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Abstract

Red grape pomace, a wine-making by-product is rich in anthocyanins and has many applications in food and pharmaceutical industry. However, anthocyanins are unstable during processing and storage. This study aimed to investigate the stability of anthocyanins obtained by hydroalcoholic extraction (with and without sorbic acid) and colloidal gas aphrons (CGA) separation; a surfactant (TWEEN20) based separation. Anthocyanins in CGA samples showed higher stability (half-life= 55 d) than in the crude extract (half-life= 43 d) and their stability increased with the concentration of TWEEN20 in the CGA fraction (6.07-8.58mM). The anthocyanins loss in the CGA sample (with the maximum content of surfactant, 8.58 mM) was 34.90%, comparable to that in the crude ethanolic extract with sorbic acid (EE-SA) (31.53%) and lower than in the crude extract (44%). Colour stabilisation was also observed which correlated well with the stability of individual anthocyanins in the EE and CGA samples. Malvidin-3-o-glucoside was the most stable anthocyanin over time.

Keywords: Grape pomace, anthocyanins stability, colloidal gas aphrons, surfactant, storage

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); AOP, antioxidant power; CGA, colloidal has aphrons; EE; ethanolic extract, EE-SA, ethanolic extract with addition of sorbic acid; GAE, gallic acid equivalents; $t_{1/2}$, half-life; glc, 3-o-glucoside; ME, malvidin glucoside equivalent; V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16.

1. Introduction

Grapes are one of the most important fruit crop cultivated across the world, whereby 80% of the grape productions are used in wine-making industry (Fontana, Antoniolli, & Bottini, 2013). Wine production is considered one of the most important agricultural activities, generating large amount of residues including grape skins, stems and seeds (Yu & Ahmedna, 2013). At the end of the fermentation process, large amounts of resiudes are being discharged containing high amount of phenolic compounds including anthocyanins, catechins, flavonol glycosides, phenolic acids and stilbenes (Kammerer, Kammerer, Valet, & Carle, 2014). This is seen by the environmental management authorities as a serious threat because they are low in pH and high in organic matter thus potentially causing a phytotoxic effect if applied to crops or wetlands (Kammerer, Claus, Carle, & Schieber, 2004; Lavelli, Harsha, Laureati, & Pagliarini, 2017). Therefore, converting and utilising this by-product to another useful product would be a solution to this problem. For instance, the anthocyanins from this pomace can be used as natural food colourant (Thakur & Arya, 1989). Anthocyanins are sensitive to thermal degradation making the recovery rather difficult and complex, but they are on demand due to their wide applications in food (already being used as food colourants, E163, approved by EC) as well as in pharmaceuticals and cosmetics. Thus, various extraction techniques have been studied and used, including acidified alcohol, sub- and supercritical fluid and high pressure processing (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Drosou, Kyriakopoulou, Bimpilas, Tsimogiannis, & Krokida, 2015; Lozovskaya, Brenner Weiss, Franzreb, & Nusser, 2012).

Food processing generally involves thermal processing prior to consumption and this process has a great influence on the anthocyanins content in the final product. Thermal processing involves high temperatures ranging from 50°C to 150°C, depending on the pH and the desired shelf life of the product. Anthocyanins are expected to degrade over time. However, the storage temperature has been found to be an important factor that is affecting anthocyanins' shelf life. Degradation of anthocyanins is greatly affected by the type of anthocyanin, the origin of the samples and the storage temperature (Hellström, Mattila, & Karjalainen, 2013). The thermal degradation of anthocyanins in extracts and model systems are reported to follow first-order reaction kinetics (Presilski, Presilska, & Tomovska, 2016).

The stability of anthocyanins can be improved, by self-association of the anthocyanins, removal of oxygen and inactivation of enzymes (Hellström et al., 2013). In the food industry, the sensitivity of bioactive compounds is addressed by incorporating edible coatings as a structural matrix, used widely to create a barrier from oxygen, moisture and solute movement (Falguera, Quintero, Jiménez, Muñoz, & Ibarz, 2011). Encapsulating methods such as spray drying/spray chilling or liposomes have been used. The former requires liquid droplets or small particles being incorporated within a continuous edible coating, thus it requires an emulsifier. Liposomes are microscopic spherical particles consisting of one or more lipid bilayers that can encapsulate or bind a variety of molecules. Therefore, particularly in food applications, food grade surfactants such as TWEEN20 have been used as emulsifying agents to fit this purpose (Quirós-Sauceda, Ayala-Zavala, Olivas, & González-Aguilar, 2014). Moreover, TWEEN20 has been seen as having a profound protective effect on five different polyphenols, by slowing down the auto-oxidation process at pH 4.5 (Lin, Wang, Qin, & Bergenståhl, 2007).

A surfactant-based separation technique, colloidal gas aphrons (CGA) has been previously studied in our group to recover various valuable bioactive compounds from

different feedstock such as astaxanthin (Dermiki, Bourquin, & Jauregi, 2010; Dermiki, Gordon, & Jauregi, 2009), proteins (Fuda & Jauregi, 2006; Fuda, Bhatia, Pyle, & Jauregi, 2005) and polyphenols (MohdMaidin, Michael, Oruna-Concha, & Jauregi, 2017; Spigno, Dermiki, Pastori, Casanova, & Jauregi, 2010; Spigno, Amendola, Dahmoune, & Jauregi, 2015). The type of surfactant (i.e cationic, anionic and non-ionic) determines the outer charge of the CGA, where molecules with the opposite charge will attract to the CGA resulting in their effective separation into the CGA phase.

In our previous work it was shown that 70% of the anthocyanins could be recovered from the ethanolic extract of grape pomace using CGA generated from TWEEN20. The CGA fraction will be rich in surfactant therefore, it will be interesting to test what will be the added value of extracting the anthocyanins in such a solution and whether this can offer any advantage to their formulation for subsequent applications. Thus the present study aimed at assessing the stability of anthocyanins in the CGA separated fraction over time in comparison with their stability in the crude ethanolic extract (EE) (before the CGA separation) as well as in the crude ethanolic extract with a commercial additive, sorbic acid (EE-SA). It is therefore hypothesised that the anthocyanins in the CGA sample will show higher stability than in the crude extract over time.

2. Materials and methods

2.1 Materials

Grape pomace (Barbera variety) was obtained from a winery in Northern Italy. All the solvents (purity of 95% and above) used in this project were obtained from Sigma-Aldrich Company Ltd., Dorset, UK. For the HPLC analysis, the solvents used were of HPLC grade (purity of 98-99.9%) also from Sigma Aldrich.

