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Postharvest Variation in Apple (*Malus* × *domestica* Borkh.) Flavonoids following Harvest, Storage, and 1-MCP Treatment

DAN D. MACLEAN,† DENNIS P. MURR,*,† JENNIFER R. DEELL,‡ AND CHRISTOPHER R. HORVATH†

Department of Plant Agriculture, University of Guelph, Guelph, Ontario, N1G 2W1, Canada, and Ontario Ministry of Agriculture, Food and Rural Affairs, 1283 Blueline Road and Highway 3, Box 587, Simcoe, Ontario, N3Y 4N5, Canada

The impact of 1-methylcyclopropene (1-MCP) on the synthesis and retention of flavonoid compounds during storage and ripening of red Delicious (*Malus* × *domestica* Borkh.) apples was investigated. Numerous anthocyanins, flavonols, flavan-3-ols, and a hydroxycinnamic acid from three different fruit harvest maturities were monitored after a 120 day storage and 1 week shelf life period using high-performance liquid chromatography/diode array detector analysis. The total flavonoid concentration was 5% greater in fruit treated with 1-MCP, whereas chlorogenic acid levels were 24% lower. All compounds analyzed increased in concentration during fruit harvest; however, the anthocyanins generally declined after storage, while chlorogenic acid levels increased. 1-MCP treatment resulted in the retention of anthocyanins in the latter stages of storage but did not affect the flavonols and flavan-3-ols. Chlorogenic acid biosynthesis from early and optimal fruit harvest maturities was greatly inhibited by 1-MCP during storage and the 1 week shelf life period. However, 1-MCP did not affect chlorogenic acid concentrations in late-harvested fruit. Results suggest that 1-MCP may inhibit the activity of phenylalanine ammonia-lyase and subsequent biosynthesis of flavonoid compounds. However, because very little postharvest biosynthesis of flavonoids occurs in apples, 1-MCP treatment may be useful for maintaining some of the intrinsic flavonoid levels of red Delicious apples, if applied at the proper harvest maturity.

KEYWORDS: Postharvest; flavonoid; chlorogenic acid; 1-methylcyclopropene; apple; delicious; maturity; cold storage; antioxidant; *Malus* × *domestica* Borkh.; HPLC-DAD

INTRODUCTION

In recent years, there has been a growing interest in flavonoids as integral antioxidants in the human diet, due in part to their demonstrated anticarcinogenic activity, inhibition of tumor cell proliferation, antioxidant and free radical scavenging capabilities, and their effectiveness as metal chelators (see reviews *1*−*3*). The major flavonoids in apple have been implicated in the prevention of lipid peroxidation of cell membranes in mammalian systems (*4*), while other studies have investigated the bioavailability of these flavonoid metabolites in human systems (*5*).

Flavonoids are polyphenolic compounds that have been subdivided into several classes based largely on the hydroxylation pattern around the central C-ring. There are six major classes of flavonoids, numerous minor classes, and over 6400 different structures identified to date (*3*, *6*). Apple flavonoids possess a high number of electron donor hydroxyl groups, a low degree of glycosylation, and a large number of double bonds throughout the 6−3−6 carbon flavan nucleus, three properties that have been shown to increase the overall antioxidant activity of flavonoids (*7*, *8*). Apples have been shown to contain high amounts of flavonoids from the flavonol (e.g., quercetin 3-glycosides), the flavan-3-ol (e.g., catechin), and anthocyanin (e.g., cyanidin 3-glycosides) classes of structures (*9*, *10*) (**Figure 1**). Furthermore, apples are known to contain high levels of some hydroxycinnamic acid derivatives, with chlorogenic acid being the predominant structure (*11*).

During cold storage of apple, flavonoid levels remain essentially constant, although some studies have reported minor fluctuations in concentrations. The major flavonoid classes were studied over a 9 month cold storage period in Granny Smith, Crofton, and Lady Williams apples, and concentrations were found to vary little (*12*). A similar study using Jonagold and Elstar reported that levels of most flavonoids and hydroxycinnamic acid derivatives were stable throughout cold or controlled atmosphere storage (*13*). A study using Delicious and Ralls apple fruits supported the stability of flavan-3-ols and flavonols in storage but indicated an increase in anthocyanin concentration with a concomitant decrease in simple phenols (*14*).

* To whom correspondence should be addressed. Tel: +1-519-824-4120 ext. 52233. Fax: +1-519-767-0755. E-mail: dmurr@uoguelph.ca.
† University of Guelph.
‡ Ontario Ministry of Agriculture, Food and Rural Affairs.

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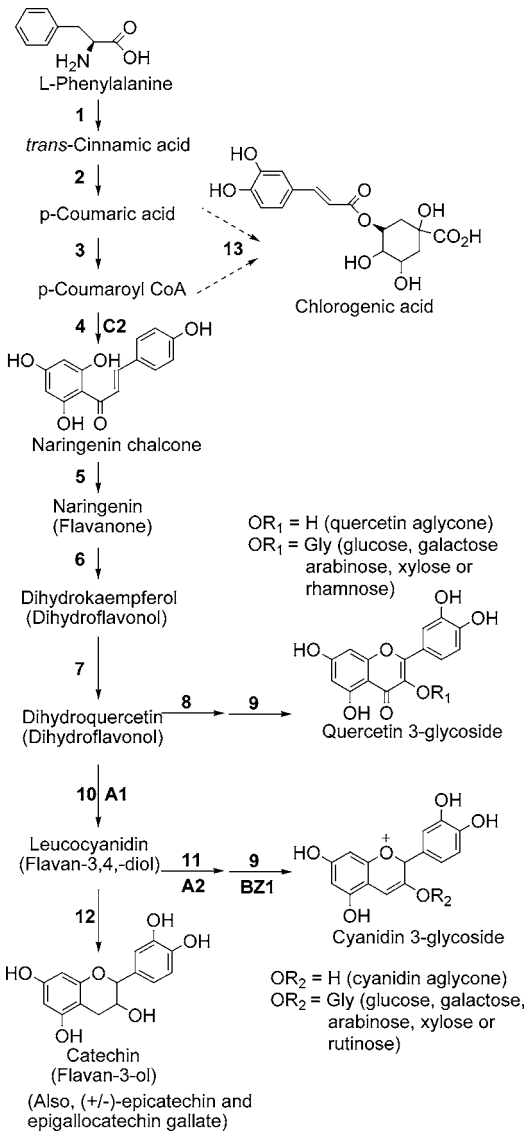

