INTERACTIONS OF NATURAL ANTIOXIDANTS WITH RED GRAPE POMACE ANTHOCYANINS IN A LIQUID MODEL MATRIX: STABILITY AND COPIGMENTATION EFFECTS

The purpose of this study was an examination of the stability and colour enhancement of red grape pomace anthocyanins in a juice model matrix, and the effect of the addition of natural antioxidants. The approach was based on a juice-like liquid medium (10.1 °Bx, pH 3.48), which was used as the model matrix to test the effect of the addition of natural antioxidants (L-cysteine, ascorbic acid, catechin and quercetin) on the degradability of anthocyanin pigments, extracted from grape pomace. It was found that treatment of the model solutions at 80 °C induced anthocyanin decomposition, which obeyed first order kinetics. Addition of increasing amounts of antioxidants, including L-cysteine, ascorbic acid, catechin and quercetin, did not provoke a proportional impact, either positive or negative, with regard to anthocyanin stability. The best stabilising effect was seen after addition of ascorbic acid and catechin at concentrations of 4 and 2 mg L⁻¹, respectively (P < 0.001). Quercetin, however, was demonstrated a very efficient co-pigment, inducing an increase in A₅₂₀ by 63%, at pH 5.6 and a copigment-to-pigment ratio of 10.

Key words: anthocyanin; co-pigmentation; model systems; natural antioxidants; thermal degradation.
ine, catechin and quercetin. The effect of these substances on the absorbance characteristics of grape pomace anthocyanins was also examined, by estimating copigmentation effects. The choice of these antioxidants was based on (i) the fact that they are natural substances and well-studied antioxidant potency, (ii) their abundance in a wide spectrum of plant foods and (iii) their proven protection of flavonols against thermal degradation [11].

MATERIALS AND METHODS

Chemicals

All solvents used for HPLC and LC/MS analyses were of HPLC grade. Ascorbic acid (AA), quercetin (Qt), catechin (Ct), and L-cysteine (Cy) were from Sigma Chemical Co (St. Louis, MO, USA). Sucrose and citric acid were from Merck (Darmstadt, Germany). For the pH range 3-6, a phosphate/citrate buffer was used.

Vinification by-product (grape pomace)

Red grape pomace was from Agiorgitiko cultivar (Vitis vinifera sp.), obtained from Gaia Winery (Neimia, Korinthia), and it was left in contact with the fermenting must for 7 days. The pomace was obtained immediately after separation from the fermenting must, strained, and transferred to the laboratory within a few hours, where it was stored at -40 °C.

Anthocyanin extracts

Grape pomace was thawed and manually de-seeded. A lot of 240 g of seed-free red grape pomace was ground in a domestic blender and then extracted with ethyl acetate (3× 700 mL), to remove non-anthocyanin phenolics. The remaining tissue was then extracted with 800 mL 0.8% HCl in MeOH (solvent-to-solid ratio 3.3:1), at ambient temperature, under stirring, for 30 min. The extract was filtered through paper filter, and this procedure was repeated twice more. The filtrates were pooled, and MeOH was removed in vacuo. The residue was made to 250 mL with distilled water, and stored at -20 °C.

HPLC determination of the anthocyanin profile

The equipment utilized was an HP 1090, series II liquid chromatograph, coupled with an HP 1100 diode array detector and controlled by Agilent ChemStation software. The column was a LiChrosphere RP18, 5 μm, 250 mm×4 mm (Merck), protected by a guard volume packed with the same material. Both columns were maintained at 40 °C. Eluents A and B were 0.1% aqueous trifluoroacetic acid and MeCN/water (6/4, v/v), containing 0.1% TFA, respectively. The flow rate was 1 mL min⁻¹, and the elution programme used was as follows: 5 min, 20% B; 30 min, 100% B; 40 min, 100% B. Monitoring of the eluate was performed at 520 nm.