105 *2.2 Extract preparation*

106 The grape pomace (Barbera variety) was kindly provided by a winery located in
107 Nothern Italy. At the winery, the fermented pomace was recovered and oven dried at 60°C
108 until the residual moisture content is <5%. The dried pomace powder was sieved with a 5mm
109 sieve to separate the skins from the seeds and milled into fine powders with particles size <
110 2mm and stored in the freezer at -20°C until further use.

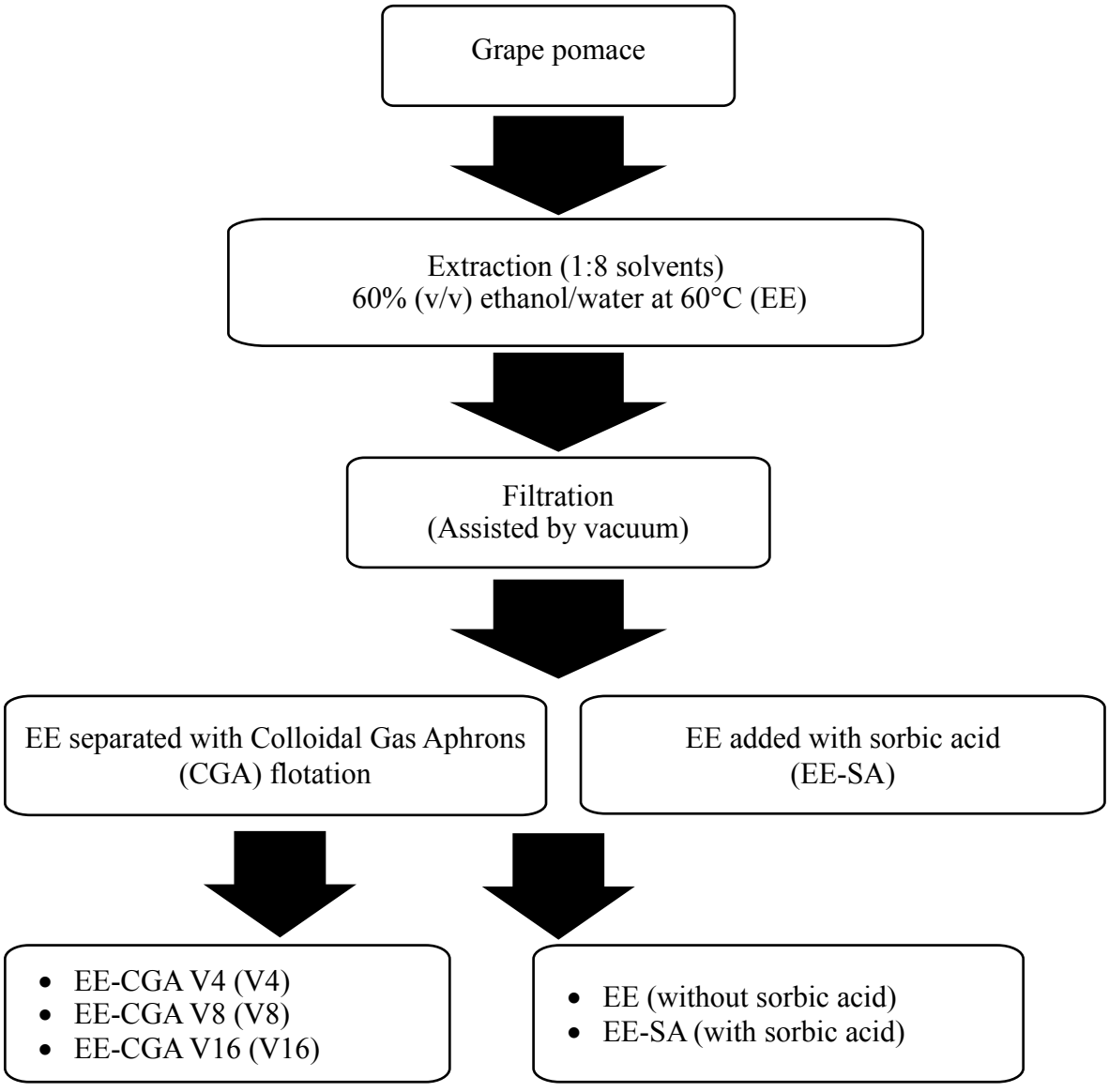
111 The extraction procedure was done in accordance to our previous study using ethanol-
112 aqueous solvent (MohdMaidin et al., 2017). The extract was filtered and two different
113 samples were produced: (1) approximately, 400mL of the ethanol extract labeled as EE and
114 (2) another 400mL ethanol extract with sorbic acid (>99%) (Sigma, UK) and labelled as EE-
115 SA. Sorbic acid was chosen in this study for its wide application as food additive, thus
116 making it closer to the formulation of most low pH food products and neutral taste (Troller
117 & Olsen, 1967). Both EE and EE-SA were considered control samples. The remaining
118 filtrate of 800mL was kept aside for CGA separation.

119

120 *2.3 CGA separation using 10mM TWEEN20*

121 The separation of polyphenols from the crude ethanolic extract was carried out at
122 different volume ratios of CGA to feed (V_{CGA}/V_{feed}). The ratios selected were 4, 8 and 16.
123 The separations were individually carried out in a flotation glass column according to the
124 method described in our previous work (MohdMaidin et al., 2017); each separation was
125 carried out in triplicate. It should be noted that as the volumetric ratio increased, so did the
126 concentration of TWEEN20 in the solution of the separated CGA fraction. The concentration
127 of TWEEN20 in each of these fractions was estimated from a knowledge of the separated
128 volume of CGA and corresponding liquid fraction which was determined from a
129 measurement of gas hold-up (gas volumetric ratio defined as the volume of air incorporated

130 in a given volume of CGA dispersion) of the CGA generated with this solution of TWEEN20
131 (61.3%). The estimated concentrations were: in V4, 6.07mM, in V8, 7.56mM and in V16,
132 8.58mM. The summary of the extraction and separation process is briefly described in Figure
133 1.
134



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136 Figure 1: Flow diagram of hydroalcoholic extraction and CGA separation processes applied
137 to grape pomace, n = 3; EE is the ethanolic extract; EE-SA is the ethanolic extract with
138 addition of sorbic acid; EE-CGA V4 is the ethanolic extract further processed with CGA at
139 CGA to feed volumetric ratio of 4; V8 and V16 correspond to the extracts further processed
140 with CGA at CGA to feed volumetric ratios 8 and 16 respectively

141 *2.4 Determination of degradation of chemical and physical properties over time*

142 Briefly, the EE, EE-SA and CGA fractions were divided in equal volumes and kept in
143 sterilised containers in the darkness. These were then stored at room temperature 20°C (SD
144 1°C) which was regularly monitored using a thermometer for 32 days. The total phenolic
145 content, total anthocyanin and antioxidant activity were determined as described in section
146 2.5-2.7. The total anthocyanins, individual anthocyanins, antioxidant capacity and the colour
147 degradation over time (32 days; every day for the first 7 days and subsequently 5 days
148 intervals) were determined.

