# Increasing catechin and procyanindin accumulation by high $CO_2$ treatments in *Fragaria vesca.*

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## ABSTRACT

This paper deals with the impact of low temperature and high $CO_2$ levels on flavonols, proanthocyanidins and anthocyanins, synthesized via branched pathways from common precursors, in strawberries (*Fragaria vesca* L.). Simultaneously extracted flavonoids from untreated (air) and $CO_2$-treated (20% or 40%) strawberries were identified with Q-TOF equipment and quantified by HPLC-quadrupole. We found that flavan 3-ols accumulated only in $CO_2$ treated strawberries, whereas in untreated fruit, flavonoid production was redirected towards anthocyanin accumulation with a sharp decrease in catechin and procyanidin B3 levels. Catechin induction could be associated with the potential of high $CO_2$ treatments to reduce the incidence and severity of decay due to its antifungal activity. Although flavan 3-ols were induced by high $CO_2$ treatments, differences were observed when comparing 20% and 40% $CO_2$ treated fruit. The accumulation of flavan-3 ols in 20% $CO_2$ treated fruit was concomitant with an increase in flavonol content (quercetin and kaempherol derivatives). By contrast, ascorbic acid levels increased in 40% $CO_2$-treated fruit. In spite of the variations in the accumulation of flavonoids, a similar antioxidant capacity was found in untreated and $CO_2$-treated Mara de Bois strawberries.



KEYWORDS: flavonoids, proanthocyanidins, strawberries, high $CO_2$, ascorbic acid, mass spectroscopy, antioxidant activity.

## INTRODUCTION

Strawberry (*Fragaria vesca* L.) is a high-value fruit due to its pleasing taste and flavour. However, there are extensive postharvest losses produced mainly by fungal attack, rapid water loss and structure deterioration. For this reason, there is a growing interest in the development of technologies for controlling fungal decay while maintaining fruit quality. The fungistatic effects of high $CO_2$ levels on strawberries are well known[1]. We previously reported that the effectiveness of high $CO_2$ treatment for controlling fungal decay in table grapes, another high $CO_2$ tolerant fruit, was not mediated by the induction of specific phenylpropanoid genes (Phenylananine ammonia lyase, PAL; Chalcone synthase, CHS; Stilbene synthase, STS)[2]. Additionally, short-term exposure to high $CO_2$ levels had no significant effect on anthocyanin content, while fruit stored in air at 0 ºC had the highest anthocyanin levels. Anthocyanin induction at low temperature was also reported in different strawberry cultivars, as well as in other plant systems[3, 4]. As anthocyanins are synthesized via branched biosynthesis pathways with proanthocyanidins, our aim in the present work was to analyse the feasibility of high $CO_2$ levels for channeling phenolic compound precursors into proanthocyanidins instead of anthocyanins. Furthermore, since proanthocyanidins, and mainly catechins[6], have been linked with protection against pathogens[5] the development of technologies that enhance its production is desirable. Proanthocyanidinds are polymeric flavonoid compounds composed of flavan-3-ol subunits. They influence the sensory properties of astringency. Nevertheless, several components, other than phenolics, are known to influence the perception of astringency, including ethanol, acidity, pH and polysaccharides among others. Although proanthocyanidin concentrations have been estimated in several plant sources using simple colorimetric methods, proanthocyanidins have also been reported in different foods by employing more sensitive, specific methods such as HPLC and Mass Spectrometry[7]. The building blocks or starter units of most proanthocyanidins are the flavan-

3-ols (+)-catechins and (-)-epicatechins, whose last common biosynthetic intermediate is leucoanthocyanidin, an intermediate in anthocyanin biosynthesis, also referred to as flavan 3,4-diol, connected with the flavonol precursor molecules[8]. Consequently, differences in proanthocyanidin levels due to the effect of high $CO_2$ levels and low temperature should be considered together with the changes in anthocyanins concentrations and also with flavonols. Additionally, flavonols and their glycosides appear to be mainly involved in response mechanisms against abiotic stress[9] and may contribute to environmental stress tolerance[10].

Furthermore, all these flavonoids act as agents against reactive oxygen species (ROS) generated during the normal ripening stage and by stressful storage conditions that cause oxidative damage in the fruit. Ascorbic acid also has the property of scavenging oxidants and free radicals, and strawberry fruit is one of the richest sources of ascorbic acid among fruits[11]. In the present work variations in ascorbic acid and antioxidant activity levels were analysed in untreated and $CO_2$-treated fruit. Ascorbic acid levels were quantified by high-performance anion-exchange chromatography (HPAEC) and antioxidant capacity was determined by means of photochemiluminescence (PCL). In the PCL assay the photochemical generation of free radicals was combined with sensitive chemiluminescence detection[14]. This method is suitable for measuring the scavenging capacity of antioxidants present in water-soluble fractions in pulp fruit extracts against the radical anion superoxide.

The objectives of this study were to identify and quantify simultaneously anthocyanidins, flavonols, proanthocyanidins and their flavan-3-ols monomers in freshly harvested strawberries, using Q-TOF equipment and quantification by HPLC-quadrupole. Second, to analyse the effect of low temperature and high $CO_2$ treatments on the accumulation of anthocyanidins, proanthocyanidins and flavonols. Third, to study the relationship between variations in flavonoid levels including ascorbic acid and the antioxidant capacity of untreated and $CO_2$-treated fruit. All the aforementioned information is essential to evaluate a beneficial

high $CO_2$ treatment able to maintain initial antioxidant capacity and avoid damage caused by fungal attack.

## MATERIALS AND METHODS

### Plant material

Organic strawberries (*Fragaria vesca* L. cv. Mara de Bois) were harvested by hand in May 2010 at the first flowering in an orchard in San Sebastian de los Reyes (Madrid, Spain). Fruit were harvested at the same mature stage when fruit have reached full size (9.8% total soluble solids, 0.8% titratable acidity as citric acid and colour parameters L* 18, a* 40, b*29). After harvest, fruit were transported to Institute of Food Science Technology and Nutrition within 2 h. Fruit selected for uniform size and colour were stored at 0 ºC (± 0.5) and >95% relative humidity in three sealed containers with a capacity of 1 $m^3$. Fifteen plastic boxes containing approximately 0.5 kg of strawberries per box were stored in each container for 3 days and exposed to a continuous flow of air (untreated fruit) or a gas mixture containing 20% or 40% $CO_2$. The same $O_2$ concentration (20%) was maintained in all three lots. $CO_2$ and $O_2$ concentrations were measured using a gas analyzer PBI Dansensor mod. Checkmate 9900. Initially and at the end of the 3-day sampling period, 45 strawberries were taken for quality analysis, and another 45 were removed at random from each of the treatment groups and divided into three batches of 15 berries. The 15 strawberries from each batch, used as a biological replicate, were mixed, frozen in liquid nitrogen and stored at -80 ºC for further analysis. From each of the three biological replicates, at least two different measurements were taken.

