Food Chemistry 228 (2017) 574–581

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

First evidences of interaction between pyranoanthocyanins and salivary proline-rich proteins

Ignacio García-Estévez, Luís Cruz, Joana Oliveira, Nuno Mateus, Victor de Freitas, Susana Soares *

REQUIMTE/LAQV, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal

ARTICLE INFO

Article history:
Received 14 November 2016
Received in revised form 19 January 2017
Accepted 7 February 2017
Available online 9 February 2017

Keywords:
Pyranoanthocyanins
Acidic proline-rich proteins
Interaction
STD-NMR
MALDI-mass spectrometry

ABSTRACT

The contribution of other classes of polyphenol compounds besides tannins to the overall perception of astringency is still poorly understood. So, this work aimed to study the interaction between a family of salivary proline-rich proteins (aPRPs) and representative pyranoanthocyanins in red wines [pyranomalvidin-3-glucoside (vitisin B), pyranomalvidin-3-glucoside-catechol, and pyranomalvidin-3-glucoside-epicatechin] using saturation transfer difference-NMR and MALDI-TOF.

For vitisin B K_D was of 1.74 mM; for pyranomalvidin-3-glucoside-catechol was 1.17 mM and for pyranomalvidin-3-glucoside-epicatechin it was 0.87 mM. The presence of the flavanol structural unit in the pyranoanthocyanins led to an increase in their interaction with aPRPs. Further, it is also interesting that the values obtained were in the range of K_D obtained previously reported for the interaction between the human saliva proline-rich peptides ($IB7_{14}$ and $IB9_{37}$) and procyanidins. Overall, the results obtained suggest that, along with tannins, other polyphenols present in red wine, namely pyranoanthocyanins, could actively contribute to red wine global astringency.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

Polyphenol compounds are synthesized by plants and are present in vegetal foodstuffs and beverages. One of the most well-known classes of polyphenols is anthocyanins, which are the pigments responsible for the colour of plants, fruits and derived products, such as red fruits juices and red wines. These compounds confer a great diversity of colours from orange and red, to purple and blue.

In nature, anthocyanins occur as glycosides of the 2-phenylbenzopyrylium cation (flavylium cation) and are usually based on six major anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. These compounds differ in the methoxyl and hydroxyl substitution pattern of the aromatic B ring (Brouillard, 1982).

Anthocyanins are the major pigments found in red wine and are responsible for their colour. During storage, red wine colour changes and evolves towards more brown-orange hues. These compounds can react with other anthocyanins but also with other red wine compounds, such as flavan-3-ols, forming anthocyanin derived co-pigmentation products. These pigments are usually more stable, with different physical-chemical features (Alcalde-Eon, Escribano-Bailón, Santos-Buelga, & Rivas-Gonzalo, 2007; Boulton, 2001; De Freitas & Mateus, 2010; Dueñas, Salas, Cheynier, Dangles, & Fulcrand, 2006; Escribano-Bailón, Álvarez-García, Rivas-Gonzalo, Heredia, & Santos-Buelga, 2001). Different classes of these pigments have been identified in the last decade, such as vitisins (Bakker & Timberlake, 1997; Bakker et al., 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998), hydroxyphenyl-pyranoanthocyanins (Cameira dos Santos, Brillouet, Cheynier, & Moutounet, 1996; Fulcrand, Cameira dos Santos, Sarni-Manchado, Cheynier, & Favre-Bonvin, 1996), methylpyranoanthocyanins (He, Santos-Buelga, Silva, Mateus, & De Freitas, 2006; Lu, Sun, & Foo, 2000), pyranoanthocyanin-flavanols (Asenstorfer, Hayasaka, & Jones, 2001; Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Mateus, Silva, Santos-Buelga, Rivas-Gonzalo, & De Freitas, 2002), portisins (Mateus, Oliveira, Haettich-Motta, & De Freitas, 2004; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003; Oliveira, De Freitas, Silva, & Mateus, 2007) and more recently a new family of pyranoanthocyanin dimers (Oliveira et al., 2010). Research has indicated that individual pyranoanthocyanins are present at ppm levels (Mateus & de Freitas, 2001; Márquez, Dueñas, Serratosa, & Merida, 2012; Morata, Gómez-Cordovés, Colomo, & Suárez, 2003). Pyranoanthocyanin formation depends on several factors, such as concentration of anthocyanins, the presence of other

* Corresponding author.
E-mail addresses: ignacio.estevez@fc.up.pt (I. García-Estévez), luis.cruz@fc.up.pt (L. Cruz), jsoliveira@fc.up.pt (J. Oliveira), nbmateus@fc.up.pt (N. Mateus), vfreitas@fc.up.pt (V. de Freitas), susana.soares@fc.up.pt (S. Soares).

http://dx.doi.org/10.1016/j.foodchem.2017.02.030
0308-8146/© 2017 Elsevier Ltd. All rights reserved.

substrates involved in the reaction and time because of long kinetics. Winemaking processes, such as using maceration enzymes (He et al., 2010) and selection of yeast strains (Morata et al., 2003), can increase the concentration of pyranoanthocyanin precursors, i.e. acetaldehyde or pyruvic acid, which, in turn, will lead to an increase in the concentration of pyranoanthocyanins in wine. Besides changing colour, these new pigments are thought to also influence other sensory properties, such as flavour. For red wine flavour, the most important sensory attributes include bitterness and astringency sensation. Astringency is described as dryness, puckering and tightening sensations perceived in the oral cavity during the ingestion of foodstuffs rich in polyphenols, and is highly related to red wine quality. Several mechanisms have been demonstrated to explain astringency onset. The most widely accepted mechanism, supported by the literature, is thought to be due to the interaction and precipitation of salivary proteins, in particular proline-rich proteins (PRPs), by tannins (Charlton et al., 2002; De Freitas & Mateus, 2012; Haslam & Lilley, 1988).

