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Interaction between red wine procyanidins and salivary proteins: Effect of stomach digestion on the resulting complexes

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Tannins, a group of polyphenols, are important at sensory (e.g. astringency sensation) and health levels (e.g., anti-cancer and cardiovascular protection). The health benefits are related to tannins' concentration that reaches the gastrointestinal tract (bioaccessible concentration) which could be affected by interaction with other biological molecules, such as salivary proteins (SP). Most of the works that study tannins health benefits do not consider these interactions. So, this work intended to mimic the ingestion of red wine condensed tannin fractions and assess the stability of the (in)soluble complexes formed between the different tannins and the different SP in a simulated stomach digestion. The results showed that some of the tannin/SP complexes could be disrupted by the gastric digestion leading to the release of tannins. This was observed for the complexes formed with the lowest polymerized tannins (monomers, dimers and trimers). Oppositely, the complexes formed by tannins tetramers and pentamers were significantly more resistant to stomach conditions. Therefore, SP probably influence negatively the concentrations at which tannins tetramers and pentamers reach the stomach and ultimately they may influence negatively some of these procyanidins biological potential health benefits. In the future, these and other biological interactions of tannin compounds should be taken into consideration in bioavailability and health benefits studies.

Introduction

Tannins are a group of polyphenol compounds that are widely distributed in vegetal foodstuffs, particularly in fruits, cereal grains and derived beverages (e.g. red wine, tea and beer)[1].

The designation "tannin" has its origin to the use of wood tannins from oak in the tanning process of animal hides into leather. Presently, tannins comprise a complex group of water-soluble phenolic compounds with a huge range of molecular weights (0.5 to around 20 kDa)[2] that have the special ability to interact with proteins, eventually leading to their precipitation. These compounds are structurally divided in two major classes, condensed tannins (polymers of flavan-3-ol units) and hydrolysable tannins (esters of glucose with gallic acid).

Tannins are important at both sensory and health levels. Regarding organoleptic properties, tannins are directly related to astringency sensation and contribute also to bitter taste. Astringency is a tactile sensation being described as dryness, tightening and puckering sensations[3] perceived in the oral cavity during the ingestion of foodstuffs rich in tannins. Several mechanisms have been proposed to its origin, however the most accepted by the scientific community arises from the interaction between tannins and salivary proteins (SP) leading to their precipitation[4]. In fact, during foodstuff consumption, tannins interact with SP, especially with proline-rich proteins (PRPs) forming (in)soluble aggregates. In general, the nature of tannin/protein interactions can be described as covalent or non-covalent based on whether the molecules are irreversibly bound to each other or not, and which could result in the formation of soluble or insoluble complexes. The interactions are thought to involve the cross-linking of separate protein molecules by the tannin which acts as a polydentate ligand on the protein surface involving hydrophobic and hydrogen bonds.

Regarding the SP, the main proteins have been grouped into six structurally related major classes: histatins, basic PRPs (bPRPs), acidic PRPs (aPRPs), glycosylated PRPs (gPRPs), statherin, and cystatins[5, 6]. The differences between the several families of PRPs depend on their charge and presence or absence of carbohydrates. All these proteins have important biological functions in saliva including calcium binding to enamel, maintenance of ionic calcium concentration, antimicrobial action or protection of oral tissues against degradation by proteolytic activity[7-12].

Additionally to the sensory properties, tannins and polyphenols in general have received high attention in the past years due to the several important health benefits associated to their ingestion. Several epidemiological studies have associated these compounds to benefic actions such as anti-cancer, anti-neurodegenerative activities, cardiovascular protection[13, 14]. One classical association is the well-known "French-paradox"[15]. The French population showed a low incidence of cardiovascular diseases despite the high consumption of saturated fat and tabaco. This fact was attributed to a regular consumption of red wine. In fact, red wine is one of the most rich and highly consumed source of tannins worldwide[16, 17].

One key aspect to study the health effect of a specific compound is to determine the amount that reaches the gastrointestinal tract (herein

referred as bioaccesible concentration) and, subsequently, the target tissue/organ (included in a more wide term, bioavailable concentration). In the case of tannins, there is only scarce quantity of data on these aspects because there are some technical increased limitations on tannin analysis (equipment sensibility, lack of standard molecules, complex polymers difficult to extract, isolate and analyze). Besides these limitations, the determination of tannins bioaccessible concentration is even more complex because some compounds have important interactions with other biological compounds, namely SP, as referred previously. In fact, most of the works that study the health benefits of these compounds do not take into account these interactions.

