JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Increasing Catechin and Procyanindin Accumulation in High-CO_2-Treated *Fragaria vesca* Strawberries

María Blanch,[†] Inma Alvarez,[‡] María T. Sanchez-Ballesta,[†] María I. Escribano,[†] and Carmen Merodio*,[†]

[†]Department of Characterization, Quality and Security and [‡]Unit Service of Analytical Techniques, Instrumentation and Microbiology (USTA), Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), Jose Antonio Novais 10, Madrid 28040, Spain

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.). Flavonoids were identified with Q-TOF equipment and quantified by HPLC-quadrupole. Proanthocyanins B1 and B3 accumulated in CO_2-treated strawberries, whereas in untreated (air) fruit, flavonoid production was redirected toward anthocyanin accumulation with a sharp decrease in catechin and procyanidin B3 levels. Moreover, in CO_2-treated fruit, mainly in those with 20% CO_2, anthocyanin accumulation did not decline. Due to its antifungal activity, catechin induction in CO_2-treated strawberries could explain the capacity of high CO_2 treatments to reduce fungal decay. Ascorbic acid content increased in 40% CO_2-treated fruits, whereas in those treated with 20% CO_2 an increase in flavonol content was observed. Despite these differences, similar antioxidant capacities were 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 flavor. 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] as is the tolerance of these fruits to high-CO_2 treatments.[2] 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).[3] Additionally, short-term exposure to high CO_2 levels had no significant effect on anthocyanin content, whereas table grapes stored in air at 0 °C had the highest anthocyanin levels. Anthocyanin induction at low temperature was also reported in different strawberry cultivars, as well as in other plant systems.[4,5] As anthocyanins are synthesized via branched biosynthesis pathways with proanthocyanidins, our aim in the present work was to analyze the feasibility of high CO_2 levels for channeling phenolic compound precursors into proanthocyanidins instead of anthocyanins. Furthermore, because flavan-3-ol monomers and their polymers have been linked with protection against pathogens,[6] the development of technologies that enhance their production without interfering with the anthocyanin levels is desirable. The subunits of most proanthocyanidins found in fruits and vegetables are the flavan-3-ol monomers, (+)-catechin and (−)-epicatechin, the astringency of which also contributes to the taste of fruits.[7] Although assessment of proanthocyanidin concentrations has been estimated in several plant sources using simple colorimetric methods, proanthocyanidins have been reported in several strawberry cultivars by employing more sensitive, specific methods such as HPLC and mass spectrometry.[8] Leucoanthocyanidins, the precursors of anthocyanidins and proanthocyanidins, are also connected with the flavonol precursor molecules.[9] 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 anthocyanin concentrations and also with flavonols. Additionally, flavonols and their glycosides appear to be mainly involved in response mechanisms against abiotic stress[10] and may contribute to environmental stress tolerance.[11] From a human point of view, several findings have demonstrated that strawberry phenolic compounds also confer protection against the environmentally adverse effects of ultraviolet radiation.[12]

Furthermore, all of 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.[13] In the present work variations in ascorbic acid levels and antioxidant activity were analyzed 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) assay.[14] This method is suitable for measuring the scavenging capacity of water-soluble antioxidants against the radical anion superoxide.

The objectives of this study were, first, to identify and quantify simultaneously anthocyanidins, flavonols, proanthocyanidins, and their flavan-3-ol monomers in freshly harvested

Received: April 10, 2012
Revised: July 11, 2012
Accepted: July 11, 2012
Published: July 11, 2012

strawberries, using Q-TOF equipment and quantification by HPLC-quadrupole. Second, to analyze the effect of low-temperature and high-CO_2 treatments on the accumulation of anthocyanidins, proanthocyanidins, and flavonols; and third, to study the relationship between variations in flavonoids and ascorbic acid levels and the antioxidant capacity of untreated and CO_2-treated fruit. All of 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 (*F. vesca* L. cv. Mara de Bois) were harvested in an orchard in San Sebastian de los Reyes (Madrid, Spain) at the ripe stage (9.8% total soluble solids; 0.8% citric acid; and L^* 18, a^* 40, and b^* 29 color values). After harvest, fruits were transported to the Institute of Food Science, Technology and Nutrition within 2 h. Fruits selected for uniform size and color were stored at 0 °C (±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. 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 °C for further analysis.

Extraction and Identification of Flavonoid Composition by MS and MS^2. For the simultaneous extraction of water-soluble flavonoids, frozen fruits were pulverized in liquid nitrogen and suspended in 30% (w/v) ultrapure water, sonicated for 10 min, and centrifuged at 30000g for 20 min at 4 °C; 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 here. Then, under the same chromatographic conditions we proceeded to quantify these compounds in selected ion monitoring (SIM) mode by HPLC-quadrupole. Analyses were performed using an Agilent 1200 series LC, composed of a quaternary pump G1311A with an integrated degasser G1322A, a thermostated autosampler G1330B, and a 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, Waldbronn, Germany). A 10 μL sample was separated in a Kromasil C18 column, 5 μm, 4.6 × 150 mm (Análisis Vínicos, S. L, Madrid, Spain) 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 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 4 GHz, mass range low m/z 1700. Ionization was achieved by atmospheric pressure electrospray ionization (ESI) with a drying gas flow rate of 10 L/min at 350 °C, a sheath gas flow of 7.5 L/min at 325 °C, nebulizer at 35 psi, cap voltage of 3500 V, nozzle voltage of 1000 V, fragmentor voltage of 150 V, and skimmer voltage of 65 V. The experiments were done with both negative polarity (for flavonols and flavanols) with reference masses (m/z 119.0363 and 966.0007) and positive polarity (for anthocyanins) with reference masses (m/z 121.0508 and 922.0097) to obtain the most sensitivity. For auto MS^2 experiments a constant collision energy of 20 eV was employed. Data Acquisition (version B.04.01) and Qualitative Analysis (version B.04.00) of MassHunter Workstation software were used (Agilent Technologies).

