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Storage stability of phenolic compounds in powdered BRS Violeta grape juice microencapsulated with protein and maltodextrin blends



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ABSTRACT

The stabilities of the phenolic compounds, antioxidant activity and colour parameters of microencapsulated powdered BRS Violeta red grape juice were evaluated throughout storage at 5, 25 and 35 °C for up to 150 days. Different soy protein (S) or whey protein (W) blends with maltodextrin (M) were used as carrier agents, added at diverse concentrations and proportions. The treatment combining S and M with the highest carrier agent concentration (1SM) preserved almost all the anthocyanins. Except for 1SM, the proportion of *p*-coumaroylated anthocyanins increased during storage, and the flavonol content of the 1SM powder decreased after 150 days. The hydroxycinnamate content decreased for all treatments, independent of storage temperature, and flavan-3-ols were lost at 35 °C. The time and temperature did not influence the antioxidant activity of the powder or the colour of the reconstituted grape juice after 150 days.

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1. Introduction

Grapes are one of the most important natural sources of phenolic compounds. The grape BRS Violeta is a hybrid cultivar, obtained in 2006 from a cross between BRS Rubea and IAC 1398-21 by the Brazilian Agricultural Research Corporation (*Empresa Brasileira de Pesquisa Agropecuária*, EMBRAPA). This cultivar contains high levels of anthocyanins and other polyphenols, becoming an alternative to produce highly coloured and antioxidant-rich grape juice (Lago-Vanzela, Da-Silva, Gomes, García-Romero, & Herмосín-Gutiérrez, 2011). A previous study demonstrated that the BRS Violeta grape is a good source of phenolic compounds, mainly found in the skin, such as anthocyanins (3930 mg/kg), flavonols (150 mg/kg), hydroxycinnamic acid derivatives (120 mg/kg) and proanthocyanidins (670 mg/kg) (Rebello et al., 2013).

Anthocyanins are red coloured pigments, and their extracts are increasingly attractive to the food industry as natural alternatives to synthetic dyes. In addition, anthocyanins and other polyphenols provide health benefits due to their high biological activity,

including antioxidant, anti-inflammatory, antibacterial and antiviral functions. Furthermore, they can provide favourable effects in the protection against chronic diseases, including cancer and cardiovascular and neurodegenerative pathologies (Fang & Bhandari, 2010).

Nevertheless, the application of anthocyanins as natural colorants on a commercial scale is limited because of their relatively low stability to processing and storage conditions. During the storage of grape juice powders the degradation of sensitive components may occur, the losses depending on the temperature, pH, exposure to oxygen, porosity, light and the presence of organic acids (Estupiñan, Schwartz, & Garzón, 2011). Knowledge of the degradation kinetics of the anthocyanins is a very important factor in the prediction of food quality loss. Several studies have shown that the degradation of anthocyanins follows first order kinetics (Flores, Singh, & Kong, 2014; Idham, Muhamad, & Sarmidi, 2012; Robert et al., 2010; Souza et al., 2014).

Microencapsulation techniques have been widely used by the industry to protect food ingredients against degradation. In this process, the immobilization and incorporation of biologically active compounds inside solid particles (microspheres) occurs, providing stability of the compound structure and its protection.

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In addition, microencapsulation facilitates light- and heat-labile molecules, such as anthocyanins, to increase their stability and lengthen their shelf life (Cavalcanti, Santos, & Meireles, 2011).

Spray drying is the most common technique applied in microencapsulation, and is an alternative for the production of grape juice powder with high anthocyanin content. The choice of polymers as the carrier agent, which acts as the wall material, strongly determines the stability of the microparticles and the degree of protection of their active core. The efficacy of maltodextrin is due to its rapid film or shell forming property and the relatively low water diffusivity in these films. On the other hand, proteins form smooth and non-sticky films or shells much earlier than maltodextrin, and the powder recovery is higher when a small amount of protein is used (Adhikari, Howes, Wood, & Bhandari, 2009). The mixture of maltodextrin and protein favours the protection of bioactive compounds (Nesterenko, Alric, Silvestre, & Durrieu, 2013) and combines the specific properties of each polymer.

Previous research has shown that, during storage at 60 °C, the degradation of polyphenols and anthocyanins in fresh juice was faster than in microencapsulated juice (Robert et al., 2010). Mahdavee Khazaei, Jafari, Ghorbani, and Hemmati Kakhki (2014) verified the strong protective effect of encapsulation and wall materials against heat with respect to the stability of anthocyanins. Idham et al. (2012) noted that the combination of two carrier agents (maltodextrin and gum Arabic) resulted in the lowest anthocyanin degradation rate, when compared to the use of a single carrier agent.

Due to all of their benefits, the use of novel and inexpensive technologies has encouraged the food industry to increase the shelf life of grape juice, as well as to preserve its unstable compounds. Based on these considerations, the aim of this study was to investigate the stability of the phenolic compounds, antioxidant activity and colour parameters of BRS Violeta grape juice microencapsulated by spray drying with whey protein concentrate/maltodextrin and soy protein isolate/maltodextrin blends. The study was extended over a storage period of 150 days at three different storage temperatures, namely 5, 25 and 35 °C.

2. Material and methods

2.1. Materials

BRS Violeta grape was provided by EMBRAPA Grape and Wine (Jales, Brazil). Whey protein concentrate 80% (Alibra, Brazil), soy protein isolate 92.8% (Tovani Benzaquen, Brazil) and maltodextrin DE-10 MOR-REX 1910 (Corn Products, Brazil) were used as the carrier agents.

All the solvents were of HPLC quality and all the chemicals used were of analytical grade (>99%). The water was of Milli-Q quality. For identification of the phenolic compounds, the following commercial standards from Phytolab (Vestenbergsgreuth, Germany) were used: malvidin 3-glucoside, malvidin 3,5-diglucoside, peonidin 3,5-diglucoside, *trans*-piceid, *trans*-caftaric acid, (–)-epigallocatechin and (–)-gallocatechin. The following commercial standards from Extrasynthese (Genay, France) were also used: cyanidin 3-glucoside, cyanidin 3,5-diglucoside, procyanidins B1 and B2, kaempferol, quercetin, isorhamnetin, myricetin, syringetin and the 3-glucosides of kaempferol, quercetin, isorhamnetin and syringetin. In addition the following commercial standards from Sigma Aldrich (Tres Cantos, Madrid, Spain) were used: *trans*-resveratrol, caffeic acid, (+)-catechin, (–)-epicatechin, (–)-epicatechin 3-gallate and (–)-gallocatechin 3-gallate. Other non-commercial flavonol standards, such as myricetin 3-glucoside, quercetin 3-glucuronide and laricitrin 3-glucoside, were previously

isolated from Petit Verdot grape skins (Castillo-Muñoz et al., 2009) and procyanidin B4 was kindly supplied by Prof. Fernando Zamora (Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Spain).

All the standards were used for identification and quantitation through calibration curves covering the expected concentration ranges. For non-available standards, the quantitation was done using the calibration curve of the most similar compound: malvidin 3,5-diglucoside for the 3,5-diglucoside anthocyanin type, malvidin 3-glucoside for the 3-glucoside type, quercetin 3-glucoside for the flavonol 3-glycosides and their free aglycones, caffeic acid for the hydroxycinnamic acid derivatives, (+)-catechin for polymeric flavan-3-ols (total proanthocyanidins), individual flavan-3-ol monomers and dimers by their corresponding standards, considering their total sum as (+)-catechin equivalents.

