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Article

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Molecular Interaction Between Salivary Proteins and Food Tannins

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Abstract

Polyphenols interaction with salivary proteins (SP) has been related with organoleptic features such as astringency. The aim of this work was to study the interaction between some human SP and tannins through two spectroscopic techniques, fluorescence quenching and Saturation Transfer Difference-Nuclear Magnetic Resonance (STD-NMR).

Generally, the results showed a significant interaction between SP and both condensed tannins and ellagitannins. Herein, STD-NMR proved to be a useful tool to map tannins' epitopes of binding, while fluorescence quenching allowed to discriminate binding affinities. Ellagitannins showed the greatest binding constants values (K_{SV} from 20.1 to 94.1 mM^{-1}; K_A from 0.7 to 8.3 mM^{-1}) in comparison with procyanidins (K_{SV} from 5.4 to 40.0 mM^{-1}; K_A from 1.1 to 2.7 mM^{-1}). In fact, punicalagin was the tannin that demonstrated the highest affinity for all three SP. Regarding SP, P-B peptide was the one with higher affinity for ellagitannins. On the other hand, cystatins showed in general the lower K_{SV} and K_A values. In the case of condensed tannins, statherin was the SP with the highest affinity, contrasting with the other two SP.

Altogether, these results are evidences that the distinct SP present in the oral cavity have different abilities to interact with food tannins class.

Keywords: ellagitannins, procyanidins, punicalagin, castalagin, vescalagin, fluorescence quenching, STD-NMR

Introduction

Polyphenols are natural phenolic compounds that result from plant secondary metabolism and that have been associated with numerous health benefits, mostly owing to their antioxidant properties.[1] Besides their benefic effects in many health disorders,[2-4] they have a wide array of applications as colorants and antioxidants in the food, beverage and cosmetic fields.[5] Therefore, polyphenols have been drawing a lot of attention from all-around researchers to expand their applicability. Polyphenols can be divided into many classes and, among them, tannins are an important group which can be further divided into two major families: condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins and gallitannins).[6] These latter are found not only in fruits, especially berries, such as pomegranate or raspberry, and nuts, but also in red wine due to their migration from oak wood during wine ageing.[7, 8] Ellagitannins differ from gallotannins in that some gallic acid moieties or galloyl groups are biaryl coupled to each other through carbon-carbon bond to form a hexahydroxydiphenyl (HHDP) moiety. Ellagitannins contain numerous of these HHDP units, as well as galloyl and/or sanguisorboyl units bound to a sugar moiety.[9] Castalagin and vescalagin are the most representative structures of ellagitannins and are the major ones found in oak wood.[7] Condensed tannins are naturally found in cereals, vegetables, fruits and also in some beverages. However, condensed tannins are much more available in the diet comparing to hydrolysable tannins, being the most abundant polyphenols in plants after lignins.[10] They are composed of flavan-3-ol units, forming dimers, oligomers and polymers with different substitution patterns (hydroxylation and galloylation). Procyanidins are a subclass of condensed tannins composed by (+)-catechin and/or (-)-epicatechin subunits linked through C4-C8 (dimers from B1 to B4) or C4-C6 (dimers from B5 to B8) interflavanic linkage. These compounds in solution present different conformers, specially resulting from the rotation of the interflavanic bond. This behavior in solution can also result in an equilibrium of different isomers forms.[11]

In a sensorial point of view, tannins are associated with the consumer's perception of astringency. Astringency has been defined as a complex group of tactile sensations involving dryness, puckering and tightening of the oral cavity, typically experienced during the ingestion of tannin-rich food and beverages. It has been generally accepted that astringency results from the tannin-induced interaction and/or precipitation of salivary proteins (SP) in the mouth.[12] Tannins interact with proteins through multiple binding sites and this association is mediated by hydrophobic and hydrogen bonds, depending on the protein size, charge and structure, as well as on tannins molecular weight.[13]

Among SP, the most important families include proline-rich proteins (PRPs), statherin, P-B peptide, cystatins and mucin.[14] Statherin (5232 Da) is known to contain a highly acidic N-terminal and for being phosphorylated at two residues (Ser2 and Ser3).[15] This protein is produced by the parotid and submandibular glands and has several isoforms.[16] Among the 43 amino acid residues of statherin, tyrosine residues are very abundant. Salivary P-B peptide (5792 Da) is usually included into the bPRPs family due to its high content in proline residues (near 50% of its sequence) but it shows higher similarities with salivary statherin.[17, 18] Statherin and P-B peptide have in common convertase consensus sequences, but their structures differ as P-B peptide present several hydrophobic amino acid residues (such as Phe, Leu and Ile) and three tyrosine residues. Besides, P-B peptide is the product of a specific gene very close to STATH gene, unlike other bPRPs which are encoded by the four genes PRB1–4. Cystatins are a group of endogenous cysteine proteinase inhibitors. They are structured proteins with molecular masses ranging from 13 to 14 kDa and two disulfide bridges.[19]

Most of astringency studies are focused on condensed tannins, due to its considerable amount in diet. Further, among SP, PRPs and especially basic PRPs have been referred as the most reactive SP toward tannins.[20, 21] However, previous *in vitro* and *in vivo* studies have gather evidences that statherin and P-B peptide are highly reactive and precipitated by tannin, while cystatins are slightly precipitated by tannins.[14, 22]

Some works point out that hydrolysable tannins tend to be more reactive toward proteins when compared with condensed tannins.[6, 23] However, in general, these works were done with model proteins.[8, 24] Therefore, the aim of this work was to study the interaction between three important SP (statherin, P-B peptide and cystatins) and five common tannins (castalagin, vescalagin, punicalagin and procyanidins B3 and B6). Tannin compounds were chosen due to particular structural features. Concerning ellagitannins, the major difference between castalagin/vescalagin (stereoisomers) and punicalagin it is in the glucose moiety, acyclic for the firsts and cyclic for the last one (Figure 1). Procyanidin dimer B3 and B6 are isomers with different position of the interflavonoid linkage, C4-C8 and C4-C6, respectively. The interaction between these compounds was studied by two molecular techniques: fluorescence quenching and Saturation Transfer Difference-Nuclear Magnetic Resonance (STD-NMR).

Materials and Methods

Reagents. All reagents used were of analytical grade or better. Acetonitrile (ACN) was purchased from Chem-Lab, Trifluoracetic acid (TFA) from Sigma-Aldrich and ethanol was acquired from AGA, Álcool e Géneros Alimentares, SA.

Salivary Proteins Isolation and Purification. The procedure of saliva collection and treatment was already described previously.[14, 18] Whole saliva was collected from healthy non-smoking volunteers at 2 p.m. after at least 1 hour without food or beverages ingestion. Then, it was treated with 10% TFA solution (final concentration 0.1%) and centrifuged at 8000 g for 5 minutes. The supernatant (acidic saliva, AS) was dialyzed (cellulose membrane, MWCO: 3.5 KDa) for 24 hours at 4ºC under constant stirring and with at least 3 water changes. After, the sample was centrifuged at 3000 rpm for 7 minutes, lyophilized, re-suspended in water and injected into semi-preparative-HPLC in order to isolate the human SP families of interest (statherin, P-B peptide and cystatins). For this, a HPLC Lachrom Merck Hitachi system (L-7100) was used equipped with a Vydac C8 column (Grace Davison Discovery Sciences, 5µm particle, 150 x 2.1 mm), detection at 214 nm, eluent A: 0.2% aqueous TFA in water, eluent B: 0.2% TFA in ACN/water 80/20 (v/v), linear gradient from 10 to 55% (eluent B) in 45 minutes, at a flow rate of 0.60 $mL.min^{-1}$. After this program, the column was washed with 100% eluent B for 10 minutes to elute other late-eluting proteins. After washing, the column was stabilized with the initial conditions (90% eluent A and 10% eluent B).

