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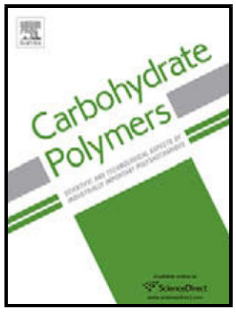

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Impact of grape pectic polysaccharides on anthocyanins thermostability

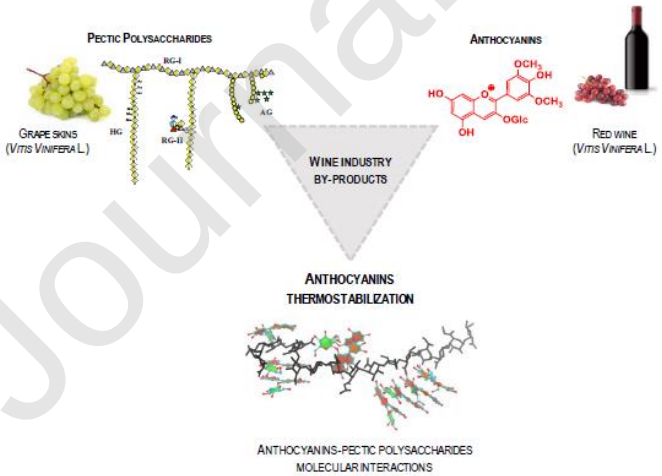
Ana Fernandes[a]* , Elsa Brandão[a], Filomena Raposo[b], Élia Maricato[b], Joana Oliveira[a], Nuno Mateus[a], Manuel A. Coimbra[b], Victor de Freitas[a]

[a]*LAQV-REQUIMTE, Departamento de Química e Bioquímica, Faculdade de Ciências, Universidade do Porto, Rua do Campo Alegre, s/n, 4169-007 Porto, Portugal.*
[b]*LAQV-REQUIMTE, Departamento de Química, Universidade de Aveiro, 3810-193, Aveiro, Portugal.*

*Author to whom correspondence should be addressed, ana.fernandes@fc.up.pt

Tel: +351.220402597

Graphical abstract



HIGHLIGHTS:

- Polysaccharides rich in uronic acids and neutral sugars were extracted from grape skins;
- Higher amount in homogalacturonan regions favors interaction with mv3Glc;
- Electrostatic interactions and hydrophobic effect mainly contribute to the binding;
- Thermal stability of mv3Glc was significantly improved by pectic polysaccharides;
- Less branched structures and pectin flexibility promote mv3Glc thermostabilization;

Abstract

The impact of grape pectic polysaccharides on malvidin-3-*O*-β-D-glucoside thermostability was evaluated in model solutions. Pectic polysaccharides richer in homogalacturonan domains, with less neutral sidechains (chelator fraction) showed higher binding with malvidin-3-*O*-β-D-glucoside, by ^{1}H-NMR (K_a=505 M^{-1}). Binding affinity with water soluble extract was estimated to be 10-fold lower, possibly due to the presence of neutral branched regions and more compacted structure, hampering the binding. Hydrophobic domains, such as rhamnogalacturonans-I domains in acid soluble polysaccharides, may participate in the formation of complexes with malvidin-3-*O*-β-D-glucoside.

The thermostability of anthocyanin-polysaccharides complexes was evaluated at different temperatures, assessing anthocyanins degradation by HPLC-DAD. Polysaccharides showed to improve anthocyanin thermostability, with chelator and acid extract having the highest impact at lowest temperatures. Electrostatic interactions, additionally stabilized by hydrophobic effect contribute to the anthocyanin-polysaccharides binding and to the consequent thermostabilization. The protection provided by grape pectic polysaccharides foresees innovative anthocyanin food products with improved thermostability and colour features.

Keywords: Anthocyanins; grape skins; HPLC-DAD; NMR; pectic polysaccharides; thermostability.

1. Introduction

Several studies have shown the formation of pectic polysaccharide-anthocyanin complexes through the establishment of weak non-covalent forces, like electrostatic interactions, hydrophobic effect and H-bonding (Buchweitz, Speth, Kammerer, & Carle, 2013a, 2013b; Koh, Xu, & Wicker, 2020a; Larsen, Buerschaper, Schieber, & Weber, 2019; Lin, Fischer, & Wicker, 2016; Padayachee, Day, Howell, & Gidley, 2017; Padayachee et al., 2012; Phan, Flanagan, D'Arcy, & Gidley, 2017). Pectic polysaccharides are one of the most complex plant heteropolysaccharides predominantly composed of homogalacturonans (HG), formed by α-1→4 linked D-galacturonic acid residues (α-D-Gal*p*A) and rhamnogalacturonan I and II domains (RG I and II, respectively) (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015). In HG domains, galacturonic acid residues can be methylated at C-6 and acetylated at positions O-2 and O-3, expressed as the degree of methylation (DM) and acetylation (DA), respectively. RGI consists of 1,4 linked α-D-Gal*p*A and 1,2 linked α–L-rhamnose (Rha*p*), substituted with neutral sugars (galactose, arabinose, glucose) (Voragen, Coenen, Verhoef, & Schols, 2009), while RGII, a minor component of pectin possesses a backbone of (1→4) linked α-D-Gal*p*A as homogalacturonans with side chains containing rhamnose and a variety of rare monosaccharides, like fucose (Pérez, Rodríguez-Carvajal, & Doco, 2003). With an apparent pK_a near 3.5-4.5, the galacturonic acid residues may be methyl esterified-uncharged, de-esterified-charged or de-esterified-protonated-uncharged, with this affecting the anionic nature of pectin (Voragen et al., 2009). These polysaccharides are typically used as hydrocolloids by the food industry as gelling, thickening and stabilizing agents (Lin et al., 2016).

Anthocyanins, one of the most important group of water-soluble pigments, are responsible for the red, purple and blue colours of flowers, fruits and vegetables. Due to their attractive colours, but also due to their broad spectrum of safety and benefitial health effects, there has been a growing interest from the industry in these pigments, particularly as food colourants (Giusti & Wrolstad, 2003). However, anthocyanin stability is affected by several factors such as pH, temperature, chemical structure, pigment concentration, light, oxygen, enzymes, the presence of

other phenolic compounds, proteins and metal ions (Castañeda-Ovando, Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Thus, retaining a strong and stable colour of anthocyanin-derived food products can be a huge challenge during processing and storage (Mateus & de Freitas, 2009). For instance, anthocyanin colour changes with the pH of the medium: the red flavylium cation at acidic pH, the colourless hemiketal and yellow-pale chalcones at slighty acidic pH and the blue-violet quinoidal bases at higher pH (Brouillard, Delaporte, & Dubois, 1978; Brouillard & Dubois, 1977). Additionally, food processing generally envolves thermal treatment prior consumption at temperatures ranging from 50 to 150º C, depending on the pH and desired shelf-life of the product. This process has a great influence on the anthocyanin content in the final product, colour quality and also affects the nutritional properties (Giusti & Wrolstad, 2003; Sadilova, Stintzing, Kammerer, & Carle, 2009). Bearing this, the evaluation of the heat-induced anthocyanin degradation is of utmost importance for the Food Industry. To overcome anthocyanin thermal instability, a series of approaches have been attempted like the addition of phenolic compounds, biopolymers or β-cyclodextrins (Buchweitz et al., 2013a; A. Fernandes et al., 2018; Gradinaru, Biliaderis, Kallithraka, Kefalas, & Garcia-Viguera, 2003; Sadilova et al., 2009).

