

Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening

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Abstract

The phenolic profile and quantitative composition of blueberries as well as the corresponding antioxidant activity of blueberries is well documented. Unfortunately, little is reported on the development of phenolic compounds and antioxidant activity during fruit maturation and ripening. In the present study, the total phenolic content and main phenolic compounds of four highbush blueberry cultivars (*Vaccinium corymbosum* L.) were analyzed at five stages of maturation and ripening. Antioxidant activity was screened with electron spin resonance spectrometry and trolox equivalent antioxidant capacity (TEAC) assay. An adequate picture of phenolic compounds developed during maturation and ripening was determined using HPLC-DAD. Anthocyanins of all varieties increased during successive harvest stages; meanwhile flavonols and hydroxycinnamic acids decreased from unripe green to ripe blue stage of berry ripening. Blueberry antioxidant activity, as well as total phenolic content tended to decrease during ripening.

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1. Introduction

Among berry fruits, blueberries (*Vaccinium corymbosum* L.) are considered to be a good source of phenolic compounds and are praised for their high antioxidant activity scores (Prior et al., 1998). Due to raised health awareness and the apparent relationship of phytochemicals in plant foods with the prevention of chronic diseases, the content and physiological activity of phenolic com-

pounds in blueberries have been studied. As a result, phenolic profile and quantitative composition of blueberries is well documented. From the existing research results, it has to be considered that they comprise not only the main blueberries cultivated, the northern and southern highbush blueberries, but also the lowbush (or wild blueberries) and rabbiteye blueberries. Between these blueberry species there are significant differences in the phenolic content and antioxidant activity, as well as between varieties and within other *Vaccinium* species (Prior et al., 1998; Taruscio, Barney, & Exon, 2004).

Of the secondary plant metabolites found in blueberries, the flavonoid subclass of the anthocyanins has received the most attention (Gao & Mazza, 1994; Wu et al., 2006);

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although flavonols (predominantly quercetin derivatives), phenolic acids (caffeic, chlorogenic, *p*-coumaric and ferulic acid) and proanthocyanidins are also present (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002; Taruscio et al., 2004) Information is also available in the literature on changes of phenolic composition and antioxidant activity during postharvest storage and handling (Kalt, 2005), as well as processing (Skrede, Wrolstad, & Durst, 2000). It is accepted, that the content of phenolics in berries is not only affected by genetic differences, preharvest environmental conditions, but also by the degree of maturity at harvest (Zadernowski, Naczek, & Nesterowicz, 2005). In this respect, it has been observed that anthocyanin accumulation continues in overripe blueberries, as well after harvest and during storage. Research groups have also analyzed the growing performance and fruit quality attributes of specific cultivars, as well as differences in growing locations in respect to phenolic compound content and antioxidant activity (Kalt, Howell, Duy, Forney, & McDonald, 2001).

The phenolic content and antioxidant activity of berry crops, including blueberries, are becoming target traits by plant breeders. Breeding programs focused for decades predominantly in creating varieties with improved commercial traits: large berry size, light-blue colour (lighter coloured berries appear fresher than darker ones), small scar (large wet scars are susceptible to infections that cause post-harvest decay), firmness, and productivity (Moore, 1965). Recently, investigations are focused on a concept of fruit quality extended to the improvement of the nutritional value. Breeding objectives not only for blueberries, but also for other berry species, include investigating the germplasm of wild species to identify phenolic rich colonies to breed cultivars with enhanced bioactivity (Scalzo, Politi, Pellegriani, Mezzetti, & Battino, 2005). This research supports the concept of an extended view of a consumer oriented quality in which health promoting bioactive substances is a desired quality attribute. This view is gaining importance among blueberry growers and actors involved in the food distribution chain.

Even though literature on blueberry issues is vast, expanding from different culture practices to the health attributes of blueberry extracts in animal studies little is reported on the development of phenolic compounds and antioxidant activity during the process of fruit maturation and ripening.

The aim of the present study was to determine changes of phenolic bioactive substances and the corresponding antioxidant activity of four northern highbush blueberry (*V. corymbosum* L.) cultivars ('Reka', 'Puru', 'Bluecrop' and 'Berkeley') during fruit maturation and ripening. The following fruit quality attributes were determined and analyzed as indicators for fruit ripening: plant phenology, berry fresh weight, fruit colour and sugar-acid ratio of fruits. These common fruit characteristics were compared with changes in the phenolic compounds (determined by HPLC): phenolic acids, flavonols and anthocyanins. The total phenolic content was determined with the Folin-Cio-

calteu method. Two different methods were used to determine the antioxidant activity – electron spin resonance (ESR) spectrometry and trolox equivalent antioxidant capacity (TEAC) assay.

2. Materials and methods

2.1. Plant material

Eight-year-old highbush blueberry plants were grown on former farmland at the experimental station in Berlin-Dahlem at the Institute of Horticultural Science, Humboldt-Universität zu Berlin. The field measured 0.3 ha: spacing was 3 × 1 m; a 3 m distance between rows and 1 m between bushes. Agrochemical characteristics of the field were as follows:

- Soil composition: 72.1% sand, 25% silt, 2.9% clay and 1.4% organic matter.
- Soil mineral composition: 31 mg P₂O₅, 62 g K₂O, and 33 mg MgO/100 g.
- Plate number lied between 25 and 35 (data from 1998 to 1999).
- Humus content: 2.97%.
- pH value: 5.58 (data from 2004).

