

## EFFECT OF CULTIVAR AND HARVEST DATE ON THIOLS, ASCORBATE AND PHENOLIC COMPOUNDS CONTENT IN BLUEBERRIES

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**Abstract.** The study was carried out on two highbush blueberry cultivars: Bluecrop and Darrow in 2001 and 2002 seasons. Analyses for phytochemical contents in berries were made during commercial maturity of fruit. Concentration of anthocyanins and low molecular weight thiol compounds was significantly higher in 'Darrow' berries in comparison to Bluecrop cv., irrespective of the harvest year. Tested cultivars did not differ in the ratio of the reduced form of glutathione in its total quantity. As opposed to this, the higher proportion of the reduced form of ascorbate in its total concentration was noted for 'Bluecrop', but it was statistically proven only in the first year of research. A very low activity of antioxidative enzymes was noted, however on the average, considerably higher activity of glutathione reductase showed 'Darrow', whereas 'Bluecrop' had higher catalase activity. The ascorbate peroxidase activity was not detected in the extracts of 'Bluecrop's' berries. Berries of 'Darrow', which exhibited, in general, the higher antioxidants content in the first year of the study, were harvested at two different dates in 2002: in the middle of July and at the end of August. The harvest date had a significant effect on the level of some phytochemicals. The fruit harvested in August had lower content of cysteine, glutathione, phenolics, flavonols and anthocyanins than the ones harvested in July. Ascorbate content was similar at both harvest dates. Growing season also had the influence on antioxidant properties, especially on the content of ascorbic acid.

**Key words:** *Vaccinium corymbosum*, cultivar, ascorbate, glutathione, phenolics, antioxidative enzyme activity

**Abbreviations:** AA – L-ascorbic acid, DHAA – dehydroascorbic acid, GSH – reduced glutathione, GSSG – oxidized glutathione, CYS – L-cysteine,  $\gamma$ -GC – glutamylcysteine, GR – glutathione reductase, APX – ascorbate peroxidase, CAT – catalase, SOD – superoxide dismutase

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## INTRODUCTION

Plants synthesize antioxidants that are used in their defence system against oxidative stress, but certain type of phytochemicals are present in sufficient amount to make a significant contribution to the antioxidant capacity necessary for health human diet [Kalt et al. 2001]. Epidemiological studies consistently show that there is cause-effect relationship between intake of fruit and vegetables and reduced rate of heart diseases mortality, common cancer and other degenerative diseases as well as aging [Kaur and Kapoor 2001]. Fruit quality has become the most important aspect of horticultural plant cultivation, recently. Hence, it is important to characterise the beneficial phytonutrients present in food and the factors that influence content as well as the mechanism of their biological action. A diet rich in plant bioactive compounds should be considered an important element in human health prophylaxis. The protective effect of fruits and vegetables is generally attributed to their content of antioxidant constituents, including ascorbate, glutathione, carotenoids, tocopherols and phenolics. These phytochemicals protect lipids, proteins and nucleic acids against oxidative damage initiated by free radicals. Phenolics are probably the largest and the most diverse group as well as possessing an ideal structure to act as antioxidant [Rice-Evans et al. 1997, Czacot 2000]. Two major classes of phenolics are common in fruits species, namely non-flavonoid e.g., phenolic acids and flavonoids. The group of flavonoids includes several subclasses such as flavonols, catechins, anthocyanins and proanthocyanidins. To the very important hydrophilic antioxidants involved in the mechanism of toxic free radical removal belong also glutathione and ascorbate [Lee and Kader 2000, Valencia et al. 2001]. These compounds may appear in two forms: biologically active reduced and oxidized ones. The level of oxidized form generally increases during environmental stresses, which generate harmful free radicals as well as during senescence [Łata 1998]. The enzymes: ascorbate peroxidase and glutathione reductase indirectly participate in free radical removal, because they are involved in glutathione/ascorbate regeneration. Moreover, catalase directly detoxifies harmful free radicals.