Identification of salivary proteins. The three SP were freeze-dried and identified by ESI-MS by flow injection analysis into an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controller by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0. The capillary voltage of the electrospray ionization (ESI) was set at 3100 V. The capillary temperature was 275ºC. The sheath gas flow rate (nitrogen) was set to 5 (arbitrary unit as provided by the software settings). The capillary voltage was 36 V and the tube lens voltage 110 V. Samples were diluted

in a methanol/CAN/TDA 0.01% (5:5:90 v/v) mixture 1:10 prior analysis. After mass spectrometry analysis, deconvolution of mass spectra was done using the MagTran 1.03 software.

Ellagitannins Extraction and Isolation. Castalagin and vescalagin were obtained from *Quercus petraea* (Matt.) Liebl wood (medium-toasted oak chips) as referred in the literature.[7] It was used a Sephadex LH-20 column and methanol:acidified water for elution to obtain different fractions which contained major ellagitannins. This composition was determined by HPLC-DAD-MS as well as the ellagitannins purity after they were isolated by semi-preparative HPLC.

Punicalagin was isolated from pomegranate, as previously reported.[25] Briefly, 1g of dried husk powder was extracted ultrasonically with 30 mL of 40% ethanol for 30 minutes twice. After ethanol evaporation, the extract was lyophilized and analyzed by LC-MS. Punicalagin purification was performed by semi-preparative HPLC and its purity was determined by LC-MS.

Procyanidin Dimers B3 and B6 synthesis. The synthesis of procyanidin dimers B3 (catechin-(4-8)-catechin) and B6 (catechin-(4-6)-catechin) followed the procedure described in the literature.[26] Briefly, a taxifolin and (+)-catechin mixture (ratio 1:3) was dissolved in ethanol under argon atmosphere and treated with sodium borohydride (in ethanol). Using CH_3COOH/H_2O 50% (v/v), the pH was adjusted to 4.5 and the mixture was kept under argon atmosphere for 30 min. The reaction mixture was extracted with ethyl acetate. After evaporation of the solvent, water was added, and the mixture was passed through reversed-phase C18 gel, washed again and recovered with methanol. After methanol evaporation, this fraction was separated through a TSK Toyopearl HW-40(s) gel column (300 mm × 10 mm i.d., 0.8 mL/min, methanol as eluent) coupled to a UV-Vis detector. From this, several fractions were recovered and analyzed by ESI-MS (Finnigan DECA XP PLUS). The fractions yielding procyanidin dimers B3 and B6 were analyzed by HPLC-MS. Spectroscopical data were in accordance with literature.[27]

133 **Fluorescence Quenching.** In all experiments, proteins and polyphenols stocks were prepared in
134 distilled water. The mixtures were prepared using 30 or 60 μM concentration of each protein
135 and tannins were added at increasing concentrations (0 to 50 μM). Statherin, P-B peptide and
136 cystatins exhibit intrinsic fluorescence due to their content in aromatic residues, such as
137 tryptophan and tyrosine. Herein, tryptophan was used as intrinsic fluorophore. The excitation
138 wavelength (λ_{ex}) was 284 nm. After mixing, the samples were transferred to the fluorimeter
139 cell and the emission spectra (from 290 to 500 nm) were measured in a Perkin-Elmer LS 45
140 Luminescence Spectrometer. After each measurement, the cell was washed with ethanol and
141 distilled water. Fluorescence intensity was always registered at the wavelength of maximum
142 fluorescence signal concerning each SP. These wavelengths were 314 nm, 313 nm and 355nm
143 for statherin, P-B peptide and cystatins, respectively. To evaluate the possibility of
144 fluorescence resonance energy transfer (FRET) between the proteins and the procyanidins, the
145 absorption spectra of both were analyzed (data not shown): the proteins studied herein
146 presents an absorption spectrum at 200 to 290 nm. At this wavelength, the procyanidins do
147 not emit light (maximum at 330 nm). Procyanidins have an absorption maximum at 270 nm,
148 and their spectrum decreases reaching residual values close to 310 nm. The protein emission
149 spectrum starts at 320 nm, and at this λ the polyphenol absorbance is small. FRET seems highly
150 unlikely at the experimental conditions used. However, it was made a blank measurement for
151 each concentration for these tannins at the λ_{ex}, where the protein was replaced by distilled
152 water.[28] These blank signals were then subtracted to the corresponding measurements of the
153 complexes SP-dimer. Castalagin, vescalagin and punicalagin did not display fluorescence in
154 these conditions.
155 Fluorescence quenching data was addressed considering the Stern-Volmer equation (equation
156 1):

157 $$\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \quad \text{(Equation 1)}$$

Where F_0 and F represent the fluorescence intensities before and after the addition of the quencher (tannin), respectively; k_q is the bimolecular quenching constant; τ_0 is the lifetime of the fluorophore (SP) in the absence of the quencher; [Q] concern the quencher concentration; and K_{SV} is the Stern-Volmer quenching constant. Taking this into account, the results obtained from the fluorimeter were represented in plots of F_0/F versus [Q] in order to determine K_{SV} by linear regression.

A linear Stern-Volmer plot generally indicates that only one kind of mechanism occurs: static, which implies the formation of a stable complex, or dynamic, which is related to collisional encounters between the fluorophore (protein) and quencher (tannin). On the other hand, a positive deviation toward the *x*-axis appears when the extent of quenching is large and it could mean two situations: either mechanisms (static and dynamic) are present simultaneously or a sphere of action exists, which means that the quencher is adjacent to the fluorophore at the moment of excitation. In this circumstance the Stern-Volmer equation is modified to (equation 2), where K_{app} represents the "apparent static quenching":

$$\frac{F_0}{F} = \left(1 + K_{app}[Q]\right) \exp([Q]VN/1000) \qquad \text{(Equation 2)}$$

The apparent static constant (K_{app}) can also be determined by linear regression from a plot of $\ln(F_0/F)$ as a function of [Q].

Determination of salivary proteins lifetime (τ_0): τ_0 was measured on a Fluoromax-4 spectrophotometer attached to a single photon counting controller (FluoroHub), both from Horiba Jobin-Yvon, at room temperature. The fluorescence excitation was performed with a Horiba Nano LED source of 290 nm, and fluorescence emission was recorded at the maximum wavelength for the protein solution (313, 314 and 355 nm). The lamp profile was recorded by placing a scatter (dilute solution of LUDOX in water) in place of the sample.[2, 29]

Saturation Transfer Difference-Nuclear Magnetic Resonance (STD-NMR): A solution of each SP was prepared in D_2O (3 μM) and the ligands were added at increasing concentrations (0.1 to 3.5 mM) as a lyophilized powder.

NMR experiments were recorded on a Bruker Avance III 600 HD spectrometer, operating at 600.13 MHz, equipped with a 5 mm CryoProbe Prodigy and pulse gradient units capable of producing magnetic field pulsed gradients in the z direction of 50 G/cm. The measurements were made with standard Bruker pulse sequences at 300 K. ^{1}H and STD spectra were recorded with a shaped pulse to suppress the water resonance using the following parameters: spectral width, 16 ppm; nutation angle, 7.08 μs and 90°; and shaped pulse duration, 2 ms. Selective saturation of the SP off-resonance at 20 ppm and on-resonance at −1 ppm was performed using a pseudo-two-dimensional (2D) sequence for STD with a shaped pulse train alternating between the on and off resonances. STD-NMR spectra were acquired using Gaus 1.1000 pulses for selective saturation (50 ms), with a total saturation time of 2.5 s. The number of scans for tannins (4), receptor gain value (250), and relaxation delay (2.5) were kept constant. To process the STD-NMR spectra it was used TopSpin 2.1 software from Bruker.

