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RELATIONSHIP BETWEEN APPLE BIOACTIVE COMPOUNDS AFTER HARVEST AND THEIR FATE IN COLD STORED FRUITS

Barbara Łata*, Aleksandra Trąmpczyńska**

*Warsaw University of Life Sciences

**Institute of Plant Physiology, University of Bayreuth (Germany)

Abstract. This study was to discover if there is any relationship between antioxidant status after harvest and bioactives fate during apple storage. The clearer link in this issue concerned enzymatic part of antioxidant apparatus, for which the particularly high year effect was noted. Except for anthocyanins, non-enzymatic bioactives end-status was not strictly related to their harvest size. However the content during the first months of storage might be closely connected with antioxidant status measured after harvest. A significantly higher concentration of majority assessed antioxidants was characterized by apple harvested and stored in 2005/2006 season, on the average. Simultaneously many, statistically proved, correlations over storage between the examined antioxidants at that time existed. Total antioxidant power (FRAP assay) significantly increased after the first storage period, probably as a result of fruit acclimatory response to storage conditions, and next decreased. In general, changes of FRAP value reflected fluctuations of individual compounds measured in this study.

Key words: *Malus domestica* Borkh, cold storage, antioxidants, FRAP assay

INTRODUCTION

Several studies proved that there is cause-effect relationship between internal (derived from genome differences) and external (particularly stress conditions existing before or after harvest) factors and chemical quality and quantity of fruit and vegetables [Lee and Kader 2000, Kalt et al. 2001, Łata 2002, Hodges et al. 2004]. The content of bioactives at harvest might be considered as important marker of fruit quality and a reflection of a tissue's ability to withstand biotic and abiotic stress conditions [Barden and Bramlage 1994, Ma and Cheng 2004, Davey et al. 2007]. Our last research made on a wide apple genetic resource demonstrated significant differences in antioxidant prop-

Corresponding author – Adres do korespondencji: Barbara Łata, Laboratory of Basic Sciences in Horticulture, Warsaw University of Life Sciences, ul. Nowoursynowska 159, 02-776 Warsaw, Poland, e-mail: barbara_lata@sggw.pl

erties of the tested genotypes and their high fluctuations in consecutive growing seasons [Łata et al. 2005a]. Apple peel was characterized by a higher, as compared to whole fruit, antioxidant concentration and skin was also in the highest degree influenced by growing season conditions [Davey et al. 2004, Łata 2007]. Simultaneously, the content of antioxidant in apple peel correlated well with whole fruit one that makes apple peel a good indicator of antioxidant metabolism in apple fruit. As relatively few apple cultivars are directly consumed after harvest (moreover, apples express high storability potential compared to other fruits), the question was about their internal quality measured as individual antioxidant content/activity as well as total antioxidant power after harvest and over cold storage. Since data presented in literature were often contradictory in this issue [Lachman et al. 2000, Avad and de Jager 2003, Leja et al. 2003, Davey and Coulemans 2004, Łata et al. 2005b], monitoring was conducted through three seasons. Another interesting question was about the link between antioxidants status after harvest and their content in stored apple, as growing season effect on bioactive compounds is frequently impressive. It was expected that in season/s favouring/inducing (also because of e.g. soft or moderate stress conditions) synthesis of bioactives, their content during storage would be higher. To reach the goal, the following was monitored over three seasons: the content of main apple active compounds such as ascorbate, thiols, anthocyanins and global phenolics, antioxidative enzyme activity: glutathione reductase (GR), ascorbate peroxidase (APX), catalase (CAT) and total antioxidant power (FRAP assay) in the apple peel of cold stored 'Šampion', 'Topaz' and 'Jonagold' cultivars.

