JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Changes in Primary Metabolites and Polyphenols in the Peel of "Braeburn" Apples (*Malus domestica* Borkh.) during Advanced Maturation

Jan Bizjak,* Maja Mikulic-Petkovsek, Franci Stampar, and Robert Veberic

Biotechnical Faculty, Department of Agronomy, Chair for Fruit, Wine and Vegetable Growing, University of Ljubljana, Jamnikarjeva 101, SI-1000 Ljubljana, Slovenia

ABSTRACT: During the two growing seasons the evolution of primary metabolites and wide range of polyphenols in the "Braeburn" apple peel during advanced maturation were investigated. During the five weeks sucrose significantly increased, whereas fructose and glucose fluctuated around the same level in one season and decreased in another. Regarding malic and citric acids, an expected decrease was recorded. The concentrations of hydroxycinnamic acids, dihydrochalcones, and flavanols remained quite constant or slightly decreased during advanced apple ripening. On the contrary an intensive accumulation of quercetin glycosides and anthocyanins took place during this period, starting with the onset of rapid formation approximately 3 weeks before the technological maturity of apples. Total phenolic content was relatively constant or slightly increased. The present results suggest that measures designed to improve the apple color and quality of "Braeburn" apples should be performed approximately 3−4 weeks before the expected technological maturity of apples.

KEYWORDS: *flavonoids, quercetin-glycosides, anthocyanins, sugars, organic acids, advanced apple ripening*

INTRODUCTION

Apart from water, apples contain different amounts of primary (sugars and organic acids) and a wide range of secondary metabolites (polyphenols). Glucose, fructose, sucrose, and organic acids represent the most important taste components in apples, and their ratio has a marked influence on the sensory quality of the fruit.[1,2] Polyphenols, together with organic acids and sugars, make an important contribution to the inner quality as well as enhance the outer appearance of the apples.[3]

Due to the high content of phenolic compounds and widely consumption, apples represent an important share of consumed flavonoids in American and European diet.[4] Many of them have been found to have strong antioxidant activity and anticancer activity[5] and thus have the potential to modulate many processes in the development of diseases, including cancer, cardiovascular disease, diabetes, pulmonary disorders, Alzheimer's disease, and other degenerative disease states.[6] Moreover, when compared to other fruits, apples have the highest portion of free phenolics, which may be more available for eventual absorption into the bloodstream.[7]

Based on molecular structure, phenolics in apples are divided into five major groups including hydroxycinnamic acids, flavanols dihydrochalcones, flavonols, and anthocyanins, mainly presented in the peel of the red apple cultivars.[8] With the exception of hydroxycinnamic acids, their accumulation is concentrated in the apple peel which contains anywhere from 2−6 times more phenolic compounds than the flesh[9,10] and 2−3 times more flavonoids in the peels when compared to the flesh.[11]

Flavonoid accumulation in the apple peel is affected by numerous factors, including light,[12] variety,[13] plant nutrition,[14] stress situations,[15] temperature,[16] cultivation type, and conditions of the plant.[17] In addition, fruit development and maturity also significantly affect the polyphenol composition of apples. Awad et al.[18] found that quercetin glycosides, catechins, phloridzin and chlorogenic acid in "Jonagold" and "Elstar" apples were mainly accumulated during the fruit development until the onset of maturation, whereas the main accumulation of anthocyanins occurred during the maturation of the fruit. Lister et al.[19] reported that concentration of quercetin glycosides and procyanidins in the peel of "Splendour" apples decreased from early to mid season but increased during apple ripening.

Despite the fact that there are several reports on the developmental changes in the concentration of polyphenols in apples during development and ripening,[15,18−20] fewer details are known on the variation of primary metabolites and polyphenolic concentrations during the advanced maturation prior to technological maturity of apples. However, in some studies the changes in polyphenols during ripening in the apple flesh were observed.[21,22]

In the present study the changes of the concentrations of sugars, organic acids and a wide range of polyphenols as well as total phenolic compounds in the "Braeburn" apple peel during the advanced maturation of apples in two growing seasons are reported. All phenolic compounds were confirmed using a mass spectrometer with an electrospray interface (ESI) operating in negative ion mode. The results will contribute information about the evolution and maximal concentration of primary and secondary metabolites in the last stages of apple ripening and their relation as well. At the same time, by knowing the time of the most intensive changes in the primary and secondary metabolites, these results could be very helpful in determining

Received: July 11, 2013
Revised: October 2, 2013
Accepted: October 9, 2013
Published: October 9, 2013

the most appropriate time to carry out the agro-technical measures designed for improving the outer and inner quality parameters of apples, such as apple coloring and the like.

MATERIALS AND METHODS

Plant Materials. Trials were conducted in the central and eastern Slovenia on striped "Braeburn" apple cultivar clone Hillwell (*Malus domestica* Borkh.) at Ljubljana location (latitude 46°2′, longitude 14°28′) in 2011 and at at the fruit growing center Maribor Gacnik location (latitude 46° 61′, longitude 15° 68′) in 2012. At both locations apple trees were grafted on M9 rootstock and grown according to the system of integrated production.

At Ljubljana location apples for measurements were taken from the 10-year-old trees planted maintaining row distances of 3.75 m and tree-to-tree distance of 1.1 m. Full bloom (>80% of the opened flower buds) occurred on the 22nd of April 2011, whereas the technological maturity, which was determined using the starch iodine test occurred on the 14th of October (175 DAFB).

At the fruit growing center Maribor Gacnik, 13-year-old trees planted maintaining row distances of 3.5 m and tree-to-tree distances of 1 m were used in the experiment. Full bloom (>80% of the opened flower buds) occurred on the 15th of April 2012, while the technological maturity, occurred on the 7th of October, (177 DAFB). At the time of technological maturity the value of firmness was approximately 9.5 kg cm^{-2} and the starch index (on the scale of 1−5) was 2.7 in both years.

Fruit Sampling. At Ljubljana the fruit sampling started on September 9th whereas at Gacnik Maribor the first sampling was carried out on September 4th. At both locations sampling continued weekly until the technological maturity of the fruit which at both locations occurred five weeks after the first sampling. At each sampling date 15 fruits from five trees were randomly harvested and combined in five samples, with 3 fruit per sample (n = 5). Immediately after the harvest, the apples were transported to the laboratory, where the tissue of samples was frozen in liquid nitrogen to prevent oxidation of phenolic substances and stored at −20 °C until the preparation of the samples.

Firmness and Soluble Solid Content Measurements. Soluble solid content (SSC) was measured using the digital refractometer ATAGO WM-7. The firmness was measured on four peeled sides in the equatorial plane of the fruit with a penetrometer (TR Italy) equipped with an 11 mm tip. The depth of tip penetration was 8 mm.

Analysis of Individual Sugars and Organic Acids. Sucrose, glucose, fructose, and sorbitol and malic and citric acids were analyzed in the whole edible part of the fruit according to Mikulic-Petkovsek et al.[9] For the extraction of individual sugars and organic acids, 10 g of the fresh mass of each sample was homogenized in 50 mL of bidistilled water using Ultra-Turrax T-25 (Ika-Labortechnik, Stauden, Germany). Samples were left for 30 min at room temperature and stirred frequently. After extraction, the homogenate was centrifuged (Eppendorf 5810 R Centrifuge, Hamburg, Germany) at 10 000 rpm for 7 min at 4 °C. The supernatants were filtered through a 0.20-μm cellulose ester filter (Macherey-Nagel, Düren, Germany) and transferred into a vial, and 20 μL of the sample was used for analysis. The analysis of sugars (fructose, glucose, and sucrose), sorbitol, malic and citric acid content was carried out using high-performance liquid chromatography (HPLC) from the Thermo Separation Products equipment.[9]

Separation of sugars and sorbitol was carried out using a Rezex RCM-monosaccharide column (300 × 7.8 mm; Phenomenex, Torrance, CA) with a flow of 0.6 mL/min and column temperature maintained at 65 °C. The mobile phase was bidistilled water; the total run time was 30 min, and a refractive index detector shodex RI-71 was used to monitor the eluted carbohydrates as described by Mikulic-Petkovsek et al.[9] Organic acids were analyzed using a RezexROA-organic acid column (300 × 7.8 mm; Phenomenex, Torrance, CA) and a UV detector set at 210 nm with a flow of 0.6 mL min^{-1} maintaining the column temperature at 65 °C. The duration of the analysis was 30 min. The concentrations of carbohydrates and organic acids were calculated with the help of corresponding external standards. The concentrations were expressed in g kg^{-1} fresh weight (FW).