The amplification factor (A_{STD}) and the association constant (K_A) were determined by equations 3 and 4, respectively[30]:

$$A_{STD} = \frac{I_0 - I_{SAT}}{I_0} \times \frac{[L]}{[P]} = \frac{I_{STD}}{I_0} \times \frac{[L]}{[P]} = \frac{\alpha_{STD}[L]}{K_D + [L]} \quad \text{(Equation 3)}$$

$$K_A = \frac{1}{K_D} \quad \text{(Equation 4)}$$

Where I_{SAT} is the signal intensity of the selectively saturated protein spectrum (*on-resonance*), I_0 represent the signal intensity of the spectrum recorded without protein saturation (*off-resonance*), [L] is the concentration of the ligand (tannin), [P] is the protein concentration and α_{STD} is the maximum amplification factor.

After the determination of A_{STD} values from the experimental results (Equation 3), a plot of A_{STD} in function of [L] was done and K_D values were determined by Solver Supplement of Microsoft Excel Office.

Statistical Analysis: All assays, except STD-NMR analysis, were performed at least in n = 3 repetitions. Values are expressed as the arithmetic means (SEM). Statistical significance of the difference between various groups was evaluated by one-way analysis variance (ANOVA) followed by the Tuckey test. Differences were considered significant when $p < 0.05$. All statistical data were processed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, USA).

Results and Discussion

In this work, the interaction between SP statherin, P-B peptide and cystatins and some representative food tannins (ellagitannins: punicalagin, vescalagin and castalagin; condensed tannins: procyanidin B3 and B6) was studied by two complementary techniques, STD-NMR and fluorescence quenching, thus allowing the study of differences in protein-tannin interaction related both with proteins and tannin structures.

Tannin compounds were chosen due to its particular structural features and to gather insights about how these features affect the interaction with proteins. Ellagitannins are more apolar compounds when compared to condensed tannins.[13] Castalagin and vescalagin are diastereoisomers which differ in the stereochemistry at C1 position of glucose moiety, with one HHDP unit in their structure (Figure 1). Punicalagin also presents a HHDP group, but oppositely to castalagin and vescalagin, its glucose moiety is cyclic. Procyanidins dimers B3 and B6 are isomers composed by two catechin units that differ only in the interflavonoid linkage, C4-C8 and C4-C6, respectively. SP were chosen based on previous studies[14, 22] that statherin and P-B peptide are also highly precipitated by tannins, opposing to cystatins.

Binding constants of the interactions between salivary proteins and tannins by fluorescence quenching. Fluorescence quenching is a spectroscopic method described as a reduction in the fluorescence intensity of a sample due to molecular interactions with a quencher.[31] In this process, the fluorescence intensity of the fluorophore (intrinsic fluorescence of statherin, P-B peptide and cystatins) decreases when it is associated with tannins. This technique has been widely used to study polyphenols interactions, providing binding affinities information of the complexes formed.[26, 32, 33] Due to its high sensitivity, this technique allowed to study the interaction with low tannins concentration. The fluorescence quenching assays permitted to establish a relation between the decrease of protein fluorescence intensity and the increase of tannins concentrations. This is observed in the spectrum presented in Figure 2 for the

238 interaction between P-B peptide and punicalagin. Similar fluorescence spectra were obtained
239 for all the other interactions (data not shown).
240 From these fluorescence spectra, the maximum fluorescence signals were acquired and Stern-
241 Volmer plots obtained. Regarding procyanidin dimers B3 and B6, the interaction with all SP
242 exhibited a linear behaviour.
243 A different behaviour was observed for ellagitannins, since castalagin, vescalagin and
244 punicalagin interaction with most proteins presented a non-linear behaviour (Figure 3). Only
245 the interaction of punicalagin with P-B peptide and statherin exhibited a linear behaviour.
246 From a linear Stern-Volmer plot and considering equation 1, the Stern-Volmer quenching
247 constant (K_{SV}) can be directly determined (Table 1). K_{SV} constants were mainly obtained for the
248 interactions with condensed tannins. In the situations where an upward-curving Stern-Volmer
249 plot is observed, a modified form of these plots was represented based on equation 2, to
250 determine the corresponding apparent static constant (K_{app}), which was mainly obtained for
251 ellagitannins (Table 1). Both constants are a measure of the interaction affinity. The different
252 form of calculation for each constant is related to the quenching mechanism.
253 The lowest affinity constants were obtained for the interaction of cystatins with condensed
254 tannins, procyanidin B3 (5.4 mM^{-1}) and B6 (6.5 mM^{-1}). On the other hand, it is also evident that
255 the affinity constants obtained for procyanidin B6 are always higher than the ones for
256 procyanidin B3. Another tendency observed by comparing the different SP was that statherin
257 showed the highest affinities for the interaction with procyanidins B3 and B6, 17.8 and 40.0
258 mM^{-1}, respectively.
259 In general, the highest affinity constants were obtained for the interaction with hydrolysable
260 tannins, in particular for punicalagin. This latter presents the highest constant (94.1 mM^{-1}) for
261 the interaction with P-B peptide. Indeed, hydrolysable tannins have been referred in other
262 studies as the ones with higher interaction when compared with condensed tannins.[6, 23, 34, 35]
263 This higher interaction could be related with the fact that hydrolysable tannins present in

general much more complex structures than the oligomeric procyanidins studied. The affinity constants obtained for the interactions with castalagin and vescalagin were significantly lower than for the ones with punicalagin. On the other hand, castalagin presented similar constants to those of vescalagin for the different proteins studied.

In general, P-B peptide was the protein with highest interaction for ellagitannins, while for condensed tannins it was statherin. This high binding affinity of P-B peptide is somehow expected if we consider the high content in proline residues of this protein. The presence of proline residues induces more rigid and open structures, and therefore more exposure of hydrophobic residues able to form hydrophobic bonds.[36-38]

In the case of statherin, the high affinity toward condensed tannins could be owed to the presence of two polar serine residues (phosphorylated amino acids) which are assumed to establish hydrophilic bonds, strengthening the interaction with those more polar tannins.

A linear Stern-Volmer plot generally indicates that only one kind of mechanism occurs: static, which implies the formation of a stable complex, or dynamic, which is related to collisional encounters between the fluorophore (protein) and quencher (tannin). To discriminate the type of mechanism involved, the calculation of the bimolecular quenching constant (k_q) (Equation 1) is required. For this, it was also necessary to determine proteins' lifetime (Table 2). The k_q values higher than the diffusion-controlled limited value (1×10^{10} $M^{-1}s^{-1}$) are usually associated with higher affinities and stronger interactions, which was observed for all linear Stern-Volmer plots. In fact, the interactions of punicalagin with statherin and P-B peptide presented the highest k_q values. These high values, about 100-fold larger than the diffusion-controlled limited value, suggests that a static mechanism is more probable to occur, where a stable complex is formed between each protein and tannin.

Therefore, the results suggest the formation of a stable complex for procyanidins B3 and B6 with statherin, P-B peptide and cystatins, as well as punicalagin with statherin and P-B peptide.

289 A positive deviation toward the *x*-axis observed for castalagin and vescalagin appears when the
290 extent of quenching is large (Figure 2) and this could mean one of two situations: both
291 mechanisms (static and dynamic) are present or a sphere of action exists, which means that
292 the quencher (tannin) is adjacent to the fluorophore at the moment of excitation. In these
293 situations, the deduction referred above (magnitude of k_q) cannot be directly applied to
294 determine the mechanism responsible for the quenching. Therefore, it becomes necessary to
295 verify whether the protein is being quenched by both mechanisms simultaneously or if a
296 sphere of action exists. For this, the proteins' lifetime was measured as a function of tannin
297 concentration and the subsequent data gathered evidences that fluorescence quenching could
298 be due to the sphere of action mechanism (data not shown). This mechanism assumes the
299 existence of a sphere of volume within which the quenching only occurs if a quencher is
300 immediately adjacent to the fluorophore when it is excited. In other words, if a tannin is
301 adjacent to a protein at the moment of excitation, the SP will not fluoresce and the quenching
302 is experienced.[26]
303 The presence of a sphere of action was observed for the interaction between cystatins and
304 punicalagin, as well as for all the interactions of castalagin and vescalagin. It is interesting that
305 these two last compounds, which are stereoisomers, present a similar mechanism of
306 interaction for the several proteins studied.
307 **STD-NMR studies.** STD technique was used to determine the tannins binding epitopes, as well
308 as to estimate association constants (K_A). The structure of castalagin, vescalagin, punicalagin
309 and procyanidins B3 and B6 were clarified by NMR ^{1}H chemical shifts described in the
310 literature.[10, 27, 39-41]
311 In STD experiments, primary control assays with high tannins concentrations were made to
312 confirm that the on-resonance irradiation frequency did not affect the tannins aromatic
313 protons and that each protein was saturated by the on-resonance irradiation. The tannins'
314 aromatic protons resonances were not visible with these control conditions.