Figure 1. Flavonoid biosynthetic pathway, modified from ref *51*. Chemical structures drawn identify the flavonoids and hydroxycinnamic acid quantified in the current study (except for naringenin chalcone). Key: 1, PAL; 2, cinnamate-4-hydroxylase (C4H); 3, 4-coumaroyl:CoA-ligase (4CL); 4, chalcone synthase (CHS); 5, chalcone isomerase (CHI); 6, flavanone 3-hydroxylase (F3H); 7, flavanone 3′-hydroxylase (F3′H); 8, flavonol synthase (FS); 9, UDPG-flavonoid glucosyl transferase (UFGT); 10, dihydroflavonol reductase (DFR); 11, leucoanthocyanidin dioxygenase (LDOX); 12, leucoanthocyanidin reductase (LCR); and 13, unknown (see ref *54* for chlorogenic acid pathway). The flavonoids controlled by C1 + R are C2 (CHS), A1 (DFR), A2 (LDOX), BZ1 (UFGT), and BZ2 (GST, not shown). Maize MYB-IF35 controls enzymes 3 and 13.

The presence of the plant hormone ethylene has been implicated in the regulation of the flavonoid biosynthetic pathway (*15*, *16*). In apple, numerous studies have demonstrated the dependence of anthocyanin accumulation on the presence of ethylene (*17*−*19*). It also has been suggested that factors other than the level of ethylene in the tissue contribute to the de novo biosynthesis of anthocyanins, including temperature (*20*), fruit maturity (*21*), and storage duration (*22*). The gaseous cyclic olefin 1-methylcyclopropene (1-MCP) is now being used extensively at the commercial level around the world as a postharvest inhibitor of ethylene action in apple fruits. A 1-MCP treatment at the preclimacteric stage of ripening results in a highly reduced rate of ethylene production as well as other processes associated with fruit ripening, such as flesh softening, sugar metabolism, volatile production, and the respiratory climacteric (*23*). However, the efficacy of the 1-MCP treatment decreases with more advanced stages of fruit ripening. More specifically, as the fruit commences autocatalytic ethylene synthesis, the beneficial effects of the 1-MCP treatment are reduced (*24*). The decreased efficacy may either be due to the increased concentrations of ethylene, allowing it to more easily compete with 1-MCP for the ethylene binding proteins, or result from the belated inhibition of ripening-related transcription factors. Itai et al. (*25*) have shown that 1-MCP inhibited expression of three ripening-induced genes of Japanese pear, indicating the dependence of these genes on the action of ethylene.

Ethylene plays a critical role in the biosynthesis of flavonoid compounds (*17*, *18*). Red color development in Rome apples, which were bagged and treated with 1-MCP in the dark, was inhibited upon exposure to fluorescent light (*26*). However, this study did not determine the effect of the 1-MCP treatment on specific flavonoid compounds. Thus, the goal of the present study was to determine the effect of 1-MCP on the concentration of numerous flavonoids and a hydroxycinnamic acid in red Delicious apples over the course of three harvest maturities, a 120 day cold storage period, and a 1 week shelf life period.

MATERIALS AND METHODS

Chemicals. Optima grade methanol (MeOH) was purchased from Fisher Scientific (Nepean, ON, Canada). Acetonitrile (ACN) and formic acid (88%) were purchased from Caledon Laboratories (Georgetown, ON, Canada). Water (18.3 MΩ resistance) was acquired from a Nanopure water filtration system (New Haven, CT). All reagents used for extraction and high-performance liquid chromatography (HPLC) analysis were vacuum filtered through a 0.45 μm nylon Whatman Filter.

Authentic standards for anthocyanin (cyanidin dihydrate; 99%), ideain (cyanidin 3-galactoside; 99%), kuramanin (cyanidin 3-glucoside; 99%), keracyanin (cyanidin 3-rutinoside; 99%), quercetin dihydrate (99%), hyperoside (quercetin 3-galactoside; 99%), isoquercetrin (quercetin 3-glucoside; 99%), and quercitrin (quercetin 3-rhamnoside; 85%) were obtained from Indofine Chemicals Co. (Somerville, NJ). Chlorogenic acid (5-*O*-caffeoylquinic acid; 95%), (+)-catechin (98%), (−)-epicatechin (99%), (+)-epicatechin (98%), and (−)-epigallocatechin gallate (95%) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All standards were dissolved in MeOH except for anthocyanins, which were dissolved in MeOH (0.1% HCl) prior to being stored in the dark (−20 °C).

Fruit Maturity. The optimal horticultural maturity was determined by monitoring internal ethylene concentration (IEC; 15 fruit total), flesh firmness, total soluble solids (TSS) content, and starch−iodine stain (*27*) of 30 fruits (10 fruits × three repetitions) in the weeks prior to harvest. The IEC was determined by withdrawing a 3 mL gas sample from the core cavity using a needle inserted through the calyx end of the fruit. The gas sample was injected into a Varian CP-3380 GC/FID equipped with a 1.8 m × 3 mm Poropak-Q stainless steel column, fitted with a 1 mL sample loop. The injector, column, and detector temperatures were 50, 60, and 340 °C, respectively. High-grade helium was used as the carrier gas at a flow rate of 22 mL min^{-1} with a typical run time of 2.30 min. Flesh firmness measurements were determined using an Effegi penetrometer (Alfosine, Italy) fitted with an 11 mm tip and were taken from pared opposite sides of the fruit. The exudates from the firmness test of 10 fruits were collected in a Petri dish for determination of TSS using a handheld refractometer. The starch−iodine stain was determined by following the standard protocol and comparing the stain pattern to the Cornell starch−iodine chart (*27*).

