JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Comparison of Phenolic Composition of Healthy Apple Tissues and Tissues Affected by Bitter Pit

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ABSTRACT: Bitter pit is an important Ca^{2+} deficiency disorder of apple fruit (*Malus domestica* Borkh.), with symptoms, necrotic spots, developing during storage. The objective of this study was to determine phenolic compounds and their contents in bitter pit in comparison to healthy skin and pulp using HPLC-MS^2. The experiment was carried out on three cultivars 'Jonagored', 'Golden Delicious' and 'Pinova'. All 15 determined phenolic compounds in pulp tissues specifically affected by bitter pit were higher than those in healthy pulp. Chlorogenic acid and catechin were to 5 times higher in those affected pulp tissues. Higher content was also determined for hydroxycinnamic acids and flavanols in the peel above the bitter pit; in contrast, flavonols and anthocyanins were higher in healthy peel. Anthocyanins in healthy peel of cultivar 'Jonagored' were 10 times higher from the content in peel above the bitter pit.

KEYWORDS: *Malus domestica, hydroxycinnamic acid, flavonols, flavanols, dihydrochalcones, anthocyanins, bitter pit, HPLC-MS*

INTRODUCTION

Bitter pit (BP) is a physiological disorder of apple (*Malus domestica* Borkh.) that has been under scientific interest for more than hundred years,[1] but the mechanisms involved in bitter pit development are still not well understood. Bitter pit is a Ca^{2+} deficiency disorder of apple fruit, which does not depend only on Ca^{2+} content, but also on its proper homeostasis at the cellular level.[2]

Bitter pit is initiated in preharvest period, but the symptoms normally develop during storage. First symptoms are water-soaked spots caused by plasma membrane breakdown, later they turn brown and in time become desiccated. Pits are mostly located in the outer cortex of the fruit and under the peel. Pitting can cause small depressions in the peel and may occur deep into the flesh. Frequency of pitting is often greater toward the calyx end of the fruit.[1−3] Bitter pit is one of the most important physiological disorders, because affected apples are declined or achieve lower prices in the market. Bitter pit occurrence can be reduced by Ca sprays, but often not entirely.[4,5] The losses in storage due to bitter pit can be really high, Hewett and Watkins[4] report up to almost 50% losses in apples with no calcium treatments against bitter pit, up to 10% losses with calcium treatment, and to 6% losses with combination of Ca sprays and vacuum-infiltration of Ca. Hopfinger and Poovaiah[6] report total prevention of bitter pit symptoms with the use of calcium chloride (2%) vacuum infiltration.

Phenolic compounds in apple are of a great importance for the tree, because they are involved in natural defensive reactions of apples against various diseases and they act as stress protective agents.[7,8] The defense related compounds are synthesized by plants in response to physical injury, infection, or stress.[8] Higher phenolic content was determined in apples infected with *Venturia inaequalis*,[9,10] cinnamic acid derivates were increased after wounding of apples[11] and chlorogenic acid, procyanidin B2 and total phenolic content increased in mechanically injured leaves.[12] Greater flavonoid synthesis, thus increased resistance, was also reported with the use of growth regulators.[13]

There are many researches about bitter pit and its connection to Ca^{2+} deficiency, but none about bitter pit in connection with phenolics. Phenolic content is a great indicator of changes in the plant or fruit that occur due to stress, pests or diseases. Knowing the phenolic content and suspected differences would improve our knowledge about bitter pit and its development. In our study, we have focused on phenolic content in bitter pit spots of tree apple cultivars 'Jonagored', 'Golden Delicious', and 'Pinova'. We analyzed healthy and bitter pit affected peel and pulp. According to the literature, we hypothesized that the peel and pulp of bitter pit contains higher content of phenolic compounds. These results will be potentially used for further research on predicting bitter pit development even before symptoms occur.

MATERIALS AND METHODS

Plant Material. The apples were acquired from orchard located in northwest part of Slovenia (lat. 46°20 N, long. 14°12 E). Orchard is at an altitude of 500 m and is influenced by mountain climate. The trees are planted on M9 rootstock and are under integrated pest management. Apples from three cultivars were harvested in technological maturity on 24th of September 2012. The harvest date was determined using starch iodine and firmness test. Apples were placed in cool storage immediately after the harvest. The storage temperatures were adjusted according to cultivar, 'Golden Delicious' was stored at 0.5 °C, 'Jonagored' and 'Pinova' at 1.5 − 2 °C. The apples were taken out from the cool storage at the beginning of January.