The blueberry fruits received much attention since having been reported to contain high level of phenolics, including anthocyanins, quercetin, kempferol, myricetin, chlorogenic acid and procyanidins and in this way high level of oxygen radical-absorbing capacity (ORAC – an aqueous assay designed to measure the peroxy-scavenging ability of water soluble antioxidants) [Cao et al. 1997, Joseph et al. 1999]. The concentration of blueberry phytochemicals depends on cultivar, maturity, geographic origin, growing season, horticultural practises, postharvest storage conditions and processing procedures [Prior et al. 1998; Kalt et al. 1999a, 1999b; Ehlenfeldt and Prior 2001; Kalt et al. 2001; Ścibisz and Mitek 2001; Howard et al. 2003]. Most of these investigations are related to phenolics concentration and composition of blueberry fruit. The objective of this study was to evaluate the content of ascorbate and glutathione (both reduced and oxidized forms), some subgroup of phenolics: flavonols, anthocyanins and total phenols content as well as enzyme activity: glutathione reductase, ascorbate peroxidase and catalase of fruit of Bluecrop and Darrow cultivars.

## MATERIAL AND METHODS

The investigation was carried out in the 2001 and 2002 seasons. Fruit was collected from two commercial cultivars of highbush blueberries: Bluecrop and Darrow, that were grown in the Blueberry Experimental Farm in Błonie near Prażmów belonging to the Department of Pomology and Basic Natural Sciences in Horticulture of Warsaw Agricultural University. Plants received standard horticultural practices. Fully ripe (not over-ripe) fruit was harvested from ten randomly selected 12–15 years old shrubs for each cultivar. In the 2002 berries of 'Darrow' (with the higher antioxidant content) were harvested at two different dates: in the middle of July and at the end of August to test the influence of harvest date on antioxidant properties.

Sample preparation: harvested samples of berries were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until analysis. Directly before analysis berries were ground (about 20 g of fruits) to a fine powder in liquid nitrogen. Chemical analyses were made in five to ten replicates for each of the cultivars.

Assays of enzyme activities: the ground tissues (200 mg) were suspended in 5 ml 100 mM potassium phosphate buffer (pH = 7.8) containing Triton X – 100 (0.5%), insoluble polyvinylpyrrolidone (400 mg) and ascorbate (5 mM). The mixture was centrifuged at  $48\,000 \times g$ , for 25 minutes at  $4^{\circ}\text{C}$ . Activity of APX, GR and CAT were carried out in a total volume of 1 ml.

Activity of APX was calculated from the decrease in absorbance at 290 nm as the ascorbate was oxidized [Nakano and Asada 1987]. Activity of GR was determined by the decrease in absorbance at 340 nm as NADPH was oxidized [Foyer and Halliwell 1976]. CAT 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  $\text{H}_2\text{O}_2$  [Beers and Sizer 1952].

Glutathione and ascorbate: frozen berries powder was extracted in 0.1 M HCl containing PVP (frozen powder : PVP in relation 2:1) and centrifuged at  $21\,900 \times g$  (glutathione) or  $48\,000 \times g$  (ascorbate), for 20 minutes at  $4^{\circ}\text{C}$ . The extract was then used in sample preparation for HPLC procedure.

GSH+GSSG were determined by spectrophotometric method (year 2001) and by HPLC in 2002. The first method based on a kinetic assay in which catalytic amounts of GSH, GSSG and GR brought about the continuous reduction of 5,5'-dithiobis (2-nitrobenzoic acid, DTNB) by NADPH [Akerboom and Sies 1981]. In the second method, GSH+GSSG was assayed in supernatant after reduction GSSG with DL-dithiothreitol (DTT) and derivatization with monobromobimane [Newton et al. 1987]. GSSG was measured after masking GSH by NEM (N-ethylmaleimid) and then their reduction to GSH form. The fluorescent thiol derivatives (GSH, CYS and  $\gamma$ -GC) were separated on a Symmetry  $\text{C}_{18}$  column (250 mm  $\times$  4,6 mm, 5  $\mu\text{m}$ , 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), flow rate 1 ml  $\text{min}^{-1}$ .

Total ascorbate was measured after complete reduction of DHAA to AA with DTT. AA was analyzed directly in the supernatant obtained after extraction [Anderson et al. 1992]. Separation was carried out using Atlantis<sup>TM</sup>  $\text{dC}_{18}$  column at 268 nm under iso-

cratic conditions. Mobile phase contain 10% of methanol and 2%  $\text{NH}_4\text{H}_2\text{PO}_4$ , pH 2.8. The results were calculated using a standard curve.

Analyses of Phenolics: for the estimation of some subgroup of phenolics extraction was made in the mixture of methanol, formic acid and distilled water (50:1.5:48.5). After centrifugation ( $24\,000 \times g$ ,  $4^\circ\text{C}$ , 10 minutes) the supernatant was filtered and diluted 1:5 with 10% ethanol. The method consisted of placing 250  $\mu\text{l}$  of sample or standard in a test tube and adding the same volume of 0.1% HCl in 95% ethanol and 4500  $\mu\text{l}$  of 2% HCl. The absorbance of the solution was then read at 280, 360 and 520 nm to measure total phenolics, flavonols, and anthocyanins, respectively. Standards used were gallic acid, quercetin, cyanidin-3,5-di-glucoside for total phenolics, flavonols, anthocyanins, respectively [Mazza et al. 1999].