Despite the potential impact of anthocyanin-pectic polysaccharide binding for anthocyanin stability and concomitant food functionality, little is known regarding anthocyanin thermostability due to pectic polysaccharides interaction and about the structural domains of polysaccharides that support these bindings and potential thermal stabilization. Additionally, the recovery of pectic polysaccharides from new natural source besides citrus and apple peels, such as grape skins and their incorporation into food products, could present an innovative and sustainable way to valorize these food industry wastes.

In this study, three pectic polysaccharide rich fractions were extracted from *Vitis Vinifera* L. white grape skins. The main goal was to evaluate the binding affinity and the potential thermostabilization of a major red wine anthocyanin, malvidin-3-*O*-β-D-glucoside, as a result of the interaction with pectic polysaccharides with different structural characteristics, extracted from grape skins, in model solution at slightly acidic pH.

2. Material and Methods

2.1. Reagents

All reagents used were analytical or HPLC grade. Folin-Ciocalteu, Bradford, bovine serum albumin and reagents for carbohydrate analysis were obtained from Sigma-Aldrich®. Acetonitrile was purchased from Panreac Quimica, methanol and formic acid from Chem-Lab NV, while ethanol was obtained from AGA. Reverse-phase C-18 silica gel (LiChroPrep, RP-18; 40-63 μm) was obtained from Merck KGaA.

2.2. Plant Materials

Pectic polysaccharide extracts were obtained from *Vitis vinifera* L. white grapes kindly provided by Lavradores de Feitoria® (Cima Corgo, Douro Region). At the laboratory, the grape skins were collected and freeze-dried. The alcohol-insoluble residue (AIR) was obtained from freeze-dried grape skins according to the methodology described by (Hernández-Hierro et al., 2014). AIR was then consecutively fractionated into water, chelator and acid soluble polysaccharides (WSP, CSP and ASP, respectively) according to an adaptation of the methodology described in the literature (Slavov et al., 2017; Slavov, Panchev, Kovacheva, & Vasileva, 2016). Briefly, AIR (7.5 g) was extracted with hot water twice (150 mL distilled water, at 90 ºC for 1 h with constant stirring). After centrifugation (15 minutes, 2862 *g*, RT) and filtrate volume reduction, the concentrated extract was precipitated with 3 volumes of absolute ethanol. The obtained precipitate was re-dissolved in water and dialysed against distilled water (Spectra/Por®, 6-8 kDa cut-off). The retentate was freeze-dried and denoted as water soluble polysaccharides (WSP). The previous residue was extracted twice with 50 mM ammonium oxalate solution at 50º C for 1h, at pH 5. The resulting residue was finally treated with dilute acid (0.1M HCl) for 1h at 85º C. Ammonium oxalate and acid diluted extract were treated in the same way as described for the water soluble extract and denoted as chelator and acid soluble polysaccharides (CSP and ASP, respectively).

Malvidin-3-*O*-β-D-glucoside was extracted from a young red wine (*Vitis vinifera* L. c. v. Touriga Nacional). Briefly, wine pre-concentrated in a nanofiltration apparatus was applied on a

low pressure C-18 gel column chromatography and a monoglucoside anthocyanin rich fraction was obtained with a 20% methanol acidic solution. After that, this fraction was subjected to a semipreparative liquid chromatography using a reversed-phase C-18 column (250 mm x 4.6 mm i.d.), as described elsewhere (Azevedo et al., 2010).

2.3. Analytical methods

Carbohydrate analysis was performed following the procedure described by (Nunes, Saraiva, & Coimbra, 2008). Results were expressed as μg sugar/mg dry sample. Neutral sugars composition were determined after conversion to their alditol acetates by GC, using 2-deoxyglucose as internal standard (Coimbra, Delgadillo, Waldron, & Selvendran, 1996). Uronic acids (UA) were determined by a modification of the 3-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973) and their content was expressed as μg galacturonic acid equivalent/mg dry sample. The determination of the degree of methyl esterification of pectic polysaccharides was based on the methanol content released after saponification (Nunes, Rocha, Saraiva, & Coimbra, 2006; Waldron & Selvendran, 1990). Methanol was quantified by gas chromatography and detected by a flame ionization detector (GC-FID), using 1-propanol as internal standard. The zeta-potential (ζ) of the pectic polysaccharides fractions were measured by dynamic light scattering in aqueous solution at pH 3.4 (200 $\mu g.mL^{-1}$), with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) at 25º C.

Average molecular weight (MW) and polydispersity analysis of WSP and CSP fractions was carried out using two PL aquagel-OH MIXED 8 μm columns (300 x 7.5 mm) protected by a PL aquagel-OH Guard 8 μm pre-column (Polymer Laboratories, UK) on a PL-Gel Permeation Chromatograph (GPC) 110 system (Polymer Laboratories, UK) equipped with a RI detector. All the analytical columns and the detector were maintained at 36 ºC during analysis. Standards and samples were dissolved in 0.1 M $NaNO_3$ aqueous solutions to reach a maximum concentration of 1%. The eluent (0.1 M $NaNO_3$ solution) was pumped at a flow rate of 0.9 mL/min. The columns

were calibrated with pullulan standards (Polymer Laboratories, UK) in the range of 5.8–1000 kDa.