Received: September 12, 2013
Revised: November 19, 2013
Accepted: November 20, 2013
Published: November 20, 2013

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

peak no.	t_R[a] (min)	λ (nm)	$[M-H]^-$ (m/z)[b]	MS^2 (m/z)	tentative identification	detected in
1	11.31	518, 280, 234	449	287	cyanidin 3-*O*-galactoside	peel
2	12.76	521, 279, 234	419	287	cyanidin 3-*O*-arabinoside	peel
3	14.60	520, 283, 234	419	287	cyanidin 7-*O*-arabinoside	peel
4	12.60	278, 234	577	425, 407, 289	procyanidin B1	peel, pulp
5	13.73	316, 287, 234	325	163, 119	*p*-coumaric acid glycoside	peel
6	14.70	279, 234	289	245	catechin	peel, pulp
7	14.85	328, 234	353	191, 179	chlorogenic acid	peel, pulp
8	16.13	278, 234	577	425, 407, 289	procyanidin dimer 1	peel
9	15.91	310, 234	517	385, 205, 223	sinapic acid glycoside	peel
10	16.13	278, 234	577	425, 407, 289	Procyanidin B2	peel, pulp
11	16.87	328, 234	353	191, 179	cryptochlorogenic acid	peel
12	17.36	279, 234	289	245	epicatechin	peel, pulp
13	17.99	278, 234	865	577, 451, 425, 407, 289	procyanidin trimer 1	peel, pulp
14	18.56	278, 234	865	577, 451, 425, 407, 289	procyanidin trimer 2	peel, pulp
15	18.56	312, 234	337	173	4-*O*-*p*-coumaroylquinic acid	peel, pulp
16	22.24	354, 234	609	301	quercetin-3-*O*-rutinoside	peel
17	22.55	278, 234	577	425, 407, 289	procyanidin dimer 2	peel
18	22.97	356, 256, 234	463	301	quercetin 3-*O*-galactoside	peel, pulp
19	23.16	356, 254, 234	463	301	quercetin 3-*O*-glucoside	peel, pulp
20	23.97	356, 255, 234	433	301	quercetin 3-*O*-xyloside	peel, pulp
21	24.11	285, 235	567	273	phloretin-2′-*O*-xylosylglucoside	peel, pulp
22	24.30	355, 255, 234	433	301	quercetin 3-*O*-arabinopyranoside	peel
23	24.68	355, 255, 234	433	301	quercetin 3-*O*-arabinofuranoside	peel, pulp
24	24.89	353, 268, 255	447	301	quercetin 3-*O*-rhamnoside	peel, pulp
25	25.80	285, 235	435	273	phloretin-2-*O*-glucoside (phloridzin)	peel, pulp

[a] t_R, retention time. [b] $[M+H]^+$ (m/z) anthocyanins were obtained in the positive ion mode, other phenolics in the negative ion mode.

Chemicals. For the quantification of phenolic compounds the following standards were used: quercetin 3-*O*-galactoside, quercetin 3-*O*-glucoside, quercetin 3-*O*-rhamnoside, (−)-epicatechin, *p*-coumaric acid, procyanidin B1 and B2, caffeic acid, cyanidin 3-*O*-galactoside, and phloridzin dihydrate from Fluka Chemie (Buch, Switzerland), chlorogenic acid and quercetin-3-*O*-rutinoside (rutin) from Sigma Aldrich Chemie, quercetin 3-*O*-arabinofuranoside and quercetin 3-*O*-xyloside from Apin Chemicals (Abingdon, UK), and (+)-catechin from Roth (Karlsruhe, Germany). Methanol and BHT for the extraction of the phenolics was acquired from Sigma-Aldrich. The chemicals for the mobile phases were HPLC-MS grade acetonitrile and formic acid from Fluka. Water for mobile phase was twice distilled and purified with a Mili-Q Millipore Direct 8 system (Merck Millipore, Billerica, MA). For the total phenolic content, Folin-Ciocalteu phenol reagent (Fluka), sodium carbonate (Merck, Darmstadt, Germany), gallic acid and methanol (Sigma-Aldrich) were used.

