

**INDIVIDUAL ANTHOCYANINS AND THEIR CONTRIBUTION TO TOTAL
ANTIOXIDANT CAPACITY IN RESPONSE TO LOW TEMPERATURE
STORAGE AND HIGH CO₂ IN CARDINAL TABLE GRAPES**

5 Irene Romero, María T. Sanchez-Ballesta, María I. Escribano, Carmen Merodio*

Departamento de Ciencia y Tecnología de Productos Vegetales, Instituto del Frío, IF-
CSIC, José Antonio Novais 10, Ciudad Universitaria, E-28040 Madrid, Spain.

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*Corresponding author. Carmen Merodio, Departamento de Ciencia y Tecnología de
15 Productos Vegetales, Instituto del Frío, IF-CSIC, José Antonio Novais 10, Ciudad
Universitaria, E-28040 Madrid, Spain. Tel: +34 91 5492300; Fax: + 34 91 5493627; E-mail
address: merodio@if.csic.es

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Abstract

In this study, we have analyzed the profiles of individual anthocyanins in the skin of Cardinal table grapes and their contribution to the total antioxidant capacity (TAC) in response to low temperature (0 °C) and high CO₂ levels (20% for 3 days). An analysis of the representative colour parameters of this red-violet variety was also determined. The anthocyanin composition was determined using High Performance Liquid Chromatography coupled to Diode Array Detector and Mass Spectrometry (HPLC-DAD-MS). The contribution of individual anthocyanins to the TAC value of table grapes was calculated on the basis of their concentration and antioxidant capacity measured as the TEAC value (slope of the anthocyanin/slope of Trolox). Chromatographic analysis identified six anthocyanins, including pelargonidin 3-glucoside (Pg-3-G), in the skin of Cardinal table grapes. Short-term storage at 0°C in air had an increasing effect on the concentration of each of the identified anthocyanins. After 3 days at 0 °C, untreated grapes had the highest anthocyanin content (27.55 mg/100gFW) and displayed the largest TAC value (52.45 mM TE/199 g FW). Peonidin 3-glucoside (Pn-3-G) was the predominant anthocyanin, and it was mainly responsible for the rise in the calculated TAC value in untreated grapes. Pn-3-G had a low average TEAC value (1.73 mM), but its contribution could be explained by the sharp increase in the content of this anthocyanin the first days of storage at 0 °C. In contrast, the Pn-3-G content in grapes at the end of the 3-day CO₂ treatment did not change, and both the total anthocyanin content and the calculated TAC value remained significantly constant in comparison to the levels in pre-stored grapes. In addition CO₂ treatment had a positive effect on the amount of Pg-3-G concomitant with a pronounced decline in delphinidin 3-glucoside (Dp-3-G) and smaller decreases in petunidin 3-glucoside (Pt-3-G) and malvidin 3-glucoside (Mv-3-G). These effects of high CO₂ levels on the profile of individual anthocyanins were progressive lost when grapes were transferred to air. Indeed, after prolonged low temperature storage when the colour of the

berries become dark violet, similar calculated TAC values were quantified in untreated grapes and in those exposed to 20% CO₂. These data provide new insights about the effect of low temperature and high CO₂ levels on the concentration of the individual anthocyanins identified in Cardinal table grapes.

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Keywords: Table grapes; Postharvest technology; Carbon dioxide; Low temperature; Anthocyanins profile; Total Antioxidant Capacity.

1. Introduction

10 Low temperature is one of the most effective technologies to extend the post-harvest storage life of table grapes. Although *Vitis vinifera* is tolerant to chilling, activation of the defense in responses to stress have been reported in Cardinal table grapes. Specifically, we observed a transitory increase in the abundance of *PAL* and *CHS* transcripts during the first three days of storage at 0 °C in the skin of untreated grapes, which was accompanied
15 by an increase in total anthocyanin content (Sanchez-Ballesta et al., 2007). Indeed, cold temperature has been seen to increase anthocyanin levels in several growing plant species (Christie et al., 1994; Faragher 1983; Oren-Shamir and Levi-Nissim 1997; Schichijo et al., 1993; Stiles et al., 2007). Although this phenomenon appears to be relatively common, there is still some uncertainty concerning the significance of increased anthocyanin
20 production during post-harvest storage at low temperature. Indeed, the effect of low temperature on the concentration and distribution of anthocyanin pigments, and on their antioxidant activity, has yet to be fully characterized.

Anthocyanins are usually present in plants as a mixture of major and minor compounds. Their separation and identification requires the use of reversed-phase high-
25 performance liquid chromatography (HPLC) with diode array detection (DAD), which is effective in analyzing these pigments (Hong and Wrolstad, 1990). However, the natural

variability of different anthocyanins is large and some of them present similar retention times and spectral characteristics, making their identification by HPLC-DAD alone difficult. Therefore other techniques are often needed to accurately identify and characterize minor pigments. Among these techniques, mass spectrometry and MSⁿ analysis, coupled to the HPLC-DAD, are widely used to identify anthocyanins in different biological tissues (De Pascual-Teresa et al., 2002).

Several assays have been introduced to measure total antioxidant activity in food extracts and as such, it is often difficult to compare the results obtained for the same product in different studies. Furthermore, since antioxidant activity is derived from the delicate balance between antioxidants and prooxidants components, the total antioxidant capacity (TAC) of biological tissues in response to environmental conditions is sometimes not uniform. Hence, to calculate the TAC of table grapes during post-harvest storage we have considered the sum of the antioxidant capacity of the individual anthocyanins determined in the sample. The antioxidant activity of many anthocyanins was previously reported in terms of their Trolox Equivalent Antioxidant Capacity (TEAC) (Awika et al., 2004; Solomon et al., 2006). However, differences in the protocols and calculation methods (De Beer et al., 2006) produce different TEAC values for the respective anthocyanins. Furthermore, while these values are useful to determine the relative importance of the respective phenolic compounds, published data cannot be used to calculate the contribution of individual compounds to the TAC of a given sample. Hence, the TEAC values of pure anthocyanin reference standards have been measured in this work using the same protocol as that employed to determine the TAC of table grapes stored under different environmental conditions.

Anthocyanins are also responsible for many red, violet and blue colors in fruits and flowers (Harbone and Grayer, 1988; Remon et al., 2004). Pelargonidins are primarily responsible for the orange, salmon, pink and red colour of the fruit, while the magenta and

crimson comes from the cyanidins, and the purple, mauve, and blue are due to the delphinidins present. Since colour is an important parameter in berry quality, the association between colour and anthocyanin pigments profiles has been evaluated during post-harvest storage of table grapes. In this work, we applied the CIELAB colorimetric system to assess the changes in skin colour of untreated and CO₂-treated grapes during low temperature storage.