### Identification of flavonoid composition by MS and-$MS^2$

For the extraction of water-soluble flavonoids, fruit samples (3 g) were homogenized in 10 mL of ultra-pure water, sonicated for 10 min and centrifuged at 30,000 x *g* for 20 min, and the supernatants were then filtered through a membrane of 0.45 μm pore size. A preliminary identification of the compounds to be studied was made by the Quadrupole Time-of-Flight (Q-TOF) mass spectrometer as described in this section. Then, under the same chromatographic conditions we proceeded to quantify these compounds in SIM (Selected Ion Monitoring) mode by HPLC-Quadrupole. Analyses were performed using an Agilent 1200 series LC, comprised of a quaternary pump G1311A with an integrated degasser G1322A, thermostated autosampler G1330B, thermostated column compartment G1316A, coupled with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS with ESI-Jet Stream Technology (Agilent Technologies, Waldbroon, Germany). A 10 μL sample was separated in a Kromasil C18 column 5μm 4.6x150mm (Análisis Vínicos, S. L, Madrid) eluted with a mobile phase made up of a mixture of deionized water (solvent A) and acetonitrile (solvent B) both containing 0.1% formic acid, at a flow rate of 0.8 mL/min. The solvent gradient changed according to the following conditions: from 90% A to 70% A in 30 min, to 65% A in 5 min, to 55% A in 5 min and then back to the initial conditions in 10 min. MS and $MS^2$ experiments were performed to identify and characterize flavonoids. The Q-TOF acquisition method was High Resolution 4GHz, mass range low 1700 m/z. Ionization was achieved by Atmospheric Pressure Electrospray Ionization (ESI) with a drying gas flow rate of 10 L/min at 350 ºC, sheath gas flow 7.5 L/min at 325 ºC, nebulizer 35 psi, cap voltage 3500V, nozzle voltage 1000V, fragmentor voltage 150V and skimmer voltage 65V. The experiments were done with both negative polarity (for flavonols and flavanols) with reference masses (119.0363 and 966.0007 m/z) and positive polarity (for anthocyanins) with reference masses (121.0508 and 922.0097 m/z)

144 to obtain the most sensitivity. For auto $MS^2$ experiments constant collision energy of
145 20eV was employed. The Data Acquisition (version B.04.01) and Qualitative Analysis
146 (version B.04.00) of MassHunter Workstation Software were used (Agilent
147 Technologies, Waldbroon, Germany).

148 **Quantification of flavonoid composition by MS.**

149 Analyses were carried out using an Agilent 1100 series LC, comprised of a quaternary
150 pump G1311A with an integrated degasser G1322A, autosampler G1313A,
151 thermostated column compartment G1316A, coupled with an Agilent G1946D
152 Quadrupole mass spectrometer (Agilent Technologies, Waldbroon, Germany). Sample
153 separation was done in the same way as that for those samples explained in previous
154 paragraphs. 20 µL or 5 µL samples were injected for the analysis of the compounds
155 quantified in negative or positive polarity respectively. Ionization was by ESI source,
156 with the electrospray capillary voltage set to 4000 V, fragmentor 150V, a nebulizing gas
157 flow rate 12 L/h at 45 psig, and a drying temperature of 350 ºC. Data acquisition and
158 analysis were carried out with Agilent ChemStation B.04.01 SP1 Software. Strawberry
159 polyphenols were quantified using data acquired in the SIM mode. For negative polarity
160 m/z 273 (afzelechin), 289 (catechin), 461 (kaempferol glucuronide), 477 (quercetin 3-
161 glucuronide), 489 (kaempferol acetylglucoside), 577 (procyanidin B1 and B3) were
162 used. For positive polarity m/z 433 (pelargonidin 3-glucoside), 475 (pelargonidin 3-
163 acetylglucoside), 519 (pelargonidin 3-malonylglucoside), 535 (cyanidin
164 malonylglucoside), 579 (pelargonidin 3-rutinoside) were employed. These compounds
165 were quantified from the areas of their chromatographic peaks in SIM mode by
166 comparison with calibration curves obtained with external standards. (+)-Catechin,
167 pelargonidin chloride and procyanidin B1 were purchased from Extrasynthese (Genay,
168 France). These standards are specific for HPLC assay and the purity of them is ≥ 95%.

169 Individual polyphenol levels were expressed as µg catechin/g fresh weight (FW) for all
170 the compounds analysed by negative polarity and as µg pelargonidin/g FW for all the
171 compounds analysed by positive polarity. From each of the three replicates two
172 different measurements were taken.

173 **Extraction and chromatographic determination of ascorbic acid.**

174 To determine ascorbic acid concentration, 3 g of frozen fruit sample was homogenized
175 in 10 mL of ultra-pure water, centrifuged at 30,000 x g for 20 min, and the supernatants
176 were then filtered through a membrane of 0.45 µm pore size as described Blanch et al.,
177 (2012). The ascorbic acid content of untreated and $CO_2$-treated fruit was analysed by
178 HPAEC using a Metrohm Advanced Compac ion chromatography instrument (867 IC.
179 Metrohm) equipped with a Metrosep Organic Acids column (7.8 x 250 mm), an IC-819
180 conductivity detector, an IC Pump 818 and a coupled IC-837 degasser. Samples were
181 eluted from the column with an isocratic gradient of 0.5 mM $HClO_4$ with 50 mM LiCl
182 suppression over 20 min at a flow rate of 0.5 mL/min. Data was acquired with ICNet
183 2.3 Metrohm software. Ascorbate acid was identified by its retention time and
184 quantified on the basis of the calibration curve derived from standard (+)-sodium L.
185 ascorbate (Sigma, Steinheim, Germany). The content was expressed as mg/g FW of the
186 sample. From each of the three replicates two different measurements were taken.

187 **Antioxidant capacity**

188 The scavenging capacity of the water-soluble fraction of untreated and $CO_2$-treated fruit was
189 determined using a photochemiluminescence (PCL) assay. In the PCL assay the
190 photochemical generation of free radicals is combined with sensitive detection by using
191 chemiluminescence. This reaction is induced by optical excitation of a photosensitizer S
192 which results in the generation of the superoxide radical $O_2^{15}$. The free radicals are visualised

with the chemiluminescent detection reagent luminol. This reaction takes place in the Photochem®. The hydrophilic antioxidants were measured with an antioxidant capacity of water-soluble substance (ACW) kit (Analytik jenaAG). 1.5 mL reagent 1 (buffer solution pH 10.5), 1 mL reagent 2 (water), 25 µL reagent 3 (photosensitizer) and 2-30 µL antioxidant solution (reagent 4, ascorbic acid) were mixed and measured. 15 µL of sample were used to determine antioxidant capacity. The results were expressed as µg ascorbic acid equivalents/ mg FW of the sample.

### Statistical analysis

One-way ANOVA and correlational analyses were performed using SPSS ver. 19.0. The multicomparison of means was assessed by the Bonferroni`s test at 0.05 significance level. The main effects of $CO_2$ treatment, storage time, and treatment time interaction on strawberry fruit were analysed.