PRPs are one of the dominant classes of salivary proteins and, as their name suggests, they are characterized by a high content in proline residues (25–42%). This family is usually divided in basic (bPRPs), acidic (aPRPs) and glycosylated (gPRPs) classes. The differences between these classes depend on their charge and the presence or absence of carbohydrate. aPRPs are characterized by a highly acidic *N*-terminal region, rich in aspartic and glutamic acid residues, whereas its C-terminal region is similar to bPRPs. They also present some phosphate groups throughout their structure (Ser 24, 33 and 38). However, gPRPs are bPRPs that present carbohydrates in their structure.

aPRPs are some of the most abundant PRPs in human saliva, and have been identified as one of the most reactive SPs towards tannins related to astringency perception (Brandão, Soares, Mateus, & de Freitas, 2014; Soares et al., 2011). However, besides tannins other polyphenols in wines, like anthocyanins, have been shown to interact with salivary aPRPs, pointing out that this family of compounds should also be taken into account to understand astringency perception (Ferrer-Gallego et al., 2015).

However, no studies about the possible interaction of PRP with anthocyanin-derived pigments, such as pyranoanthocyanins, which appear (and are more stable) during red wine aging, have been performed. Moreover, the presence of phenolic moeities in some pyranoanthocyanin structures, such as phenol, catechol or even flavanols structures, would have an important effect on the interactions with salivary proteins. The understanding of these aspects will enable growers and winemakers to have more control over the characteristics of their wines.

So, the present work aims to study the interaction between a family of salivary proteins (aPRPs) and representative pyranoanthocyanins in wines (Mateus & de Freitas, 2001; Morata et al., 2003), from the simplest one, the pyranomalvidin-3-glucoside (1) to more complex structures, such as pyranomalvidin-3-glucoside-catechol (2), and pyranomalvidin-3-glucoside-epicatechin (3) (Fig. 1).

2. Material and methods

2.1. Reagents

All reagents used were of analytical grade.

2.2. Isolation and identification of salivary proteins

Saliva was isolated and treated as reported previously (Soares et al., 2011). Large volumes of saliva from several volunteers were mixed with 10% trifluoroacetic acid (TFA, final concentration = 0.1%), to precipitate several high molecular weight SP (such as α-amylases, mucins, carbonic anhydrase, and lactoferrin) that were not significant for our study, and to preserve sample protein composition by partial inhibition of intrinsic protease activity. This saliva is rich in histatins, PRPs, statherins and cystatins proteins. After mixing, samples were centrifuged (8000*g*, 5 min) and the supernatant (acidic saliva) was used to isolate the different families of salivary proteins.

This acidic saliva was dialyzed for 24 h (cellulose membrane, MWCT 3.5 kDa) against water at 4 °C with stirring. Water was replaced several times. Then, saliva was centrifuged and the supernatant was freeze-dried. The resulting powdered saliva was solubilized in the minimal possible volume of water and filtered. The resulting solution was separated into the different families of SP by semi-preparative HPLC with the following conditions: HPLC equipped with reversed-phase C8 column (150 × 2.1 mm), solvents were: 0.2% aqueous TFA (A) and 0.2% TFA in ACN/water 80/20 (v/v) (B). The gradient applied was linear from 10 to 40% of B in 60 min, at a flow rate of 0.30 ml/min. Salivary proteins detection was carried out at 214 nm.

The fractions of salivary proteins (SP) were freeze-dried and the major families of salivary proteins present in each fraction were identified by ESI-MS, by flow injection analysis into an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0. The capillary voltage of the electrospray ionization (ESI) was set to 3100 V. The capillary temperature was 275 °C. The sheath gas

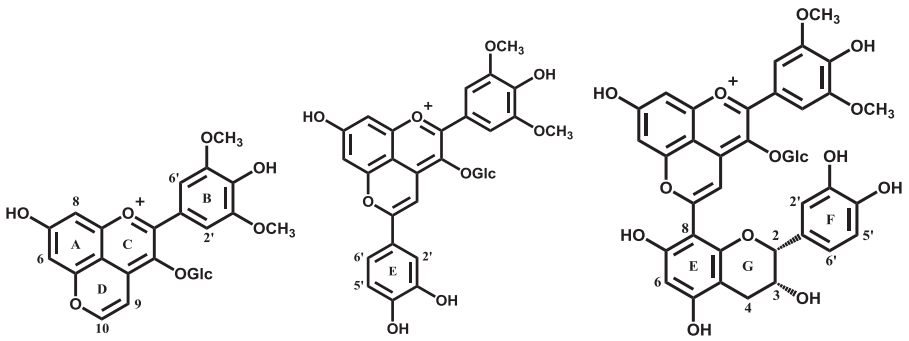


Fig. 1. Chemical structures of the pyranoanthocyanin pigments (1–3) studied. Pymv3glc: pyranomalvidin-3-*O*-glucose.

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 into a methanol/acetonitrile/TFA 0.01% (5:5:90 v/v) mixture 1:10 prior to analysis. Because proteins acquire different charge states in the interface, after the mass spectrometry analysis, spectra were subjected to a deconvolution process using the charge ratio analysis method by MagTran 1.03 software.

The study was conducted according to the Declaration of Helsinki and was submitted to the local Ethics Committee.

2.3. Hemisynthesis of pyranomalvidin-3- glucoside (Pymv3glc) derivatives

The hemisynthesis of the pymv3glc-type pigments followed the procedures described elsewhere (Cruz et al., 2008; Hakansson, Pardon, Hayasaka, de Sa, & Herderich, 2003; Oliveira, De Freitas, & Mateus, 2009), Briefly, solutions containing malvidin 3-glucoside (mv3glc, 1.4 mM) were incubated with 8-vinyl-(-)-epicatechin, 4-vinylcatechol (2–3 eq.) and vinyloxy-trimethylsilane (220 eq.) in 5–12% ethanol/water at pH 3.2 (adjusted with dilute HCl or NaOH) at room temperature. The formation of the respective pigments was monitored by high-performance liquid chromatography (HPLC) with diode array detection (DAD) at 500 nm. When their formation reached the maximum, the reactions were stopped and the purification performed.

