



Effect of flavonols on wine astringency and their interaction with human saliva



Raúl Ferrer-Gallego^{a,b}, Natércia F. Brás^c, Ignacio García-Estévez^b, Nuno Mateus^d, Julián C. Rivas-Gonzalo^b, Victor de Freitas^d, M. Teresa Escribano-Bailón^{b,*}

^a Parc Tecnològic del Vi – VITEC, Ctra Porrera, 43730 Falset, Spain

^b Grupo de Investigación en Polifenoles, Facultad de Farmacia, University of Salamanca, Campus Miguel de Unamuno, E 37007 Salamanca, Spain

^c REQUIMTE-UCIBIO, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal

^d REQUIMTE-LAQV, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal

ARTICLE INFO

Article history:

Received 3 September 2015

Received in revised form 12 April 2016

Accepted 20 April 2016

Available online 21 April 2016

Keywords:

Wine

Flavonol

Human saliva

Astringency

Fluorescence quenching

Molecular dynamic simulations

ABSTRACT

The addition of external phenolic compounds to wines in order to improve their sensory quality is an established winemaking practice. This study was aimed at evaluating the effect of the addition of quercetin 3-O-glucoside on the astringency and bitterness of wines. Sensory results showed that the addition of this flavonol to wines results in an increase in astringency and bitterness.

Additionally, flavonol-human salivary protein interactions were studied using fluorescence spectroscopy, dynamic light scattering and molecular dynamic simulations (MD).

The apparent Stern-Volmer (K_{svApp}) and the apparent bimolecular quenching constants (k_{qApp}) were calculated from fluorescence spectra. The K_{svApp} was $12620 \pm 390 \text{ M}^{-1}$, and the apparent biomolecular constant was $3.94 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$, which suggests that a complex was formed between the human salivary proteins and quercetin 3-O-glucoside.

MD simulations showed that the quercetin 3-O-glucoside molecules have the ability to bind to the IB9₃₇ model peptide.

© 2016 Elsevier Ltd. All rights reserved.

1. Introduction

Exogenous phenolic compounds are often added to must or wines in order to mask faults and to improve the sensory quality of these beverages by, for example, modifying the mouth-feel or stabilizing the colour. For this purpose, many extracts and isolated compounds obtained from different sources have been tested (Alcalde-Eon et al., 2014; Alexandre-Tudo et al., 2013; Cliff, Stanich, Edwards, & Saucier, 2012; Harbertson, Parpinello, Heymann, & Downey, 2012). In this way, it has been stated that flavonols are of crucial importance to the colour quality of red wine because they act as good co-pigments (Escribano-Bailón & Santos-Buelga, 2012; Gomez-Míguez, González-Manzano, Escribano-Bailón, Heredia, & Santos-Buelga, 2006). Recently, the addition of extracts from white wine by-products containing glycosylated flavonols has been suggested as a good practice to stabilize red wine colour (Gordillo et al., 2014; Jara-Palacios et al., 2014). Additionally, flavonols are of great interest for the food

industry because of their well-known beneficial effects, for example, antioxidant properties, anticancer activities, and their roles in reducing inflammation and reducing the risk of chronic disease. Therefore, the addition of these compounds to beverages and food may also be an interesting practice. Nevertheless, the subject of possible changes in the mouth-feel perception due to exogenous addition of flavonols is not adequately addressed in these studies.

The determination of the relationship between phenolic structures and the sensory perceptions elicited by them is one of the top current challenges in food chemistry and, in particular, in oenology research. This goal is closely related to the mechanisms implicated in the astringency perception, which remain unclear. It has been established that the phenolic-salivary protein interaction is not the only mechanism involved in astringency. Some sensory receptors may also participate in this oral perception (Carpenter, 2013; Gibbins & Carpenter, 2013). Recently, it has been stated that the chemosensory detection of astringent phenols together with the stimulation of trigeminal mechanosensors create the entire sensation of astringency (Schobel et al., 2014). The simultaneous activation of both chemosensory and

* Corresponding author.

E-mail address: escriban@usal.es (M.T. Escribano-Bailón).

mechanosensory receptors could be the reason for some synergisms of astringency shown by certain phenolic compounds (Ferrer-Gallego, Hernandez-Hierro, Rivas-Gonzalo, & Escribano-Bailon, 2014).

A combination of sensory analysis and analytical techniques is necessary for developing a proper understanding of the astringency phenomenon. Over the years, astringent properties of many phenolic compounds have been described. However, little research has been reported in relation to the astringency of wine flavonols (Hufnagel & Hofmann, 2008). These compounds are present in numerous fruits and beverages, grapes and wines being one of their most important sources in our diet (Makris, Kallithraka, & Kefalas, 2006).

Red wine and tea contain up to 45 mg/L of flavonols, but the richest sources are onions, curly kale, leeks, broccoli and blueberries (Manch, Scalbert, Morand, Rémésy, & Jiménez, 2004). Flavonols are present as aglycones, and 3-*O*-glucosides are their most common conjugated forms. Quercetin and its conjugated derivatives are frequently the main flavonols in both red and white grapes. In red varieties, they can represent up to 87% of the total flavonol content, while in white varieties, they can form up to 97% of the total flavonol content (Mattivi, Guzzon, Vrhovsek, Stefanini, & Velasco, 2006).

Other beverages such as cider, tea and beer also contain flavonols that may contribute to the mouth-feel sensation. Global flavonol content in apple juice ranges from 5 to 16 mg/L, which represents, on average, only 1–3% of the total phenolic concentration. Apple juice presents very low levels of quercetin glycosides (Ramírez-Ambrosi et al., 2015), and their astringency has been mainly attributed to the hydroxycinnamic acids (Mangas, Rodríguez, Suárez, Picinelli, & Dapena, 1999). However, flavonol content has been associated with mouth-drying astringency in blackcurrant juices (Laaksonena, Mäkilä, Tahvonen, Kallio, & Yang, 2013) and black tea. The flavonol glycosides were found to induce a mouth-drying and mouth-coating sensation at very low threshold concentrations. Particularly, the threshold of quercetin 3-*O*-glucoside has been established as 0.65 μM , its concentration in black tea being around 6 μM (Scharbert & Hofmann, 2005).