315 Figure 4 presents a STD-NMR spectra titration obtained for the interaction between the three
316 SP studied and increasing concentrations of tannin. After establishing the experimental
317 parameters and test several protein and ligand concentrations, it was only possible to obtain
318 STD-NMR signals in the tannins concentration range from 0.1 mM to 3.5 mM, depending on
319 the tannin. From the results presented in Figure 4, it is possible to observe that at low
320 concentrations (i.e. 0.1 to 0.5 mM) the signals of protons that appear first are near 7.0 ppm,
321 which correspond to the HHDP and NHTP moieties' protons. When concentration increases,
322 other protons signals emerge namely in 4.0 and 3.0 ppm regions, which correspond to glucose
323 moiety protons. This was observed for the interaction between all hydrolysable tannins and
324 proteins studied.
325 Regarding condensed tannins, it was also observed a similar tendency. For procyanidin B3, at
326 low concentrations the firsts sites of interaction are rings B and E while other regions of the
327 molecule interact only at higher concentrations (rings A and D). Interestingly, the same
328 tendency was not observed for procyanidin B6. In this case, the only molecular regions that
329 interacted were rings B and E even for the highest concentrations.
330 The engagement of procyanidin B3 rings B and E was already observed for the interaction with
331 other proteins, namely trypsin enzyme and small PRP peptides ($IB9_{37}$ and $IB7_{14}$).[28, 38, 42] When
332 concentration increases, it was observed that rings A, D and F also seem to be involved in
333 these interactions. To our knowledge, there is no data in the literature about the involvement
334 of procyanidin dimer B6 (C4-C6 linkage) epitopes in the interaction with proteins.
335 From these results, evidences that tannins could act as multidentate ligands were gathered,
336 wherein one region of the molecule is favoured for the initial interaction and the other parts
337 participate in the interaction when concentration increases. The same does not exactly
338 happens for procyanidin B6, which seems to be more selective. This could be related to a more
339 elongated and flexible structure of C4–C6 dimers.

340 **Binding constants of the interaction between salivary proteins and tannins by STD-NMR.** The
341 protons that presented the highest intensities on the STD spectrum were used to perform a
342 STD-NMR titration for each protein/tannin pair. The STD amplification factor (A_{STD}) was
343 calculated from the differences between the off-ressonance and the on-ressonance spectra
344 with increasing procyanidins concentration, according to equation 1 (Experimental Section).
345 The plots of each tannin titration regarding A_{STD} values as a function of ligand concentration at
346 a fixed SP concentration (3 μM) are represented in Figure 5.
347 Based in these graphics, the STD signal increases systematically with concentration until
348 reaching a plateau of maximum interaction, which depends on the protein. Each
349 corresponding K_A was estimated according to equations 3 and 4 (Experimental Section), using
350 the Solver tool of Microsoft Excel software. The K_A obtained range from 0.7 to 8.3 mM (Table
351 3).
352 In general, and although the K_A values have the same magnitude, there is an increase of K_A in
353 the order procyanidin B3 < procyanidin B6 and vescalagin < castalagin < punicalagin. Overall,
354 hydrolysable tannins present higher K_A than procyanidins. The highest K_A value was obtained
355 for punicalagin interaction with P-B peptide (8.3 mM), while procyanidins maximum value was
356 only 2.7 mM (for interaction with statherin).
357 The K_A values obtained by STD-NMR are lower than the values obtained by fluorescence
358 quenching assays. However, it must be noted that the concentrations imposed by each
359 technique were different. Fluorescence quenching allowed the use of low tannins
360 concentrations (up to 50.0 μM), while STD-NMR focused on higher concentrations (up to 3.5
361 mM). The interaction between tannins (T) and proteins (P) are highly dependent on the T and
362 P concentration and molar ratio T/P.[13]
363 The K_A values obtained for procyanidins are similar to the ones reported in literature for similar
364 interactions using STD-NMR (proline-rich peptide $IB7_{14}$ interaction with procyanidin dimers B1
365 to B4 and trimers).[42]

To the best of our knowledge, with exception of procyanidin B3,[38] there is no data in the literature about the affinity toward SP or similar proteins. Thus, this is the first time that the affinity of statherin, PB- peptide and cystatins towards different food tannins have been studied.

In summary, regarding the influence of each SP, P-B peptide presents K_A values higher than the other proteins for the interactions with ellagitannins, except vescalagin. In fact, this ellagitannin showed very similar K_A values for the three SP. Cystatins are the SP with the lowest K_A values for almost all tannins, except for the interaction with procyanidin B3 and castalagin. On the other hand, statherin was the SP with higher K_A when the interaction involved procyanidins. This is in agreement with previous studies in which statherin has been reported as one of the SP with higher interaction for condensed tannins.[14] Overall, the combination of fluorescence quenching and STD-NMR techniques allowed the characterization of the tannin-SP interaction. It was observed that ellagitannins (vescalagin, castalagin and punicalagin) interact better with the three SP than procyanidins. This could be explained since ellagitannins are globally more apolar molecules than condensed tannins, being probably more able to stablish hydrophobic interactions,[13] which have been described as the main driving forces involved in tannin-protein interaction.[14] Their higher interaction was observed for P-B peptide, which is the only one rich in proline residues among the studied proteins.[18] These amino acids have been pointed as crucial for interaction with tannins and are able to stablish hydrophobic stacking.[13] Thus, the results point out that hydrophobic bonds may be more significant for hydrolysable tannins interaction than for condensed tannins, as it was already proposed by other authors.[13, 43] However, in the case of vescalagin, the affinity is significant lower than in the case of the other ellagitannins and close to that shown by B3 and statherin. Since vescalagin is more hydrophilic than its stereoisomer castalagin,[9] the hydrophobic interactions with protein could be less important, which may explain the lower affinity of vescalagin regarding the other ellagitannins.

The high affinity of procyanidin B6 towards tannins comparatively to procyanidin B3 was also observed. This could be related to a more extended structure of procyanidin B6 than procyanidin B3. Molecular mechanistic studies have shown that C4-C6 linkage presents a large number of conformers and a more extended structure, which results in a higher flexibility of these structures comparing to the C4-C8 isomers.[44, 45]

Moreover, it seems that protein structure could play an important role in the interaction with tannins. It has been observed that proteins with structure have low affinity toward tannins, probably because the interaction is thought to involve only surface exposed residues.[46, 47] This could explain the lowest affinity for tannins of cystatins, since they are the only structured proteins here studied.

Based in the results herein, it seems that the ability of tannins to interact with SP is highly related with tannin structure, which in turn is related to the hydrophobicity of these compounds. Moreover, the existence of more extended tannin structures may favor the interaction with SP. Regarding proteins, the abundance of some specific amino acids such as proline in the structure and the fact that they are structured proteins are the main factors affecting the interaction with tannins. Further studies must be done in order to explore the interaction between other proteins and tannins and also to try to stablish a relationship with sensorial properties.