Quantification of Flavonoid Composition by MS. Analyses were carried out using an Agilent 1100 series LC, composed of a quaternary pump G1311A with an integrated degasser G1322A, an autosampler G1313A, and a thermostated column compartment G1316A, coupled with an Agilent G1946D Quadrupole mass spectrometer (Agilent Technologies). Sample separation was done in the same way as for those samples explained in previous paragraphs. Samples of 20 or 5 μL were injected for the analysis of the compounds quantified in negative or positive polarity, respectively. Ionization was by ESI source, with the electrospray capillary voltage set to 4000 V and fragmentor to 150 V, a nebulizing gas flow rate of 12 L/h at 45 psig, and a drying temperature of 350 °C. Data acquisition and analysis were carried out with Agilent ChemStation B.04.01 SP1 software. Strawberry polyphenols were quantified using data acquired in the SIM mode. For negative polarity m/z 273 (afzelechin), 289 (catechin), 461 (kaempferol glucuronide), 477 (quercetin 3-glucuronide), 489 (kaempferol acetylglucoside), 577 (procyanidins B1 and B3) were used. For positive polarity m/z 433 (pelargonidin 3-glucoside), 475 (pelargonidin 3-acetylglucoside), 519 (pelargonidin 3-malonylglucoside), 535 (cyanidin malonylglucoside), and 579 (pelargonidin 3-rutinoside) were employed. These compounds were quantified from the areas of their chromatographic peaks in SIM mode by comparison with calibration curves obtained with external standards. (+)-Catechin, pelargonidin chloride, and procyanidin B1 were purchased from Extrasynthese (Genay, France). These standards are specific for HPLC assay, and their purity was ≥95%. All of the compounds analyzed by negative polarity are expressed as micrograms catechin per gram fresh weight (FW) and those analyzed by positive polarity as micrograms pelargonidin per gram FW. Data represent the means of the three biological replicates with two different technical measurements for each.

Chromatographic Determination of Ascorbic Acid. The ascorbic acid content was determined by an HPAEC system using a Metrohm Advanced Compac ion chromatography instrument (867 IC Metrohm) following the previously published procedure for organic acid determination in Mara de Bois strawberries.[15] Samples were eluted from the column with an isocratic gradient of 0.5 mM $HClO_4$ with 50 mM LiCl suppression over 20 min at a flow rate of 0.5 mL/min. Data were acquired with ICNet 2.3 Metrohm software. Ascorbic acid was identified by its retention time and quantified on the basis of the calibration curve derived from standard (+)-sodium L-ascorbate (Sigma, Steinheim, Germany). The content was expressed as milligrams per gram FW, and the data represent the means of the three biological replicates with two different technical measurements for each.

Antioxidant Capacity. The scavenging capacity of water-soluble compounds of untreated and CO_2-treated strawberry fruit was determined using a PCL assay. In the PCL assay the photochemical generation of free radicals is combined with sensitive detection by using chemiluminescence. This reaction is induced by optical excitation of a photosensitizer S, which results in the generation of the superoxide radical O_2.[14] The free radicals are visualized 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). Amounts of 1.5 mL of reagent 1 (buffer solution pH 10.5), 1 mL of reagent 2 (water), 25 μL of reagent 3 (photosensitizer), and 2−30 μL antioxidant solution (reagent 4, ascorbic acid) were mixed and measured. Fifteen microliters of sample was used to determine antioxidant capacity. The results were expressed as micrograms ascorbic acid equivalents per milligram FW.

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

Table 1. Characterization of Flavonoid Compounds in *Fragaria vesca* cv. Mara de Bois Strawberries by Mass Spectrometry Detection in Positive and Negative Modes[a]

		MS identification			MS² identification	
peak	t_R (min)	exact mass molecular ion [M⁺]	tentative molecular formula	score (%)	MS² (m/z)	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″-malonylglucoside)
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)
		MS identification			MS² identification	
peak	t_R (min)	exact mass molecular ion [M − H]	tentative molecular formula	score (%)	MS² (m/z)[b]	tentative identification
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] t_R (min), retention time. [b] The most abundant ions are shown in bold. These ions are isolated for fragmentation in positive or negative mode.