The northern highbush blueberry cultivars used were: 'Reka' and 'Puru' (both early season ripening cultivars), and 'Bluecrop' and 'Berkeley' (both mid season ripening cultivars). Plants were mulched with pine bark and fertilised twice a week with the fertilising dose Manna Lin M, comprising of: 10 g N, 29 g P₂O₅, 83 g K₂O, 13 mg MgO and 66 mg B per plant and year.

2.2. Determination of maturity stage

For sample collection of each cultivar, three ripening stages (unripe green (RS1), unripe purple (RS2) and ripe blue (RS3)) and three harvest dates (H1, H2 and H3) were distinguished as described in Table 1. Maturity stages were

Table 1
Description of the selected maturity stages for sample collection of highbush blueberry cultivars

| Denomination | Abbreviation | Maturity Stage | Fruit color (%) | Phenology ^b (%) |
|----------------------------|--------------|----------------|--------------------------|----------------------------|
| Ripening stage 1 | RS1 | Unripe green | 100% whitish green | 70–90 |
| Ripening stage 2 | RS2 | Unripe purple | ≥60% purple ^a | 5–20 |
| Ripening stage 3/Harvest 1 | RS3/H1 | Ripe blue | 100% blue | 50–70 |
| Harvest stage 2 | HS2 | Ripe blue | 100% blue | 20–40 |
| Harvest stage 3 | HS3 | Ripe blue | 100% blue | ≤15 |

^a At RS2 the coloration of berries varied from light pink to violet to a deep purple hue; the rest was still whitish green.

^b Approximate percentage range of berries on the bush at the particular maturation stage. Variations were due to cultivar differences.

sorted according to the progressive fruit development by the following parameters: colour changes, percentage of berries on the bush (phenology) at the particular maturity level; and considering, as well, the characteristic harvest begin of each of the analyzed cultivars observed in previous years at the experimental station in Berlin-Dahlem.

2.3. Sample collection

Fruit samples were harvested from each variety at weekly intervals (6–8 day) from late June till early August 2005 ($n = 186$ berries/variety/treatment). Especial attention was taken on picking berries of the appropriate maturity stage according to the chosen sorting classification (Table 1) and considering sample collection from all sections of the bush. Three replicates were collected per maturity stage and cultivar ($n = 60$).

2.4. Sample preparation

After picking the appropriate maturity and harvest stages, samples of each of the four cultivars were divided in two parts. One part of the samples was used immediately for determination of fresh weight, fruit colour, soluble solids and titratable acidity. The other part was shock-frozen in liquid nitrogen and kept at $-20\text{ }^{\circ}\text{C}$ before freeze drying (Christ Alpha 1–4, Christ; Osterode, Germany). Freeze-dried samples were ground for subsequent extraction and compound analysis.

2.5. Determination of fruit quality attributes

The average fresh weight of berries was determined by the quotient between the weight of whole berry samples and number of berries per sample. Soluble solids were determined using a hand refractometer (Leo Kübler GmbH, Germany) from the juice of 10 berries crushed with mortar and pestle. Five replications were conducted and values were adjusted to room temperature.

Titration of total acidity was conducted with 0.1 N NaOH until pH 8.1 (Microprocessor pH Meter 539 WTW) was expressed as percent total organic acid on basis of malic acid, being the predominant organic acid in blueberries. Berry samples ($n = 25$ –30 per treatment) were homogenised (Ultra-Turrax T25, Ika-Labor Technik, Staufen, Germany) for these determinations. Three grams of the blueberry homogenate was dissolved with 15 mL of distilled water.

2.6. Determination of the total phenolic content

According to Slinkard and Singleton (1997), total phenolic content in the fruit extracts were determined using the Folin–Ciocalteu method with results expressed as milligrams gallic acid equivalents (GAE) per gram of dry matter. Absorbance was measured at 765 nm (LKB-Novaspek II, Pharmacia, Freiburg, Germany). Extraction was con-

ducted following the method described by Connor, Luby, and Tong (2002) using acidified methanol (0.1% hydrochloric acid). 0.5 g grinded sample was mixed vigorously with 3 mL of acidified methanol (0.1% hydrochloric acid) and centrifuged for 15 min at 3000 rpm. Supernatant was collected in a 10 mL volumetric flask. The residue was treated again twice with 3 mL acidified methanol and 15 min centrifugation. Supernatants were collected and standardised to a final volume of 10 mL. Appropriate dilutions of extracts were prepared in duplicate with distilled water. Duplicate lecture of samples were measured spectrophotometrically at 765 nm. The extracts obtained were used for the determination of the total phenolic content, phenolic compound composition and antioxidant activity.