*Statistical analysis.* The results were elaborated by one – factor analysis of variance (Anova-1), separately for each tested year. To calculate the percentage of reduced forms of ascorbate and glutathione in the total quantity arc sin transformation was applied. The significance of the differences between cultivars and harvest dates means was evaluated using the Tukey test at  $\alpha = 0.05$ .

## RESULTS AND DISCUSSION

The significant differences between cultivars of four *Vaccinium species* [*V. corymbosum* L., (Highbush)], *V. angustifolium* (Lowbush), *V. ashei* (Rabbiteye) and *V. myrtillus* L. (Bilberry)] in phenols, anthocyanins and ascorbate content as well as the ORAC values were noted by Prior et al. [1998]. Ascorbate concentration ranged from 13 to 164  $\mu\text{g}$  per g f.w. showing a significant variability between species and cultivars. Among highbush cultivars, berries of ‘Bluecrop’ had 81  $\mu\text{g g}^{-1}$  f.w. of ascorbic acid. Compared to this results, both of the tested by us cultivars showed higher ascorbate content, on the average: 236 and 207  $\mu\text{g g}^{-1}$  f.w. for ‘Darrow’ and ‘Bluecrop’, respectively (tab. 1). It could be partially explained that both forms of ascorbate: reduced and oxidized were analysed. Especially high ascorbate concentration was measured in ‘Darrow’ in the 2002 year. Besides, it is worth considered very low proportion of reduced form of ascorbate in its total quantity in this year, on the average approximately 33%. High ratio DHAA:AA indicate more stressful conditions in this vegetation period in comparison to the former one. In the first season, both tested cv. had similarly level of total ascorbate content, but ‘Bluecrop’ exhibited significantly higher concentration of AA. It may also prove about high variability of these hydrophilic antioxidant in plant tissues.

Ehlenfeld and Prior [2001] reported the content of phenolics, anthocyanins as well as total antioxidant activity in Darrow cv. was considerably higher when set beside ‘Bluecrop’.

Likewise results were obtained in the present study. Darrow cv. had the significantly higher anthocyanins and flavonols content and in consequence of this higher total phenols concentration in comparison to ‘Bluecrop’ one (tab. 1). Compared to Bluecrop cv. the increase of concentration of phenolics, flavonols and anthocyanins, on the average, in ‘Darrow’ berries was as follows: 13.4%, 22.1%, and 40.0%, respectively. Out of

explored by Kalt et al. [1999b] cultivars 'Bluecrop' had the lowest content of anthocyanins. In general, among tested by these authors commercial and non-commercial highbush blueberries, wild genotypes had 2-fold higher content of anthocyanins than cultivated one.

Table 1. Content of some antioxidants and enzyme activity depending on cultivar and growing season

Tabela 1. Stężenie antyoksydantów oraz aktywność enzymów oksydacyjnych w zależności od odmiany i roku badań

Year – Rok	2001		2002	
Parameter Wskaźnik	Darrow	Bluecrop	Darrow	Bluecrop
Content of some subgroup of phenolics <sup>1</sup> and their total amount ( $\mu\text{g g}^{-1}$ f.w.)				
Stężenie różnych podgrup związków fenolowych oraz ich całkowita zawartość ( $\mu\text{g g}^{-1}$ św.m.)				
Flavonols	401.7 a <sup>2</sup>	408.4 a	597.4 b	409.6 a
Anthocyanins	1255.8 b	881.2 a	1071.4 b	781.1 a
Phenolics	1960.9 a	1769.4 a	1999.6 a	1724.1 a
Ascorbate content ( $\mu\text{g g}^{-1}$ f.w.)				
Askorbinian ( $\mu\text{g g}^{-1}$ św.m.)				
AA +DHAA	166.2 a	162.1 a	305.7 b	251.7 a
%AA	54.7 a	70.5 b	32.8 a	34.3 a
Thiols content ( $\text{nmol g}^{-1}$ f.w.)				
Związki tiolowe ( $\text{nmol g}^{-1}$ św.m.)				
GSH + GSSG	34.0 b	15.3 a	57.2 b	15.3 a
CYS			8.4 b	3.6 a
$\gamma$ -GC			0.8 b	0.2 a
% GSH			70.2 a	62.2 a
Enzyme activity ( $\text{nkat g}^{-1}$ f.w.)				
Aktywność enzymów ( $\text{nkat g}^{-1}$ św.m.)				
GR	4.3 b	2.7 a	4.6 a	4.9 a
APX	3.3	ND	3.6	ND
CAT	2.6 a	2.8 a	2.3 a	3.2 b