2.4. Pigments purification and characterization

The ethanol in each solution was eliminated by evaporation, and liquid-liquid extraction with ethyl acetate was performed in order to remove the catechins and other organic impurities. The aqueous phases containing the pyranoanthocyanin derivatives were applied onto a TSK Toyopearl gel HW-40 (S) column (250 mm × 16 mm i.d.) and the pigments were eluted with 40–50% aqueous methanol acidified with 2% HCl at flow rate at 0.8 ml/min. The fractions collection was made upon visual detection of the coloured bands. The methanol was removed under vacuum, protected from light and at room temperature. The pyranoanthocyanin pigments were freeze-dried and then stored at −18 °C until use. When necessary, preparative HPLC was used in order to obtain pure pigments. The compounds were further characterized by LC-DAD/ESI-MS (Fig. 1) and their structures and purity were assessed by NMR analyses (Hakansson et al., 2003; Mateus et al., 2002; Mateus et al., 2003).

2.5. HPLC conditions

The samples were analyzed by HPLC (Merck-Hitachi L-7100) on a 150 mm × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany) at 25 °C; detection was carried out at 500 nm using a diode array detector (Merck-Hitachi L-7450A). Solvents were (A) water/formic acid 9:1 (v:v) and (B) acetonitrile, with the following gradient: 10–35% B over 50 min at a flow rate of 0.5 ml/min. The sample injection volume was 20 µl. The chromatographic column was washed with 100% B during 10 min and then stabilized with the initial conditions during another 10 min. The preparative HPLC system (Elite LaChrom) was composed of a L-2130 quaternary pump, a manual injector with loop of 250 µl and a L-2420 UV–vis detector. The stationary phase was composed of a reversed-phase C18 column (Merck, Darmstadt, Germany) (150 mm × 4.6 mm i.d., 5 µm pore size) thermostatted at 25 °C and the eluents were (A) water/formic acid 9:1 (v:v) and (B) methanol, with the following gradient: 20–100% B over 60 min at a flow rate of 1 ml/min. Detection wavelength was set to 500 nm.

2.6. LC-DAD/ESI-MS analysis

LC-DAD/ESI-MS analysis was done on a Finnigan Surveyor series liquid chromatograph, equipped with a Thermo Finnigan (Hypersil Gold) reversed-phase column (150 mm × 4.6 mm, 5 µm, C18) at 25 °C. The samples were analyzed using the same solvents, gradients, injection volume and flow rate referred above for the HPLC analysis. Double-online detection was done by a photodiode spectrophotometer and mass spectrometry. The mass detector was a Finnigan LCQ DECA XP MAX (Finnigan Corp., San Jose, Calif., USA) quadrupole ion trap equipped with an atmospheric pressure ionization (API) source, using an electrospray ionization (ESI) interface. The vaporizer and the capillary voltages were 5 kV and 4 V, respectively. The capillary temperature was set at 325 °C. Nitrogen was used both as sheath and auxiliary gas at flow rates of 90 and 25, respectively (in arbitrary units). Spectra were recorded in positive ion mode between m/z 250 and 1500.

2.7. NMR

^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were recorded in CD_3OD/ TFA (98:2) on a Bruker-Avance 500 spectrometer at 303 K and with TMS as an internal standard (chemical shifts (σ) in parts per million, coupling constants (J) in hertz). ^{1}H chemical shifts were assigned using 2D NMR (COSY) experiment while ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC) (Bax & Subramanian, 1986; Bax & Summers, 1986).

2.8. Saturation transference difference (STD)-NMR

Protein samples containing 3 µM of aPRPs were prepared in D_2O and the pigments were progressively added to the protein samples in the 0.12 to 3.7 mM ranges. To maintain the protein concentration constant throughout the experiments, the pigments were lyophilized and added as a powder allowing for the same 5 mm NMR tube to be used for all the experiments for each pigment experiment.

STD-NMR experiments were recorded on a Bruker Avance III 600 HD spectrometer, operating at 600.13 MHz, equipped with a 5 mm PATXI 1H/D-13C/15N 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 peptide 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 Gauss 1.1000 pulses for selective saturation (50 ms), with a total saturation time of 2.5 s. The number of scans (16), receptor gain value (2030), and relaxation delay (3.5 s) were kept constant. To subtract the unprocessed on and off-resonance spectra, and to baseline correct the resulting difference spectrum as well as to integrate the areas, TopSpin 2.1 software from Bruker was used.

The dissociation constants (K_D) were determined using the following equations:

$$A_{STD} = \frac{I_0 - I_{Sat}}{I_0} \times L/P\ molar\ ratio = \frac{I_{STD}}{I_0} \times L/P\ molar\ ratio \quad (1)$$

$$A_{STD} = \frac{\propto_{STD} \times [L]}{K_D + [L]} \quad (2)$$

where A_{STD} is the amplification factor, [L] is the ligand concentration, P is the protein, $\propto_{STD}$ is the maximum amplification and I_0

and I_{sat} are the off-ressonance and on-ressonance intensities, respectively.

2.9. MALDI-TOF analysis

The complexes resulting from the interaction between aPRPs and anthocyanin-derived pigments and also control conditions (aPRPs and each pigment alone) 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. Aqueous solutions of pigments adjusted at pH 3 were incubated with aPRPs in a molar ratio 20:1 pigment:protein for 10 min. Then, samples were mixed (1:1) with a matrix solution (3 mg/ml) of super-DHB matrix (sDHB: 9:1 mixture of DHB (2,5-dihydroxybenzoic acid) and 2-hydroxy-5-methoxybenzoic acid) prepared in 0.1% aqueous TFA. Aliquots of samples (2 μl) were spotted onto the MALDI sample target plate, and spectra were obtained in the mass range between 10,000 and 200,000 Da with ca. 2000 laser shots.