Chemicals. For the quantification of phenolic compounds the following standards were used: chlorogenic acid (3-caffeoylquinic acid), phloretin, and rutin (quercetin 3-*O*-rutinoside) from Sigma Aldrich Chemie (Steinheim, Germany), cyanidin 3-*O*-galactoside chloride, quercetin 3-*O*-rhamnoside, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B1, B2, and phloridzin dihydrate from Fluka Chemie (Buchs, Switzerland), quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside from Apin Chemicals (Abingdon, U.K.), and (+)-catechin from Roth (Karlsruhe, Germany). Methanol for the extraction of phenolics was acquired from Sigma. Chemicals for the mobile phases were the high performance liquid chromatography−mass spectrometry (HPLC-MS) grade acetonitrile and formic acid from Fluka Chemie. Water for mobile phase was bidistilled and purified with the Milli-Q system (Millipore, Bedford, MA, USA). For determination of the total phenolic content, Folin−Ciocalteu phenol reagent (Fluka Chemie GmbH), sodium carbonate (Merck, Darmstadt, Germany), gallic acid, and ethanol (Sigma) were used.

Extraction and Determination of Phenolic Compounds. The extraction of fruit samples for phenolic compounds was done as described by Mikulic-Petkovsek et al.[17] with some modification. Apple samples were ground to a fine powder in a mortar chilled with liquid nitrogen. A total of 5 g of apple peel was extracted with 10 mL of methanol containing 3% (v/v) formic acid and 1% (w/v) 2.6-ditert-butyl-4-methylphenol (BHT) in a cooled ultrasonic bath for 1 h. BHT was added to the samples in order to prevent oxidation. After extraction, the treated samples were centrifuged for 5 min at 10 000 rpm. The supernatant was filtered through a Chromafil AO-20/25 polyamide filter (Macherey-Nagel, Düren, Germany) and transferred to a vial before the injection into the HPLC system.

The individual phenolic compounds were analyzed using a Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA) with a diode array detector at 280, 350, and 530 nm. The hydroxycinnamic acids, dihydrochalcones, and flavanols were detected at 280 nm, flavonols at 350 nm, and anthocyanins at 530 nm. For the separation of phenolic compounds a Phenomenex (Torrance, CA) HPLC column C18 (150 × 4.6 mm, Gemini 3 μ) protected with a Phenomenex security guard column operated at 25 °C was used. The injection volume for the fruit extract was 20 μL, and the flow rate maintained at 1 mL min^{-1}. The elution solvents were aqueous 1% formic acid and 5% acetonitril (A) and 100% acetonitrile (B). Samples were eluted according to the linear gradient described by Marks et al.[23] 0−5 min, 3% to 9% B; 5−15 min, 9% to 16% B; 15−45 min, 16% to 50% B; 45−50 min, 50% isocratic; this step was followed by the washing and reconditioning of the column. Compound identification was achieved by comparing the retention times and their UV−vis spectra from 200 to 600 nm, as well as by the addition of an external standard. The identity of the phenolic compounds was also confirmed using a mass spectrometer (Thermo Scientific, LCQ Deca XP MAX) with an electrospray ionization (ESI) operating in the negative and positive ion mode. For the analyses full-scan data dependent MS scanning from m/z 115 to 2000 was used. Quantification was achieved according to the concentrations of a corresponding external standard. For the compounds lacking reference compounds, related compounds were used as standards for the quantification. Therefore 4-*O*-*p*-coumaroylquinic acid was quantified in equivalents of *p*-coumaric acid, phloretin-2-*O*-xylosylglucoside in equivalents of phloridzin, quercetin 3-*O*-arabinopyranoside in equivalents of quercetin 3-*O*-arabinofuranoside or anthocyanins (cyanidin 3-arabinoside, cyanidin 7-arabinoside, cyanidin 3-glucoside, and cyanidin 3-xyloside) were quantified in equivalents of cyanidin 3-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (FW).

Determination of the Total Phenolic Content. Extracts of the samples were obtained in the same way as for the individual phenolic compounds, with the difference that no BHT was added. The total phenolic content of extracts was assessed using the Folin-Ciocalteu phenol reagent method, as described by Singleton and Rossi.[24] To 100 μL of sample extracts were added 6 mL of bidistilled water and 500 μL

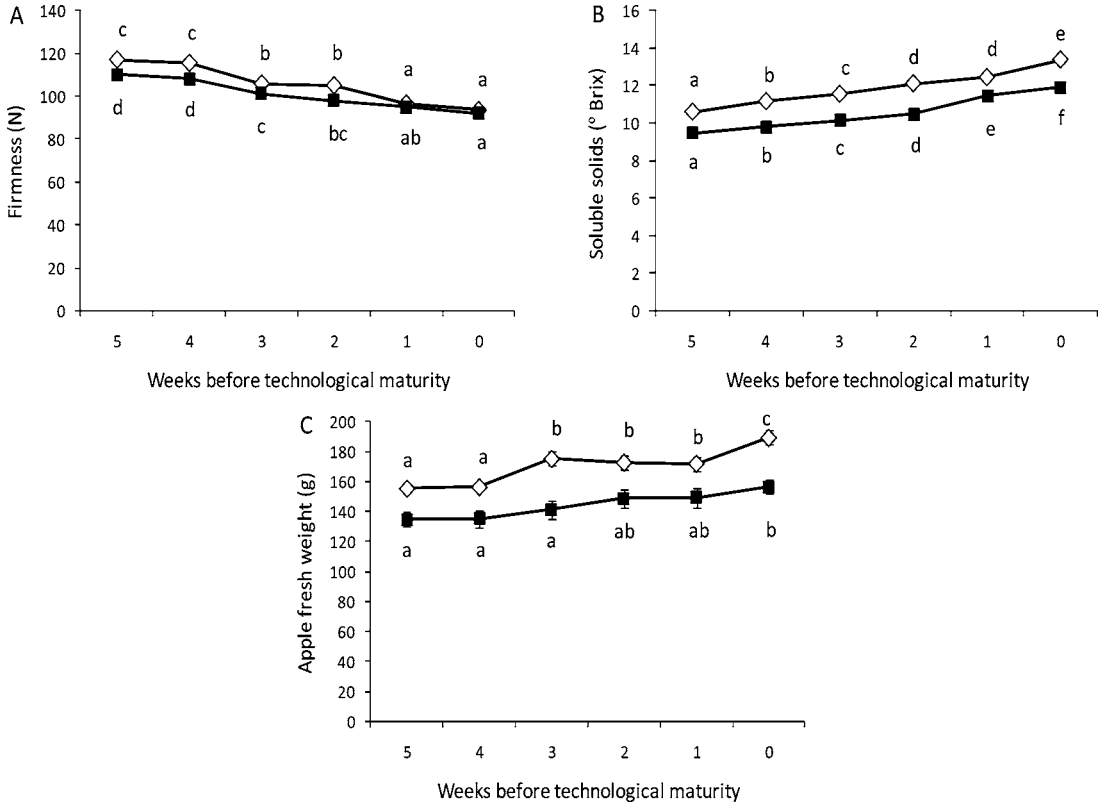

Figure 1. Mean (±SE) firmness (A), soluble solids (B), and fruit fresh weight (C) of "Braeburn" apples in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Different letters (a–f) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$, $n = 15$).

of Folin-Ciocalteau reagent; after resting between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) and 1.9 mL of bidistilled water were added. The extracts were mixed and allowed to stand for 30 min at 40 °C. After that the absorbance was measured in a spectrophotometer (Perkin-Elmer, UV/vis Lambda Bio 20) at 765 nm. A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in mg kg^{-1} fresh weight (FW). Absorption was measured in three replications.