2.2. Grape juice preparation

The grape juice was prepared by steam extraction at temperatures in the range of 75–85 °C for 60 min. The juice was filtered through a 270 mesh on the Tyler standard screen scale to remove potassium bitartrate crystals, and then stored in a freezing chamber at –18 °C until used. The grape juice had a total solids content of 15.18 ± 0.02 g/100 g (w/w), 14.0 ± 0.1 °Brix, pH 3.84 ± 0.006 , acidity (% tartaric acid) of 0.60 ± 0.004 , ash of 0.43 ± 0.004 g/100 g (w/w) (AOAC, 2006), reducing sugar content of 12.32% and $1965.38 \text{ mg/l} \pm 3.74$ of total anthocyanins.

2.3. Microencapsulation

The grape juice was spray dried in a mini spray dryer (model B-290, Büchi, Switzerland) using the following processing conditions: inlet air temperature 140 °C, feed flow rate 2 ml/min and air flow 500 l/h.

Blends of whey protein concentrate (W) or soy protein isolate (S) with maltodextrin (M) were used as the carrier agents. The following carrier agent concentration (CAC = g of carrier agent/g of juice soluble solids) and the percentage (w/w) of protein in the carrier agent (g dry protein/100 g total carrier agent) used were: 1SM, CAC 1.25, 10.00%; 2SM, CAC 1.00, 5.86%; 1WM, CAC 0.75, 30.00%; and 2WM, CAC 0.85, 20.00%. The carrier agents were added to the juice with magnetic stirring until complete dissolution.

These protein/maltodextrin systems were selected based on a previous study in which the carrier agent concentrations and proportion of protein in the microencapsulated grape juice were evaluated (Moser, Souza, & Telis, 2016). In the samples formulated with the WM blend, the CAC varied between 0.15 and 0.85, whereas with the SM blend the CAC values ranged from 0.65 to 1.35. In this study, it was observed that high carrier concentrations resulted in higher drying yields and better powder properties, such as an increase in anthocyanin retention. Moreover, higher carrier concentrations improved the encapsulation efficiency and resulted in a more suitable morphology, whereas lower carrier concentrations showed poor microcapsule formation ability. Increasing the carrier concentration and protein/carrier agent ratio resulted in brighter powders, but the rehydrated juice presented a colour similar to that of the fresh juice. The use of larger amounts of carrier resulted in better anthocyanin microencapsulation of properties.

2.4. Storage stability of the grape juice powders. Influence of time and temperature on phenolic composition, antioxidant activity and colour

One gram portions of the powders were packed into metallized plastic bags, sealed under vacuum, and stored in a refrigerator or incubator at three different temperatures: 5 °C, representing the

refrigeration temperature (aiming at the use of the powder as an additive in low temperature stored products), 25 °C, representing room temperature, and 35 °C, which is one of the temperatures recommended for accelerated shelf life studies (Tonon, Brabet, & Hubinger, 2010).

The samples were analyzed after the following storage times: 0, 7, 15, 30, 45, 60, 90, 120, and 150 days, with respect to the contents of individual, total and polymeric anthocyanins, the antioxidant capacity and the colour parameters. Flavonols, hydroxycinnamic acid derivatives, flavan-3-ol monomers, B-type dimers and the total proanthocyanidin content were only analyzed at zero time (0 day) and the final (150 days) storage time. The analyses were carried out in triplicate (three bags were used for each storage time analyzed) and the results were expressed as mg/100 g of dry juice matter (excluding the mass of the carrier agents). Just before the analyses, the juice was reconstituted by mixing the necessary mass of juice powder with distilled water in order to always maintain the same proportion between the solids coming from the fresh grape juice and water. Before carrying out any tests, the residual insoluble particles of the carrier agents were removed from the juice by centrifugation (14,000 rpm) or filtration (0.20 µm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany).

2.4.1. Anthocyanins

The polymeric anthocyanin content (expressed as a percentage with respect to the total anthocyanins) was estimated using the sulfur dioxide bleaching method with a Shimadzu UV spectrophotometer (UV-1800, Kyoto-Japan) (Ribéreau-Gayon & Stonestreet, 1965). The concentration (mg/l) was determined from a calibration curve obtained with standard malvidin-3,5-diglucoside solutions.

The degradation kinetics was calculated from the total anthocyanins as analyzed by HPLC. The reaction rate constants (k) and half-life time ($t_{1/2}$) were calculated from the first-order kinetics using the following equations:

$$-\ln\left(\frac{C_t}{C_0}\right) = kt \quad (1)$$

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

where C_0 is the initial anthocyanin content, and C_t is the anthocyanin content at reaction time (t).

The variation of k as a function of the temperature fitted the Arrhenius-type equation well (Eq. (3)), allowing for the calculation of the activation energy (E_a).

$$\ln k = \ln k_0 - \frac{E_a}{RT} \quad (3)$$

where E_a is the activation energy, R is the ideal gas constant and T is the absolute temperature.

2.4.2. HPLC–DAD–ESI–MSⁿ analysis of phenolic compounds

The HPLC separation, identification and quantitation of the grape juice phenolic compounds were carried out using an Agilent 1100 Series system (Agilent, Germany) equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI–MSⁿ) system, coupled to an Agilent ChemStation (version B.01.03) data-processing unit. The mass spectra data were processed using the Agilent LC/MS Trap software (version 5.3).

Anthocyanins, flavonols and hydroxycinnamic acid derivatives from the microencapsulated grape juice were separately analyzed after the adaptation of previously described methods (Castillo-Muñoz, Gómez-Alonso, García-Romero, & Hermosín-Gutiérrez,

2007; Castillo-Muñoz et al., 2009) to the use of narrow bore, smaller particle size, chromatography columns (Rebello et al., 2013).

For the analysis of anthocyanins, 20 µl of the reconstituted juice was injected directly into the HPLC system after filtration (0.20 µm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany) and dilution (1:4) with 0.1 N HCl. For flavonols and hydroxycinnamic acid derivatives, anthocyanin-free extracts were obtained by SPE on Bond Elute Plexa PCX (Agilent; 6 cm³, 500 mg of adsorbent) cartridges (Castillo-Muñoz et al., 2007).

The anthocyanin and non-anthocyanin compounds were analyzed according to a previously described method (Lago-Vanzela et al., 2011). The juice samples were injected (10 µl for anthocyanin analysis and 20 µl for non-anthocyanin flavonol analysis) into a Zorbax Eclipse XDB-C18 reversed-phase column (2.1 × 150 mm; 3.5 µm particle; Agilent, Germany) maintained at 40 °C.

An ion trap ESI–MS/MS detector was used in both positive (anthocyanins) and negative (non-anthocyanin) ion modes for identification with the following parameters: dry gas, N₂, 8 l/min; drying temperature, 325 °C; nebulizer, N₂, 50 psi; ionization and fragmentation parameters optimized by direct infusion of appropriate standard solutions (malvidin 3,5-diglucoside in positive ionization mode; quercetin 3-glucoside and caffeic acid in negative ionization mode); scan range, 50–1200 m/z . The identification was based mainly on spectroscopic data (UV–vis and MS/MS) obtained from authentic standards or using previously reported data (Barcia, Pertuzatti, Gómez-Alonso, Godoy, & Hermosín-Gutiérrez, 2014; Lago-Vanzela et al., 2013, 2014; Nixdorf & Hermosín-Gutiérrez, 2010; Rebello et al., 2013). For quantification, DAD-chromatograms were obtained at 520 nm (anthocyanins), 360 nm (flavonols) and 320 nm (hydroxycinnamic acid derivatives) and calibration curves were obtained for the most representative compound of each type of phenolic compound: malvidin 3,5-diglucoside for anthocyanins, quercetin 3-glucoside for flavonols and caffeic acid for hydroxycinnamic acid derivatives. The analyses were carried out in duplicate for each of the triplicate samples.