Explanations: <sup>1</sup> Concentration based on gallic acid for total phenols content, quercetin for flavonols and cyanidin-3,5-di – glucoside for anthocyanins – Stężenie wyrażono w  $\mu\text{g}$  kwasu galusowego, kwercetyny i 3,5-di-glukozydu cyjanidyny odpowiednio dla fenoli, flawonoli i antocyjanów

<sup>2</sup> Means followed by the same letters do not differ significantly – średnie oznaczone tą samą literą nie różnią się istotnie;

ND – activity was not detected – aktywności nie stwierdzono

The greater differences were obtained with respect to thiol compounds (tab. 1). Compared to 'Bluecrop', fruit of 'Darrow' contained 3.7-fold, 2.3-fold and 4.0-fold higher content of GSH+GSSG, CYS and  $\gamma$ -GC, respectively, based on results from the 2002 year. The difference in the proportion of reduced form of glutathione in overall concentration between cultivars was negligible and amounted, on the average, 66.2%. To our knowledge there is no information on thiols content in blueberries.

Measured enzyme activity, compared to other fruit and vegetables tested by us (date not shown) was very low in the ripe berries, however differences between tested cultivars were also noted. 'Darrow' had significantly higher activity of GR in comparison to

'Bluecrop', but only in the first year of the study. As opposed to this, 'Bluecrop' had significantly higher CAT activity in comparison with 'Darrow', but in the 2002 year. The APX activity in 'Bluecrop' berries was not found.

The fruit maturity or harvest date may also have an influence on the content of health promoting compounds in small fruits [Prior et al. 1998, Kalt et al. 2001]. Taking into account the harvest date fruit of 'Darrow' collected in the middle of July had significantly higher concentration of flavonols, GSH+GSSG and lower CAT activity (tab. 2). Minor differences between the rest of the tested parameters were noted. Fruit was harvested at the same level maturity, but probably some differences in the weather factors could influence synthesis and accumulation of some tested compounds. Course of the weather is also a great factor explaining the year to year differences in antioxidant content [Lee and Kader 2000, Kähkönen et al. 2001, Howard et al. 2003]. The considerably increase of flavonols, ascorbate, glutathione and GR activity in 2002 in comparison to former year was noted (tab. 1). Particularly, high increase took place in the case of total ascorbate and glutathione concentrations. The increase was as follows: 75.2 and 47.1%, respectively. Effect of the year on phenolics content was less visible.

Table 2. Content of some antioxidants and enzyme activity depending on harvest date in Darrow cultivar

Tabela 2. Stężenie antyoksydantów oraz aktywność enzymów oksydacyjnych w zależności od terminu zbioru dla odmiany Darrow

Parameter Wskaźnik	July Lipiec	August Sierpień	Average Średnio
Content of some subgroup of phenolics <sup>1</sup> and total ( $\mu\text{g g}^{-1}$ f.w.) Stężenie różnych podgrup związków fenolowych oraz ich całkowita zawartość ( $\mu\text{g g}^{-1}$ św.m.)			
Flavonols	597.5 b <sup>2</sup>	357.7 a	477.6
Anthocyanins	1071.4 a	1050.2 a	1060.8
Phenolics	1999.9 a	1901.0 a	1950.4
Ascorbate content ( $\mu\text{g g}^{-1}$ f.w.) Askorbinian ( $\mu\text{g g}^{-1}$ św.m.)			
AA + DHAA	305.7 a	307.3 a	306.5
% AA	29.5 a	28.8 a	29.1
Thiols content (nmol $\text{g}^{-1}$ f.w.) Związki tiolowe (nmol $\text{g}^{-1}$ św.m.)			
CYS	8.4 a	7.8 a	8.1
$\gamma$ -GC	1.0 a	1.0 a	1.0
GSH + GSSG	57.1 b	37.4 a	47.2
% GSH	70.2 a	80.6 a	75.4
Enzyme activity (nkat $\text{g}^{-1}$ f.w.) Aktywność enzymów (nkat $\text{g}^{-1}$ św.m.)			
GR	4.6 a	4.9 a	4.7
APX	3.1 a	3.0 a	3.0
CAT	2.3 a	3.0 b	2.6

Explanations:<sup>1,2</sup> the same as for table 1. Objasnienia jak dla tabeli 1.

