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Effects of Sampling Time and Nitrogen Fertilization on Anthocyanidin Levels in *Vaccinium myrtillus* Fruits

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Vaccinium myrtillus berries (bilberries) contain antioxidants, in particular anthocyanins, which are secondary metabolites that have proven health-promoting effects. Bilberries were collected at the Svartberget research forest in northern Sweden from plots with no, low, and high applications of NH_4NO_3 on three replicated dates in each year from 2005−2007, and their anthocyanidin contents were analyzed by high performance liquid chromatography. Their mean total anthocyanidin contents were 9.0, 6.2, and 22.7 mg/g DW in 2005, 2006, and 2007, respectively. The values were significantly higher in 2005 than in 2006 and significantly higher in 2007 than in both previous years, across all three sampling dates. In addition, anthocyanidin contents were significantly affected by sampling date in all years ($P < 0.001$); they were linearly correlated with the thermal sum in 2005 and 2007 but rose between the first and second sampling occasions and subsequently declined in 2006. No significant effect of nitrogen fertilization on total anthocyanidin levels was detected in any of the studied years. The results indicate that climatic factors and yearly fluctuations influence anthocyanidin biosynthesis and degradation more strongly than nitrogen availability. To our knowledge, this is the first time this effect of sampling time on anthocyanins in mature bilberries has been shown.

KEYWORDS: Aglycone; anthocyanin; antioxidant; bilberry; boreal; climate; cyanidin; delphinidin; fertilizer; malvidin; NH_4NO_3; petunidin; peonidin; *Vaccinium myrtillus*

INTRODUCTION

Free oxygen radicals or reactive oxygen species (ROS) are involved in several types of human diseases, for example, cardiac and vascular diseases (*1*), various types of cancer (*2*, *3*), and eye diseases (*4*). Thus, substances with the capacity to reduce ROS, known as antioxidants, are considered to be essential components of the human diet. Antioxidants are abundant in many fruits and vegetables (*4*), and particularly potent sources are bilberries (fruits of *Vaccinium myrtillus* L.), which have high contents of a number of health-promoting compounds, including antioxidants such as anthocyanins and vitamin C, and thus may be excellent ingredients of functional foods. For instance, bilberry enriched diets have been shown to enhance cell survival in the treatment of Parkinson's disease (*5*).

The *Vaccinium* genus is widespread throughout the world, being represented by more than 200 species of woody plants, including many economically important cultivated small fruit species. *Vaccinium myrtillus* is a long-lived ericaceous dwarf shrub that grows wild in Europe and Asia, although it is most abundant in northern and eastern Europe. Both the plant and the berry it produces are commonly known as bilberry; therefore, hereafter the berries are referred to as bilberries and the plant solely as *V. myrtillus* for convenience. Bilberries contain several substances with antioxidant capacity. The most interesting of these substances are the anthocyanins, flavonoids that give the mature bilberries their characteristic blue color. Like other flavonoids, anthocyanins have a C6−C3−C6 flavone skeleton and are glycosylated phenolic compounds that release their corresponding aglycones, called anthocyanidins, upon hydrolysis. The aglycones of anthocyanins are usually analyzed rather than the parent compounds since the sugars attached to the anthocyanidins can interfere with their analysis (*6*). Anthocyanins are plant secondary metabolites (*7*, *8*), and both their concentration and function within plants are influenced by several abiotic and biotic factors (*9*). For example, UV light

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has been shown to influence the content of flavonoids in several types of plants (*10*, *11*) and the total phenolic content (*12*) in *V. myrtillus*. In addition, rates of anthocyanin synthesis and degradation are correlated with temperature (*13*), and total phenol contents of *V. myrtillus* shoots and leaves tend to be increased by high water availability during the growth season (*14*).

Nitrogen (N) fertilization, and thus plant N status, may also affect berry chemistry. N fertilization has well-documented effects on boreal forest ecosystems in terms of tree growth and shifts in ground vegetation (*15*, *16*), and some information is also available on the effects of fertilization on ground vegetation chemistry in boreal forests (*17*). However, there are conflicting reports regarding the effects of N fertilization on the plants' antioxidant concentrations and capacities. For example, Azaizeh et al. (*18*) found a fertilizer concentration-dependent increase in antioxidant activity in *Teucrium polium*, while studies on other plant species did not detect any significant connections between fertilization and either antioxidant activity (*19*) or flavonol content (*20*). In addition, Okamoto et al. (*21*) found higher anthocyanin concentrations in samples of grape skins from moderately N fertilized plots (1.5 times higher than normal levels for the studied site) than in samples from both unfertilized and more heavily fertilized plots (2.0 times higher than normal levels for the studied site).