Supporting Information description

Figure S1. A. RP-HPLC profile (214 nm) of the whole human saliva used to isolate the different SP (statherin, P-B peptide and cystatins). **B.** Deconvolution of the mass spectra outlining the major SP, their molecular weight and isoforms.

Figure S2. RP-HPLC profile (280 nm) of each tannin compound to assess its purity. **a)** castalagin, **b)** vescalagin, **c)** punicalagin – isomers α and β, **d)** procyanidin B3, **e)** procyanidin B6

Figure S3. Stern-Volmer plots representative of the fluorescence quenching of (△) statherin, (□) P-B peptide and (●) cystatins in the presence of increasing concentrations of condensed tannins [a) procyanidin dimer B6] and ellagitannins [b) vescalagin, c) punicalagin]. Each curve results from a triplicate assay.

Figure S4. Modified form of Stern-Volmer plots representative of the fluorescence quenching of (△) statherin, (□) P-B peptide and (●) cystatins in the presence of increasing concentrations of ellagitannins [a) castalagin, b) vescalagin, c) punicalagin]. Each curve results from a triplicate assay.

Figure S5. STD amplification factor (A_{STD}) for the interaction between the three different SP (3 μM) [a) P-B peptide, b) statherin, c) cystatins] and increasing concentrations of procyanidin B3. Symbols represent experimental values and lines represent theoretical values by equation 1.

Figure S6. STD amplification factor (A_{STD}) for the interaction between the three different SP (3 μM) [a) P-B peptide, b) statherin, c) cystatins] and increasing concentrations of procyanidin B6. Symbols represent experimental values and lines represent theoretical values by equation 1.

Figure S7. STD amplification factor (A_{STD}) for the interaction between the three different SP (3 μM) [a) P-B peptide, b) statherin, c) cystatins] and increasing concentrations of castalagin. Symbols represent experimental values and lines represent theoretical values by equation 1.

Figure S8. STD amplification factor (A_{STD}) for the interaction between the three different SP (3 μM) [a) P-B peptide, b) statherin, c) cystatins] and increasing concentrations of vescalagin. Symbols represent experimental values and lines represent theoretical values by equation 1.

436 **REFERENCES**

437 (1) Ross, J. A.; Kasum, C. M., Dietary flavonoids: bioavailability, metabolic effects and safety.
438 *Annu. Rev. Nutr.* **2002**, *22*, 19-34.
439 (2) Dias, R.; Perez-Gregorio, R.; Mateus, N.; De Freitas, V., The interaction between tannins and
440 gliadin derived peptides in a celiac disease perspective. *RSC Adv.* **2015**, *5*, 32151-32158.
441 (3) Santos-Buelga, C.; Scalbert, A., Proanthocyanidins and tannin-like compounds - nature,
442 occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*,
443 1094-1117.
444 (4) Visioli, F.; de la Lastra, C. A.; Andres-Lacueva, C.; Aviram, M.; Calhau, C.; Cassano, A.;
445 D'Archivio, M.; Faria, A.; Favé, G.; Fogliano, V.; Llorach, R.; Vitaglione, P.; Zoratti, M.; Edeas, M.,
446 Polyphenols and human health: A prospectus. *Crit. Rev. Food Sci.* **2011**, *51*, 524-546.
447 (5) Zillich, O. V.; Schweiggert-Weisz, U.; Eisner, P.; Kerscher, M., Polyphenols as active
448 ingredients for cosmetic products. *Int. J. Cosmet. Sci.* **2015**, *37*, 455-464.
449 (6) Soares, S.; Mateus, N.; de Freitas, V., Interaction of different classes of salivary proteins
450 with food tannins. *Food Res. Int.* **2012**, *49*, 807-813.
451 (7) García-Estévez, I.; Escribano-Bailón, M. T.; Rivas-Gonzalo, J. C.; Alcalde-Eon, C.,
452 Development of a fractionation method for the detection and identification of oak
453 ellagitannins in red wines. *Anal. Chim. Acta* **2010**, *660*, 171-176.
454 (8) Chira, K.; Teissedre, P. L., Relation between volatile composition, ellagitannin content and
455 sensory perception of oak wood chips representing different toasting processes. *Eur. Food Res.*
456 *Technol.* **2013**, *236*, 735-746.
457 (9) Vivas, N.; Laguerre, M.; Pianet de Boissel, I.; Vivas de Gaulejac, N.; Nonier, M.-F.,
458 Conformational Interpretation of Vescalagin and Castalagin Physicochemical Properties. *J.*
459 *Agric. Food. Chem.* **2004**, *52*, 2073-2078.
460 (10) Esatbeyoglu, T.; Wray, V.; Winterhalter, P., Dimeric procyanidins: screening for B1 to B8
461 and semisynthetic preparation of B3, B4, B6, And B8 from a polymeric procyanidin fraction of
462 white willow bark (Salix alba). *J. Agric. Food. Chem.* **2010**, *58*, 7820-30.
463 (11) Balas, L.; Vercauteren, J., Extensive high-resolution reverse 2D NMR analysis for the
464 structural elucidation of procyanidin oligomers. *Magn. Reson. Chem.* **1994**, *32*, 386-393.
465 (12) Bajec, M. R.; Pickering, G. J., Astringency: Mechanisms and perception. *Crit. Rev. Food Sci.*
466 **2008**, *48*, 858-875.
467 (13) de Freitas, V.; Mateus, N., Protein/Polyphenol Interactions: Past and Present
468 Contributions. Mechanisms of Astringency Perception. *Curr. Org. Chem.* **2012**, *16*, 724-746.
469 (14) Soares, S.; Vitorino, R.; Osorio, H.; Fernandes, A.; Venancio, A.; Mateus, N.; Amado, F.; de
470 Freitas, V., Reactivity of human salivary proteins families toward food polyphenols. *J. Agric.*
471 *Food. Chem.* **2011**, *59*, 5535-47.
472 (15) Schlesinger, D. H.; Hay, D. I.; Levine, M. J., Complete Primary Structure of Statherin, A
473 Potent Inhibitor of Calcium-Phosphate Precipitation, From the Saliva of the Monkey, Macaca-
474 Arctoides. *Int. J. Pept. Protein Res.* **1989**, *34*, 374-380.
475 (16) Helmerhorst, E. J.; Traboulsi, G.; Salih, E.; Oppenheim, F. G., Mass Spectrometric
476 Identification of Key Proteolytic Cleavage Sites in Statherin Affecting Mineral Homeostasis and
477 Bacterial Binding Domains. *J. Proteome Res.* **2010**, *9*, 5413-5421.
478 (17) Isemura, S.; Saitoh, E., Nucleotide sequence of gene PBI encoding a protein homologous
479 to salivary proline-rich protein P-B. *J. Biochem.* **1997**, *121*, 1025-1030.
480 (18) Messana, I.; Cabras, T.; Inzitari, R.; Lupi, A.; Zuppi, C.; Olmi, C.; Fadda, M. B.; Cordaro, M.;
481 Giardina, B.; Castagnola, M., Characterization of the human salivary basic proline-rich protein
482 complex by a proteomic approach. *J. Proteome Res.* **2004**, *3*, 792-800.
483 (19) Henskens, Y. M. C.; Veerman, E. C. I.; Mantel, M. S.; Velden, U. v. d.; Amerongen, A. V. N.,
484 Cystatins S and C in Human Whole Saliva and in Glandular Salivas in Periodontal Health and
485 Disease. *J. Dent. Res.* **1994**, *73*, 1606-1614.