MATERIAL AND METHODS

The investigation was carried out in 2003/2004, 2004/2005 and 2005/2006 seasons. Apple of three commercial cultivars: 'Šampion', 'Topaz' and 'Jonagold' (all on M9 rootstock) grown in the experimental orchard of the Department of Pomology of Warsaw University of Life Sciences (Warsaw Wilanow, 52^{o}N, 21^{o}E) were tested. All trees received standard horticultural practices. The harvest dates, designed following ethylene release analysis, were: September 19, 24 and 27 ('Šampion'), September 30, October 6 and 5 ('Jonagold'), and September 30 October 13 and 14 ('Topaz'), in 2003, 2004 and 2005, respectively. Fruits were picked from the outer layer, from the designated five trees, avoiding the tops and bottoms of the trees. Apples were stored in boxes and for each tested time of storage healthy fruits, with similar size, were selected for chemical analysis. There were no significant differences in the mean fresh weight of the fruit analyzed at each time point (data not shown), suggesting that any differences in metabolite concentrations were not related to changes in the fresh weight of fruits. Assays for phytochemical content in apple peels were made after commercial harvest time and next after 1.5 and 3 moths after common cold storage (CS) at 1°C.

Sample preparation: Fruits were cleaned with tissue paper, weighted, peeled and the skin was immediately frozen in liquid nitrogen and stored in –80°C until analysis. Directly before analysis apple tissue was ground to a fine powder in liquid nitrogen. Chemical analyses were made in five replicates for each of the cultivars and every point time, and all of them included peel tissue from two fruits.

Assays of enzyme activities: activity of APX (ascorbate peroxidase) was calculated from the decrease in absorbance at 290 nm as the ascorbate was oxidized. Activity of GR (glutathione reductase) was determined by the decrease in absorbance at 340 nm as NADPH was oxidized. CAT (catalase) activity was calculated from the fall in absorbance at 240 nm in the supernatant containing 50 mM potassium phosphate buffer (pH = 7.0) and 10 mM H_2O_2.

Glutathione and ascorbate: Total glutathione content (GSH+GSSG, reduced and oxidized, respectively) and their precursors: L-cysteine (L-cys) and γ-glutamylcysteine (γ-GC) were assayed in supernatant after reduction GSSG with DL-dithiothreitol (DTT) and derivatization with monobromobimane. The fluorescent derivatives were separated on a Symmetry C_{18} column (250 mm × 4.6 mm, 5 μm, Waters) applying a solution of 10% methanol containing 0.25% (v/v) glacial acetic acid (solvent A, pH 4.3) and 90% methanol with the same acetic acid concentration (solvent B, pH = 3.9), the flow rate was 1 ml min^{-1}.

Total ascorbate content (AA+DHA, ascorbic and dehydroascorbic acid, respectively) was measured after complete reduction of DHA to AA with DTT, as in the case of glutathione. Separation was carried out using Atlantis^{TM} dC_{18} column at 268 nm under isocratic conditions. Mobile phase contained 10% of methanol and 2% $NH_4H_2PO_4$ (pH 2.8). The HPLC results were calculated using a standard curves.

Spectrophotometric measurement of phenolics: for the estimation of phenolics extraction was made in the mixture of methanol, formic acid and distilled water (50:1.5:48.5). The absorbance of the solution was then read at 280, and 520 nm to measure total phenolics and anthocyanins, respectively. Standards used were gallic acid, and cyanidin-3,5-di-glucoside for the phenolics and anthocyanins, respectively. More detailed description of aforementioned methods used were previously described [Łata et al. 2005a, b].

Ferric reducing/antioxidant power (FRAP assay): this procedure involved the reduction of ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) to a blue colored complex Fe^{2+}/TPTZ [(4,6-tri(pyridyl-S-triazine)] in the presence of bioactive compounds (antioxidants), what was monitored as increase of absorption at 593 nm. Determination was carried out according to procedure described in details by Benzie and Strain [1999].