Flavonols also seem to have some influence on the astringency perception in legumes (Troszynska et al., 2011).

In this work, the addition of quercetin 3-*O*-glucoside in wines has been evaluated in terms of astringency and bitterness. For this, two wines, seven sensory attributes, five different concentrations and two sensory evaluation times were considered.

To obtain further insights into the flavonol-human salivary protein interactions, experiments between quercetin 3-*O*-glucoside and saliva were performed using fluorescence spectroscopy, dynamic light scattering (DLS) and molecular dynamics simulations (MD).

This study combines analytical techniques and sensory analysis, in order to understand the molecular mechanism of the flavonol-salivary protein interaction and its relationship with the sensory perception of wine. To our knowledge, this is the first time that sensory analysis of flavonols and their molecular interactions with saliva have been studied.

2. Materials and methods

All solvents were of HPLC grade, and all chemicals were of analytical reagent grade. Quercetin 3-*O*-glucoside was supplied by Sigma-Aldrich (St. Louis, MO, USA).

Fluorescence and DLS measurements were performed in 0.1 M acetate buffer (12% ethanol) at pH 5.0. At this pH, salivary proteins have been shown to interact strongly with phenolic compounds (Soares, Mateus, & De Freitas, 2007).

2.1. Sensory assessment

A sensory panel was composed of 10 panellists previously trained in the quantitative and qualitative description of astringency. They were aged from 23 to 60 years old. Eight of them were professional tasters, and the others were students of Oenology. The panellists attended four preliminary training sessions in order to be familiarized with the sensory attributes and to standardize the use of terms and the scale. All of them had previously participated in analogous sensory tests, and the consistency of the trained panel was determined during this time (Ferrer-Gallego et al., 2014; Ferrer-Gallego et al., 2015a, 2015b).

The intensity of astringency, bitterness, velvety, dryness, dynamic, greenness, roughness and persistence of astringency were evaluated according to Gawel, Oberholster, and Francis (2000). Tests were carried out using a red wine and a white wine, both selected for their low astringency. Nineteen wines from different Spanish regions were previously evaluated for this purpose. The selected red wine was a young wine elaborated with 'Tempranillo' (2012) from Rioja D.O.C. The selected white wine was a young wine elaborated with 'Macabeo' (2012) from Vino de la Tierra de Castilla I.G.P.

Tests were carried out in individual booths in a professional tasting room at 20 °C. The addition of five different concentrations (0.25, 0.5, 1, 1.5 and 2 g L^{-1}) of quercetin 3-*O*-glucoside was evaluated in both wines. In the case of the red wine, the sensory analysis was also performed three months after storage at 4 °C.

A labelled magnitude scale (LMS) was used for rating the intensity of each attribute. This scale is characterized by a quasi-logarithmic spacing of its verbal labels from 0 ("barely detectable" oral sensation) to 100 ("strongest imaginable" oral sensation) (Fig. 1) (Green et al., 1996). Samples were tested in 25-mL screw neck glass bottles and were randomly served. Tasters took 5 mL of the sample and 15 s and after this, rated the intensity for each attribute. This protocol was repeated two times per sample to assign the final value in the mentioned scale. They rinsed with deionized water and waited for 2 min between samples.

2.2. Saliva collection

A saliva pool was collected according to Soares et al. (Soares, Mateus, & de Freitas, 2012), from healthy, non-smoking volunteers aged from 22 to 32 years. All panellists held saliva inside their mouth for 10 min and spit it all at once; they had previously avoided food and beverages for at least 1 h before collection. The standardized collection time was approximately 2 p.m. to reduce variability linked to circadian rhythms (Messana, Inzitari, Fanali, Cabras, & Castagnola, 2008). Saliva was treated with 10% of trifluoroacetic acid (final concentration 0.1%) and centrifuged. Finally, supernatant was used for fluorescence and DLS analyses.

2.3. Fluorescence spectroscopy

A quercetin 3-*O*-glucoside solution (0.25 mM) was prepared in 0.1 M acetate buffer (12% ethanol) at pH 5.0. Then, 100 μL of saliva was added to different volumes of this stock solution (from 5 to 100 μL). All solutions were brought up to a final volume of 200 μL by addition of acetate buffer. The final flavonol concentrations were 0, 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 μM . All samples were mixed in a vortex for 10 s. Spectra were recorded at a controlled temperature 24 ± 0.1 °C. A Varian Cary Eclipse spectrofluorometer equipped with a "single cell peltier accessory" in 1-cm quartz cuvettes, with a slit width of 10 nm, scan rate of 600 nm/min, and data range of 1 nm was used. The excitation wavelength was set to 282 nm, and the emission spectrum was recorded from 300 to 500 nm. The assay was performed in triplicate.