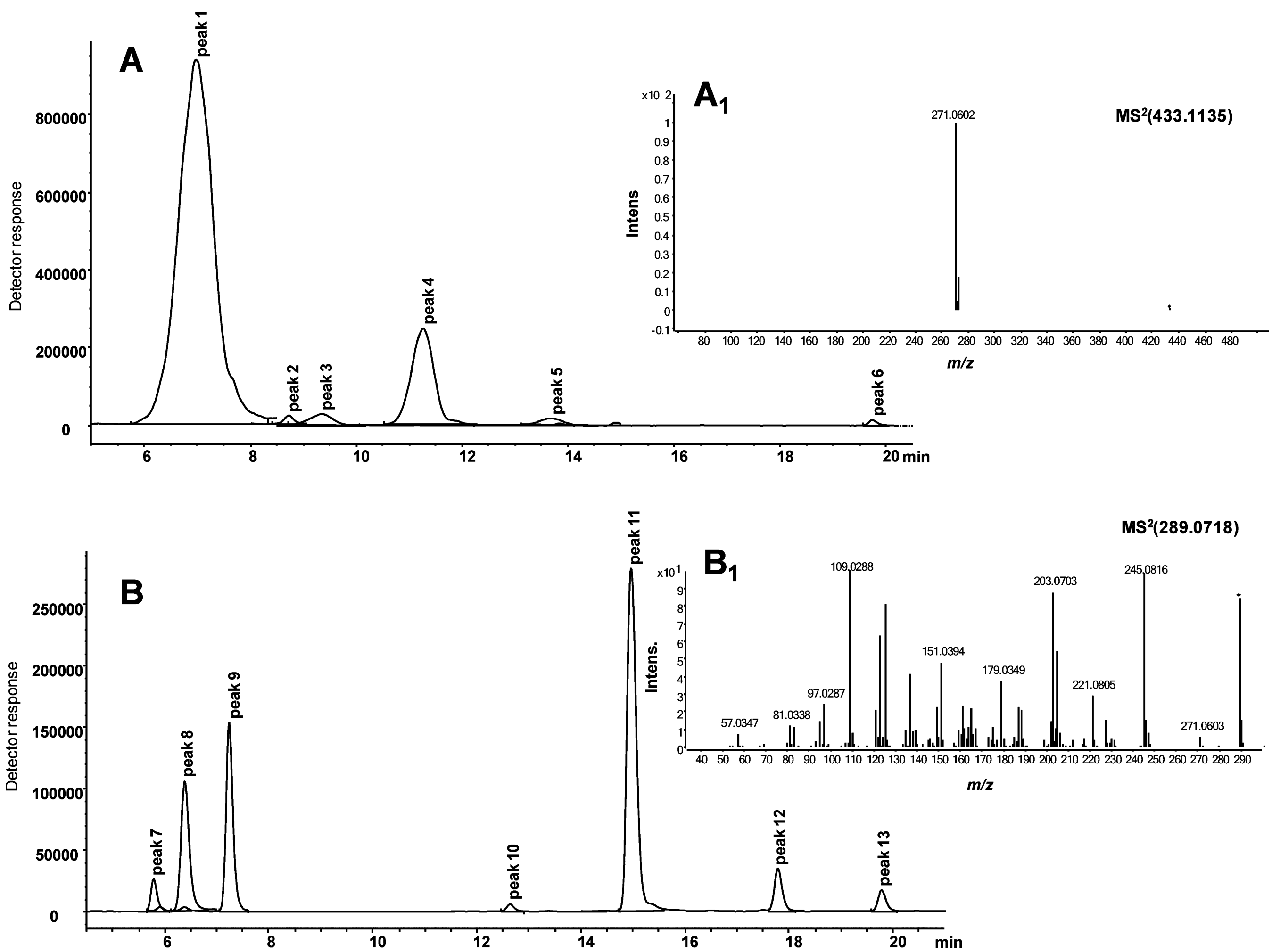

Figure 1. Extracted ion chromatograms from HPLC-ESI-MS analysis corresponding to (A) flavonoids quantified in positive polarity (peaks 1–6, identified in Table 1; (inset) MS² scan spectra at m/z 433 of pelargonidin 3-glucuronide (peak 1), collision energy = 20 eV) and (B) flavonoids quantified in negative polarity (peaks 7–13, identified in Table1; (inset) MS² scan spectra at m/z 289 of (+)-catechin (peak 9), collision energy = 20 eV).

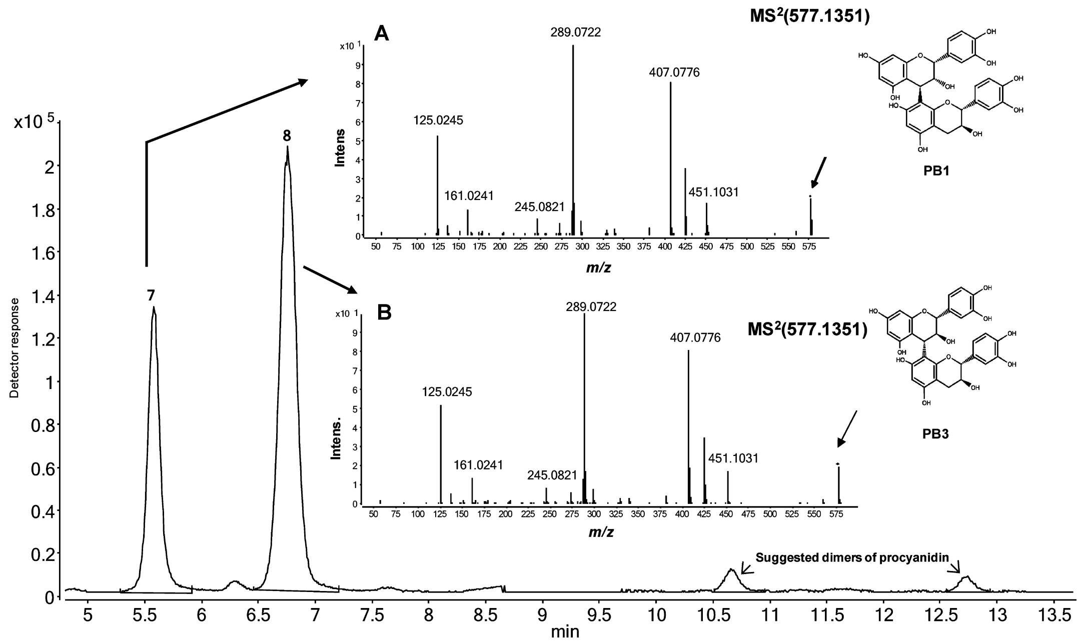

Figure 2. Extracted ion chromatograms from HPLC-ESI-MS analysis corresponding to procyanidin B1 (peak 7) and procyanidin B3 (peak 8). (Inset A) MS^2 scan spectra at *m/z* 577 of procyanidin B1 (EC-4,8-C) (peak 7). (Inset B) Procyanidin B3 (C-4,8-C) (peak 8). Collision energy = 20 eV. A fragment ion of *m/z* 289 was found in the MS^2 spectra of these two procyanidins corresponding to a catechin molecule.