The aim of the present work was to analyze the individual anthocyanins in red-violet table grapes and to define their changes in response to low temperature storage (0 °C) after harvest. We also assessed how table grapes respond to high CO₂ levels (3 days, 20%) at 0 °C, both at the end of the treatment and when grapes were transferred to air. To associate anthocyanin production with important metabolic aspects of the fruit, we analyzed the contribution of individual anthocyanins to the TAC, as well as their implication in the colour of the skin of the grapes when exposed to CO₂ or not. This information is essential to evaluate the efficiency of post-harvest technologies whose goals should be to maintain the health and quality of the intact fruit.

2. Materials and Methods

2.1. Plant Material

Early-harvesting mature table grapes (*Vitis vinifera* L. cv. Cardinal) from Sevilla (Spain) were used (12.7% total soluble solids, 0.81% tartaric acid). Forced-air precooled bunches free from physical and pathological defects were randomly divided into two lots and stored at 0 ± 0.5 °C and 95% relative humidity (RH) in two sealed neoprene containers of 1 m³ capacity. Ten plastic boxes containing about 3 kg of table grapes per box were stored in each container. One lot was stored in air for 33 days (untreated fruit) and the other under a

gas mixture of 20% CO₂ + 20% O₂ + 60% N₂ (CO₂-treated fruit) for 3 days. The CO₂ concentration was maintained throughout the pretreatment and was measured daily using an automated gas chromatography system equipped with a thermal conductivity detector and a Poraplot Q column (Varian Chrompack CP20033P). After 3 days, the CO₂-treated
5 grapes were transferred to air under the same conditions as the untreated fruit until the end of the storage period. Ten clusters were sampled periodically. The berries from five clusters (approx. 300 g each cluster) were peeled and the skin was mixed, frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C for further analysis. For colour analysis, 45 berries were used that were randomly removed from the other five clusters and distributed
10 into three replicates of 15 berries each.

2.2. Extraction of anthocyanins

Frozen skin samples (0.5 g) were homogenized with 0.5 mL methanol containing 0.01% HCl by ultra-sonication for 10 min. The extract was centrifuged at 4000 x g and the
15 supernatant was removed. This step was repeated twice with the same solvent system until no more pigment was extracted and the solvent remained clear. The combined supernatants were filtered through a 0.45 µm nylon membrane filter (Millipore), to remove the solids residues, and the filtered material was evaporated to dryness with N₂ gas before resuspending in an equal volume of acidified water containing 4.5% formic acid (v/v) and
20 acetonitrile (95:5, v/v) for later HPLC-DAD-MS analysis.

2.3. HPLC-DAD-MS analyses

Aliquots of extracted anthocyanins (50 µL) were analyzed using a liquid chromatography/mass selective detector (LC/MSD) system coupled in series to a
25 photodiode array detector (DAD, G1315B) consisting of a quaternary pump (G1311A), a

vacuum degasser, a well-plate autosampler (G1313A) and a thermostat controlled column compartment controlled by software LC/MS ChemStation Revision A.08.03 from Agilent Technologies 1100 series (Waldbronn, Germany). Samples were injected at room temperature (20 °C) and the components were separated using a reverse-phase C₁₈ column (150 × 4.6 mm, 5 µm particle size, Scharlab, Barcelona, Spain). The mobile phase consisted of acidified water containing 4.5 % formic acid (v/v, A) and acetonitrile (B). The gradient used commenced with 5% phase B at time 0, 5-8% at 5-10 min, 8-10% at 10-15 min, 10-15% at 15-22 min, 15-25% at 22-27 min, 25-40% at 27-30 min, 40-73% at 30-33 min, 73-95% at 33-34 min, 95-5% at 34-41min. The flow rate was 0.9 mL/min, and the wavelength of detection was set at 520 nm. Scanning was also performed from 450 to 800 nm in 2 nm steps. Electrospray mass spectrometric analyses were carried out in a positive ion mode using a quadrupole mass spectrometer (G1946D) fitted with an atmospheric pressure electrospray ionization source (API-ES) with a fragmentation voltage of 140 V. The conditions of the spray chamber were: nebulizer, 45 psig; dry gas (N₂), 12 L/min; gas temperature, 300 °C; capillary voltage (positive), 3000 V. Spectra were recorded between m/z 287 and 494. The anthocyanin peaks were identified by comparison with molecular and main fragment ion values previously described in the literature (Muñoz-Espada et al., 2004; De Beer et al., 2006) and confirmed by comparison of commercially available anthocyanins standards. The anthocyanin concentrations were determined by the peak areas of the respective ion chromatograms extracted for Delphinidin 3-*O*-glucoside (Dp-3-G), Cyanidin 3-*O*-glucoside (Cy-3-g), Petunidin 3-*O*-glucoside (Pt-3-G), Pelargonidin 3-*O*-glucoside (Pg-3-G), Peonidin 3-*O*-glucoside (Pn-3-G) and Malvidin 3-*O*-glucoside (Mv-3-G), and using a standard curve derived from the commercial anthocyanins (Extrasynthese, France). The range of the calibration curves, with at least 5 points for each standard anthocyanin, took into account the relative abundance of table grape anthocynins: 0-2 µg/mL for Pg-3-G, 1-10 µg/mL for Cy-3-G, 2-20 µg/mL for Dp-3-G and Mv-3-G and 2-

300 µg/mL for Pn-3-G. The quantification for Pt-3-G was carried out in Pg-3-G equivalents and individual anthocyanin levels were expressed as mg/100 g fresh weight (FW).

5 2.5. Colour

Berry skin colour was measured at three different positions around the equator of the grape using the Hunter Lab System and a Minolta CR200™ colorimeter (Minolta Camera Co., Osaka, Japan). Results were given in Commission Internationale de l'Eclairage L^* (Lightness), a^* , and b^* (CIELAB) colour space coordinates. The chroma $(a^2 + b^2)^{1/2}$ and hue angle $(\tan^{-1} b/a)$ were calculated.