Extraction and Determination of Individual Phenolic Compounds. The extraction of fruit samples was done as described by Mikulic-Petkovsek et al.[14] with some modifications. Pits were cut out from fruits, all the brown tissue with thin layer of healthy pulp (about 1 mm), skins above pits were separated and both, peel and bitter pit, immediately immersed in liquid nitrogen. The healthy parts of apple, with no sign of physiological disorder, were peeled and both pulp and peel were also immersed in liquid nitrogen.

For the analysis 20 apples with bitter pit were chosen for each cultivar. From each apple, peel above the bitter pit and core of the pit were cut out, and also unaffected (healthy) peel and pulp. All sampling material was immediately flash-frozen in liquid nitrogen and stored at −80 °C until it was analyzed. The samples were divided into five repetitions and ground in separate mortars with the help of liquid nitrogen. For the peel 0.3 g of sample powder was extracted with 2.1 mL of methanol containing 3% (v/v) formic acid and 1% 2.6-ditert-butyl-4-methylphenol (BHT) (w/v) and for pulp and pit 0.8 g sample powder was extracted with 3.2 mL of methanol containing 3% (v/v) formic acid and 1% BHT (W/v) in a cooled ultrasonic bath for 1 h. The samples were centrifuged for 10 min at 10 000*g* at 4 °C. The supernatant was filtered through a Chromafil AO-20/25 polyamide filter (Macherey-Nagel, Düren, Germany) to a vial. The vials were placed in Thermo Finnigan Surveyor HPLC system (Thermo Scientific, San Jose, CA). The detection was made with diode array detector at 280, 350, and 530 nm. At 280 nm the hydroxycinnamic acids, dihydrochalcones and flavanols, at 350 nm flavonols and at 530 nm anthocyanins, were detected. For the separation of phenolic compounds Phenomenex (Torrance, CA) HPLC column C18 (150 × 4.6 mm, Gemini 3 μ) with attached Phenomenex security guard column was used. The column temperature was 25 °C. The injection volume for extracted samples was 10 μL, and the flow rate maintained at 0.6 mL min^{-1}. The elution solvents were aqueous 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). Samples were eluted according to the linear gradient from 5% to 20% B in the first 15 min, followed by a linear gradient from 20 to 30% B for 5 min, then an isocratic mixture for 5 min, followed by a linear gradient from 30 to 90% B for 5 min, and then an isocratic mixture for 15 min before returning to the initial conditions.[15]

All phenolic compounds presented in our results were identified by an HPLC-Finnigan MS detector and an LCQ Deca XP MAX (Thermo Finigan, San Jose, CA) instrument with electrospray interface (ESI) operating in positive (for anthocyanins) and negative (other phenolic groups) ion mode. The analyses were carried out using full scan data-dependent MS^2 scanning from m/z 110 to 1300. Column and chromatographic conditions were identical to those used for the HPLC-DAD analyses. The injection volume was 10 μL and the flow rate maintained at 0.6 mL min^{-1}. The capillary temperature was 250 °C, the sheath gas and auxiliary gas were 60 and 15 units, respectively; the source voltage was 3 kV for negative ionization and 4 kV for positive ionization and normalized collision energy was between 20 and 35%. Spectral data were elaborated using the Excalibur software (Thermo Scientific).

The identification of compounds was confirmed by comparing retention times and their spectra as well as by adding the standard solution to the sample and by fragmentation. Quantification was achieved according to the concentrations of corresponding external standard. For the compounds for which the standards were not available, related compounds were used as standards. Therefore 4-*O*-*p*-

Table 2. Content of Individual Phenolic Compounds (mean ± SE in mg kg^{-1} FW) and Total Phenolic Content (mean ± SE in mg GAE kg^{-1} FW) in Healthy Apple Pulp and Pulp Affected by Bitter Pit for Three Cultivars