3. Results and discussion

3.1. General

Previous studies on astringency comprehension have identified aPRPs as one of the most reactive salivary proteins towards tannins. As previously discussed, although anthocyanins are one of the major classes of polyphenols present in red wine, there is a total lack of information about their possible interaction with salivary proteins. The first evidence that anthocyanins could interact with salivary proteins (aPRPs) appeared from Ferrer-Gallego and co-workers (Ferrer-Gallego et al., 2015). However, anthocyanins

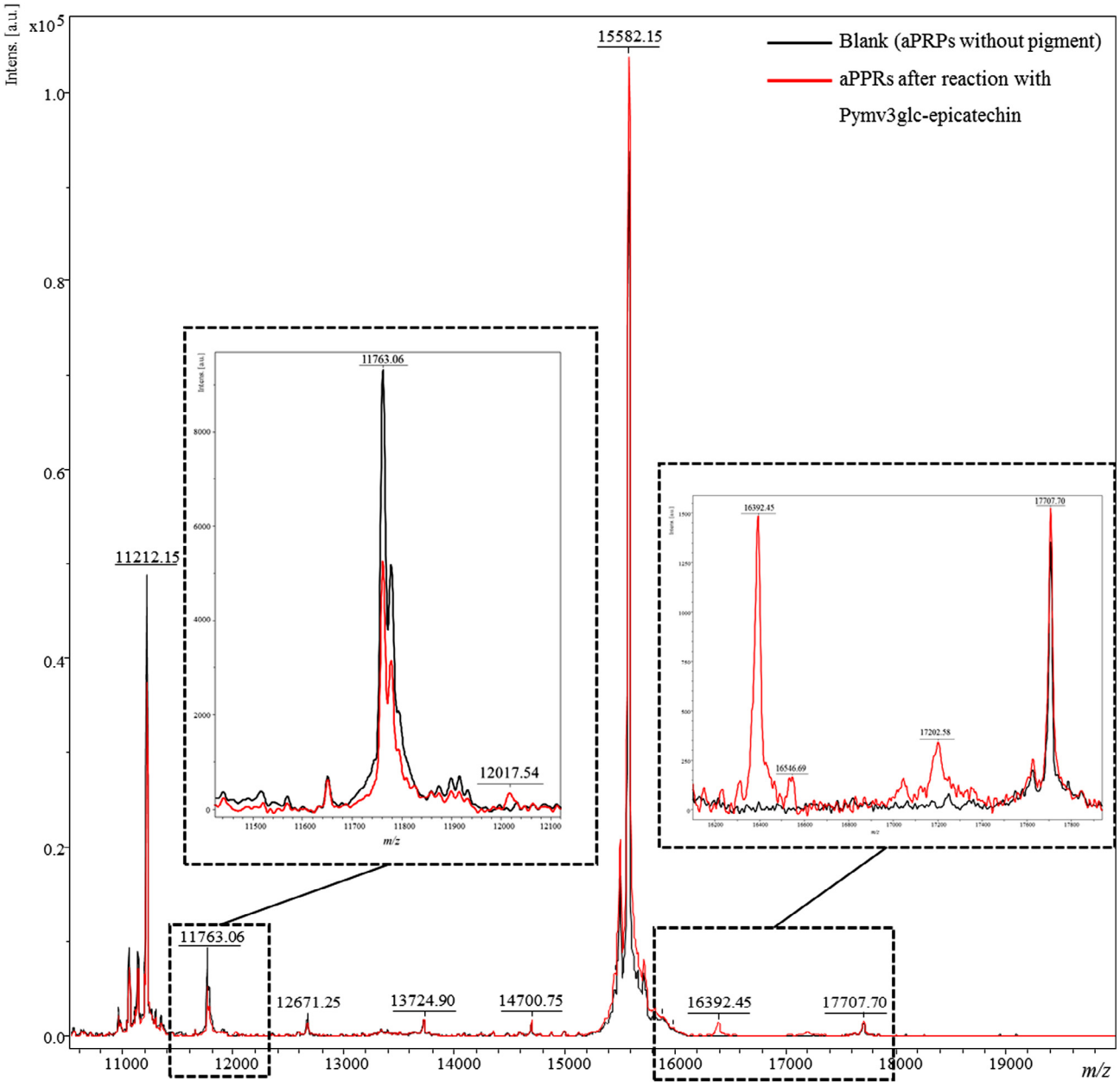


Fig. 2. MALDI-TOF mass spectrum obtained for aPRP fraction analysis (blank) and resulting of the analysis of the mixture aPRPs + Pymv3glc-epicatechin (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

participate in several reactions during wine ageing giving rise to other derived pigments. So, this work aimed to provide new molecular insights about the interaction between a salivary protein (aPRPs) and three representative pyranoanthocyanins: the simplest structure, [pyranomalvidin-3-glucoside (Vitisin B)] and two pyranoanthocyanin derivatives, pyranomalvidin-3-glucoside-epicathecin and pyranomalvidin-3-glucoside-catechol], all three are reported to be present in red wine (Rentzsch, Schwarz, & Winterhalter, 2007). This interaction was studied by MALDI-TOF in order to have molecular information about the complexes formed, and also by STD-NMR to determine the interaction dissociation constants.

3.2. Study of pyranoanthocyanins – aPRPs interaction by MALDI-TOF

In order to identify the aPRPs present, the acidic fraction isolated from human saliva in non-stimulated conditions was initially analyzed by MALDI-TOF. The human salivary protein HPLC profile has already been well characterized regarding their elution. Two major aPRPs were identified, 80% of PRP1 di-phosphorylated (MW 15582) and 20% PRP3 di-phosphorylated (MW 11212) (Fig. 2).