2.6. Radical cation ABTS scavenging capacity

Trolox (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid, Hoffman-La Roche) and standard anthocyanins, Dp-3-G, Cy-3-G, Pn-3-G, Pg-3-G and Mv-3-G (Extrasynthese, France) were diluted with ethanol at different concentrations to evaluate their antioxidant capacity, in a linear range, by the radical cation 2,2'-azinobis(3-ethylbenzothiazolone 6-sulphonate) ($ABTS^+$) as described by Re et al. (1999). The $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30 °C. After adding 990 µL of diluted $ABTS^+$ solution to 10 µL aliquots of different concentrations of Trolox and standard anthocyanins (µM), the absorbance reading was taken at 30 °C exactly 1 min after the initial mixing, and then for up to 6 min. Appropriate solvent blanks were run in each assay. The inhibition of absorbance at 734 nm is calculated and plotted as a function of the antioxidant concentration, and of the inhibition produced by Trolox as the standard reference compound.

The TAC contribution (mM TE/100g FW) of individual anthocyanins in the skin of table grape was calculated from their anthocyanins content (mg/100g FW) and TEAC values (mM) as described by De Beer et al. (2006):

$$\text{TEAC} = \text{slope (test compound)} / \text{slope (Trolox)}$$

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$$\text{TAC contribution} = [\text{compound}] \times \text{TEAC}$$

2.7. Statistical analyses

Data from at least three replicates per sampling period were subjected to an analysis of variance (ANOVA, Statgraphics program, STSC, Rockville, Md.), and a multiple
10 variance analysis was used to determine the significance of the data at $p \leq 0.05$. Two-way analysis of variance was performed using the LSD test procedure with type III sums of squares and a confidence level of 95% (Statgraphics program, STSC, Rockville, Md). The main effects of high CO₂ treatment, time of storage at 0 °C, and the CO₂ treatment x Time interaction on fruit were assessed.

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3. Results

3.1. Identification of anthocyanins

By combining HPLC-DAD and the analysis of the MS spectra, six anthocyanins
20 were identified in Cardinal table grapes (Fig. 1), whose retention times, molecular ions and main fragment ions are summarized in Table 1. Of these, peak 5 was associated with a strong signal in the MS detector and it corresponded to Pn-3-G. The MS spectrum revealed a molecular ion at m/z 433.3 associated with peak 4 and the main ion fragment produced was 271, corresponding to Pg-3-G. Additionally, a comparison of the molecular
25 ions and ion products of these anthocyanins with those of commercially available

anthocyanin standards confirmed the identification of Dp-3-G, Cy-3-G, Pn-3-G, Pg-3-G, Mv-3-G and Pt-3-G.

3.2. *Content and distribution of individual anthocyanins in table grapes in response to low temperature and short-term exposure to high levels of CO₂.*

The change in the anthocyanin content as result of the sum of the six individual anthocyanins during storage at 0 °C was examined (Fig. 2), and the anthocyanin levels in CO₂ treated grapes were quantified at the end of the treatment and when grapes were transferred to air. While 3 days of storage in air induced a sharp increase in the anthocyanin content from 14.55 to 27.55 mg/100 g FW, exposure to high levels of CO₂ did not cause any change with respect to the values of pre-stored grapes. After 6 days storage at 0 °C, the elevated anthocyanin content observed in untreated grapes decreased, reaching values similar to those found in CO₂-treated ones. In contrast, the anthocyanin content of CO₂-treated grapes transferred to air increased after 12 days and was still higher than that in untreated grapes at the end of the storage period.

The individual concentration of anthocyanins found in table grapes at the end of the CO₂ treatment and in those stored in air was determined at the same chronological age (3 days) (Table 2). Pn-3-G was the predominant anthocyanin found in this variety, with values of 12.78 mg/100 g FW in pre-stored table grapes. In untreated fruit stored in air for 3 days, there was an important increase in Pn-3-G content (+97%), reaching values of 25.20 mg/100 g FW. There was also a sharp increase in the minor anthocyanin Pg-3-G (+141%), from 0.061 to 0.147 mg/100 g FW. The first days of storage at 0°C had a less pronounced effect on Cy-3-G (+49%), Dp-3-G (+48%) and Pt-3-G (+25%), and only a moderate effect on the Mv-3-G content (+18%). While exposure for 3 days to high CO₂ treatment did not effect the Pn-3-G content (0%) and it had only a moderate effect on the Cy-3-G content (+24%), it sharply increased the Pg-3-G (+200%). In contrast to the

increase observed in grapes stored in air, high CO₂ treatment caused a pronounced decrease in Dp-3-G (-64%) and smaller decreases in Mv-3-G (-21%) and Pt-3-G (-27%) with respect to the content in pre-stored grapes

The change in the proportion of individual anthocyanins relative to the total anthocyanin content was determined in untreated grapes during storage at 0 °C and in CO₂ treated grapes at the end of the treatment and when the grapes were transferred to air (Fig. 3). Pn-3-G was the main anthocyanin, accounting for 87.8% of anthocyanin content in pre-stored grapes. After 3 days storage at 0 °C, a rise in the percentage of Pn-3-G was observed in untreated grapes. By contrast, Pn-3-G content was similar to the values found in pre-stored grapes after the CO₂-treatment. Interestingly, at the end of the 3 day CO₂ treatment there was a pronounced decrease in the anthocyanins synthesized directly from dihydromyricetin in the grapes, namely Dp-3-G, Mv-3-G and Pt-3-G, concomitant with a marked increase in the 3-O-glucoside of pelargonidin. After 6 days storage at 0 °C, the sharp rise both in the anthocyanin content and in the percentage of Pn-3-G observed in the first days of storage in untreated grapes decreased, reaching levels similar to those observed in the CO₂-treated fruit. Thus, our results showed that when CO₂-treated grapes were transferred to air, the percentage of Cy-3-G and Pg-3-G dropped sharply while the proportion of Mv-3-G, Dp-3-G and Pt-3-G began to increase, reaching similar values to those detected in untreated grapes after 12 days in storage. Thereafter, the trend in the percentage of individual anthocyanins was virtually identical in untreated grapes and those exposed to CO₂, finally reaching the same proportions.

3.3. Skin colour changes in untreated grapes and those exposed to CO₂ during storage at 0 °C

We determined the evolution of the parameters L^* , a^* , b^* , chroma and the hue angle in untreated and CO₂-treated grapes during storage at 0 °C (Fig. 4). The skin of untreated and CO₂-treated berries showed a similar evolution in their red-violet colour

during low temperature storage. The values of parameter a^* and chroma showed a progressive decrease in the first days of storage at 0 °C, and were higher in untreated than in CO₂-treated grapes. This decrease was particularly notable after 22 days when they reached their lowest values. On the contrary, the values of the parameter b^* and the hue angle undergo an opposite effect over the storage period and they were higher in CO₂-treated than in untreated grapes. After 22 days of storage at 0°C, the values of the parameter b^* and hue angle were very near 0, and the L^* parameter reached minimum values, indicating that at this time the colour of untreated and CO₂-treated berries was dark violet.