Statistics. Statistical analysis was conducted in order to evaluate the changes of primary and secondary metabolites in "Braeburn" apples during the advanced ripening. The data was analyzed using the Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, Maryland, USA). Data from all of the analyses was tested for any differences among sampling dates within each year using one-way analysis of variance (ANOVA). The differences were tested using the Duncan test with the significance level 0.05. In diagrams, the means as well as their standard errors are presented (mean ± SE).

RESULTS

Firmness decreased gradually whereas soluble solids content (SSC) increased linearly during the advanced maturation of "Braeburn" apples in both years (Figure 1A,B). During the 5 weeks of ripening SSC increased by 26% in both years, while firmness decreased in 2011 by 20% and in 2012 by 16%. A similar percentage also increased the fruit weight, namely by 22% in 2011 and 16% in 2012 (Figure 1C).

The changes of sugars (sucrose, glucose, and fructose), sorbitol, and organic acids (citric and malic acid) are presented in Figure 2. At the technological maturity of "Braeburn" apples, fructose was the predominant sugar representing 48/51% of the total sugars in 2011/2012, followed by sucrose (39/33%), glucose (9/14%), and the main sugar alcohol sorbitol (4/2%). Our results are in agreement with those obtained on other apple varieties.[9,25−27] Sucrose increased during the advanced ripening, reaching the highest concentration at technological maturity of "Braeburn" apples in both years. In the five weeks of fruit ripening the concentration increased from 23% in 2011 to 51% in 2012. On the contrary, fructose and glucose in 2011 decreased by 20%, whereas in 2012 it decreased prior to 3 weeks before technological maturity and recovered afterward (Figure 2B,C). The concentration of sorbitol fluctuated during fruit ripening in 2012 and increased prior to 1 week before technological maturity in both years (Figure 2D). Regarding the total sugars their concentration decreased from 5 to 2 weeks before the technological maturity of apples and increased afterward, reaching the peak at the technological maturity of apples (data not shown). Regarding the organic acids, malic and citric acids were quantified. The level of shikimic and fumaric acids were of the order of 10 mg kg^{-1} of fresh weight, thus their concentrations are not reported. Malic acid was the main acid contributing about 82–85% of the total acid concentrations. Its concentration significantly decreased during the 5 weeks of advanced ripening in 2012, whereas no significant changes were observed in 2011 (Figure 2F). Significant changes were observed also in the case of citric acid (Figure 2E). When comparing the concentrations measured at the beginning of the advanced ripening and concentrations noted at technological maturity of apples, the concentration of citric acid in 2012 decreased while it remained unchanged in 2011.

A total of 21 phenolics (Table 1) were identified and quantified in "Braeburn" apple peels belonging to five groups: hydroxycinnamic acids, dihydrochalcones, flavonols, flavanols,

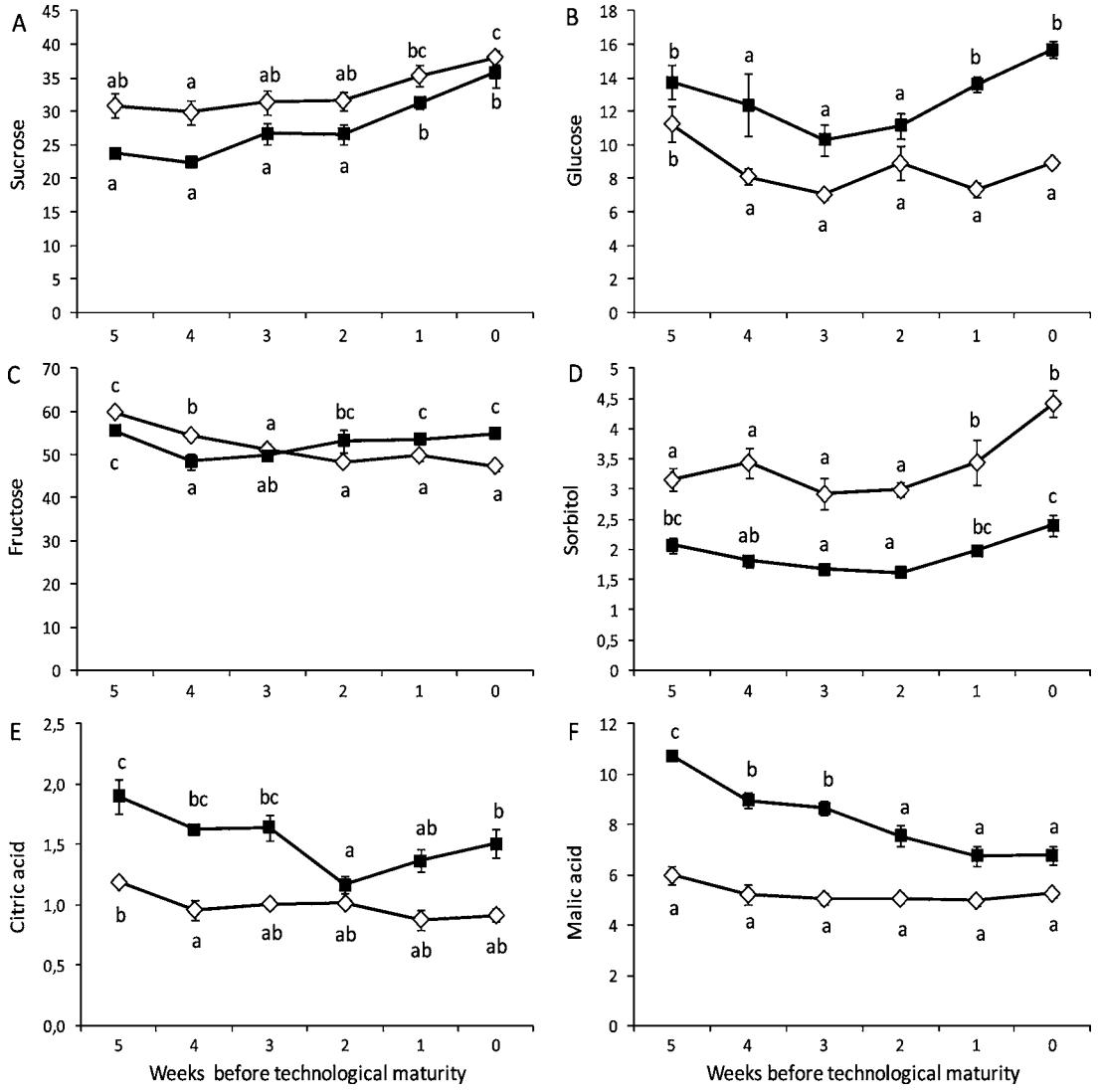

Figure 2. Concentration (g kg^{-1} FW) of sucrose (A), glucose (B), fructose (C), sorbitol (D), citric acid (E), and malic acid (F) in "Braeburn" apples in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a−c) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

and anthocyanins. With the exception of flavonols the concentrations of all the phenolic groups was in 2012 much higher than in 2011. Great differences up to 70% in the phenolics between seasons have already been noted in the study of Lata et al.[13] in several other cultivars.

