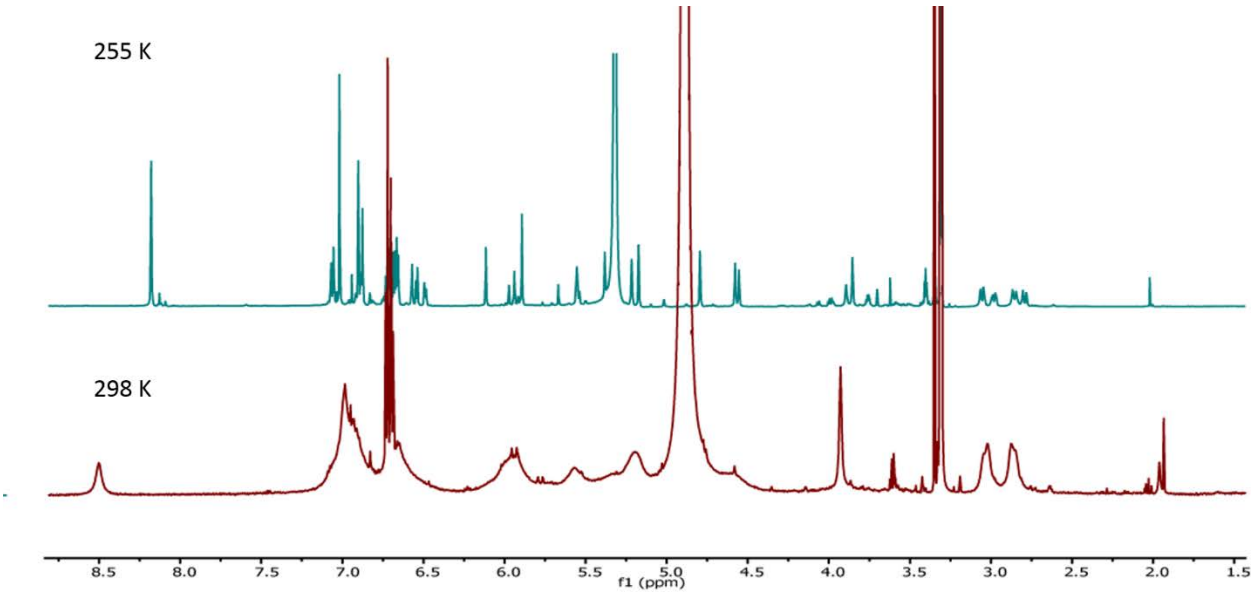


**Fig. 4.** $^{1}$H NMR spectra of compound 1 at 255 K (low temperature) and 298 K (room temperature). Restricted rotation around the interflavanyl bond in proanthocyanidins results in complex and uninterpretable NMR spectra at room temperature. This phenomenon is called atropisomerism. The above figure highlights the difference between the room temperature and low temperature $^{1}$H NMR spectrum of **1**. The low temperature NMR shows signals from both major and minor rotamer but the resolution is drastically improved and allows chemical shift assignment and coupling constant determination of the signals.