2.4. ^{1}H-NMR studies

For the ^{1}H-NMR studies, 1.0 mM of malvidin-3-*O*-β-D-glucoside solution was prepared in D_2O and the pH was adjusted to 3.0 with DCl or NaOD and transferred into 5 mm NMR tubes (pD 3.4, with pD = pH-meter reading for solutions in D_2O+ 0.40) (Popov, Rönkkömäki, & Lajunen, 2006). Sodium trimethylsilyl-[2,2,3,3-d4]-propionate (TSP) was used as an internal standard for chemical shift measurements (5 μL TSP 2 mg.mL^{-1} in D_2O, calibrated at 0.00 ppm). After mv3Glc spectrum acquisition, pectic polysaccharide solutions (45 and 32 mM galA equivalents for WSP and CSP/ASP, respectively) in D_2O at pH 3.0, were independently titrated into the anthocyanin solution and ^{1}H-NMR spectra were acquired at each titration point, aiming to obtain different mv3Glc:galA equivalents molar ratios. All ^{1}H NMR spectra were acquired at 298 K on a Bruker Avance III 600 HD spectrometer, operating at 600.13 MHz, equipped with 5 mm CryoProbe Prodigy and pulse gradient units, capable of producing magnetic field pulsed gradients in the z-direction of 50 G/cm. The measurements were done with standard Bruker pulse sequences at 298 K. ^{1}H NMR experiments were performed with water suppression using excitation sculpting with gradients, acquisition time 1.70 s, relaxation delay 2 s and 128 transients of a spectral width of 8000 Hz collected into 32 K time domain points.

2.5. NMR data analysis

Chemical shifts of individual assigned resonances of the mv3Glc protons were analyzed as a function of mv3Glc:galA equivalents molar ratios. Chemical shift data were used to calculate the association constant (K_a), number of binding sites per molecue (n) and the maximum chemical shift change ($\Delta\delta_{max}$) for mv3Glc protons using the equation previously described (*equation (1)*) (Hu, Xu, & Cheng, 2012):

$$\Delta\delta = \frac{\Delta\delta_{max}}{2}\left\{\left(1 + \frac{1}{K[A]} + \frac{n[P]}{[A]}\right) - \left[\left(1 + \frac{1}{K[A]} + \frac{n[P]}{[A]}\right)^2 - 4\frac{n[P]}{[A]}\right]^{1/2}\right\} \qquad (1)$$

where [A] is the total anthocyanin concentration (M) and [P], total concentration of each pectic polysaccharide fraction (M galacturonic acid equivalents). Values of K_a, n and $\Delta\delta_{max}$ were calculated using a nonlinear least-squares-fitting routine within the software program Microsoft EXCEL.

2.6. Anthocyanin-pectic polysaccharide binding

To study the impact of pectic polysaccharide-anthocyanin interactions for mv3Glc thermostability, a stock solution of mv3Glc was prepared in aqueous solution (pH 3.4). Interacting samples (Mv3Glc-WSP, Mv3Glc-CSP and Mv3Glc-ASP) were obtained by mixing aliquots of each polysaccharide fraction, also prepared in aqueous solution at pH 3.4 (0.2% w/v final concentration) with the mv3Glc stock solution (200 $mg.L^{-1}$, final concentration). Control sample (mv3Glc alone) was prepared at the same concentration and pH. Samples were mixed with a vortex and the pH of each solution was re-adjusted to pH 3.4 with HCl (0.1 M) or NaOH (0.1 M) whenever necessary. In order to guarantee total pH equilibration, all solutions were left to equilibrate overnight. The pH was measured in a Radiometer Copenhagen PHM240 pH/ion meter (Brønshøj, Denmark). The thermostability assays of malvidin-3-*O*-β-D-glucoside (CONTROL) and anthocyanin binding systems with pectic polysaccharide fractions were performed at three different temperatures (60, 80 and 100º C) at pH 3.4. Both samples were placed into a glass flask with a screw-cap at different temperatures and aliquots were collected over time (0, 30, 60, 90, 180, 240 minutes). These aliquots were immediatly cooled in an ice bath and subsequently frozen at -20º C prior further analysis. All samples were performed in triplicate. Prior to anthocyanin content determination, both solutions of anthocyanins and anthtocyanins-biopolymers, were acidified (pH≈1) and were left to equilibrate for at least 4 hours before anthocyanins analysis. The remaining anthocyanin amount was monitored by HPLC-DAD as a function of time.

2.7. Evaluation of thermal degradation kinetics

Anthocyanin residual content, obtained by HPLC-DAD analysis was fitted through a first-order reaction kinetics (equation (*2*)), where C_0 is the initial anthocyanin content and C is the anthocyanin content after heating at a predetermined time (t) (Wang & Xu, 2007). The logarithm of the anthocyanin residual content ($\ln(C/C_0)$) was plotted versus time (t) and the degradation rate constants (k) were obtained from the slope of this graphical representation. For a first order reaction, half-life values ($t_{1/2}$, the time needed for 50% degradation of anthocyanins) were determined by equation *(3)*:

$$ln(C/C_0) = -kt \qquad (2)$$

$$t_{1/2} = ln(2)/k \qquad (3)$$

The effect of temperature on the degradation rate constants was expressed by the linearized Arrhenius equation (equation (*4*)), by plotting ln$k$ against 1/T in which the temperature dependence of k was quantified by the activation energy Ea according to:

$$lnk = lnk_0 - Ea/RT \qquad (4)$$

where Ea is the activation energy (kJ/mol), R is the universal gas constant (8.314 J/mol/K), k0 is the frequency factor and T is the absolute temperature (Kelvin, K). The Ea value was calculated from the slop of the straight lines given by equation *(4)*.

2.8. HPLC-DAD analysis

The residual anthocyanin content after each thermal treatment was analyzed and quantified using a HPLC-DAD system (Merck Hitachi) consisting on an Elite Lachrom L-2130 pump, with a reversed-phase C_{18} column (250 mm x 4.6 mm i.d., 5 μm, Merck, Lichrospher), thermostatized at 25 ºC. Detection was carried out at 520 nm on an Elite Lachrom L-2455 Diode Array Detector. 20 μL of each sample was injected using an Elite Lachrom L-2200 autosampler and anthocyanins were separated and quantified using a mobile phase, a flow rate and an elution gradient as described elsewhere (Oliveira et al., 2013)

2.9. Statistical analysis

Analysis were performed with n=3 and the values obtained was expressed as mean±standard deviation (SD). Statistical significance was detected by analysis of variance (ANOVA), followed by the Tukey's test; Differences were considered to be statistically significant at $p < 0.05$. All statistical data were processed using GraphPad Prism version 5.0 for Windows.