2.4.3. Flavan-3-ol monomers and B-type procyanidin dimers

For the analysis of the flavan-3-ol monomers and procyanidin B-type dimers from microencapsulated grape juice, 0.30 ml of the reconstituted juice was diluted with 1.5 ml of water:formic acid (98.5:1.5) in a chromatographic vial that was sealed and used for direct injection into the HPLC system. The analysis was carried out using a HPLC Agilent 1200 series system equipped with DAD (Agilent, Germany) and coupled to an AB Sciex 3200 TRAP (Applied Biosystems) with a triple quadrupole, turbo spray ionization (electrospray assisted by thermo-nebulization) mass spectroscopy system (ESI–MS/MS). The chromatographic system and mass spectra data treatment were managed by Analyst[®] Software (Applied Biosystems, version 1.5). The diluted samples were injected (20 µl) into an Ascentis C18 reversed-phase column (150 mm × 4.6 mm with 2.7 µm of particle size), maintained at 16 °C. The solvents and gradients used for this analysis, the two types of MS scan used (Enhanced MS – EMS and Multiple Reaction Monitoring – MRM), as well as all the mass transitions (m/z) for identification and quantitation were those previously described (Lago-Vanzela, Da-Silva, Gomes, García-Romero, & Hermosín-Gutiérrez, 2011). For quantification, an external catechin standard was injected at the beginning and end of every samples injection sequence. The mean value of the areas obtained for this external standard was used for the correction of the response factors previously obtained for every compound analyzed (flavan-3-ol monomers and procyanidin B-type dimers) with respect to catechin in a series of

standard solutions containing a fixed concentration of catechin and variable concentrations of each compound analyzed.

2.4.4. Antioxidant activity

The antioxidant capacity was determined by reaction with a methanolic solution ($6 \times 10^{-5} \text{ mol l}^{-1}$) of the DPPH (2,2-diphenyl-1-picrylhydrazyl, Fluka Chemie) radical after 25 min of reaction, and quantified using calibration curves prepared with methanolic solutions of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Fluka Chemie), under the same conditions (Brand-Williams, Cuvelier, & Berset, 1995).

2.4.5. Colour parameters

The colour parameters of the reconstituted grape juice were measured using a Portable Spectrophotometer CM-2500c (Konica Minolta Inc.) calibrated for the CIE Lab colour coordinates, with a D65 illuminant and 10° observation angle. In this system, L^* denotes lightness on a 0–100 scale from black to white; a^* , (+) red and (–) green colour components; and b^* , (+) yellow and (–) blue colour components. The total colour differences (ΔE^*) were calculated at each storage time evaluated, with respect to the colour at the start of storage, using the following equation:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (4)$$

2.5. Statistical analysis

Statistical analyses were carried out using the SAS System for Windows version 9.0. The regression analysis was used to correlate the total or polymeric anthocyanins, antioxidant activity and colour with respect to the time of storage at different temperatures. The ANOVA analysis, followed by the Student t test, was applied to discriminate the differences between the concentrations of flavonols, hydroxycinnamic acid derivatives and flava-3-ols (monomers and dimers) at the start (0 days) and end of the storage period (150 days). The relationship between individual anthocyanins and the storage conditions was determined using the principal component analysis (PCA).

3. Results and discussion

3.1. Evolution of phenolic compounds during storage

3.1.1. Anthocyanins

The grape juice was microencapsulated with carrier agents of varied compositions, thus presenting different values for the retention and encapsulation efficiency of the anthocyanins. The total anthocyanins in the grape juice powders, obtained at the start of the storage time (0 day), were analyzed by HPLC and expressed as mg/100 g powdered juice, with: 1SM = 764; 2SM = 993; 1WM = 970; 2WM = 585. In order to study the effect of time and temperature separately, the amounts of anthocyanins at the start of the storage time were considered as 100%, excluding the differences in anthocyanin concentrations among the four treatments.

All the powdered juices stored at 5°C showed the smallest losses of anthocyanins (2–11%), which suggested that microencapsulation, combined with storage at low temperature, helped maintain the majority of the anthocyanins contained in the product (Fig. 1). Only the 1WM treatment presented a correlation ($p < 0.05$) between the decrease of the anthocyanin content and the storage time at 5°C .

For all treatments, storage at 25 and 35°C showed linear correlations between time and the decrease of anthocyanin content. After 120 days of storage, the 1SM treatment maintained more

than 90% of the initial content of anthocyanins but, after 150 days, a more pronounced reduction was observed (Fig. 1A).

The grape juice powder formulated with 1SM, which had the largest amount of carrier agent ($\text{CAC} = 1.25$) and a relatively low protein ratio (10%) when compared with the WM treatments (30% in 1WM and 20% in 2WM), appeared to show the best ability for the preservation of the anthocyanins. On the other hand, the 1WM treatment, which had the lowest amount of carrier agent ($\text{CAC} = 0.75$) and the highest whey protein ratio (30%), did not protect the anthocyanins well. The improvement in stability of the samples formulated with larger amounts of carrier agents and lower protein proportions and, consequently, with a greater amount of maltodextrin, can be associated to the complexing of the flavylum cation form of the anthocyanins with dextrans, preventing their transformation to other less stable forms. Furthermore, maltodextrin has an additional stabilizing effect due to its ability to reduce reactant mobility (Estupiñan, Schwartz, & Garzón, 2011). According to Souza et al. (2014) the samples containing less carrier agent are more hygroscopic and therefore absorb more water, facilitating the degradation reactions. In a previous study (Moser et al., 2016), a greater encapsulation efficiency of the anthocyanins was observed when using SM blends instead of WM blends. These results may help to explain why the use of SM showed greater protection of the anthocyanins than samples formulated with WM.

Understanding degradation mechanisms is important to maximize the nutritional and sensory quality of products. Thus, the degradation kinetics of the anthocyanins was monitored throughout the storage period, and the reaction rate constant (k) and half-life time ($t_{1/2}$) parameters are shown in Table 1. The anthocyanins of the juice powder followed first-order kinetics, showing linear degradation with respect to time. The treatments 2SM, 1WM and 2WM stored at 35°C showed greater anthocyanin degradation than samples stored at 25°C . The opening of the pyrylium ring and formation of a chalcone can be the first step in the degradation of anthocyanins (Patras, Brunton, O'Donnell, & Tiwari, 2010).

The 1WM treatment, which contained the largest amount of whey protein in the blend, presented the highest degradation rate, suggesting that this carrier agent is not sufficiently effective to protect the anthocyanins. Flores et al. (2014) observed the disadvantage of using only whey protein as the wall material as compared to combining it with polysaccharides. Comparing the temperatures of 25 and 35°C , the 1SM treatment stored at 35°C presented the lowest degradation rate and the longest half-life time (approximately 18 months). These results suggest that 1SM is more suitable for microencapsulating and preserving the anthocyanins of grape juice stored at temperatures of 25 and 35°C . Lago-Vanzela et al. (2014) studied the accelerated aging of wines made from BRS Violeta grapes for 120 days, and found higher reaction rate constants than found in the present study, with k values of 0.0078 and 0.0140 at storage temperatures of 25 and 35°C , respectively.

The activation energy (E_a) values for the microencapsulated grape juice are shown in Table 1. The highest E_a value was found for the 1WM treatment, followed by 2WM, 1SM and 2SM, respectively. The results suggest that the activation energy was influenced by the concentration of maltodextrin. The two soy protein/maltodextrin blends, which had higher concentrations of carrier agents and higher ratios of maltodextrin in the mixture, presented smaller E_a values, when compared to the whey protein/maltodextrin blends.