Fruit Harvest and Treatment. Delicious apple fruits (*Malus* × *domestica* Borkh., strain Red Chief) were harvested randomly from five trees in a commercial orchard near Simcoe (ON, Canada) at an early (7 days before optimal horticultural harvest date; −7 days), optimal (0 days), and late (+7 days) in the 2002 harvest year. After

harvest, fruits were transported to the University of Guelph cold storage facility and placed immediately in air storage at 3 °C and treated with 1 μL L^{-1} of 1-MCP for 24 h. Similar nontreated control fruits were held in air for 24 h at 3 °C. Following treatment, fruits were placed at 0−1 °C and 98−99% relative humidity for 0, 30, 60, 90, or 120 days. After each storage time, fruit samples were removed and allowed to equilibrate at room temperature for 1 or 8 days (∼21 °C). The skin and some underlying cortical tissue (∼1−2 mm) from five fruits (three repetitions, 15 fruits total) were removed with a mechanical peeler, combined, frozen, and ground under liquid nitrogen and then held at −80 °C until preparation for HPLC analysis.

Tissue Preparation for HPLC Analysis. Approximately 1.00 g (±0.020 g) of finely ground frozen tissue from five fruits was placed in a 12.5 mL disposable glass culture vial containing 5 mL of MeOH (0.1% HCl). The sample was sonicated at 20 °C for 30 min, and then, the headspace of the vial was flushed with $N_{2(g)}$. The vial was capped with a rubber septa-seal and subsequently placed in complete darkness at 4 °C for overnight extraction (∼16 h). The following morning, the sample was centrifuged at 1000*g* for 5 min at 4 °C. The supernatant was decanted and set aside, while the pelleted residue was further extracted with a 3 mL wash of MeOH (0.1% HCl). After a 15 min sonication, the sample was placed in complete darkness for 3 h and centrifuged at 1000*g* for 5 min at 4 °C, and then, the supernatant was added to the first supernatant for a final total extraction volume of ∼8 mL. The headspace of the vial was flushed with $N_{2(g)}$, and the vial was then capped with a rubber septa-seal and placed at −20°C for no more than 12 h. The sample extract was concentrated to near dryness using a rotary evaporator held at 60°C and then resuspended with 0.7 mL of MeOH (0.1% HCl), vortexed for 10 s, and centrifuged at 16000*g* for 20 min. After centrifugation, the sample was decanted directly into a 1.5 mL amber autosampler vial and the final volume was brought to 1 mL with MeOH (0.1% HCl). The vial was sealed with a PTFE autosampler vial cap and stored at −80 °C until analyzed by HPLC.

HPLC/Diode Array Detector (DAD) Analysis. Flavonoids were separated and identified using an Agilent 1100 series HPLC (Mississauga, ON, Canada) system equipped with an inline continuous vacuum solvent degasser, binary pump, temperature-controlled autosampler and column compartments, and a photodiode array detector (PDA), all controlled by Chemstation (version 4.0) software package. Solvents used were as follows: A, 5% formic acid; and B, ACN at a flow rate of 0.300 mL min^{-1}. The gradient (expressed as %B) was as follows: 0−1 min, 5%; 1−8 min, 5−13%; 8−12 min, 13−15%; 12−26 min, 15−22%; 26−26.5 min, 22−40%; 26.5−30.5 min, 100%; and 30.5−35 min, 5%. The autosampler compartment was maintained at 4 °C. The volume of injection for both samples and standards was 5 μL. The flavonoid compounds were retained using a Waters Symmetry Shield RP-18 column (100 mm × 2.1 mm × 3.5 μm; Mississauga, ON, Canada) protected by a guard column (10 mm × 2.1 mm × 3.5 μm) all held at 30 °C within the column compartment. Eluted compounds were detected using a PDA equipped with a semimicro flow cell with a full spectral scan set from 210 to 700 nm (1 nm steps) and monitored at 530 nm for the detection of anthocyanins, 350 nm for flavonols, 280 nm for flavan-3-ols, and 330 nm for chlorogenic acid, all with a bandwidth of 4 nm (**Table 1**).

Standard Curves and Method Validation. Compound identification and quantification were performed by PDA (as described earlier) by comparing spectral characteristics and retention times of the eluted compounds with those of authentic standards. The various flavonoids were quantified using an external standard curve produced from the authentic standards, consisting of five different standard concentrations over a linear range of 0.2−100 μg mL^{-1}. Anthocyanin standards were diluted in MeOH (0.1% HCl), while all others were diluted in MeOH.

Extraction efficiency and repeatability, as well as instrument repeatability studies, were performed. A sample of tissue was sequentially extracted three times (as described previously), but the supernatants were kept separate for analysis by HPLC-DAD ($n = 3$) in order to determine the minimum number of extractions required to remove all detectable flavonoids. Similarly, three samples were extracted once and analyzed by HPLC-DAD in order to determine the repeatability of the extraction technique. Furthermore, a tissue sample was injected 10 times in order to test the instrument repeatability of the HPLC-DAD.