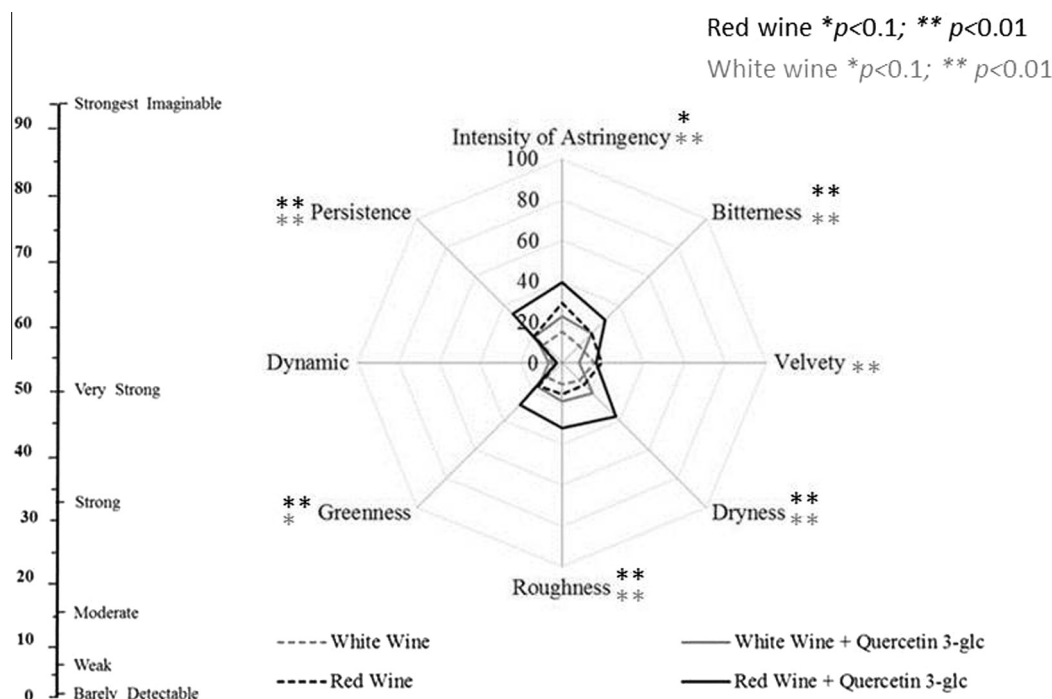


Fig. 1. Sensory analysis of white and red wines before and after the addition of quercetin 3-O-glucoside (2 g/L).

The saliva fluorescence lifetime was measured at 24 ± 0.1 °C with a Fluoromax-4 spectrophotometer attached to a single photon counting controller (FluoroHub), both from Horiba Jobin-Yvon. The experiments were performed with 100 μ L of the saliva pool. The fluorescence excitation was performed with a Horiba Nano LED source of 290 nm, and fluorescence emission was recorded at the maximum wavelength (337 nm). The lamp profile was recorded by placing a scatter (dilute solution of LUDOX in water) in place of the sample.

Decay curves were analysed by a nonlinear least-squares iteration procedure using DAS6 v6.5 decay analysis software. Two exponentials were required to fit the decay curves, and the quality of the fits were assessed from the global χ^2 value ($\chi^2 \leq 1.3$), weighted residuals and autocorrelation plots.

For a two-exponential decay, the average fluorescence lifetime of a fluorophore is given by Eq. (1):

$$\bar{\tau} = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (1)$$

where τ_i represents each component of the fluorescence lifetime and α_i the respective pre-exponential factor (Lakowicz, 2006).

2.4. Dynamic light scattering

The size of the complexes formed between salivary proteins and quercetin 3-O-glucoside was determined by dynamic light scattering (DLS, Zetasizer Nano S, Malvern, UK). This analysis provides information about the particle size and polydispersity of the sample dispersed or dissolved in a liquid. The Brownian motion of particles causes laser light to be scattered in all directions at different intensities. Analysis of these intensity fluctuations enables the determination of the diffusion coefficients of particles, which are then converted into a size distribution.

Sample solutions of human saliva with different concentrations of quercetin 3-O-glucoside were illuminated by a 633 nm laser at 25 °C. The intensity of the scattered light was detected at an angle of 173° (standard measurement) and measured using an avalanche

photodiode. In general, data of the average size were considered suitable when the polydispersity of solutions was below 0.5. All samples were vortexed and measured 10 min after the addition of the flavonol in order to guarantee the interaction. Intensity of light scattered by a saliva solution was used as the reference.

2.5. Optimization and molecular dynamics simulations

A system representative of a peptide model (one proline-rich protein (PRP) fragment called IB9₃₇) and quercetin 3-O-glucoside molecules was built. The amino acid sequence of IB9₃₇ is SPPGKPQGPPQGGNQPPPPGKPPQGGNR. The initial structure of quercetin 3-glc was determined by the GaussView software (Gaussian Inc.). The Gaussian 09 suite of programs was used to obtain optimized geometries and electronic properties for subsequent parameterization. These calculations were performed with the restricted Hartree-Fock method and with the 6-31G(d) basis set. Atomic charges were further recalculated using the RESP algorithm (Bayly, Cieplak, Cornell, & Kollman, 1993). Geometry optimization and MD simulations were performed using GAFF (Wang, Wolf, Caldwell, Kollman, & Case, 2004) and parm99 (Cornell et al., 1995) force fields for polyphenols and peptides, respectively. Explicit solvation (TIP3P water model) was included as a rectangular box with a 20 Å distance between the box edges and any atom within each system. MD simulations were performed with an IB9₃₇ fragment and four quercetin 3-O-glucoside molecules. The flavonol molecules were randomly placed around the peptide, reproducing the sensory experimental conditions used ([flavonol] = 2.3 g/L). All complex geometries were minimized in two stages. Subsequently, an MD simulation of 100 ps was performed at constant volume and temperature and considering periodic boundaries conditions. This was followed by an MD simulation of 40 ns with the NPT ensemble (constant number of particles, pressure, and temperature), in which Langevin dynamics were used (collision frequency of 1.0 ps⁻¹) to control the temperature at 303.15 K. All simulations were carried out using the AMBER 12.0 simulations package. Bond lengths involving hydrogen atoms

were constrained using the SHAKE algorithm, and the equations of motion were integrated with a 2 fs time-step using the Verlet leap-frog algorithm (Ryckaert, Ciccotti, & Berendsen, 1977). The Particle-Mesh Ewald (PME) method (Essmann et al., 1995) was used to treat long-range interactions, and the nonbonded interactions were truncated with a 10 Å cutoff. The MD trajectories were saved every 2 ps and were analysed with the PTRAJ module of AMBER 12.0 (Case et al., 2012).

2.6. Statistical analysis

A principal component analysis (PCA) was used for data analysis. PCA was applied from the correlation matrix of original variables constituted by the sensory scores. Student's *t*-test was used to evaluate the statistical significance of the analysis. The software package SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL) was used for data processing.

2.7. Ethics statements

This study complies with the policies laid down by the Helsinki declaration and has been approved by the Bioethics Committee of the University of Salamanca. All of the panellists were informed about the aim of the research and all of them expressed their consent to participate in this study.