Liquid chromatography-mass spectrometry (LC/MS)

A Finnigan MAT Spectra System P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18, 125 mm×2 mm, 4 μm, column (Macherey-Nagel, Germany), protected by a guard column packed with the same material, and maintained at 40 °C. Analyses were carried out employing electrospray ionization (ESI) at the positive ion mode, with acquisition set at 12 and 70 eV, capillary voltage 4 kV, source voltage 45 V, detector voltage 650 V and probe temperature 400 °C. Eluents A and B were 2.5% acetic acid and MeOH, respectively. The flow rate was 0.33 mL min⁻¹, and the elution programme used was as described elsewhere [12].

Juice model matrix and thermal treatments

The juice-like solution was composed of sucrose (93 g L⁻¹, 10.1 °Bx), citric acid (5 g L⁻¹), and SO₂ (30 mg L⁻¹), adjusted to pH 3.48. A suitable volume of anthocyanin extract was dissolved in the model solution to give an approximate total anthocyanin concentration of 30 mg L⁻¹ and then filtered to eliminate suspended particles. This matrix was used for kinetic studies. For the examination of the effect of antioxidants (Cy, AA, CT and Qt), samples were spiked with antioxidant solutions to give final concentrations of 2, 4, 8 and 16 mg L⁻¹. Aliquots of 1 mL of the juice model matrix were placed in 2-mL microcentrifuge tubes, and heated at 80 °C, in a preheated water bath. At predetermined intervals, the tubes were withdrawn and immediately cooled with tap water.

Total anthocyanin determination

The pH-differential methodology was used [13]. An aliquot of sample was mixed with an appropriate volume of potassium chloride buffer (pH 1) and the absorbance was read at 520 (A₅₂₀) and 700 nm (A₇₀₀). Extracts were also combined similarly with sodium acetate buffer (pH 4.5) and the absorbance was obtained at the same wavelengths. Total anthocyanin content was determined as oenin (malvidin 3-O-glucoside) equivalents (MvE) using $\varepsilon = 28000$ and MW = 529, as follows:

$$TA (mg L^{-1}) = \frac{10^3 AMWF_{\varepsilon}}{\varepsilon}$$

(1)
where $A = (A_{520} - A_{700})_{pH \ 1} - (A_{520} - A_{700})_{pH \ 4.5}$, and $F_0$ the dilution factor. For all measurements, an HP8452A diode array spectrophotometer was used.

**Kinetics**

The degradation kinetics of anthocyanins fitted a first-order kinetic model. The reaction rate constant ($k$) and half-life ($t_{1/2}$) were determined using the following equations:

$$\ln\left(\frac{c_t}{c_0}\right) = -kt$$  \hspace{1cm} (2)

$$t_{1/2} = \frac{\ln 0.5}{-k}$$  \hspace{1cm} [3]

Where $c_0$ is the total anthocyanin concentration of the matrix and $c_t$ is the total anthocyanin concentration after time $t$ of incubation. The equilibration time was not accounted for the calculation of $t_{1/2}$.

**Copigmentation effects**

Pigment extract and solutions of antioxidants were combined suitably with buffers, at pH values ranging from 3.6 to 5.6. The final total anthocyanin concentration was 0.025 mM and the copigment-to-pigment ratio was 10. The mixtures were shaken vigorously and then the absorbance at 520 nm was recorded ($A$). The magnitude of copigmentation was calculated as follows:

$$\frac{A - A_0}{A_0}$$  \hspace{1cm} (4)

where $A_0$ is the absorbance of the pigment solution without copigment.

**Statistical analyses**

All determinations were carried out at least in triplicate and values were averaged. For all statistics, Microsoft Excel™ 2000 and SigmaPlot™ 9.0 were used.