3. Results and Discussion

3.1. Pectic polysaccharide characterization

Pectic polysaccharide fractions were recovered from white grape skins alcohol insoluble residue with water, chelating agent (oxalate aqueous solution) and acidic solution (HCl), allowing to obtain extracts WSP, CSP and ASP, respectively, with 89, 96 and 97 molar % of galacturonic acid (UA) residues and a sugar content of 777, 848, and 898 μg/mg dry matter (**Table 1**), characteristic of homogalacturonans, the dominant polysaccharides of grape skin cell walls (Hernández-Hierro et al., 2014). Arabinose, galactose and to a lesser extent rhamnose could also be detected (**Table 2**), which is characteristic of pectic polysaccharides (Nunes et al., 2008). The total neutral sugars content, although significantly higher for WSP than for CSP and ASP extracts, was lower than the values reported in the literature (Hernández-Hierro et al., 2014; Vicens et al., 2009). This may be due to the high temperature and acidic conditions used for extraction, combined with the ethanol precipitation step performed after the recovery of the extracted polysaccharides. Under the hot water extraction conditions, pectic polysaccharides can be debranched by β-elimination reactions (Voragen et al., 2009) and the acidic conditions used in CPS and ASP extractions are known to promote the hydrolysis of the acid labile arabinan-rich side-chains. As arabinans are polysaccharides highly soluble in ethanol/water solutions (Cardoso, Silva, & Coimbra, 2002; P. A. R. Fernandes, Le Bourvellec, et al., 2019), they probably have been lost in the supernatant during the precipitation of the pectic polysaccharides in 3 volumes of ethanol. The presence of Ara and Gal is characteristic of arabinan and galactan-type sidechains, solubilized during extraction. Glucose and xylose were also present in the water soluble fraction (6 and 4 μg/mg, respectively), as reported for skin HEPES-soluble polysaccharides (Vidal, Williams, O'Neill, & Pellerin, 2001).

The estimation of the linearity of these fractions, determined by the ratio of (UA+Rha)/(Ara+Gal) increased from 14 to 37 and 84 for WSP, CSP, and ASP, respectively, showing that the pectic polysaccharides recovered with a chelator or acidic aqueous solution were much more linear than those extracted with water (WSP). The arabinose to galactose ratio (Ara/Gal) was close to 1 for ASP, suggesting the presence of arabinogalactan-type sidechains (Slavov et al., 2017). For the water and chelator fractions an increase in this ratio could be noticed (2 and 3, respectively), showing the higher proportion of Ara compared to Gal in these fractions, characteristic of the predominance of arabinan structural features. These results are consistent with a previous study showing that pectic sidechains for grape berry skin tissue are composed of arabinans and galactans (Vidal et al., 2001). The highest ratio between GalA to Rha (GalA/Rha) for the chelator soluble polysaccharides suggested that this fraction was richer in homogalacturonan, compared to ramnogalacturonan like structures (Arnous & Meyer, 2009). Oppositely, the acid soluble fraction, which contained significantly higher amounts of rhamnose, showed a lower ratio (≈ two-fold lower), which suggests of the presence of RGI-type domains. Also, the (Ara+Gal)/Rha ratio decreased from 14 to 8 and 2 (WSP, CSP and ASP), reflecting the increased proportion of Rha compared to the Ara and Gal in ASP fraction, possibly resultant from the acid conditions used.

The methanol content released from the extracts was similar for CSP and ASP, 38 μg/mg dry sample, while WSP showed lower amounts of released methanol (31 μg/mg dry sample), representing a similar degree of methylesterification for all fractions, with about 25 % of the total uronic acid methylesterified. The zeta-potential (ζ) values were close to -12 mV for all fractions, not evidencing differences in the pectic polysaccharides superficial charge. These results are different than the ones reported for water soluble blueberry pectins, showing that this fraction consisted on highly methoxylated pectin, with less negative charges (Lin et al., 2016). The heating treatment to obtain WSP fraction, possibly resulted in the degradation of highly methylated regions of pectic polysaccharides due to β-elimination (Stolle-Smits, Beekhuizen, Recourt, Voragen, & Van Dijk, 1997).

Average molecular weight (Mw) and polydispersity were also determined for the major eluting peaks for WSP and CSP fractions (**Table 1**). These fractions showed an average molecular weight of 519 and 1089 kDa, and a polydispersity index of about 19 and 18, respectively. The molecular weight for the grape skins pectic polysaccharides extracted in this work are substantially higher than those reported for commercial citrus pectin of about 100 kDa (A. Fernandes, Brás, Mateus, & de Freitas, 2014). The mild extraction conditions used probably maintained the integrity of the carbohydrate polymers (Lin et al., 2016), resulting in higher sizes.

Table 1. Chemical composition of grape skins extracts obtained by sequential fractionation with water (WSP), chelator (CSP) and acidic solutions (ASP). Results expressed as average±standard deviation (μg/mg dry matter).

	TS (μg/mg)	NS (μg/mg)	ζ (mV)	Mw (KDa)	PDI
WSP	777 ± 136[a]	74 ± 12[a]	-12.5 ± 0.9[a]	519	18.5
CSP	848 ± 42[a]	31 ± 3[b]	-12.9 ± 0.3[a]	1089	17.6
ASP	898 ± 241[a]	23 ± 1[b]	-11.7 ± 0.7[a]	-	-

TS, total sugars; NS, neutral sugars; ζ, zeta potential; DM, degree of methyl esterification; Mw, molecular weight; PDI, polydispersity index. In the same column, samples with different letters are significantly different ($p < 0.05$).

Table 2. Neutral sugar and uronic acids composition of grape skins extracts obtained by sequential fractionation with water (WSP), chelator (CSP) and acidic solutions (ASP). Results are expressed as average±standard deviation (μg/mg dry matter).

	Rha (μg/mg)	Fuc (μg/mg)	Ara (μg/mg)	Xyl (μg/mg)	Man (μg/mg)	Gal (μg/mg)	Glc (μg/mg)	UA (μg/mg)
WSP	3.8±0.7[a]	0.2±0.1[a]	32.0±5[a]	6.0±2[a]	2.5±0.6[a]	21±0.8[a]	4.0±1[a,c]	709±149[a]
CSP	2.6±0.1[a]	0.2±0.03[a]	15.8±0.4[b]	1.8±0.2[b]	0.5±0.01[b]	6±2[b]	1.5±0.2[b]	820±44[a]
ASP	6.4±0.1[b]	*n.d.*	5.5±0.3[c]	0.9±0.1[c]	0.6±0.1[b]	5±0.3[b]	4.6±0.01[a]	875±241[a]

Rha, rhamnose; Fuc, Fucose; Ara, Arabinose; Xyl, Xylose; Man, Mamnose; Gal, Galactose; Glc, Glucose, UA, Uronic Acid; *n.d.*, non detected; In the same column, samples with different letters are significantly different ($p < 0.05$).

3.2. Pectic polysaccharides-anthocyanin binding

3.2.1. ^{1}H-NMR titration

The intermolecular interactions between malvidin-3-*O*-β-D-glucoside and grape pectic polysaccharide fractions were investigated by ^{1}H-NMR spectroscopy, following mv3Glc protons chemical shift variation as a function of mv3Glc:galA molar ratio. These experiments were performed in order to establish the binding affinity of these pectic polysaccharides to malvidin-

3-*O*-β-D-glucoside, aiming also to establish a structure-affinity relationship involved in these bindings.