149 The kinetics of degradation of total anthocyanins and individual anthocyanins were
150 assessed; the natural logarithms of these were plotted against time in order to test for first-
151 order kinetics as described by the equation below:

152

153 $$-ln\left(\frac{A_t}{A_0}\right) = k * t$$ (Equation 1)

154

155 Where A_0 is the initial anthocyanin content, A is the anthocyanin content at time *t*, *t* is the
156 storage time and *k* is the rate constant. The degradation rate constant (*k*) was determined
157 from the slope of the straight line obtained when plotting *Ln* (At/A_0) vs *t*. From the equation
158 above, the time taken for the anthocyanin content to halve, the half-life ($t_{1/2}$), can be derived
159 as:

160

161 $$t\frac{1}{2} = \frac{Ln(2)}{k}$$ (Equation 2)

162

2.5 Total phenolic content

Folin Ciocalteu (FC) colorimetry method (Singleton & Rossi, 1965) was employed to determine the total phenolic content of the EE and EE-SA (control samples) and also in all of the CGA processed samples. This method involves the oxidation of phenols using a molybdotungstate reagent to yield a coloured product which can be measured at 760nm using a spectrophotometer (Biotech Ultrospec 1100 pro UV spectrophotometer). Gallic acid (Sigma-Aldrich, UK) standards with concentrations ranging from 0-1000mg/L were used to generate standard plots ($R^2 = 0.9881$) and an equation for the calculation of the total phenolic concentration in each extract. The analysis was done in triplicate. The total phenolic content in the CGA processed samples were compared to the controlled samples over time.

2.6 Evaluation of in vitro antioxidant activity

The antioxidant activity of the control samples (EE and EE-SA) along with the CGA processed samples were evaluated according to Re et al., (1999) by the ABTS assay. This method assesses the ability of the antioxidants to scavenge the radical (ABTS) which was determined by measuring the decrease in its absorbance at 734nm using a spectrophotometer (Biotech Ultrospec 1100 pro UV spectrophotometer). Different concentrations (0-2000µM) of Trolox standard were used to construct a calibration curve ($R^2 =0.9991$). The analysis was done in triplicate. The antioxidant activity of the CGA processed samples was compared against that of the control samples, expressed as µM Trolox equivalent. The ratio of percentage inhibition to the total phenolic content of all samples, termed as specific antioxidant power (AOP), was calculated.

186 *2.7 Total anthocyanins content*

187 The total monomeric anthocyanins of control samples, EE and EE-SA along with the
188 CGA processed samples were determined over time using the pH differential method
189 approved by AOAC (Lee, Rennaker, & Wrolstad, 2008). This method is based on the
190 anthocyanins structural transformation that occurs with a change in pH. Briefly, the extract
191 was mixed individually with pH 1.0 and 4.5 buffer solutions in a ratio of 1:5 and left for 20
192 minutes. The absorbance of the test portions at both pHs were determined
193 spectrophotometrically (Biotech Ultrospec 1100 pro UV spectrophotometer) at a wavelength
194 of 520nm and 700nm. The results of the anthocyanin pigment were expressed as malvidin-
195 3-glucoside equivalents (ME) according to equation 3.

196

197 $$Total\ Anthocyanins\ \left(ME, \frac{mg}{L}\right) = \frac{A*MW*DF*10^3}{\varepsilon*1}$$ (Equation 3)

198

199 Where A = $(A_{520nm}\text{-}A_{700nm})_{pH1.0} - (A_{520nm}\text{-}A_{700nm})_{pH4.5}$; MW (molecular weight of malvidin-
200 3-glucoside = 493.43g/mol; DF = dilution factor; 1 = path length in cm; Ɛ = 28000 molar
201 extinction coefficient and 10^3 = factor for conversion from g to mg and cm.

202

203 *2.8 Identification and Quantification of Anthocyanins by HPLC*

204 The separation of the polyphenols was performed using an Agilent HPLC 1100 series
205 equipped with a degasser, a quaternary pump and a photodiode array detector model
206 (Agilent, Waldbronn, Germany) with Chemstation software. The column used was a C18
207 HiChrom (150 mm x 4.6 mm i.d; 5µm particle size and 100 Å pore size; part no.EXL-121-
208 1546U) operated at 30°C. The separation method was the same as described in our previous
209 paper (MohdMaidin et al., 2017). The polyphenols were monitored at 280nm and the UV/Vis

210 spectra were recorded in the range of 200 to 760nm. The main anthocyanins were detected
211 at 520nm and identified based on the retention times and by comparing the spectra with that
212 of the external standards which were: delphinidin-3-o-glucoside (>99%) (RT= 4.8;
213 calibration curve R^2 = 0.8771); cyanidin-3-o-glucoside (>98%) (RT= 7.8; calibration curve
214 R^2=0.98744); petunidin-3-o-glucoside (>98%) (RT= 8.5; calibration curve R^2=0.99702) and
215 malvidin-3-o-glucoside (>99%) (RT= 7.8; calibration curve R^2= 0.99994); all supplied by
216 Extrasynthese, Paris, France.

217

218 *2.9 Determination of CIELab colour parameters and pH*

219 The changes in colour of the EE, EE-SA and the CGA processed samples were
220 measured using a CT-1100 ColourQuest HunterLab by taking the measurements in
221 transmittance mode. Standard black plates were used for standardization. L*, a* and b*
222 measurements were obtained and used to calculate chroma and hue angles based on
223 equations 4 and 5 below. Delta E (ΔE) was calculated based on the changes of the values of
224 L*, a* and b* at a given time, in comparison to these values at day 0 and applying equation
225 6.

226 $$Hue\ angle\ (°) = \left[180 * \left(\frac{ATAN\left(\frac{b}{a}\right)}{\pi}\right)\right] + \left(\frac{b}{a}\right)$$ (Equation 4)

227 $$Chroma = \sqrt{a^2 + b^2}$$ (Equation 5)

228 $$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{\frac{1}{2}}$$ (Equations 6)

229

The hue angle and chroma may be used on a CIE 1979 L*a*b* colourimetric system diagram to identify colour and monitor changes. The changes of colour in all the CGA processed samples over time were compared to EE and EE-SA as the control samples.

The pH of all samples was monitored regularly with a pH meter (Mettier-Toledo SevenEasy), which was calibrated by using pH 4.0 and 7.0 buffer solutions (Sigma, UK).