3. Results and discussion

3.1. Sensory analysis

Fig. 1 shows the astringency and bitterness scores for the red and white wines in absence and in presence of quercetin 3-*O*-glucoside. The results show that the addition of this flavonol clearly affects the sensory analysis of both red and white wines. The intensity of the astringency sub-qualities and bitterness underwent a significant increase when the flavonol was added, except in the case of dynamic nature, which did not show any change. Additionally, velvety texture, commonly considered as a good attribute in the sensory analysis of wines, showed a significant decrease in the case of the white wine.

In general, wines were smooth-tasting before the flavonol addition and became more astringent, rough, green, dry, bitter and persistent in its presence. The addition of the flavonol to the white wine resulted in greenness, persistence and bitterness qualities close to the red wine characteristics.

The increase in the intensity of astringency and bitterness after the addition was similar in both red and white wines. The red wine

showed a greater increase in persistence, greenness, harshness and dryness; and the white wine was clearly perceived as less ripe (velvety) when the flavonol was added. The dynamic parameter showed the lowest intensity and the least variation in both cases.

Fig. 2 shows the principal component analysis from the sensory data of the red wine with the addition of increasing concentrations of quercetin 3-*O*-glucoside. Wines were evaluated just after the addition of the flavonol (F) and three months after storage (2F). F0 corresponds to the wine in absence of the flavonol and F1 to F5 correspond to the concentrations added (0.25, 0.5, 1, 1.5 and 2 g L⁻¹, respectively). PC1 and PC2 explain the 88.9% of the variability of the samples. The control (wine without flavonol; F0) showed the most negative value in PC1 followed by the control three months after storage (2F0). This indicates a lower intensity of astringency and its sub-qualities (except velvety) for the wines in the absence of the flavonol. When quercetin was added, wine samples tended to be placed at more positive values of PC1, indicating a tendency towards increased values for variables contributing the most to the positive values in this PC1 (mainly greenness and bitterness; but also persistence, intensity, drying and roughness), and a decrease in the variable contributing to negative values of PC1, i.e., the velvety perception. A distinguishable pattern of wines after storage was shown along PC2. After storage, samples of wines moved to negative values in PC2, meaning that they became more dynamic and velvety, with a slight loss of astringency and bitterness.

The results of the sensory analysis suggest that the addition of flavonols may not be an appropriate winemaking practice considering their strong contribution to the astringency perception. Hence, the impact in the wine sensory properties needs to be carefully evaluated when extracts with significant amounts of flavonols are added for other purposes, for example, to stabilize colour or to improve the antioxidant properties. Wine colour enhancement by the addition of flavonols could be at the expense of the mouth-feel quality. Further research should be performed in order to evaluate more factors, such as the addition time, concentrations, and storage conditions.

3.2. Fluorescence quenching analysis

Fluorescence quenching is a sensitive technique that is frequently used to evaluate interactions between phenolic compounds and proteins (Dias, Perez-Gregorio, Mateus, & de Freitas, 2015; Ferrer-Gallego, Gonçalves, Rivas-Gonzalo, Escribano-Bailon, & de Freitas, 2012; Guharay, Sengupta, & Sengupta, 2001; Harbertson, Kilmister, Kelm, & Downey, 2014; Hu, Cui, & Liu, 2010; Papadopoulou, Green, & Frazier, 2005; Soares et al., 2007; Xiao et al., 2008). In this study, fluorescence quenching was used

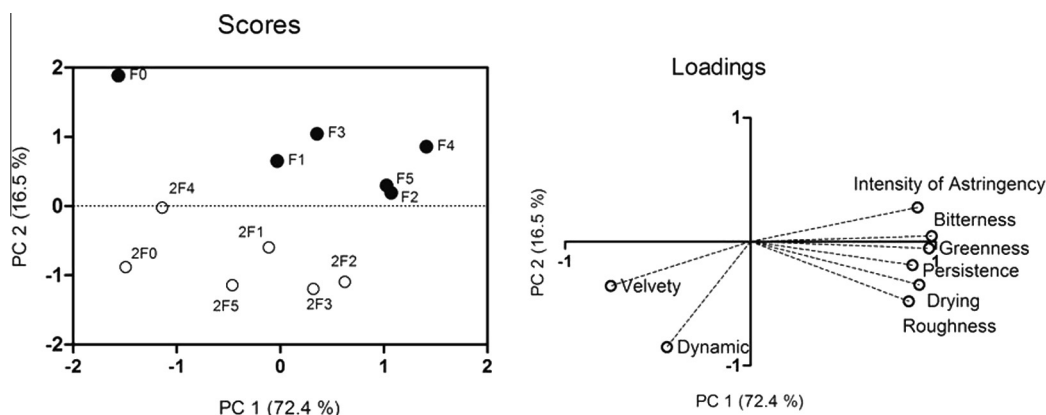


Fig. 2. Principal component analysis performed from the sensory analysis of red wine just after the addition of quercetin 3-*O*-glucoside (F) and 3 months after storage (2F). Numbers from 0 to 5 correspond to the wine at increasing concentrations of quercetin 3-*O*-glucoside: 0, 0.25, 0.5, 1, 1.5 and 2 g L⁻¹, respectively.