	'Jonagored'		'Pinova'		'Golden Delicious'	
	healthy	bitter pit	healthy	bitter pit	healthy	bitter pit
chlorogenic acid	71.99 ± 3.73 a[a]	300.06 ± 11.01 b	92.40 ± 9.04 a	413.90 ± 3.23 b	52.76 ± 8.07 a	274.37 ± 7.7 b
4-*O*-*p*-coumaroylquinic acid	1.25 ± 0.09 a	1.78 ± 0.07 b	0.10 ± 0.01 a	0.24 ± 0.00 b	1.42 ± 0.16 a	3.29 ± 0.09 b
total hydroxycinnamic acids	**73.24 ± 3.79 a**	**301.84 ± 11.06 b**	**92.51 ± 9.1 a**	**414.14 ± 3.24 b**	**54.18 ± 8.22 a**	**277.65 ± 7.77 b**
catechin	9.99 ± 0.52 a	41.63 ± 1.53 b	23.95 ± 2.34 a	107.27 ± 0.84 b	8.98 ± 1.37 a	46.72 ± 1.31 b
procyanidin B2	40.80 ± 2.92 a	107.44 ± 5.20 b	62.74 ± 8.96 a	192.64 ± 3.26 b	7.47 ± 2.00 a	25.08 ± 1.42 b
epicatechin	28.16 ± 2.53 a	40.85 ± 2.22 b	50.44 ± 4.48 a	87.16 ± 1.42 b	23.74 ± 4.37 a	56.76 ± 2.91 b
procyanidin trimer 1	50.25 ± 3.53 a	71.65 ± 2.71 b	28.24 ± 3.66 a	65.89 ± 1.19 b	48.89 ± 5.32 a	113.17 ± 3.22 b
procyanidin trimer 2	4.65 ± 0.53 a	7.70 ± 0.44 b	ND	ND	ND	ND
procyanidin B1	12.34 ± 0.94 a	32.98 ± 1.14 b	15.37 ± 1.66 a	38.36 ± 0.67 b	11.63 ± 1.61 a	37.76 ± 0.79 b
total flavanols	**146.19 ± 10.72 a**	**301.25 ± 12.65 b**	**180.74 ± 21.02 a**	**491.33 ± 6.48 b**	**100.70 ± 14.51 a**	**279.49 ± 8.54 b**
phloretin-2-*O*-(2″-*O*-xylosilglucoside)	7.93 ± 0.94 a	13.05 ± 0.47 b	3.84 ± 0.28 a	13.51 ± 0.36 b	3.64 ± 0.62 a	14.05 ± 0.48 b
phloridzin	5.92 ± 1.69 a	11.27 ± 0.76 b	3.36 ± 0.40 a	6.81 ± 0.28 b	5.37 ± 1.06 a	36.12 ± 1.33 b
total dihydrochalcones	**13.85 ± 2.63 a**	**24.32 ± 0.87 b**	**7.19 ± 0.67 a**	**20.32 ± 0.52 b**	**9.00 ± 1.58 a**	**50.17 ± 1.5 b**
quercetin 3-galactoside	0.37 ± 0.02 a	0.72 ± 0.03 b	0.38 ± 0.07 a	2.58 ± 0.14 b	0.21 ± 0.03 a	1.38 ± 0.07 b
quercetin 3-glucoside	0.26 ± 0.03 a	0.55 ± 0.02 b	0.49 ± 0.06 a	5.70 ± 0.23 b	0.23 ± 0.02 a	1.41 ± 0.03 b
quercetin 3-xyloside	0.08 ± 0.01 a	0.17 ± 0.01 b	0.10 ± 0.01 a	0.57 ± 0.02 b	0.08 ± 0.01 a	0.35 ± 0.01 b
quercetin 3-arabinofuranoside	0.70 ± 0.13 a	1.36 ± 0.11 b	0.69 ± 0.06 a	4.05 ± 0.17 b	0.67 ± 0.08 a	2.38 ± 0.07 b
quercetin 3-rhamnoside	2.43 ± 0.33 a	4.00 ± 0.23 b	1.96 ± 0.16 a	6.88 ± 0.09 b	2.07 ± 0.18 a	5.49 ± 0.07 b
total flavonols	**3.84 ± 0.51 a**	**6.80 ± 0.39 b**	**3.62 ± 0.33 a**	**19.78 ± 0.62 b**	**3.25 ± 0.31 a**	**11.00 ± 0.22 b**
total phenolic content (TPC)	**364.62 ± 16.71 a**	**747.47 ± 28.79 b**	**452.88 ± 30.01 a**	**1080.35 ± 14.93 b**	**302.68 ± 36.26 a**	**840.67 ± 13.40 b**

[a]Different letters (a, b) among healthy apple pulp and pulp affected by bitter pit denote significant differences (LSD test, $p < 0.05$) within cultivar.

coumaroylqunic acid was quantified in equivalent of *p*-coumaric acid, sinapic acid glycoside in equivalent of caffeic acid, cryptochlorogenic acid in equivalent of chlorogenic acid, phloretin-2-*O*-xylosylglucoside in equivalent of phloridzin, procyanidin trimer 1 and 2 in equivalent of procyanidin B1, quercetin 3-*O*-arabinopyranoside in equivalent of quercetin-3-*O*-arabinofuranoside and anthocyanins cyanidin 3-*O*-arabinoside and cyanidin 7-*O*-arabinoside in equivalent of cyanidin 3-*O*-galactoside. Concentrations of the phenolic compounds were expressed in mg kg^{-1} of fresh weight (FW).