A similar result was found by Carrillo-Navas et al. (2011) for passion fruit juice microencapsulated using mesquite gum, gum arabic and maltodextrin blends, and stored at 25, 35 and 40°C . The authors observed that for concentrations of 17% and 66% of maltodextrin in the blends, the E_a values were 30.6 kJ/mol and

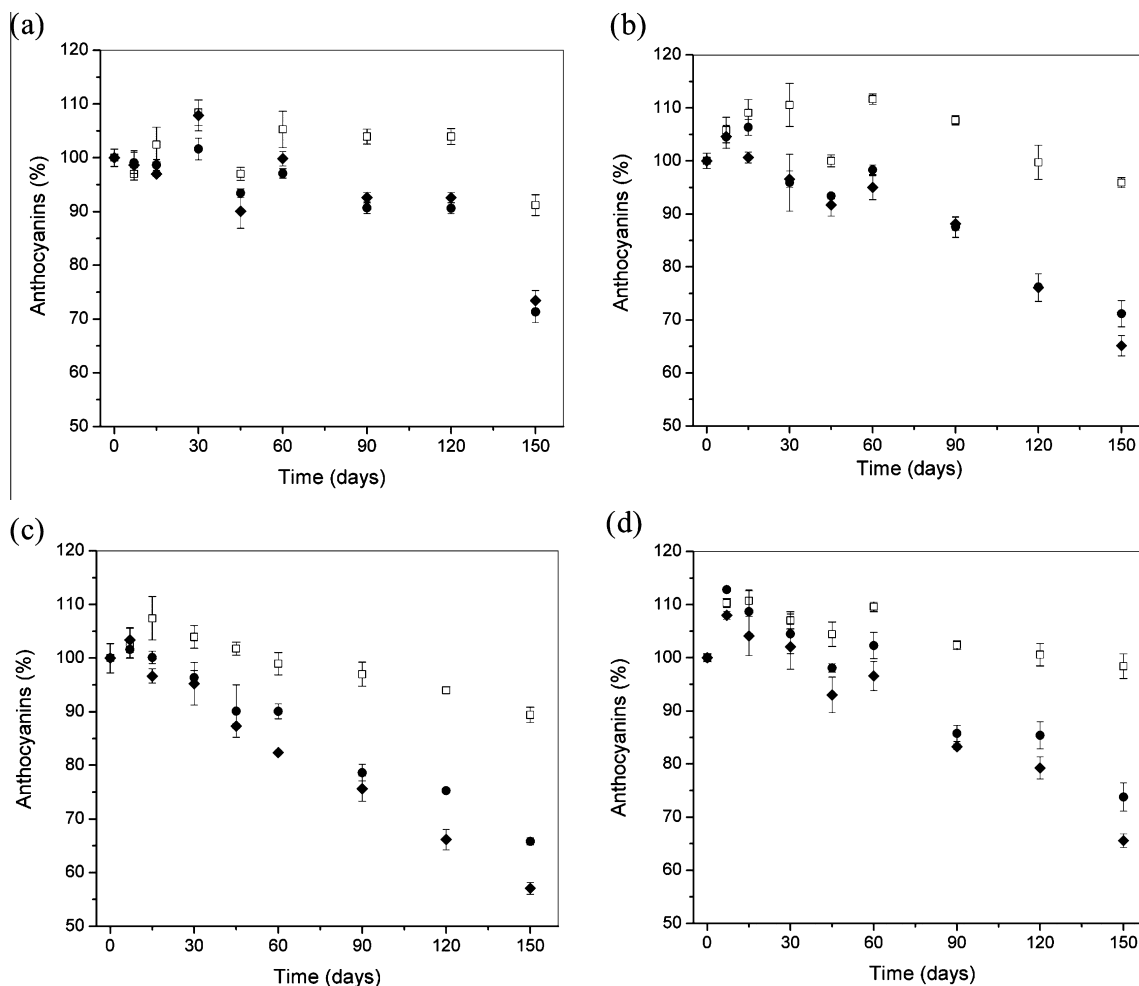


Fig. 1. Total anthocyanins in grape juice microencapsulated with: (a) 1SM; (b) 2SM; (c) 1WM; and (d) 2WM, storage at \square 5, \bullet 25 and \blacklozenge 35 °C, for 150 days.

Table 1

Degradation kinetic parameters and activation energy (E_a) of microencapsulated anthocyanins in powdered grape juice stored at 5, 25 and 35 °C for 150 days.

Treatment	Temperature	k (days ⁻¹)	$t_{1/2}$ (days)	R^2	E_a (kJ/mol)
1SM	5 °C	0.0006	1195	0.90	20.85
	25 °C	0.0015	471	0.77	
	35 °C	0.0013	545	0.62	
2SM	5 °C	0.0012	573	0.93	14.83
	25 °C	0.0019	361	0.86	
	35 °C	0.0022	309	0.89	
1WM	5 °C	0.0011	654	0.95	29.12
	25 °C	0.0027	252	0.97	
	35 °C	0.0035	198	0.98	
2WM	5 °C	0.0007	950	0.83	24.66
	25 °C	0.0014	495	0.60	
	35 °C	0.0021	330	0.83	

19.9 kJ/mol, respectively. The activation energy for diffusion may be described as the energy required to create a “hole” large enough to accommodate a diffusing molecule. The hole is created by the separation of polymer segments as the diffusing molecule moves through them (Soottitantawat et al., 2005).

The contribution of polymeric anthocyanins to the total anthocyanin content was estimated from the degree of bisulfite bleaching, which is greatly affected by the protein type used as the carrier agent in conjunction with maltodextrin. In the SM treatments (from 4 to 10.8% of total anthocyanins), the low polymerization

did not present a linear correlation between the polymeric anthocyanins and the storage time or temperature (see Supplementary material, Fig. S1).

In contrast, for the treatment with WM, there was a positive linear correlation between the polymerized anthocyanins and the storage time, but no correlation with temperature was found. The storage time resulted in an increase in anthocyanin polymerization from 8.7 to 33.6%, which was greater in the first 60 days of storage, and then followed by an apparent stabilization. Nevertheless, at the end of the storage time, the amount of polymerized anthocyanins increased again. An increase in polymeric pigments and losses of monomeric anthocyanins may be due to several factors, including residual enzymatic activity or condensation reactions between the anthocyanins and other phenolic compounds, such as flavan-3-ols (Brownmiller, Howard, & Prior, 2008). The residual enzymatic activity can be discarded because of the probable destruction of the enzymes by the heat treatments used during the grape juice extraction and drying processes.

The evolution of the main individual anthocyanins in the grape juice powder during storage was evaluated by HPLC. In both the fresh grape juice and the microencapsulated grape juice, 21 monomeric anthocyanins derived from delphinidin, cyanidin, petunidin, peonidin and malvidin were found. The chromatographic anthocyanin profile was practically the same at the start and end of storage, although a decrease in the intensity of the signal was evident (Fig. 2).