**RESULTS**

**Pigment composition of the grape pomace extract**

The analytical anthocyanin composition of the grape pomace extract is illustrated in Figure 1. Two major pigments were detected at 520 nm, and LC/MS analyses were carried out to confirm their structure.
Peak 1 gave a molecular ion at m/z 493, and upon increased collision energy (70 eV) it yielded one major fragment at m/z 331. In a same fashion, peak 2 had a molecular ion at m/z 639 and a major fragment at m/z 331. On the basis of this set of data and previously published information [14,15], peaks 1 and 2 were identified as malvidin 3-O-glucoside, and its corresponding conjugate with p-coumaric acid (Figure 1).

Effect of antioxidant addition on the thermal degradation kinetics

For reasons of obtaining a measurable degradation rate of anthocyanins within a relatively short period of time, an accelerated assay at 80 °C was established. This specific temperature was chosen on the basis of published data [16,17] and preliminary experimentation. The thermal degradation of anthocyanins in the juice model matrix used was found to follow first order kinetics, which produced a good fit of the data (Figure 2). The reaction rate constants (k) and the half-life (t½) were calculated graphically and given analytically in Table 1. The addition of Cy appeared to accelerate the decomposition of anthocyanins, as k values increased from 1.46 (control sample) up to 1.77 × 10⁻³ min⁻¹ at the level of 2 and 8 mg L⁻¹ (P < 0.001). However, no consistent trend was recorded, since increasing amounts of Cy did not result in concomitant increases in k. This lack of consistency in the degradation behaviour was also observed in the case of AA, Ct and Qt addition. However, statistically lower k values (P < 0.001) were obtained upon addition of AA and Ct at levels of 4 and 2 mg L⁻¹, respectively. Contrary to that, Ct and Qt at a concentration of 8 mg L⁻¹ induced significant acceleration of pigment degradation (P < 0.001).

Co-pigmentation effects

The effect of co-pigmentation of each of the antioxidants tested, was evaluated by measuring the fractional increase in A₅₂₀ (Eq. (4)) within a pH range typical to food matrices, such as fruit juices. The copigment-to-pigment ratio of 10 was chosen as a mean value of ratios that usually trigger a measurable copigmentation effect, which is manifested as hyperchromic shift [18]. In Figure 3 it can be seen that strong copigmentation effects could be induced by Qt, whereas the effect of AA, Ct and Cy was of considerably lower magnitude. The pH value for maximal effect was 5.6 for Qt, AA and Ct, but Cy gave optimum results at pH 4.8 (Table 2). At the corresponding pH values, the fractional increase in A₅₂₀ induced by Qt was 1.84-fold higher than that of AA, 7.41-fold of Ct and 6.85-fold of Cy.

DISCUSSION

Anthocyanin degradation and the effect of antioxidants

Anthocyanins are known to be thermally labile pigments and their stabilisation pertains not only to
the preservation of their original colour [19], but also to their functional properties, such as the antioxidant activity [20]. However, the regulation of pH, which is long before known as a major stability parameter [21,22], as well as the avoidance of exposure to high temperatures during various modes of processing, is not always feasible. Thus, the search for techniques of stabilising mainly anthocyanins occurring indigenously in food matrices, such as fruit juices, becomes imminent.

In the study presented herein, it was shown that addition of selected natural antioxidants in a juice-like model solution does not always result in increased anthocyanin stability, although oxygen has been prominently implicated in anthocyanin degradation [23]. Only AA and Ct at specific ratios delayed anthocyanin degradation in a statistically significant manner, whereas Cy and Qt were proven ineffective in this regard.

Failure to enhance anthocyanin stability has been reported for other phenolics, such as chlorogenic acid [17] and procyanidins [24], but flavone C-glycosides were shown to afford significant protection of açai fruit anthocyanins in model systems [24]. In a similar fashion, polyphenol-containing rose extracts were also demonstrated to exert protective effect in a strawberry beverage [25], and orange flavonoids in blood orange juice [26].