In order to obtain useful insights on the interaction between the pyranoanthocyanin pigments and aPRPs, the studied pyranoanthocyanins were incubated, each one in a single experiment, with aPRPs and then analyzed by MALDI-TOF-MS to identify the possible formation of aggregates. MALDI-TOF analysis without use of the matrix (i.e. LDI-TOF-MS) was also tested, as well as the use of different MALDI matrices in different solvents, i.e. 2,5-dihydroxybenzoic acid (DHB) and super-DHB (sDHB) both in 0.1% aqueous TFA or in TFA30 solvent (0.1% acid TFA in H_2O:Acetonitrile 70:30) and 2′,4′,-dihydroxyacetophenone (DHAP) both in water or in acetonitrile. LDI-TOF-MS analysis allowed detecting only the pigment and no signal corresponding to proteins could be detected, thus pointing out that a matrix is needed to analyze aPRPs. Regarding the different matrices and different solvents, only DHB and super-DHB provided satisfactory results, with the latter allowing higher intensities of the peaks. However, the use of TFA30 as solvent did not allow the detection of any protein-pigment aggregates, whereas analysis using aqueous matrix allowed detecting new peaks that could correspond to possible protein-pigment aggregates. Thus, the use of organic solvents in MALDI matrixes seems to affect the interaction of protein-pigments, and consequently MALDI analyses were done using super-DHB in 0.1% aqueous TFA.

Moreover, to study these interactions, different concentrations of pyranomalvidin-3-glucoside (Vitisin B), pyranomalvidin-3-gluco side-catechol (Pymg3glc-catechol) and pyranomalvidin-3-gluco side-epicatechin (Pymv3glc-epicatechin) were mixed with aPRPs in order to get pigment:protein ratios between 15:1 and 1:50, and then analyzed by MALDI-TOF-MS. Finally, a pigment:protein ratio 1:20 was selected since it allowed the detection of a higher number of aggregates. Fig. 2 shows, as an example, the MALDI-TOF saliva mass spectrum after the incubation with Pymv3glc-epicatechin. From this figure it is possible to observe the appearance of new peaks in the mass spectrum comparatively to that obtained from the analysis of aPRPs solution at the same

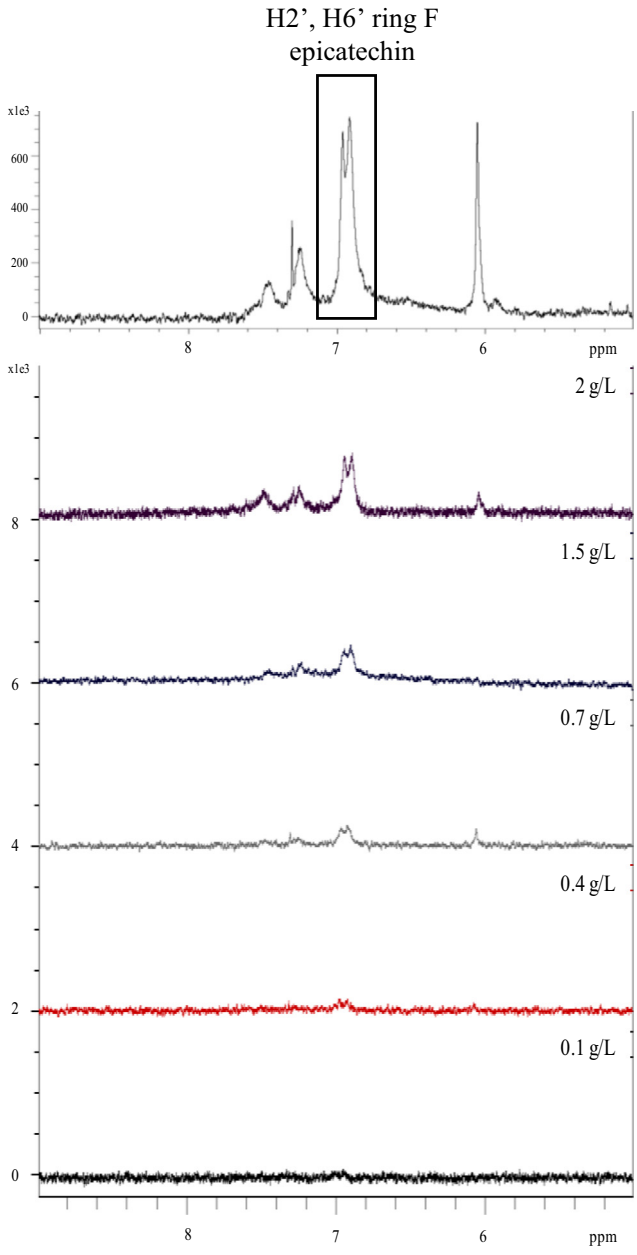


Fig. 3. Proton spectrum of Pymv3glc-epicatechin (up) and STD spectra at different concentrations (0.1–2.0 g/l) (bottom) showing the 9.0–5.0 ppm region where most protons resonate. Spectrum was recorded at 600 MHz and 300 K in deuterium oxide (D_2O).

Table 1
Putative identification of new aggregates between pyranoanthocyanins and the acidic Proline-Rich Proteins (aPRPs: PRP1 and PRP3) by MALDI-TOF.