In this way, one important consideration for the bioaccessibility of tannins, it is their ability to interact forming (in)soluble complexes with SP present in human saliva and the stability of these complexes in stomach conditions. This can modify the accessibility of tannins in the gastrointestinal tract.

So, this work intended to mimic the ingestion of condensed tannin fractions with increasing degrees of polymerization prepared in a wine model solution (since red wine is a natural, highly consumed and rich source of procyanidins) and assess the stability of the insoluble complexes eventually formed between the different tannins and the different classes of SP in a simulated stomach digestion mode.

Results and Discussion

In the oral cavity, tannins or procyanidins have the characteristic property to interact with SP forming (in)soluble complexes and leading to astringency sensation, as already referred. However, there is not much knowledge on how this interaction affects tannins accessibility (bioaccessibility) in the gastric system. So, in this work it was intended to simulate the ingestion of red wine procyanidins to study the stability of the eventually formed complexes (procyanidins/SP) in a simulated gastric digestion environment.

Grape Seed Fraction (GSF) characterization

GSF where characterized by reaction with phloroglucinol in order to determine the mDP of each fraction. Based on previous works it is expected that the polymerization degree increases with the fractions obtained[18]. Fraction 1 was found to contain mainly catechins and gallic acid but also a small quantity of procyanidin dimers (mean DP 1.1). F2 contains essentially catechins and galloyl derivatives as well as procyanidin dimers and galloyl derivatives (mean DP 1.4). F3 contains mainly procyanidin dimers and trimers and their galloyl derivatives but also a small quantity of procyanidin tetramers (mean DP 2). F4 contains mainly procyanidin trimers and tetramers, their galloyl derivatives and also procyanidin pentamers (mean DP 4). F5 contains mainly procyanidin tetramers and pentamers and their galloyl derivatives but also hexamers galloylated (mean DP 5).

SP:procyanidins ratio and interaction between SP and procyanidins

The SP:procyanidins ratio was chosen according to the literature[19] and considering that a volume of 10 mL is usually used when simulating the drinking process.

Regarding the saliva and in order to make the *in vitro* interaction similar to what happens in the oral cavity, it was considered that saliva is dynamic being continuously produced during the tasting of wine. The volume of saliva normally present in mouth (residual saliva) is around 0.75 mL and the continuous flow of further saliva secreted by the salivary glands in responsive to wine is known to be 1 mL.min^{-1}. According to sensory protocols[20], when wine is introduced in the mouth, the maximum astringency is reached only after 15 s, thus the volume of saliva produced within 15 s resulted to be 0.25 mL. The total salivary volume that comes in contact with wine and produced within 15 s resulted in 1 mL (0.75 + 0.25 mL) or 4 mL.min^{-1}. However, some *in vitro* studies and also some sensory studies usually use 5 min to study this interaction and then the volume of saliva in this time becomes 20 mL. Since 10 mL of wine is exposed to 20 mL (4 mL.min^{-1} x 5 min) of saliva for 5 min, a saliva:wine ratio of 2:1 seems closer to reality[19].

In order to compare the ability of the different GSF to interact and precipitate the different families of SP, all fractions reacted with saliva at the minimum and maximum concentrations studied (20.0 mg.L^{-1} and 60.0 mg.L^{-1}, respectively). After the interaction, the precipitates were removed by centrifugation and the procyanidins that remain in the supernatant were determined by reaction with 3-[4-(dimethylamino)phenyl]prop-2-enal (DMACA). The procyanidins concentration present in the precipitate was calculated by subtracting the supernatant concentration value to the initial procyanidins concentration (Figure 1).