Regarding AA, the findings available point to a rather controversial role with respect to anthocyanin protection. The presence of AA was found to be beneficial in elderberry juice during processing [22], but detrimental effects were shown for anthocyanin extracts from acerola [26] and blood orange juice anthocyanins [27]. According to previous investigations [28], this might be caused by oxidation reactions, in which AA acts as an activator of molecular oxygen, producing free-radicals that cleave the pyrilium ring.

Further, it should be stressed that Cy, Ct and Qt at the level of 8 mg L⁻¹ induced a statistically important acceleration of anthocyanin loss. This fact highlights the significance of the relative concentrations of compounds to be used as anthocyanin stabilisers, since it appears that addition at certain proportions might have detrimental effects.

### Co-pigmentation effects

The magnitude of copigmentation effect has been found to be dependent upon a range of factors, including the nature of both the pigment and co-pigment, and can be estimated by the parameter \( \frac{(A - A_0)}{A_0} \). For certain phenolics, the pH values within

<table>
<thead>
<tr>
<th>Concentration, mg L⁻¹</th>
<th>( k \times 10^3 ) / min⁻¹</th>
<th>( t_\frac{1}{2} ) / min</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.46±0.01</td>
<td>475</td>
<td>0.997</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.77±0.01a</td>
<td>392</td>
<td>0.988</td>
</tr>
<tr>
<td>4</td>
<td>1.62±0.01</td>
<td>428</td>
<td>0.995</td>
</tr>
<tr>
<td>8</td>
<td>1.77±0.04a</td>
<td>392</td>
<td>0.986</td>
</tr>
<tr>
<td>16</td>
<td>1.61±0.01</td>
<td>430</td>
<td>0.992</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.47±0.03</td>
<td>471</td>
<td>0.998</td>
</tr>
<tr>
<td>4</td>
<td>1.43±0.03a</td>
<td>485</td>
<td>0.996</td>
</tr>
<tr>
<td>8</td>
<td>1.70±0.01</td>
<td>408</td>
<td>0.987</td>
</tr>
<tr>
<td>16</td>
<td>1.56±0.01</td>
<td>444</td>
<td>0.994</td>
</tr>
<tr>
<td>Catechin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.41±0.02a</td>
<td>491</td>
<td>0.993</td>
</tr>
<tr>
<td>4</td>
<td>1.78±0.04a</td>
<td>389</td>
<td>0.999</td>
</tr>
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<td>8</td>
<td>1.71±0.02a</td>
<td>405</td>
<td>0.997</td>
</tr>
<tr>
<td>16</td>
<td>1.46±0.04</td>
<td>475</td>
<td>0.988</td>
</tr>
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<td>Quercetin</td>
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<tr>
<td>2</td>
<td>1.46±0.01</td>
<td>475</td>
<td>0.992</td>
</tr>
<tr>
<td>4</td>
<td>1.48±0.01</td>
<td>468</td>
<td>0.997</td>
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<tr>
<td>8</td>
<td>1.73±0.06a</td>
<td>401</td>
<td>0.992</td>
</tr>
<tr>
<td>16</td>
<td>1.51±0.02</td>
<td>459</td>
<td>0.998</td>
</tr>
</tbody>
</table>

\( \text{aValues statistically significant at a 99.9% level (student’s } t\text{-test)} \)
which optimum effects were seen varied from 3.2 to 4.7 [18].

Table 2. Optimal co-pigmentation effects of the antioxidants used, in relation to pH (all measurements were performed at a co-pigment-to-pigment ratio of 10. Total pigment concentration: 0.025 mM MvE)

<table>
<thead>
<tr>
<th>Co-pigment</th>
<th>pH</th>
<th>( \frac{A - A_0}{A_0} )_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cysteine</td>
<td>4.8</td>
<td>0.092</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>5.6</td>
<td>0.342</td>
</tr>
<tr>
<td>Catechin</td>
<td>5.6</td>
<td>0.085</td>
</tr>
<tr>
<td>Quercetin</td>
<td>5.6</td>
<td>0.630</td>
</tr>
</tbody>
</table>