Pymv3glc-epicatechin		Pymv3glc-catechol		Vitisin B	
$[M]^+$, m/z	Putative identification	m/z	Putative identification	m/z	Putative identification
12017	PRP3 + P	16209	PRP1 + P	16099	PRP1 + P
16392	PRP1 + P	16531	PRP1 + P + sDHB		
16546	PRP1 + P + DHB	17156	PRP1 + P + P + sDHB		
17202	PRP1 + 2P				

P: pigment. DHB: 2,5-dihydroxybenzoic acid: sDHB: DHB+2-hydroxy-5-methoybynzoic acid.

concentration. These new masses detected were studied trying to assign their possible identity. Table 1 shows the new peaks that were identified as possible protein-pigment aggregates for the three pigments assayed. The observed interaction between aPRPs and Pymv3glc-epicatechin led to the formation of a higher number of aggregates, whereas in the case of vitisin B only one aggregate could be detected. The minor aPRP, PRP3, only interacts with Pymv3glc-epicatechin leading to a dimer protein-pigment adduct, whereas the major aPRP, PRP1, interacts with the three pyranoanthocyanins assayed, although in a different way. This aPRP leads to 2 different aggregates, (with a 1:1 and a 1:2 protein:pigment stoichiometry, respectively), when it interacts with Pymv3glc-epicatechin or with Pymv3glc-catechol, only one aggregate (a dimer protein:pigment) was detected in the case of vitisin B. This can point out a higher affinity between Pymv3glc-epicatechin and aPRPs, which may be related to the presence of the epicatechin moiety in the molecule, which could have a greater affinity for the proteins. Pymv3glc-catechol formed a greater number of aggregates with aPRPs than vitisin B but lower than in the case of Pymv3glc-epicatechin, which may indicate that the catechol in the molecule is also involved in the interaction with the protein but to a lesser extent than the epicatechin. In order to quantify these differences observed in the formation of soluble aggregates, STD-NMR were carried out with the three pigments assayed.

3.3. STD-NMR pyranoanthocyanins – aPRPs binding studies

STD-NMR experiments have proved useful to characterize ligand-protein complexes. So, in order to quantify the interaction between pyranoanthocyanin and its derived pigments and aPRPs solution, the dissociation constant (K_D) was measured using STD-NMR spectroscopy.

To achieve this, preliminary control experiments were made. To confirm that the on-resonance irradiation frequency did not affect the anthocyanins' aromatic protons and that the protein was saturated by the on-resonance irradiation, experiments were performed at high anthocyanin concentrations. For pyranoanthocyanin alone, the STD spectrum showed the anthocyanins' aromatic resonances were not visible under these experimental conditions, which indicates that the on-resonance irradiation frequency does not overlap with these resonances. Based on the STD-NMR results, the protons that presented the highest intensities on the STD spectrum were measured to follow the binding affinity between the aPRPs and vitisin B, and derived pigments at increasing concentrations. The chosen protons were H2′and H6′ of ring B of the vitisin B and of pyranomalvidin-3-glucoside-catechol, and H2′and H6′ of ring F of the epicatechin moiety of pyranomalvidin-3-glucoside-epicatechin (Fig. 1). Fig. 3 presents, as an example, the ^{1}H NMR spectrum of Pymv3glc-epicatechin pigment and the difference STD spectra for some of the levels of concentration of the pigment assayed, both recorded in deuterium oxide. The proton resonances were assigned to the molecular structures based on the literature (Mateus et al., 2002; Oliveira, Mateus, Silva, & De Freitas, 2009).

The STD amplification factor (A_{STD}) was calculated from the differences between off-ressonance and on-ressonance spectra with increasing anthocyanin concentration (Fig. 4), according to Eq. (1) and the corresponding dissociation constant of the interaction was calculated according to the Eq. (2) using Solver tool of the Microsof Excel software.

Fig. 4 shows the observed (symbols) and fitted (line) STD amplification factor for vitisin B (1), pyranomalvidin-3-glucoside-catechol (2) and pyranomalvidin-3-glucoside-epicatechin (3). It is noticeable that the STD signal of the elicited pyranoanthocyanin pigments resonances increases systematically as concentrations increase. The maximum *plateau* of the interaction was reached at different concentrations depending on the pyranoanthocyanin. The dissociation constants obtained range from 0.8 to 1.7 mM depending on the pyranoanthocyanin pigment (Table 2). For vitisin B it was obtained the K_D of 1.74 mM, followed by pyranomalvidin-3-glucoside-catechol 1.17 mM and then by pyranomalvidin-3-glucoside-epicatechin 0.87 mM.

From these results it is observed that vitisin B, the molecular structure common to all the studied pigments, has the highest value of K_D therefore the lowest binding to aPRPs. It is also observed that the inclusion of any other of the studied groups (catechol or epicatechin) into the structure led to a decrease of the K_D, hence an increase in the interaction with aPRPs.

Furthermore, for pyranomalvidin-3-glucoside-epicatechin it is the epicatechin moiety the region of the molecule that presented highest interaction. But in this case, the protons of ring B of vitisin B also presented a significant intensity on STD spectrum. These results are in accordance with those obtained by MALDI-TOF-MS,

Table 2
Dissociation constants (K_D) of human saliva acidic proline-rich proteins and pyranoanthocyanins interactions obtained by STD-NMR for: Vitisin B, Pymv3glc-catechol, and pyranomalvidin-3-glucoside.

Pigment	K_D/mM
Vitisin B (**1**)	1.74
Pymv3glc-catechol (**2**)	1.17
Pymv3glc-(-)-epicatechin (**3**)	0.87

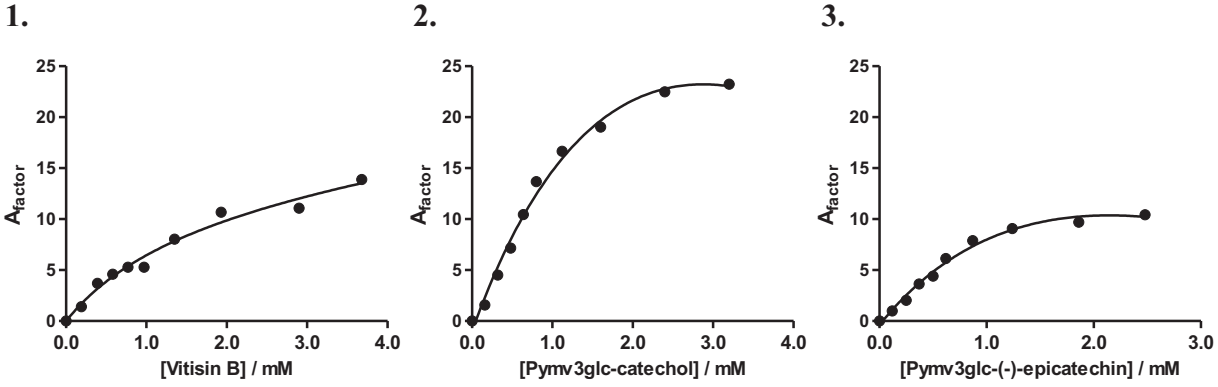


Fig. 4. STD amplification factor of vitisin B (1), pyranomalvidin-3-glucoside-catechol (2) and pyranomalvidin-3-glucoside-epicatechin (3) at pH 3.0. Symbols represent experimental values and lines represent calculated values by Eq. (1).