2.10 Statistical analysis

All the experiments were performed in triplicate. The data were subjected to the analysis of variance using IBM® SPSS® Statistics 21 software program where statistical differences were noted. Differences among the different treatments were determined by using the Tukey test. The significance level was defined at $p<0.05$. The results were reported as means ± SD.

3. Results and Discussion

3.1. Changes of total phenolic content over storage time

Initial values for total phenolic content was measured in control samples (EE and EE-SA) and three of the CGA processed samples at day 0. The total phenolic content for all samples range was 285-2080 mg GAE/L. The TP content for EE-SA was higher ($p = 0.0371$) than EE, which can possibly be explained by the presence of sorbic acid. The total monomeric anthocyanin range was 99.1-422.9 mg ME/L. The antioxidant activity range was 2299-14469 µM Trolox equivalent.

Over a storage period of 32 days, the losses in the TP content were minimal in all the samples (Fig. 2A). The maximum degradation observed in EE-SA and EE was not more than 10%. Among all the CGA processed samples, the lowest losses of the TP content was in V16

(4.91%), followed by V8 (5.44%) and finally V4 (6.42%), although they were not significantly different (p = 0.062).

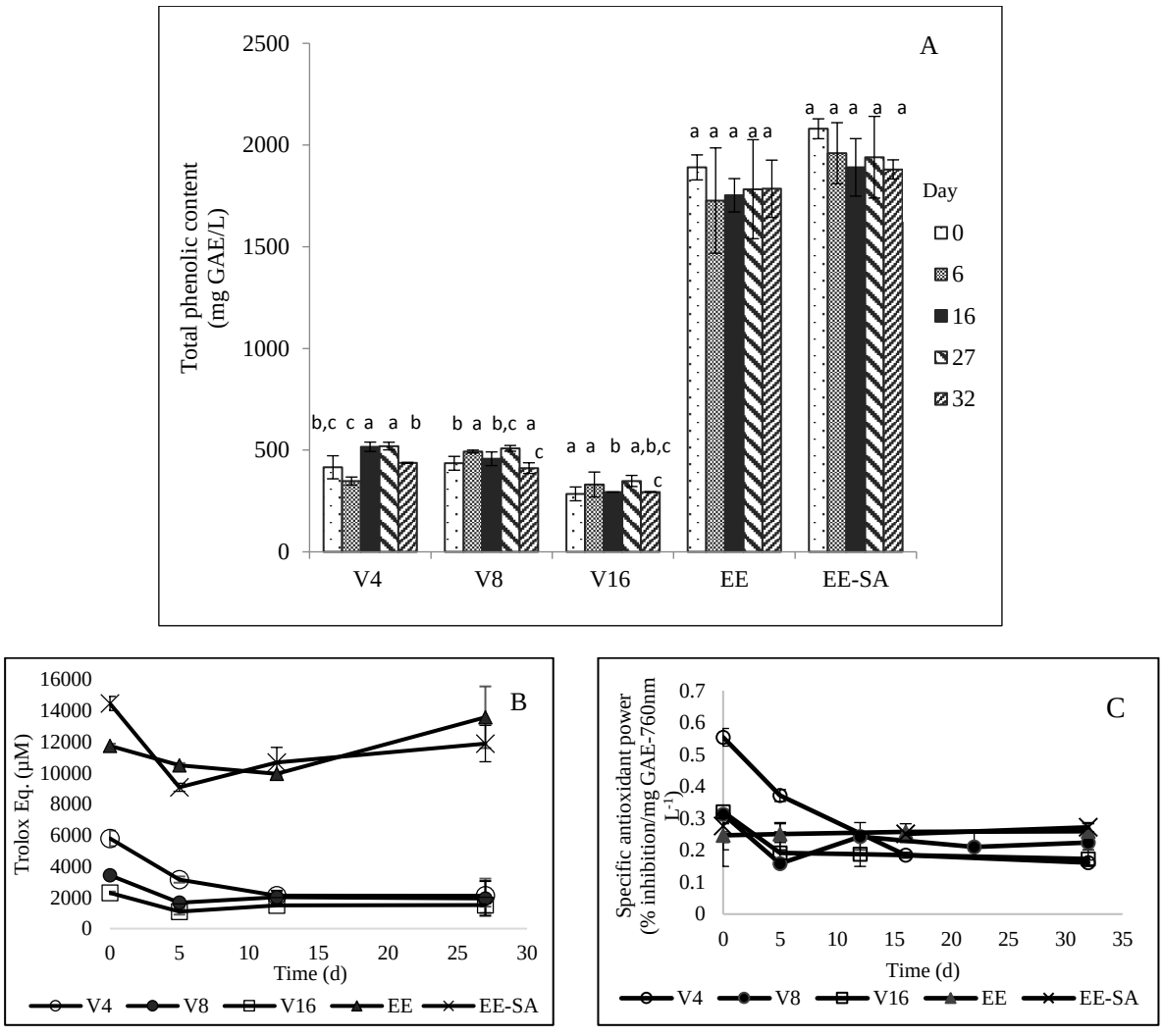


Figure 2. Total phenolic content (A), antioxidant activity profile expressed as Trolox Equivalent (µM) (B), and specific antioxidant power (% inhibition/mg GAE-760nm L^{-1}), (C) of CGA processed and control samples over time. Error bars represent means ± SD (n=3). V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated

264 at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.
265 Different letter within each series indicates significant differences using Tukey's test ($P<0.05$).

266

267

268 *3.2. Changes in antioxidant activity over storage time*

269 The antioxidant activity of the control samples and CGA processed samples stored
270 over time was evaluated using ABTS assay. Figure 2 (B) shows a decrease in antioxidant
271 activity in both, the control samples and the CGA processed samples over time. The
272 reduction in antioxidant activity was clearly observed for all samples during the first 5 days.
273 Further decrease was observed in V4 after 5 days, however in V8, V16 and control samples
274 no further reductions or even slight increases in antioxidant activity was observed after this
275 time.

276 Moreover, when the specific antioxidant power (calculated as percentage of inhibition
277 per total phenol content) was calculated, a more distinct pattern was observed (Fig. 2C). In
278 general, the specific antioxidant power decreased over time at a higher rate than the total
279 phenols (Fig. 2A). Rapid loss of antioxidant power was observed particularly in V4 from
280 day 0 to day 16, and in V8, V16 and EE-SA only over the first 5 days. It was interesting to
281 note that there was no specific antioxidant loss in EE. This could be related to the total
282 phenolic content as depicted in Figure 2A where the losses in EE were not significantly
283 different ($p\leq0.05$) between the time points. This implied that the losses in TP and antioxidant
284 activity in EE were in the same proportion hence the antioxidant efficiency was almost
285 constant over time (Fig. 2B). However, this was not the case for the CGA samples where
286 minimal losses of total phenolic content were noted but important changes in antioxidant
287 activity were observed. Therefore, this suggests that in these samples the antioxidant activity
288 may not be solely derived from the total phenolic content and/or that the phenolics undergo
289 some chemical changes that affect their antioxidant activity. Over estimation of total

phenolic content could possibly happen by the high sugar content or ascorbic acid in the crude extracts and the CGA processed samples (Ainsworth & Gillespie, 2007).