Chemical shift displacement can be used to describe the chemical environment of specific nuclei. It is assumed that due to charge interactions, the electron density around the cation will decrease and, as a result, an increase of the chemical shift (high-frequency shift) will occur (Hu, Cheng, Ma, Wu, & Xu, 2009). Formation of hydrogen bonds also leads to the decrease of electron density around the nucleus, resulting in a high-frequency shift (Boas, Karlsson, de Waal, & Meijer, 2001; Boas, Söntjens, Jensen, Christensen, & Meijer, 2002). In the case of hydrophobic interactions, chemical shift variation will depend on the type of functional groups (*i.e.* deshielding or shielding groups) (Hu et al., 2012). **Figure 1** shows the ^{1}H-NMR spectra of 1.0 mM mv3Glc solution, evidencing the resonance signals related to the presence of the flavylium cation (AH^{+}) and the hemiketal equilibrium forms (B) which are in equilibrium at pD 3.4. Other signals present were attributed to other anthocyanin equilibrium forms in solution (*cis*- and *trans*-chalcones). As pectic polysaccharides (WSP, CSP and ASP) were added to mv3Glc solution, the resonance signals belonging to the flavylium cation protons (AH^{+} signs) were systematically shifted to high-frequency fields (deshielding) and progressively broadened, for all interacting systems. Additionally, the intensity of these peaks also decreased during titration although no precipitation was observed. Due to the observed chemical shift behavior, and due to the slightly acidic pH in which these experiments were performed, electrostatic interactions between the positively charged anthocyanin (flavylium cation) and negatively charged galacturonic acid residues are presumed to be the main event in anthocyanin-pectin interaction. Previously, electrostatic interaction between anionic pectin and cationic anthocyanins has also been proposed to be the fundamental driving force at pH 3.0-3.5 (Koh et al., 2020a; Lin et al., 2016; Padayachee et al., 2012; Phan et al., 2017). In addition to electrostatic interactions, the binding towards anthocyanins can occur by hydrogen bonds formed by hydroxyl groups of the anthocyanin rings and hydroxyl groups accessible on pectin surface (Buchweitz et al., 2013a; A. Fernandes et al., 2014) and through hydrophobic effect. In mv3Glc, bearing two methoxyl groups on the B-ring, besides electrostatic interactions, binding should occur through hydrophobic effect (like π-interactions)

in addition to hydrogen bonds, further contributing to the stabilization of the anthocyanin-pectic polysaccharides complexes.

The chemical shift of protons belonging to other anthocyanin forms (hemiketal) were almost unchanged during the titration experiments, which seemed to indicate the preferential interaction between pectic polysaccharides and anthocyanins' flavylium cation form. Protons belonging to mv3Glc chalcone forms (C*t* and C*c* at 7.2 and 6.6 ppm, respectively) also evidence a small chemical shift variation to high-frequency fields. This chemical shift variation could also result from the establishement of a molecular interaction between anthocyanin equilibrium forms and the pectin structure.

Analysis of the ^{1}H-NMR titration data (for the flavylium cation protons) through equation *(1)* was used to estimate the association constant (K_a), number of binding sites (n) and the maximum chemical shift change ($\Delta\delta_{max}$). Flavylium cation protons observed chemical shift change ($\Delta\delta_{obs}$) was plotted against the mv3Glc:GalA molar ratio (WSP, CSP or ASP) and this data was fitted using equation *(1)*. From the fitting of the experimental data (obtained with an estimated error of ≈10%), the association constant could be determined, with mv3Glc evidencing different binding affinities towards individual pectic polysaccharides. This binding was higher for CSP fraction and in the order CSP > ASP > WSP (505, 133 and 57 M^{-1}, respectively) (**Figure 2**). From the calculated *n* values (obtained through the fitting), it was estimated that one mv3Glc molecule interacts with approximately 3.3, 1.1 and 0.6 GalA molecules in pectic polysaccharide structure (CSP, ASP and WSP, respectively), reflecting the higher affinity of CSP fraction to mv3Glc. These results were slightly lower than those reported by Koh, Xu, & Wicker (2020a), estimating that one anthocyanin molecule bound to every 4-5 galacturonic acid molecules of blueberry pectin chelating soluble polysaccharides.

From the binding parameters obtained, it can be assumed that specific pectic polysaccharide structures (specific hydrophilic and hydrophobic domains, and sugars composition) can influence anthocyanin binding. According to the sugar analysis, there were no significant differences in the total sugars, uronic acid content (expressed as galacturonic acid) and ζ-potential. Conversely,

statistical differences (p<0.05) were observed in the neutral sugar composition between the three fractions, with water soluble polysaccharides evidencing significantly higher amounts of Ara, Xyl, Man, Gal and Glc. WSP also presents a decreased linearity, compared to the chelator and acid soluble pectic polysaccharides. Due to the higher neutral sugar branches on pectic polysaccharides structure, a more compact conformation is expected, with this possibly limiting the accessibility of binding sites in WSP (Alba, Bingham, & Kontogiorgos, 2017). The amounts of neutral monosaccharides such as Xyl and Glc were also significantly higher in WSP fractions compared to CSP and ASP fractions, possibly hampering the binding with mv3Glc. Characteristic monosaccharide ratios also evidenced the predominance of specific pectic domains that can be important for anthocyanin-pectic polysaccharides binding. For CSP fraction, the higher GalA/Rha ratio, reflecting the richness in HG in relation to RG-I type domains and the less branched structure compared to WSP fraction, can be associated to the higher binding affinity with mv3Glc approximately by ten-fold at pH 3.4. As a consequence of the lower amonts of neutral sugar side-chains (RG-I regions) than those rich in galacturonic acid (HG regions), this fraction has a greater flexibility, possibly favouring the binding with mv3Glc (Morris & Ralet, 2012). In the case of ASP fraction, the lowest (Ara+Gal)/Rha ratio, significantly higher amount of Rha, showing the increased proportion of RG-I type domains and the presence of arabinogalactan sidechains (Ara/Gal=1), seemed to be beneficial to the binding to mv3Glc, compared to WSP fraction. These findings suggest that these specific hydrophobic domains of the hairy region can participate in the complex formation with mv3Glc, a hydrophobic anthocyanin. Similar results were observed for the complexation of monoglucoside anthocyanins with the hairy region of ultrasound modified sugar beet pectins (Larsen et al., 2019), as well as for the interaction of arabinan-rich pectic polysaccharides with procyanidins (P. A. R. Fernandes, Le Bourvellec, et al., 2019; P. A. R. Fernandes, Silva, et al., 2019). Although no significant differences could be observed in the ζ-potential of the three pectic polysaccharide fractions, indicating similar negative superficial charge, the more prevalent linearity of the CSP and ASP fractions could possibly result in the localization of the negative charges in micro-enviroments, allowing a more efficient interaction with mv3Glc, compared to WSP (Koh, Xu, & Wicker, 2020b).