■ RESULTS

Identification of *F. 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 (percent), the main fragments observed in MS^2, and the tentative identified flavonoid compounds. With 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-malonylglucoside. Peak 5 lost 204 amu (acetylglucoside) during fragmentation and was identified as pelargonidin 3-acetylglucoside. Peaks 3 and 6, both with $[M^+]$ at *m/z* 535 and a subsequent loss of 248 amu, suggested the presence of cyanidin malonylglucoside isomers. This fragmentation behavior is in accordance with literature data for cyanidin 3-(3″-malonyl)glucoside.[16,17] The more stable cyanidin 3-(3″-malonyl)glucoside yielded only a product ion at *m/z* 287, not at 3-(6″-malonylglucoside), showing that the acyl linkage to the 3″-position of the sugar was more stable than the corresponding linkage to the 6″-position.

Table 1 also gives the results for flavanols from negative mode MS^2. Flavan 3-ol monomers and proanthocyanidins were identified and have been included in Table 1 as follows: catechin, peak 9; afzelechin, peak 10; and two B type procyanidin dimers, peaks 7 and 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.[18] 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.[19] 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. 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 204 amu (acetylglucoside), was identified as kaempferol 3-acetylglucoside[20,21] (Table 1).

Figure 1 shows the extracted ion chromatogram (EIC) from HPLC-ESI-MS analysis corresponding to flavonoid compounds quantified in positive polarity (peaks 1−6 indicated in Table 1) (Figure 1A) or negative polarity (peaks 7−13 indicated in Table 1) (Figure 1B). Inset A_1 represents the fragmentation pattern of pelargonidin 3-glucoside (peak 1), the most abundant anthocyanin in strawberries. Inset B_2 represents the fragmentation pattern of (+)-catechin (peak 9).

Figure 2 represents the EIC from HPLC-ESI-MS analysis corresponding to procyanidins B1 and B3 and two other suggested minor dimers. Insets show fragmentation patterns in negative mode from procyanidin B1 (peak 7) (EC-4, 8C) after comparison with the authentic standard (inset A) and peak 8 containing B3 (C-4, 8-C), one of the most abundant flavanols in strawberries (inset B).

Changes in the Levels of Anthocyanidin-Specific Branch Products of the Flavonoid Pathway. The

Table 2. Anthocyanin Content in *Fragaria vesca* cv. Mara de Bois Strawberries after Harvesting (Pre- stored, 0 Days) and after 3 Days of Storage at 0 °C in Air (Untreated), 20% CO_2, or 40% CO_2[a]

	pre-stored	untreated	CO_2-treated	
	0 days	3 days, air	3 days, 20% CO_2	3 days, 40% CO_2
pelargonidin 3-glucoside	842.09 ± 46.41 ab	973.45 ± 42.24 c	917.58 ± 12.70 bc	772.03 ± 16.86 a
pelargonidin 3-rutinoside	6.24 ± 0.45 a	7.33 ± 0.37 b	7.46 ± 0.35 b	5.70 ± 0.53 a
cyanidin 3-malonylglucoside (1)	9.80 ± 1.15 b	13.24 ± 0.99 c	11.67 ± 0.29bc	6.44 ± 0.45 a
pelargonidin 3-malonyl-glucoside	96.24 ± 3.26 a	115.59 ± 8.47 b	102.29 ± 2.05 b	86.53 ± 7.61 a
pelargonidin 3-acetylglucoside	7.59 ± 0.67 a	6.43 ± 0.48 a	7.18 ± 0.47 a	9.49 ± 0.63 b
cyanidin 3-malonylglucoside (2)	0.82 ± 0.18 b	0.83 ± 0.16 b	1.17 ± 0.01 b	0.40 ± 0.07 a

[a]Data are expressed as μg pelargonidin equivalents per g FW. 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 Content in *Fragaria vesca* cv. Mara de Bois Strawberries after Harvesting (Pre-stored, 0 Days) and after 3 Days of Storage at 0 °C in Air (Untreated), 20% CO_2, or 40% CO_2[a]

	pre-stored	untreated	CO_2-treated	
	0 days	3 days, air	3 days, 20% CO_2	3 days, 40% CO_2
quercetin 3-glucuronide	39.14 ± 2.44 a	51.73 ± 1.91 b	49.61 ± 2.61 b	39.10 ± 2.41 a
kaempferol 3-glucuronide	3.99 ± 0.21 a	4.98 ± 0.20 b	5.16 ± 0.34 b	3.81 ± 0.21 a
kaempferol 3-acetylglucoside	1.67 ± 0.01 ab	1.81 ± 0.08 ab	1.89 ± 0.22 b	1.36 ± 0.24 a

[a]Data are expressed as μg catechin equivalents per g FW. The data are presented as the mean ± SE of th ree replicates (n = 6), and the letters indicate significant differences ($p < 0.05$).

anthocyanins in *F. vesca* cv. Mara de Bois were pelargonidin and cyanidin glycosides. Delphinidin derivatives were not detected. Pelargonidin-based anthocyanin content was much higher than that of cyanidin-based anthocyanins (Table 2). The main anthocyanin, pelargonidin 3-glucoside, in freshly harvested fruit was 842.09 ± 46.41 μg/g FW, whereas 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 of the major anthocyanins detected in freshly harvested fruit increased significantly in untreated fruit stored in air. In 40% CO_2-treated fruit the anthocyanin content was significantly reduced as compared with air-stored fruit or with 20% CO_2-treated ones (Table 2).