Statistical analysis. Obtained results were elaborated by multifactor ANOVA of Statgraphics Plus 4.1., LSD test was used to check the significance of differences between means of main effects (cultivar, growing season and time of storage) at 5% probability level. Out of interactions the highest one appeared between growing season and time of storage, and these data are presented in this paper. Correlation coefficients were calculated separately for each tested year to notice the expected differences between years. Linear regression analyses were computed using the regression procedure in Microsoft Excel for Windows.

RESULTS AND DISCUSSION

Enzyme activity. Time dependent fluctuations of antioxidative enzyme activity expressed certain similarity, although an impact of growing season conditions strongly

depended on enzyme being analysed (tab. 1). The first storage period resulted in the decrease in activity of the tested enzymes in the first two examined seasons. Contrary to it, during the last experiment (2005/2006) meaningful increase of GR, followed by APX and CAT activity was noted. In both aforementioned instances, the GR activity was subjected to the biggest fluctuations between the harvest and the first analysed point time. The decreases or increase of its activities in the succesive seasons were the following: 62, 340 and 390%, respectively. Relatively high stability over storage expressed CAT, as compared to GR or APX activity. Growing season effect (the highest differences between years) measured after harvest was also the highest for GR, followed by CAT and APX activity. According to our previous comprehensive studies [Łata et al. 2005a, Łata 2007], GR activity was also the most sensible, and strongly defined by conditions of growing season, as compared to other enzymatic and non-enzymatic antioxidant components. In relation to this finding, GR activity as possible environmental stress marker was proposed. Enzyme status (high or extremely low enzyme activity) measured after harvest can be closely related to stress degree that plant/fruit encountered before that date. Unfortunately, it was difficult to indicate an unstressful level, since all seasons differed significantly. However, in this study low enzymes activity after harvest (year 2005) resulted in their better keeping and/or activating during storage. Enzyme activity as factor determining the susceptibility of fruit to mechanical damage or infection during post-harvest storage were indicated in the past [Kochhar et al. 2003, Torres et al. 2003].

Table 1. Antioxidative enzyme activity (nkat g^{-1} FW) after fruit harvest and during storage depending on tested season

Tabela 1. Aktywność enzymów antyoksydacyjnych (nkat g^{-1} św.m.) po zbiorze owoców i w czasie przechowywania w zależności od sezonu

Enzyme Enzym	Years Lata	Harvest Zbiór	Months of storage Miesiące przechowywania		Mean Średnio
			1.5	3	
Glutathione reductase Reduktaza glutationowa	2003/04	5.52	3.40	3.24	4.05 a
	2004/05	9.10	2.07	3.26	4.81 b
	2005/06	1.80	8.83	10.0	6.89 c
Mean – Średnio		5.47 b[a]	4.77 a	5.51 b	
Ascorbate peroxidase Peroksydaza askorbinianowa	2003/04	127	111	154	131 c
	2004/05	128	111	74	104 a
	2005/06	93	120	136	117 b
Mean –Średnio		116 a	114 a	121 a	
Catalase Katalaza	2003/04	6.37	6.15	7.19	6.57 a
	2004/05	9.12	8.77	9.89	9.26 c
	2005/06	7.26	8.64	7.52	7.81 b
Mean – Średnio		7.58 a	7.86 ab	8.20 b	

[a]Mean separation for time of storage or season by LSD test ($p < 0.05$)

[a]Istotność różnic między średnimi w zależności od czasu przechowywania lub sezonu określono stosując test LSD ($p < 0,05$)