Among hydroxycinnamic acids chlorogenic, caffeic, and 4-coumaroylquinic acids were analyzed. Hydroxycinnamis acids were the lowest represented group of phenolics in "Braeburn" apple peels ranging from 6% of the total analyzed phenolic concentration at the beginning of ripening to 3% at the technological maturity of apples. During the 5 weeks of advanced ripening the total hydroxycinnamic acids concentration decreased by 27% in both years (Figure 3A). However, the decrease was significant just in 2012 and was mainly the consequence of the decrease of the 4-*p*-coumaroylquinic acid (Figure 4B). Viewed from the perspective of the individual acids significant changes during the ripening were found only in the case of chlorogenic acid in 2011 (Figure 4A).

Phloridzin was the main dihydrochalcone in "Braeburn" apple peels, accounting for approximately 65% of the total dihydrochalcones. Besides phloridzin, phloretin 2-xylosylglucoside was also identified and quantified. Since the beginning of ripening till the technological maturity of apples concentration of the dihydrochalcones decreased by 18% in both years; however, the decrease was not significant (Figure 3B). The decrease was more pronounced in the case of phloridzin than phloretin (data not shown).

The analysis of flavonols covered 7 types of quercetin glycosides. At technological maturity the major flavonol species was quercetin 3-galactoside, accounted for approximately 35−40% of total flavonols concentration in "Braeburn" apple peels, followed by quercetin 3-*O*-arabinofuranoside (15−21%), quercetin 3-*O*-rhamnoside (15−18%), quercetin 3-*O*-xyloside (12−14%), quercetin 3-*O*-glucoside (7−8%), quercetin 3-*O*-rutinoside (4%), and quercetin 3-*O*-arabinopyranoside (2%). Similar findings in "Braeburn" apples were already reported in the previous study of Bizjak et al.[28]

In both years 2011 and 2012, the developmental pattern of flavonols was very similar (Figure 3C). From 5 to 3 weeks before the technological maturity, a slight increase in the concentration was observed, followed by a significant increase which was completed approximately 1 week before the

Table 1. Identification of Phenolic Compounds in Apple Peels in Positive and Negative Ions With HPLC-MS and MS^2

peak no.	$[M-H]^-$ (m/z)	Rt[b]	MS^2 (m/z)	tentative identification	standard	expressed as	compound class	detection (nm)
1	577	6.33	425, 407, 289	procyanidin B1	yes	procyanidin B1	flavanols	280 nm
2	289	7.70	245	catechin	yes	catechin	flavanols	280 nm
3	353	7.84	191, 179	chlorogenic acid	yes	chlorogenic acid	hydroxycinnamic acids	280 nm
4	577	8.83	425, 407, 289	procyanidin B2	yes	procyanidin B2	flavanols	280 nm
5	179	9.64	135	caffeic acid	yes	caffeic acid	hydroxycinnamic acids	280 nm
6	289	10.13	245	epicatechin	yes	epicatechin	flavanols	280 nm
7	337	10.82	173	4-*O*-*p*-coumaroylquinic acid	no	*p*-coumaric acid	hydroxycinnamic acids	280 nm
8	567	19.2	273	phloretin-2′-*O*-xylosylglucoside	no	phloridzin	dihydrochalcones	280 nm
9	435	21.6	273	phloridzin	yes	phloridzin	dihydrochalcones	280 nm
10	609	16.44	301	quercetin-3-*O*-rutinoside	yes	quercetin-3-*O*-rutinoside	flavonols	350 nm
11	463	17.09	301	quercetin-3-*O*-galactoside	yes	quercetin-3-*O*-galactoside	flavonols	350 nm
12	463	17.54	301	quercetin-3-*O*-glucoside	yes	quercetin-3-*O*-glucoside	flavonols	350 nm
13	433	18.69	301	quercetin-3-*O*-xyloside	yes	quercetin-3-*O*-xyloside	flavonols	350 nm
14	433	18.19	301	quercetin-3-*O*-arabinopyranoside	no	quercetin-3-*O*-arabinofuranoside	flavonols	350 nm
15	433	19.86	301	quercetin-3-*O*-arabinofuranoside	yes	quercetin-3-*O*-arabinofuranoside	flavonols	350 nm
16	447	20.24	301	quercetin-3-rhamnoside	yes	quercetin-3-rhamnoside	flavonols	350 nm
17[a]	449	6.56	287	cyanidin-3-galactoside	yes	cyanidin-3-galactoside	anthocyanins	530 nm
18[a]	449	7.07	287	cyanidin-3-glucoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
19[a]	419	7.65	287	cyanidin-3-arabinoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
20[a]	419	9.29	287	cyanidin-7-arabinoside	no	cyanidin-3-galactoside	anthocyanins	530 nm
21[a]	419	9.56	287	cyanidin-3-xyloside	no	cyanidin-3-galactoside	anthocyanins	530 nm

[a] $[M+H]^+$ (m/z) anthocyanins were obtained in the positive ion mode. [b] Retention time.

technological maturity of apples. During the last week of ripening a decrease was found, which was more pronounced in 2011 (Figure 3C).

Among the individual quercetin glycosides, quercetin 3-*O*-galactoside contributed the largest share to the increase of total flavonols. During the 5 weeks of ripening its concentration increased by 35% in 2011 and almost 5-fold in 2012 (Figure 4E). However, it is necessary to take into account that in 2012 its concentration at the beginning of the advanced ripening (29.15 mg kg^{-1} FW) was very low in comparison with 2011 (93.14 mg kg^{-1} FW), thus increases were so different sizes. The significant increase from 3 to 1 week before the technological maturity was observed in the case of all the other individual quercetin glycosides, except quercetin 3-*O*-rutinoside (rutin) in 2011 (Figure 4D). In the case of quercetin 3-*O*-rhamnoside (Figure 4F) as well as quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside (data not shown), a significant decrease in the last week of the advanced ripening in 2011 was detected.

Within flavanols the monomeric catechin and epicatechin and dimeric flavanol procyanidin B1 and B2 were analyzed. Procyanidin B2 was the most abundant individual flavanol in the "Braeburn" apple peels, representing approximately 55% of the total flavanol concentration. Other flavanols listed in descending order, were epicatechin, catechin and procyanidin B1. With regard to quantity flavanols were the most represented phenolic group in "Braeburn" apple peels. Their concentration ranged from 50% and 65% of the total analyzed phenolic concentration at the beginning of the advanced ripening to 38% and 47% at the technological maturity of apples in 2011 and 2012, respectively. Flavanols have already been reported to be quantitatively the most abundant phenolic groups by numerous of authors in several other cultivars.[17,29,30] No significant changes in flavanols were observed during the 5 weeks of the advanced ripening (Figure 3D), however flavanol concentration slightly decreased in both years. Among individual flavanols, significant changes during ripening were observed only in the case of procyanidin B1 in 2012, when a decrease was found (Figure 4C).

Among anthocyanins cyanidin 3-*O*-galactoside, cyanidin-3-*O*-arabinoside, cyanidin 7-*O*-arabinoside, cyanidin 3-*O*-xyloside, and cyanidin 3-*O*-glucoside were identified and quantified. Cyanidin 3-*O*-galactoside was the main cyanidin glycoside in the "Braeburn" apple peels, while the cyanidin 3-*O*-glucoside level was the lowest, findings which are in accordance with those obtained by other autors.[20,28,31] Cyanidin 3-*O*-galactoside ranged from 89% at the beginning of ripening to 81% of the total anthocyanin concentration at the technological maturity of apples. Cyanidin 3-*O*-glucoside with the concentration of 1% of total anthocyanins became detectable 4 weeks before technological maturity of the apples. Other cyanidin glycosides were each present at 8% or less of the total anthocyanin concentration and their percent increase during the advanced ripening. In 2012 anthocyanin synthesis probably started shortly prior to our first sampling, since at first sampling the measured concentration of total anthocyanin was just 0.89 mg kg^{-1} FW. However, the intensive onset of anthocyanin accumulation occurred in both years approximately at the same time somewhere between 3 and 2 weeks before the technological maturity of apples (Figure 3C,E).