Changes in the Levels of Flavonol-Specific Branch Products of the Flavonoid Pathway. The flavonol profile in *F. vesca* cv. Mara de Bois consisted of quercetin derivatives, mainly quercetin 3-glucuronide with a value of 39.14 ± 2.44 μg/g FW, followed by minor amounts of other kaempferol derivatives such as kaempferol 3-glucuronide and kaempferol 3-acetylglucoside (Table 3). The quercetin 3-glucuronide content increased in both untreated and 20% CO_2-treated fruits, 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). 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 °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.

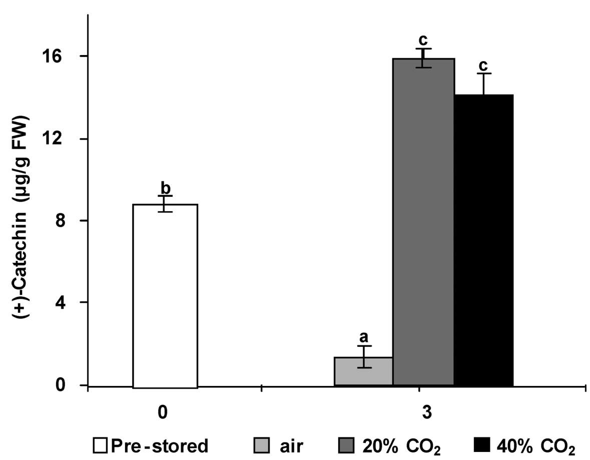

Figure 3. Catechin content (μg/g FW) in *Fragaria vesca* strawberries after harvesting (pre-stored, 0 days) and after 3 days of storage at 0 °C in air (untreated), 20% CO_2, or 40% CO_2. The data are presented as the mean ± SE of three replicates (n = 6), and the letters indicate significant differences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

Procyanidin B3 was the predominant proanthocyanidin with an initial value of 10.05 ± 1.95 μg/g FW, followed by procyanidin B1 and minor amounts of other two proanthocyanidins. 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%), for which 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).

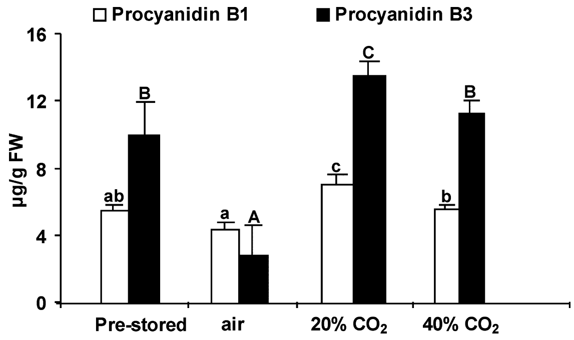

Figure 4. Procyanidin B1 and procyanidin B3 contents (μg/g FW, expressed as catechin equivalents) in *Fragaria vesca* strawberries after harvesting (prestored, 0 days) and after 3 days of storage at 0 °C in air (untreated), 20% CO_2, or 40% CO_2. The data are presented as the mean ± SE of three replicates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

Antioxidant Capacity and Changes in Ascorbic Acid Levels. The antioxidant capacity of untreated and CO_2-treated fruit, as indicated by micrograms of ascorbic acid equivalents per milligram FW, is shown in Table 4. In this table, the amount of ascorbic acid (mg/g FW) and the calculated sum of the identified and quantified flavonols, flavanols, and anthocyanins are also shown. In the case of CO_2-treated fruit, although catechin and procyanidin B1 and B3 accumulation was detected in both 20 and 40% CO_2-treated fruits, 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 (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 sum of 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. Despite 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 antioxidants, as defense 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] However, knowledge of 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-diol) can be directly reduced to 2,3-*trans*-flavan-3-ol, such as catechin, or converted to 3-OH-anthocyanidin molecules. Therefore, in the present work we analyzed the effect of low temperature and high CO_2 levels in directing the metabolic flux to either anthocyanins or flavan-3-ols. Moreover, because the production of anthocyanidins and proanthocyanidins shares didydroflavonols as precursor molecules, we also analyzed alterations in the amounts of flavonols. Thus, we developed a water-soluble extraction method for the analysis of simultaneous anthocyanins, flavonols, and proanthocyanidins, for preserving the extracted flavonoids better in their original form and to give good peak resolution. With regard to the anthocyanidin profile, in *F. vesca* cv. Mara de Bois strawberries (Tables 1 and 2), pelargonidin 3-glucoside is the main anthocyanin as in other cultivars and species.[23] There are many papers indicating that anthocyanin levels are affected by a variety of chemical and environmental factors,[24] and, specifically, that exposure to low temperatures produces a rapid increase in anthocyanin accumulation. In the present work a significant accumulation of anthocyanins occurred in strawberry fruit stored in air at low temperature. Moreover, the increase in anthocyanin levels in untreated fruit stored in air was associated with a sharp decrease in the amounts of catechin and procyanidins. These data suggest that in untreated fruit there is a redirection of the metabolic flux from leucoanthocyanidins toward anthocyanins. Interestingly, in CO_2-treated fruits, mainly those with 20% CO_2, anthocyanin accumulation did not decline, whereas an increase in the accumulation of catechin (Table 1; Figure 3) and procyanindins B1 and B3 (Table 1; Figure 4) was quantified. The fact that catechin and procyanidin 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 proanthocyanidin branches. Differences in the detection and amounts of flavan 3-ols among the different strawberry (*Fragaria* × *ananassa*) cultivars have been reported.[8,25−27] Comparative studies for another complex group of polyphenols in *F. vesca* and *Fragaria* × *ananassa* have also been reported.[28] Furthermore, the reported inducible accumulation of procyanidins and catechin in high-CO_2-treated strawberries could be one of the factors explaining the reduction of incidence and severity of fungal decay. In this sense, flavan-3-ols, such as catechins, epicatechins, and oligomeric proanthocyanidins, have been shown to have