Extraction and Determination of Total Phenolic Content (TPC). The extraction of samples for the determination of total phenolics was made according to the same protocol as for phenolics, with the difference that no BHT was added. Total phenolic content of extracts was assessed using the Folin-Ciocalteu reagent method.[16] Into 6 mL of twice distilled water, 100 μL of extract and 500 μL of Folin-Ciocalteu reagent were added. After a period between 8 s and 8 min at room temperature, 1.5 mL of sodium carbonate (20% w/v) and 1.9 mL twice distilled water were added. The extracts were mixed and allowed to stand for 30 min at 40 °C. Afterward 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) of apple peel and pulp. Absorbance was measured in three replications.

Statistics. The data was analyzed with the help of Statgraphics Plus 4.0 program (Manugistics. Inc.; Rockville, MD). One-way analysis of variance (ANOVA) and least significant difference test (LSD) were used to determine the differences among healthy skin and skin above the bitter pit and between healthy pulp and pit for each cultivar separately.

RESULTS AND DISCUSSION

Fifteen and 25 phenolic compounds were detected in pulp and peel tissues, respectively. These compounds were identified using HPLC-MS, by comparison of their retention times and PDA spectra with standards and mass spectral data. The identification was also carried out on the basis of molecular ion identification, relative position in the chromatogram, and UV and MS spectra for those flavonols for which commercial standards were not available. LC-MS with subsequent fragmentation of the dominant ions in MS^2 and further fragmentation were used to obtain additional information on the molecular masses of conjugates, masses of the sugars bonded to the aglycones, and the structures of aglycones (Table 1). Identified phenolic compounds in apple are in accordance with previous researches.[17,18]

Phenolic Compounds in Apple Pulp and Peel. In apple pulp phenolic compounds belonging to four groups, were identified: hydroxycinnamic acids, flavonols, flavanols, and dihydrochalcones (Table 2). Phenolics of the same groups were also identified in the peel, with addition of anthocyanins (Table 3).

In the pulp chlorogenic acid and 4-*O*-*p*-coumaroylqunic acid were identified from the group of hydroxycinnamic acids (Table 2). Both were significantly higher in the pulp affected by bitter pit in all three cultivars. Particularly large differences were in chlorogenic acid content, up to 5 times higher content was determined in bitter pit tissue, and consequently also the content of total hydroxycinnamic acids was higher. In the peel three additional hydroxycinnamic acids were determined: *p*-coumaric acid glycoside, sinapic acid glycoside and cryptochlorogenic acid (Table 3). There were also differences in total hydroxycinnamic acids, due to higher levels of chlorogenic acid and *p*-coumaric acid glycoside content in peel above the bitter pit. The accumulation of hydroxycinnamic acids shows defense reactions of the plant. This is consistent with the literature reviewed about plant response to stress, injury or pathogen

Table 3. Content of Individual Phenolic Compounds (mean ± SE in mg kg^{-1} FW) and Total Phenolic Content (mean ± SE in mg GAE kg^{-1} FW) in Healthy Apple Skin and Skin Affected by Bitter Pit for Three Cultivars