which showed a higher interaction of Pymv3glc-epicatechin with aPRPs than of the rest of pigments.

It is also interesting that the values obtained are in the range of dissociation constants obtained by other authors (0.4 to 8 mM) for the interaction between the human saliva proline-rich peptides ($IB7_{14}$ and $IB9_{37}$) and procyanidins (Cala et al., 2010).

Overall, the results obtained suggest that along with tannin compounds, other polyphenols present in red wine could contribute to its global astringency. As discussed, anthocyanin-derived pigments present dissociation constants similar to those obtained for the interaction between human saliva proline-rich peptides and procyanidins, which are the key class of polyphenols responsible for astringency sensation. It is also interesting that the presence of the tannins' "structural unit" in the pyranoanthocyanins molecule induces an increase in the interaction with acidic proline-rich proteins. So, it seems that overall astringency comprehension should be extended to other classes of polyphenols beside tannins which is of great interest for wine industry and producers.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank Dr. Mariana Andrade for the NMR analysis and Dr. Silvia Maia for MALDI-TOF analysis. Funding: This work was supported by a research project grant (PTDC/AGR-TEC/6547/2014) with financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 (UID/QUI/50006/2013 - POCI/01/0145/FEDER/007265); by two Post-Doctoral grants from FCT (SFRH/BPD/88866/2012 – Susana Soares and SFRH/BPD/72652/2010 – Luis Cruz); by an investigator contract from FCT (IF/00225/2015 – Joana Oliveira).



References

Alcalde-Eon, C., Escribano-Bailón, M. T., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2007). Identification of dimeric anthocyanins and new oligomeric pigments in red wine by means of HPLC-DAD-ESI/MSn. *Journal of Mass Spectrometry, 42*(6), 735–748.

Asenstorfer, R. E., Hayasaka, Y., & Jones, G. P. (2001). Isolation and structures of oligomeric wine pigments by bisulfite-mediated ion-exchange chromatography. *Journal of Agricultural and Food Chemistry, 49*(12), 5957–5963.

Bakker, J., Bridle, P., Honda, T., Kuwano, H., Saito, N., Terahara, N., & Timberlake, C. F. (1997). Isolation and identification of a new anthocyanin occurring in some red wines. *Phytochemistry, 44*, 1375–1382.

Bakker, J., & Timberlake, C. F. (1997). Isolation, identification, and characterization of new color-stable anthocyanins occurring in some red wines. *Journal of Agricultural and Food Chemistry, 45*, 35–43.

Bax, A., & Subramanian, S. (1986). Sensitivity enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *Journal of Magnetic Resonance, 67*, 565–569.

Bax, A., & Summers, M. F. (1986). ^{1}H and ^{13}C assignments from sensitivity-enhanced detection of heteronuclear multiple-bond connectivity by 2D multiple quantum NMR. *Journal of the American Chemical Society, 108*, 2093–2094.

Boulton, R. (2001). The copigmentation of anthocyanins and its role in the color of red wine: A critical review. *American Journal of Enology and Viticulture, 52*(2), 67–87.

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*(39), a9562–e9568.

Brouillard, R. (1982). Chemical structures of anthocyanins. In P. Markakis (Ed.), *Anthocyanins as food colors*. New York: Academic Press.

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. *The FASEB Journal, 24*(11), 4281–4290.

Cameira dos Santos, P. J., Brillouet, J. M., Cheynier, V., & Moutounet, M. (1996). Detection and partial characterisation of new anthocyanins derived pigments in wine. *Journal of the Science of Food and Agriculture, 70*, 204–208.

Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J. G., Haslam, E., Davies, A. P., & Williamson, M. P. (2002). Polyphenol/peptide binding and precipitation. *Journal of Agricultural and Food Chemistry, 50*(6), 1593–1601.

Cruz, L., Teixeira, N., Silva, A. M. S., Mateus, N., Borges, J., & De Freitas, V. (2008). Role of vinylcatechin in the formation of pyranomalvidin-3-glucoside-(+)-catechin. *Journal of Agricultural and Food Chemistry, 56*(22), 10980–10987.

De Freitas, V., & Mateus, N. (2010). Updating Wine Pigments. In C. Santos-Buelga, M. T. Escribano-Bailon & V. Lattanzio (Eds.), Recent Advances in Polyphenol Research, (pp. 59–80).

De Freitas, V., & Mateus, N. (2012). Protein/polyphenol interactions: Past and present contributions. Mechanisms of astringency perception. *Current Organic Chemistry, 16*(6), 724–746.

Dueñas, M., Salas, E., Cheynier, V., Dangles, O., & Fulcrand, H. (2006). UV-visible spectroscopic investigation of the 8,8-methylmethine catechin-malvidin 3-glucoside pigments in aqueous solution: Structural transformations and molecular complexation with chlorogenic acid. *Journal of Agricultural and Food Chemistry, 54*(1), 189–196.

Escribano-Bailón, T., Álvarez-García, M., Rivas-Gonzalo, J. C., Heredia, F. J., & Santos-Buelga, C. (2001). Color and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin 3-O-glucoside and (+)-catechin. *Journal of Agricultural and Food Chemistry, 49*(3), 1213–1217.