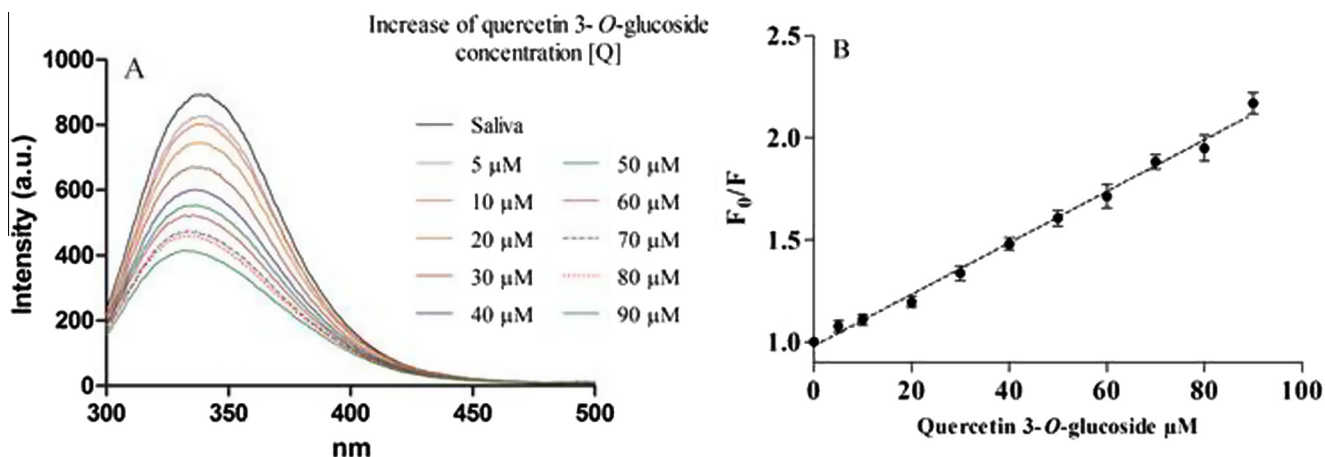


Fig. 3. Fluorescence emission spectra (at λ_{ex} 282 nm) of saliva in the presence of increasing concentrations of quercetin 3-O-glucoside (A) and the corresponding Stern-Volmer plot (B).

to evaluate the interaction between the flavonol quercetin 3-O-glucoside and human salivary proteins. Fluorescence intensity of saliva was studied in 0.1 M acetate buffer at pH 5. Fig. 3A shows the fluorescence emission spectra (at λ_{ex} 282 nm) of saliva in the absence and presence of increasing concentrations (from 5 μM to 90 μM) of quercetin 3-glucoside (quencher [Q]).

A decrease in the fluorescence intensity was observed because of quenching of emission spectra. No significant shift was observed in the maximum wavelength of the saliva emission spectra, which suggests that the structural conformation of the salivary proteins was not affected by the mechanism of flavonol interaction. At the studied concentrations, flavonol samples showed negligible fluorescence in comparison to the fluorescence of saliva.

In fluorescence quenching studies, it is important to understand of the type of interactions taking place between the quencher and the fluorophores. In this case, the Stern–Volmer plot for quercetin 3-O-glucoside and saliva is linear (Fig. 3B) indicating that only one type of quenching mechanism occurs (dynamic or static). The linearity of the plot also implies that all fluorophores of the saliva are equally accessible to the flavonol. In the case of a dynamic mechanism for fluorescence quenching, the diffusion-limited collision between the quencher and the fluorophore molecules allows energy-transfer without radiation. A static mechanism involves the formation of a complex, and in such cases, the bimolecular quenching constant (k_q) is calculated by dividing the obtained Stern–Volmer constant (K_{sv}) values by the lifetime (τ) of the protein. The maximum possible value for diffusion-limited quenching (dynamic mechanism) in water is $10^{10} \text{ M}^{-1} \text{ s}^{-1}$ (Lakowicz, 2006). Therefore, the bimolecular quenching constant (k_q) can be used to determine whether the quenching can be attributed to a static or dynamic mechanism.

For this, the apparent Stern–Volmer quenching constant was calculated from the slope of the F_0/F vs. [Q], where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. The lifetime (τ) of the saliva fluorophore was determined by time-resolved fluorescence analysis and $k_{q\text{App}}$ was then obtained.

The value of the apparent quenching constants (K_{svApp}) for the interaction between quercetin 3-O-glucoside and saliva was $12620 \pm 390 \text{ M}^{-1}$ with a 95% confidence level. To the best of our knowledge, there are no data related to the apparent Stern–Volmer constant for interactions between phenolic compounds and whole saliva. However, it should be noted that the K_{sv} for the interaction between catechin and α -amylase isolated from human saliva was $14100 \pm 3656 \text{ M}^{-1}$ at the same pH (pH 5.0) (Soares et al., 2007).

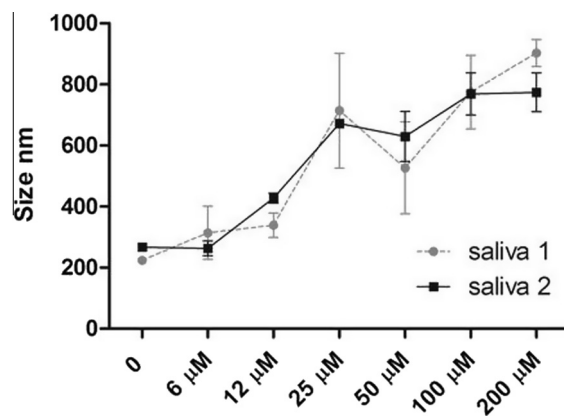


Fig. 4. Average size of soluble flavonol-saliva aggregates measured by DLS with increasing quercetin 3-O-glucoside concentration.

The obtained value for the saliva lifetime was $3.20 \pm 0.05 \text{ ns}$. As mentioned above, when the value of the bimolecular quenching constant is higher than $10^{10} \text{ M}^{-1} \text{ s}^{-1}$, it could mean that there is complex formation between the protein and quencher, corresponding to a static mechanism. The resulting $k_{q\text{App}}$ is $3.94 \times 10^{12} \text{ M}^{-1} \text{ s}^{-1}$, suggesting complex formation (static mechanism) between the flavonol and saliva.

3.3. Dynamic light scattering

The size of the soluble flavonol-saliva aggregates was measured by dynamic light scattering with increasing concentration of quercetin 3-glc. The results were obtained by independent experiments with saliva collected on two different days. Fig. 4 shows the apparent increase in particle size when different concentrations of flavonol were added to saliva solutions. The size of the molecules in saliva solution increased when increasing concentrations of flavonol were added and seemed to reach a maximum size close to 700–800 nm at 25 μM . Beyond this, the size tended to stabilize despite the increase in the flavonol concentration. Lower maximum average sizes of saliva complexes, approximately 200 nm, were found with procyanidin trimers (400 μM), pentagalloylglucose (200 μM) and commercial procyanidins by other authors (Brandão, Soares, Mateus, & de Freitas, 2014; Soares et al., 2012). DLS results suggest the formation of complexes or metastable aggregates, which is in good agreement with the results showed in the MD simulations.