Table 4. Changes in the Content of Ascorbic acid Antioxidant Capacity and in the Calculated Sum of Anthocyanins Flavonols and Flavanols in *Fragaria vesca* cv. Mara de Bois Pre-stored, Untreated, and 20 or 40% CO_2-Treated Stored for 3 Days at 0 °C[a]

	pre-stored	untreated	CO_2-treated	
	0 days	3 days, air	3 days, 20% CO_2	3 days, 40% CO_2
sum of anthocyanins (mg/g FW)	0.96 ± 0.34 ab	1.12 ± 0.42 c	1.05 ± 0.37 bc	0.88 ± 0.31 a
sum of flavonols (μg/g FW)	44.80 ± 1.18 a	58.52 ± 0.70 b	56.66 ± 0.32 b	45.35 ± 0.38 a
sum of flavan-3-ols and dimers (μg/g FW)	26.39 ± 1.40 b	11.03 ± 0.78 a	38.52 ± 0.34 c	35.01 ± 0.41 c
ascorbic acid (mg/g FW)	1.11 ± 0.11 a	0.79 ± 0.11 a	0.85 ± 0.08 a	1.94 ± 0.41 b
antioxidant capacity (μg ascorbic acid equiv/mg FW)	5.29 ± 0.64 a	7.43 ± 0.50 b	6.21 ± 0.80 ab	6.60 ± 0.61 ab

[a]Different letters indicate significant differences at $p < 0.05$.

dx.doi.org/10.1021/jf301547t | J. Agric. Food Chem. 2012, 60, 7489−7496

inhibitory and antifungal properties toward different fungi, including *Botrytis cinerea*.[6] Furthermore, anthocyanidin and/or proanthocyanidin accumulation did not impair flavonol production of quercetin and kaempferol derivatives in either untreated (air) or 20% CO_2-treated fruit, (Tables 1 and 3). On the contrary, no increase in kaempferol 3-glucuronide and quercetin 3-glucoronide levels occurred in fruit treated with 40% CO_2, although these levels did not differ from those found at time of harvest. It is well-known that the amounts of flavonols vary substantially among the different strawberry cultivars, although, in general, quercetin and kaempferol are the major flavonols and occur as 3-glucosides and 3-glucuronides.[27−30] It has been reported that flavonol glucosides of quercetin and kaempferol have multiple functional roles in plant responses to abiotic stress,[31] including water deficit stress in response to UV-B radiation, and may contribute to ozone tolerance.[32,33] Moreover, studies with strawberries grown under UV-blocking film exhibited lower contest of quercetin 3-glucuronide and kaempferol 3-glucoside than those grown in the open field.[34] In accordance with our results, the increase in flavonols in response to 20% CO_2 treatment may reflect a CO_2 tolerance strategy, and so no such accumulation was observed in 40% CO_2-treated fruit.

Furthermore, flavonoids also act as antioxidants.[35] Our results indicate (Table 4) that untreated fruit stored in air or with high CO_2 exhibited a similar antioxidant capacity, and when compared with freshly harvested fruit a significant increase was observed only in untreated fruit. Considering the changes in the amounts of ascorbic acid and the specific flavonoids quantified, the anthocyanin accumulation seems to be responsible for the increase in antioxidant content in untreated fruit. In this respect, both the highest anthocyanin content and antioxidant capacity, determined by TEAC assay,[36] have previously been reported in other fruits such as table grapes stored in air at 0 °C. In 40% CO_2-treated strawberries, ascorbic acid levels increased significantly, whereas in untreated fruit and those exposed to 20% CO_2, the levels of this metabolite did not differ from those at the time of harvest. 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 the high levels of ascorbic acid and the very high antioxidant capacity of catechin (data not shown) could be sufficient to prevent modifications in their antioxidant capacity. Moreover, the increase in ascorbic acid levels with too high a level of CO_2 (40% CO_2), as opposed to 20% CO_2, could be associated with susceptibility to CO_2 injury, as supported by the results of previously published studies dealing with water status and cellular structure.[37] Indeed, increases in levels of ascorbate under stress conditions[38] and in strawberry fruit (*Fragaria* × *ananassa* cv. Camarosa) stored in an atmosphere containing ozone[39] have been reported. The increase in ascorbic acid in 40% CO_2-treated fruit may result from activation of its multiple biosynthetic pathways and also through increased ascorbate recycling. Further analyses should be carried out to see if this increase in ascorbic acid was accompanied by changes in the glutathione system and in the redox state of 40% CO_2-treated tissues. In 20% CO_2-treated fruit, the increase in catechin, procyanidin (B1 and B3), and flavonol glycoside (quercetin 3-glucoronide and kaempferol 3-glucuronide) levels seems to explain their antioxidant capacity values. Moreover, these flavonols, besides acting as antioxidant agents against ROS,[40] are also involved in the response against abiotic stress[10] and fungal decay. 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 accumulation of oligomeric and polymeric flavan-3-ols in CO_2-treated fruit.