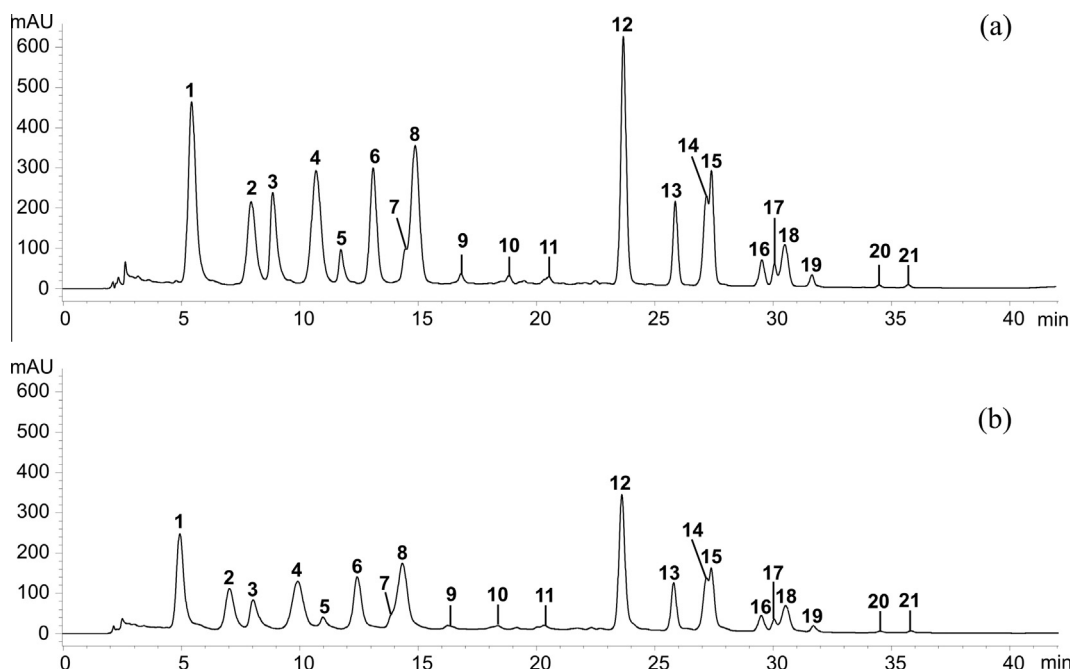


Fig. 2. Chromatographic profile obtained at 520 nm of the 1WM treatment stored at 35 °C: (a) zero storage time and (b) final storage time. Peak assignment: 1, dp-3,5-diglc; 2, cy-3,5-diglc; 3, dp-3glc; 4, pt-3,5-diglc; 5, cy-3glc; 6, pn-3,5-diglc; 7, dp-3-acglc-5glc; 8, mv-3,5-diglc; 9, pt-3glc; 10, mv-3glc; 11, dp-3-cfglc-5glc; 12, dp-3-cmglc-5glc; 13, cy-3-cmglc-5glc; 14, pt-3-cmglc-5glc; 15, dp-3cmglc; 16, pn-3-cmglc-5glc; 17, cy-3cmglc; 18, mv-3-cmglc-5glc; 19, pt-3cmglc; 20, mv-3cmglc. Abbreviations: dp, delphinidin; cy, cyanidin; pt, petunidin; pn, peonidin; mv, malvidin; 3,5-diglc, 3,5-diglucosides; 3-glc, 3-glucoside; 3-acglc-5-glc, 3-(6'-acetyl)-glucoside-5-glucoside; 3-cfglc-5-glc, 3-(6'-caffeoyl)-glucoside-5-glucoside; 3-cmglc-5-glc, 3-(6'-*p*-coumaroyl)-glucoside-5-glucoside; 3-cmglc, 3-(6'-*p*-coumaroyl)-glucoside.

Principal Component (PC) analysis was applied to the data with the aim of highlighting the relationships between individual anthocyanin percentages and the storage time or temperature. Fig. 3 shows the representation of samples corresponding to the treatments 1SM, 2SM, 1WM and 2WM in the planes determined by the first Principal Component (PC1) and second Principal Component (PC2), as well as the percentage of variance explained by each PC.

In the 1SM treatment, the percentages of *p*-coumaroylated derivatives of anthocyanidin-3-glucosides (3-glc) were more correlated with PC2. The mixture of these anthocyanins decreased in relation to the initial sample for all the temperatures and storage times, except for the sample stored for 90 days at 25 °C, which appears to be an anomaly. On the other hand, some *p*-coumaroylated derivatives of anthocyanidin-3,5-diglucosides (3,5-diglc) with negative loadings, together with non-acylated derivatives of both 3,5-diglc and 3-glc, with positive loadings, were more correlated with PC1. The mixture of anthocyanins correlated with PC1 is difficult to interpret, in addition, they are not grouped with a time criteria: at 5 °C there were no differences between these variables; at 25 °C the temporal logic was reversed, with samples that appeared after 150 days now appearing between 30 and 90 days; and at 35 °C there was no differentiation between the samples stored for 90 and for 150 days, and they showed a greater proportion of *p*-coumaroylated derivatives of anthocyanins 3,5-diglc than after only 30 days of storage.

In general, for the 2SM, 1WM and 2WM treatments some of the *p*-coumaroylated derivatives of the anthocyanins were more correlated with PC1 (positive loadings). Thus, with the increase in storage time, the proportion of *p*-coumaroylated anthocyanins in the anthocyanin profile increased. This trend was only observed for the temperature of 5 °C, but began to be apparent at a temperature of 25 °C and was clearly apparent after storage at 35 °C. This behaviour regarding the variables more correlated to PC1 can be explained by the greater stability of the acylated anthocyanins, and certainly by the ability of the *p*-coumaroyl residues to be

involved in the formation of inter- and intra-molecular copigmentation complexes (Lago-Vanzela et al., 2013; Patras, Brunton, O'Donnell, & Tiwari, 2010), which could contribute to an improvement in the stability of the anthocyanins. These results were in agreement with those observed by Lago-Vanzela et al. (2014) in their study of the accelerated aging of wines from BRS Violeta grapes. The authors reported that the half-life values for *p*-coumaroylated anthocyanidin 3,5-glucosides, aged at 50 °C, almost doubled in relation to their corresponding non-acylated derivatives.

In the 2SM treatment some non-acylated derivatives of anthocyanidin-3,5-diglucosides and anthocyanidin-3-glucosides were more correlated with PC1 and presented negative loadings, thus, as the storage time increased, these anthocyanins decreased their proportion in the anthocyanin profile. The *p*-coumaroylated anthocyanidin-3-glucosides and the non-acylated malvidin-3,5-diglucoside were more correlated to PC2, and presented similar behaviour to 1SM, which did not show any logical differentiation with temperature.

In the 1WM treatment, the PC2 allowed one to evaluate the effect of storage temperature on the anthocyanins. It was observed that each group of samples corresponding to a determined storage temperature was in the same PC axis, regardless of storage time. The anthocyanins that could differentiate the control (0 days of storage) from the stored samples, and could differentiate the storage temperature, were minority anthocyanins with common structural features: they are 3-glucosides and belong to the series of B-ring tri-substituted anthocyanins (pt-3-glc and dp-3-glc), having at least one *o*-diphenol grouping (1 in petunidin; 2 in delphinidin). The proportion of these two anthocyanins decreased as compared to the control, the decrease being similar at 5 °C and 25 °C, and greater at 35 °C.