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3.4. The antioxidant capacity of table grapes in response to low temperature and short-term exposure to high CO₂

The antioxidant capacity of Trolox and reference standard solutions of anthocyanins was determined using the ABTS⁺ scavenging assay. Inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of each compound (Fig. 5). Subsequently, the data were adjusted by linear regression and the slope relation between reference standards and Trolox were used to determine the antioxidant capacity measured as TEAC for individual anthocyanins. A comparison between the antioxidant capacity of the anthocyanins indicated that Dp-3-G had the highest antioxidant capacity (6.99 mM) followed by Pg-3-G (3.68 mM). On the other hand, Mv-3-G had the lowest TEAC value (1.37 mM). According to these TEAC values (mM) and the concentration of the individual anthocyanins (mg/100 g FW), the contribution of the major individual anthocyanins to the TAC value of untreated and CO₂-treated table grapes was calculated (mM TE/100g FW, Table 3). Our results indicate that after 3 days storage at 0 °C the untreated grapes had the highest anthocyanin content (27.55 mg/100g FW) and displayed the largest TAC value (52.45 mM TE/100g FW). Although an increase in each of the

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anthocyanins contributed to this high TAC value, Pn-3-G was mainly responsible for this sharp early rise in the TAC value of untreated grapes. Pn-3-G had a low average TEAC value (1.73 mM) but its strong contribution could be explained by the drastic increase in the content of this predominant anthocyanin after 3 days in storage at low temperature (Table 3). Conversely, no significant difference was observed in the total TAC value in 3 day CO₂-treated grapes and the Pn-3-G values remained at similar values to those observed in pre-stored grapes. Pg-3-G was present in relatively small amounts in these berries but it had a very high average TEAC value (3.68 mM) and thus, it was an important contributor to the TAC value in CO₂-treated grapes. Additionally, our results indicated that as result of the changes in the concentration of individual anthocyanins, the TAC contribution showed significant changes throughout low temperature storage (Table 3). The LSD test confirmed that the factors time (D) and time x CO₂-treatment interaction (DxT), significantly affected the TAC contribution of individual anthocyanins and the TAC value of untreated and CO₂-treated table grapes (P<0.05). The highest F value for the factor time was found for the contribution of Pg-3-G TAC (F value = 79.49), followed by Mv-3-G (F value= 15.26). The effect of exposure to high CO₂ throughout low temperature storage was only significant on the TAC contribution of Dp-3-G. The decrease in the levels of Dp-3-G up to 6 days in storage at 0 °C seemed to explain the significant effect of CO₂ on the TAC contribution of this anthocyanin.

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4. Discussion

Cold-stressed growth conditions are known to produce an increase in anthocyanin synthesis, although the mechanisms underlying temperature sensitivity in anthocyanin production are poorly understood (see reviews by Mol et al., 1996; Chalker-Scott, 1999). In general, the effects of low temperature storage on anthocyanin production have been assessed in terms of total anthocyanin levels rather than changes in the levels of specific

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anthocyanins. In the present work, we have identified the individual anthocyanin pigments in Cardinal table grapes using mass spectrometry and we have examined the effects of low temperature (0 °C) with and without high CO₂ (20%) on each of these. Moreover, instead of relative peak areas, the precise determination of the concentration of the individual anthocyanins in this variety has been calculated. We identified six anthocyanins and in contrast to wine grapes in which the main anthocyanin has been reported to be Mv-3-G (Muñoz-Espada et al., 2004), Pn-3-G was the predominant anthocyanin in the skin of table grapes. It is also important to note the presence of Pg-3-G in the skin of Cardinal table grapes. The presence of 3-*O*-glucoside of pelargonidin was also previously found in Concord, Rubired, and Salvador grape juices. Rubired and Salvador grapes are hybrids from *Vitis vinifera* and *Vitis rupestris* and Concord is a grape from the native American cultivar *Vitis labrusca* (Wang et al., 2003). The amount of the six anthocyanins found in untreated grapes all increased after storage for 3 days at 0 °C. In accordance with the anthocyanin biosynthetic pathway in plants (Schijlen et al., 2004, Winkel-Shirley, 2001; Stiles et al., 2007), we suggest that the cyanindin, delphinidin and pelargonidin branches seem to be involved in anthocyanin accumulation during the first days of storage at 0 °C. Although further work is needed to know the mechanism implicated in the induction of anthocyanins in grapes at very low temperature, our results indicate that at least one of the temperature control points could be upstream of dihydrokaempferol, where this branching occurs. This is consistent with the induction of *PAL* and *CHS* mRNA in untreated grapes after 3 days at 0 °C (Sanchez-Ballesta et al., 2007), which may lead to increased upstream enzyme activity. In contrast, short-term exposure to high CO₂ levels (20% for 3 days) had no significant effect on anthocyanin content as the observed values were similar to those in pre-stored grapes. Moreover, in grapes treated for 3 days in CO₂ the increase in pelargonidin, an anthocyanin synthesized directly from dihydrokaempferol, was concomitant with the decrease in anthocyanins synthesized directly from dihydromyricetin,

namely delphinidin, petunidin and malvidin. It is known that the anthocyanin biosynthesis pathway is controlled in response to different developmental and environmental cues (Holton and Cornish, 1995; Mol et al., 1998). Another challenging aspect would be to clarify the detailed course of the reactions and control systems involved in the effects of
5 low temperature and high CO₂ on anthocyanins biosynthesis in fruit tissues.

Anthocyanins are the most prominent pigments in grape skin and they are strong antioxidants. Their double bond conjugate systems allow electron delocalization, resulting in very stable structures and a powerful antioxidant activity. Furthermore, the extent and position of hydroxylation and methoxylation in the B ring modulates their stability and
10 reactivity (Muñoz-Espada et al., 2004). Differences in antioxidant activities between various anthocyanins have been noted in several studies (Tsuda et al., 1994; Wang et al., 1997; Deighton et al., 2000). Moreover, the increase in anthocyanin content in table grapes measured by the pH differential method was not always associated with a similar proportional increase in antioxidant capacity measured by ABTS assay (Sanchez-Ballesta et
15 al., 2007). It has been reported that pH differences have a major influence on scavenging capacity of wine anthocyanins (Borkowski et al., 2005) and that the presence of acid in the solvent has a influence negative in the antioxidant capacity of samples measured by ABTS procedure (Pérez-Jiménez and Saura-Calixto, 2006). In the present work we decided to calculate the TAC value of the samples considering the contribution of individual
20 anthocyanins to the TAC on the basis of their concentration and antioxidant capacity. We found that the calculated TAC values were higher in untreated grapes stored for 3 days at 0°C than in pre-stored grapes. The rise in Pn-3-G levels, the predominant anthocyanin in this variety, contributed to the peaking of TAC value in grapes at the beginning of low temperature storage. Moreover, the fact that the calculated TAC values were lower in
25 grapes treated with CO₂ seems to be due to the maintenance of Pn-3-G levels.