Ascorbate and thiols. Ascorbate and glutathione levels might be considered as important markers of fruit quality and they can influence apple storage ability [Davey and Coulemans 2004, Davey et al. 2007]. In the present study, differences in ascorbate contents after harvest in consecutive years did not exceed 13% (tab. 2). The loses of total ascorbate content after the last sampled time were lesser than 10%, as compared to harvest, regardless of the examined season. The higher drop of ascorbate concentration, 23% on the average, was described by Davey and Coulemans [2004], but more cultivars were examined. Moreover, a strong correlation between commercial harvest date and mean vitamin C content and total antioxidant ability over different production years was also reported. Later harvested cultivars were richer in ascorbate and no substantial loses or slight increases of it after 3 months of cold storage were noted. In our previous study, also concerning later harvested apple cultivars, at the end of storage, the total ascorbate content considerably increased, as compared to harvest [Łata 2005b]. Contrary to this, Lachman et al. [2000] described subsequent significant decrease of ascorbic acid content during six months of storage over two seasons, however apples were stored at a higher temperature (5°C). Fruits might accumulate L-AA during ripening on or of the plant, but the increase od ascorbate content was frequently higher for fruit left on the plant [Lee and Kader 2000]. Davey et al. [2004] recently demonstrated that apple exocarp and mesocarp tissues were incapable of L-AA biosynthesis and therefore they were dependent upon phloem transport of L-AA to maintain cellular concentration.

Table 2. Concentrations of ascorbate and thiols (nmol g^{-1} FW) after fruit harvest and during storage depending on tested season

Tabela 2. Zawartość askorbinianu i związków tiolowych (nmol g^{-1} św.m.) po zbiorze owoców i w czasie przechowywania w zależności od sezonu

Constituent Składnik	Years Lata	Harvest Zbiór	Months of storage Miesiące przechowywania		Mean Średnio
			1.5	3	
Ascorbate Askorbinian	2003/04	3725	2930	3396	3350 a
	2004/05	4089	4111	4032	4077 b
	2005/06	3634	4407	3668	3901 b
Mean –Średnio		3816 a[a]	3816 a	3697 a	
L-cysteine L-cysteina	2003/04	6.63	7.31	3.32	5.75 a
	2004/05	5.32	6.54	5.68	5.85 a
	2005/06	5.15	7.84	6.79	6.59 b
Mean –Średnio		5.70 a	7.23 b	5.26 a	
γ-glutamycysteine γ-glutamylocysteina	2003/04	0.20	0.34	0.34	0.29 b
	2004/05	0.08	0.11	0.00	0.06 a
	2005/06	0.11	2.03	1.40	1.18 c
Mean –Średnio		0.13 a	0.83 c	0.58 b	
Glutathione Glutation	2003/04	52.0	69.2	49.1	56.8 b
	2004/05	53.5	55.8	36.0	48.5 a
	2005/06	46.8	74.3	49.9	57.0 b
Mean –Średnio		50.8 b	66.5 c	45.0 a	

[a]Mean separation for time of storage or season by LSD test ($p < 0.05$)

[a]Istotność różnic między średnimi w zależności od czasu przechowywania lub sezonu określono stosując test LSD ($p < 0,05$)

It should be stressed however, the pattern of bioactive compound metabolism might be strictly connected to the behaviour of other active constituent/s. In the present study, similar concentrations of ascorbate were characterised by apple harvested in 2003 and 2005 growing seasons, but its fluctuations after the first storage period were completely different. In the first season ascorbate concentration considerably decreased (ca 30%), while during the last one meaningful increase was noted (ca 20%). The explanation can be derived from the interaction of the constituents of ascorbate – glutathione cycle known also as Halliwell-Asada cycle [Noctor et al. 2002], which was more distinct at that time in the season 2005/2006. A high increase of glutathione was accompanied by the increase of its precursors (especially γ-GC), what suggested synthesis of this compound de novo. The similar successive increase of γ-GC content during cold storage was already described [Łata et al. 2005b]. Simultaneously with the content of thiols, increased GR and APX activity (tab. 1), what might be noticed as a symptom of accelerating demand of the regeneration of oxidized forms of ascorbate (DHA) and glutathione (GSSG). A high capacity of the regeneration of oxidized forms of these antioxidants is a condition of preserving their antioxidant activity. Compared to harvest, the glutathione concentration after three months of apple storage markedly decreased only in the second series of our experiment. According to Davey and Keulemans [2004] cultivars with a higher storage ability (up to 6 months at 1°C) were characterised by a slight increase of both ascorbate and glutathione concentrations during apple cold storage.