(20) Lu, Y.; Bennick, A., Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717-728.
(21) Charlton, A. J.; Baxter, N. J.; Lilley, T. H.; Haslam, E.; McDonald, C. J.; Williamson, M. P., Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats. *FEBS Lett.* **1996**, *382*, 289-292.
(22) Brandao, E.; Soares, S.; Mateus, N.; de Freitas, V., In vivo interactions between procyanidins and human saliva proteins: effect of repeated exposures to procyanidins solution. *J. Agric. Food. Chem.* **2014**, *62*, 9562-8.
(23) Obreque-Slier, E.; López-Solís, R.; Peña-Neira, Á.; Zamora-Marín, F., Tannin–protein interaction is more closely associated with astringency than tannin–protein precipitation: experience with two oenological tannins and a gelatin. *Int. J. Food Sci. Tech.* **2010**, *45*, 2629-2636.
(24) Hofmann, T.; Glabasnia, A.; Schwarz, B.; Wisman, K. N.; Gangwer, K. A.; Hagerman, A. E., Protein binding and astringent taste of a polymeric procyanidin, 1,2,3,4,6-penta-O-galloyl-β-D-glucopyranose, castalagin, and grandinin. *J. Agric. Food. Chem.* **2006**, *54*, 9503-9509.
(25) Lu, J.; Ding, K.; Yuan, Q., Determination of Punicalagin Isomers in Pomegranate Husk. *Chromatographia* **2008**, *68*, 303-306.
(26) Bras, N. F.; Goncalves, R.; Fernandes, P. A.; Mateus, N.; Ramos, M. J.; de Freitas, V., Understanding the binding of procyanidins to pancreatic elastase by experimental and computational methods. *Biochemistry* **2010**, *49*, 5097-108.
(27) Tarascou, I.; Barathieu, K.; Simon, C.; Ducasse, M.-A.; André, Y.; Fouquet, E.; Dufourc, E. J.; de Freitas, V.; Laguerre, M.; Pianet, I., A 3D structural and conformational study of procyanidin dimers in water and hydro-alcoholic media as viewed by NMR and molecular modeling. *Magn. Reson. Chem.* **2006**, *44*, 868-880.
(28) Gonçalves, R.; Mateus, N.; Pianet, I.; Laguerre, M.; de Freitas, V., Mechanisms of Tannin-Induced Trypsin Inhibition: A Molecular Approach. *Langmuir* **2011**, *27*, 13122-13129.
(29) Ferreira, M.; Gameiro, P., Ciprofloxacin Metalloantibiotic: An Effective Antibiotic with an Influx Route Strongly Dependent on Lipid Interaction? *J. Membr. Biol.* **2015**, *248*, 125-136.
(30) Viegas, A.; Manso, J.; Nobrega, F. L.; Cabrita, E. J., Saturation-Transfer Difference (STD) NMR: A Simple and Fast Method for Ligand Screening and Characterization of Protein Binding. *J. Chem. Educ.* **2011**, *88*, 990-994.
(31) Lakowicz, J. R., *Principles of Fluorescence Spectroscopy* Springer US: 2006; Vol. 3rd Edition.
(32) Soares, S.; Mateus, N.; Freitas, V., Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. *J. Agric. Food. Chem.* **2007**, *55*, 6726-35.
(33) Papadopoulou, A.; Green, R. J.; Frazier, R. A., Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *J. Agric. Food Chem.* **2005**, *53*, 158-163.
(34) Bacon, J. R.; Rhodes, M. J. C., Development of a Competition Assay for the Evaluation of the Binding of Human Parotid Salivary Proteins to Dietary Complex Phenols and Tannins Using a Peroxidase-Labeled Tannin. *J. Agric. Food. Chem.* **1998**, *46*, 5083-5088.
(35) Deaville, E. R.; Green, R. J.; Mueller-Harvey, I.; Willoughby, I.; Frazier, R. A., Hydrolyzable tannin structures influence relative globular and random coil protein binding strengths. *J. Agric. Food Chem.* **2007**, *55*, 4554-4561.
(36) Lu, Y.; Bennick, A., Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717-728.
(37) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J. M.; Fouquet, E.; Pianet, I.; Dufourc, E. J., Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385-10395.
(38) Cala, O.; Dufourc, E. J.; Fouquet, E.; Manigand, C.; Laguerre, M.; Pianet, I., The colloidal state of tannins impacts the nature of their interaction with proteins: The case of salivary proline-rich protein/procyanidins binding. *Langmuir* **2012**, *28*, 17410-17418.

537 (39) Kraszni, M.; Marosi, A.; Larive, C. K., NMR assignments and the acid-base characterization
538 of the pomegranate ellagitannin punicalagin in the acidic pH-range. *Anal. Bioanal. Chem.* **2013**,
539 *405*, 5807-16.
540 (40) Zaruwa, M. Z.; Manosroi, J.; Akihisa, T., Castalagin from *Anogeissus acuminata*
541 (Roxb.ex.DC) Guill. Ex. Perr, a potent Hypoglycaemic Agent. *International Journal of Current*
542 *Research and Academic Review* **2015**, *3*, 147-152.
543 (41) Du Penhoat, C. L. M. H.; Michon, V. M. F.; Peng, S.; Viriot, C.; Scalbert, A.; Gage, D.,
544 Structural elucidation of new dimeric ellagitannins from Quercus robur L. roburins A-E. *J.*
545 *Chem. Soc., Perkin Trans. 1* **1991**, 1653-1660.
546 (42) Cala, O.; Pinaud, N.; Simon, C.; Fouquet, E.; Laguerre, M.; Dufourc, E. J.; Pianet, I., NMR
547 and molecular modeling of wine tannins binding to saliva proteins: revisiting astringency from
548 molecular and colloidal prospects. *FASEB J.* **2010**, *24*, 4281-4290.
549 (43) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T., Mechanisms of protein precipitation for two
550 tannins, pentagalloyl glucose and epicatechin$_{16}$ (4-8) catechin (procyanidin). *J. Agric. Food*
551 *Chem.* **1998**, *46*, 2590-2595.
552 (44) Ricardo-da-Silva, J. M.; Cheynier, V.; Souquet, J.-M.; Moutounet, M.; Cabanis, J.-C.;
553 Bourzeix, M., Interaction of grape seed procyanidins with various proteins in relation to wine
554 fining. *J. Sci. Food Agr.* **1991**, *57*, 111-125.
555 (45) Porto, P. A. L. d. S.; Laranjinha, J. A. N.; de Freitas, V. A. P., Antioxidant protection of low
556 density lipoprotein by procyanidins: structure/activity relationships. *Biochem. Pharmacol.*
557 **2003**, *66*, 947-954.
558 (46) Frazier, R. A.; Deaville, E. R.; Green, R. J.; Stringano, E.; Willoughby, I.; Plant, J.; Mueller-
559 Harvey, I., Interactions of tea tannins and condensed tannins with proteins. *J. Pharm. Biomed.*
560 *Anal.* **2010**, *51*, 490-495.
561 (47) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P., Multiple interactions between
562 polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation.
563 *Biochemistry* **1997**, *36*, 5566-5577.

564

565 **FIGURE CAPTIONS**

566 **Figure 1.** Molecular structure of condensed tannins: a) procyanidin B3 and b) procyanidin B6]
567 and ellagitannins [c) vescalagin, d) castalagin and e) punicalagin.

568 **Figure 2.** Fluorescence spectra of P-B peptide (30 μM) recorded at λ_{ex} 284 nm with increasing
569 concentrations of punicalagin (0 to 30 μM).

570 **Figure 3.** Stern-Volmer plots representative of the fluorescence quenching of (△) statherin,
571 (□) P-B peptide and (●) cystatins in the presence of increasing concentrations of a)
572 procyanidin dimer B3 and b) castalagin. Each curve results from a triplicate assay.

573 **Figure 4.** STD-NMR spectra for the interaction with increasing concentrations of each tannins
574 and proteins (3 μM). It is presented the 8.0-2.0 ppm region, where most protons resonate.
575 Spectra were recorded at 600 MHz and 300 K in deuterium oxide (D_2O). Interaction between
576 statherin with a) procyanidin B3 and b) procyanidin B6. Interaction between P-B peptide and c)
577 punicalagin, d) castalagin and e) vescalagin.