2.7. Analysis of the phenolic compounds using HPLC-DAD

In the present study, the concentrations of two major flavonoid subclasses, flavonols and anthocyanins, and one phenolic acid group, hydroxycinnamic acids derivatives, were determined by HPLC. An analytical Hewlett Packard 1100 series HPLC instrument equipped with an autosampler, quaternary HPLC pump and diode array detector was used. Analytical separation of the phenolic compounds was carried out on a 250×4.6 mm i.d., $5\text{ }\mu\text{m}$, Fluofix 120E column (Wako Pure Chemical Industries, Osaka, Japan) with a two solvent mobile phase (eluent A = water/acetic acid/acetonitrile (94.5/0.5/5 v/v/v); eluent B = acetonitrile). The eluent gradient used for all extracts was described as follows: 0–4 min, 0–4% B; 4–5 min, 4–2% B; 5–10 min, 2–4% B; 10–25 min, 4–8% B; 25–40 min, 8–22% B; 40–45 min, 22–28% B; 45–50 min, 28% B; 50–55 min, 28–45% B; 55–60 min, 45% B; 60–61 min, 45–0% B. The identification of phenolic compounds on the basis of the compound group's characteristic absorption wavelength was done according to Kähkönen, Hopia, and Heinonen (2001). Two milliliter aliquots of extract (50 mg DM mL^{-1}) were concentrated by drying with nitrogen and then re-dissolved to a volume of 500 μL with HPLC grade methanol. The injection volume was 20 μL , and the flow rate 1 mL min^{-1} . Anthocyanin (detection wavelength = 520 nm), flavonol (365 nm), and hydroxycinnamic acid (320 nm) contents were quantified as cyanidin-3-glucoside, rutin, and chlorogenic acid equivalents, respectively. Total concentration of each representative subclass compound was calculated from a calibration curve of the compounds mentioned above. Results were expressed as milligrams per gram dry matter (DM).

2.8. Determination of the antioxidant activity

2.8.1. Trolox equivalent antioxidant capacity (TEAC) assay

Antioxidant capacity was monitored using the trolox equivalent antioxidant capacity (TEAC) assay and measured by spectrophotometry (Specord 40, analytik jena AG, Jena, Germany) at 734 nm (method modified by Rohn, Rawel, & Kroll, 2004).

According to the total phenolic content, extracts are adequately diluted in duplicate with PBS buffer. A 500 μL aliquot of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, 500 mM L^{-1}) is added to 100 μL of diluted extract, or rather the blank value of PBS buffer, in disposable plastic cuvettes. To initiate the reaction, 200 μL of potassium persulphate are added to the samples in a regular interval of 15 s. The mixture developed a dark green colour and after 6 min, the reading of each cuvette was done again in the same regular time intervals. The procedure was also conducted for the Trolox calibration curve: 10 different concentrations of Trolox (0.05–4.5 mM/L) were used. Results were expressed as mM Trolox equivalents (TE) per gram DM.

2.8.2. Electron spin resonance (ESR) spectrometry

The degradation of a stable synthetic radical, Fremy's salt (potassium nitrosodisulfonate), in presence of antioxidants in the blueberry extracts was monitored with ESR as applied by Roesch, Bergmann, Knorr, and Kroh (2003). Appropriate extract dilutions were prepared and 100 μL aliquots were allowed to react for 5 min with an equal volume of a solution of Fremy's salt (1 mM in phosphate buffer, pH 7.4). ESR spectra of Fremy's radical were obtained with a Miniscope MS100 spectrometer (Magnetech GmbH, Berlin, Germany). The antioxidant activity expressed as mM Fremy's salt reduced by 1 g dried sample (mM per g DM), was calculated by comparison with a control reaction with 100 μL Fremy's salt 1 mM and 100 μL phosphate buffer.

2.9. Statistical analysis

The statistic calculations were performed with SPSS 11.0 by SPSS Inc. (2001). Significances of differences were conducted with a Tukey-HSD multiple-comparison test ($p \leq 0.01$).

3. Results and discussion

3.1. Fruit weight

During maturation and ripening there was a considerable increase in fruit weight. The highest increase in weight occurred in unripe berries (stages RS1 and RS2) until reaching ripeness (stage RS3/H1) (data not shown). This increase was in average more than the double (56%) of the unripe green berries. After reaching horticultural maturity and throughout successive harvest (stages HS2 and HS3), all cultivars decreased in average weight around 12% ('Puru' only 5% of weight loss, meanwhile 'Berkeley' 30%). Berries of the cultivar 'Bluecrop' were the largest of the four cultivars, whereas 'Reka', 'Puru' and 'Berkeley' were of similar size.

Blueberry fruit exhibits a double-sigmoidal curve in weight increase (Tamada, 2002). The maturity stages selected for this study represented more the second growth

period of blueberry fruit which leads to ripe fruits. This corresponded to the third stage of fruit development described by Gough (1991).

3.2. Fruit quality attributes

For the common assessment of horticultural maturity of many fruit crops such as blueberries, the ratio between the total soluble solids and titratable acidity, also called maturity index, was determined during maturation and ripening (Fig. 1).

During fruit ripening, soluble solids increased from 9% to 15% from the unripe to the ripe fruit stage (RS3/H1),