Total phenolic content was relatively constant during advanced ripening, and no significant changes were observed neither in 2011 nor in 2012 (Figure 3F). The average content (mg GAE kg^{-1} FW) in 2012 ranged from 1578.33 to 1923.73 and was higher than in 2011 when varied between 1062.40 and

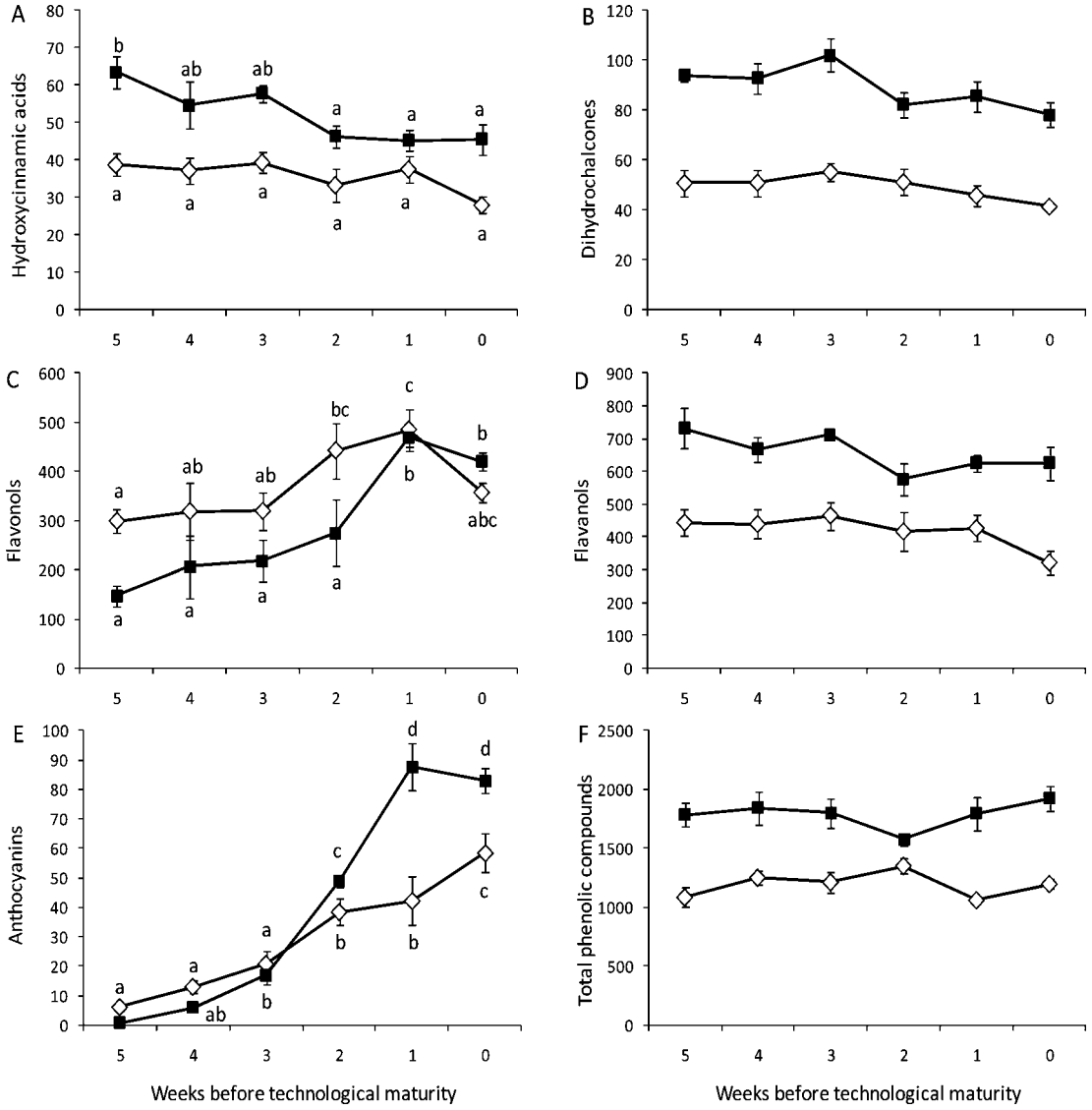

Figure 3. Concentration (mg kg^{-1} FW) of total hydroxycinnamic acids (A), dihydrochalcones (B), flavonols (C), flavanols (D), anthocyanins (E), and total phenolic compounds (mg GAE kg^{-1} FW) in "Braeburn" apple peels in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a–d) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

1347.24. Differences in the TPC between the years were so similar to those previously observed in several phenolic groups. Great differences in the total phenolic content between the seasons have already been reported by Lata et al.[13] in many apple cultivars.

■ DISCUSSION

Firmness and soluble solids content (SSC) are two important quality parameters in determining fruit maturity and harvest time, and represent key parameters in assessing and grading the postharvest quality of apples.[32] The loss of fruit firmness during advanced ripening is a consequence of many cellular events which are influenced by external factors,[33] whereas the increases in soluble solids during the ripening would likely be attributed to the hydrolysis of the starch component in the unripe apples.[34] The increase of the fruit weight during the fruit development of the apples is due to the cell division and cell enlargement and its pattern shows a typical sigmoid pattern.[35] However, during the advanced maturation, the growth curve becomes much more flat, which is largely conditioned by apple cultivar, growth conditions and number of fruits harvested on the tree.

Sugars (glucose, sucrose, and fructose) and organic acids (citric and in particular malic acid) represent the most important taste components in apples.[1,2] The concentration of glucose and fructose in 2012 fluctuated during the 5 weeks of advanced ripening, whereas it decreased in 2011 (Figure 2B,C). Our results, especially those in 2012 are consistent with the findings of Ackermann et al.,[25] who also found a decrease of glucose and fructose during the advanced ripening, followed by an increase approximately 2 weeks before the technological maturity of apples. Sorbitol is the primary product of photosynthesis mainly present in apple leaves and is transported to sink tissues such as fruit where being converted to fructose.[36] The increase in the fructose concentration during the fruit ripening corresponded well with the decrease in sorbitol concentration,[21] which was not confirmed by our results where no interconnection between the concentration of