## RESULTS

### Identification of *Fragaria vesca* flavonoids

Flavonoid identification was performed by employing exact mass and fragmentation characteristics provided by Q-TOF using positive and negative modes according to analytes. The following peak data obtained in the Q-TOF analysis are summarized in Table 1 including the exact mass molecular ion, the tentative molecular formula, the score (%), the main fragments observed in $MS^2$ and the identified flavonoid compounds. Respect to the results obtained for anthocyanins from positive mode $MS^2$, four peaks with $MS^2$ fragmentation ions at *m/z* 271 were identified as derivatives of pelargonidin. Peak 1 with $[M^+]$ at *m/z* 433 and a subsequent loss of 162 amu (hexose) was pelargonidin-3-glucoside, the major anthocyanin in strawberries. Peak 2 with $[M^+]$ at *m/z* 579 and a loss of 308 amu (deoxyhexose-hexose) upon fragmentation was

assigned as pelargonidin 3-rutinoside. Less polar pelargonidin glycosides, peaks 4 and 5, had [$M^+$] at *m/z* 519 and 475, respectively (Table 1). During fragmentation peak 4 lost 248 amu which most likely represented the residue composed of hexose and malonic acid, (malonylglucoside) and was identified as pelargonidin 3-malonyl-glucoside. Peak 5 lost 204 amu (acetylglucoside) during fragmentation and was identified as pelargonidin-3-acetyl-glucoside. Peaks 3 and 6, both with [$M^+$] at *m/z* 535 and a subsequent loss of 248 amu suggested the presence of cyanidin-malonyl-glucoside isomers. This fragmentation behaviour is in accordance with literature data for cyanidin 3-(3″-malonyl) glucoside[16,17]. The more stable cyanidin 3-(3″-malonyl) glucoside only yielded a product ion at m/z 287, not at 3-(6”-malonyl glucoside), showing that the acyl linkage to the 3″-position of the sugar was more stable than the corresponding linkage to the 6″-position**.** We further observed the absence of anthocyanins derived from leucodelphinidin.

Table 1 also gives the results for flavanols form negative mode $MS^2$. Flavan-3-ols and dimeric proanthocyanidins polyphenols were identified and have been included in Table 1 as follows: (+)-catechin peak 9, afzelechin peak 10, and two B types proanthocyanidin dimers peaks 7 and peak 8. Peak 7 had [M-H] at *m/z* 577 and was identified as B1 (EC-4, 8-C) after comparison with the authentic standard. Peak 8 with [M-H] at *m/z* 577 probably contained B3 (C-4, 8-C), the most abundant flavanol after (+)-catechin in strawberries, with a fragmentation pattern in negative mode consistent with that of a flavanol[20]. Peak 9 which had [M-H] at m/z 289 was identified as (+)-catechin. Peak 10 with [M-H] at m/z 273 was identified as afzelechin with a fragmentation pattern in negative mode in accordance with that of a flavanol [21]. Table 1 also gives the results for flavonols from negative mode $MS^2$. Peak 11 with [M-H] at *m/z* 477 and the elimination of a glucurone unit (176 amu) during fragmentation was

identified as quercetin 3-glucuronide (Figure 1A), previously reported to be the major flavonol in strawberries [18,19]. Two peaks (12 and 13) were identified as kaempferol derivatives due to $MS^2$ fragmentation ions at *m/z* 285 in negative mode MS. Peak 12, identified as kaempferol 3-glucuronide, with mass 461 lost a glucurone unit (176 amu) during fragmentation, and peak 13 with mass 489, which lost a 204 amu (acetylglucoside) was identified as kaempferol 3-acetylglucoside [19,20] (Table 1).

Figure 1 shows the Extracted Ion Chromatogram (EIC) from HPLC-ESI-MS analysis corresponding to flavonoids compounds quantified in positive polarity (peaks 1 to 6) Figure 1A or negative polarity (peaks 7 to 13) Figure 1B. Insert $A_1$ represents the fragmentation pattern of pelargonidin 3-*O*-glucoside (peak 1), the most abundant anthocyanin in *Fragaria vesca* cv. Mara de Bois. Insert $B_2$ represents the fragmentation pattern of (+)-catechin (peak 9) one of the most important flavanols in strawberry.

Figure 2 represents the Extracted Ion Chromatogram (EIC) from HPLC-ESI-MS analysis corresponding to dimeric proanthocyanidins polyphenols (procyanidins B1 and B3) and other two suggested dimmers of procyanidin. Inserts shows fragmentation pattern in negative mode from procyanidin B1 (peak 7) (EC-4, 8C) after comparison with the authentic standard (insert A) and peak 8 probably contained B3 (C-4, 8-C) one of the most abundant flavanol in *Fragaria vesca* (insert B).

**Changes in the levels of anthocyanidin-specific branch products of the flavonoid pathway**.

The anthocyanins in *Fragaria vesca* cv. Mara de Bois were pelargonidin and cyanidin glycosides. Delphinidin derivatives were not detected. Pelargonidin-based anthocyanin content in was much higher than that of cyanidin-based anthocyanins (Table 2). The main anthocyanin, pelargonidin 3-glucoside in freshly harvested fruit was 842.09 ±

46.41µg/g FW, while that of cyanidin 3-malonylglucoside, the main cyanidin-based anthocyanin was 9.80 ± 1.1.5 µg/g FW. The same anthocyanidin profile was identified in fruit with and without $CO_2$ treatment. The results indicated that all the different anthocyanins detected in freshly harvested fruit increased significantly in untreated fruit stored in air, except for pelargonidin 3-acetyl-glucoside which only increased in 40% $CO_2$ -treated fruit (Table 2).

**Changes in the levels of flavonol-specific branch products of the flavonoid pathway.**

The flavonol profile in *Fragaria vesca* cv. Mara de Bois consisted of quercetin derivatives, mainly quercetin 3-glucuronide with values of 39.14 ± 2.44 µg/g FW, followed by minor amounts of other kaempferol derivatives such as kaempferol 3-glucuronide and kaempferol 3-acetyl-glucoside (Table 3). The quercetin 3-glucuronide content increased in both untreated and 20% $CO_2$ treated fruit, whereas in the case of 40% $CO_2$ treated fruit the values exhibited were similar to those found in freshly harvested fruit. Kaempferol 3-glucuronide content also increased in untreated and 20% $CO_2$-treated fruit unlike that in 40% $CO_2$-treated fruit (Table 3). The myricetin derivatives were not detected.

**Changes in the levels of proanthocyanidin-specific branch products of the flavonoid pathway**.

The levels of catechin in freshly harvested strawberries were 10.33 ± 3.00 µg/g FW. The content of catechin increased after 3 days of 20% $CO_2$ treatment and reached levels 1.54 times higher than those found in freshly harvested fruit (Figure 3). In contrast, storage at 0 $^{o}$C in air caused a marked decrease in catechin content reaching values that were only 8% of those found in $CO_2$-treated fruit. Additionally, 40% $CO_2$-treated fruit

had enhanced catechin levels compared with freshly harvested strawberries, reaching values of 14. 14 ± 0.97 µg/g FW (Figure 3). Epicatechin was not detected and the levels of afzelechin did not change.