The purposes of the study presented here were to evaluate the effect of sampling time and the possible influence of N fertilization on anthocyanidin concentrations in bilberries. Hence, the following hypotheses were tested: the relative contents of anthocyanidins in mature berries will increase (i) with picking time during the season, and (ii) with N-fertilization.

MATERIALS AND METHODS

The experiment was conducted in a late successional Norway spruce (*Picea abies* L.) forest in the Svartberget research forest (*Picea abies* - *Vaccinium myrtillus* type (*22*)) at Vindeln, Sweden (64° 14′ N, 19° 46′ E), where a long-term fertilization experiment was established in 1996. Since the beginning of the experiment, granulate NH_4NO_3 has been applied to plots of various sizes, at levels of 12.5 (N1) or 50.0 (N2) kg ha^{-1} $year^{-1}$, at the start of each year's growing season, while other plots have been left unfertilized as controls (C). For further details on the experimental site and design see refs *23* and *24*. The experimental area consisted of approximately 1 ha of closed canopy forest with a slightly sloping forest floor and good access to lateral groundwater. It was divided into two blocks, with differing degrees of water availability as judged by the ground vegetation, and each block contained three randomly scattered plots per treatment (C, N1, and N2). To reduce the risk of false conclusions due to variations in the plants' responses associated with genetic variations, small fixed sampling spots of 50 cm radii were used within the plots rather than scattered random sampling. The repeated samplings over time, see below, were consequently made from plants within the same sampling spot. In addition, plants from within the same sampling spots were selected during each sampling period, as follows. In 2005, one sample spot was laid out per treatment plot, with an additional sample spot added per treatment plot during early spring 2006. However, visual inspection on the first sampling date 2006 indicated that the pollination success was low, and three additional sampling spots were selected in each block (two in N1 and one in N2 plots). All of the established spots were sampled in 2007. At least 10 berries of a uniform dark blue color were selected from each sampling spot on three occasions in each year (27th of July, and 11th and 25th of August 2005; 24th of July, and seventh and 21th of August 2006; and 24th of July, and seventh and 22nd of August 2007). However, it was not always possible to obtain berry samples from all of the spots at each sampling date (see **Table 1** for numbers of samples taken per block, treatment, and sampling date each year).

Immediately after harvesting, berries were put in sealable plastic bags, which were placed in an ice-filled cooler for transportation to the laboratory, whereupon they were transferred to a freezer and stored at −20 °C until analyzed. In addition, a weather station within the experimental site automatically collected climatic data, air temperature (°C), relative air humidity (%), precipitation (mm), and light radiation (MJ), every 10 min at a point 1.7 m above the ground.

Table 1. Number of Samples Collected at Each Sampling Date (Date 1/Date 2/Date 3) and Year (2005, 2006, and 2007), per Nitrogen Treatment (C, N1, and N2) and Block (I and II)

treatment	block	2005	2006	2007
C	I	3/2/3	5/5/6	4/4/3
	II	3/2/3	5/6/6	2/4/4
N1	I	3/1/2	6/8/8	6/7/5
	II	3/3/3	4/5/6	4/5/4
N2	I	3/3/3	4/7/6	4/3/3
	II	2/2/2	7/7/6	3/4/6
total		17/13/16	31/39/38	22/27/25

All extractions and analyses were performed at the Swedish University of Agricultural Sciences (SLU), Department of Plant Breeding and Biotechnology, Balsgård. For analysis, 8 to 10 berries from each sample (except for one control treatment sample, collected on the 24th of July 2006, for which only five berries could be obtained) were selected and placed in a plastic jar prior to fresh weight measurements. The material from 2005 was then freeze-dried in a laboratory freeze-drier (Edwards, Moduylo) for 24 h before being finely hand milled with a glass piston (method FDG 1). In contrast, material from 2006 and 2007 was freeze-dried in a semi-industrial freeze-drier (Ehrist, LC3) for seven days prior to being finely milled with an electric grinder (IKA A10, Yellow line) (method FDG 2). Eighteen samples from 2005 were also treated in the same way as samples from 2006 and 2007, thereby allowing a comparison of the different freeze-drier and milling methods. Fifty milligrams of the milled powder from each sample was then transferred to sampling tubes (Sarstedt, 13 mL) and mixed with 2 mL of 2 M HCl to extract the five anthocyanidin compounds known to occur in bilberries: (delphinidin, cyanidin, petunidin, peonidin, and malvidin (*9*)).