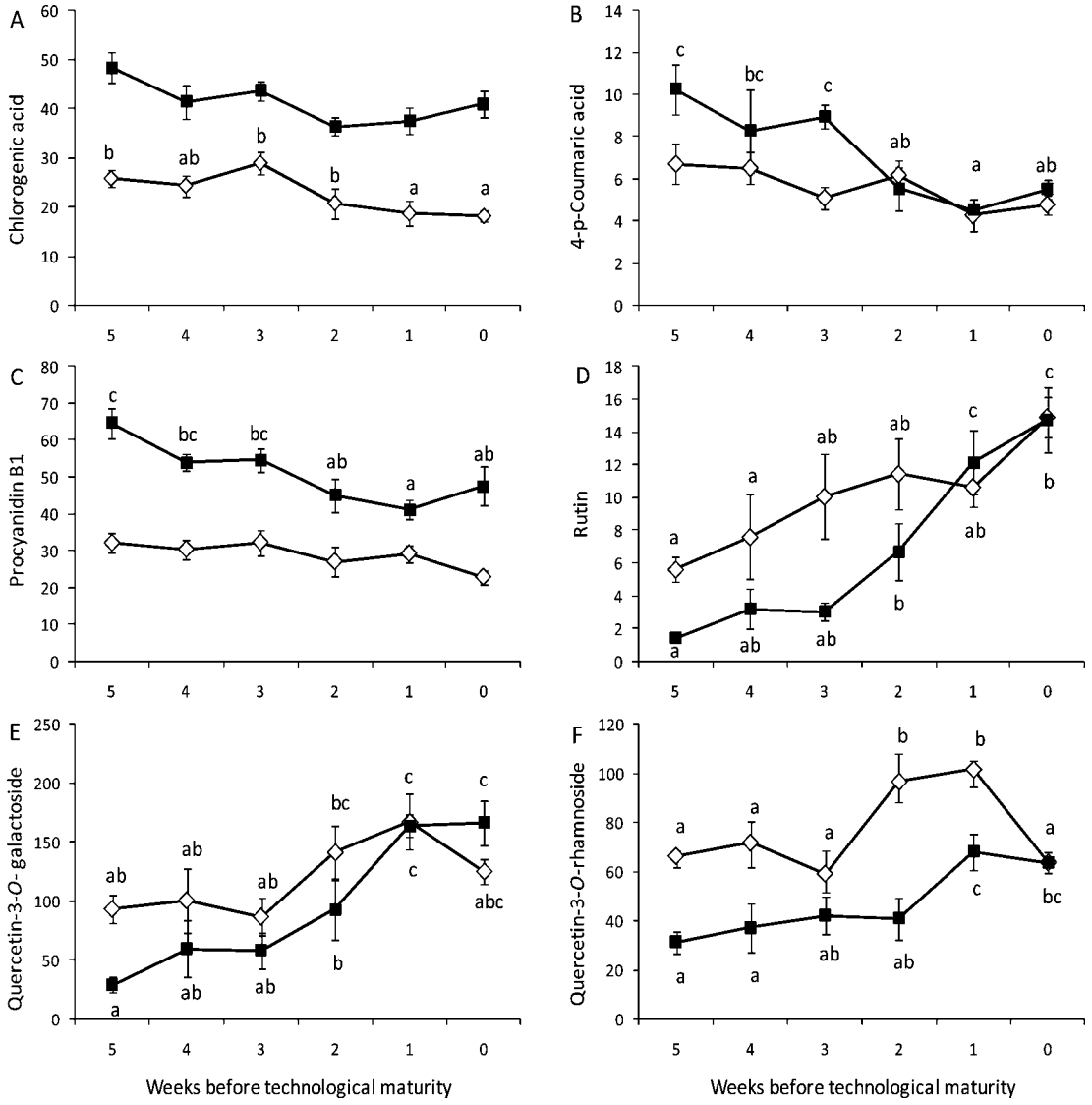

Figure 4. The concentration (mg kg^{-1} FW) of chlorogenic acid (A), p-coumaric acid (B), procyanidin B1 (C), rutin (D), quercetin-3-*O*-galactoside (E), and quercetin-3-*O*-rhamnoside in "Braeburn" apple peels in 2011 (◊) and 2012 (■) at different sampling dates during the advanced maturation of apples. Means and their standard errors are presented (n = 5). Different letters (a–c) denote statistically significant differences between sampling dates within each year (Duncan test, $p \leq 0.05$).

sorbitol and fructose was found. However, the continued increase in the sucrose concentration after the fructose accumulation stopped support the suggestion of Berüter[37] that part of the increase in sucrose before the fruit harvest might have been synthesized from fructose via sucrose synthase. In our study sucrose was the only individual sugar which increased during the advanced maturation and also the only one with the same developmental pattern in both years (Figure 2A). The accumulation of the sucrose during advanced ripening resulted most probably from sucrose that was transported from the leaves on the one hand and newly synthesized sucrose via sucrose phosphate synthetase and/or sucrose synthase on the other hand.[21] The sucrose increase during apple ripening has also been observed in some other apple varieties.[21,25] So it appears that among individual sugars sucrose is at least influenced upon many external factors, including apple cultivar, climate, and growth conditions.

Malic and citric acid concentrations decreased during advanced ripening in 2012, whereas they showed less significant changes in 2011 (Figure 2E,F). The decrease could be attributed to a dilution effect caused by the mass increase during the cell growth phase and/or increased respiration, since malic acid is the principal metabolic substrate used in this process.[25,38,39] The developmental pattern of malic and citric acids during advanced ripening was similar to those observed from other researchers.[21,25]

With respect to hydroxycinnamic acids, dihydrochalcones, and flavanols, no significant changes have been observed during advanced ripening of "Braeburn" apples (Figure 3B,D) with the exception of hydroxycinnamic acids in 2012 (Figure 3A), when a significant decrease was found. Developmental patterns of above-mentioned phenolic groups were very similar in both years, showing small fluctuations, but the general tendency was to slightly decrease during the advanced ripening of apples. A relatively constant level of total catechins (catechin plus epicatechin), phloridzin, and chlorogenic acid was observed also by Awad et al.[12] in the skin of "Elstar" and "Jonagold" apples. With respect to oligomeric procyanidins generally a

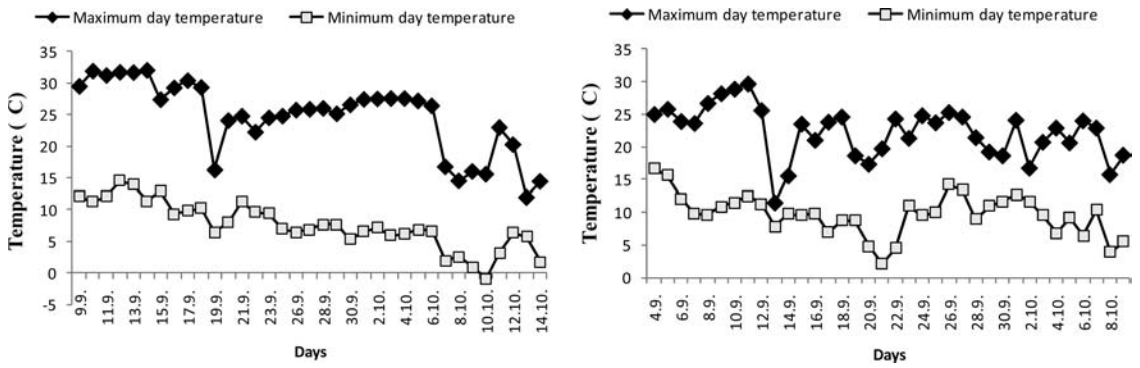

Figure 5. Maximum day and minimum night temperatures recorded during the advanced ripening of "Braeburn" apples in the orchard of the Biotehnical faculty in Ljubljana in 2011 (left) and growing center Maribor Gacnik in 2012 (right).

slight decrease was observed in our study, which is consistent with the findings of Macheix et al.,[40] but not in the accordance with the result of Lister et al.,[19] who found an increase of procyanidins during the advanced ripening of "Splendour" apples. Oligomeric procyanidins have attracted increasing attention in the fields of nutrition and medicine due to their potential health benefits observed in vitro and vivo.[41]

The concentration of anthocyanins and flavonols significantly increased during the advanced ripening of "Braeburn" apples. In both years the onset of rapid flavonol and anthocyanin formation occurred somewhere between 3 to 2 weeks before the technological maturity (Figure 3C,E). Similar to our findings Lister et al.[19] also reported that flavonol synthesis coincided with an increased accumulation of anthocyanins. Similar observations regarding the onset of anthocyanins were also reported by Bizjak et al.[28] and Awad and de Jager,[14] who also detected rapid anthocyanin accumulation in "Jonagold" apples and "Braeburn" apples around 20 days before the harvest. The accumulation of anthocyanins is rather dependent on the temperature.[42] Approximately three weeks before the harvest the night temperatures dropped, whereas the day temperatures still remained quite high (Figure 5). And just the drop in the night temperatures may have stimulated anthocyanins biosynthesis. Cold night temperatures followed by warm day temperatures have already been reported to stimulate anthocyanin synthesis in some apple cultivars.[43] Awad and de Jager[14] and Whale and Singh[44] suggested endogenous ethylene being the trigger for anthocyanin biosynthesis. Similar to our findings Lister et al.[19] also reported that the increased flavonol synthesis coincided with an increased accumulation of anthocyanins. Regarding the co-ordination of synthesis it can be assumed that during the advanced ripening the pool of hydroxycinnamic acids and dihydrochalcones decreased in favor with an increased synthesis of flavonols. A decrease in the pool of hydroxycinnamic acids in favor of flavonols has already been suggested by Mayr et al.[45] Since leucocyanidin (flavan-3,4-diol) is a common precursor for anthocyanidins and flavanols,[3] the slight decrease of flavanols found in our study could be on account of the increased accumulation of anthocyanins. Finally, a decrease of the astringent procyanidins coincided with an increase of flavonols and anthocyanins indicate people and fruit eating animals that an apple is suitable for use which makes an important contribution to the seed dispersal and spread of apple trees.