Procyanidin B3 was the predominant proanthocyanidin with an initial value of 10.05 ± 1.95 µg/g FW. However, procyanidin B3 content was found to decrease markedly to 28% of its initial value during storage at low temperature for 3 days in air. (Figure 4). A slight decrease in procyanidin B1 content was also found in untreated fruit. The opposite was observed in the case of high $CO_2$ treated fruit (in both 20% and 40%), where there was a sharp increase in procyanidin B3, reaching values at least 4 times higher than those found in untreated fruit. Procyanidin B1 levels also significantly increased in 20% $CO_2$-treated fruit reaching values of 7.08±0.61 µg/g FW (Figure 4).

**Antioxidant capacity and changes in ascorbic acid and total flavonoids.**

The antioxidant capacity of untreated and $CO_2$-treated fruit, as indicated by µg of ascorbic acid/mg FW, is shown in Table 4. In this table, the amount of ascorbic acid (mg/g FW) and the calculated sum of total flavonoids (mg/g FW), including the identified and quantified flavonols, flavanols and anthocyanins, is also shown. Our results indicate that the total sum of flavonoids is quite similar in untreated and $CO_2$-treated fruit, as well as in freshly harvested strawberries. In the case of $CO_2$-treated fruit, although an accumulation in flavan-3-ols and dimmers was detected in both 20 and 40% $CO_2$-treated fruit, there were differences with regard to ascorbic acid, anthocyanins and flavonols dependent on $CO_2$ levels. The highest amount of ascorbic acid content was detected in fruit treated with 40% $CO_2$ for 3 days (Table 4) whereas in 20% $CO_2$-treated fruit the ascorbic acid levels were similar to those found in freshly harvested fruit. Furthermore, in strawberries treated with 20% $CO_2$, the total

anthocyanins and of flavonols increased significantly, whereas in those exposed to 40% $CO_2$ no differences were observed when compared with those at time of harvesting. In spite of these differences, similar antioxidant capacities were detected in both 20% and 40% $CO_2$-treated fruit, as well as in untreated fruit, and the only significant increase was observed in untreated fruit compared with freshly harvested fruit.

## DISCUSSION

Regulation of anthocyanin and proanthocyanidin production in fruit during storage is of special interest because of the significant role they have as antioxidant, as defence compounds against fungal diseases, and as protection against environmental stress conditions. Advances have been made with regard to factors involved in proanthocyanidin and anthocyanidin gene regulation in different plant systems, including strawberry fruits [22,23]. However, flux control and the manner in which low temperature and high $CO_2$ levels influence anthocyanin production with respect to alterations in proanthocyanidin levels is still incomplete and not well understood. Anthocyanins and proanthocyanins are produced by two related but distinct branches of the flavonoid pathway, so leucoanthocyanidin (flavan 3, 4 diols) can be directly reduced to 2, 3 *trans*-flavan 3-ols, such as (+)-catechins, or converted to 3-OH-anthocyanidin molecules[24]. Therefore, in the present work we analysed the effect of low temperature and high $CO_2$ levels in directing the metabolic flux to either anthocyanins or flavan-3-ols. Moreover, since the production of anthocyanidins and proanthocyanidins share didydroflavonols as precursor molecules, we also analysed alterations in the amounts of flavonols.

As regards the anthocyanidin profile, in *Fragaria vesca* cv. Mara de Bois strawberries (Table 1 and Table 2), pelargonidin 3-glucoside is the main anthocyanin as in other cultivars and species[25], followed by cyanindin 3-glucoside and minor amounts of other pelargonidin derivatives. There are many reports indicating that anthocyanin levels are affected by a variety of chemical and environmental factors[26], and specifically, that exposure to low temperatures produces a rapid increase in anthocyanin accumulation [3,4-13]. In the present work a significant accumulation of anthocyanins occurred in strawberry fruit stored in air at low temperature, this accumulation was limited in $CO_2$-treated fruit, and even more highly restricted in 40%-$CO_2$ treated fruit. Moreover, the increase in anthocyanin levels in untreated fruit stored in air was associated with a sharp decrease in the amount of flavan-3-ols monomers and procyanidin dimers. These data suggest that in untreated fruit there is a redirection of the metabolic flux from leucoanthocyanidins towards anthocyanins. Interestingly, $CO_2$-treated fruit, mainly those with 20% $CO_2$, anthocyanin accumulation in pulp tissues of strawberries did not decline, while an increase in the accumulation of procyanindins B1 and B3 was quantified (Table 1, Figure 4). Furthermore, in the case of monomeric subunits the levels of free 2, 3 *trans*-flavan 3-ols also increased significantly in $CO_2$-treated fruit (20 and 40%) (Table 1, Figure 3). Conversely, a pronounced decrease in catechin levels was quantified in untreated fruit stored in air at low temperature. In *Fragaria x annassa* cv. Camarosa epicatechin and catechin were not detectable, even though moderate levels have been reported for other cultivars such as Camp Dover[27,28]. The fact that catechin and proanthocyanidin levels in $CO_2$-treated strawberries increased but anthocyanin accumulation did not decline, would seem to indicate an apparent lack of competition between the anthocyanidin and proanthocyanidins branches. The reported inducible accumulation of procyanidins and catechins in high $CO_2$-treated strawberries,

362 could be one of the factors explaining the resistance to fungal decay. In this sense,
363 flavan-3-ols, such as catechins, epicatechins and oligomeric proanthocyanidins have
364 been shown to have inhibitory and antifungal properties towards different fungi,
365 including *Botrytis cinerea*[6], and against insects[29]. Proanthocyanidin accumulation has
366 also been associated with high ambient $CO_2$ concentrations (70 Pa), suggesting a
367 potential positive effect on proanthocyanidin production as a result of greenhouse gas
368 emission[30,31]. Additionally, since procyanidins are either metabolized or strongly bound
369 to other components of the plant, we cannot rule out the possibility that the increase in
370 procyanidins could be contributed to the reported beneficial effect of high $CO_2$
371 treatment on strawberry storage. In line with this, the enhancement of other polymers,
372 fructo-oligosaccharides, in 20% $CO_2$-treated Mara de Bois strawberries, polymers that
373 showed a high ability to bind water molecules resulted in protective benefits in
374 maintaining water status and cell integrity[32].

375 Anthocyanidin and/or proanthocyanidin accumulation did not impair flavonol
376 production of quercetin and kaempferol derivatives in either untreated (air) or 20%
377 $CO_2$-treated fruit, (Table 1 and Table 3). It is well known that the amounts of flavonols
378 vary substantially among the different strawberry cultivars, although in general,
379 quercetin and kaempferol are the major flavonols and occur as 3-glucosides and 3-*O*-
380 glucuronides[34,35]. For the most part, quercetin derivative levels were significantly
381 higher than those of kaempferol derivatives[28]. Flavonols and their glycosides appear to
382 be mainly involved in response mechanisms against abiotic stress[9]. Numerous studies
383 suggest that flavonol glucosides of quercetin and kaempferol have multiple functional
384 roles in plant responses to abiotic stress[24,36-37], including water deficit stress in response
385 to UV-B radiation[38], and may contribute to ozone tolerance[39]. We have shown that the
386 magnitude of flavonol accumulation in untreated fruit and 20% $CO_2$-treated fruit stored

387 at 0 ºC was similar but, no increase in kaempferol 3-glucuronide and quercetin 3-
388 glucoronide levels occurred in fruit treated with 40% $CO_2$. Therefore it was not
389 possible to quantify significant differences with respect to freshly harvested fruit.