The lower binding affinity of mv3Glc to water soluble polysaccharides was similar to a previous research which showed that pectic polysaccharides with branched structures and higher amounts of neutral sugars limited the interaction with monoglucoside anthocyanins, with this interaction affecting its *in vitro* gastrointestinal stability (Koh et al., 2020b). Binding can also be attributed to electrostatic interactions between anthocyanin flavylium cation and the negatively charged carboxyl groups, anthocyanin stacking (Lin et al., 2016) and due to the higher flexibility of the blueberry chelator pectin chain (Lin, Pattathil, Hahn, & Wicker, 2019).

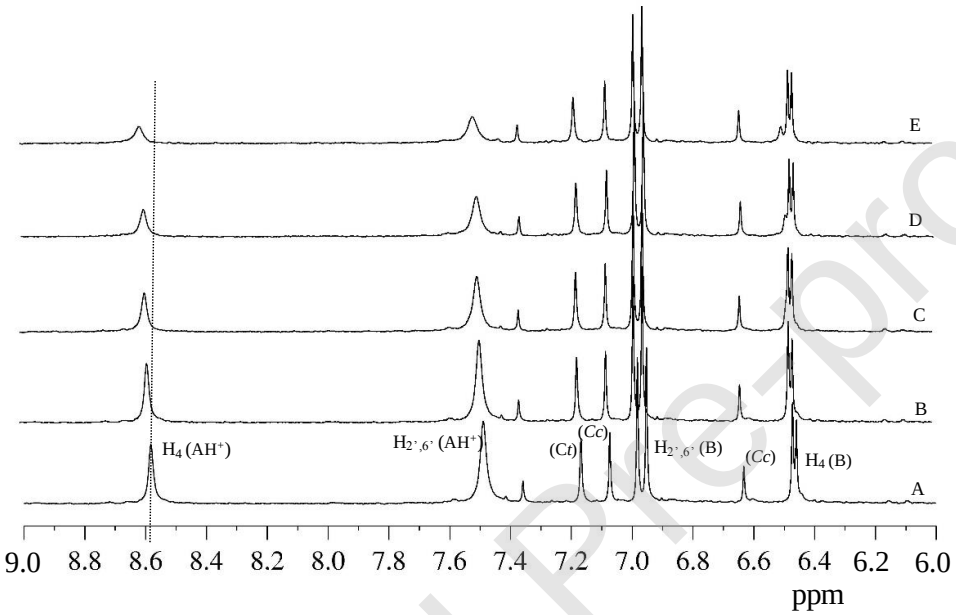


Figure 1. ^{1}H-NMR spectra region (9-6 ppm) of mv3Glc at 1mM in the absence (A) and with increasing CSP concentration (B-E). Spectra were recorded in D_2O, at pD 3.4. Assignment of the different anthocyanin equilibrium forms, from Houbiers, et al (Houbiers, Lima, Maçanita, & Santos, 1998).

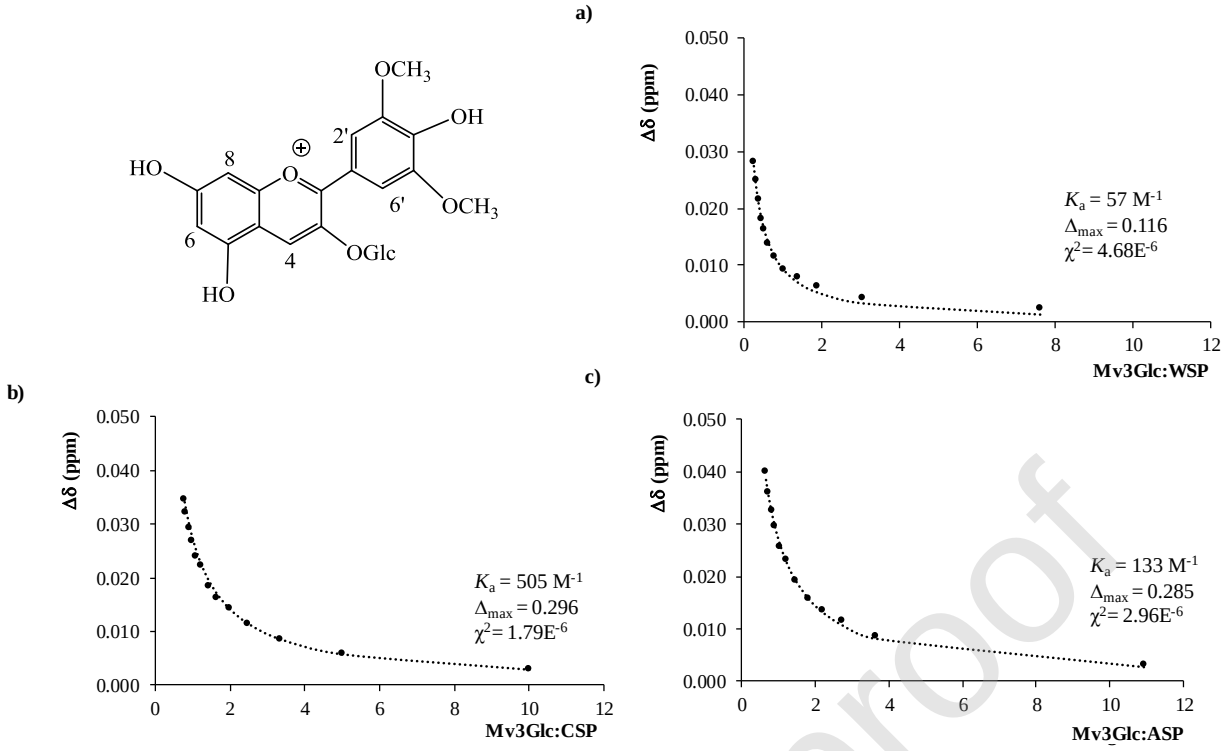


Figure 2. Representation of the chemical shift variations of H_4 proton of mv3Glc ($\Delta\delta_{obs}$) as a function of the mv3Glc:pectic polysaccharides ratio (*a*-WSP, *b*-CSP and *c*-ASP). Fitting was achieved with equation *(1)*.

3.3. Thermostability assays

To investigate the effect of pectic polysaccharides fortification on the thermostability of anthocyanins, the binding systems were prepared in aqueous solutions at pH 3.4 and the kinetic parameters of the thermal degradation of malvidin-3-*O*-β-D-glucoside were determined at 60, 80 and 100º C. Both control and fortified samples were acidified (pH≈1) and left to equilibrate for at least 12h before anthocyanin quantification, by HPLC-DAD. Acidification of the samples allowed the quantification of the residual flavylium cation ion, from the conversion of the colourless forms (hemiketal and chalcones). This allows the selective monitoring of the irreversible auto-oxidation contribution to anthocyanins colour loss (Fenger, Moloney, Robbins, Collins, & Dangles, 2019). In this way, the kinetics of anthocyanin loss and effective pigment degradation could be determined for each interaction system.