We suggest that the accumulation of anthocyanins at the beginning of storage at 0° C may be a response to a burst in free radicals originated by the fruits' own metabolism in response to the temperature shift. At this low temperature, the untreated fruit triggers this natural antioxidant defense mechanism to reduce the severity of stress. Since the calculated
5 antioxidant capacity in our CO₂-treated table grapes was similar to that found in pre-stored grapes, we can conclude that CO₂ reduces the sensitivity of these grapes to low temperature rather than activating this defense mechanism. In this sense, we previously reported that the effectiveness of this gaseous treatment was not mediated by the induction of either *STS* gene expression (Sanchez-Ballesta et al., 2006) or that of PR genes (Romero
10 et al., 2006). Indeed, a decrease or no change in antioxidant activity was reported in fruits stored at low temperature under controlled atmospheric conditions (van der Sluis 2001). Our results indicate that after prolonged low temperature storage similar calculated TAC values were obtained from untreated grapes and those exposed to short-term high CO₂.

The colour of red and black grapes results from the accumulation of the
15 corresponding pigments, the orange to red pelargonidin, the red to magenta cyanidin, and the violet to blue delphinidin. Each variety of grapes has a unique set of anthocyanins (Mazza and Miniati, 1993) and the external colour of the skin of the grapes is used to classify grape varieties into the following groups: green-yellow, pink, red, red-grey, red-dark violet, red-black and blue-black. The mature berries of Cardinal table grapes usually possess
20 red or violet tones of greater or lesser intensity, and the amount and nature of its anthocyanins could situate this variety in the red-black group (Carreño et al., 1997). These authors also reported that the *L** hue angle and chroma values in grapes with violet tints were closely correlated, both between each other and also with the visual color, while the value of *a** was not a representative parameter of color. Taking into account the results
25 obtained here, it seems that the most significant differences in the L, chroma and hue angle values between untreated and CO₂ treated grapes appear during the initial days of storage at

0°C. The fact that the reduction in the blue tones was greater in CO₂-treated than in untreated grapes in the first 6 days of storage at 0 °C could be associated with the decrease in the amount of delphinidin pigments. However, the possible relationship between colour and the changes in the levels of Dp-3-G and Pg-3-G after exposure to high CO₂ levels should be further studied. After 22 days storage at 0°C, the colour of untreated and CO₂-treated berries becomes dark violet with red-violet tints of lesser intensity. At this time, the *L**, *b**, chroma and hue angle values are in accordance with those reported by Carreño et al., (1995) for ripe dark violet grapes.

Until now, the presence of anthocyanins characteristic of any particular species has been useful in testing or confirming the interspecies origin of the genotypes that produce the characteristics of grapes and blackberries. The data we have obtained provide a new framework for the effect of high CO₂ treatment and low temperature on the induction of specific anthocyanins in table grapes. Furthermore, the present results show that the analysis of the profiles of individual anthocyanins and their contribution to the TAC value represents a valuable tool to define their tolerance to specific environmental storage conditions.

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Table 1. Chromatographic and spectrophotometric characteristics of each identified anthocyanins in Cardinal table grape

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Peak number	Retention time (min)	Molecular ion (<i>m/z</i>)	Main fragment ions	Peak assignment
1	4.78 ± 0.16	465.3	303	Dp-3-G
2	7.14 ± 0.32	449.2	287	Cy-3-G
3	8.95 ± 0.39	479.4	317	Pt-3-G
4	10.35 ± 0.40	433.3	271	Pg-3-G
5	12.12 ± 0.39	463.3	301	Pn-3-G
6	14.17 ± 0.35	493.4	331	Mv-3-G

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Table 2. Individual anthocyanin content (mg/100 g FW) in untreated and CO₂-treated grapes after 3 days at 0 °C.

Days at 0 °C	0d	3d air	Δ (%) ²	3d CO ₂	Δ (%) ³
Pn-3-G	12.78 ± 3.09a ¹	25.20 ± 0.33b	+97	13.08 ± 0.84a	0
Cy-3-G	0.41 ± 0.01a	0.61 ± 0.12b	+ 49	0.51 ± 0.06ab	+ 24
Pg-3-G	0.061 ± 0.003a	0.147 ± 0.007b	+141	0.183 ± 0.009c	+200
Mv-3-G	1.14 ± 0.10ab	1.35 ± 0.14b	+18	0.90 ± 0.18a	-21
Dp-3-G	0.123 ± 0.015b	0.182 ± 0.004c	+48	0.044 ± 0.015a	-64
Pt-3-G	0.048 ± 0.002b	0.060 ± 0.005c	+25	0.035 ± 0.002a	-27

¹Different letter in a row indicate a significant difference ($P \leq 0.05$).

²delta between those two time points t=0 and t= 3d air

5 ³delta between those two time points t=0 and t= 3d CO₂

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Table 3. TAC contribution (mM TE/100 g FW) of the individual anthocyanins and calculated TAC value of untreated and CO₂-treated Cardinal table grapes during storage at 0 °C

Days	Pn-3-G		Mv-3-G		Cy-3-G		Pg-3-G		Dp-3-G		TAC value ^a	
	air	CO ₂	air	CO ₂	air	CO ₂	air	CO ₂	air	CO ₂	air	CO ₂
0	23.97±5.79	23.97±5.79	1.65±0.15	1.65±0.15	0.84±0.01	0.84±0.01	0.23±0.01	0.23±0.01	0.95±0.11	0.95±0.11	27.63±6.09	27.63±6.09
3	47.27±0.62	24.53±1.57	1.95±0.20	1.30±0.26	1.25±0.25	1.04±0.12	0.58±0.02	0.72±0.03	1.38±0.03	0.30±0.11	52.45±1.12	27.91±2.10
6	28.15±8.14	22.28±5.59	1.56±0.37	1.31±0.28	0.97±0.17	0.65±0.11	0.40±0.04	0.30±0.05	0.75±0.10	0.21±0.04*	31.84±8.83	24.77±6.08
12	19.34±0.10	33.13±5.91	1.31±0.12	2.30±0.08	0.70±0.03	1.05±0.27	0.29±0.03	0.31±0.05	0.63±0.04	1.11±0.33	22.27±0.34	37.89±6.66
22	24.89±3.37	22.24±1.97	1.48±0.20	1.34±0.33	1.02±0.03	0.61±0.03	0.31±0.04	0.29±0.07	0.92±0.06	0.69±0.06	28.61±3.72	25.18±1.97
33	31.94±6.22	37.59±4.51	1.54±0.37	2.01±0.25	0.93±0.11	1.01±0.19	0.29±0.05	0.33±0.05	0.86±0.36	0.92±0.10	35.55±7.12	41.86±5.11
Significance	D*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*	D*, T*, DxT*	D*, DxT*	D*, DxT*	D*, DxT*

*Significant at P≤0.05, where D=days and T=CO₂-treatment.