390 Apart from the advantages mentioned above, flavonoids also act as antioxidants[40]. Our
391 results indicate (Table 4) that fruit stored in air or with high $CO_2$ levels exhibited a
392 similar antioxidant capacity, although compared with freshly harvested fruit a
393 significant increase was only observed in untreated fruit stored in air. This indicated
394 that strawberry fruit stored in air at severe low temperature had high scavenging
395 activity for generated active oxygen species. Considering the changes in the specific
396 flavonoids quantified, including that of ascorbic acid, the increase in anthocyanin
397 content seems to be responsible for the increase in antioxidant content in untreated
398 fruit. In this respect, untreated table grapes stored in air at 0 ºC had both the highest
399 anthocyanin content and antioxidant capacity, determined by TEAC assay[41]. Ascorbic
400 acid levels increased significantly in strawberries treated with 40% $CO_2$, whereas in
401 untreated fruit and fruit exposed to 20% $CO_2$ the levels of this metabolite did not differ
402 from those at the time of harvest. Variations in ascorbic acid levels in strawberries in
403 response to temperature storage have already been reported by other authors[11, 43]. We
404 suggest that the increase in ascorbate levels with too high $CO_2$ levels (40% $CO_2$), as
405 opposed to 20% $CO_2$, could be associated with susceptibility to $CO_2$ injury. Indeed,
406 increases in levels of ascorbate under stress conditions have been reported[44]. The
407 increase in ascorbic acid in 40% $CO_2$-treated fruit may result from an activation of its
408 multiple biosynthetic pathways and also through increased ascorbate recycling. Further
409 analyses should be carried out to see if this increase in ascorbic acid was accompanied
410 by changes in the glutathione pool, thus resulting in a substantial change in the
411 intracellular redox state of 40% $CO_2$-treated tissues. Furthermore, the increase in the

content of ascorbic acid in these treated fruits was concomitant with the lowest values registered for total flavonoids, except for catechin and procyanidins that showed a very high antioxidant capacity (data not shown). As no differences in antioxidant capacity were observed between fruit treated with 40% $CO_2$ and freshly harvested fruit, we reasoned that the antioxidant and radical scavenging activities of ascorbic acid and catechins could be sufficient to prevent modifications in their antioxidant capacity, although the underlying mechanism remains unclear. 20% $CO_2$ treatment led to an increase in catechin, proantocyanidin and flavonol glycoside (quercetin 3-glucoronide and kaempferol 3-glucuronide) levels. These flavonols, besides acting as antioxidant agents against ROS[45], are also involved in the response against abiotic stress and fungal decay[46]. Undoubtedly, given the antibacterial and antifungal activities exhibited by (+)-catechin[6], its induction by high $CO_2$ treatments would serve to protect strawberries from fungal attack and makes this procedure an attractive alternative to other chemical treatments. Additionally, $CO_2$ treatment of fruit is a good system that clearly shows the connection between proanthocyanidins, anthocyanidins and flavonols. Further studies will be necessary to determine the metabolic implications and to identify the mechanisms underlying the redirection of anthocyanidin into flavan-3-ols in $CO_2$-treated fruit.

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## FIGURE CAPTIONS

**Figure 1:** Extracted Ion Chromatogram (EIC) from HPLC-ESI-MS analysis corresponding to (A) flavonoids quantified in positive polarity (peaks 1 to 6) from *Fragaria vesca* strawberry. Insert $A_1$: $MS^2$ Scan spectra at *m/z* 433 of pelargonidin 3-*O*-glucuronide (peak 1), collision energy 20eV. (B) Flavonids quantified in negative polarity (peaks 7 to 13) from *Fragaria vesca* strawberry. Insert $B_1$: $MS^2$ Scan spectra at *m/z* 289 of (+)-catechin (peak 9), collision energy 20eV.

**Figure 2:** Extracted Ion Chromatogram (EIC) from HPLC-ESI-MS analysis corresponding to procyanidin dimers B1 (peak 7) and B3 (peak 8). (Insert A): $MS^2$ Scan spectra at m/z 577 of procyanidin dimer B1(EC-4,8-C) (peak 7). (Insert B): procyanidin dimer B3(C-4,8-C) (peak 8). Collision energy 20eV. A fragment ion of m/z 289 was found in the $MS^2$ spectra of these two procyanidins corresponding to a catechin molecule.

**Figure 3:** Catechin content (µg/g FW) in *Fragaria vesca* strawberries after harvesting (pre-stored, 0 days) and after 3 days of storage at 0 ºC in air (untreated), 20% $CO_2$ or 40% $CO_2$. The data are presented as the mean ± SE of three replicates (n=6) and the letters indicate significant differences ($p$<0.05).

**Figure 4:** Procyanidin B1 and Procyanidin B3 content µg/g FW (expressed as catechin equivalents) in *Fragaria vesca* strawberries after harvesting (pre-stored, 0 days) and after 3 days of storage at 0 ºC in air (untreated), 20% $CO_2$ or 40% $CO_2$. The data are presented as the mean ± SE of three replicates (n=6) and the letters indicate significant differences ($p$<0.05).

**Table 1: Characterization of flavonoids compounds in water extracts of *Fragaria vesca* cv. Mara de Bois strawberries by Mass Spectrometry detection in positive and negative mode. Rt (min), Retention time.**