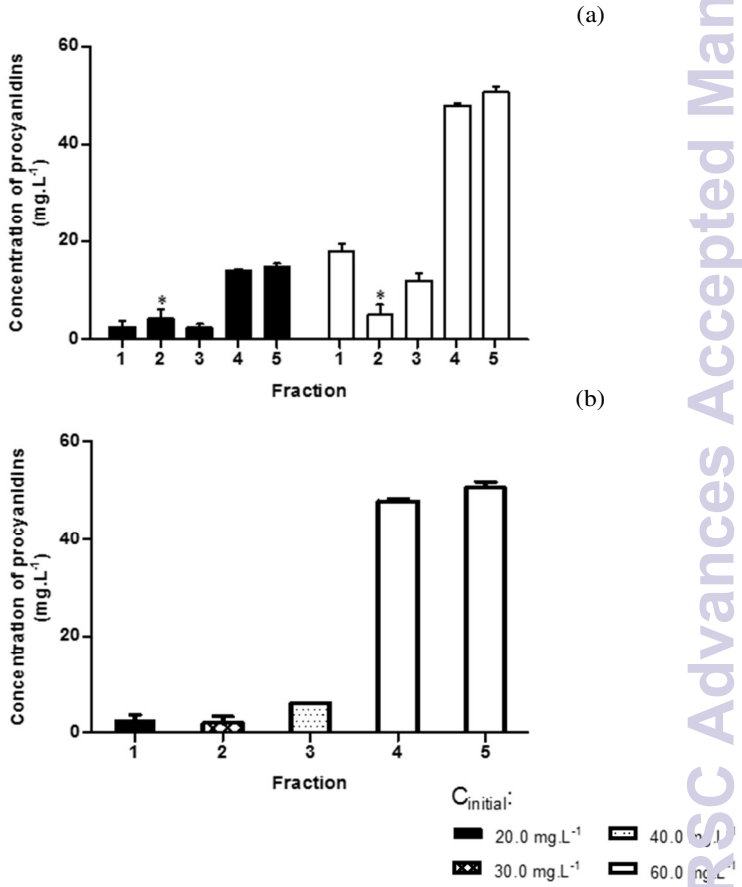


Figure 1. Concentration of each procyanidin fraction in the precipitate for (a) the minimum and maximum concentrations and (b) for the concentrations of each fraction reported in red wine considering the mDP of each fraction. These concentrations were calculated by subtraction of the initial procyanidins concentration from the procyanidins concentration in the supernatant (determined by DMACA method) after reaction with SP. Each value represents the mean ± SEM (n =

3). The only non-significantly different pairs are assigned (*). The other values are significantly different $p < 0.01$.

From the results presented in Figure 1(a) it is possible to observe that for both (minimum and maximum) concentrations, the most reactive fractions toward SP precipitation were F4 and F5. This means that the most polymerized fractions are the most effective ones in the interaction/precipitation of SP. In fact, it is possible to observe that for the same concentration increasing the mDP (F1<F2<F3<F4<F5) increases interaction with SP. This fact is also visible in the results for the other tested concentrations (30.0 and 40.0 mg.L^{-1} for F2 and F3, respectively) (Figure 1(b)). The only unexpected result it was that obtained with the F1 at 60.0 mg.L^{-1}. The fact that tannins interaction with proteins increases either with concentration, polymerization degree and galloylation has been well supported in the literature[21-23].

After comparing the reactivity of the different GSF towards SP, the following experiments were done only for the concentrations that were reported to exist in red wine taking into consideration the mDP: F1, 20.0 mg.L^{-1}; F2, 30.0 mg.L^{-1}; F3, 40.0 mg.L^{-1}; F4 and F5, 60.0 mg.L^{-1} [24].

The protein profile of the supernatants was analyzed by HPLC before (control) and after the interaction with GSF in order to determine which SP reacted and were precipitated by procyanidins (Figure 2(a)).

The HPLC chromatogram of the supernatant control solution at 214 nm is presented in Figure 2. The top of the figure shows the distribution of the different families of SP along the chromatogram that were established previously by proteomic approaches, namely ESI-MS and MALDI-TOF/TOF[21].