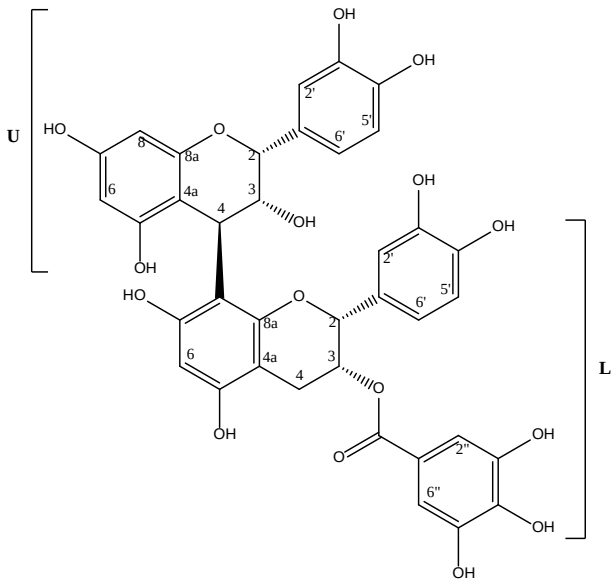


**Fig 5.** Epicatechin-(4β→8)-epicatechin-3-*O*-gallate (1); U - upper unit, L - lower unit.

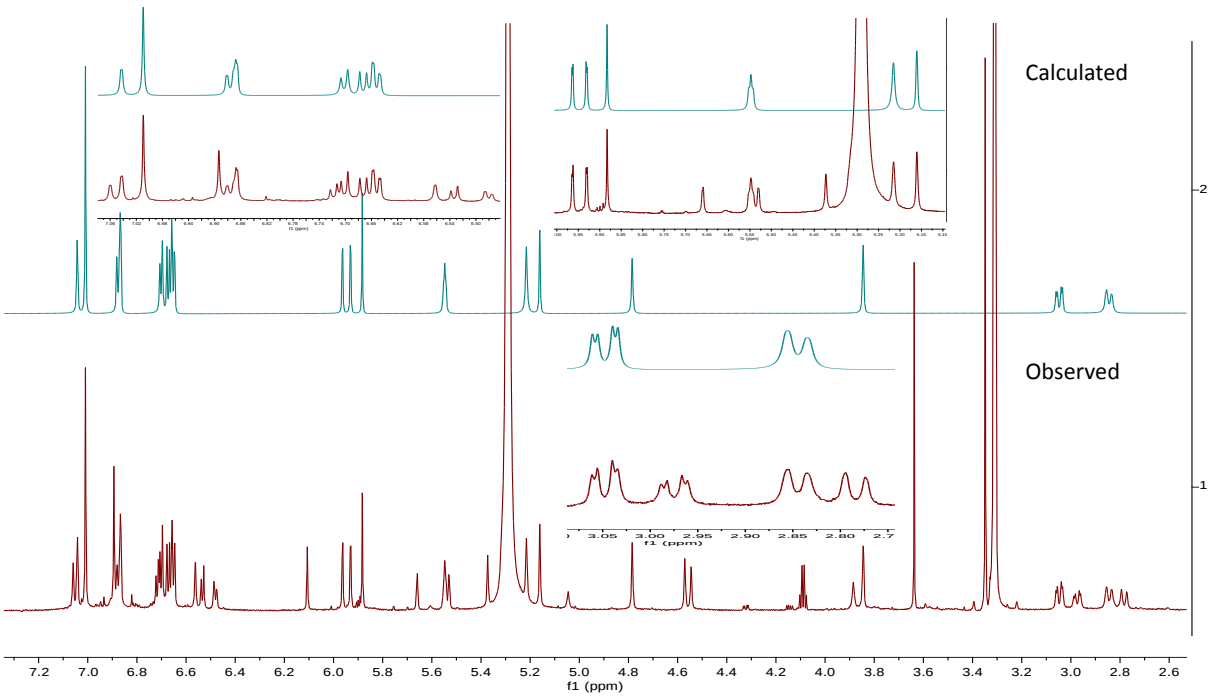


**Fig. 6.** Comparison between the simulated $^{1}$H NMR spectrum generated by PERCH (in green), and the experimental $^{1}$H NMR spectrum (in red) of **1**. Stack plots of the simulated and experimental spectra were generated in MestReNova. PERCH iteration was performed using both D-mode (overall integral fitting) and T-mode (total line shape fitting). The experimental $^{1}$H NMR spectrum was recorded at 800 MHz at 255 K.

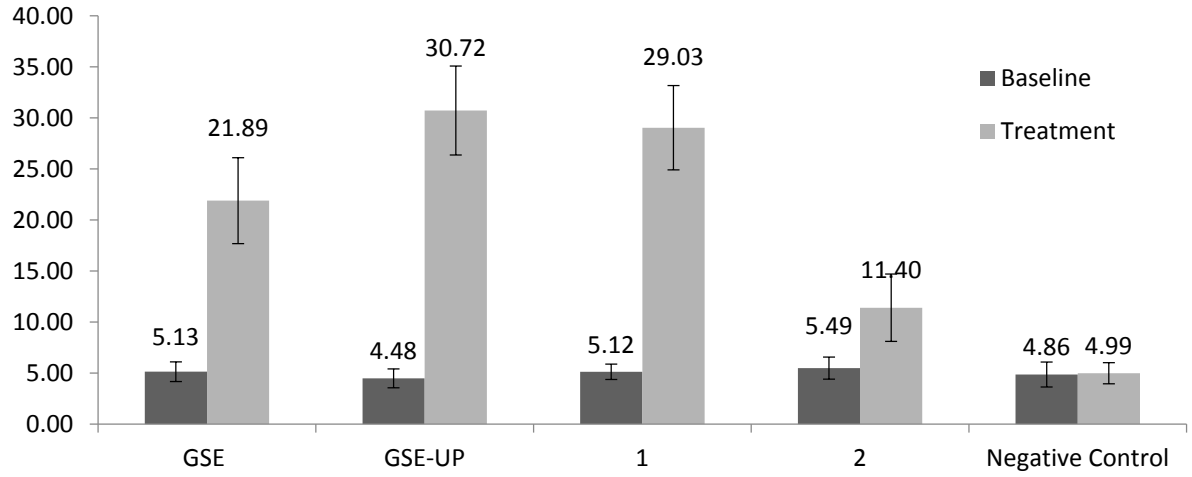


**Fig. 7.** The dentin mechanical bioassay data for **1** and **2** in comparison to the crude GSE, GSE-UP and negative control (no treatment). Y–axis represents the mean values of the modulus of elasticity (expressed in MPa; bars depict the standard deviations; N: 15 per group).