^a TAC value was calculated as the sum of the contribution of individual anthocyanins. The contribution of Pt-3-G to the TAC value is not included.

Figure Legends

Fig. 1. HPLC-DAD chromatogram (520 nm) and the ion chromatograms extracted at m/z corresponding to the molecular ion of each identified anthocyanins in Cardinal table grape:

- 5 (1) Delphinidin-3-glucoside; (2) Cyanidin-3-glucoside; (3) Petunidin-3-glucoside; (4) Pelargonidin-3-glucoside; (5) Peonidin-3-glucoside; (6) Malvidin-3-glucoside.

Fig. 2. Changes in anthocyanin content as result of the sum of the individual anthocyanins identified and quantified in untreated and CO₂-treated Cardinal table grapes during storage
10 at 0 °C. The dotted line indicates the short period of high CO₂ treatment. The row indicates the transfer of CO₂-treated fruit to air. The results are the mean of three replicate samples ± SE.

Fig. 3. Percentage of individual anthocyanins relative to the total anthocyanin content in
15 untreated and CO₂-treated Cardinal table grapes during storage at 0 °C. The dotted line indicates the short period of high CO₂ treatment and the row indicates the transfer of CO₂-treated fruit to air.

Fig. 4. Evolution of the L^* , a^* , b^* , chroma and hue angle in untreated and CO₂-treated
20 Cardinal table grapes during storage at 0 °C. The dotted line indicates the short period of high CO₂ treatment and the row indicates the transfer of CO₂-treated fruit to air. The results are the mean of three replicate samples ± SE.

Fig. 5. Antioxidant capacity of Trolox and standard reference anthocyanin solutions using
25 the ABTS⁺ scavenging assay. The TEAC values (mM) were calculated from the standard and Trolox slope ratios.

Figure 1

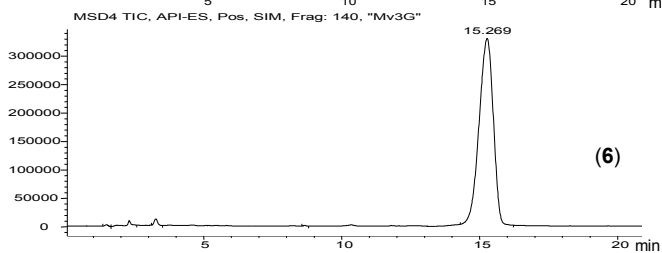
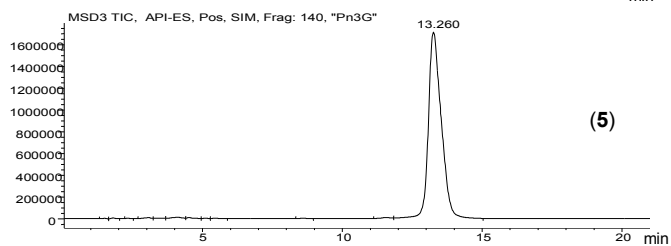
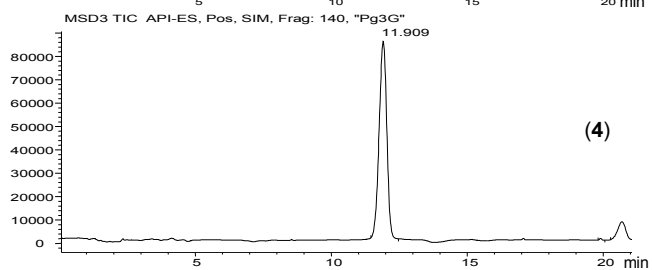
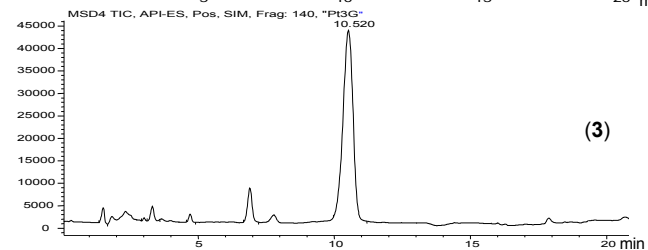
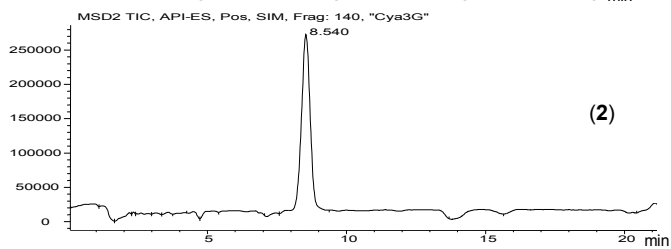
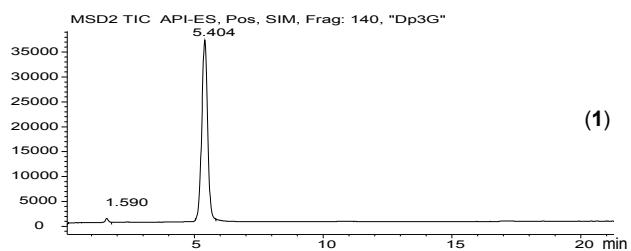
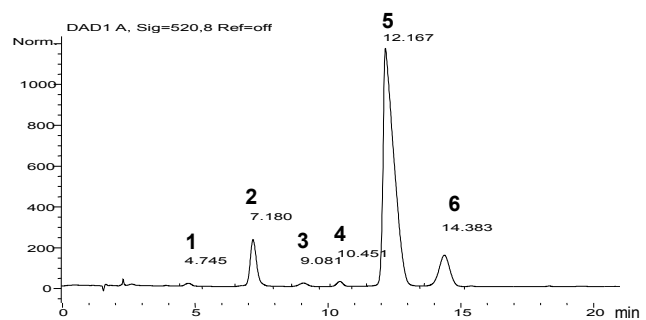