578 **Figure 5.** STD amplification factor (A_{STD}) for the interaction between increasing concentrations
579 of punicalagin and the three different proteins (3 μM): [a) P-B peptide, b) statherin, c)
580 cystatins]. Symbols represent experimental values and lines represent theoretical values by
581 equation 1.

582 **Abbreviations**

583 ACN, Acetonitrile

584 AS, Acidic Saliva

585 A_{STD}, Amplification Factor

586 ESI, Electrospray Ionization

587 FRET, Fluorescence Resonance Energy Transfer

588 K_A, Association Constant

589 K_{APP}, Apparent Static quenching constat

590 K_D, Dissociation Constant

591 k_q, Bimolecular quenching constant

592 K_{SV}, Stern-Volmer quenching constant

593 PRPs, Proline-Rich Proteins

594 SP, Salivary Proteins

595 STD-NMR, Saturation Transfer Difference-Nuclear Magnetic Resonance

596 TFA, Trifluoracetic Acid

597 τ_0, lifetime

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Table 1. Stern-Volmer (K_{SV}) and apparent static quenching (K_{app}) constants for the interaction of statherin, P-B peptide and cystatins with the five studied tannins. Values with the different letter are significantly different (p<0.01)

	CONDENSED TANNINS					
	B3			B6		
Protein	**Statherin**	**P-B**	**Cystatins**	**Statherin**	**P-B**	**Cystatins**
K_{SV} /mM^{-1}	17.8 ± 1.2^{f,g}	11.8 ± 0.7^{h}	5.4 ± 0.3^{j}	40.0 ± 4.1^{c}	14.9 ± 0.8^{g}	6.5 ± 0.2^{i}

	ELLAGITANNINS								
	Castalagin			Vescalagin			Punicalagin		
Protein	**Statherin**	**P-B**	**Cystatins**	**Statherin**	**P-B**	**Cystatins**	**Statherin**	**P-B**	**Cystatins**
K_{SV} /mM^{-1}	-	-	-	-	-	-	77.9 ± 4.3^{b}	94.1 ± 3.0^{a}	-
K_{app} /mM^{-1}	25.3 ± 0.7^{d}	27.2 ± 0.8^{d}	25.9 ± 0.6^{d}	20.2 ± 0.9^{e,f}	21.1 ± 0.6^{e}	20.1 ± 0.6^{e,f}	-	-	78.2 ± 2.4^{b}

Table 2. Proteins' lifetime and biomolecular quenching constants (k_q) for the interaction of statherin, P-B peptide and cystatins with the five studied tannins.

		k_q (10^{13})/ $M^{-1}s^{-1}$				
SP	**τ_0 / ns**	**B3**	**B6**	**Castalagin***	**Vescalagin***	**Punicalagin**
Statherin	1.73	1.03 ± 0.07	2.32 ± 0.02	-	-	4.51 ± 0.25
P-B peptide	2.55	0.46 ± 0.03	0.59 ± 0.03	-	-	3.69 ± 0.12
Cystatins	4.02	0.135 ± 0.009	0.16 ± 0.01	-	-	*

*These compounds present a non-linear Stern-Volmer plot

Table 3. Association constant (K_A) values estimated for the interaction between each SP and the different tannins determined by equations 2 and 3.

	K_A (mM^{-1})				
SP	**B3**	**B6**	**Castalagin**	**Vescalagin**	**Punicalagin**
P-B peptide	1.1**	1.8*	3.3*	1.4*	8.3*
Statherin	1.7***	2.7*	1.7***	1.6*	3.3*
Cystatins	1.2**	1.6**	2.2**	0.7**	2.6*

Confidence of the fitting: *≥97%, **≥93%, ***≥80%

Figure 1.

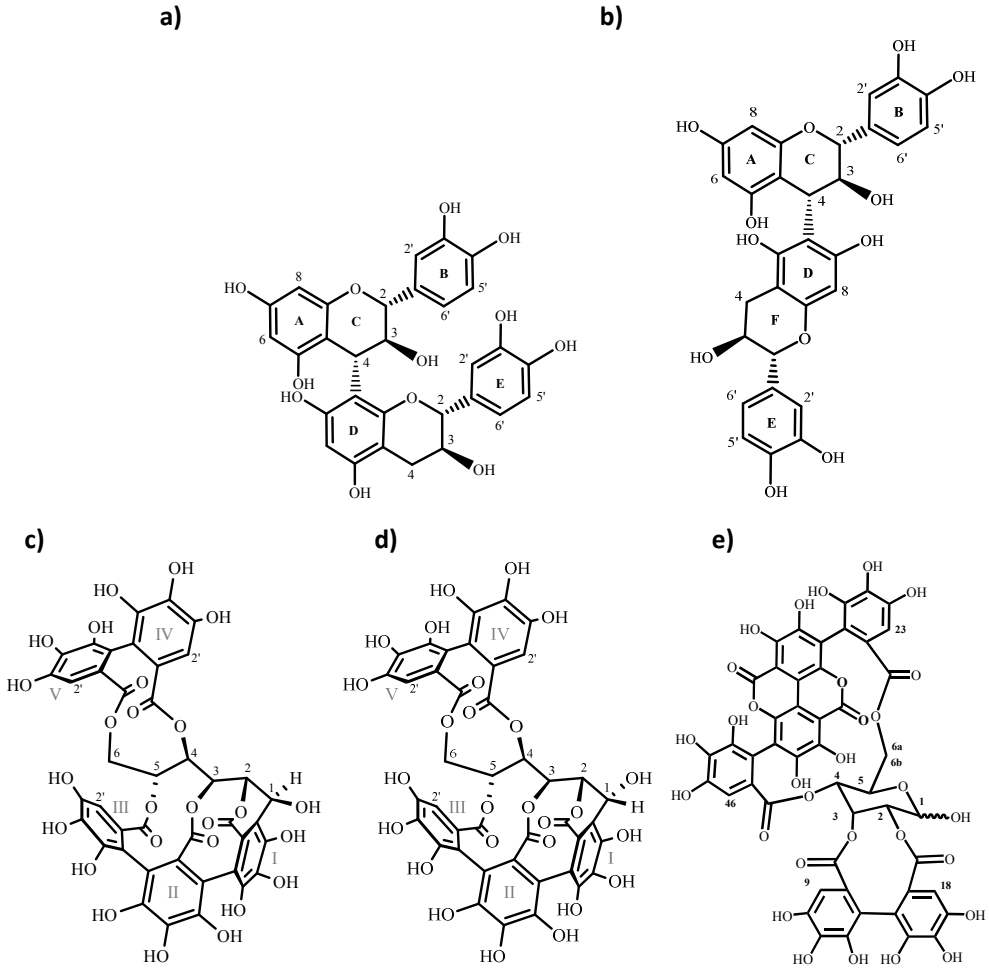


Figure 2.

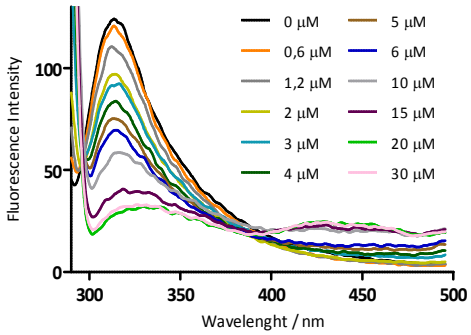

0 µM
0,6 µM
1,2 µM
2 µM
3 µM
4 µM
5 µM
6 µM
10 µM
15 µM
20 µM
30 µM
Fluorescence Intensity
100
50
0
300
350
400
450
500
Wavelenght / nm

Figure 3.