3.4. Molecular dynamics simulations

Additional computational studies were carried out in order to gain further insights into the conformational behaviour of the complexes formed as a consequence of the interaction between quercetin 3-*O*-glucoside and salivary proteins. MD simulations of 40 ns were performed. It was observed that after the first 5 ns, the root-mean-square (RMSD) values stabilized and then showed small variations during the remaining time. This supports the overall stability and equilibration of the peptide structure. Fig. 5 shows the formation of stable complexes involving quercetin 3-glc molecules and some residues of the IB9₃₇ model peptide at 33 ns.

The occurrence of an assembly of two quercetin molecules was initially detected after 4.8 ns, perfectly aligned between themselves by their large planar surfaces. Further, at 33 ns, one molecule of quercetin was bound to the peptide (Fig. 5). This structural arrangement, with 25% of quercetin molecules bound to the IB9₃₇ peptide and the complex formed by the two quercetin molecules, was maintained throughout the remaining 7 ns of simulation, revealing its higher stability.

A recent study showed that binding was maintained between 25% of the catechin molecules and the IB7₁₄ peptide, throughout 20.2 ns of simulation (Ferrer-Gallego et al., 2015). It should be noted that the affinity for tannin molecules increases with the peptide chain length. However, Cala and co-workers verified that the number of binding sites found for IB9₃₇ was slightly lower than for IB7₁₄ (Cala et al., 2012). A similar behaviour has been stated for procyanidin dimers and trimers with peptides of proline-rich protein in which the cooperative linkage of molecules to the peptide was also observed (Cala et al., 2010; Canon et al., 2015). The interaction of quercetin 3-glc molecules to IB9₃₇ occurs mainly by hydrophobic contacts (direct and perpendicular π - π stacking and van der Waals interactions) between the large, accessible and planar surfaces of polyphenols, and the ring planes of several amino acids (e.g., Pro, Phe, Tyr, Trp). The small proximity between some of these planes allows the establishment of optimized π -stacking interactions that contribute to the higher stability of the global complex. With this interaction, there is a concomitant high-energy release of water molecules from the solvation shells (hydrophobic effect). Further, the binding could also be strengthened by H-bonds involving numerous hydroxyl groups from these

phenolic units and polar groups of IB9₃₇ peptide. In the complex with IB9₃₇ peptide, the quercetin molecule specifically interacts with Gln18, Gly19, Pro20 and Pro21 residues. Similar sites involving Pro and Gly residues have been reported for the binding of tannin molecules on peptides IB9₃₇ and IB7₁₄ (Simon et al., 2003).

The Solvent-Accessible Surface Area (SASA) values were determined during the MD simulation. This value evaluates the surface area of the peptide that is accessible to a solvent probe, which could indicate the extension of quercetin' binding. It was observed that the SASA value of the IB9₃₇ peptide decreases from $3843.5 \pm 230.2 \text{ \AA}^2$ (first 7 ns) to $3623.7 \pm 159.1 \text{ \AA}^2$ (last 7 ns) in the presence of the polyphenol.

Overall, all of these computational data suggest that quercetin 3-*O*-glucoside molecules have the ability to bind to IB9₃₇ peptide, which is in agreement with the experimental studies performed and may justify the astringent ability of this phenolic compound.

4. Conclusions

Sub-qualities of astringency and bitterness of quercetin 3-*O*-glucoside have been assessed in wines by sensory analysis. The results showed noticeable increase in astringency and bitterness perception when this flavonol was added to wine. Oral sensations elicited by quercetin 3-*O*-glucoside may be explained by its interaction with human salivary proteins.

The fluorescence quenching results indicate the formation of complexes (static mechanism) between flavonols and human saliva. The average size of saliva complexes increased with increasing concentrations of flavonol, reaching a maximum size of approximately 800 nm at 200 μM . MD simulations verify the ability of flavonols to bind to the model peptide IB9₃₇.

This work may also contribute to understanding the role of flavonols in the sensory perception of food and wine. The importance of sensory analysis in studies of addition of phenolic compounds has been highlighted.

The evidence of complex formation between flavonols and saliva opens potential opportunities for future research focusing on the importance of this interaction from a nutritional point of view.

Acknowledgements

The work was supported by Ministerio de Economía y Competitividad (Project ref. AGL2014-58486-C2-1-R), Fundação para a Ciência e Tecnologia (ref. IF/01355/2014 and NORTE-07-0162-FEDER-000048) UID/Multi/04378/2013 and UID/QUI/50006/2013. Raúl Ferrer-Gallego thanks Fundación Alfonso Martín Escudero for the post-doctoral fellowship and Natércia F. Brás also thanks FCT for her IF starting grant (IF/01355/2014).

References

- Alcalde-Eon, C., García-Estévez, I., Ferreras-Charro, R., Rivas-Gonzalo, J. C., Ferrer-Gallego, R., & Escribano-Bailon, M. T. (2014). Adding oenological tannin vs. overripe grapes: Effect on the phenolic composition of red wines. *Journal of Food Composition and Analysis*, 34, 99–113.
- Alexandre-Tudo, J. L., Álvarez, I., Lizama, V., García, M. J., Alexandre, J. L., & Du Toit, W. J. (2013). Impact of caffeic acid addition on phenolic composition of tempranillo wines from different winemaking techniques. *Journal of Agricultural and Food Chemistry*, 61, 11900–11912.
- Bayly, C. I., Cieplak, P., Cornell, W. D., & Kollman, P. A. (1993). A Well-Behaved electrostatic potential based method using charge restraints for deriving atomic charges – The Resp model. *Journal of Physical Chemistry*, 97, 10269–10280.
- Brandão, E., Soares, S., Mateus, N., & de Freitas, V. (2014). In vivo interactions between procyanidins and human saliva proteins: Effect of repeated exposures to procyanidins solution. *Journal of Agricultural and Food Chemistry*, 62, 9562–9568.
- Cala, O., Dufourc, E. J., Fouquet, E., Manigand, C., Laguerre, M., & Pianet, I. (2012). The Colloidal state of tannins impacts the nature of their interaction with proteins:

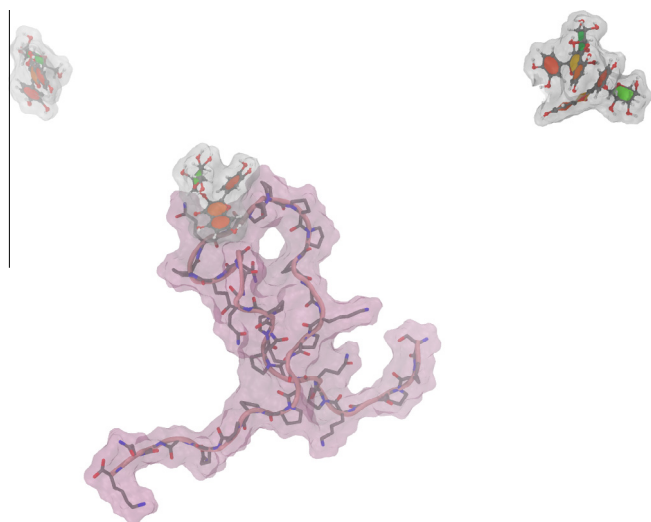


Fig. 5. Representation of geometries of IB9₃₇: (quercetin)₄ system extracted from MD simulations (33 ns). The peptide is depicted as a surface, balls and sticks and a cartoon and is coloured pink, whilst the quercetin 3-glc molecules are depicted with balls and sticks and are coloured by atom type.