Our, as well as referred studies, confirmed that total pool of ascorbate and glutathione does not change significantly during cold storage, but their level and quality (the reduced/oxidized ratio) might be strictly connected with regeneration efficiency e.g. in the glutathione-ascorbate cycle.

Phenolics. Growing season effect, measured as phenolics content after the harvest time, was more emphasized on its subgroup, anthocyanins synthesis (tab. 3). All seasons significantly differed in their content and a bigger anthocyanins pool after harvest resulted in its higher concentration over storage. Despite the great differences in anthocyanins content after harvest between consecutive seasons, their growth at the end of storage was at comparable degree level, between 25–35%, on the average. As for previously related compounds, the third season diverged highly (but in a similar way) from the two first ones. The high increase in 2005/2006 season concerned of both, phenolics and anthocyanins content.

Lachman et al. [2000] observed successively significant decrease in the total polyphenol concentration. The opposite results were described by Leja et al. [2003], where during long-term storage, total phenolics content of 'Jonagold' and 'Šampion' apples increased considerably, regardless of storage conditions (CA or CS). However, contrary to present results, apple stored in air showed a slight decrease in anthocyanins, as compared to harvest. According to Avad and de Jager [2000] during long-term storage of 'Jonagold' and ´Elstar´ the concentration of cyanidin-3-galactoside (the main out of apple anthocyanins) was relatively constant, and increased only when apple was being exposed to white light during shelf life following storage. The initial increase in peel phenolics concentration may be an effect of ethylene action in the apple fruit, which initiates ripening. Ethylene affected activity of phenylalanine ammonia lyase, a key enzyme in the pathway of phenolic synthesis and next their accumulation [Leja et al.

2003]. According to the present study, an effect of increasing of total phenolics and anthocyanins content, particularly distinct in 2005/2006, was maintained throughout seasons.

Table 3. Concentrations of anthocyanins and phenolic compounds (µg g^{-1} FW) after fruit harvest and during storage depending on tested season

Tabela 3. Zawartość antocyjanów i związków fenolowych (µg g^{-1} św.m.) po zbiorze owoców i w czasie przechowywania w zależności od sezonu

Compound Związek	Years Lata	Harvest Zbiór	Months of storage Miesiące przechowywania		Mean Średnio
			1.5	3	
Anthocyanins[b] Antocyjany	2003/04	103	112	129	115 a
	2004/05	124	159	167	150 b
	2005/06	151	312	203	222 c
Mean –Średnio		126 a[a]	195 c	167 b	
Phenolics[c] Fenole	2003/04	981	1234	1209	1141 a
	2004/05	1074	1273	1284	1210 a
	2005/06	925	2361	1817	1701 b
Mean –Średnio		993 a	1623 c	1437 b	

[a]Mean separation for time of storage or season by LSD test ($p < 0.05$)
[a]Istotność różnic między średnimi w zależności od czasu przechowywania lub sezonu określono stosując test LSD ($p < 0,05$)
[b]Expressed as equivalent of cyanidin-3.5-di-glucoside; [b]w przeliczeniu na di-glukozyd – 3,5-cyjanidyny;
[c]Expressed as equivalent of gallic acid; [c]w przeliczeniu na kwas galusowy.