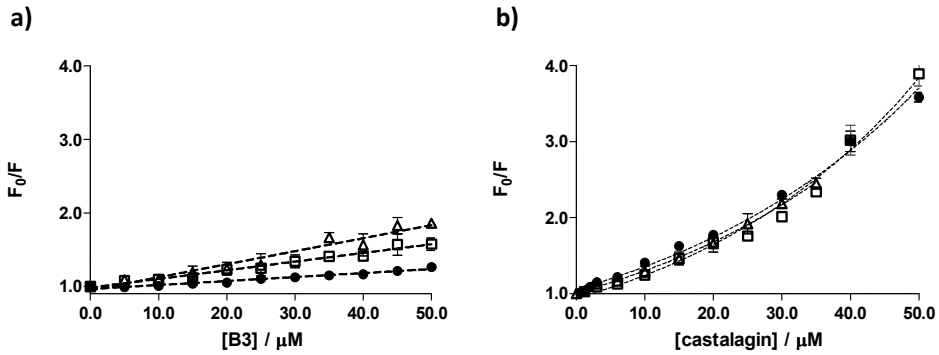
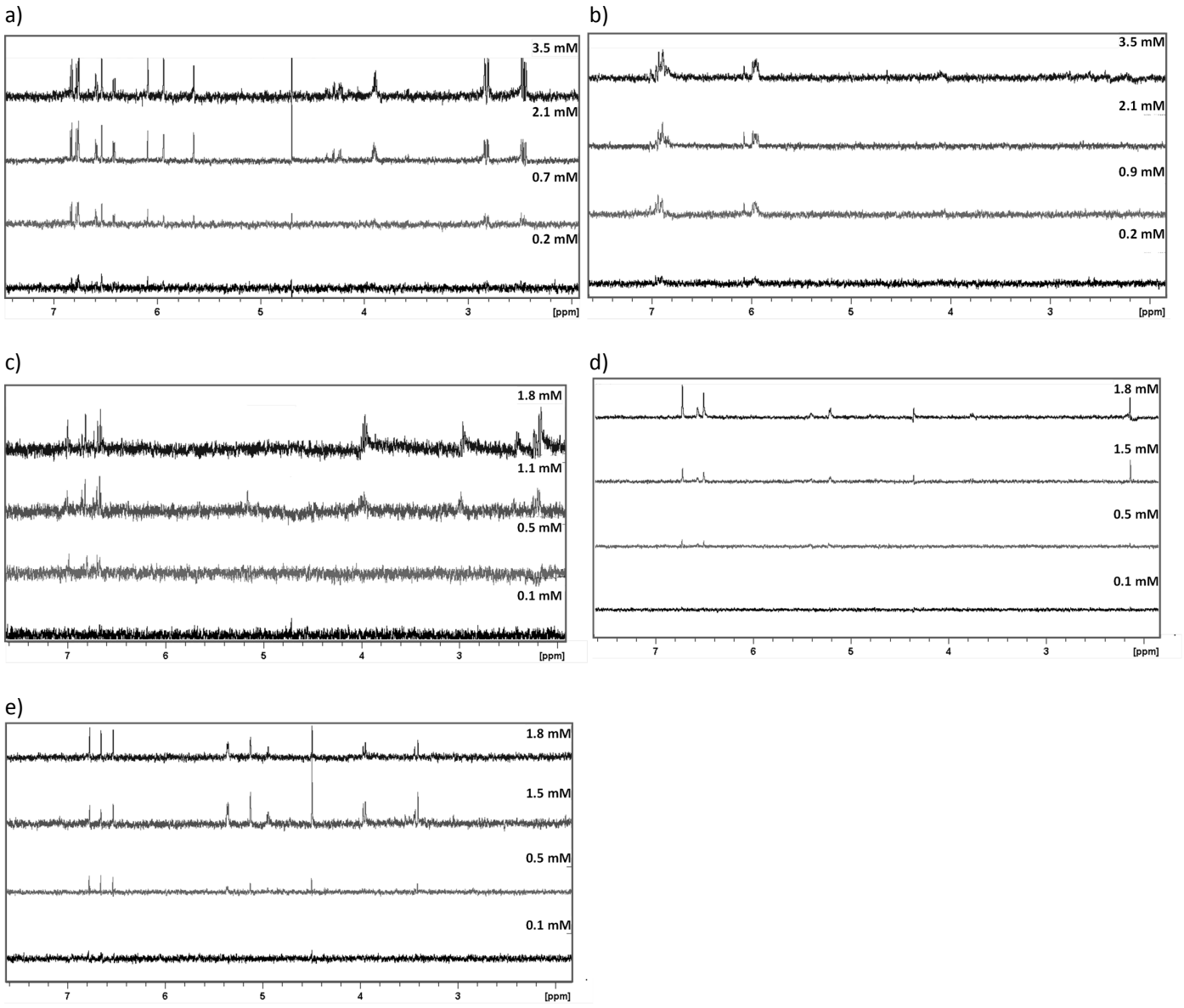


Figure 4.


a)
3.5 mM
2.1 mM
0.7 mM
0.2 mM
7
6
5
4
3
[ppm]
b)
3.5 mM
2.1 mM
0.9 mM
0.2 mM
7
6
5
4
3
[ppm]
c)
1.8 mM
1.1 mM
0.5 mM
0.1 mM
7
6
5
4
3
[ppm]
d)
1.8 mM
1.5 mM
0.5 mM
0.1 mM
7
6
5
4
3
[ppm]
e)
1.8 mM
1.5 mM
0.5 mM
0.1 mM
7
6
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4
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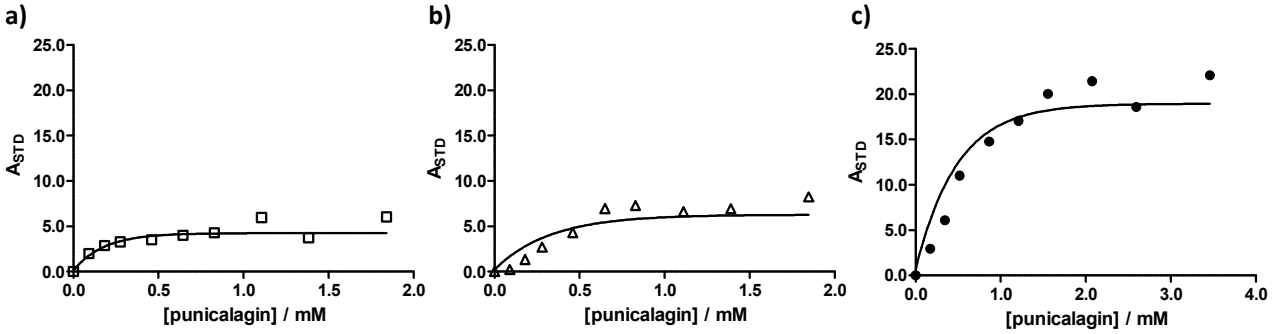
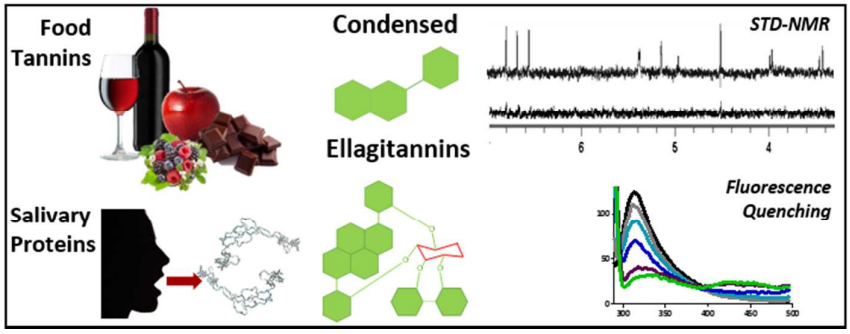
[ppm]

1

Figure 5.



TOC Graphic


Food
Tannins
Salivary
Proteins
Condensed
Ellagitannins
STD-NMR
6
5
4
Fluorescence
Quenching
100
50
0
300
350
400
450
500

1