For the 2WM treatment, the non-acylated anthocyanin derivatives (3,5-diglc and 3-glc) were more correlated with PC1 and presented negative loadings, thus their proportions decreased with

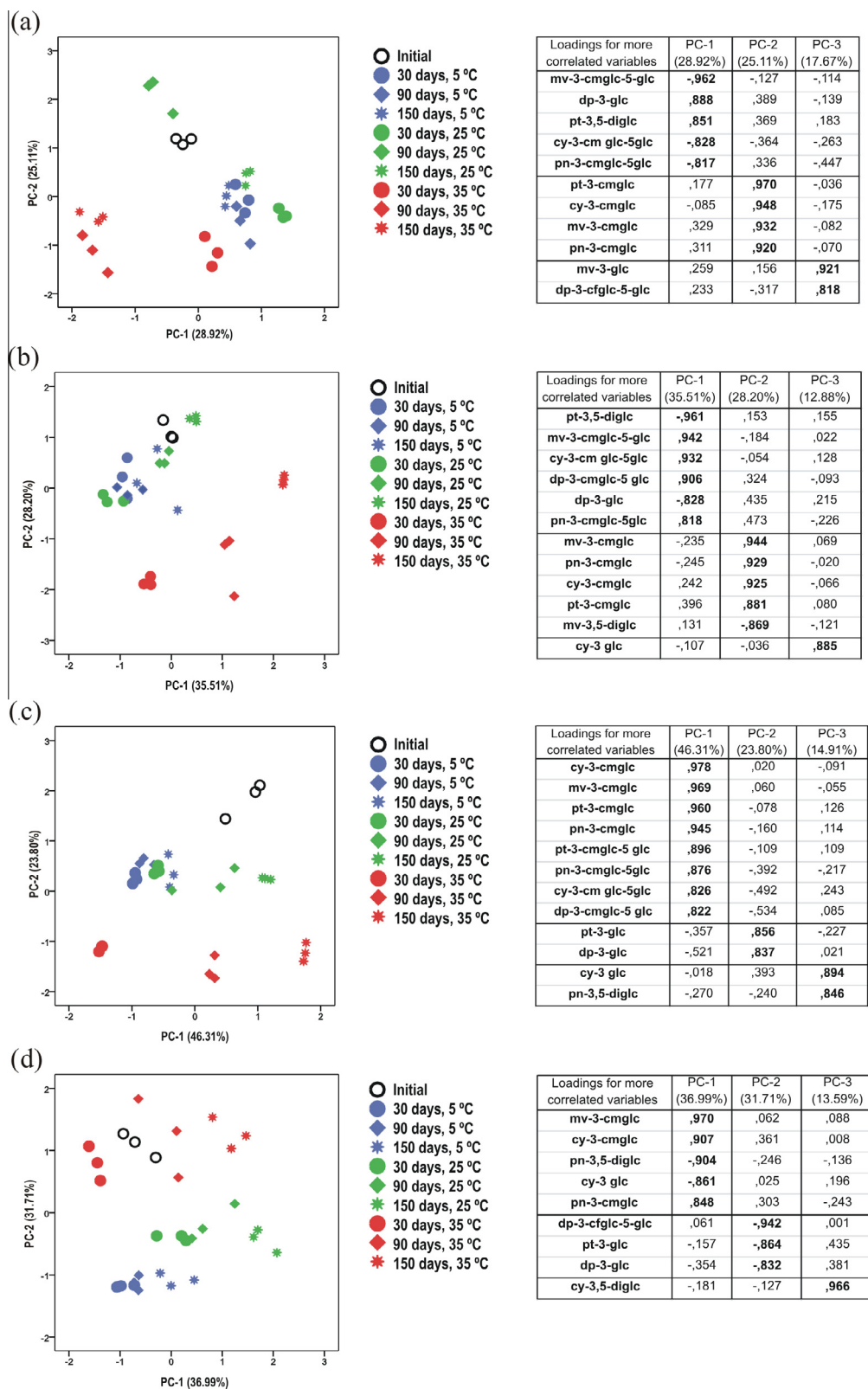


Fig. 3. Principal component analysis (plot of the samples in the PC-1 vs. PC-2 graph; tables of the “loadings” of the most correlated variables and percentage of total variance explained by each PC) as applied to individual anthocyanin percentages in relation to storage time and temperature in the treatments: (a) 1SM, (b) 2SM, (c) 1WM, (d) 2WM.

storage time. The caffeoyl derivatives of both anthocyanidin-3,5-glucosides and 3-glucosides were more correlated with PC2 and presented negative loadings, their proportions reducing with

increase in storage temperature. However, the anthocyanins in the samples stored at 35 °C did not differ from those of the control, which seems to be anomalous behaviour.

3.1.2. Flavonols

At zero time (0 days) seven flavonols were found in the microencapsulated grape juice: myricetin 3-glucoside, quercetin 3-galactoside, quercetin 3-glucuronide, quercetin 3-glucoside, free myricetin, laricitrin 3-glucoside, isorhamnetin 3-glucoside and syringetin 3-glucoside. The most important type of flavonol in the microencapsulated grape juice was based on myricetin (average of 77%), myricetin 3-glucoside being the main individual flavonol found (average of 70%), followed by the quercetin-based flavonols as the second most important type (average of 16%). The flavonol profile was the same at zero time and at the end of storage.

Table 2 shows the amounts of flavonols in the powdered grape juice at zero time and at the end of storage. The 1SM treatment showed a significant loss of flavonols at the end of storage at the three temperatures. The latter treatment contained the highest amount of added carrier agent together with a relatively high proportion of protein. Under these conditions, flavonols are limited in their ability to form copigment complexes with anthocyanins because the competing stabilizing action of maltodextrin and the presence of aromatic amino acid residues that can also act as copigments. Therefore, the flavonols could be more exposed to oxidation over storage, especially the most abundant myricetin-based flavonols (Barcia et al., 2014; Castillo-Muñoz et al., 2009; Lago-Vanzela et al., 2014). The treatments with 2SM and 2WM only showed significant losses when stored at 25 °C. On the other hand, the 1WM treatment did not lead to a significant loss of flavonols at any of the three storage temperatures, which means that this formulation was the most efficient in protecting this phenolic compound during the shelf life. An evaluation of the effect of temperature at the end of the storage period showed that temperature did not influence the amount of flavonols within the same treatment.

Table 2

Flavonol contents (as quercetin 3-glucoside equivalents), hydroxycinnamic acid derivatives contents (as caffeic acid equivalents) and flavan-3-ol monomer and dimer contents (B-type procyanidins) (as catechin equivalents) in microencapsulated grape juice stored at 5, 25 and 35 °C, at the start and end of storage.