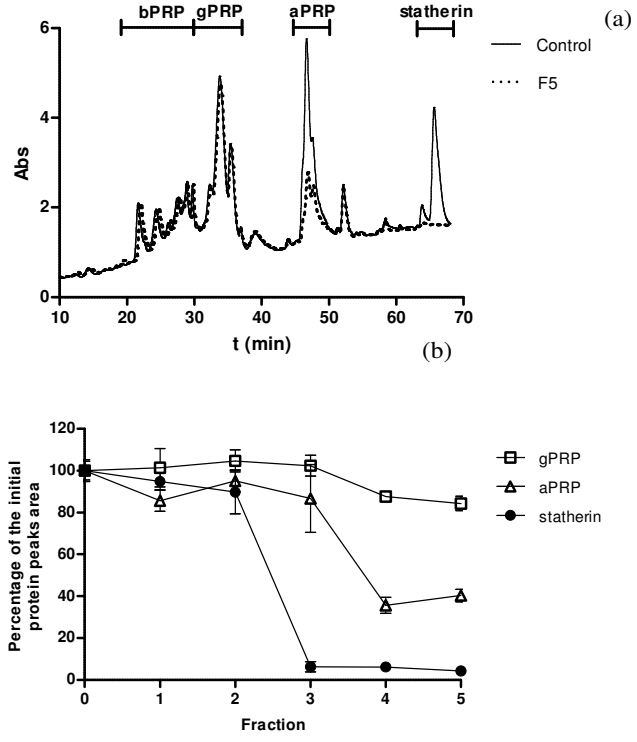


Figure 2. Influence of GSF fraction (F1, 20.0 mg.L^{-1}; F2, 30.0 mg.L^{-1}; F3, 40.0 mg.L^{-1}; F4 and F5, 60.0 mg.L^{-1}) on SP precipitation. (a) Chromatogram of saliva after the interaction with F5 (60.0 mg.L^{-1}). (b) The observed changes in the chromatographic peaks area were calculated as percentage of the control condition. gPRP – glycosylated proline-rich proteins, aPRP – acidic proline-rich proteins. These results represent the average of three independent experiments.

The HPLC chromatogram of the control solution is roughly divided into four SP family regions: the first region comprises proteins that belong to the classes of bPRPs and histatins. The bPRPs identified in this region include IB-8b, IB-8c, IB-9, IB-4 and P-J and the histatins include histatins 3, 5, 7, 8 and 9. The second region comprises mainly one gPRPs, the bPRP3. The next region corresponds entirely to aPRPs, namely PRP1 and PRP3, and the last region has phosphorylated and non-phosphorylated forms of statherin and peptide P-B.

As an example of the observed changes in SP HPLC profile, the results for the interaction with 60.0 mg.L^{-1} of F5 are presented in Figure 2(a). The observed changes of the chromatographic peaks area of the different SP with the different GSF were expressed in percentage of the area of these proteins relatively to the respective area in the control saliva (Figure 2(b)).

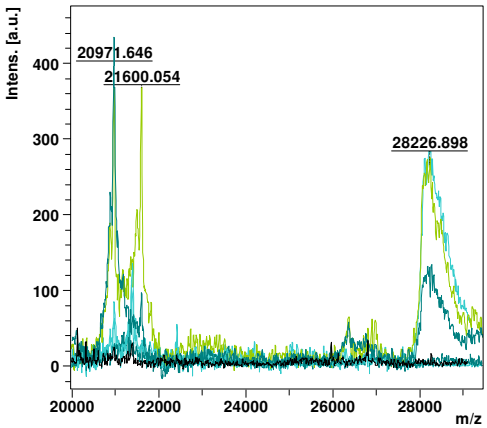
From the presented results it is possible to observe that the most precipitated proteins were statherin and aPRP, in particular for F3, F4 and F5. bPRP are not affected for any GSF even for the most polymerized ones while gPRPs start to be precipitated for the most polymerized fractions. Probably it would be necessary a higher concentration of GSF to precipitate these proteins, as observed in previous works[21, 25].

The analyses of the SP are in accordance with the quantification of GSF in the supernatant for the correspondent concentration. For instance, F4 and F5 at 60.0 mg.L^{-1} were the most reactive and most precipitated GSF (Figure 1(b)) and also in the HPLC SP profile where they were the ones that depleted almost completely statherin and reduced significantly aPRPs. However, F5 also starts to reduce gPRP (~20%). Oppositely, F1 and F2 at 20.0 and 30.0 mg.L^{-1}, respectively, showed a very small interaction with SP by GSF quantification (Figure 1(b)) and in the HPLC profile analysis of SP (Figure 2(b)).