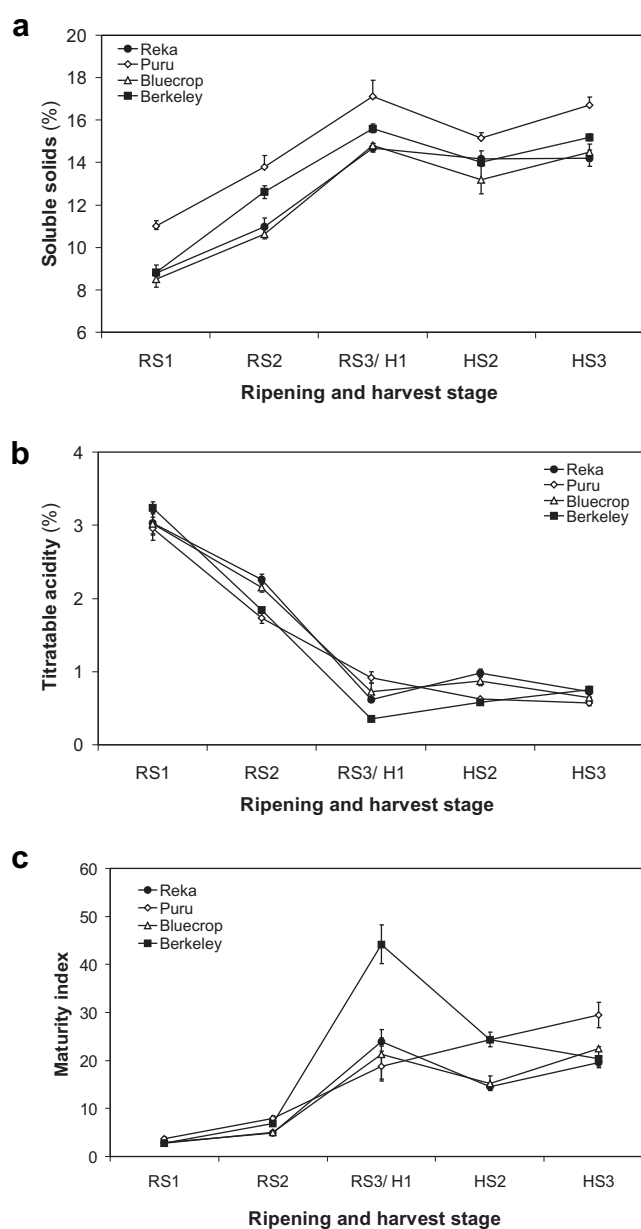


Fig. 1. Chemical composition of blueberry cultivars during fruit ripening and at selected harvest dates: (a) soluble solids, (b) titratable acidity, and (c) maturity index.

respectively. Meanwhile, titratable acidity was reduced to approx. 78%. For all cultivars a decrease in total soluble solids was observed after the first harvest with a subsequent increase thereafter until the third harvest date. At the first harvest date (RS3/H1) the highest values of soluble solids were found in ‘Puru’ reaching 17.1%, and the lowest in ‘Reka’ with 14.7%. Shutak, Hindle, and Christopher (1956) reported an increase in soluble solids of berries of the cvs. ‘Pioneer’, ‘Pemberton’, ‘Atlantic’, and ‘Dixi’ in different stages of development as they changed in colour from green to red to blue. They found a direct association between the differences among varieties and blueberry quality.

‘Berkeley’ had the second highest soluble solids values and due to the low percent of titratable acid, the maturity index ratio was the highest of all cultivars analyzed. This ratio doubled in average from the green berries to the purple coloured berries, and increased 4.5-fold till ripeness. At the first harvest the ratio soluble solids:titratable acidity ranged from 18.8 (‘Puru’) to 44.2 (‘Berkeley’). The significant higher value of ‘Berkeley’ is not surprising. This variety is characterized by its high soluble solids:titratable acidity ratio, resulting from a low acidity (Remberg, Rosenfeld, Haffnier, Gronnerod, & Lea, 2006).

For all cultivars, the maturity index ratio tended to decrease at following harvests, except for ‘Puru’ berries which by the third harvest date, increased to 36%. Berries reached horticultural maturity (RS3/H1) between 12 and 14 weeks after flowering (the last week of April 2005).

Pearson correlations were made for soluble solids and titratable acidity; these attributes correlated negatively ($r = -0.911$; $p \leq 0.01$). Consequently, the maturity index had also high correlations with both soluble solids ($r = 0.806$; $p \leq 0.01$), and titratable acidity ($r = -0.870$; $p \leq 0.01$).

3.3. Total phenolic content

The changes in total phenolic content during maturation and ripening are presented in Fig. 2. The extracts of all four cultivars had higher concentrations of total phenolic content at the maturity stage RS1 (unripe green berries). After this stage, total phenolic content decreased during colour break and ripening, particularly at stage RS2 (unripe purple berries). Then in cultivars ‘Reka’, ‘Bluecrop’ and ‘Berkeley’ total phenolics maintained stable till reaching horticultural maturity (RS3); but at successive pickings of full blue ripe berries, cultivars showed different patterns. ‘Reka’s total phenolic content decreased from RS3/H1 to HS3 (1. and 3. harvest, respectively) in almost half (48%), meanwhile the content of ‘Puru’ almost doubled by the third harvest (49%). The largest differences in total phenolic content among cultivars was found at the first harvest with values from 17.3 (‘Puru’) to 52.6 (‘Reka’) mg GAE/g DM. The average total phenolic content in ripe berries in the three harvest dates was 33 mg GAE/g DM.