In this case Qt was shown to be the best co-pigment, giving a fractional increase in the anthocyanin solution of 0.63, at pH 5.6 and a pigment-to-co-pigment ratio of 10. Qt is known to be a good malvin (malvidin 3,5-diglucoside) copigment [30], an anthocyanin which is structurally similar with malvidin 3-glucoside, the major anthocyanin in the pomace extract used, hence the effect observed. Very satisfactory copigmentation by Qt was also reported in wine-like model systems containing malvidin 3-glucoside [31]. By contrast, Ct was a rather poor copigment, an outcome that confirms the results given in Figure 3.

On the other hand, copigmentation of malvin was proven to occur also with simpler phenolics, such as caffeic and ferulic acids [32,33], isoflavonoids [34], and non-phenolic food constituents [35]. In Figure 3 can be seen that indeed AA was the second more efficient copigment after Qt. This is particularly important, considering that AA was one of the most protective compounds tested. On such a basis, it could be hypothesised that additives providing both colour enhancement and stabilisation might be attractive alternatives for food fortification. Contrary to that, Cy was shown nor protective neither colour enhancer, and therefore its value as a potential additive is rather questionable.

**CONCLUSIONS**

The most important findings of this study could be summarised as follows.

The analysis of an anthocyanin-containing grape pomace from the Hellenic native *V. vinifera* variety Agioritiko revealed that the major anthocyanin pigments were malvidin 3-glucoside and its corresponding p-coumaric acid ester.

The prevention of the thermal degradation of anthocyanins in a juice-like model matrix by the addition of natural antioxidants indicated that only AA and Ct can be effective at specific concentrations, whereas no consistent trend was observed with regard to the effect of concentration for any of the antioxidants tested.

The estimation of the co-pigmentation effects of the antioxidants used showed that Qt might be a good colour enhancer, but AA might be preferable since under specific conditions, could also exert better anthocyanin retention.
Abbreviations
AA - Ascorbic acid;
Ct - Catechin;
Cy - L-Cysteine;
MvE - Malvidin 3-O-glucoside equivalents;
Qt - Quercetin.

REFERENCES
INTERAKCIJA PRIRODNIH ANTIOXIDANATA SA ANTOCIJANINIMA U KAŠI CRVENOG GROŽĐA U TEČNOM MODEL MARIKSU: STABILNOST I KOPIGMENTACIONI EFEKTI

Cilj ovog rada bio je ispitivanje stabilnosti i poboljšanje boje antocijanina kaše crvenog grožđa u matričnom modelu soka, kao i uticaj dodatih prirodnih antioksidanata. Kao matrični model za testiranje uticaja dodatih prirodnih antioksidanata (L-cistein, askorbinska kiselina, katehin i kvercetin) na razgradnju antocijaninskih pigmenta, ekstrahovanih iz grožđane kaše, korišćen je sok (10,1 °Bx, pH 3,48). Utvrđeno je da tretiranje modelnog rastvora na 80 °C dovodi do procesa dekompozicije antocijanina, koji se odigrava po kinetički prvog reda. Dodavanje veće količine antioksidanata, uključujući L-cistein, askorbinsku kiselinu, katehin i kvercetin, nije izazvalo proporcionalni uticaj na stabilnost antocijanina, bilo pozitivan ili negativan. Najbolji stabilizujući efekat je uočen nakon dodavanja askorbinske kiseline i katehina u koncentraciji od 4 i 2 mg L⁻¹, respektivno (P < 0.001). Kvercetin se pokazao kao efikasan kopigment, koji indukuje povećanje vrednosti A520 za 63% pri pH 5,6 i pri odnosu kopigment-pigment 10.

Ključne reči: antocijanin; kopigmentacija; model sistemi; prirodni antioksidansi; termalna degradacija.