During the heating process, degradation occurred for both control and anthocyanin-binding systems and the red colour of all samples tended to disappear mainly due to anthocyanins thermal

degradation. This occurred through the formation of chalcone glycosides, which further degraded to hydroxybenzaldehyde and hydroxybenzoic acid derivatives (O. Andersen & Jordheim, 2013). The stabilising effect of different pectic fractions on the total anthocyanin content was primarily determined by the susceptibility of mv3Glc to the pectin type (water, chelator and acid solube pectins). Anthocyanin residual content was monitored over a period of 4 hours by HPLC-DAD analysis. The logarithms of both mv3Glc and mv3Glc-binding systems content ($\ln(C/C_0)$) were plotted versus time (t) for each heat treatment at 60, 80 and 100º C. The kinetic plots of mv3Glc and binding systems at the three temperatures tested, are shown in **Figure 3**. Overall, linear relationships were observed between $\ln(C/C_0)$ and time ($R^2 > 0.9681$) at all temperatures tested, indicating that the degradation processes followed a first-order reaction kinetics.

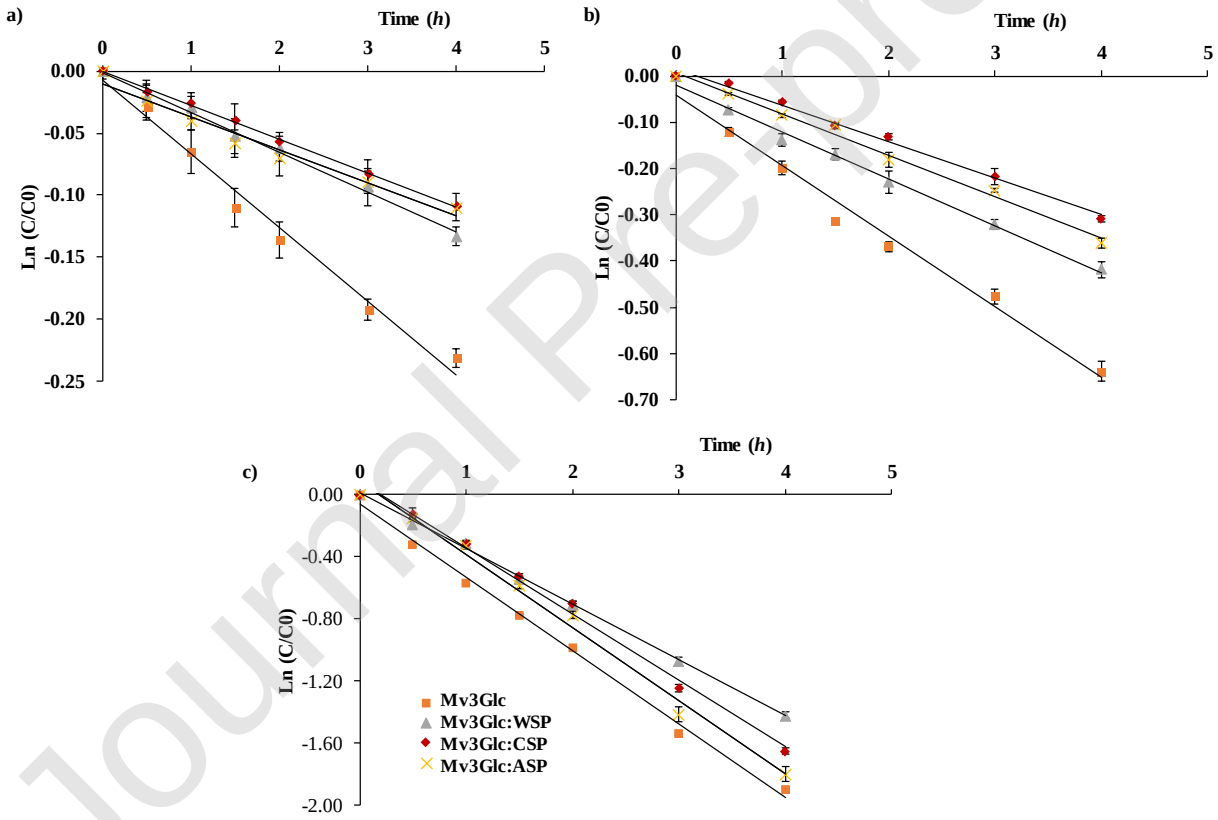


Figure 3. Degradation of mv3Glc and mv3Glc-pectic polysaccharides (WSP, CSP and ASP) during thermal treatment at 60 (**a**), 80 (**b**) and 100 ° C (**c**). Data is presented as mean values ± standard deviation of n=3 trials.

The kinetic parameters of the thermal degradation of the four systems, rate constant (k), half-life value ($t_{1/2}$, the time needed for 50% degradation of anthocyanins), activation energy (Ea) are

summarized in **Table 3**. The increase of the degradation rate constant, *k* and the corresponding decrease of the $t_{1/2}$ values could be observed at 60, 80 and 100ºC temperature for the four systems (both control and binding systems). At 60º C, anthocyanins thermal stability was significantly improved in the presence of the three pectic polysaccharides compared to stability of the model solutions with malvidin-3-*O*-β-D-glucoside alone (control). This resulted on lower *k* values and consequently higher $t_{1/2}$ values, demonstrating mv3Glc significantly higher thermostability in the presence of pectic polysaccharide fractions. The stability ranking was in agreement with the affinity constants measured with chelator and acid soluble polysaccharide fractions showing the highest thermal protection (no significant differences), followed by water soluble fraction (CSP≈ASP>WSP>Mv3Glc) (**Table 3**). After 4 hours, the relative total anthocyanin degradation (expressed relatively to anthocyanin initial content) in the presence of pectic polysaccharides was about 10-12% compared to 21% of degradation for the corresponding model solution without pectic polysaccharides. At 80º C, best stabilization was obtained with model solutions containing chelator soluble pectic polysaccharides, followed by acid and water soluble fractions (CSP>ASP>WSP>Mv3Glc). The relative total anthocyanin degradation was approximately two-fold lower with pectic polysaccharides at 60 and 80º C. The formation of anthocyanin-pectin complexes allowed the chemical protection of mv3Glc chromophore, possibly shielding the highly electrophilic C2-position of the flavylium cation, easily attacked by water and subsequent chemical degradation (Ø. Andersen & Markham, 2006). Electrostatic interactions, as the starting event followed by hydrophobic π-interactions and hydrogen bonds with pectic polysaccharides backbone are responsible for anthocyanin-pectic polysaccharides interactions and higher thermal stability of mv3Glc. Additionally, the less branched polysaccharides structures should allow the creation of some nucleation sites for anthocyanin π–π stacking (A. Fernandes et al., 2014; Padayachee et al., 2012; Phan et al., 2017). For more branched polysasaccharide structures, steric hindrance or entanglements due to side chains should impair the binding resulting in the lack of protection (Larsen et al., 2019).