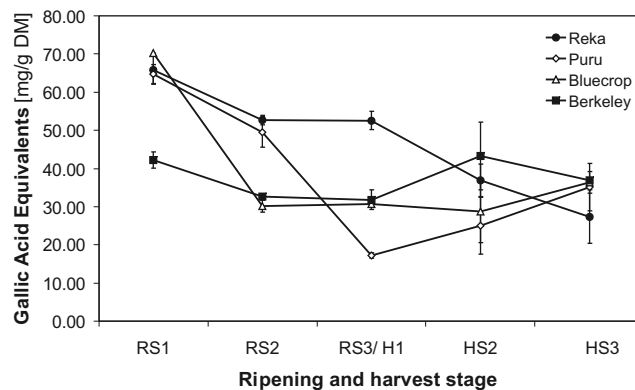


Fig. 2. Total phenolic content of blueberry cultivars as measured by the Folin–Ciocalteu method during fruit ripening and at selected harvest dates [mg gallic acid/g DM].

Kalt, Lawand, Ryan, McDonald, and Donner (2003) examined anthocyanins, total phenolic content and antioxidant activity of highbush blueberries (cvs. ‘Bergitta’, ‘Bluegold’ and ‘Nelson’) at different stages of ripeness: At comparable maturity stages as in the present study, total phenolic content ranged in average from 21.36 (green unripe berries) to 15.13 (ripe blue) mg GAE/g DM. A decrease during ripening was also observed. However, the values in the present study ranged in average from 60.76 (green unripe berries) to 33 (ripe blue) mg GAE/g DM. Ripe ‘Bluecrop’ berries had more than double total phenolic content (712 mg GAE/100 g FW at RS3/H1) than that reported by Moyer et al. (2002): They found 304 mg/100 g FW for ‘Bluecrop’ blueberries. Differences of the present results in comparison to the one reported in the literature may arise due to different cultivation and climate conditions or using different extraction solvents in sample preparation. The solvents used in the present study were optimized for the samples investigated and resulted in the highest phenolic content.

3.4. Phenolic compound composition

The profile of phenolic compounds of the investigated blueberry cultivars at different stages is shown in Fig. 3. From the phenolic compound groups analyzed, the hydroxycinnamic acids were the most predominant group at all stages of ripening. This was observed even when concentrations decreased in ripening berries. Total flavonol values at the first harvest date (RS3/H1) were less than half of those reported for five highbush blueberry varieties (Cho, Howard, Prior, & Clark, 2004). Flavonols showed significant differences only between the first two maturity stages, meanwhile hydroxycinnamic acids contents were significantly different between ripening stages RS1 and RS2, and between RS1 and the first harvest, RS3/H1. Anthocyanins were not expected to be found in green unripe berries. Kalt et al. (2003) reported almost undetectable anthocyanin contents in green unripe berries. Prior et al. (1998) reported anthocyanin contents of six highbush

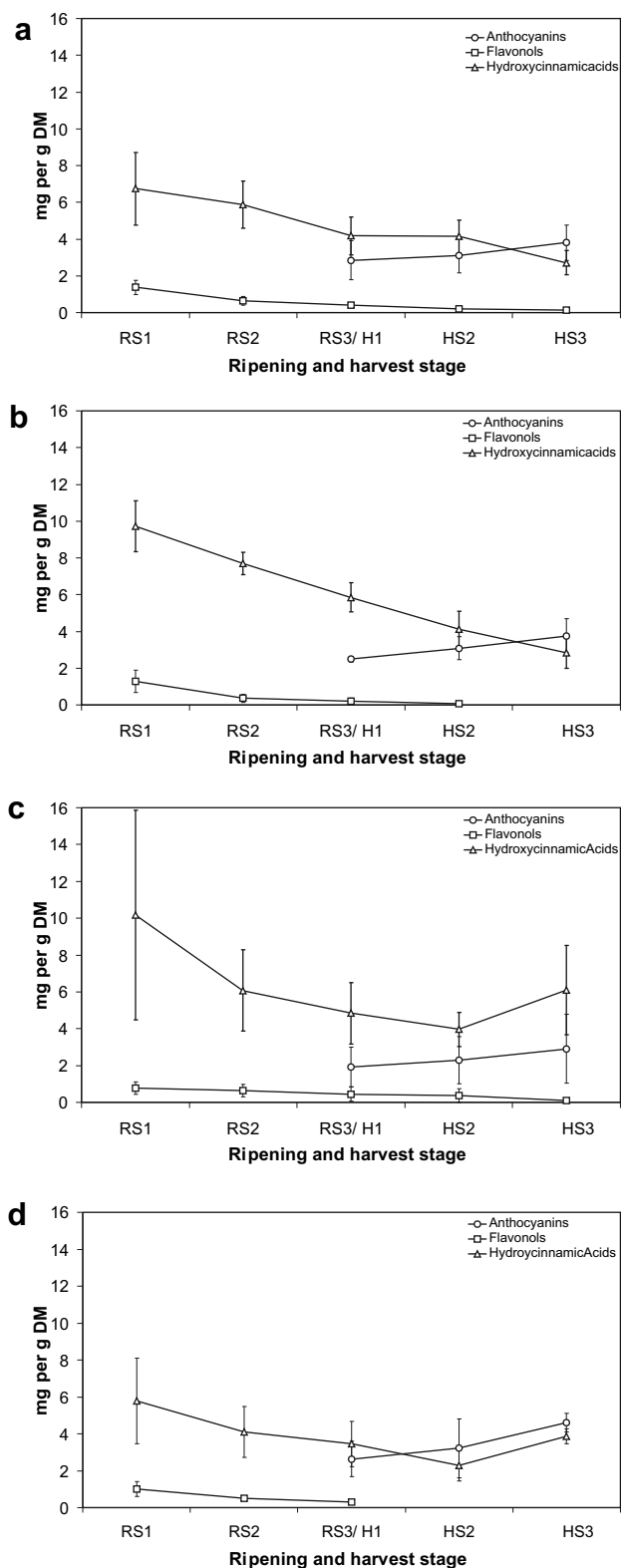


Fig. 3. Content of flavonoid subclasses (anthocyanins and flavonols) and phenolic acids (hydroxycinnamic acids) during fruit ripening and at selected harvest dates of different blueberry cultivars [mg phenolic compound per g DM]. (a) cv. Reka, (b) cv. Puru, (c) cv. Bluecrop, (d) cv. Berkeley.