AUTHOR INFORMATION

Corresponding Author

*Phone: +34 91 5492300. Fax: + 34 91 5493627. E-mail: merodio@ictan.csic.es.

Funding

This work was financed by CICYT Projects AGL2008-02949 and AGL2011-26742. M.B. was supported by a postdoctoral JAE contract from the CSIC (Spain).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Estela Vega for her helpful support. Thanks are also due to grower Hugo Vela nursery for providing certified organic strawberries with requisites in compliance with CE Regulations 834/2007 and 889/2008.

REFERENCES

(1) Wszelaki, A. L.; Mitcham, E. J. Effect of combinations of hot water dips, biological control and controlled atmospheres for control of gray mold on harvested strawberries. *Postharvest Biol. Technol.* **2003**, *27*, 255−264.

(2) Bodelon, O.; Blanch, M.; Sanchez-Ballesta, M. T.; Escribano, M. I.; Merodio, C. The effects of high CO_2 level on anthocyanin composition, antioxidant activity and soluble sugar content of strawberries stored at low non-freezing temperature. *Food Chem.* **2010**, *122*, 673−678.

(3) Sanchez-Ballesta, M. T.; Romero, I.; Jiménez, J. B.; Orea, J. M.; González-Ureña, A.; Escribano, M. I.; Merodio, C. Involvement of the phenylpropanoid pathway in the response of table grapes to low temperature and high CO_2 levels. *Postharvest Biol. Technol.* **2007**, *46*, 29−35.

(4) Gil, M. I.; Holcroft, D. M.; Kader, A. A. Changes in strawberry anthocyanins and other polyphenols in response to carbon dioxide treatments. *J. Agric. Food Chem.* **1997**, *45*, 1662−1667.

(5) Christie, P. J.; Alfenito, M. R.; Walbot, V. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: Enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. *Planta* **1994**, *194*, 541−549.

(6) Veluri, R.; Weir, T. L.; Bais, H. P.; Termitz, F. R. S.; Ivanco, J. M. V. Phytotoxic and antimicrobial activities of catechin derivatives. *J. Agric. Food Chem.* **2004**, *52*, 1077−1082.

(7) Peleg, H.; Gacon, K.; Schlich, P.; Noble, A. C. Bitterness and astringency of flavan-3-ol monomers, dimmers and trimers. *J. Sci. Food Agric.* **1999**, *79*, 1123−1128.

(8) Buendia, B.; Gil, M. I.; Tudela, J. A.; Gady, A. L.; Medina, J. J.; Soria, C.; López, J. M.; Tomás-Barberán, F. A. HPLC-MS analysis of proanthocyanidin oligomers and other phenolics in 15 strawberry cultivars. *J. Agric. Food Chem.* **2010**, *58*, 3916−3926.

(9) Dixon, R. A.; Xie, D. Y.; Sharma, S. B. Proanthocyanins − a final frontier in flavonoid research? *New Phytol.* **2005**, *165*, 9−28.

(10) Agati, G.; Biricolti, S.; Guidi, L.; Ferrini, F.; Fini, A.; Tattini, M. The biosynthesis of flavonoids is enhanced similarly by UV radiation

and root zone salinity in *L. vulgare* leaves. *J. Plant Physiol.* **2011**, *168*, 204−212.

(11) Foy, D.; Lee, E. H.; Rowland, R.; Deune, T. E.; Buzzel, R. I. Ozone tolerance related to flavonol glycoside genes in soybean. *J. Plant Nutr.* **1995**, *18*, 637−647.

(12) Giampieri, F.; Alvarez-Suarez, J. M.; Tilipani, S.; Gonzàles-Paramàs, A. M.; Santos-Buelga, C.; Bompadre, S.; Quiles, J. L.; Mezzetti, B.; Battino, M. Photoprotective potential of strawberry (*Fragaria* × *ananassa*) extract against UV-A irradiation damage on human fibroblasts. *J. Agric. Food Chem.* **2012**, *60*, 2322−2327.

(13) Cordenunsi, B. R.; Genovese, M. I.; Nascimiento, J. R. O.; Hassimtto, N. M. A.; dos Santos, R. J.; Lajolo, F. M. Effect of temperature on the chemical composition and antioxidant activity of three strawberry cultivars. *Food Chem.* **2005**, *91*, 113−121.

(14) Popov, I.; Lewin, G. Antioxidative homeostasis: characterization by means of chemiluminescent technique. *Methods Enzymol.* **1999**, *300*, 437−456.

(15) Blanch, M.; Sanchez-Ballesta, M. T.; Escribano, M. I.; Merodio, C. Water distribution and ionic balance in response to high CO_2 treatments in strawberries (*Fragaria vesca* L. cv Mara de Bois). *Postharvest Biol. Technol.* **2012**, *73*, 63−71.

(16) Wu, X.; Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 2589−2599.

(17) Andersen, O. M.; Fossen, T. Anthocyanins with an unusual acylation pattern from stem of *Allium victorialis*. *Phytochemistry* **1995**, *40*, 1809−1812.

(18) De Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C. Quantitative analysis of flavan-3-ols in spanish foodstuffs and beverages. *J. Agric. Food Chem.* **2000**, *48*, 5331−5337.