3.3. Kinetics of total anthocyanins degradation over storage time

Degradation of anthocyanins has been previously studied in wine and its residues (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Clemente & Galli, 2011, 2013; Lavelli et al., 2017). The patterns of degradation involving anthocyanins are complex, but the degradation rate generally follows first-order kinetics (Amendola, De Faveri, & Spigno, 2010; Buckow, Kastell, Terefe, & Versteeg, 2010). However, the information on anthocyanin degradation in the presence of surfactant is lacking. In this study, the degradation of anthocyanins in the control samples (EE and EE-SA) were compared with the CGA processed samples stored at 20°C, which also followed first-order kinetics (Fig. 3).

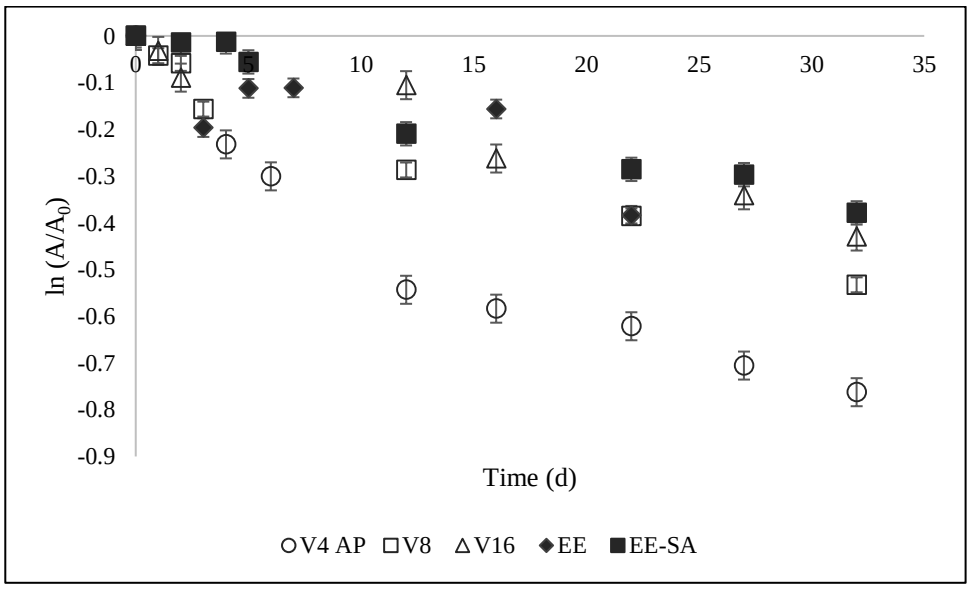


Figure 3. Time course for the decrease of anthocyanins represented here as the natural logarithm of the ratio of anthocyanins concentration at a given time and at time zero (A_0) during storage at 20°C. Error bars represent means ± SD. V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

Degradation of anthocyanins in all samples followed first order kinetics as shown by data in Figure 3. In Table 1A the first-order rate constant (k) and the linear regression coefficient (R^2) of all samples are shown. The first-order rate constant for anthocyanins degradation ranged between 0.0124 and 0.0217 d^{-1}. Although the R^2 values for V4 and EE were lower than the others, first-order kinetics were assumed. This was also based on the assumption that since the pH of these samples ranged 3.5-3.8 the degradation of the anthocyanins and thus the ionization of flavillium ion followed first-order kinetics as found by West & Mauer (2013). The first-order rate constant of EE was higher (k = 0.0159 d^{-1}) than the one of EE-SA; the first-order rate constant of EE-SA was the lowest among all samples (k = 0.0121 d^{-1}). This suggests that the addition of sorbic acid increased the stability of anthocyanins during storage although sorbic acid has only been reported to prevent microbial growth during storage (Troller & Olsen, 1967).

As shown in Table 1A the first-order rate constant decreased with the increase in volumetric ratio and thus with an increase in surfactant content in the CGA fractions (ranging from 6.07-8.58mM TWEEN20). The samples with the highest stability were the EE-SA and the CGA fraction with the highest surfactant concentration, V16, followed by V8 and EE which had very similar stability. The sample with the lowest concentration of surfactant V4, was found to degrade the fastest over time.

The extraction of grape pomace with water containing 3% of citric acid has also been proposed to recover phenolic-rich coloured extracts with 36-62% of total anthocyanins composition (Cardona, Lee And, & Talcott, 2009). However, the colour degradation of these water-based extracts at 30°C is fast, with first-order rate constants of 0.0364 and 0.038 for cold and hot pressed extractions, respectively. In the present study, the first-order rate constants were lower indicating more stable extracts. The most stable sample was the EE-SA (k = 0.0121d^{-1}) suggesting a stabilisation effect of sorbic acid. Comparable results were

obtained for V16 CGA with $k = 0.0124 d^{-1}$. However, interestingly the stabilisation effect in the CGA processed samples was only achieved above a certain concentration of surfactant as in V4 and V8 CGA samples the observed stabilisation effect when compared against the EE sample was minimal. It was estimated that the surfactant concentration in V16, V8 and V4 was 8.58mM, 7.56mM and 6.07mM respectively (see Methods). Therefore the concentration of surfactant in the samples should be at or above 8.58mM (about 1%) in order to have a stabilisation effect.

Table 1A. First-order empirical rate constants (k) and half-life for anthocyanins.