Within flavonoids, anthocyanins are the most important group for the production of the red color of the apple peel, which is of great commercial importance determining market acceptance of apples.[46] Although most of the phenolics are reported to have an antioxidant activity, quercetin, a major representative of the flavonol subclass, has been reported to have structural advantages as antioxidant[47] and contributes the largest share to the total antioxidant capacity of apples.[5] Recently, additional considerable attention has been paid to this phenol since quercetin and its sugar-bound, or glycosilated forms represent 60−75% of flavonoid intake.[48] In the present study we have demonstrated that in the "Braeburn" apple peel anthocyanins as well as quercetin glycosides are mainly accumulated during the last few weeks of advanced ripening until the onset of the technological maturity of apples. Therefore, viewed from marketing as well as from the health points of view it is very important to pick the apples at their proper technological maturity. Since accumulation of quercetin glycosides and anthocyanin are strongly light/tree position dependent,[12] it is additionally very important that trees are properly pruned and illuminated. Use of reflective foil represents one of the efficient agro-technical measures that can effectively increase light utilization in the apple orchard.[49] On the basis of our results, we suggest that summer pruning, orchard floor covering, applications of certain elements or plant regulators and similar measures that have been designed to improve the color and quality of the fruit should be performed approximately 3−4 weeks before the expected technological maturity of "Braeburn" apples, depending on the speed of an effect of each measure.

With respect to the total phenolic compounds (TPC) no significant changes during the advanced ripening were noticed in our experiment in both years (Figure 2F). The polyphenol content in the apples is strongly dependent upon their growth conditions; thus, it is not surprising that different results have been reported concerned with this issue. Similar to us, Zheng et al.[50] found out that TPC in the apples did not change significantly and remained nearly constant during the last weeks of apple ripening. On the other hand Blanco et al.[51] and Campo et al.[52] reported different behaviors, depending on the apple variety considered. However, when comparing and evaluating these results it must be taken into account that in the study of Blanco et al.[51] and Campo et al.[52] experiments have been done on the cider apple varieties which generally contain much higher levels of phenolics than table apples.[23]

In conclusion during the 5 weeks of the advanced ripening and technological maturity as well the monomeric and polymeric flavanols were the most abundant phenolic group in the "Braeburn" apple peel, followed by flavonols, dihydrochalcones, hydroxycinnamic acids, and anthocyanins,

the concentration of which in the last 2 weeks prior to technological maturity exceeded the concentration of dihydrochalcones and hydroxycinnamic acids. We demonstrate that during advanced ripening the concentrations of hydroxycinnamic acids, dihydrochalcones, and flavanols remain quite constant or slightly decrease. On the contrary an intensive accumulation of quercetin glycosides and anthocyanins takes place during this period, starting with the onset of rapid formation approximately 3 weeks before the technological maturity of apples. On the basis of our results, on summer pruning of "Braeburn" apples, the orchard floor covering and foliar applications of certain elements and similar measures designed to improve the color and quality of the fruit should be performed approximately 3−4 weeks before the expected technological maturity of apples. However, similar studies over a number of years should be performed on the basis of which it could be more accurately determined when during the advanced maturation of apples would be the best time that these technological measures should be carried out.

AUTHOR INFORMATION

Corresponding Author

*E-mail: jan.bizjak@bf.uni-lj.si. Tel.: +386 1320 3150. Fax: +386 14231088.

Funding

This work is part of program Horticulture No. P4-0013-0481, funded by the Slovenian Research Agency (ARRS).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Füleki, T.; Pelayo, E.; Palabay, R. B. Sugar composition of varietal juices produced from fresh and stored apples. *J. Agric. Food Chem.* **1994**, *42*, 1266−1275.

(2) Del Campo, G.; Santos, J. I.; Berregi, I.; Munduate, A. Differentiation of Basque cider apple juices from different cultivars by means of chemometric techniques. *Food Control* **2005**, *16*, 551−557.

(3) Treutter, D. Biosynthesis of phenolic compounds and its regulation in apple. *Plant Growth Regulat.* **2001**, *34*, 71−89.

(4) Boyer, J.; Liu, R. H. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, *3* (5), 1−15.

(5) Lee, K. W.; Kim, Y. J.; Kim, D.-O.; Lee, H. J.; Lee, C. Y. Major phenolics in apple and their contribution to the total antioxidant capacity. *J. Agric. Food Chem.* **2003**, *51*, 6516−6520.

(6) Hyson, D. A. A comprehensive review of apples and apple components and their relationship to human health. *Adv. Nutr.* **2011**, *2*, 408−420.

(7) Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **2002**, *50*, 7449−7454.

(8) Tsao, R.; Yang, R.; Xie, S.; Sockovie, E.; Khanizadeh, S. Which polyphenolic compounds contribute to the total antioxidant activities of apple? *J. Agric. Food Chem.* **2005**, *53*, 4989−95.

(9) Mikulic-Petkovsek, M.; Stampar, F.; Veberic, R. Parameters of inner quality of the apple scab resistant and susceptible apple cultivars (*Malus domestica* Borkh.). *Sci. Hort.* **2007**, *114*, 37−44.

(10) Lata, B.; Trampczynska, A.; Paczesna, J. Cultivar variation in apple peel and whole fruit phenolic composition. *Sci. Hort.* **2009**, *121*, 176−181.

(11) Wolfe, K.; Wu, X.; Liu, R. H. Antioxidant activity of apple peels. *J. Agric. Food Chem.* **2003**, *51*, 609−614.

(12) Awad, M. A.; de Jager, A.; van der Plas, L. H. W.; van der Krol, A. R. Flavonoid and chlorogenic acid changes in the peel of Elstar and Jonagold apples during development and ripening. *Sci. Hort.* **2001**, *90*, 69−83.

(13) Łata, B.; Przeradzka, B.; Bińkowska, M. Great differences in antioxidant properties exist between 56 apple cultivars and vegetation seasons. *J. Agric. Food Chem.* **2005**, *53*, 8970−8978.

(14) Awad, M. A.; de Jager, A. Relationships between fruit nutrients and concentrations of flavonoids and chlorogenic acid in Elstar apple skin. *Sci. Hort.* **2002**, *92*, 265−276.

(15) Mayr, U.; Batzdorfer, R.; Treutter, D.; Feucht, W. Surfactant-induced changes in phenol content of apple leaves and fruit skins. *Acta. Hort.* **1994**, *381*, 479−487.

(16) Lancaster, J. E. Regulation of skin color in apples. *Crit. Rev. Plant Sci.* **1992**, *10*, 487−502.

(17) Mikulic-Petkovsek, M.; Slatnar, A.; Stampar, F.; Veberic, R. The influence of organic/integrated production on the content of phenolic compounds in apple leaves and fruits in four different varieties over a 2-year period. *J. Sci. Food. Agric.* **2010**, *90*, 2366−2378.