Figure 2

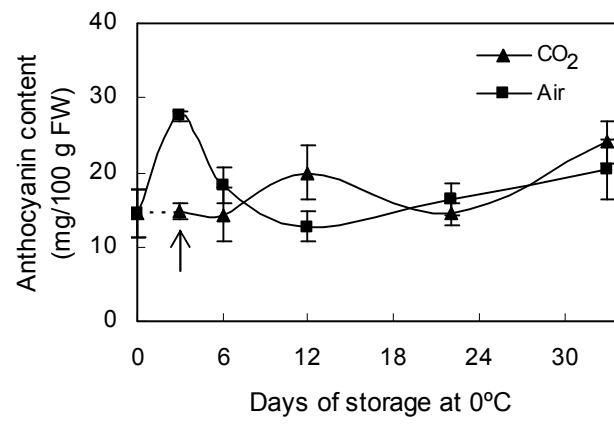


Figure 3

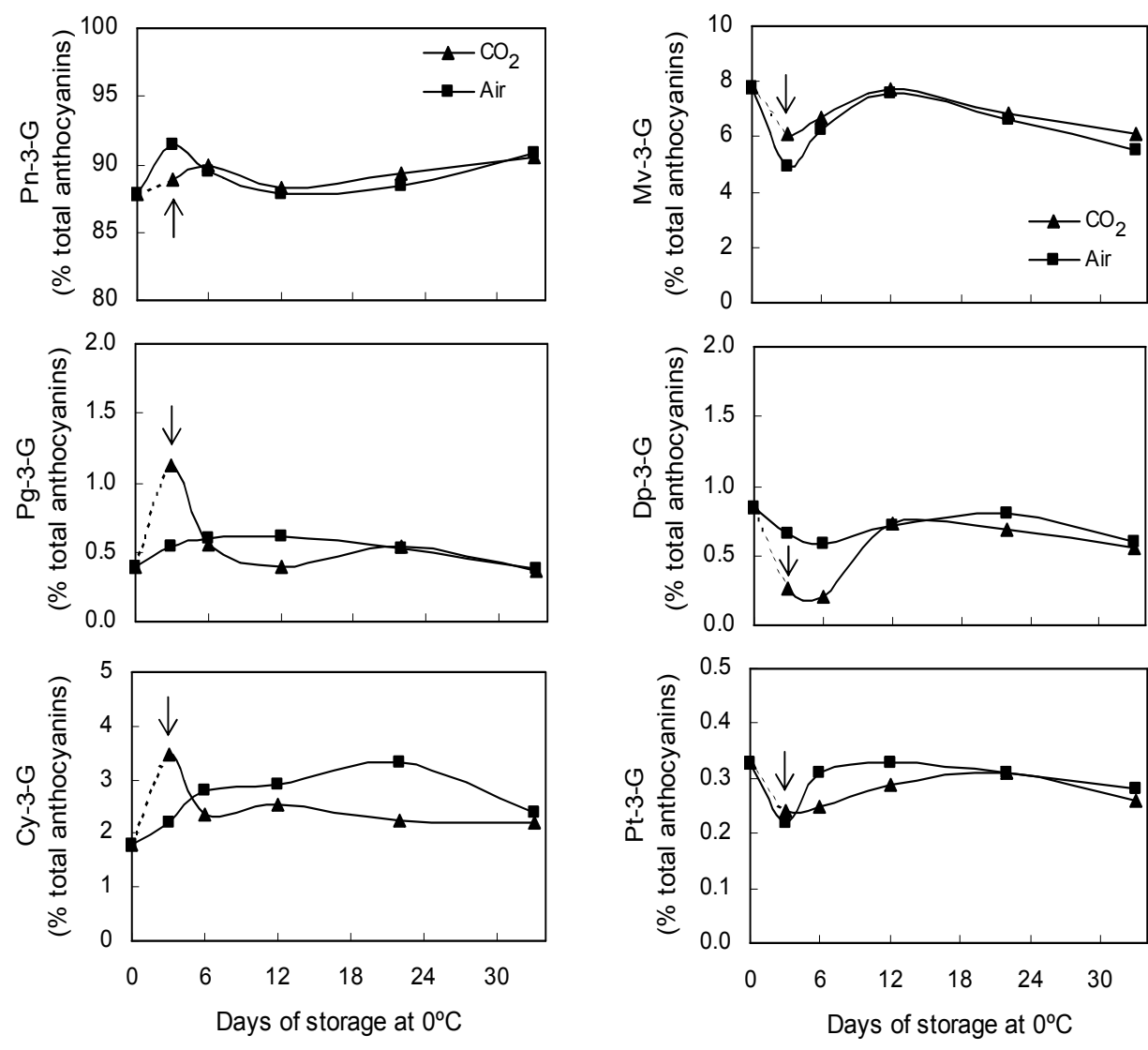


Figure 4

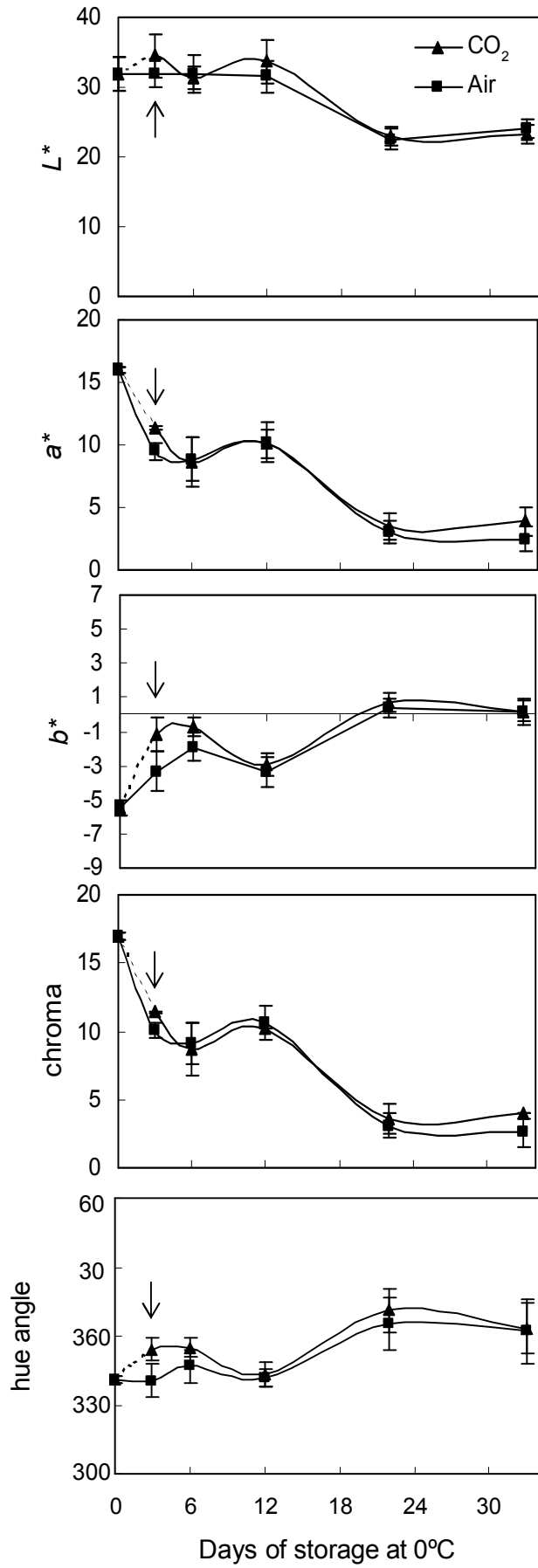
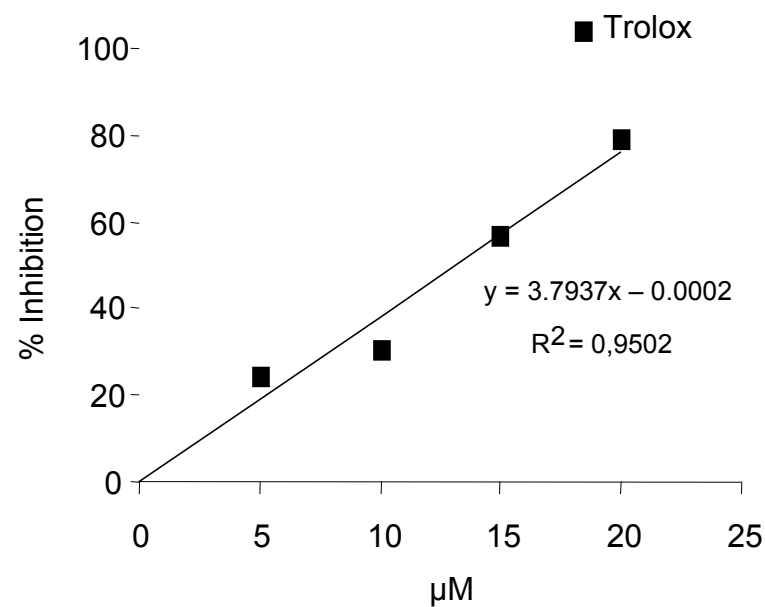
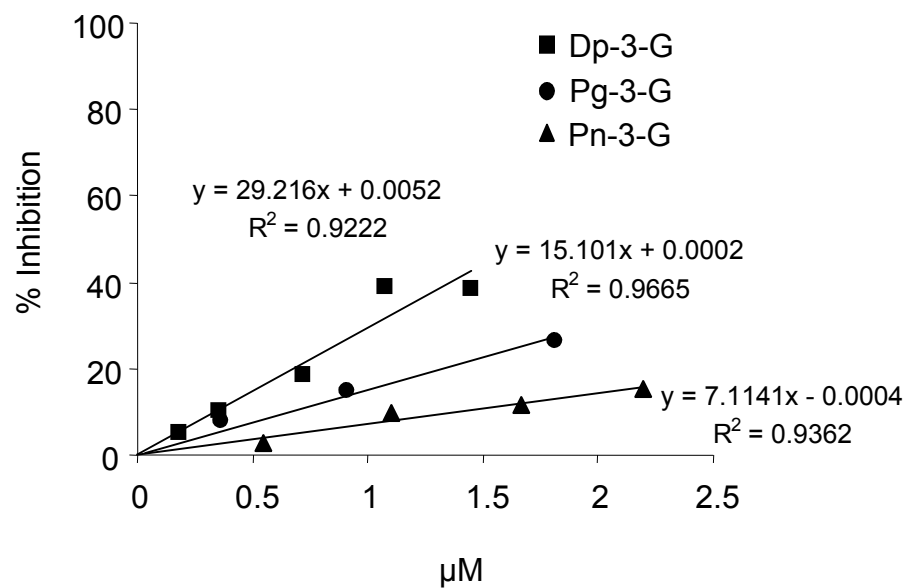
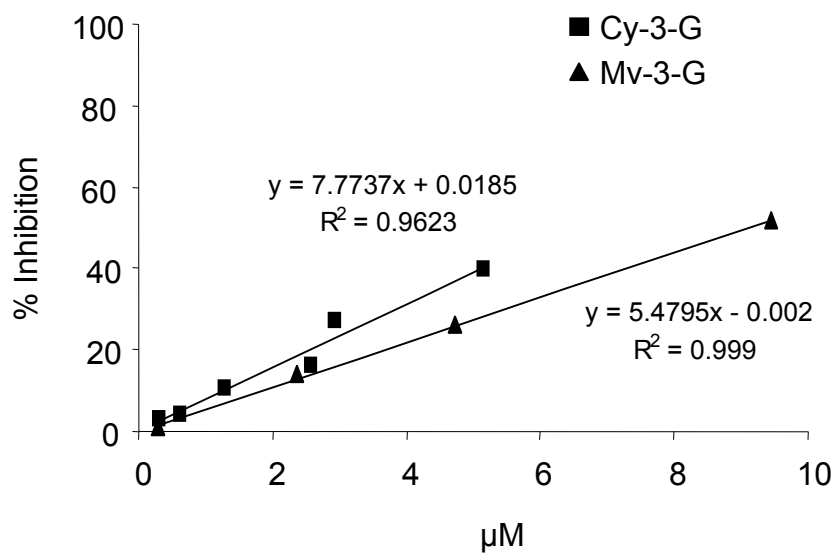


Figure 5

Anthocyanin	TEAC (mM)
Pn-3-G	1.73
Mv-3-G	1.37
Cy-3-G	1.89
Pg-3-G	3.68
Dp-3-G	6.99



Dear Dr. Ferguson

Thanks to you and the reviewers for the timely examination of our manuscript. We have considered the issues noted by the reviewers and have carefully revised the manuscript following the Reviewers' valuable suggestions.

Best regards,

Carmen Merodio