	'Jonagored'		'Pinova'		'Golden Delicious'	
	healthy	bitter pit	healthy	bitter pit	healthy	bitter pit
p-coumaric acid glycoside	4.6 ± 0.2 a[a]	7.8 ± 0.2 b	11.7 ± 0.5 a	23.8 ± 0.3 b	5.7 ± 0.3 a	12.9 ± 0.9 b
chlorogenic acid	39.9 ± 1.9 a	162.5 ± 7.1 b	276.8 ± 11.4 a	550.3 ± 11.3 b	107.8 ± 3.9 a	228.1 ± 6.4 b
Sinapic acid glycoside	15.6 ± 0.9 a	13.6 ± 0.7 a	27.6 ± 1.5 a	29.8 ± 1.5 a	38.6 ± 1.4 a	31.7 ± 3.8 a
cryptochlorogenic acid	37.1 ± 1.7 a	35.4 ± 2.3 a	70.3 ± 2.5 a	88.1 ± 4.1 b	25.7 ± 1.2 a	20.6 ± 2.1 a
4-*O*-*p*-coumaroylquinic acid	0.2 ± 0.0 a	0.2 ± 0.0 a	ND	ND	ND	ND
total hydroxycinnamic acids	**97.3 ± 4.7 a**	**219.5 ± 10.1 b**	**386.4 ± 15.4 a**	**692.0 ± 15.6 b**	**177.8 ± 6.5 a**	**291.5 ± 10.2 b**
catechin	125.9 ± 6.1 a	512.4 ± 22.5 b	211.2 ± 8.7 a	419.9 ± 8.6 b	28.9 ± 1.1 a	61.1 ± 1.7 b
procyanidin B2	37.3 ± 1.6 a	42.5 ± 2.2 a	437.4 ± 18.3 a	777.4 ± 34.9 b	56.1 ± 2.1 a	53.7 ± 7.3 a
epicatechin	208.8 ± 9.7 b	168.0 ± 11.8 a	356.8 ± 12.6 a	433.4 ± 19.0 b	190.9 ± 5.9 a	154.3 ± 33.0 a
procyanidin trimer 1	40.3 ± 2.6 a	60.7 ± 1.2 b	64.8 ± 2.7 a	102.6 ± 7.5 b	6.6 ± 0.4 a	14.5 ± 1.1 b
procyanidin trimer 2	155.5 ± 8.6 a	159.3 ± 7.8 a	316.5 ± 14.1 a	491.5 ± 18.3 b	183.3 ± 6.9 a	174.2 ± 28.3 a
procyanidin B1	57.4 ± 3.9 a	98.4 ± 4.0 b	ND	ND	51.9 ± 4.9 a	79.5 ± 8.8 b
procyanidin dimer 1	97.4 ± 5.1 a	93.9 ± 1.3 a	ND	ND	ND	ND
procyanidin dimer 2	45.5 ± 2.7 a	54.1 ± 1.8 b	ND	ND	ND	ND
total flavanols	**768.1 ± 39.3 a**	**1189.4 ± 50.4 b**	**1386.6 ± 55.4 a**	**2224.7 ± 85.4 b**	**517.7 ± 19.8 a**	**546.3 ± 86.1 a**
phloretin-2-*O*-(2″-*O*-xylosilglucoside)	63.4 ± 3.2 b	47.0 ± 2.7 a	36.1 ± 1.4 a	43.0 ± 1.5 b	49.1 ± 1.7 a	40.9 ± 3.5 a
phloridzin	126.5 ± 6.8 b	97.5 ± 5.8 a	126.5 ± 4.6 a	176.0 ± 8.6 b	125.6 ± 3.8 a	96.9 ± 12.8 a
total dihydrochalcons	**189.9 ± 9.9 b**	**144.5 ± 8.5 a**	**162.6 ± 6.0 a**	**219.0 ± 10.0 b**	**174.3 ± 5.4 a**	**137.8 ± 16.2 a**
quercetin 3-rutinoside	18.3 ± 1.2 b	6.2 ± 0.3 a	36.9 ± 1.8 b	12.0 ± 0.5 a	37.5 ± 1.8 b	21.0 ± 1.7 a
quercetin 3-galactoside	394.6 ± 22.7 b	92.4 ± 1.3 a	837.2 ± 23.4 b	450.1 ± 19.7 a	469.2 ± 15.3 a	400.3 ± 31.0 a
quercetin 3-glucoside	61.6 ± 3.4 b	27.9 ± 0.5 a	197.1 ± 7.6 a	183.9 ± 5.4 a	104.8 ± 4.2 a	98.3 ± 7.3 a
quercetin 3-xyloside	184.6 ± 10.5 b	81.7 ± 1.7 a	297.8 ± 11.4 b	253.4 ± 9.0 a	150.8 ± 4.5 a	178.7 ± 14.0 a
quercetin 3-arabinopyranoside	30.4 ± 1.9 b	12.4 ± 0.2 a	47.8 ± 1.8 b	33.2 ± 1.1 a	24.9 ± 0.9 a	24.9 ± 1.9 a
quercetin 3-arabinofuranoside	399.7 ± 22.1 b	163.9 ± 2.87 a	591.2 ± 20.9 b	479.7 ± 19.0 a	297.8 ± 9.2 a	347.3 ± 25.1 a
quercetin 3-rhamnoside	186.6 ± 10.6 b	78.6 ± 1.3 a	162.9 ± 6.2 b	140.4 ± 5.3 a	152.8 ± 4.6 a	190.0 ± 13.2 b
total flavonols	**1275.9 ± 72.2 b**	**463.1 ± 8.0 a**	**2170.8 ± 72.8 b**	**1552.7 ± 59.6 a**	**1237.7 ± 40.0 a**	**1260.5 ± 94.1 a**
cyanidin 3-galactoside (idaein)	631.9 ± 29.1 b	67.6 ± 4.8 a	434.4 ± 3.5 b	90.6 ± 3.2 a	ND	ND
cyanidin 3-arabinoside	60.2 ± 1.9 b	4.7 ± 0.4 a	15.8 ± 0.2 b	2.8 ± 0.2 a	ND	ND
cyanidin 7-arabinoside	47.3 ± 1.4 b	4.1 ± 0.2 a	5.9 ± 0.1 b	1.1 ± 0.1 a	ND	ND
total anthocyanins	**739.4 ± 31.7 b**	**76.4 ± 5.4 a**	**456.1 ± 3.8 b**	**94.5 ± 3.3 a**	**ND**	**ND**
total phenolic content (TPC)	**3531.0 ± 169.9 b**	**2661.2 ± 38.2 a**	**5403.3 ± 184.3 a**	**5755.3 ± 215.6 a**	**3116.8 ± 68.8 a**	**3120.1 ± 143.7 a**