MALDI-TOF analysis of the precipitates

In order to obtain information and characterize the insoluble complexes formed by interaction of SP with F3, F4 and F5, the precipitates were resuspended or resolubilized for MALDI-TOF analysis. In this experiment some difficulties were observed: the total resolubilization was extremely difficult and in the case of F4 and F5 it was only possible to do a parcial resolubilization and the resuspension of precipitates was not homogenous. These difficulties suggest that the insoluble complexes formed are extremely stable and so probably they are originated by covalent bonds with tannins and proteins being irreversibly bound to each other.

Neverthless, the complexes that were resuspended or resolubilized were analyzed and the results are presented in Figure 3.



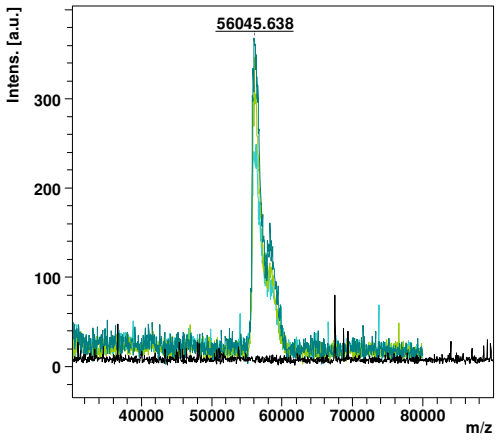


Figure 3. MALDI-TOF analysis of control condition (saliva) and of the precipitates resultant from the interaction between F3, F4 and F5 with SP. The experimental conditions were adapted to the mass range between (a) 20 – 30 kDa and (b) 30 – 80 kDa.

The results showed clearly the appearance of new and high molecular weight peaks that were not present in the control condition (saliva and model wine solution). In Figure 3 it is possible to observe the appearance of peaks with small masses around 20 and 28 kDa but also with higher masses around 60 kDa.
As referred herein and in previous work[21], most of the SP analyzed by the described methodology are indeed peptides with small molecular weights and with different isoforms and post-translational modification, such as phosphorylation. Staherin have a molecular weight around 5 kDa, aPRP have a molecular weight around 11 – 15 kDa, histatins have a molecular weight around 3 kDa and bPRP have a molecular weight around 4 – 6 kDa. As the identified masses are significantly higher than these values, this analysis supports that SP and tannins interact forming a huge network of protein/tannin complexes with very high molecular weights.
However, it is important to refer that the analysis of the precipitates is very difficult and the solutions were not homogenous. So, it is extremely difficult to obtain information about the molecular composition of these precipitates.

Stability of tannins/SP complexes to gastric digestion

After comparing the reactivity of the different GSF toward SP and determining which SP were precipitated by the different GSF, it was intended to study the stability of the formed tannin/SP complexes during stomach digestion. So, the precipitates were incubated for 2h in a solution simulating stomach conditions with continuous shaking. After this incubation the procyanidins eventually released to the solution (supernatant) were quantified by reaction with DMACA. The obtained results are presented in Figure 4.
From the results presented in Figure 4 it is possible to observe that for F1, F2 and F3 the concentration of procyanidins in the precipitates (before) and supernatant (after) digestion are not-significantly different. Therefore, it seems that for the lowest polymerized fractions the complexes formed with SP could be disrupted by the stomach environment leading to the release of procyanidins to the supernatant. On the other hand, the results for F4 and F5 are quite different. From Figure 4 it is possible to observe that after digestion the concentration of procyanidins is approximately half or less of the concentration initially present in the precipitates. This means that the complexes formed by these fractions with SP are more resistant to the digestion conditions.