Table 1. Flavonoids and Hydroxycinnamic Acid Quantified in This Study with the Retention Times (t_r) and Standard Curve Linearity Measurements (r^2)

peak	flavonoid	t_r (min)	r^2
	anthocyanins, λ_{max} 530 nm		
3	cyanidin 3-galactoside[a]	9.8	0.999
5	cyanidin 3-glucosidea	10.8	0.999
6	cyanidin 3-rutinosidea	11.6	0.999
8	cyanidin 3-arabinoside[b]	13.4	
9	cyanidin 3-xyloside[b]	13.9	
10	cyanidin aglycone[a]	16.6	0.999
	flavonols, λ_{max} 350 nm		
11	quercetin 3-galactoside[a]	20.8	0.999
12	quercetin 3-glucoside[a]	21.2	0.998
13	quercetin 3-xyloside[b]	23.3	
14	quercetin 3-arabinoside[b]	24.0	
15	quercetin 3-rhamnoside[a]	25.3	0.999
16	quercetin aglycone[a]	30.5	0.995
	flavan-3-ols, λ_{max} 280 nm		
1	catechin[a]	6.8	0.996
4	(±)-epicatechin[a]	10.1	0.998
7	epigallocatechin gallate[a]	13.3	0.998
	cinnamic acid, λ_{max} 330 nm		
2	chlorogenic acid[a]	8.3	0.999

[a] Quantified by comparison with authentic standards. [b] Quantified by comparison of absorption spectra and relative retention times with published data.

Statistical Analysis. Each main effect of maturity × removal × shelf life × 1-MCP treatment contained three repetitions of five fruits per repetition. The experimental setup was a completely randomized design with fruit maturity, storage removal time, shelf life, and 1-MCP treatment designated as fixed effects. Least squares means, standard error of the mean (SEM), analysis of variance, tests for normality, and outliers were performed using the General Linear Model Procedure (Proc GLM) of the Statistical Analysis System (SAS) software (version 8.2, SAS Institute, Cary, NC) at a significance level of $P \leq 0.05$.

RESULTS AND DISCUSSION

HPLC-DAD. Extraction efficiency was determined by sequentially extracting a tissue sample three times. The first extraction removed 93.1% of the detectable flavonoids present in the tissue, with the balance being extracted after the second extraction, except for ≤0.24% of cyanidin 3-galactoside (expressed as percent of total concentration). Instrument repeatability varied between 0.15 and 3.40% for the flavonoids quantified in the study for a sample that was repeatedly injected 10 times, while the variation in flavonoid concentration between repeated extractions varied between 1.7 and 7.5% (expressed as percent of highest extraction concentration).

The gradient used for the HPLC-DAD analysis resulted in a comparatively short run time of 35 min and resulted in good separation between the compounds of interest. The short runtime effectively doubled the sample throughput when compared to other published run times typical for studies of apple flavonoid matrices. The anthocyanins cyanidin 3-galactoside and cyanidin 3-glucoside were resolved to the baseline even when high levels of the former were present in the tissue sample. Satisfactory separation between quercetin 3-galactoside and quercetin 3-glucoside was also achieved, although baseline resolution was not obtained during analysis of the tissue samples. The HPLC solvent gradient was also designed to eliminate the coelution of major compounds of interest to the study, regardless of the wavelength of maximum absorption. Particular care was

Table 2. Selected Flavonoids and Hydroxycinnamic Acids of Delicious Skin Tissue after Various Harvest Maturity, Cold Storage Removal, Shelf-Life Period, and 1-MCP Treatments[a]

		total flavonoids and hydroxycinnamic acids (μg g^{-1} fw)										
		anthocyanins			flavonols				flavan-3-ols			hydroxycinnamic acid
		cyanidin 3-galactoside	cyanidin 3-rutinoside	cyanidin 3-arabinoside	quercetin 3-galactoside	quercetin 3-arabinoside	quercetin 3-rhamnoside	quercetin aglycone	catechin	epicatechin	total flavonoids	chlorogenic acid
		factor										
cultivar	Delicious	833	68	59	216	196	163	94	97	797	2672	332
maturity (days)	early (−7)	531 a	37 a	33 a	168 a	157 a	138 a	83 a	80 a	712 a	2062	257 a
	optimal (0)	732 b	59 b	53 b	187 b	169 a	138 a	77 a	95 b	755 b	2394	264 a
	Late (+7)	1237 c	108 c	92 c	294 c	260 b	214 b	122 b	117 c	925 c	3562	474 b
removal (days)	0	874 a	71 b	62 b	224 a	190 a	169 a	91 b	85 b	791 b	2756	185 a
	30	877 a	76 a	64 a	231 a	203 a	169 a	110 a	108 a	837 a	2830	238 b
	60	821 b	69 b	61 b	206 a	192 a	157 a	100 b	102 a	798 b	2645	295 c
	90	796 b	63 c	55 c	208 a	198 a	159 a	83 c	94 b	771 c	2576	435 d
	120	798 b	62 c	54 c	213 a	194 a	162 a	88 b	97 ab	791 b	2608	504 e
shelf (days)	+1	868 a	71 a	62 a	212 a	192 a	160 a	95 a	98 a	799 a	2704	287 a
	+8	798 b	65 b	57 b	220 a	199 a	166 a	94 a	96 a	795 a	2641	376 b
treatment	1-MCP	860 a	73 a	63 a	221 a	197 a	162 a	95 a	97 a	813 a	2732	286 a
	control	806 b	64 b	56 b	212 a	194 a	164 a	93 a	97 a	782 b	2613	
		significance [b]										
cultivar (CV)												
maturity (MAT)		***	***	***	***	***	***	***	***	***		***
removal (REM)		***	***	***	NS	NS	NS	***	***	*		
shelf (SHLF)	***	***	***	NS	NS	NS	NS	NS	NS	***		
treatment (TRT)	***	***	***	NS	NS	NS	NS	NS	*		***	
MAT × TRT	***	***	***	***	***	***	***	NS	***	***		
REM × TRT	***	***	***	***	***	***	***	***	***	***		
SHLF × TRT	NS	NS	NS	*	NS	NS	NS	*	NS	***		
MAT × REM × TRT	***	NS	***	***	**	NS	NS	NS	***			
MAT × SHLF × TRT	NS	NS	NS	NS	NS	NS	NS	NS	NS	***		
REM × SHLF × TRT	***	NS	***	***	NS	NS	NS	***				

[a] Compounds listed represent a subset of the total flavonoids analyzed in study. [b] NS, *, **, and ***: not significant and significant at $P = 0.05$, 0.01, and 0.001, respectively. Means followed by the same letter within a factor are not significantly different ($P = 0.05$).

taken to avoid the coelution of chlorogenic acid (λ_{max} 330 nm, t_r 8.3 min) with cyanidin 3-galactoside (λ_{max} 530 nm, t_r 9.8 min).