blueberry varieties from 93 to 235 mg per 100 g FW. Nevertheless, at the harvest stages in the present study, antho-

cyanins were the second most prevalent phenolic subclass and concentrations were higher in extracts of successive picking dates. From the maturity stages RS3/H1 to HS3 total anthocyanins increased in average by 34.5%. During this period values ranged from 1.91 ('Bluecrop') to 4.61 ('Berkeley') mg cyanidin-3-glucoside equivalents per g DM. Between the first and third harvest date mean differences were significant. The lowest anthocyanin values were found in 'Bluecrop' which accordingly also had the berries with the lowest hue value (data not shown). The berries of the other cultivars had darker blue hue values, which are the result of the anthocyanin compound mix, concentration and pH (Clifford, 2000). Comparing the contents of flavonols to the total anthocyanins of thornless blackberry, no correlation had been found between the accumulation of flavonols and anthocyanins (Bilyk & Sapers, 1986). Gao and Mazza (1994) noted a relationship of anthocyanin accumulation with the berry size in different blueberry cultivars. Studies of 10 lowbush blueberry cultivars and hybrids showed that smaller berries had higher anthocyanin contents in comparison to larger berries of 'Bluecrop' highbush blueberries. Moyer et al. (2002) found that fruit size was highly correlated ($r = 0.84$) with total anthocyanin content in *V. corymbosum*, but was not correlated across eight other *Vaccinium* species. There was also no relationship between fruit weight and anthocyanin content among either 135 lowbush clones or the 80 highbush clones studied by Kalt, Ryan, et al. (2001). However, in the present study, anthocyanins which were found only in ripe berries correlated significantly with fruit weight ($r = -0.521$, $p \leq 0.01$). This may be partially explained by an increase in total anthocyanins with successive harvests, while fruit weight decreased on the other hand.

There is evidence that flavonoid biosynthesis is tightly associated with the development stages of the fruit. The enzyme activities are controlled in response to different developmental and environmental cues. Exemplarily, in bilberry (*Vaccinium myrtillus*) it has been demonstrated a coordinated expression of flavonoid biosynthetic genes in relation to the accumulation of anthocyanins, proanthocyanidins, and flavonols in developing fruits (Jaakola et al., 2002). In strawberry (*Fragaria × ananassa*) it has been shown that flavonoid biosynthesis has two distinct key flavonoid enzymes activity peaks during fruit ripening at early and late development stages (Halbwirth et al., 2006). This two phase flavonoid biosynthesis has also been observed in grapes. If this applies to other berry fruits, it may be speculated from our results of flavonoid and non-flavonoid content by HPLC that during maturation and ripening the first flavonoid biosynthesis peak corresponded to flavonols and hydroxycinnamic acids, while the second peak related to anthocyanin accumulation. This pattern can be observed to some extent also by changes in the total phenolic content (mg GAE/g DM) of the cultivars 'Puru' and 'Berkeley' where after a decrease in total phenolic content from the green berries (RS1) till horticultural maturity (RS3/H1); an increase in total phenolic content was observed at

successive harvest of ripe berries. Kalt et al. (2003) also reported an increase of anthocyanin content and a decrease of total phenolics during fruit ripening. They suggest that during the ripening of highbush blueberry fruits there is a shift in the pool of total phenolics toward anthocyanin synthesis, and an overall decline in the content of other phenolic components.

The HPLC analysis method developed detected almost no flavonols in ripe berries (RS3/H1, HS2, and HS3); nevertheless, other authors have reported quercetin glycosides in ripe berries: Bilyk and Sapers (1986) found likewise small quantities of quercetin in the blueberry cultivars ‘Earliblue’, ‘Weymouth’, ‘Coville’ and ‘Bluetta’.

3.5. Antioxidant activity during maturation and ripening

3.5.1. Trolox equivalent antioxidant capacity (TEAC) assay

The relative antioxidant activity as measured by the TEAC assay is presented in Fig. 4a. The antioxidant activity of blueberry extracts was in accordance to the results of total phenolic content for all cultivars studied. That means that the differences in total phenolic content between cultivars and maturity stages reflected differences in the obtained antioxidant activity by the TEAC assay (Figs. 2 and 4a). This is in accordance with the observations of Kalt et al. (2003) who assessed antioxidant activity in highbush blueberries during ripening with the ORAC assay.