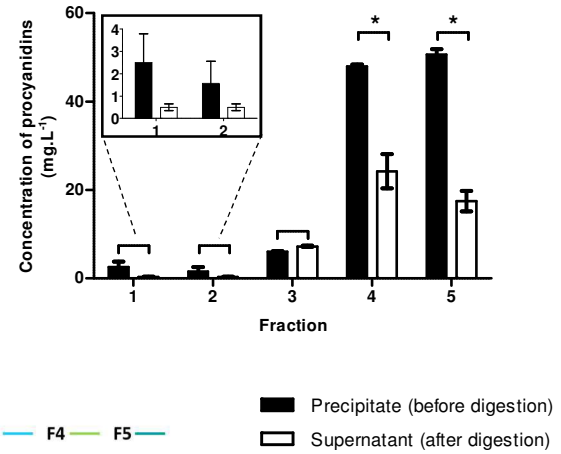
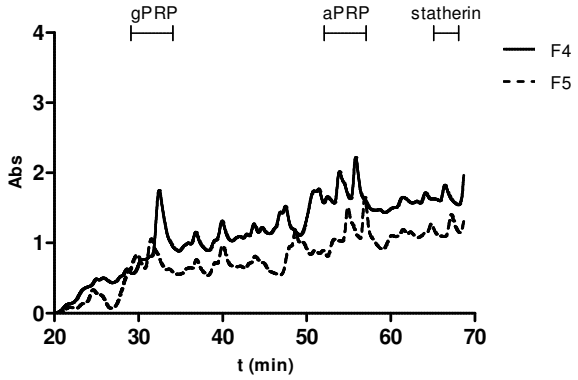


Figure 4. Concentration of each procyanidin fraction before and after digestion [pepsin (315 units.mL^{-1}), pH 1.7] of the precipitates. The concentration of procyanidins before digestion were calculated by subtraction of the initial procyanidins concentration from the procyanidins concentration in the supernatant (determined by DMACA method) after reaction with SP. The concentration of procyanidins after digestion were obtained directly in the solution. Each value represents the mean ± SEM (n = 3). The significantly different pairs are assigned (*p < 0.01).

The supernatants after the simulated digestion of the precipitates of the most reactive fractions (F4 and F5) were also analyzed by HPLC in order to determine which SP were released from the complexes.



The results are presented in Figure 5.

Figure 5. Influence of simulated stomach digestion on the release of SP from the precipitates formed for F4 and F5 (60.0 mg.L^{-1} for both). gPRP – glycosylated proline-rich proteins, aPRP – acidic proline-rich proteins.

From the results presented in Figure 5 it is possible to observe that the major SP that are released from the complexes are gPRP and aPRP. In fact, statherin it seems to be released in a very small extent. Somehow the complexes statherin/procyanidins are more stable and resistant than the ones formed with proline-rich proteins family.
These results have important consequences at the biological level. Although the lowest polymerized procyanidins (F1, F2 and F3) are the ones that are present in red wine in lowest concentrations, they seem to be the ones more readily bioaccessible. They have the

lowest interaction with SP and when they interact with SP the eventually formed insoluble complexes are disrupted by the gastric digestion. So, overall, the monomeric and dimeric procyanidins probably reach the gastrointestinal environment in small protein/procyanidins complexes or intact at the concentrations that exist in red wine and could display beneficial biological activities such as anticancer activity[26] or cardiovascular protection but also harmful actions such as inhibition of digestive enzymes[27].
Regarding the most polymerized fractions (F4 and F5), at the concentration present in red wine they are practically depleted by SP and they reach the gastrointestinal environment in the form of insoluble complexes. As the results showed some of these complexes could be disrupted by the gastric digestion releasing these procyanidins to exert some biological activities or they could be catabolized into small phenolic molecules by the intestinal microflora[28].

Experimental

Materials and methods

Reagents. All reagents used were of analytical grade or better. Acetonitrile (ACN) and hydrochloric acid were purchased from Panreac Quimica, acetic acid (HOAc) was purchased from Carlo Erba Reagents, trifluoracetic acid (TFA) were purchased from Fluka Biochemica (Switzerland), ethanol (EtOH) was purchased from AGA, Álcool e Géneros Alimentares, SA., sodium hydroxide was purchased from Laboratório Maialab, Lda (Maia, Portugal), sodium carbonate was purchased from Sigma-Aldrich, tartaric acid was purchased from Aldrich, MeOH, ascorbic acid, phloroglucinol, acetate sodium, catechin, 4-(dimethylamino)cinnamaldehyde (DMACA), pepsin, H_2SO_4