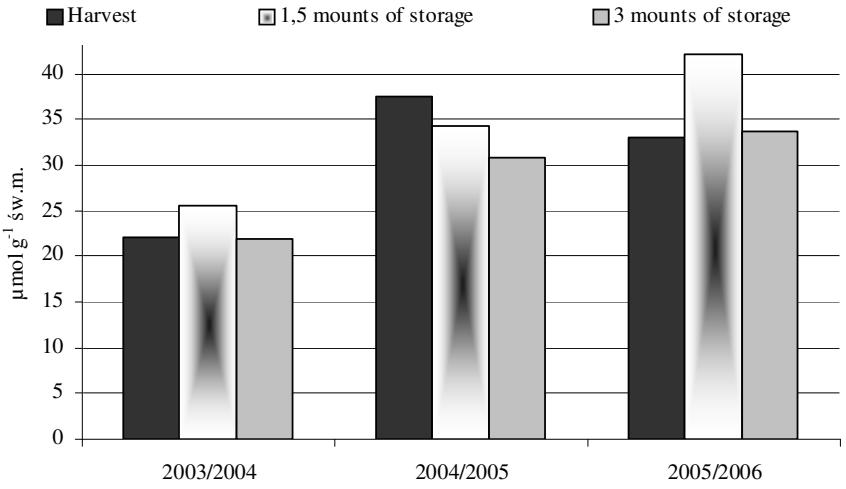

Fig. 1. Total antioxidant power (FRAP assay, µmol g^{-1} FW) after fruit harvest and during storage depending on the tested season

Rys. 1. Całkowita aktywność przeciwutleniająca (wskaźnik FRAP, µmol g^{-1} św.m.) po zbiorze owoców i w czasie przechowywania w zależności od sezonu

FRAP assay. In principle, one clear difference between examined seasons was noted in total antioxidant power (FRAP assay) of apple peel with respect to the harvest time (fig. 1). Total antioxidant activity after fruit harvest in 2003/2004 season was considerably lower as compared to the FRAP value of the next two seasons, ca. 70 and 49%, respectively. High increase of total antioxidant power between harvest and the first sampling time in 2005/2006 confirmed fluctuations in individual compounds discussed above, and caused that FRAP index expressed the highest value in this season. Rather low variability of total antioxidant ability were indicated by other authors [van der Sluis et al., 2001, Leja et al. 2003, Trieerweiler et al. 2004]

Correlation found between chosen antioxidants during storage. Obtained correlations reflected the data received after analysis of individual components of antioxidant apparatus (tab. 4). Moreover, they were strictly connected with an antioxidant size. There were lack of correlations in the first season and, in general, the lowest antioxidant content was noted. Contrary to it, the highest antioxidant concentrations in the last examined season and most of the relationship between bioactives became significantly

Table 4. Correlation coefficient between antioxidants through storage period, depending on tested season

Tabela 4. Zależności między antyoksydantami w czasie przechowywania w zależności od sezonu

	2003/2004	2004/2005	2005/2006
Glutathione-L-cysteine	0.684 [a]	0.737 [a]	0.728 [a]
Glutathione-γ-glutamylcysteine	- 0.005 [d]	0.108 [d]	0.433 [c]
Glutathione-glutathione reductase	-0.239 [d]	0.392 [c]	0.624 [a]
Glutathione-ascorbate	0.326 [d]	0.191 [d]	0.231 [d]
Ascorbate-glutathione reductase	- 0.171 [d]	0.276 [d]	0.489 [b]
Ascorbate-phenolics	0.242 [d]	0.230 [d]	0.398 [c]
Ascorbate-anthocyanins	-0.189 [d]	0.432 [c]	0.563 [b]
Phenolics-anthocyanins	0.138 [d]	0.365 [d]	0.548 [b]
Catalase-glutathione reductase	0.484 [c]	0.610 [a]	0.736 [a]
Catalase-glutathione	- 0.191 [d]	0.086 [d]	0.358 [d]
Catalase-ascorbate	- 0.301 [d]	0.437 [c]	0.608 [b]

[a] significant at $\alpha = 0.001$; [b] significant at $\alpha = 0.01$; [c] significant at $\alpha = 0.05$; [d] – insignificant.