| | | MS IDENTIFICATION | | | $MS^2$ IDENTIFICACTION | |
|---|---|---|---|---|---|---|
| **Peak** | **Rt (min)** | **Exact Mass Molecular ion [$M^+$]** | **Tentative Molecular Formula** | **Score %** | **$MS^2$ *(m/z)*[a]** | **Tentative identification** |
| **1** | 8.4 | 433.1135 | $C_{21}H_{21}O_{10}^+$ | 95.88 | 271 | **Pelargonidin 3-*O*-glucoside** |
| **2** | 9.1 | 579.1714 | $C_{27}H_{31}O_{14}^+$ | 98.13 | 271 | **Pelargonidin 3-*O*-rutinoside** |
| **3** | 11.4 | 535.1088 | $C_{24}H_{23}O_{14}^+$ | 97.23 | 287 | **Cyanidin 3-*O*-(6”-malonyl)glucoside (1)** |
| **4** | 13.4 | 519.1139 | $C_{24}H_{23}O_{13}^+$ | 95.71 | 271 | **Pelargonidin 3-*O*-(6”-malonyl-glucoside)** |
| **5** | 15.9 | 475.1240 | $C_{23}H_{23}O_{11}^+$ | 99.17 | 271 | **Pelargonidin 3-*O*-(6”-acetyl)glucoside)** |
| **6** | 23.2 | 535.1088 | $C_{24}H_{23}O_{14}^+$ | 92.17 | 287 | **Cyanidin 3-*O*-(6”-malonyl)glucoside (2)** |
| | | **Exact Mass Molecular ion [M-H]** | | | | |
| **7** | 5.6 | 577.1351 | $C_{30}H_{26}O_{12}$ | 86.48 | 451, 407, **289,** 125 | **Procyanidin B1** |
| **8** | 6.8 | 577.1351 | $C_{30}H_{26}O_{12}$ | 89.80 | 451, 407, **289,** 125 | **Procyanidin B3** |
| **9** | 7.7 | 289.0718 | $C_{15}H_{14}O_6$ | 92.26 | 245, 203, 179, 151,**109** | **(+)-Catechin** |
| **10** | 14.3 | 273.0768 | $C_{15}H_{14}O_5$ | 84.76 | 255, 229, 187,**137,** 97 | **Afzelechin** |
| **11** | 17.5 | 477.0675 | $C_{21}H_{18}O_{13}$ | 83.75 | 301 | **Quercetin 3-*O*-glucuronide** |
| **12** | 20.8 | 461.0725 | $C_{21}H_{18}O_{12}$ | 97.80 | **285,** 113, 59 | **Kaempferol 3-*O*-glucuronide** |
| **13** | 23.2 | 489.1038 | $C_{23}H_{22}O_{12}$ | 94.42 | 285 | **Kaempferol 3-*O*-acetyl-glucoside** |

[a]The most abundant ions are shown in bold. These ions are isolated for fragmentation in positive or negative mode.

**Table 2: Anthocyanin contents in *Fragaria vesca* cv. Mara de Bois strawberries after harvesting (pre-stored, 0 days) and after 3 days of storage at 0 ºC in air (untreated), 20% $CO_2$ or 40% $CO_2$. Data are expressed as µg pelargonidin equivalent per g FW.**

| | pre-stored | untreated | $CO_2$-treated | |
|---|---|---|---|---|
| | 0d | 3d air | 3d 20% $CO_2$ | 3d 40% $CO_2$ |
| **Pelargonidin 3-*O*-glucoside** | 842.09±46.41$^{ab}$ | 973.45±42.24$^{c}$ | 917.58±12.70$^{bc}$ | 772.03±16.86$^{a}$ |
| **Pelargonidin 3-*O*-rutinoside** | 6.24±0.45$^{a}$ | 7.33±0.37$^{b}$ | 7.46±0.35$^{b}$ | 5.70±0.53$^{a}$ |
| **Cyanidin 3-*O*-(6"-malonyl)glucoside (1)** | 9.80±1.15$^{b}$ | 13.24±0.99$^{c}$ | 11.67±0.29$^{bc}$ | 6.44±0.45$^{a}$ |
| **Pelargonidin 3-*O*-(6"-malonyl-glucoside)** | 96.24±3.26$^{a}$ | 115.59±8.47$^{b}$ | 102.29±2.05$^{b}$ | 86.53±7.61$^{a}$ |
| **Pelargonidin 3-*O*-(6"-acetyl-glucoside)** | 7.59±0.67$^{a}$ | 6.43±0.48$^{a}$ | 7.18±0.47$^{a}$ | 9.49±0.63$^{b}$ |
| **Cyanidin 3-*O*-(6"-malonyl)glucoside (2)** | 0.82±0.18$^{b}$ | 0.83±0.16$^{b}$ | 1.17±0.01$^{b}$ | 0.40±0.07$^{a}$ |

The data are presented as the mean ± SE of three replicates (n=6) and the letters indicate significant differences ($p$<0.05).

**Table 3: Flavonol contents in *Fragaria vesca* cv. Mara de Bois strawberries after harvesting (pre-stored, 0 days) and after 3 days of storage at 0 ºC in air (untreated), 20% $CO_2$ or 40% $CO_2$. Data are expressed as µg catechin equivalent per g FW.**

| | pre-stored | untreated | $CO_2$-treated | |
|---|---|---|---|---|
| | 0d | 3d air | 3d 20% $CO_2$ | 3d 40% $CO_2$ |
| **Quercetin 3-*O*-glucuronide** | 39.14±2.44$^{a}$ | 51.73±1.91$^{b}$ | 49.61±2.61$^{b}$ | 39.10±2.41$^{a}$ |
| **Kaempferol 3-*O*-glucuronide** | 3.99±0.21$^{a}$ | 4.98±0.20$^{b}$ | 5.16±0.34$^{b}$ | 3.81±0.21$^{a}$ |
| **Kaempferol 3-*O*-acetyl-glucoside** | 1.67±0.01$^{ab}$ | 1.81±0.08$^{ab}$ | 1.89±0.22$^{b}$ | 1.36±0.24$^{a}$ |

The data are presented as the mean ± SE of three replicates (n=6) and the letters indicate significant differences ($p$<0.05).

**Table 4: Changes of the content of anthocyanins, flavonols, flavanols, ascorbic acid and antioxidant capacity of strawberry *Fragaria vesca* cv. Mara de Bois pre-stored, untreated and 20%$CO_2$ or 40%$CO_2$-treated stored for 3 days at 0 ºC.**

| | pre-stored | untreated | $CO_2$-treated | |
|---|---|---|---|---|
| | 0d | 3d air | 3d 20% $CO_2$ | 3d 40% $CO_2$ |
| **Sum of anthocyanins (mg/g FW)** | $0.96\pm0.34^{ab}$ | $1.12\pm0.42^{c}$ | $1.05\pm0.37^{bc}$ | $0.88\pm0.31^{a}$ |
| **Sum of flavonols (µ/g FW)** | $44.80\pm1.18^{a}$ | $58.52\pm0.70^{b}$ | $56.66\pm0.32^{b}$ | $45.35\pm0.38^{a}$ |
| **Sum of flavan-3-ols and dimers (µ/g FW)** | $26.39\pm1.40^{b}$ | $11.03\pm0.78^{a}$ | $38.52\pm0.34^{c}$ | $35.01\pm0.41^{c}$ |
| **Sum of Total flavonoids (mg/g FW)** | 1.03±8.82 | 1.18±8.09 | 1.14±2.37 | 0.962±3.19 |
| **Ascorbic acid (mg/g FW)** | $1.11\pm0.11^{a}$ | $0.79\pm0.11^{a}$ | $0.85\pm0.08^{a}$ | $1.94\pm0.41^{b}$ |
| **Antioxidant capacity (µg ascorbic acid equivalent /mg FW)** | $5.29\pm0.64^{a}$ | $7.43\pm0.50^{b}$ | $6.21\pm0.80^{ab}$ | $6.60\pm0.61^{ab}$ |