On the other hand, at 100º C, only fortification with water and chelator soluble polysaccharides resulted on a significant mv3Glc thermostabilization, with the total anthocyanin degradation being only 10 and 5% lower than the control sample (WSP>CSP>ASP≈Mv3Glc). Addition of acid soluble polysaccharides had no effect on preventing mv3Glc thermal degradation. In general, it is accepted that the maximum stability of pectic polysaccharides is found around pH 4. However, at elevated temperatures, pectic polysaccharide degradation may occur due to the hydrolytic cleavage of the glycosidic linkages, eventually also through β-elimination of high methylesterified galacturonic acid residues (Lopes da Silva & Rao, 2006). At 100º C, cleavage of pectic polysaccharides, even if only containing 25% of methylesterified galacturonic acid residues, could potentially be occurring, resulting on a lower mv3Glc thermal protection. On the other hand, mv3Glc-pectic polysaccharides intermolecular binding could also be affect by the increase of temperature, resulting on a weaker thermal protection due to the destruction of anthocyanin-pectic polysaccharides complexes. In fact, temperature increase could adversely affect electrostatic interactions and hydrogen bonding, with this resulting on a lower association between these biomolecules and consequently a lower thermal protection.

The dependence of the temperature degradation of mv3Glc in the presence of pectic polysaccharides was also determined through the calculation of the activation energy (*Ea*). Generally, higher *Ea* reaction values are more susceptibe to temperature alteration (Li et al., 2013). The *Ea* value of the binding systems increased from 62.3 kJ.mol^{-1} (WSP) to 71.9 and 77.7 kJ.mol^{-1} for CSP and ASP, respectively. Thus, it seemed that the susceptibility to temperature alteration of mv3Glc in the presence of chelator and acid soluble pectic polysaccharides is higher than for water soluble. In comparison to mv3Glc alone (52.5 kJ.mol^{-1}), these values were superior, which might indicate that they had faster degradation kinetics whith temperature increase. However, the *k* and $t_{1/2}$ parameters demonstrated higher thermostability of the interacting systems than that of mv3Glc. It can be assumed that the mechanism of thermal degradation of mv3Glc is also affected by the chemical degradation of pectic polysaccharides.

Table 3. Kinetic parameter of the thermal degradation of malvidin-3-*O*-β-D-glucoside and the binding systems with water (WSP), chelator (CSP) and acid (ASP) pectic polysaccharides fractions at different temperatures (60, 80 and 100º C); rel. TAD (%), relative total anthocyanin degradation expressed relative to the initial anthocyanin content; Results are expressed as mean values ± standard deviation of *n*=3 trials; For each temperature and column, different letters are statistically different.

Temperature	Samples	k (h^{-1})	$t_{1/2}$ (h)	R^2	rel. TAD (%)
60º C	Mv3Glc	0.060 ± 0.002^{a}	11.6 ± 0.5^{a}	0.9851	20.7 ± 0.6^{a}
	Mv3Glc-WSP	0.032 ± 0.002^{b}	22 ± 2^{b}	0.9936	12.5 ± 0.7^{b}
	Mv3Glc-CSP	$0.027 \pm 0.002^{b,c}$	$25 \pm 2^{b,c}$	0.9980	10 ± 1^{c}
	Mv3Glc-ASP	0.027 ± 0.002^{c}	26 ± 2^{c}	0.9681	$10.5 \pm 0.8^{b,c}$
80º C	Mv3Glc	0.152 ± 0.003^{a}	4.5 ± 0.1^{a}	0.9826	47 ± 1^{a}
	Mv3Glc-WSP	0.102 ± 0.003^{b}	6.8 ± 0.2^{b}	0.9936	34 ± 1^{b}
	Mv3Glc-CSP	0.079 ± 0.002^{c}	8.8 ± 0.2^{c}	0.9921	26.5 ± 0.5^{c}
	Mv3Glc-ASP	0.089 ± 0.001^{d}	7.75 ± 0.09^{d}	0.9912	30.3 ± 0.7^{d}
100º C	Mv3Glc	0.474 ± 0.002^{a}	1.464 ± 0.007^{a}	0.9956	85.1 ± 0.1^{a}
	Mv3Glc-WSP	0.358 ± 0.003^{b}	1.94 ± 0.01^{b}	0.9984	75.9 ± 0.3^{b}
	Mv3Glc-CSP	0.428 ± 0.004^{c}	1.62 ± 0.01^{c}	0.9923	80.9 ± 0.4^{c}
	Mv3Glc-ASP	0.47 ± 0.01^{a}	1.47 ± 0.03^{a}	0.9899	83.5 ± 0.8^{d}

4. Conclusions

The thermal stability of malvidin-3-*O*-β-D-glucoside due to grape skin pectic polysaccharides interaction was demonstrated in this study. From the degradation kinetic parameters, this protection was more significant at 60 and 80º C and with the chelator soluble fraction. This higher thermal stability could be attributed to the increased binding affinity of this fraction to mv3Glc as a result of specific structural features such as high linearity and less neutral sugar sidechains. According to the ^{1}H-NMR data, these biomolecules interacted through electrostatic interactions, possibly further stabilized through hydrophobic effect (π-interaction).

The potential use of grape skins as an innovative source of pectic polysaccharides was demonstrated. This represent an additional economic and environmental valorization of wine industry byproducts, besides their traditional use as anthocyanins source. The results obtained reinforce the importance of the detailed knowledge of pectic polysaccharides structure-

functionality aiming to predict and optimize anthocyanin-polysaccharides interaction possibly leading to better processing methodologies and food products with increased anthocyanins stability.

Author Statement

- Ana Fernandes conceptualized and performed the experiments described in this paper, analyze the data and wrote the paper.
- Elsa Brandão, Filomena Raposo and Élia Maricato performed sugar analysis and characterization.
- Joana Oliveira analyzed the data.
- Manuel A. Coimbra supervised the experiments related to polysaccharides characterization, analyzed the data and co-wrote the paper.
- Nuno Mateus and Victor de Freitas supervised the experiments, analyzed the data and co-wrote and edited the paper.

All authors discussed the results and commented on the manuscript. No conflicts of interest were identified.

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