Cyanidin 3-arabinoside (λ_{max} 530 nm, t_r 13.4 min) and cyanidin 3-xyloside (λ_{max} 530 nm, t_r 13.9 min) have been previously reported in apple tissue (*10*, *28*). These two peaks were tentatively identified based on the comparison of retention times with these published works, as well as on the UV/vis spectral absorption pattern characteristic of the anthocyanins. Similarly, two flavonols were also tentatively identified as quercetin 3-xyloside (λ_{max} 350 nm, t_r 23.3 min) and quercetin 3-arabinoside (λ_{max} 350 nm, t_r 24.0 min) (*29*, *30*). For quantification of these peaks, cyanidin 3-rutinoside and quercetin 3-rhamnoside authentic standards were used for the anthocyanin and flavonol compounds, respectively.

Total Flavonoids. The mean total flavonoid concentration, averaged over all harvest maturities, cold storage removals, shelf life periods, and 1-MCP treatment, in Delicious apple tissue was 2672 μg g^{-1} fw (**Table 2**). This value is consistent with those previously reported for Delicious apples, where similar flavonoid compounds and classes were quantified (*14*, *31*). Of all of the anthocyanins present in Delicious, cyanidin 3-galactoside was the most abundant, accounting for approximately 82% of the total detectable anthocyanins and contributing 31% toward the total detectable flavonoid concentration (**Table 2**). Numerous groups have reported similar disproportionate amounts of cyanidin 3-galactoside in red apple fruits with values ranging from 80 to 95% for Delicious as well as for other cultivars studied (*19*, *32*).

The flavonol and flavan-3-ol concentrations of the tissue were 715 and 894 μg g^{-1} fw and contributed 27 and 33% toward the total flavonoid complement quantified in this study, respectively (**Table 2**). Total flavonol and flavan-3-ol concentrations are consistent with numerous studies reported for Delicious (*11*, *19*), Granny Smith (*12*), and Jonagold and Elstar (*13*) but are higher than those levels reported for Golden Delicious, Empire, and Rhode Island Greening (*33*) and lower than those levels reported for Granny Smith and Splendor (*34*).

The only hydroxycinnamic acid measured in this study was chlorogenic acid at a total concentration of 332 μg g^{-1} fw. Chlorogenic acid is the major simple phenol present in both apple and pear tissue (*35*) and has been of great interest with respect to human health (*36*). Similar levels of chlorogenic acid were found in Jonagold (*13*), Crofton (*12*), Reinata (*31*), Jonagold and Golden Delicious (*37*), and Delicious, Empire, and Cortland apples (*38*), although they were approximately 2−10-fold higher than in numerous cultivars reported from other studies (*11*, *33*).

Maturity. Over the 2 week harvest period, the expected maturation and ripening pattern for apple fruit was observed (**Table 3**). Fruit firmness decreased while TSS, starch score, and IEC all increased. Although flesh firmness and TSSs proceeded in a near linear manner, starch and ethylene levels lagged behind during the first week of harvest and then increased in the rate of degradation or production, respectively, during the second week. This is indicative of a typical climacteric fruit pattern. On the basis of the IEC, the early maturity (−7 days) fruits were harvested near the preclimacteric minimum stage, while the late maturity (+7 days) fruits were well into the climacteric period and the autocatalytic rise of ethylene production.

Table 3. Initial Harvest Maturity Data for Delicious Apple (2002)

harvest maturity (days)	harvest date	firmness (N)	soluble solids (°Brix)	starch–iodine	IEC (μL L^{-1})
–7 (early)	October 3	78.5 ± 0.26	11.1 ± 0.15	3.2 ± 0.62	0.2240 ± 0.35
0 (optimal)	October 10	75.6 ± 0.27	11.4 ± 0.10	3.9 ± 0.31	2.8002 ± 3.12
+7 (late)	October 17	72.9 ± 0.29	11.8 ± 0.12	5.4 ± 0.17	14.9913 ± 13.37