The different letters indicate significant differences at $p< 0.05$

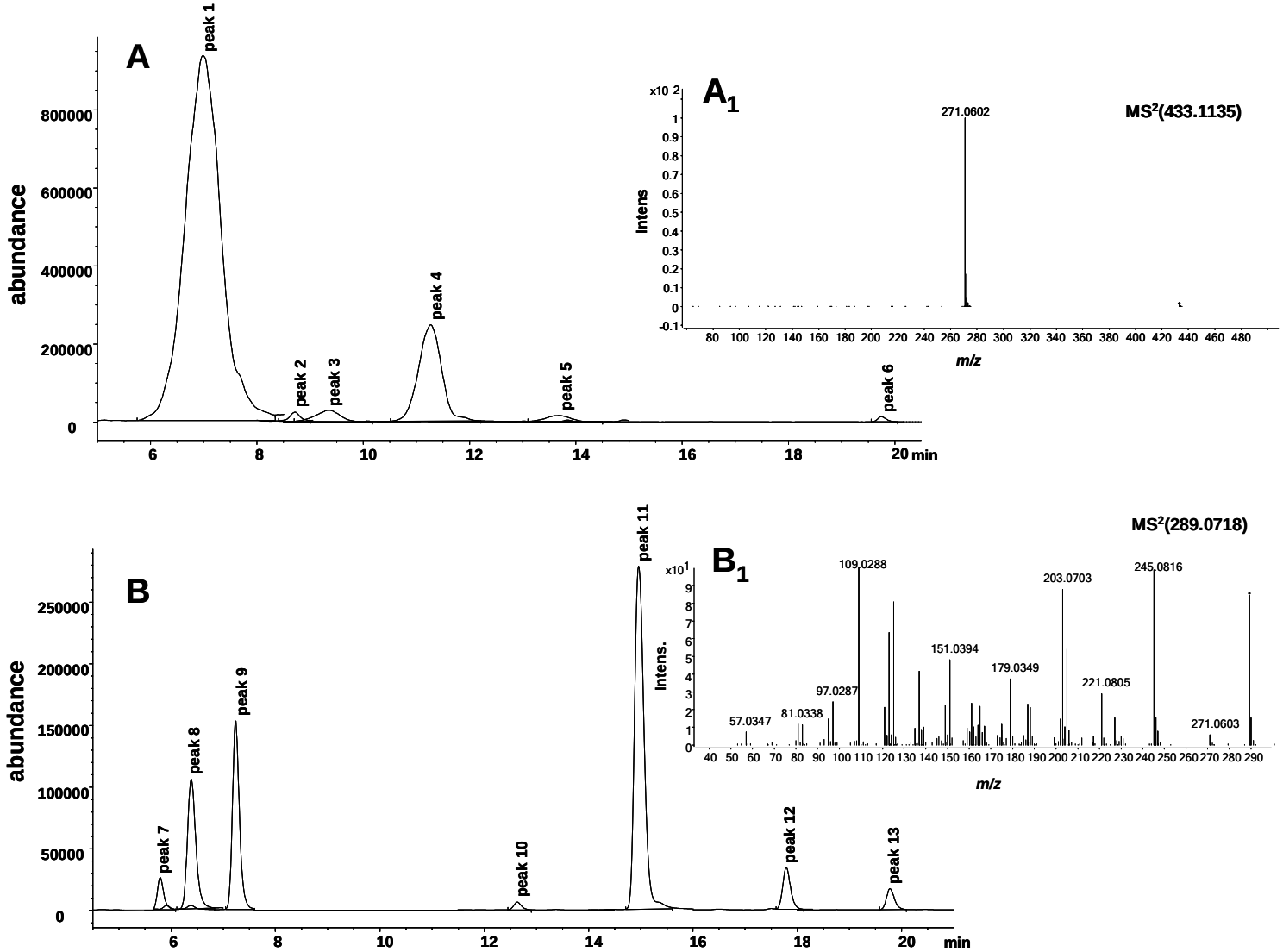

A
peak 1
peak 2
peak 3
peak 4
peak 5
peak 6
abundance
min
A1
271.0602
MS²(433.1135)
Intens
m/z
B
peak 7
peak 8
peak 9
peak 10
peak 11
peak 12
peak 13
abundance
min
B1
MS²(289.0718)
109.0288
203.0703
245.0816
151.0394
179.0349
221.0805
97.0287
81.0338
57.0347
271.0603
Intens.
m/z