[a]Different letters (a, b) among healthy apple skin and skin affected by bitter pit denote significant differences (LSD test, $p < 0.05$) within cultivar.

attack. Abdallah et al.[11] report increase of cinnamic acids after wounding of apples, similarly Slatnar et al.[12] report higher chlorogenic acid contents in wounded apple leaves, Mikulic-Petkovsek et al.[9] and Slatnar et al.[10] report higher phenolic content in apples infected with *Venturia inaequalis*, and Latreche and Rahmania[19] report higher *p*-hydroxycinnamic acids in date palm leaves with presenting sympthoms of Brittle leaf disease.

In the group of flavanols, six compounds in pulp: catechin, epicatechin, procyanidin B1, procyanidin B2, two procyanidin trimers were determined (Table 2) and additional two procyanidin dimers in apple skin (Table 3). All individual compounds, and consequently total flavanols, were significantly higher in pulp affected by bitter pit in all three cultivars. Catechin content increased the most, as it was 4−5 times higher in pulp affected by bitter pit. Total flavanols were more than 2 times higher in pulp affected by bitter pit. There were differences in the flavanol content in the peel as well, again the greatest in catechin content, cv. 'Jonagored' with 4 times higher content in peel above the bitter pit than in healthy peel, 512.4 mg kg^{-1} FW and 125.9 mg kg^{-1} FW, respectively. In the peel of cv. 'Pinova' there were differences in all flavanols determined; there was the greatest difference between healthy peel and peel above the bitter pit, in total flavanols among cultivars. Mayr et al.[20] report accumulation of flavanols and chlorogenic acid after several types of wounding of apple fruits and leaves. Bitter pit could induce flavanol and chlorogenic acid synthesis in a similar way, because once the plasma in cells disintegrates and cell dies as a result of Ca^{2+} imbalance,[2] plant accumulates phenols for defense against pathogen invasion.

Phloridzin and phloretin-2-*O*-(2″o-xylosylglucoside), dihydrochalcones, also statistically differed between treatments. Total dihydrochalcone content was from 2 to more than 5 times higher in pulp affected by bitter pit; in 'Golden Delicious' the content of dihydrochalcones rose from 9 to 50.2 mg kg^{-1} FW. The changes in the peel in dihydrochalcone content between healthy and peel above the bitter pit were different among cultivars. Cv. 'Jonagored' healthy peel had higher contents and 'Pinova' had lower contents of dihydrochalcones, as for 'Golden Delicious' there were no statistical differences. The contents of dihydrochalcones in healthy tissue are comparable with does cited by Treutter[21] and Mikulic-Petkovsek et al.[14] The increase of dihydrochalcones in pulp could be explained by bitter pit development, but for the results of dihydrochalcones in peel further research should be done, to make some conclusions.