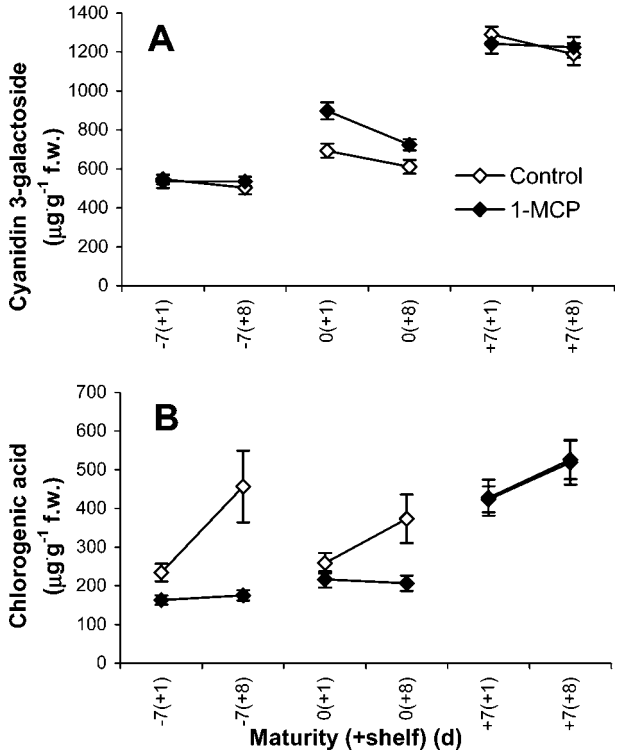

Figure 2. Changes in the concentration of cyanidin 3-galactoside (**A**) and chlorogenic acid (**B**) (μg g^{-1} fw) in the skin of red Delicious apples, with harvest maturity (early, –7 days; optimal, 0 days; and late, +7 days), shelf life periods (+1 or +8 days), and 1-MCP treatment effects over all cold storage removals (0, 30, 60, 90, and 120 days). Fruits were treated with 1-MCP (1 μL L^{-1}) immediately after harvest for 24 h at 3 °C and then stored at 0–1 °C. The legend is the same for both **A** and **B**. The mean and SEM of each point are comprised of 15 values, while differences between points, when present, are significant at $P \leq 0.05$.

Flavonoid levels in the fruit increased during the 2 week harvest period, with the greatest increase occurring in the early stages of fruit ripening, paralleling the degradation of starch and the increase in IEC. Over the course of the week from the early to optimal harvest, total fruit flavonoid levels increased 16%, while in the second week from optimal to late harvest, the flavonoid levels increased a further 49% or a total of 73% from the first to last experimental harvest date (**Table 2**). Similar trends have been reported elsewhere (*39*, *40*). This trend can also be seen in **Figure 2** where the concentration of both cyanidin 3-galactoside and chlorogenic acid increased with harvest maturity.

This aforementioned trend was highly significant and consistent for all of the compounds analyzed in this study, including chlorogenic acid, suggesting that the flavonoid biosynthetic pathway is extremely active during fruit maturation and does not preferentially synthesize one specific class of flavonoids over another. Furthermore, the fact that the increase in flavonoid concentration is in parallel with the increase in ethylene suggests a dependence of flavonoid biosynthesis on the presence of ethylene.

Storage. Although all flavonoids increased significantly during harvest, the level of flavonoids during cold storage did not consistently change. In general, the levels of flavonoids remained near harvest levels over the course of the 120 days of cold storage. This case is particularly true for the flavonol glycosides (**Table 2**). However, a subtle trend was present for both the anthocyanins and the flavan-3-ols. The flavonoid levels in these classes tended to increase for the first 30−60 days of storage followed by a transient decline in levels thereafter. It has been shown previously that cold temperatures can induce anthocyanin production and that levels can increase during short-term storage at temperatures from 0 to 4 °C (*41*, *42*). For the anthocyanin compounds, the statistical difference was most attributable to the decreasing levels of these compounds in the latter stages of storage.

The biosynthesis of flavonoids early in storage is supported by the work of Golding et al. (*12*) and Burda et al. (*33*), who also found a general increase in the concentration of epicatechins and catechins, as well as other flavonoids through the first few months of cold storage. Awad and de Jager (*13*) found few fluctuations of note in the levels of flavonols and anthocyanins in Elstar and Jonagold during 6 and 8 month storage periods in air or ultralow oxygen. However, the levels of anthocyanins in our study appear not to be stable in apple fruit throughout storage but rather are supported by the findings of Ju et al. (*14*), who also found a decreasing trend of total anthocyanin concentration in apples as storage progressed. These findings also may help to confirm the tentative conclusions of Reay (*43*), who had noted a slight decrease in anthocyanins during storage of Gala fruit.

In contrast to the trend observed for the flavonoids during storage, chlorogenic acid levels increased in a linear manner by approximately 2.5-fold ($R^2 = 0.9686$) (**Table 2**). This trend was also found in Lady Williams apples (*12*), but other studies have reported that chlorogenic acid levels remain stable or decrease during storage (*14*, *35*).

Shelf Life. During the 1 week shelf life period at room temperature, the physiological condition of the fruit changes significantly. As with cold storage, the response of the fruit to the elevated temperatures experienced during this period resulted in a decrease in anthocyanins, no change in the levels of flavonols and flavan-3-ols, and a highly significant increase in the levels of chlorogenic acid. With respect to harvest maturity, total cyanidin 3-galactoside concentration, across all removals, either remained stable or declined slightly during the shelf life period, independent of the fruit maturity at harvest (**Figure 2A**). However, total chlorogenic acid concentration from control fruit increased irrespective of harvest maturity, with the greatest increase occurring at the early harvest maturity (**Figure 2B**). As seen in **Figure 3A**, the levels of cyanidin 3-galactoside in the control fruit during the 1 week at room temperature once removed after 60−120 days of storage, declined in total concentration. In contrast, chlorogenic acid levels increased significantly in the control fruit after 90−120 days of storage (**Figure 3B**). The changes seen in the poststorage time frame are in contrast with a study that found no significant changes in the concentration of flavonoids or chlorogenic acid in Jonagold and Elstar apple fruits over a 2 week shelf period (*13*).