The anthocyanidin extraction protocol was based on that described in 2001 by Nyman and Kumpulainen (*25*), after optimizing the hydrolysis time. The results obtained using a range of hydrolysis durations indicated that 40 min incubation at 90 °C, in a water bath, were optimal for bilberry anthocyanidin extraction (rather than the 50 min at 90 °C proposed in the cited article); therefore, these hydrolysis conditions were used for further extractions. After a single hydrolysis, the amounts of anthocyanidins extracted were ≥95% of the amounts extracted by two repeated hydrolyses; therefore, single hydrolyses were regarded as providing quantitative yields. The hydrolyzed samples were filtered (Micron, Nylon 0.45 nm), 0.8 mL of each filtered sample was transferred to a 1.5 mL amber glass vial (VWR), and the vials were placed in a Kontron Instruments 460 HPLC autosampler. The amounts of delphinidin, cyanidin, petunidin, peonidin, and malvidin in each sample were then determined by HPLC, using a Shimatzu 460 system with a SPD-M10Avp diode array detector and a Phenomenex Synergi 4 μm Hydro-RP 80A, 250 × 4.60 mm column. Gradient elution was performed using 10% formic acid (A) and 100% acetonitrile (B) from 96:4 (A/B) to 20:80 (A/B) and at time intervals of 0, 8, 23, 24, 33, and 37 min. Flow rate was 0.8 mL/min, and the injection volume was 10 μL for all samples. The wavelengths used for quantification were 530 nm for delphinidin and 510 nm for cyanidin, petunidin, peonidin, and malvidin. Standard curves were prepared using pure anthocyanidins supplied by Extrasynthese, Lyon, France, dissolved in acidic MeOH (0.1% HCl) in four serial dilutions, with concentrations ranging from 9.0 to 291.0 μg/mL, depending on the anthocyanidin. The identities of the compounds corresponding to putative anthocyanin peaks in the resulting chromatograms were confirmed by comparing their spectra and retention times with those of the pure compounds. Total anthocyanidin contents in the samples were calculated as sum contents of all five anthocyanidins analyzed, and amounts are reported as mg/g dry weight (DW).

The effects of sampling time, N fertilization level, and preparation method on the measured anthocyanidin levels were tested using SPSS

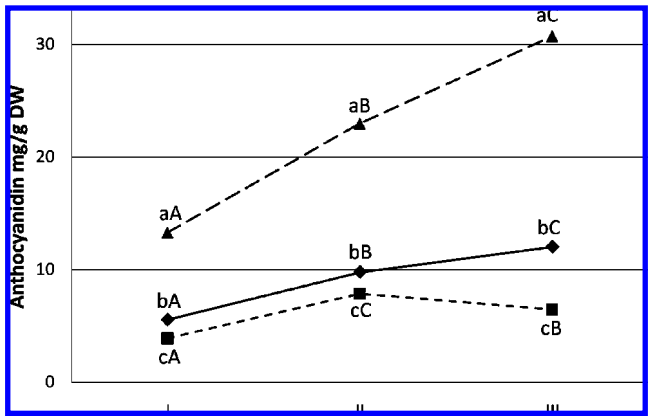

Figure 1. Total anthocyanidin levels (mg/g dry weight) in bilberries harvested on the three sampling occasions in 2005 (-◆-), 2006 (-■-), and 2007 (-▲-). Significant between-year differences at the 95% probability level at specific sampling dates, irrespective of nitrogen treatment, are indicated by lowercase letters (a,b,c), and significant within-year differences by capital letters (A,B,C).

16.0 and Minitab 15.0 for Windows software. All data from 2005 and 2006 were square root transformed prior to analysis, while the data from 2007 were $-\log_{10}$ transformed, to meet the distribution and homogeneity assumptions of univariate general linear models (GLM). The effects of the two different sample preparation methods were examined by comparing the total bilberry anthocyanin levels using a balanced ANOVA, with preparation method (FDG 1 and 2), sampling date (*1*−*3*), and treatment (C, N1, and N2) as factors. Field data were tested statistically for variations in delphinidin, cyanidin, petunidin, peonidin, malvidin, and total bilberry anthocyanidin levels using the GLM (unbalanced ANOVA) procedure, with year (2005, 2006, and 2007) and block (I and II) as random factors, and sampling date and treatment as fixed factors. Data from each year were also examined separately in a similar manner. Tukey's post hoc tests were used to determine differences between means at the 95% probability level. Despite the data transformation, variances in individual and total anthocyanidin levels proved to be nonhomogenous when data for all of the studied years were tested together and for some compounds when data for 2006 and 2007 were examined in isolation. Hence, a robust one-way ANOVA was used to examine the total and individual anthocyanidins, and the Tukey post hoc test was complemented with Tamhane's T2 test (equal variances not assumed), to reduce the risk of potential type I and II errors. Climate data were also used in a regression analysis of total anthocyanidin contents on the accumulated thermal sum (°C) in SPSS 16.0, using linear curve estimation.