It is known that flavonols are synthesized by plants in response to the pathogen attack,[22] while the mechanical injury

of leaves has no influence on the changes in quercetin content.[12] The following compounds from the group of flavonols were identified in apple pulp: quercetin 3-galactoside, quercetin 3-glucoside, quercetin 3-xyloside, quercetin 3-arabinofuranoside and quercetin 3-rhamnoside (Table 2). They were all in very low amounts. All individual quercetins were significantly higher in pulp affected by bitter pit. The content of individual quercetins was from 1.5 to more than 10 times higher in pulp affected by bitter pit than in healthy pulp. 'Pinova' had the largest increase in total flavonol content, more than 5 times higher contents in pulp affected by bitter pit, with the highest increase of quercetin 3-glucoside from 0.49 to 5.70 mg kg^{-1} FW. The content of flavonols in peel was markedly higher than in the pulp; however there was a reverse effect on flavonol content, since there were higher contents of flavonols in healthy peel than in peel above the bitter pit (Table 3). Slatnar et al.[23] also found higher values of flavonols in the healthy peel and lowest in the apple scab symptomatic spot. The greatest difference in our study was with cv. 'Jonagored', where healthy peel contained 1275.9 mg kg^{-1} FW total flavonols and peel above the bitter pit just 463 mg kg^{-1} FW. Cv. 'Pinova' had the highest content of total flavonols, 2170.8 mg kg^{-1} FW in healthy peel, but the differences between treatments were smaller. There were no differences in flavonol content in the peel of cv. 'Golden Delicious'. The accumulation of flavonols in the bitter pit, could again be attributed to protective accumulation of phenols against possible invasion of fungi trough the death cells. The content of flavonols in peel above the bitter pit could be lower, because the synthesis was directed more to flavanols.

Cyanidin 3-galactoside (idaein) is the leading individual anthocyanin in apple peel;[21,24] in addition we also determined cyanidin 3-arabinoside and cyanidin 7-arabinoside. There were no anthocyanins determined in apple pulp. We determined anthocyanins in the peel of cultivars 'Jonagored' and 'Pinova'. Anthocyanins were also higher in healthy peel (Table 3). Differences between treatments were high, with 'Jonagored' about 10 times higher contents of total anthocyanins in healthy peel (739.4 mg kg^{-1} FW). This difference in anthocyanin content could be a result of cell death and discoloration of the peel above the bitter pit, and thus reduced anthocyanin content above the bitter pit.

Total phenolic content (TPC) in pulp was, as all groups and individual phenols, statistically different, higher, in pulp affected by bitter pit. Phenolic content differs among cultivars,[25] cultivar 'Pinova' had the highest TPC in affected pulp tissue, 1080.4 mg GAE kg^{-1} FW (Table 2). In pulp, hydroxycinnamic acids and flavanols present about 90% of all phenolics, thus difference in TPC occurs mainly because of their increase. TPC of healthy pulp is comparable with results of other researches,[14,26] while TPC in the peel is slightly higher. There are statistical differences in TPC only with cv. 'Jonagored', where TPC is higher in healthy peel, due to higher anthocyanins, flavonols, and dihydrochalcones. There were no differences in the peel of other two cultivars, because of the equalization of content of phenolic groups, on the one hand higher hydroxycinnamic acids and flavanols in peel above the bitter pit, on the other hand higher flavonols and anthocyanins in healthy peel.

Our results showed higher levels of all 15 determined phenolic compounds in pulp tissue affected by bitter pit. Chlorogenic acid, catechin and procyanidins have contributed the most to phenolic content increase. The reason for this increase could be the combination of phenol accumulation for defense against pathogen invasion and release of phenols from vacuoles, due to cell disintegration and its death as a result of Ca^{2+} imbalance. The same pattern was detected also in the peel with hydroxycinnamic acids and flavanols, which are known groups of stress and defense induced phenols.[10,20,22] Flavonols and anthocyanins were higher in healthy peel, due to discoloration of peel above the bitter pit. The knowledge of phenolic compound content in the bitter pit could help us to better understand and maybe predict bitter pit occurrence according to phenolic changes.

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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.

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