- The case of salivary proline-rich protein/procyanidins binding. *Langmuir*, 28, 17410–17418.
- Cala, O., Pinaud, N., Simon, C., Fouquet, E., Laguerre, M., Dufourc, E. J., & Pianet, I. (2010). NMR and molecular modeling of wine tannins binding to saliva proteins: Revisiting astringency from molecular and colloidal prospects. *Food Research International*, 42, 4281–4290.
- Canon, F., Ployon, S., Mazauric, J. P., Sarni-Manchado, P., Réfrégiers, M., Giuliani, A., & Cheyrier, V. (2015). Binding site of different tannins on a human salivary proline-rich protein evidenced by dissociative photoionization tandem mass spectrometry. *Tetrahedron*, 71, 3039–3044.
- Carpenter, G. H. (2013). Do transient receptor protein (TRP) channels play a role in oral astringency? *Journal of Texture Studies*, 44, 334–337.
- Case, D. A., Darden, T. A., Cheatham, T. E., III, Simmerling, C. L., Wang, J., Duke, R. E., Luo, R., et al. (2012). *AMBER 12*. San Francisco: University of California.
- Cliff, M. A., Stanich, K., Edwards, J. E., & Saucier, C. T. (2012). Adding grape seed extract to wine affects astringency and other sensory attributes. *Journal of Food Quality*, 35, 263–271.
- Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Ferguson, D. M., Spellmeyer, D. C., et al. (1995). A 2nd generation force-field for the simulation of proteins, nucleic-acids, and organic-molecules. *Journal of the American Chemical Society*, 117, 5179–5197.
- Dias, R., Perez-Gregorio, R., Mateus, N., & de Freitas, V. (2015). The interaction between tannins and gliadin derived peptides in a celiac disease perspective. *RSC Advances*, 5, 32151–32158.
- Escribano-Bailón, M. T., & Santos-Buelga, C. (2012). Anthocyanin copigmentation – Evaluation, mechanisms and implications for the colour of red wines. *Current Organic Chemistry*, 16, 715–723.
- Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., & Pedersen, L. G. (1995). A smooth particle mesh Ewald method. *Journal of Chemical Physics*, 103, 8577–8593.
- Ferrer-Gallego, R., Gonçalves, R., Rivas-Gonzalo, J. C., Escribano-Bailón, M. T., & de Freitas, V. (2012). Interaction of phenolic compounds with bovine serum albumin (BSA) and alpha-amylase and their relationship to astringency perception. *Food Chemistry*, 135, 651–658.
- Ferrer-Gallego, R., Hernandez-Hierro, J. M., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2014). Sensory evaluation of bitterness and astringency sub-qualities of wine phenolic compounds: Synergistic effect and modulation by aromas. *Food Research International*, 62, 1100–1107.
- Ferrer-Gallego, R., Quijada-Morín, N., Brás, N. F., Gomes, P., de Freitas, V., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2015a). Characterization of sensory properties of flavanols—A molecular dynamic approach. *Chemical Senses*, 6, 381–390.
- Ferrer-Gallego, R., Soares, S., Mateus, N., Rivas-Gonzalo, J. C., Escribano-Bailón, M. T., & de Freitas, V. (2015b). New anthocyanin-human salivary protein complexes. *Langmuir*, 31, 8392–8401.
- Gaussian Inc., C. O. P., Bldg. 6, Pittsburgh, PA 15106, USA.
- Gawel, R., Oberholster, A., & Francis, L. (2000). A 'Mouth-feel Wheel': Terminology for communicating the mouth-feel characteristics of red wine. *Australian Journal of Grape and Wine Research*, 6, 203–207.
- Gibbins, H. L., & Carpenter, G. H. (2013). Alternative mechanisms of astringency – What is the role of saliva? *Journal of Texture Studies*, 44, 364–375.
- Gomez-Míguez, M., González-Manzano, S., Escribano-Bailón, M. T., Heredia, F. J., & Santos-Buelga, C. (2006). Influence of different phenolic copigments on the color of malvidin 3-glucoside. *Journal of Agricultural and Food Chemistry*, 54, 5422–5429.
- Gordillo, B., Jesus Cejudo-Bastante, M., Rodriguez-Pulido, F. J., Jose Jara-Palacios, M., Ramirez-Perez, P., Lourdes Gonzalez-Miret, M., & Heredia, F. J. (2014). Impact of adding white pomace to red grapes on the phenolic composition and color stability of syrah wines from a warm climate. *Journal of Agricultural and Food Chemistry*, 62, 2663–2671.
- Green, B. G., Dalton, P., Cowart, B., Shaffer, G., Rankin, K., & Higgins, J. (1996). Evaluating the 'labeled magnitude scale' for measuring sensations of taste and smell. *Chemical Senses*, 21, 323–334.
- Guharay, J., Sengupta, B., & Sengupta, P. K. (2001). Protein-flavonol interaction: Fluorescence spectroscopic study. *Proteins-Structure Function and Bioinformatics*, 43, 75–81.
- Harbertson, J. F., Kilmister, R. L., Kelm, M. A., & Downey, M. O. (2014). Impact of condensed tannin size as individual and mixed polymers on bovine serum albumin precipitation. *Food Chemistry*, 160, 16–21.
- Harbertson, J. F., Parpinello, G. P., Heymann, H., & Downey, M. O. (2012). Impact of exogenous tannin additions on wine chemistry and wine sensory character. *Food Chemistry*, 131, 999–1008.
- Hu, X., Cui, S., & Liu, J. Q. (2010). Fluorescence studies of interaction between flavonol p-coumaroylglucoside tiliroside and bovine serum albumin. *Spectrochimica Acta Part A-Molecular and Biomolecular Spectroscopy*, 77, 548–553.
- Hufnagel, J. C., & Hofmann, T. (2008). Quantitative reconstruction of the nonvolatile sensometabolome of a red wine. *Journal of Agricultural and Food Chemistry*, 56, 9190–9199.
- Jara-Palacios, M. J., Gordillo, B., González-Miret, M. L., Hernanz, D., Escudero-Gilete, M. L., & Heredia, F. J. (2014). Comparative study of the enological potential of different winemaking byproducts: Implications in the antioxidant activity and color expression of red wine anthocyanins in a model solution. *Journal of Agricultural and Food Chemistry*, 62, 6975–6983.
- Laaksonena, O., Mäkilä, L., Tahvononb, R., Kallio, H., & Yang, B. (2013). Sensory quality and compositional characteristics of blackcurrant juices produced by different processes. *Food Chemistry*, 138, 2421–242.
- Lakowicz, J. (2006). *Principles of fluorescence spectroscopy*. .
- Makris, D. P., Kallithraka, S., & Kefalas, P. (2006). Flavonols in grapes, grape products and wines: Burden, profile and influential parameters. *Journal of Food Composition and Analysis*, 19, 396–404.
- Manch, C., Scalbert, A., Morand, C., Révész, C., & Jiménez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition*, 79, 727–747.
- Mangas, J. J., Rodríguez, R., Suárez, B., Picinelli, A., & Dapena, E. (1999). Study of the phenolic profile of cider apple cultivars at maturity by multivariate techniques. *Journal of Agricultural and Food Chemistry*, 47, 4046–4052.
- Mattivi, F., Guzzon, R., Vrhovsek, U., Stefanini, M., & Velasco, R. (2006). Metabolite profiling of grape: Flavonols and anthocyanins. *Journal of Agricultural and Food Chemistry*, 54, 7692–7702.
- Messana, I., Inzitari, R., Fanali, C., Cabras, T., & Castagnola, M. (2008). Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? *Journal of Separation Science*, 31, 1948–1963.
- Papadopoulou, A., Green, R. J., & Frazier, R. A. (2005). Interaction of flavonoids with bovine serum albumin: A fluorescence quenching study. *Journal of Agricultural and Food Chemistry*, 53, 158–163.
- Ramírez-Ambrosi, M., López-Márquez, D., Abad-García, B., Dapena, E., Berrueta, L., & Gallo, B. (2015). Comparative study of phenolic profile of fruit and juice samples of a progeny of 'Meana' × 'Florina' from an Asturian cider apple breeding program. *European Food Research and Technology*, 241, 769–784.
- Ryckaert, J. P., Ciccotti, G., & Berendsen, H. J. C. (1977). Numerical-integration of cartesian equations of motion of a system with constraints – Molecular-dynamics of N-alkanes. *Journal of Computational Physics*, 23, 327–341.
- Scharbert, S., & Hofmann, T. (2005). Molecular definition of black tea taste by means of quantitative studies, taste reconstitution, and omission experiments. *Journal of Agricultural and Food Chemistry*, 53, 5377–5384.
- Schobel, N., Radtke, D., Kyereme, J., Wollmann, N., Cichy, A., Obst, K., Kallweit, K., et al. (2014). Astringency is a trigeminal sensation that involves the activation of g protein-coupled signaling by phenolic compounds. *Chemical Senses*, 39, 471–487.
- Simon, C., Barathieu, K., Laguerre, M., Schmitter, J. M., Fouquet, E., Pianet, I., & Dufourc, E. J. (2003). Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry*, 42, 10385–10395.
- Soares, S., Mateus, N., & De Freitas, V. (2007). Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary alpha-amylase (HSA) by fluorescence quenching. *Journal of Agricultural and Food Chemistry*, 55, 6726–6735.
- Soares, S., Mateus, N., & de Freitas, V. (2012). Interaction of different classes of salivary proteins with food tannins. *Food Research International*, 49, 807–813.
- Troszyńska, A., Estrella, I., Lamparski, G., Hernández, T., Amarowicz, R., & Pegg, R. B. (2011). Relationship between the sensory quality of lentil (*Lens culinaris*) sprouts and their phenolic constituents. *Food Research International*, 44, 3195–3201.
- Wang, J. M., Wolf, R. M., Caldwell, J. W., Kollman, P. A., & Case, D. A. (2004). Development and testing of a general amber force field. *Journal of Computational Chemistry*, 25, 1157–1174.
- Xiao, J., Suzuki, M., Jiang, X., Chen, X., Yamamoto, K., Ren, F., & Xu, M. (2008). Influence of B-ring hydroxylation on interactions of flavonols with bovine serum albumin. *Journal of Agricultural and Food Chemistry*, 56, 2350–2356.