Grape Seed Fractions (GSF) Isolation. Procyanidins were extracted from grape seeds (*Vitis vinifera)* with an ethanol/water/chloroform solution (1:1:2, v/v/v). The resulting solution was centrifuged and the chloroform phase, containing chlorophylls, lipids and other undesirable compounds was rejected. The hydroalcoholic phase was then extracted with ethyl acetate, and the organic phase was evaporated using a rotary evaporator (30 ºC). The resulting residue corresponding essentially to oligomeric procyanidins was fractionated through a TSK Toyopearl HW-40(s) gel column (100 mm x 10 mmi.d., with 0.8 $mL.min^{-1}$ methanol as eluent), yielding five fractions according to the method described in the literature [29]. The first 30 min of elution were rejected. The first (F1), second (F2) and third (F3) fractions were obtained after elution with 99.8% (v/v) methanol during 15 min (12 mL), other 15 min (12 mL) and other 4 h (192 mL), respectively. The fourth fraction (F4) was eluted with methanol/5% (v/v) acetic acid during the next 14 h (670 mL) and the fifth fraction (F5) was eluted with methanol/10% (v/v) acetic acid during the next 8 h (384 mL). All fractions were mixed with deionized water, and the organic solvent was eliminated using a rotary evaporator under reduced pressure at 30 ºC and then freeze-dried.

Analysis and characterization of GSF. The procyanidin composition of fractions was determined by direct analysis by ESI-MS (Finnigan DECA XP PLUS) and subsequent analysis of the average full mass spectra. The mean degree of polymerization (mDP) was determined by acid-catalysis reaction in presence of phloroglucinol as described in the literature followed by LC-MS (Finnigan DECA XP PLUS) and HPLC analysis[30]. Briefly, a solution of 0.1 N HCl in MeOH, containing 50 $g.L^{-1}$ and 10 $g.L^{-1}$ ascorbic acid was prepared. The GSF of interest was reacted in this solution (5 $g.L^{-1}$) at 50^0C for 20 min, and then it was added 5 volumes of 40 mM aqueous sodium acetate to stop the reaction. The resulting solutions were analyzed either by LC-MS to identify the flavan-3-ols monomers and the monomeric phloroglucinol adducts as well as by HPLC to determine the moles of each monomer by a calibration curve obtained from (+)-catechin.

Saliva Collection. Saliva was collected from six healthy non-smoking volunteers and 2 mL of saliva from each volunteer were used to make a saliva pool (whole saliva). Collection time was standardized at 2 p.m. in order to reduce concentration variability connected to circadian rhythms of secretion[31]. The saliva pool was mixed with 10% TFA (final concentration 0.1%) to precipitate several high molecular weight SP (such as α-amylases, mucins, carbonic anhydrase and lactoferrin) and to preserve sample protein composition, since TFA partially inhibits intrinsic protease activity. However, peptides and proteins like histatins, basic, acidic and glycosylated PRPs, statherin, cystatins, and defensins are soluble in acidic S solution and may be directly analyzed by RP-HPLC, as previously described. After the centrifugation (8000 g for 5 min), the supernatant (referred subsequently as saliva, S) was separated from the precipitate and used for the following experiments. The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of Medical School of University of Porto (EK84032011).

SP analysis (HPLC analysis). 100 µL of each solution were injected on a HPLC Lachrom system (L-7100) (Merck Hitachi) equipped with a Vydac C8 column (Grace Davison Discovery Sciences), with 5 µm particle diameter (column dimensions 150 x 2.1 mm); detection was carried out at 214 nm, using a UV-Vis detector (L-7420). The HPLC solvents were 0.2% aqueous TFA (eluent A) and 0.2% TFA in ACN/water 80/20 (v/v) (eluent B). The gradient applied was linear from 10 to 40% (eluent B) in 60 min, at a flow rate of 0.30 $mL.min^{-1}$. After this program the column was washed with 100% eluent B for 20 min in order to elute S-type cystatins and other late-eluting proteins. After washing, the column was stabilized with the initial conditions.