important. All season differed substantially in the mean temperature and rainfall (data not shown). The growing seasons in 2004 and 2005, as compared to 2003, were rather cold and dry. The mean temperature of August 2005 was especially low, as compared to previous seasons. Fluctuations in temperature and light (increased anthocyanins accumulation) probably affected the content and antioxidant behaviour in consecutive seasons. All these events confirmed the meaningful involvement of these compounds in apple maturation, storage behaviour, signalling and acclimation responses, although mechanism of action is still explored. Changes in activity of hydrogen peroxide scavenging enzymes (in this study significant correlation between GR and CAT existed over all seasons) and of redox status of antioxidants such as GSH and AA may act as signal transducing molecules toward starting defence mechanism [Noctor et al. 2002]. Some indirect proof might be used of 1-MCP (1-methylcyclopropene) in keeping fruit quality.

Treated fruits had lower level of hydrogen peroxide and ascorbate during air storage, but enzymatic antioxidants rose, indicating beneficial effect of increasing antioxidative enzyme activity [Larrigaudiere et al. 2005, Zubini et al. 2007].

CONCLUSIONS

The content of bioactives at harvest might be considered as important marker not only in relation to internal fruit quality and a reflection of a tissue ability to withstand stress conditions during storage, but also can be strictly connected with growing season conditions before harvest date. In this study, the link between antioxidant enzyme activity after harvest and bioactives fate during apple storage occurred. Except for ascorbate, the highest concentration of non-enzymatic antioxidants was characterised by apple harvested and stored in 2005/2006 season. Simultaneously many statistically proved correlations between examined non-enzymatic and/or enzymatic antioxidants over storage at that time existed. Enzyme activity, especially glutathione reductase was very low this year after fruit harvest, and significantly increased during storage. Compared to harvest, cold stored apple maintained their ascorbate and glutathione content, whereas the concentration of anthocyanins and global phenolics even increased and the end of the storage. With an exception of anthocyanins, end-status of other bioactives was not strictly related to harvest size. Whereas mid-status of antioxidant compounds in cold stored apple might be closely related to their harvest content (an example was third season in our study). It seemed that this status was supported by increasing concentration of antioxidant precursors and high enzyme activity involved in antioxidant regeneration, especially in the first months of storage. The comparison of antioxidant content/activity after the harvest time and over storage in later harvested apple cultivars provided arguments about a high efficiency of their antioxidant apparatus and its involvement in fruit maturation and storage.

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POZIOM ANTYOKSYDANTÓW PO ZBIORZE JABŁEK A ICH ZAWARTOŚĆ W CZASIE PRZECHOWYWANIA OWOCÓW W CHŁODNI

Streszczenie. Celem badań było określenie, czy istnieje związek między stężeniem antyoksydantów po zbiorze jabłek a ich poziomem w czasie przechowywania. Pewną zależność można było odnotować dla antyoksydantów enzymatycznych, których aktywność była szczególnie zależna od warunków wegetacji w danym sezonie. Z wyjątkiem antocyjanów, stężenie antyoksydantów nieenzymatycznych oznaczone po zakończeniu przechowywania nie było ściśle związane z ich poziomem odnotowanym po zbiorze owoców. Jednakże ich zawartość w pierwszym miesiącu (miesiącach) przechowywania może mieć związek ze stanem pozbiorczym. Istotnie wyższe, przeciętne stężenie większości związków biologicznie aktywnych, a jednocześnie wiele istotnych statystycznie korelacji między nimi odnotowano w sezonie 2005/2006. Całkowita aktywność przeciwutleniająca istotnie wzrosła po pierwszym etapie przechowywania owoców w chłodni (prawdopodobnie jako efekt aklimatyzacji owoców do warunków przechowywania), a następnie obniżyła się. Zmiany tego wskaźnika odzwierciedlały na ogół wahania indywidualnych składników aparatu antyoksydacyjnego.

Słowa kluczowe: *Malus domestica* Borkh, chłodnia, antyoksydanty, wskaźnik FRAP

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