Howard et al. [2003] reported that variation in total concentration of phenolics, anthocyanins, flavonols, hydroxycinnamic acid between tested genotypes was much greater than that observed between growing season, indicating that genetics plays more important role than growing season in influencing phenolic content and total antioxidant

potential (ORAC) in blueberries. In the present study berries of 'Darrow', based on the average for both seasons values, had higher content almost all tested compounds and enzyme activity. Nevertheless, as could be see, concentration of ascorbate or flavonols clearly depended on growing season. Environmental growing conditions can impact antioxidant properties of blueberries fruit and it is why blueberry genotypes should be screened over multiple seasons in order to identify antioxidant-rich germplasm.

Moreover, in general, genotypes with smaller berries had higher ORAC values and levels of individual subgroup of phenolics than large-berried genotypes as reported Howard et al. [2003]. It was not consistent with our results, 'Darrow' had higher amounts of antioxidant having simultaneously greater fruit size.

## CONCLUSIONS

Our study showed that blueberries contain not only different phenolic compounds, what is frequently being discussed in the literature in relation to this plant, but some cultivars may possess also other important antioxidants such as glutathione and its precursors. Total ascorbate content was higher, then reported in the literature cited, especially for 'Darrow' in the year 2002. It is important to measure both forms of ascorbate, because AA and DHAA exhibits biological activity, moreover DHAA can be converted to AA by reduced glutathione in the presence of some enzymes (e.g. APX, GR). In general 'Darrow' exhibited higher content of bioactive compounds. The distinct effect of growing season and harvest date on some antioxidants was also noted. Antioxidants as oxidative stress metabolites are subjected to very dynamic fluctuations in plant tissues what is a reason of difficulties in explanation of factors influence their content and redox ratio.

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## WPLYW ODMIANY I TERMINU ZBIORU NA ZAWARTOŚĆ ZWIĄZKÓW TIOLOWYCH, ASKORBINIANU ORAZ FENOLI W OWOCACH BÓRÓWKI WYSOKIEJ

**Streszczenie:** Badania przeprowadzono w latach 2001–2002. Ocenie poddano dwie odmiany borówki wysokiej: Bluecrop i Darrow. Analizy chemiczne dotyczące zawartości fitozwiązków w owocach wykonano w fazie dojrzałości zbiorczej. Stężenie antocyjanów oraz niskocząsteczkowych związków tiolowych było istotnie wyższe w owocach odmiany Darrow w porównaniu z odmianą Bluecrop, niezależnie od roku badań. Udział zreduko-



wanego glutationu w jego całkowitej zawartości kształtował się na zbliżonym poziomie u obu ocenianych odmian. Przeciwnie, istotnie wyższą zawartością zredukowanego askorbinianu charakteryzowała się odmiana Bluecrop, ale tylko w pierwszym roku badań. Aktywność enzymów oksydacyjnych w dojrzałych owocach borówek była bardzo niska. Jakkolwiek przeciętnie, wyższą aktywnością reduktazy glutationowej charakteryzowała się odmiana Darrow, natomiast ekstrakt z owoców odmiany Bluecrop wykazywał wyższą aktywność katalazy. Ekstrakty z owoców odmiany Bluecrop nie wykazywały aktywności peroksydazy askorbinianowej. Owoce odmiany Darrow, które charakteryzowały się na ogół wyższym stężeniem antyoksydantów w pierwszym roku badań, w roku kolejnym poddano analizie w dwóch terminach: w połowie lipca i końcu sierpnia. Termin zbioru wpłynął istotnie na poziom fitozwiązków. W owocach zebranych w sierpniu stwierdzono niższą zawartość cysteiny, glutationu, związków fenolowych ogółem jak i flawonoli oraz antocyjanów w porównaniu do zbioru wcześniejszego. Poziom askorbinianu był zbliżony, niezależnie od terminu zbioru. Stwierdzono wyraźny wpływ sezonu wegetacyjnego na poziom analizowanych antyoksydantów, szczególnie dotyczyło to stężenia askorbinianu.

**Słowa kluczowe:** borówka wysoka, odmiana, glutation, askorbinian, fenole, aktywność enzymów oksydacyjnych

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