Sample	R^2	K (d^{-1})	$t_{1/2}$ (d)	Loss (%)*
V4	0.8861	0.0217 ± 0.0019	31	53.35
V8	0.9585	0.0157 ± 0.0024	44	41.30
V16	0.9385	0.0124 ± 0.0015	55	34.90
EE	0.8131	0.0159 ± 0.0012	43	41.04
EE-SA	0.9583	0.0121 ± 0.0011	57	31.53

Rate constants are expressed as means ± SD. V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.
*anthocyanins loss calculated after day 32

Table 1B. Half-lives ($t_{1/2}$, day) and degradation rate (k, d^{-1}) of different anthocyanins in control and CGA samples, stored at 20°C

Compound/Sample	V4 $T_{1/2}/K$ (d/ d^{-1})	V8 $T_{1/2}/K$ (d/ d^{-1})	V16 $T_{1/2}/K$ (d/ d^{-1})	EE $T_{1/2}/K$ (d/ d^{-1})	EE-SA $T_{1/2}/K$ (d/ d^{-1})
Delphinidin 3-o-glucoside	35 0.0195	36 0.0190	29 0.0233	41 0.0168	41 0.0168
Cyanidin 3-o-glucoside	42 0.0163	55 0.0126	52 0.0132	33 0.0204	42 0.0162
Petunidin 3-o-glucoside	50 0.0136	44 0.0155	52 0.0132	50 0.0136	49 0.0139
Malvidin 3-o-glucoside	55 0.0126	57 0.0121	71 0.0097	59 0.0116	65 0.0116

A study conducted by Lavelli et al., (2017) in an ethanolic extract of grape pomace maltodextrin-encapsulated showed a low first-order rate constant, 0.0033-0.0014 d^{-1}. This might be due to lower water activity content and therefore these results are not comparable with the present results, as this study assessed the stability of anthocyanins in a liquid form. Moreover, the drying process will require higher overhead costs, and needs high energy and pressure input which would add up greatly to the overall cost.

The half-lives of anthocyanins of EE and EE-SA stored at 20°C were 43 and 57 d, respectively (Table 1A). As discussed above, the degradation is faster in the CGA samples with lowest surfactant concentration so V4 had the shortest half-life of 31 d, followed by V8 (44 d) and V16 (55 d). Moreover the half-life of V16 was almost similar to EE-SA's, but longer than that of EE. These half-life values were higher than the ones reported for the blueberry juice stored at 25°C ($t_{1/2}$ = 4.4 d), possibly due to the different types of anthocyanins present (Buckow et al., 2010). Similarly, when the percentage of anthocyanin losses after 32 days of storage was determined, the EE-SA had the least loss (31.53%), closely followed by V16 (34.90%).

In summary, from all the above data it can be concluded that the surfactant had a stabilisation effect on anthocyanins and this effect was comparable to that observed in extracts with sorbic acid. To the best of our knowledge, only one report by Thakur & Arya (1989) assessed the stability of anthocyanins in grape juice preserved with sorbic acid and their result agreed with the findings in this study. This further confirmed that the surfactant might play an important role in protecting the anthocyanins from oxidation, thus extending the half-life.

3.4. Anthocyanins Identification and Quantification

The HPLC-DAD analysis showed that all samples had 13 anthocyanins identified at the beginning and at the end of storage study (Figure S1), which was in agreement with our previous study (MohdMaidin et al., 2017). The identified anthocyanins were: delphinidin, cyanidin, petunidin, peonidin and malvidin with different glycosyl acylation attached. In red wines and their pomace made from *V. vinifera* grapes, the main anthocyanins detected were of 3-o-monoglucosides of the free anthocyanidins including pelargonidin-3-o-glucoside, cyanidin-3-o-glucoside, delphinidin-3-o-glucoside, peonidin-3-o-glucoside, petunidin-3-o-glucoside and malvidin-3-o-glucoside (Drosou et al., 2015; He et al., 2012; Kammerer et al., 2004). However, in this study, pelargonidin-3-o-glucoside was not detected and four anthocyanins (malvidin 3-o-glucoside, cyanidin 3-o-glucoside, delphinidin-3-o-glucoside, and petunidin-3-o-glucoside) were quantified as these are the most abundant anthocyanins present.

In all the samples, the most abundant anthocyanin was malvidin-3-o-glucoside (0.68mg/g) which was in agreement with other studies (Bimpilas, Panagopoulou, Tsimogiannis, & Oreopoulou, 2016; Morais, Ramos, Forgács, Cserháti, & Oliviera, 2002) followed by delphinidin3-o-glucoside (0.58mg/g). Both pigments were typically responsible for the purple and purple-blue which could be seen in the colour of the ethanolic extract.

Different anthocyanins had different degradation kinetics in each sample (Table 1B). Among the four anthocyanins, delphinidin was the least stable anthocyanin in all the samples except in EE. This can be seen in their short half-lives (29-41d). Fleschhut, Kratzer, Rechkemmer, & Kulling (2006) reported that an increase in hydroxyl groups in the B ring of the anthocyanin nucleus could result in a decrease in the stability which could possibly account for the anthocyanins loss. However, this was not observed in EE where cyanidin degraded faster than delphinidin, but both of them seemed to be less stable than petunidin

and malvidin indicating that methylation of hydroxyl-groups in B ring increased the stability of anthocyanins. Our results were comparable to those reported by Helllstrom et al., (2013) for delphinidin and cyanidin in the blackcurrant and chokeberry juices stored at 21°C with half-lives between 16-44 days.

Malvidins are known to be the most stable as compared to other anthocyanins due to the absence of two hydroxyl groups in the B ring structure. This was clearly evident as they had the longest half-life as compared to other anthocyanins across all samples. Interestingly, malvidin in V16 sample had longer half-life (71d), with slowest degradation rate (k = 0.0097 d^{-1}) than any of the control samples including EE-SA (k = 0.0116d^{-1}, $t_{1/2}$ = 65d) which agrees with the above observation on the protecting effect of the surfactant.

According to Hellström et al., (2013), the effect of the sugar moiety was minor as compared to the effect induced by the type of the core anthocyanidin. In these extracts, malvidin- and delphinidin 3-glucosides were the two predominant anthocyanins. Delphinidin glucosides exhibited greater temperature sensitivity due to their three hydroxyl group in the B ring in comparison to malvidin derivative which had only one –OH group attached to it (Buckow et al., 2010). This can be clearly seen in the half-life of malvidin-3-glucoside ($t_{1/2}$ = 55-71 days) across all samples. Moreover, the stability of anthocyanins was also reduced by the number of hydroxyl groups in the A ring with the absence of dihydroxyl group in the B ring (Buckow et al., 2010). The matrix of samples also has been reported to have a major impact on the stability of anthocyanins where anthocyanins in juices were more prone to degradation as compared to those in smoothies, where the anthocyanins may be protected by other phenolic compounds but the concrete reasons of this impact remained unclear (Hellström et al., 2013). A study on anthocyanins stability from encapsulated grape skin showed significant increase in the half-life of anthocyanins up to 452 days. The study also proved that lowering the water activity of the encapsulated grape skin powder can

415 double the half-life up to 998 days (Lavelli et al., 2017). In fruit juices, several factors can
416 influence the stability of anthocyanins, such as pH, presence of ascorbic acid and
417 anthocyanin degrading enzymes (Buckow et al., 2010). Finally, the degree of glycosylation
418 also might possibly affect anthocyanins stability; the higher the degree of glycosylation, the
419 more stable they became.