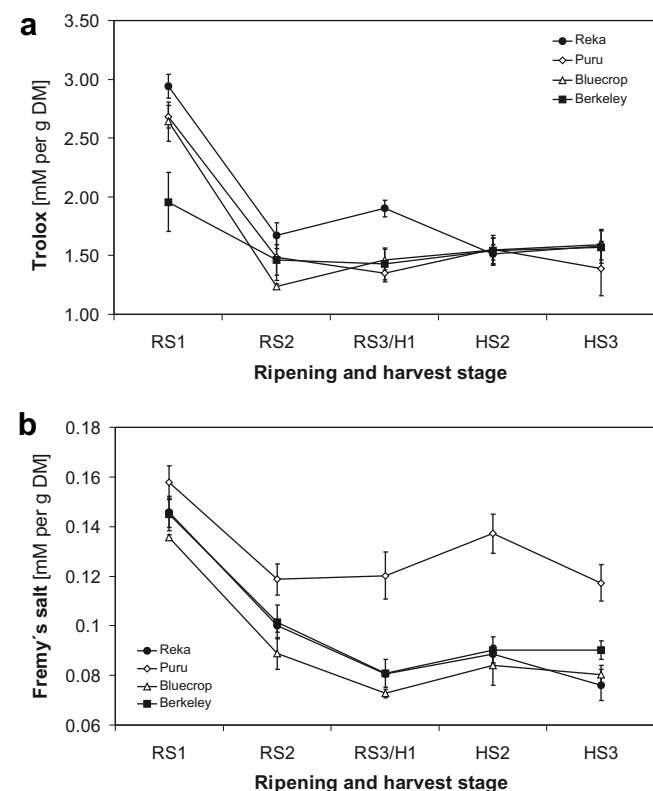


Fig. 4. Antioxidant activity of blueberry cultivars as measured (a) by the Trolox equivalent antioxidant capacity (TEAC) assay and (b) electron spin resonance (ESR) during fruit ripening and at selected harvest dates.

The antioxidant activity of the cultivar ‘Reka’, as well as ‘Bluecrop’ decreased from ripening stage RS1 to RS2 (43% and 53%, respectively). Towards RS3/H1, there was a slight increase in antioxidant activity (12% and 15%, respectively). During successive picking dates (RS3/H1 to HS3), ‘Reka’ tended to decrease in antioxidant activity ranging from 1.90 to 1.58 Trolox equivalents, meanwhile the other cultivars revealed small increases in antioxidant activity. In ‘Berkeley’, the antioxidant activity changed only to a small extent during maturation from the green unripe stage to the ripe purple one in comparison with the other cultivars, but increased also with successive picking dates. The light increase in antioxidant activity observed in ‘Bluecrop’ and ‘Berkeley’ may be due to the increased content of total anthocyanins at these harvest stages (Fig. 3).

Antioxidant activity measured with TEAC had a high correlation with total phenolic content calculated for all maturity stages and cultivars ($r = 0.828$; $p \leq 0.01$). The correlation remained unchanged when considering the results on a 100 g fresh weigh basis ($r = 0.718$; $p \leq 0.01$). The Pearson correlation coefficients between the antioxidant activity and the different phenolic compounds are summarized in Table 2.

The contribution of particular phenolic compound groups to the total antioxidant activity may vary. It was suggested that anthocyanins in lowbush and highbush blueberries contributed more to ORAC values than other phenolics (Kalt, Ryan, et al., 2001). The antioxidant activity results of the present study, although screened with another assay, showed that phenolic compounds other than the anthocyanins may have contributed positively to the total antioxidant activity. Decreasing contents of hydroxycinnamic acids during maturation and ripening mirrored more or less the pattern of antioxidant activity independent of increasing contents of anthocyanins during the harvest periods. Not to be underestimated in the total antioxidant activity is the contribution of the flavonols which among the flavonoids, are considered good antioxidants due to the particular substitution pattern of free

Table 2

Correlation coefficients (r) between phenol compound analysis (TPH and HPLC) and antioxidant activity assays (TEAC and ESR) on a 100 g fresh weight (FW) basis

| | TPH r ($n = 60$) | ACY r ($n = 35$) | FLAV r ($n = 48$) | HCA r ($n = 59$) | TEAC r ($n = 60$) | ESR r ($n = 60$) |
|------|-------------------------|-------------------------|--------------------------|-------------------------|--------------------------|-------------------------|
| TPH | – | 0.341* | 0.662** | 0.503** | 0.829** | 0.516** |
| ACY | | – | –0.459* | 0.010 | 0.397* | 0.259 |
| FLAV | | | – | 0.402** | 0.718** | 0.578** |
| HCA | | | | – | 0.506** | 0.499** |
| TEAC | | | | | – | 0.718** |
| ESR | | | | | | – |

*,** significant at $p \leq 0.05$, and 0.01, respectively.

TPH = total phenolic content, ACY = total anthocyanins; FLAV = total flavonoids; HCA = total hydroxycinnamic acids.

TEAC = Trolox equivalent antioxidant capacity; ESR = electron spin resonance spectrometry.

hydroxyl groups on the flavonoid skeleton (Roesch et al., 2003). The contribution of phenolic compounds other than anthocyanins to overall antioxidant activity in blueberry was also recognized by Connor, Luby, Hancock, Berkheimer, and Hanson (2002).

3.5.2. Electron spin resonance (ESR) spectrometry

The use of ESR applied to assessing antioxidant activity in food systems is not commonly, although its application might be advantageous. This accounts especially for blueberries, due their dark fruit colour which might influence other antioxidant activity assays (Rohn & Kroh, 2005). Antioxidants in blueberry acidified methanol extracts were assessed by electron spin resonance (ESR) spectrometry to evaluate their efficiency to reduce a synthetic free radical species, i.e. the semi-stable nitroxide radical Fremy's salt (potassium nitrosodisulfonate) (Fig. 4b).