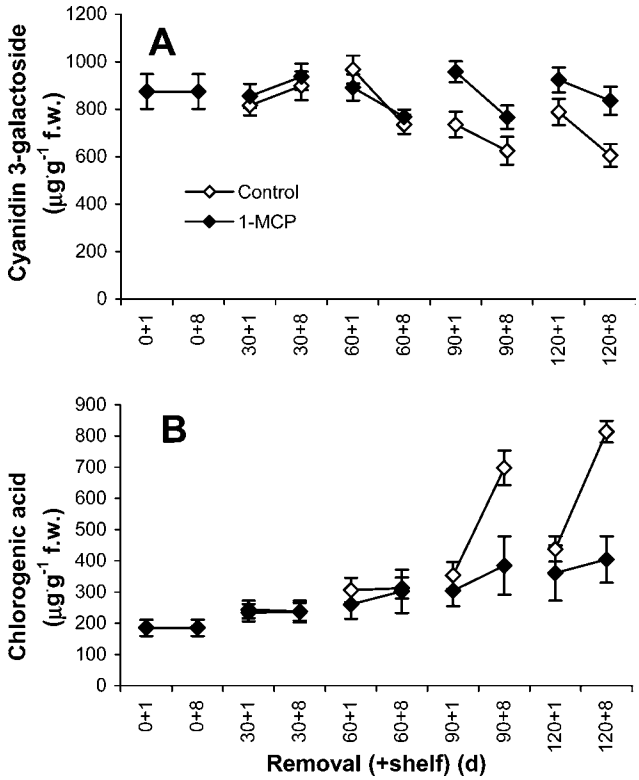

Figure 3. Changes in the concentration of cyanidin 3-galactoside (**A**) and chlorogenic acid (**B**) (μg g^{-1} fw) in the skin of red Delicious apples, with cold storage removal date (0, 30, 60, 90, or 120 days), shelf life periods (+1 or +8 days), and 1-MCP treatment effects over all harvest maturities (early, −7 days; optimal, 0 days; and late, +7 days). Fruits were treated with 1-MCP (1 μL L^{-1}) immediately after harvest for 24 h at 3 °C and then stored at 0–1 °C. The legend is the same for both **A** and **B**. The mean and SEM of each point are comprised of nine values, while differences between points, when present, are significant at $P \leq 0.05$.

However, they are in agreement with the results found for Granny Smith (*44*), Delicious, and Ralls (*14*) apple fruits, where a significant decrease in flavonoid and phenolic acids was reported during a 1 week simulated marketing period.

1-MCP and Flavonoids. Across all classes of flavonoids, a treatment of 1-MCP resulted in an apparent retention of flavonoids, with the amount in treated fruit being 5% higher than that of the control fruit. The treatment with 1-MCP had a pronounced effect at the optimal harvest fruit maturity date, but there was seldom a difference at the early or late harvest maturities (**Figure 2A**). The final level of flavonols and flavan-3-ols in both the 1-MCP-treated and the control fruit varied little from the initial flavonoid concentration at harvest. However, the apparent retention of cyanidin 3-galactoside levels due to 1-MCP treatment was evident over the course of cold storage. Similar to the response of flavonols and flavan-3-ols to cold storage, the anthocyanin levels in treated fruit were also stable throughout storage. However, these levels were significantly higher than the control fruit after the 120 day storage period, which exhibited a 20% loss of total anthocyanin concentration (**Figure 3A**).

Phenylalanine ammonia-lyase (PAL) is generally regarded as the first enzyme of the flavonoid biosynthetic pathway, catalyzing the conversion of l-phenylalanine to *trans*-cinnamic acid (**Figure 1**). It has been shown that ethylene induces the activity of PAL in root (*16*) and vegetative tissues (*45*, *46*). In Jonathan apples, the induction of PAL coincides with the presence and accumulation of both ethylene and anthocyanins (*17*). If ethylene is the regulator of the flavonoid biosynthetic pathway, then 1-MCP treatment should result in lower levels of flavonoids, simply because the action of ethylene in a ripening fruit is inhibited by such a treatment. However, we found that 1-MCP treatment resulted in higher flavonoid levels.

1-MCP is believed to inhibit the action of ethylene by binding irreversibly to the ethylene receptor (*23*) and the presence of 1-MCP bound to any member of the ethylene receptor protein family (e.g., ETR1 or ERS1) should result in the inhibition of all downstream transcription factors up-regulated by ethylene. Faragher and Chalmers (*17*) and Blankenship (*18*) have demonstrated in apple the dependence of PAL activity on the presence of ethylene. Other enzymes in the flavonoid biosynthetic pathway have been shown to be coordinately expressed in ripening apple tissue, suggesting the absence of a rate-limiting enzyme in the flavonoid pathway, thereby implicating ethylene as the primary or sole regulator of flavonoid biosynthesis (*47*). Therefore, the difference in anthocyanin levels between control and 1-MCP-treated fruit is most likely attributable to the retention of the flavonoids synthesized in the fruit up to the time of harvest, as opposed to significant de novo biosynthesis during storage.

The decline in concentration of cyanidin 3-galactoside in the latter stages of storage may be attributable to the control fruit being subjected to higher levels of reactive oxygen species formed in response to the prolonged physiological stress of cold storage. It has been shown in Blanquilla pear that a treatment of 1-MCP will result in lower levels of cellular oxidative stress and an increase in the enzymatic antioxidant capacity of the tissue (*48*). As a consequence, these flavonoids may be degraded through oxidation by reactive oxygen species or, alternatively, may be polymerized into larger compounds (*44*). However, another possibility is the differential control of the anthocyanin class of compounds during storage over the other classes of flavonoids. It has been observed previously that only the anthocyanins and chlorogenic acid compounds increased in response to various treatments (e.g., ethephon) during growth and development of Jonagold apples (*49*). Such differential control may be due to the activation of a cold-induced transcription factor in the nontreated fruit.

1-MCP and Chlorogenic Acid. While a treatment with 1-MCP was effective in the retention of flavonoid compounds, it also resulted in an inhibition of the synthesis of chlorogenic acid. Across all treatment effects, the 1-MCP-treated fruit contained 24% less chlorogenic acid. The inhibition was greatest in early harvested fruit and was reduced at optimal maturity, and by late harvest, there was no effect of 1-MCP on the tissue levels of chlorogenic acid (**Figure 2B**). Furthermore, the synthesis of chlorogenic acid only commenced once fruits were warmed to room temperature after 90 or 120 days of cold storage (**Figure 3B**). As seen in **Figure 4**, fruits from all harvest maturities had similar levels of chlorogenic acid after 120 days of storage. However, the inhibition caused by 1-MCP was most dramatic in the early harvest maturity (**Figure 4A**), less severe in the optimal maturity (**Figure 4B**), and completely lost at the late harvest maturity (**Figure 4C**). These results indicate that cold storage can induce chlorogenic acid biosynthesis and also suggest that induction of PAL activity may be inhibited by 1-MCP.