420 Co-pigmentation of anthocyanins with other compounds is considered as an
421 important mechanism of colour stabilisation in plants. Anthocyanins can form co-pigments
422 with metal ions, other phenolic compounds or through self- association (Castañeda-Ovando,
423 Pacheco-Hernández, Páez-Hernández, Rodríguez, & Galán-Vidal, 2009). Co-pigmentation
424 through self-association is unlikely because in order for it to take part, the concentration of
425 the anthocyanin should be greater than 1mmol//L. Hydroxycinnamic acids and flavonols
426 were reported as the best cofactors in wine (Bimpilas et al., 2016). Co-pigmentation can be
427 influenced by the anthocyanins and co-pigment structure, and also by the concentration of
428 anthocyanins to the co-pigment (Eiro & Heinonen, 2002). In the present study, the ratio of
429 anthocyanins and co-pigments might not be sufficient for the co-pigmentation to occur since
430 no additional phenolic acids were added to the samples. Thus, the stabilisation effect
431 observed in this study was solely due to the surfactant and the addition of sorbic acid.

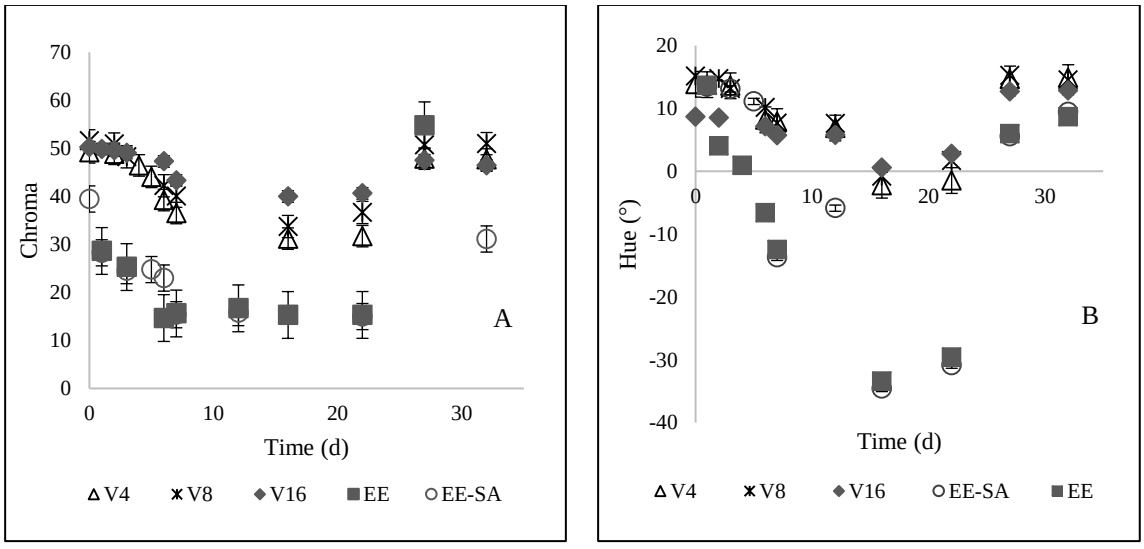
432

433 *3.5. Colour stability and pH*

434 Grape pomace extract had high levels of anthocyanins. However, anthocyanins
435 undergo degradation during processing and storage, thus affecting colour characteristics. In
436 the present study, the colour stability of the control samples and the CGA processed samples
437 was investigated and compared against EE and EE-SA. Colourimetric parameters chroma
438 (C) and hue (h) and ΔE were determined to assess colour changes over time. The effects
439 were comparable to those observed in anthocyanins, yet with some exceptions.

Figure 4 (A and B) showed the changes of colour in chroma and hue angle for the control samples and the CGA processed samples over time. The results obtained showed that EE and EE-SA had a similar trend; ΔE values overlapped with each other. At day 0, both samples had dark red colour (c = 39.4, hue = 6.9). Over time, both chroma and hue values decreased rapidly by day 22, from dark red tending towards blue-black shade (c = 14.9, hue = -29.6).

The same trend was observed in all of the CGA processed samples, although the chroma and hue angles decreased steadily as compared to EE and EE-SA. At day 0, all of the CGA processed samples had almost similar colour of dark red shade (c = 49.2-51.5; hue = 8.7-15.1). However, the chroma (c = 31.7-40.6) and hue angle (hue = -1.4-2.8) values decreased in all of the CGA processed samples over time. In short, V4 turned from dark red to light red, tending towards blackish; V8 turned from dark red to light red, tending towards browning and finally, V16 turned from dark red to light red, tending towards pinkish. Therefore, these results showed that minimum colour changes were observed in V16, which correlated with the lower degradation rate of anthocyanins determined above and confirms the stabilisation effect by the surfactant.



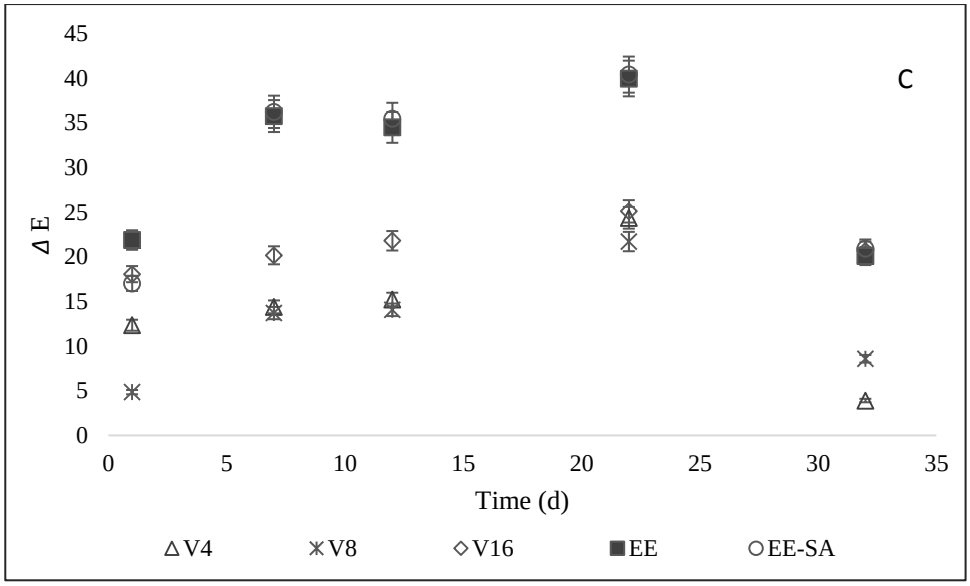


Figure 4. Chroma and hue values of samples during storage (A and B), total colour difference (ΔE) between samples (C). Error bars represent means ± SD (n = 3). V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

Furthermore, the colour changes of samples can be further explained by ΔE (Fig. 4C). ΔE indicates the magnitude of the colour difference between fresh and stored grape extracts for different time points. Higher colour differences were measured for the control samples than for the CGA samples. In all the CGA processed samples, the changes were not significantly different ($p>0.05$) in the first 12 days of storage. However, higher magnitude of changes was observed in day 22, and minimal changes were observed in day 32.