Protein and Tannin Interaction. The S sample was analyzed by HPLC before and after the interaction with different concentrations of GSF. These experiments were made in order to simulate the ingestion of red wine. So all the GSF solutions were made in a wine model solution (tartaric acid 5 g/L, 12% EtOH, pH adjusted to 3.5 with NaOH). The chosen GSF concentrations are in accordance to the previously reported concentrations in red wine taking into account the mean DP of each fraction [24]: F1, 20.0 $mg.L^{-1}$; F2, 30.0 $mg.L^{-1}$; F3, 40.0 $mg.L^{-1}$; F4 and F5, 60.0 $mg.L^{-1}$. In addition and in order to compare the reactivity of the different fractions towards SP, all fractions were also tested at the minimum and maximum concentrations (20.0 and 60.0 $mg.L^{-1}$).
The control condition was a mixture of S (200 µL) and model solution (100 µL) (final volume 300 µL). Different volumes of GSF stock solutions (400 $mg.L^{-1}$) prepared in model solution were added to S (200 µL) to obtain the desired final concentrations. The final volume was adjusted to 300 µL with model solution. The ratio used (2:1 saliva:wine) has been used previously as a model closer to reality of wine ingestion [19]. The mixture was shaken and kept for 5 min at room temperature (± 20 ºC) and then centrifuged (8000 g, 5 min). The supernatant was separated from the precipitate. Part of the

supernatant was injected into the HPLC to monitor the SP present and other part was analyzed by spectrometry after reaction with DMACA to measure the procyanidin content. The precipitate was subjected to simulated stomach/gastric digestion.

Analysis of the precipitates by MALDI-TOF. The precipitates resultant from the interaction between F3, F4 and F5 and SP and also a control condition (saliva in wine model solution) were analyzed by MALDI-TOF, using a 4800 MALDI-TOF analyzer (Applied Biosystems, Foster City, CA) in the linear mode to obtain the molecular weight of larger species. All samples were mixed (1:1) with a matrix solution (3 mg/mL) of α-cyano-4-hydroxycinnamic acid matrix prepared in 50% methanol. Aliquots of samples (2 µL) were spotted onto the MALDI sample target plate, and spectra were obtained in the mass range between 1500 and 200000 Da with ca. 2000 laser shots.

Gastric digestion. The procedure was adapted from Coates and co-workers[32]. The method consists on a pepsin/HCl digestion with shaking to simulate gastric conditions. A pepsin solution (315 units.mL^{-1}) was prepared in water and then the pH was adjusted to 1.7 with HCl. 300 µL of this solution was added to the precipitates originated from the reaction between S and GSF. The pellet was ressupended and the digestion occurred for 2 h at 37 °C with shaking. After the 2h, the procyanidin content was quantified by DMACA either before or after solution centrifugation.

DMACA assay to measure the procyanidin content. The method used was similar to the one described previously[33]. Briefly, a 0.1% solution of DMACA was prepared in acidified (0.75 M H_2SO_4) methanol. On a 96 well plate, 50 µl of a standard or test solution were incubated with 50 µl of DMACA solution for 20 min at room temperature, and the absorbance of each well was determined at 640 nm in a µQuant microtitre plate reader. Each fraction was used as a standard, and the concentration of procyanidin in (a) supernatant after reaction with S, and (b) precipitate after digestion and after centrifugation, was calculated from the appropriate standard curve, using Graphpad Prism software.

Statistical Analysis. All assays were performed in n = 3 independently repetitions. The mean values and standard deviations were evaluated using analysis of variance (ANOVA); all statistical data were processed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com).

Procedure outline:

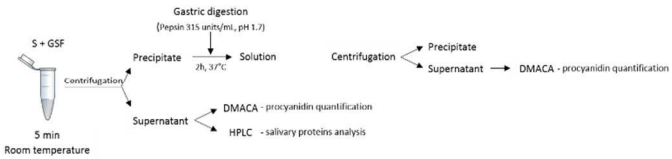


Figure 7. Procedure outline: summary of the treatment and analysis of the whole experiment.

Conclusions

This work provided evidences that SP probably influence negatively the concentration at which tetramers and pentamers of procyanidins reach the stomach due to their precipitation and ultimately they may influence negatively some of these procyanidins biological potential health benefits. This effect was opposite for procyanidin monomers, dimers and trimmers which reach the gastrointestinal environment at the concentrations they exist in red wine or in small protein/procyanidins complexes that are disrupted by stomach digestion.

These results also support the hypothesis that the high and resistant ability of some SP to precipitate the most polymerized tannins could be related to the prevention of deleterious effects of tannin compounds in the digestive tract (e.g., inhibition of digestive enzymes).

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Notes and references

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