| Unit | Position | epicatechin-4β→8-epicatechin-3-*O*-gallate, $\delta$ (*J*; mult) | |
|---|---|---|---|
| | | **Major rotamer** | **Minor rotamer** |
| **U** | 2 | 5.1607 (1.47; d) | 5.3730 (s) |
| | 3 | 3.8451 (1.89, 1.47; dd) | 3.8854 (1.67; d) |
| | 4 | 4.7846 (1.89; d) | 4.5453 (1.67; d) |
| | 6 | 5.9635 (2.08; d) | 5.5302 (2.3; d) |
| | 8 | 5.9306 (2.08; d) | 5.6600 (2.3; d) |
| | 2' | 6.8661 (2.10, 0.87; dd) | 7.0598 (2.00, 0.09; dd) |
| | 5' | 6.7008 (8.22; d) | 6.7168 (8.32, 0.09; dd) |
| | 6' | 6.6529 (8.22, 2.10; dd) | 6.6528 (2.00, 8.32; dd) |
| **L** | 2 | 5.2153 (1.04, 0.2; dd) | 4.5708 (1.75, 0.94; dd) |
| | 3 | 5.5474 (4.64, 3.76, 1.04; ddd) | 5.2968 ( 1.5.69, 2.62, 1.75; ddd) |
| | 4a,4b | 3.0466 (-17.00, 4.64, 0.20; ddd),<br>2.8451 (-17.00, 3.76; dd) | 2.9738 (-17.36, 5.69, 0.94; ddd)<br>2.7845 (-17.36, 2.62; dd) |
| | 6 | 5.8829 (s) | 6.1067 (s) |
| | 2' | 7.0425 (1.86, 0.18; dd) | 6.5626 (2.02, 0.88; dd) |
| | 5' | 6.6727 (8.31, 0.18; dd) | 6.5329 (8.07, 0.88; dd) |
| | 6' | 6.8758 (8.31, 1.86; dd) | 6.4814 (8.07, 2.02; dd) |
| | 2" and 6" | 7.0093 (s) | 6.8936 (s) |

**Table 1.** $^{1}$H NMR chemical shifts (δ, in ppm), coupling constants (*J*, in Hz) and multiplicities for **1** as generated by HiFSA using the PERCH NMR software for iterative analysis of the experimental spectrum. NMR signals at positions 2, 3, and 4 of both the U and L units appear as broad singlets. The underlying small coupling constants, which cannot be evaluated visually, were extracted using the HiFSA method of $^{1}$H NMR analysis.

| Fraction no. | *m/z* value of major ions | | DP | DG | ID |
|---|---|---|---|---|---|
| | Negative mode | Positive mode | | | |
| 1 | 441 | 443 | 1 | 1 | ECG |
| 2 and 3 | 289 | 291 | 1 | | Catechin or epicatechin |
| 4 | 301,463 | 303, 465 | 1* | | Quercetin and quercetin glucoside |
| 5 and 6 | 729 | 731 | 2 | 1 | Mono-galloylated dimer |
| 7 | 577 | 579 | 2 | | Non galloylated PAC dimers |
| 8 | 577, 865 | 579, 867 | 2,3 | | PAC dimers and trimers |
| 9 an 10 | 593, 865, 881, 1017 | 595, 867, 883, 1017 | 2,3 | 0,1 | PAC dimer with an EGC unit(593), trimers, mono-galloylated trimer (1017) |
| 11 to 15 | 865, 1017, 1153, 1305 | 867, 1019, 1155, 1307 | 3,4 | 1 | PAC trimers and tetramers, mono-galloylated tetramer (1305) |

*Flavonoids but not flavan-3-ols or PACs

**Table 2.** The *m/z* values of the major parent ions present in the CPC fractions F1-F15.The *m/z* values were obtained from IT-TOF MS analysis using full scan method in both positive and negative mode. MS analysis enabled the classification of these fractions according to their degree of polymerization (DP) and degree of galloylation (DG). A greater overlap between the OPACs is observed after fraction F8.

## Graphical abstract