Anthocyanins differ from each other by the number and position of the hydroxyl, and methoxyl substituent groups in the B ring of the molecule. The hydroxylation pattern of the anthocyanins in the B ring can directly affect the hue and colour stability due to the effect on the delocalized electron path length in the molecule (He et al., 2012). Anthocyanins with more hydroxyl groups in the B ring can contribute more to blueness, meanwhile the degree of methylation in the B ring can increase redness. The rapid decrease in red colour of EE and EE-SA might be explained by the degradation of a particular anthocyanin. In both

474 control samples, cyanidin-3-glucoside and petunidin-3-glucoside had the shortest half-lives
475 between 16-21 days (Table 1B). Both anthocyanins were responsible for the red and dark
476 red colour respectively, which could explain the losses of dark red colour in both controlled
477 samples after 21 days. Both anthocyanins had two hydroxyl groups attached to the B ring,
478 which increased the blueness of the colour, as found in these samples. In the case of the CGA
479 processed samples, V4 appeared to have the same result as EE and EE-SA, which is
480 supported by the short half-life of cyanidin-3-glucoside determined in this sample. However,
481 in the case of V8 and V16, delpinidin-3-glucoside had the shortest half-life, 31 and 36 days
482 respectively. This could have contributed to the colour changes observed, from dark red to
483 light red, tending towards brownish and pinkish. Delphinidin-3-glucoside is responsible for
484 the blueness as it has three hydroxyl groups attached to the B ring.

485 Although most studies showed that delphinidin-3-glucoside exhibited a greater
486 thermal sensitivity due to their three hydroxyl substitution group, this was not clearly
487 observed in this study; thus the correlation between anthocyanin stability and chemical
488 structure is still unclear (Rice-Evans, Miller, & Paganga, 1996). Moreover, the colour
489 changes in EE-SA could not be explained by the slowest anthocyanins loss in this sample.
490 This suggests that the mechanism of colour stabilisation in this sample needs further study
491 as colour change does not correlate with anthocyanins degradation. The mechanisms of
492 stabilisation of anthocyanins by TWEEN20 are yet to be determined, but we propose that
493 the micelles might play a role in encapsulating the anthocyanins protecting them against
494 oxidation during storage.

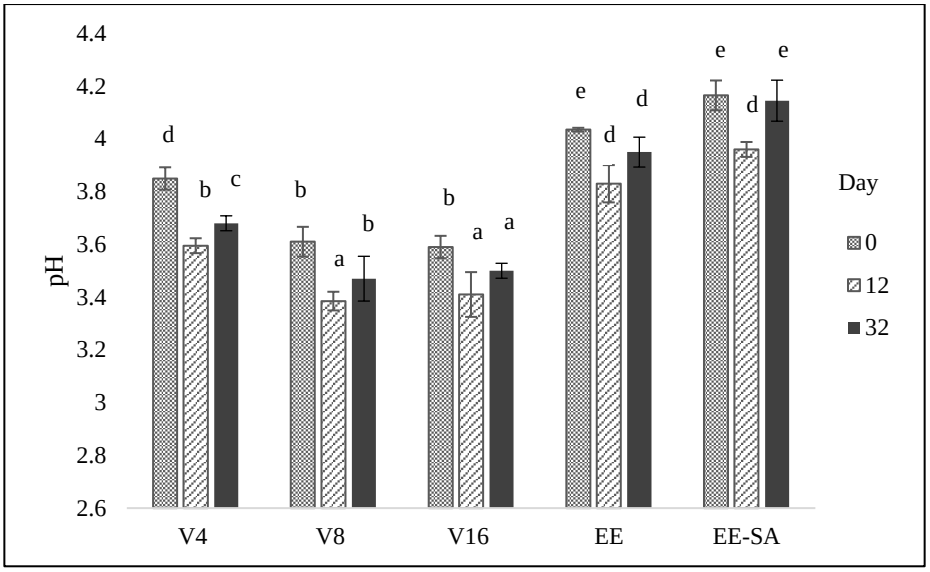


Figure 5. pH values of all samples on selected days. Error bars represent means ± SD, n = 3. V4, CGA fraction separated at volumetric ratio 4; V8, CGA fraction separated at volumetric ratio 8; V16, CGA fraction separated at volumetric ratio 16; EE, crude ethanolic extract and EE-SA, ethanolic extract with sorbic acid.

Overall, the pH of all samples dropped and then increased slightly (Fig. 5). Although it is known that pH plays an important role in determining the state of the flavylium ion, the trend was unclear in the present study. This could possibly be due to the presence of TWEEN20 which could have a stabilisation effect as shown by the reduction of pH after CGA separation. The increased in pH values later throughout the end of storage may be due to the formation of phenolic acids like gallic acid; this can be supported by the fluctuations in total phenolic content over storage which may be also an indication of the formation of these intermediate compounds (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010).

4. Conclusions

The stability of the ethanolic raw extract from grape marc was compared with that of the further processed sample after applying the CGA separation. The main effect was found on the stability of anthocyanins. Anthocyanins stability in CGA fraction V16 was higher

than in the raw extract based on the comparison of the first order kinetics of anthocyanins degradation followed by all the samples. The stability in CGA samples increased with an increase in surfactant concentration, V16 sample having the highest half-life (55 d) and similar to the raw extract's with sorbic acid (57 d). Thus these results show that the surfactant has a stabilization effect on the anthocyanins and the sorbic acid seems to have a similar effect. Moreover a good correlation between the colour changes and degradation rate of individual anthocyanins was observed whereby malvidin-3-o-glucoside was found to be the most stable anthocyanin at all the studied conditions with the highest half-life found in V16. Overall, this study shows that the surfactant has a stabilisation effect on the anthocyanins and half-lives determined here were higher than others reported for wet formulations of anthocyanins. The mechanism of stabilisation of anthocyanins by TWEEN20 may be related to the solubilisation of the anthocyanins within the micelles. Furthermore, the main findings in this study have shown the advantages of CGA as a separation method that can also integrate a pre-formulation step.

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Conflict of interest

The authors declare no conflict of interest.

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