Days	T (°C)		1SM	2SM	1WM	2WM	
<i>Flavonols (mg/100 g)</i>							
0	–		26.72 ± 0.68 ^a	29.68 ± 1.20 ^a	31.60 ± 1.62 ^a	29.89 ± 0.57 ^a	
	5		21.95 ± 0.40 ^b	25.06 ± 1.20 ^{ab}	25.95 ± 0.19 ^a	26.68 ± 1.48 ^{ab}	
	150	25	20.76 ± 1.63 ^b	22.68 ± 0.80 ^b	26.79 ± 1.46 ^a	25.58 ± 0.85 ^b	
		35	20.96 ± 1.62 ^b	26.38 ± 2.30 ^{ab}	25.29 ± 1.51 ^a	25.96 ± 1.99 ^{ab}	
<i>Hydroxycinnamic acid derivatives (mg/100 g)</i>							
0	–		32.75 ± 0.68 ^a	30.68 ± 1.18 ^a	24.26 ± 3.82 ^a	31.25 ± 1.51 ^a	
	5		22.54 ± 0.78 ^b	23.51 ± 1.57 ^b	14.72 ± 0.73 ^b	21.91 ± 2.03 ^b	
	150	25	21.94 ± 0.92 ^b	22.56 ± 1.74 ^b	11.47 ± 0.78 ^b	22.82 ± 1.49 ^b	
		35	19.30 ± 3.65 ^b	24.66 ± 1.06 ^b	15.27 ± 3.88 ^b	21.72 ± 1.99 ^b	
<i>Flavan-3-ol (mg/100 g)</i>							
0	–	Catechin	31.85 ± 3.47 ^a	89.63 ± 19.08 ^a	58.93 ± 6.37 ^a	37.20 ± 4.70 ^a	
	5		24.13 ± 3.05 ^b	31.17 ± 2.35 ^b	58.13 ± 4.76 ^a	35.38 ± 1.35 ^a	
	150		25	21.52 ± 5.89 ^b	22.88 ± 7.23 ^b	58.69 ± 3.74 ^a	36.32 ± 2.09 ^a
			35	19.78 ± 0.99 ^b	34.99 ± 1.77 ^b	42.74 ± 5.76 ^b	26.04 ± 1.71 ^b
0	–	Epicatechin	7.15 ± 1.09 ^a	18.64 ± 2.99 ^a	12.91 ± 1.88 ^a	8.39 ± 1.09 ^a	
	5		5.99 ± 1.20 ^{ab}	7.99 ± 0.37 ^b	13.55 ± 1.27 ^a	8.61 ± 0.61 ^a	
	150		25	5.57 ± 1.58 ^{ab}	4.31 ± 1.61 ^c	11.69 ± 0.95 ^a	6.79 ± 0.38 ^b
			35	4.87 ± 0.01 ^b	6.85 ± 0.80 ^{bc}	7.63 ± 1.24 ^b	4.86 ± 0.27 ^c
0	–	Procyanidin B1	19.62 ± 2.19 ^a	59.95 ± 9.09 ^a	43.67 ± 3.43 ^a	39.14 ± 5.19 ^a	
	5		15.53 ± 2.02 ^{ab}	25.88 ± 2.57 ^b	47.49 ± 3.73 ^a	33.56 ± 0.64 ^b	
	150		25	12.89 ± 3.95 ^b	15.06 ± 5.31 ^c	34.64 ± 1.87 ^b	26.88 ± 1.93 ^c
			35	11.06 ± 0.78 ^b	21.89 ± 2.31 ^{bc}	19.23 ± 2.74 ^c	17.50 ± 0.69 ^d
0	–	Procyanidin B2	6.31 ± 1.11 ^a	17.26 ± 2.08 ^a	13.69 ± 1.64 ^{ab}	12.68 ± 1.54 ^a	
	5		5.68 ± 0.90 ^{ab}	9.24 ± 0.90 ^b	15.60 ± 1.14 ^a	11.48 ± 0.86 ^a	
	150		25	4.48 ± 1.36 ^{ab}	4.66 ± 1.75 ^c	11.81 ± 0.19 ^b	8.67 ± 0.23 ^b
			35	4.21 ± 0.23 ^b	7.09 ± 0.93 ^{bc}	6.95 ± 0.77 ^c	5.51 ± 0.43 ^c

Means with different letters in the same column, for each type of compound, indicate significant differences ($p < 0.05$) between the beginning and end of the storage time according to the Student *t* test.

3.1.3. Hydroxycinnamic acid derivatives (HCADs)

The following nine hydroxycinnamic acid derivatives (HCADs) were found in the microencapsulated grape juice before the storage period (0 days): *trans*-caftaric acid, three isomers of caffeoyl-glucose (caffeoyl-glucose-1, caffeoyl-glucose-2 and caffeoyl-glucose-3), *trans*-coutaric acid, three isomers of *p*-coumaroyl-glucose (*p*-coumaroyl-glucose-1, *p*-coumaroyl-glucose-2 and *p*-coumaroyl-glucose-3) and free caffeic acid. The most important types of hydroxycinnamic acid derivatives found in the microencapsulated grape juice were *trans*-caftaric acid and *p*-coumaroyl-glucose. The HCADs profile did not change during storage.

All the samples showed significant losses of HCADs in the powder at the end of the storage period (Table 2). When the 1WM treatment was stored at 25 °C, a reduction of 56% was observed in the amount of hydroxycinnamic acid, showing that this carrier agent and temperature were not suitable for preserving HCADs during the shelf life. The explanation to these results could be related to the lowest CAC value in the 1WM powder that makes it more hygroscopic and, consequently, their HCADs are more exposed to oxidation, especially those derived from caffeic acid, which are the most abundant HCADs. In addition, the storage temperature did not influence the amount of remaining HCADs within the same treatment.

3.1.4. Flavan-3-ol monomers and dimers

The total amount of flavan-3-ols in grape juice is expected to be low. This kind of phenolic compound can be found as monomers, B-type procyanidin dimers and oligomeric and polymeric proanthocyanidins (also called condensed tannins). The latter compounds are poorly soluble in water, mainly located in solid parts of the grapes, namely the seeds and skins, and the grape juices obtained using the steam extraction method usually contain low amounts of condensed tannins (Yamamoto et al., 2015). The main

flavan-3-ol monomer found in microencapsulated grape juice was catechin, while the main flavan-3-ol dimer was procyanidin B1 (Table 2).

All the samples showed significant losses of catechin, epicatechin, procyanidin B1 and procyanidin B2 in the powder at the end of storage when stored at 35 °C. In general, the 2SM treatment presented the greatest loss in the amount of flavan-3-ols as compared to the other treatments, showing that this carrier agent is not suitable for preserving this kind of phenolic compound during the shelf life.

On the other hand, the 1WM and 2WM treatments showed no significant losses of catechin at the end of the storage time when stored at 5 and 25 °C. In addition, the 1WM treatment showed no loss of epicatechin under the same storage conditions and the 2WM treatment preserved the initial epicatechin content when stored at 5 °C. Thus, the samples formulated with WM were more efficient in protecting the flavan-3-ol monomers during the shelf life than the samples formulated with SM.

Similar results were found by Spanos and Wrolstad (1990) for grape juice during processing. The authors observed that the procyanidins were sensitive to heat degradation. Procyanidins become sensitive to non-enzymatic condensation and polymerization at elevated temperatures, resulting in decreases in the contents.

3.2. Antioxidant activity

Since the different microencapsulation treatments led to differences in the phenolic composition of the powdered grape juices obtained, differences in their antioxidant activities (expressed as

mmoles Trolox/100 g powdered juice) were also expected: 1SM = 9.83; 2SM = 11.06; 1WM = 7.94; and 2WM = 8.60. To study the effects of time and temperature separately, the antioxidant activity at the start of storage was considered as 100%, thus excluding the differences among the four treatments.

The storage temperature and time did not influence the antioxidant activity of the powdered grape juices (Fig. 4). For the SM treatments, there was a reduction in antioxidant activity (approximately 25%) after 45 days, followed by a recovery and then another fall at the end of the storage period. For the WM treatments, no tendency for degradation with time was verified and the decrease in antioxidant activity was small, being only 6% for 1WM and 14% for 2WM at the end of storage.

On the other hand, during storage the antioxidant activity appeared to have had no correlation with the observed decrease in the total anthocyanin content, which showed linear degradation with time. Fracassetti et al. (2013), studying the storage of freeze-dried wild blueberry powder, also found a reduction in total anthocyanins with maintenance of the antioxidant activity. According to these authors, this was probably due to the formation of antioxidant polymers, such as low molecular weight procyanidins, or the formation of degradation products of the anthocyanins or phenolic acids, which also show antioxidant activity.