(19) De Souza, L. M.; Cipriani, T. R.; Iacomini, M.; Gorin, P. A. J.; Sassaki, G. L. HPLC/ESI-MS and NMR analysis of flavonoids and tannins in bioactive extract from leaves of *Maytenus ilicifolia*. *J. Pharm. Biomed. Anal.* **2008**, *47*, 59−67.

(20) Määttä-Riihinen, K. R.; Kamal-Eldin, A.; Törrönen, A. R. Identification and quantification of phenolic compounds in berries of *Fragaria* and *Rubus* species (family Rosaceae). *J. Agric. Food Chem.* **2004**, *52*, 6178−6187.

(21) Ryan, J. J. Flavonol glycosides of cultivated strawberry. *J. Food Sci.* **1971**, *36*, 867−870.

(22) Griesser, M.; Hoffmann, T.; Bellido, M. L.; Rosati, C.; Fink, B.; Kurtzer, R.; Aharoni, A.; Muñoz-Blanco, J.; Schwab, W. Redirection of flavonoid biosynthesis through the down-regulation of an anthocyanidin glucosyltransferase in ripening strawberry fruit. *Plant Physiol.* **2008**, *146*, 1528−1539.

(23) Lopes da Silva, F.; Escribano-Bailón, M. T.; Pérez-Alonso, J. J.; Rivas-Gonzalo, J. C.; Santos-Buelga, C. Anthocyanin pigments in strawberry. *LWT*−*Food Sci. Technol.* **2007**, *40*, 374−382.

(24) Dixon, R. A.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085−1097.

(25) Da Silva Pinto, M.; Kwon, Y. I.; Apostolidis, E.; Lajolo, F. M.; Genovese, M. I.; Shetty, K. Functionality of bioactive compounds in Brazilian strawberry (*Fragaria* × *ananassa* Duch.) cultivars: evaluation of hyperglycemia and hypertension potential using in vitro models. *J. Agric. Food Chem.* **2008**, *56*, 4386−4392.

(26) Arts, I. C.; Van de Putte, B.; Hollman, P. C. Catechin contents of foods commonly consumed in The Netherlands. 1. Fruits, vegetables, staple foods, and processed foods. *J. Agric. Food Chem.* **2000**, *48*, 1746−1751.

(27) Aaby, K.; Mazur, S.; Nes, A.; Skredea, G. Phenolic compounds in strawberry (*Fragaria x ananassa* Duch.) fruits: composition in 27 cultivars and changes during ripening. *Food Chem.* **2012**, *132*, 86−97.

(28) Vrhovsek, U.; Guella, G.; Gasperotti, M.; Pojer, E.; Zancato, M.; Mattivi, F. Clarifying the identity of the main ellagitannin in the fruit of the strawberry, *Fragaria vesca* and *Fragaria ananassa* Duch. *J. Agric. Food Chem.* **2012**, *60*, 2507−2516.

(29) Downey, M. O.; Rochfort, S. Simultaneous separation by reversed-phase high-performance liquid chromatography and mass spectral identification of anthocyanins and flavonols in Shiraz grape skin. *J.Chromatogr., A* **2008**, *1201*, 43−47.

(30) Häkkinen, S. H.; Kärenlampi, S. O.; Heinonen, M.; Mykkänen, H. M.; Törrönen, A. R. Content of the flavonols quercetin, myricetin, and kaempherol in 25 edible berries. *J. Agric. Food Chem.* **1999**, *47*, 2274−2279.

(31) Buer, C. S.; Imin, N.; Djordjevic, M. A. Flavonoids: new roles for old molecules. *J. Integr. Plant Biol.* **2010**, *52*, 98−111.

(32) Foy, D.; Lee, E. H.; Rowland, R.; Deune, T. E.; Buzzel, R. I. Ozone tolerance related to flavonol glycoside genes in soybean. *J. Plant Nutr.* **1995**, *18*, 637−647.

(33) Steger-Hartman, T.; Koch, U.; Dunz, T.; Wagner, E. Induced accumulation and potential antioxidative function of rutin in two cultivars of *Nicotiana tabacum* L. *Z. Naturforsch. C.* **1994**, *49*, 57−62.

(34) Josuttis, M.; Dietrich, H.; Treutter, D.; Will, F.; Linnemannstöns, L.; Krüger, E. Solar UVB response of biactives in strawberries (*Fragaria ananassa* Duch. L.): a comparison of protected and open-field cultivation. *J. Agric. Food Chem.* **2010**, *58*, 12692−12702.

(35) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(36) Romero, I.; Sanchez-Ballesta, M. T.; Escribano, M. I.; Merodio, C. Individual anthocyanins and their contribution to total antioxidant capacity in response to low temperature and high CO_2 in stored Cardinal table grapes. *Postharvest Biol. Technol.* **2008**, *49*, 1−9.

(37) Blanch, M.; Goñi, O.; Sanchez-Ballesta, M. T.; Escribano, M. I.; Merodio, C. Characterization and functionality of fructo-oligosaccharides affecting water status of strawberry fruit (*Fragaria vesca* cv. Mara de Bois) during postharvest storage. *Food Chem.* **2012**, *134*, 912−919.

(38) Noctor, G.; Foyer, C. H. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249−279.

(39) Pérez, A. G.; Sanz, C.; Ríos, J. J.; Olías, R.; Olías, J. M. Effects of ozone treatment on postharvest strawberry quality. *J. Agric. Food Chem.* **1999**, *47*, 1652−1656.

(40) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radical Biol. Med.* **1997**, *22*, 749−760.