Ferrer-Gallego, R., Soares, S., Mateus, N., Rivas-Gonzalo, J., Escribano-Bailón, M. T., & Freitas, V. d. (2015). New anthocyanin-human salivary protein complexes. *Langmuir, 31*(30), 8392–8401.

Francia-Aricha, E. M., Guerra, M. T., Rivas-Gonzalo, J. C., & Santos-Buelga, C. (1997). New anthocyanin pigments formed after condensation with flavanols. *Journal of Agricultural and Food Chemistry, 45*, 2262–2265.

Fulcrand, H., Benabdeljalil, C., Rigaud, J., Cheynier, V., & Moutounet, M. (1998). A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins. *Phytochemistry, 47*, 1401–1407.

Fulcrand, H., Cameira dos Santos, P., Sarni-Manchado, P., Cheynier, V., & Favre-Bonvin, J. (1996). Structure of new anthocyanin-derived wine pigments. *Journal of the Chemical Society, Perkin Transactions, 1*, 735–739.

Hakansson, A. E., Pardon, K., Hayasaka, Y., de Sa, M., & Herderich, M. (2003). Structures and colour properties of new red wine pigments. *Tetrahedron Letters, 44*(26), 4887–4891.

Haslam, E., & Lilley, T. H. (1988). Natural astringency in foodstuffs – A molecular interpretation. *Critical Reviews in Food Science and Nutrition, 27*(1), 1–40.

He, J., Santos-Buelga, C., Silva, A. M. S., Mateus, N., & De Freitas, V. (2006). Isolation and structural characterization of new anthocyanin-derived yellow pigments in aged red wines. *Journal of Agricultural and Food Chemistry, 54*(25), 9598–9603.

He, J. J., Han, F. L., Yu, Q. Q., Pan, Q. H., Duan, C. Q., & Cheng, G. L. (2010). Effects of the maceration enzymes on evolution of pyranoanthocyanins and cinnamic acids during the cabernet gernischet (Vitis vinifera L. cv.) red wine making. *Food Science and Biotechnology, 19*(3), 603–610.

Lu, Y. R., Sun, Y., & Foo, L. Y. (2000). Novel pyranoanthocyanins from black currant seed. *Tetrahedron Letters, 41*(31), 5975–5978.

Márquez, A., Dueñas, M., Serratosa, M. P., & Merida, J. (2012). Formation of vitisins and anthocyanin-flavanol adducts during red grape drying. *Journal of Agricultural and Food Chemistry, 60*(27), 6866–6874.

Mateus, N., Carvalho, E., Carvalho, A. R. F., Melo, A., Gonzalez-Paramas, A. M., Santos-Buelga, C., ... De Freitas, V. (2003). Isolation and structural characterization of new acylated anthocyanin-vinyl-flavanol pigments occurring in aging red wines. *Journal of Agricultural and Food Chemistry, 51*(1), 277–282.

Mateus, N., & de Freitas, V. (2001). Evolution and stability of anthocyanin-derived pigments during port wine aging. *Journal of Agricultural and Food Chemistry, 49*(11), 5217–5222.

Mateus, N., Oliveira, J., Haettich-Motta, M., & De Freitas, V. (2004). New family of bluish pyranoanthocyanins. *Journal of Biomedicine and Biotechnology, 5*, 299–305.

Mateus, N., Silva, A. M. S., Rivas-Gonzalo, J. C., Santos-Buelga, C., & De Freitas, V. (2003). A new class of blue anthocyanin-derived pigments isolated from red wines. *Journal of Agricultural and Food Chemistry, 51*(7), 1919–1923.

Mateus, N., Silva, A. M. S., Santos-Buelga, C., Rivas-Gonzalo, J. C., & De Freitas, V. (2002). Identification of anthocyanin-flavanol pigments in red wines by NMR

and mass spectrometry. *Journal of Agricultural and Food Chemistry, 50*(7), 2110–2116.
Morata, A., Gomez-Cordoves, A. C., Colomo, B., & Suarez, J. A. (2003). Pyruvic acid and acetaldehyde production by different strains of Saccharomyces cerevisiae: Relationship with vitisin A and B formation in red wines. *Journal of Agricultural and Food Chemistry, 51*(25), 7402–7409.
Oliveira, J., Azevedo, J., Silva, A. M. S., Teixeira, N., Cruz, L., Mateus, N., & De Freitas, V. (2010). Pyranoanthocyanin dimers: A new family of turquoise blue anthocyanin-derived pigments found in port wine. *Journal of Agricultural and Food Chemistry, 58*(8), 5154–5159.
Oliveira, J., De Freitas, V., & Mateus, N. (2009). A novel synthetic pathway to vitisin B compounds. *Tetrahedron Letters, 50*(27), 3933–3935.
Oliveira, J., De Freitas, V., Silva, A. M. S., & Mateus, N. (2007). Reaction between hydroxycinnamic acids and anthocyanin-pyruvic acid adducts yielding new portisins. *Journal of Agricultural and Food Chemistry, 55*(15), 6349–6356.
Oliveira, J., Mateus, N., Silva, A. M. S., & De Freitas, V. (2009). Equilibrium forms of vitisin B pigments in an aqueous system studied by NMR and visible spectroscopy. *The Journal of Physical Chemistry B, 113*(32), 11352–11358.
Rentzsch, W., Schwarz, M., & Winterhalter, P. (2007). Pyranoanthocyanins – An overview on structures, occurrence, and pathways of formation. *Trends in Food Science & Technology, 18*(10), 526–534.
Soares, S., Vitorino, R., Osório, H., Fernandes, A., Venâncio, A., Mateus, N., ... de Freitas, V. (2011). Reactivity of human salivary proteins families toward food polyphenols. *Journal of Agricultural and Food Chemistry, 59*(10), 5535–5547.