**Figure 2**

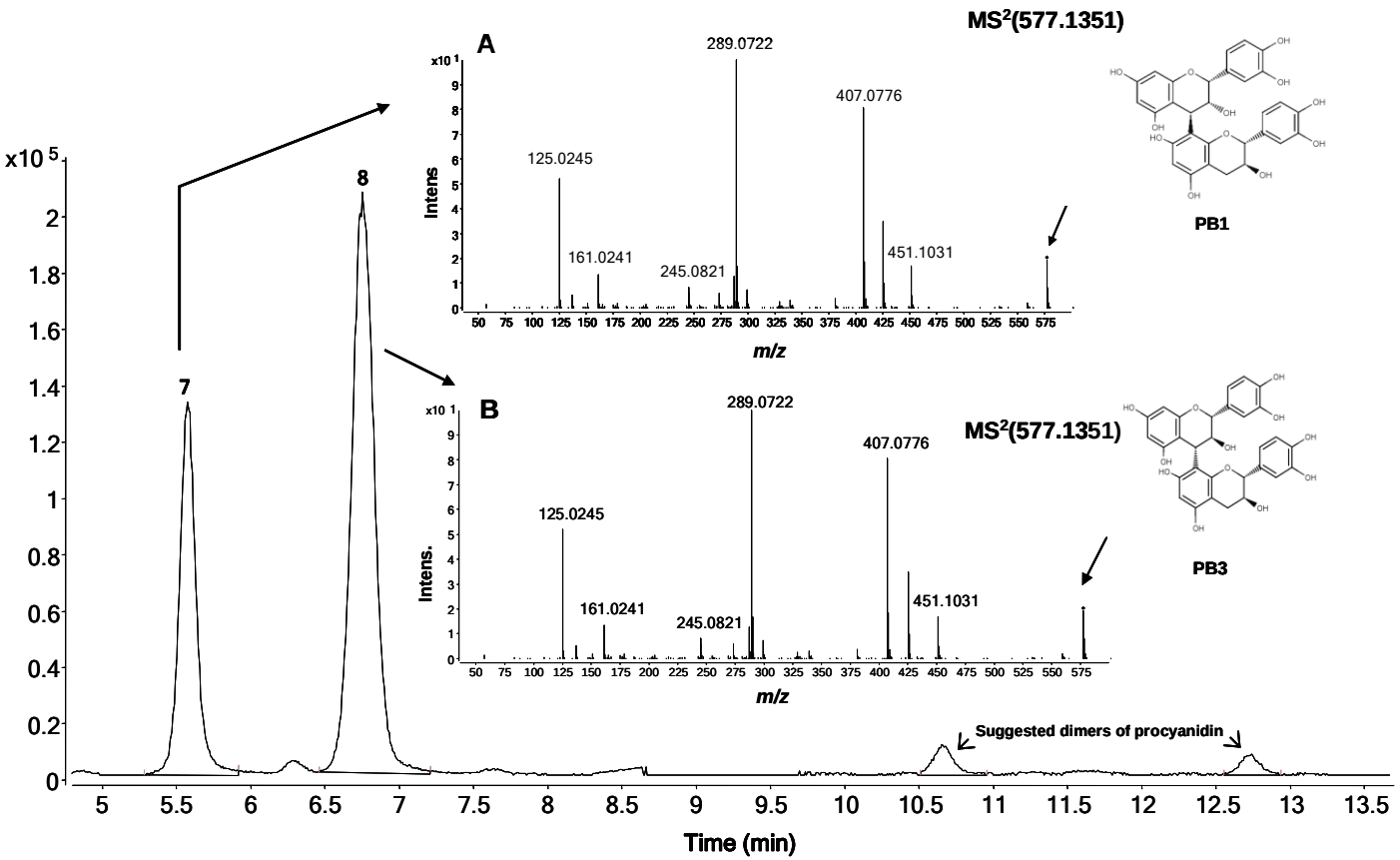

MS²(577.1351)
A
289.0722
407.0776
125.0245
161.0241
245.0821
451.1031
Intens
m/z
PB1
B
MS²(577.1351)
289.0722
407.0776
125.0245
161.0241
245.0821
451.1031
Intens.
m/z
PB3
7
8
Suggested dimers of procyanidin
Time (min)

**Figure 3**

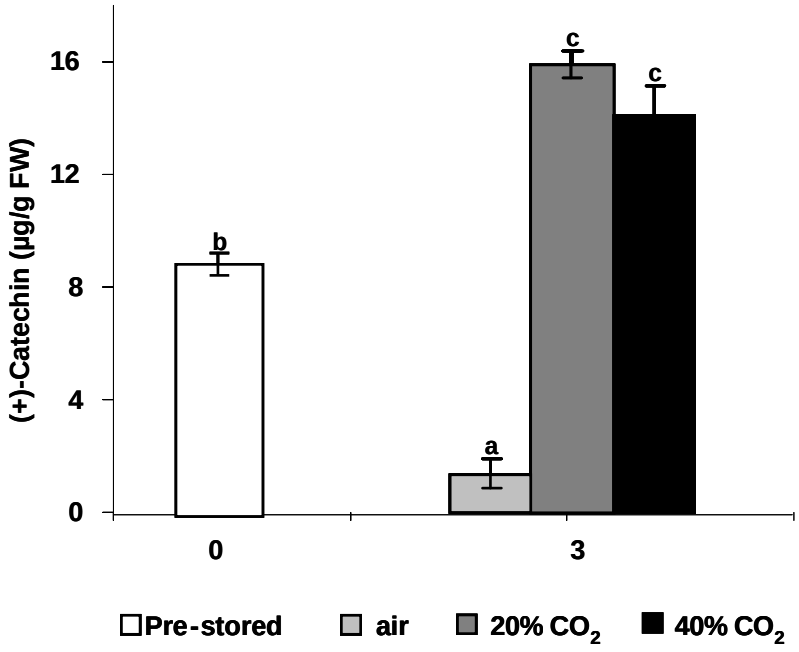
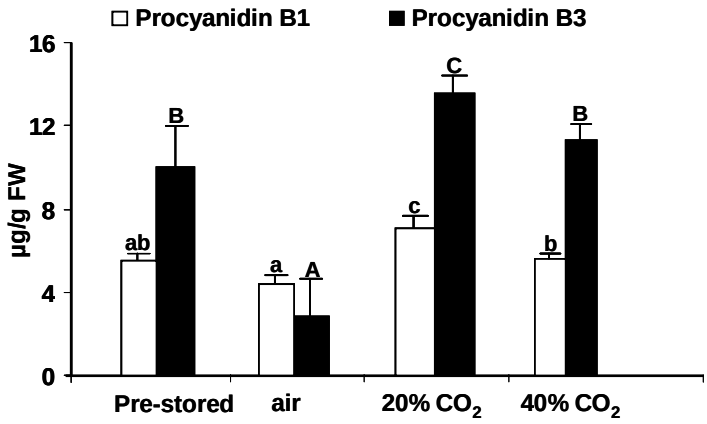
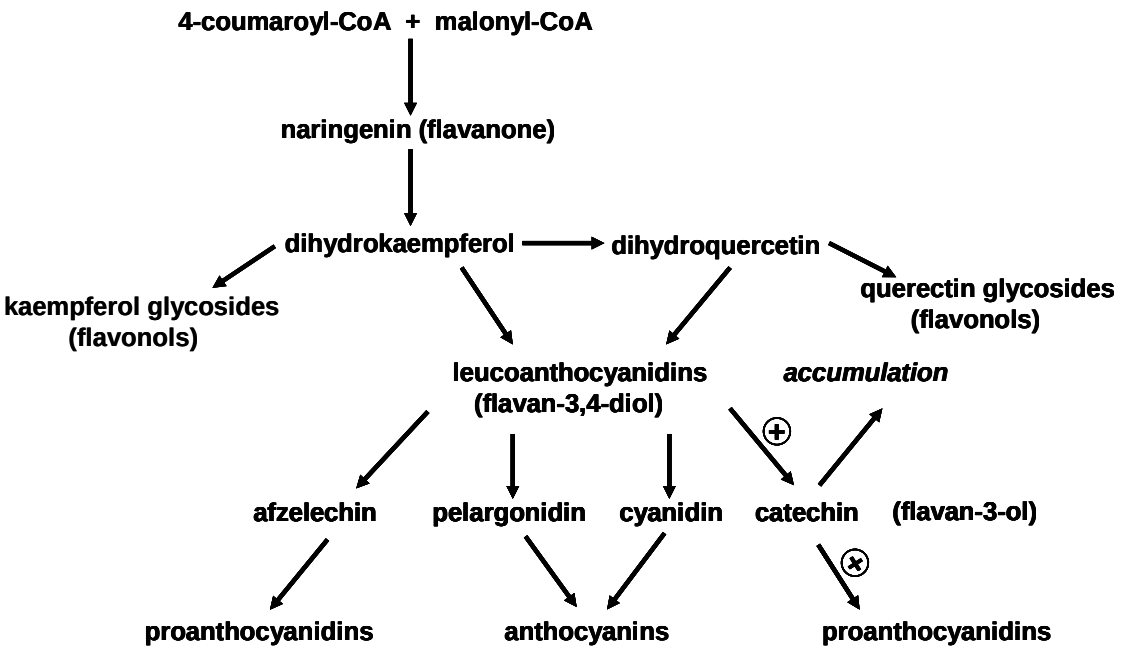


**Figure 4**


Procyanidin B1
Procyanidin B3
16
12
8
4
0
µg/g FW
ab
B
a
A
c
C
b
B
Pre-stored
air
20% CO2
40% CO2

## TOC graphic




Simplified schematic of the flavanolos, anthocyanins and proanthocyanidins production, account for the major products found in *Fragaria vesca* strawberries as proposed to be regulated by high $CO_2$ atmospheres.