It appears that the antioxidant activity has some correlation with the polymerized anthocyanins. The treatment with WM showed greater anthocyanin polymerization during storage, and also showed greater antioxidant activity. According to Laine et al. (2008), the phenolic compounds are typically “team players”, meaning that they work synergistically by supporting each other's

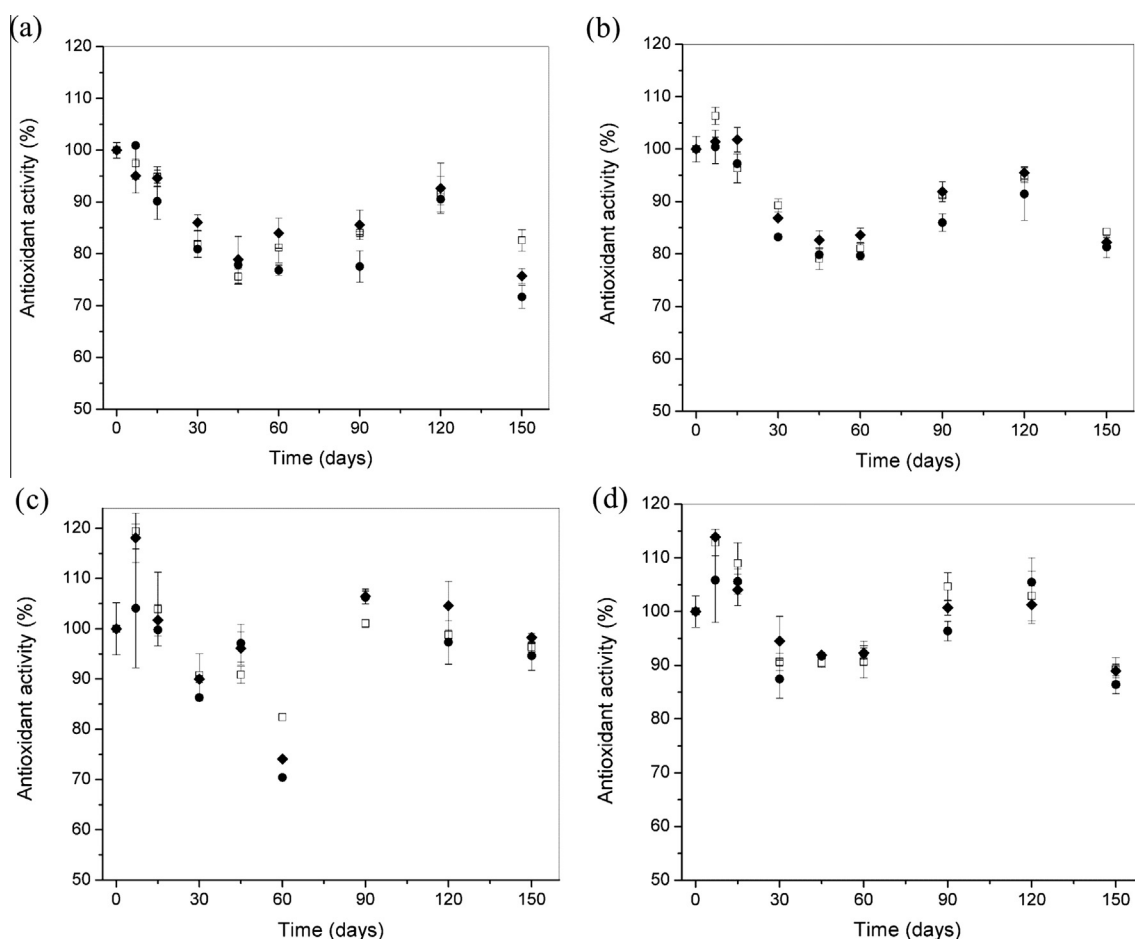


Fig. 4. Antioxidant activity of the microencapsulated grape juice with (a) 1SM; (b) 2SM; (c) 1WM; and (d) 2WM and stored at □ 5 °C, ● 25 °C, ◆ 35 °C for 150 days.

antioxidant activities. The loss of original phenolics might be compensated by newly formed phenolics with equal or improved antioxidant activities.

3.3. Colour parameters

Independent of the type of carrier agent used, the temperature and storage time did not influence the colour of the corresponding reconstituted grape juice, the differences being smaller than 1.5 for any time/temperature combination, except for the 1WM treatment stored for 150 days at 35 °C (data not shown). This result is important because it demonstrates that microencapsulated grape juice can maintain the colour of the original product during its shelf life, and hence its sensory aspects. According to Obón, Castellar, Alacid, and Fernández-López (2009), a total colour difference from 0 to 1.5 indicates that, visually the sample is almost identical to the original one, while in the range from 1.5 to 5, the difference in colour can be distinguished.

Reconstituted grape juice from the 1WM treatment presented a colour difference value of 1.79 at the end of the storage time at 35 °C. The lightness (L^*) increased slightly from 26.75 at zero time to 28.05 at the end, implying in a brighter juice with less colour. Changes in the contribution of different colour components were observed. Thus the red colour component (a^* , positive value) increased during storage at 35 °C from 4.35 (0 days) to 4.53 (150 days). This behaviour could be related to the formation of anthocyanin-derived pigments that stabilize the flavylium red-coloured form (Lago-Vanzela et al., 2014). The blue colour component (b^* , negative value), associated with purplish nuances, decreased its initial negative value from -3.28 to -2.07 at the end of storage. The evolution from negative to positive or less negative b^* values is associated with the loss of copigmentation effects accompanied by the formation of anthocyanin-derived red-orange pigments, such as pyranoanthocyanins (Lago-Vanzela et al., 2014), even though the latter compounds were not detected in the samples of reconstituted grape juices analyzed.

The greatest loss of total anthocyanins during storage was already observed in the 1WM treatment as compared to the other treatments, and this was accompanied by the same behaviour in the colour of the reconstituted juice. Thus, the 1WM treatment does not appear to be a good option for the microencapsulation of anthocyanins. Idham et al. (2012), studying the colour of spray-dried rosele powder, observed that the storage period and encapsulating agent significantly affected the colour change, whereas the storage temperature had no effect.

4. Conclusions

The 1SM treatment stored at 5 °C presented the lowest anthocyanin degradation rate and consequently the longest half-life time, whereas the 1WM treatment stored at 35 °C presented the highest degradation rate and the shortest half-life time, suggesting that this carrier agent is not effective in protecting anthocyanins. It is very likely that the largest amounts of carrier agent and highest ratios of maltodextrin also improved the stability of the anthocyanins. The anthocyanins polymerized more in WM than in SM. The WM treatments presented a positive linear correlation between the polymerized anthocyanin content and the storage time. The individual anthocyanin profile changed slightly during storage. In the 2SM, 1WM and 2WM treatments, an increase in storage time resulted in a greater proportion of *p*-coumaroylated anthocyanins. This behaviour can be explained by the greater stability of the acylated anthocyanins, especially those acylated with *p*-coumaroyl residues. The 1SM treatment led to significant losses of flavonols after 150 storage days, but this did not happen

with the 1WM treatment. All the samples showed significant losses of HCADs in the powdered juice during storage. The loss of flavan-3-ols occurred in the powdered juice at the end of storage when stored at 35 °C and the 2SM treatment presented the greatest loss of this kind of phenolic compound.

Considering a single treatment, the time/temperature combination did not influence the antioxidant activity of the powdered grape juice after 150 days of storage. The storage time and temperature did not influence the colour of the reconstituted grape juice, the colour difference being less than 1.5 at the end of storage, except for the 1WM treatment stored at 35 °C for 150 days.

The most remarkable result of this study was the thermal stability of the 1SM treatment. Microencapsulation was an effective way of stabilizing phenolic compounds, ensuring a longer shelf life as well as a range of possible industrial applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.081>.

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