(18) Awad, M. A.; Wagenmakers, P. S.; de Jager, A. Effects of light on flavonoid and chlorogenic acid levels in the skin of Jonagold apples. *Sci. Hort.* **2001**, *88*, 289−298.

(19) Lister, C. E.; Lancaster, J. E.; Sutton, K. H.; Walker, J. R. L. Developmental changes in the concentration and composition of flavonoids in skin of a red and a green apple cultivar. *J. Sci. Food Agric.* **1994**, *64*, 155−161.

(20) Kondo, S.; Hiraoka, K.; Kobayashi, S.; Honda, C.; Terahara, N. Changes in the expression of anthocyanin biosynthetic genes during apple development. *J Am. Soc. Hort. Sci.* **2002**, *127*, 971−976.

(21) Zhang, Y.; Li, P.; Cheng, L. Developmental changes of carbohydrates, organic acids, amino acids, and phenolic compounds in ‘Honeycrisp’ apple flesh. *Food Chem.* **2010**, *123*, 1013−1018.

(22) Renard, C. M. G. C.; Dupont, N.; Guilermin, P. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. *Phytochemistry* **2007**, *68*, 1128−1138.

(23) Marks, S. C.; Mullen, W.; Crozier, A. Flavonoid and chlorogenic acid profiles of English cider apples. *J. Sci. Food Agric.* **2007**, *87*, 719−728.

(24) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phospholungistic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.

(25) Ackermann, J.; Fischer, M.; Amado, R. Changes in sugars, acids, and amino acids during ripening and storage of apples (Cv. Glockenapfel). *J. Agric. Food Chem.* **1992**, *40*, 1131−1134.

(26) Hecke, K.; Herbinger, K.; Veberic, R.; Trobec, M.; Toplak, H.; Štampar, F.; Keppel, H.; Grill, D. Sugar-, acid- and phenol contents in apple cultivars from organic and integrated fruit cultivation. *Eur. J. Clin. Nutr.* **2006**, *60*, 1136−1140.

(27) Veberic, R.; Zadravec, P.; Stampar, F. Fruit quality of ‘Fuji’ apple (*Malus domestica* Borkh.) strains. *J. Sci. Food. Agric.* **2007**, *87*, 593−599.

(28) Bizjak, J.; Weber, N.; Mikulic-Petkovsek, M.; Slatnar, A.; Stampar, F.; Zobayer, A.; Stich, K.; Heidi, H.; Veberic, R. Influence of phostrade Ca on color development and anthocyanin content of ‘Braeburn’ apple (*Malus domestica* Borkh.). *Hortscience* **2013**, *48*, 193−199.

(29) Tsao, R.; Yang, R.; Young, J. C.; Zhu, H. Polyphenolic profiles in eight apple cultivars using high-performance liquid chromatography (HPLC). *J. Agric. Food Chem.* **2003**, *51*, 6347−6353.

(30) Slatnar, A.; Stampar, F.; Veberic, R. Influence of bicarbonate salts, used against apple scab, on selected primary and secondary metabolites in apple fruit and leaves. *Sci. Hort.* **2012**, *143*, 197−204.

(31) Ben Yehuda, G.; Korchinsky, R.; Redel, G.; Ovadya, R.; Oren-Shamir, M.; Cohen, Y. Colour accumulation patterns and the anthocyanin biosynthetic pathway in´Red Delicious´ apple variants. *J. Hort. Sci. Biotechnol.* **2005**, *80*, 187−192.

(32) Peng, Y.; Lu, R. Prediction of apple fruit firmness and soluble solids content using characteristics of multispectral scattering images. *J. Food Eng.* **2007**, *82*, 142−152.

(33) Rato, A. E.; Nunes, C.; Agulheiro-Santos, A. C.; Barroso, J. M.; Riquelme, F.; Coimbra, M. A. Influence of cell wall calcium content in

fruit firmness during the ripening of plums (*Prunus domestica* L.). *Acta Hort.* **2012**, *934*, 1047−1054.
(34) Bowen, J. H.; Watkins, C. B. Fruit maturity, carbohydrate and mineral content relationships with watercore in 'Fuji' apples. *Postharvest Biol. Technol.* **1997**, *11*, 31−38.
(35) Combe, B. G. The development of fleshy fruits. *Ann. Rev. Plant Physiol.* **1976**, *27*, 207−228.
(36) Teo, G.; Suzuki, Y.; Uratsu, S. L.; Lampinen, B.; Ormonde, N.; Hu, W. K.; DeJong, T. M.; Dandekar, A. M. Silencing leaf sorbitol synthesis alters long- distance partitioning and apple fruit quality. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, 18842−18847.
(37) Berüter, J. Carbohydrate metabolism in two apple genotypes that differ in malate accumulation. *J. Plant Physiol.* **2004**, *161*, 1011−1029.
(38) Sturm, K.; Stampar, F. Seasonal variation of sugars and organic acids in apple (*Malus domestica* Borkh.) in different growing systems. *Phyton-Ann. Bot.* **1997**, *39*, 91−96.
(39) Róth, E.; Berna, A.; Beullens, K.; Yarramraju, S.; Lammertyn, J.; Schenk, A.; Nicolaï, B. Postharvest quality of integrated and organically produced apple fruit. *Postharvest Biol. Technol.* **2007**, *45*, 11−19.
(40) Macheix, J. J.; Fleuriet, A.; Billot, J. Changes and metabolism of phenolic compounds in fruits. *In Fruit Phenolics*; CRC PressL Boca Raton, FL, 1990; Chapter 3, p 378.
(41) Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **2000**, *130*, 2086S−2092S.
(42) Arakawa, O. Effect of temperature on anthocyanin accumulation in apple fruit as affected by cultivar, stage of fruit ripening and bagging. *J. Hort. Sci.* **1991**, *66*, 763−768.
(43) Reay, P. F. The role of low temperatures in the development of the red blush on apple fruit ('Granny Smith'). *Sci. Hort.* **1999**, *79*, 113−119.
(44) Whale, S. K; Singh, Z. Endogenous ethylene andcolor development in the skin of 'Pink Lady' apple. *J. Amer. Soc. Hort. Sci.* **2007**, *132*, 20−28.
(45) Mayr, U.; Treutter, D.; Santos-Buelga, C.; Bauer, H.; Feucht, W. Developmental changes in the phenol concentrations of Golden Deliciouś apple fruits and leaves. *Phytochemistry* **1995**, *38*, 1151−1155.
(46) Telias, A.; Bradeen, J. M.; Luby, J. J.; Hoover, E. E. Regulation of anthocyanin accumulation in apple peel. *Hort. Rev.* **2011**, *38*, 357−391.
(47) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.
(48) Bouktaib, M.; Atmani, A.; Rolando, C. Regio- and stereoselective synthesis of the major metabolite of quercetin, quercetin-3-O-b-D-glucuronide. *Tetrahedron Lett.* **2002**, *43*, 6263−6266.
(49) Blanke, M. M. Alternatives to reflectivemulch cloth (ExtendayTM) for apple under hail net? *Sci. Hort.* **2008**, *116*, 223−226.
(50) Zheng, H.-Z.; Kim, Y.-ll.; Chung, S.-K. A profile of physicochemical and antioxidant changes during fruit growth for the utilisation of unripe apples. *Food Chem.* **2012**, *131*, 106−110.
(51) Blanco, D.; Morán, M. J.; Gutiérrez, M. D.; Moreno, J.; Dapena, E.; Mangas, J. Biochemical study of the ripening of cider apple varieties. *Z. Lebensm. Unters. Forsch.* **1992**, *194*, 33−37.
(52) Del Campo, G.; Berregi, I.; Iturriza, N.; Santos, J. I. Ripening and changes in chemical composition of seven cider apple varieties. *Food Sci. Tech. Int.* **2006**, *12*, 477−487.