It is known that ethylene, when bound to an ethylene receptor, results in the up-regulation of transcription factors (*50*). Recent work has also demonstrated the intertwined regulation of flavonoid biosynthesis by numerous R2R3 MYB transcription factors (*51*). In maize and other plants, the presence of the MYB

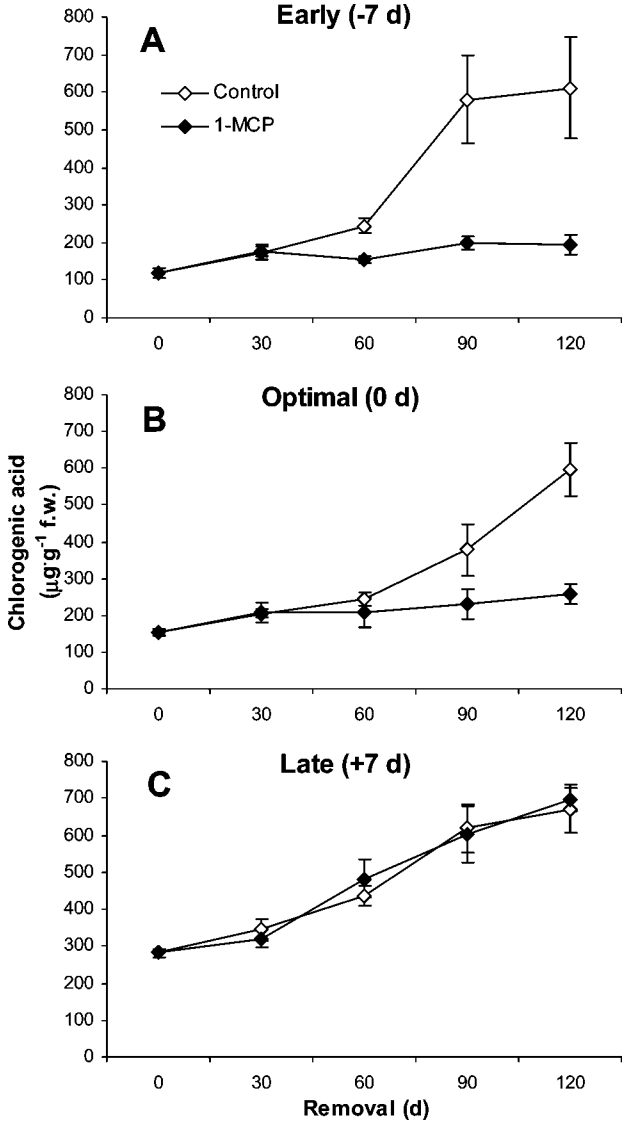

Figure 4. Changes in chlorogenic acid concentration (μg g^{-1} fw) in the skin of red Delicious apples, with harvest maturity [(**A**) early, −7 days; (**B**) optimal, 0 days; and (**C**) late, +7 days], cold storage removal date (0, 30, 60, 90, or 120 days), and 1-MCP treatment effects over all shelf life periods (+1 and +8 days). Fruits were treated with 1-MCP (1 μL L^{-1}) immediately after harvest for 24 h at 3 °C and then stored at 0–1 °C. The legend is the same for all three figures. The mean and SEM of each point are comprised of six values, while differences between points, when present, are significant at $P \leq 0.05$.

transcription factor C1 and basic helix−loop−helix (bHLH) factor R results in anthocyanin biosynthesis through the up-regulation of CHS, DFR, LDOX, UFGT, and GST enzymes (*52*) (**Figure 1**) and can be regulated by environmental stimuli, light, and plant hormones (*51*). The rapid increase in chlorogenic acid levels independent of similar increases in flavonoid levels may be due to the activation of an alternate transcription factor, such as MYB-IF35 found in maize (*53*). Such a transcription factor may be up-regulated when the other regulatory genes (e.g., C1 + R) fail, in this case, to successfully ripen the fruit due to prolonged exposure to low temperatures of storage. In maize, the MYB-IF35 transcription factor has lost the capacity to synthesize any compounds downstream from the enzyme CHS but has retained the capacity to synthesize chlorogenic acid (**Figure 1**). If an MYB-IF35-like transcription factor is present in apple, it would likely be regulated by the ethylene signal transduction pathway, as 1-MCP resulted in the inhibition of the accumulation of chlorogenic acid that such an MYB factor should control. Therefore, the increase in chlorogenic acid in the latter stages of storage is possibly due to up-regulation of an MYB-IF35-like transcription factor in apple and may explain the different response of flavonoid and chlorogenic acid metabolism to the low storage temperature. Similarily, the differential response of anthocyanins to cold storage when compared to the other classes of flavonoids (i.e., the former declines while the others remain stable) may also be due to the action of this or an alternate transcription factor.

The present study demonstrated that a treatment of 1-MCP is effective at maintaining the intrinsic flavonoid levels present at harvest in stored apple fruit but concurrently inhibits the biosynthesis of chlorogenic acid. Furthermore, it reiterates the importance of treating with 1-MCP at the proper harvest maturity in order to ensure the desired effects from the treatment are achieved.

ABBREVIATIONS USED

1-MCP, 1-methylcyclopropene; HPLC-DAD, high-performance liquid chromatography/diode array detector; IEC, internal ethylene concentration; TSS, total soluble solids content; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; bHLH, basic helix−loop−helix.

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