By looking at the graphic of antioxidant activity by electron spin resonance (ESR) the results are not as comparable to those of TEAC when compared with the curve of total phenolic content. There is indeed a lower antioxidant activity during maturation and ripening from green unripe berries to ripe blue ones and small increases from one harvest date to the other. However, the antioxidant activity curves obtained by ESR of particular cultivars showed differences as compared with those by TEAC. Cultivar 'Puru' had a higher antioxidant activity measured by ESR than the other three cultivars, and revealed a peak at the second harvest. This peak at the second harvest (HS2) was also observed in the cultivars 'Reka' and 'Bluecrop'. The extracts of 'Reka', 'Bluecrop', and 'Berkeley' had a marked reduction in the efficiency of scavenging the Fremy's salt radical from the green unripe berries (RS1) to the ripe blue berries (RS3/H1), afterwards values remained comparatively unchanged.

Antioxidant activity measured with ESR had a medium correlation with that of TEAC calculated for all maturity stages and cultivars ($r = 0.647$; $p \leq 0.01$). The correlation increased when considering the antioxidant activity of both methods on a 100 g fresh weigh basis ($r = 0.718$; $p \leq 0.01$) (Table 2). As for total phenolic content, the correlation with ESR was both low on a dry weight basis ($r = 0.487$; $p \leq 0.01$) as on fresh weight basis ($r = 0.516$; $p \leq 0.01$) (Table 2).

It must be considered that these two antioxidant activity assays differed, among other things, first of all in the nature of the radicals used. Fremy's salt is a stable radical with a low deterioration rate and reactivity towards most compounds (Rohn & Kroh, 2005; Schwarz et al., 2001). Other authors have reported high correlations between total anthocyanins and/or total phenol content with different antioxidant screening methods like Moyer et al. (2002) who evaluated 15 *V. corymbosum* cultivars and hybrids with ORAC and FRAP assays. Roesch et al. (2003) studied structure-antioxidant efficiency of phenolic substances by ESR and found the highest antioxidant capacity (mol Fremy's salt per mol antioxidant) for the hydroxycinnamic

acids caffeic and chlorogenic acid (7.0 and 6.7 mol Fremy's salt per mol, respectively). The flavonol quercetin had an antioxidant capacity of 8.0 mol Fremy's salt per mol.

Fruits of the highbush blueberry cultivars examined were characterized, as it is common in ripening fruit, by increasing fresh weight gain, tissue softening, changes in soluble solids and titratable acidity, and colour changes during maturation and ripening. Therefore, the selected maturity stages represented an accurate picture of the physical and chemical changes occurring during this period of maturation and ripening of highbush blueberry irrespectively of the genetic differences of the varieties.

The colorimetric quantification of total phenolic content revealed a decline in total phenolic compounds particularly from green unripe berries to mature ripe berries of all blueberry varieties studied. However, the HPLC method developed provided more reasonable amount of information within a single analysis of the major flavonoid (flavonols and anthocyanins) and non-flavonoid (hydroxycinnamic acids) compound classes found in blueberry. The phenolic compound group with the highest concentrations comprised of hydroxycinnamic acids at all maturity stages analyzed regardless the genetic differences of the varieties. Thus, the total quantification of these major compound classes permitted a very adequate picture of phenolic metabolism during ripening. All highbush blueberry cultivars examined presented the same pattern of phenol biosynthesis characterized by decreasing flavonols and hydroxycinnamic acids at early maturation and through ripening, meanwhile accumulation of anthocyanins increased only during the successive harvest. On the one hand this might be associated with a concentration effect as fruit weight declined almost to the same extent. On the other hand it might be suggested that during ripening there was a shift in the pool of total phenolics toward anthocyanin synthesis. However, these changes occurred cultivar-specific and serve as valuable information for plant breeding traits.

The assessment of the antioxidant capacity of blueberry fruits during five stages of maturation and ripening using electron spin resonance spectrometry and TEAC assay revealed that antioxidant activity was strongly related to the total phenolic content ($r = 0.516$, $p \leq 0.01$) in all blueberry cultivars. Tendentiously, it was higher in early maturation and during initial pigmentation than at ripeness. This may be attributed to the higher concentrations of hydroxycinnamic acids and flavonols before ripening; whereas lower antioxidant activity of horticultural mature berries may suggest that anthocyanins have less antioxidant potential than other phenolic compounds such as flavonols which among the flavonoids are considered good antioxidants. However, it was found that antioxidant activity of fruits seems to be more influenced by genetic differences than physiological ripening changes. These findings will be investigated in more detail specifically in terms of the increasing focus on the impact on health promoting antioxidative compounds in fruit and vegetables during

food supply chain management. For associated breeding purposes the present results revealed that maturation and ripening processes as well as climate conditions appeared to have a more pronounced effect on flavonoid biosynthesis and phenolic composition in comparison to genetic differences of highbush blueberry varieties. Health promoting phenolic compounds other than anthocyanins, e.g. flavonols and hydroxycinnamic acids have contributed positively to the total antioxidant activity and thus might be implemented in further breeding study programs.

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