RESULTS

There were no significant differences in measured anthocyanidin levels between replicate samples prepared using the two different preparation methods ($P = 0.147$, data not shown). However, total anthocyanidin concentrations significantly differed between years (**Figure 1** and **Table 2**), with means of 8.99, 6.20, and 22.73 mg/g DW in 2005, 2006, and 2007, respectively (**Table 3**). Furthermore, the anthocyanidin levels were significantly higher in 2007 than in the other years and significantly higher in 2005 than in 2006 for all sampling occasions (**Figure 1**). However, in 2005 and 2007 anthocyanidin levels successively increased from the first to second and from the second to third sampling dates, whereas in 2006, the levels were highest at the second date (**Figure 1** and **Table 3**). The relative concentrations of individual anthocyanidins were similar between years (**Table 3**), with delphinidin being the most abundant, accounting for 40−44% of the total content, followed by cyanidin (28−34%), while malvidin, petunidin, and peonidin contents were ca. 12% or lower. The individual anthocyanidin levels also generally followed the same pattern as the total anthocyanidin levels in each year. Furthermore, the only anthocyanidins that did not differ significantly in concentration between sampling dates 2 and 3 were delphinidin and cyanidin in 2005; malvidin in 2006; and delphinidin in 2007 (**Table 3**).

Table 2. Summary Statistics (GLM Unbalanced ANOVA Complemented with a Robust One-Way ANOVA When Values Were Nonhomogenous) from the Analysis of Total Anthocyanidin Levels in Bilberries, with Year (2005, 2006, and 2007), Treatment (Control and Nitrogen Loads of 12.5 and 50.0 kg ha^{-1} $year^{-1}$), and Sampling Date (*1*−*3*) as Factors

all years	df	mean square	*F*-value	*P*-value
year	2	5342.12	26.61	0.017
block	1	251.11	1.18	0.396
treatment	2	41.13	8.44	0.674
date	2	121.61	10.86	0.046
year · treatment	2	25.86	3.24	0.107
year · date	2	331.25	3.57	0.158
treatment · date	4	9.67	2.36	0.613
2005				
block	1	6.11	0.47	0.495
treatment	2	20.10	1.62	0.169
date	2	182.85	37.68	<0.001
treatment*date	4	0.20	1.94	0.125
2006				
block	1	0.02	0.01	0.951
treatment	2	3.37	0.62	0.319
date	2	137.24	47.48	<0.001
treatment · date	4	0.04	0.37	0.839
2007				
block	1	1208.10	12.61	0.001
treatment	2	44.74	0.40	0.387
date	2	1783.67	27.90	<0.001
treatment · date	4	0.02	0.90	0.475

There was no significant sampling date−N-treatment interaction or N-fertilization effect on the total anthocyanidin levels in any of the years (**Table 2**). The highest mean total anthocyanidin levels in each year were observed in the berries from N2 plots in 2005 and 2007, and from the N1 plots in 2006 (**Table 3**).

Mean bilberry water contents were 88% in 2005 and 2007, and 81% in 2006. Mean dry weight (g) at the different sampling dates (1/2/3) were 0.51/0.46/0.54, 0.46/0.41/0.43, and 0.59/0.49/0.49 in 2005, 2006, and 2007, respectively. In addition, the collected climate data showed that air temperatures and solar radiation levels were generally higher, while precipitation and relative air humidity were generally lower, in the May−September period of 2006 than in the corresponding periods of 2005 and 2007 (**Figure 2**). Total anthocyanidin contents increased linearly with the thermal sum (calculated from May first) in 2005 ($R^2 = 0.919$; $P < 0.001$; $Y = 0.017x - 10.65$) and 2007 ($R^2 = 0.969$; $P < 0.001$; $Y = 0.040x - 24.18$), but they decreased between the second and third dates of 2006 ($R^2 = 0.104$; $P = 0.104$; $Y = 0.005x - 0.70$) (**Figure 3**). Data from all of the studied years were then combined in a regression analysis of total anthocyanidin content on accumulated thermal sum, and this gave a good quadratic fit ($R^2 = 0.889$; $P = 0.001$; $Y = -1310 + 2.267x - 0.000847x^2$) with a maximum at around 1350 degree sums. Furthermore, sequential analysis of the quadratic curve showed that the linear and quadratic parts of the curve were of roughly equal importance (linear $P = 0.006$ and quadratic $P = 0.016$) (**Figure 3**).

DISCUSSION

The content of phenolic compounds in a fruit varies strongly depending on the species and maturity of the fruits (which is

Table 3. Overall Mean Specific and Total Anthocyanidin Concentrations (mg/g Dry Weight) from Bilberries Sampled during 2005, 2006, and 2007[a]

	delphinidin	cyanidin	petunidin	peonidin	malvidin	total
			2005			
sampling date						
1	2.61 a	1.73 a	0.53 a	0.20 a	0.40 a	5.47 a
2	4.36 b	2.80 b	1.08 b	0.50 b	1.16 b	9.90 b
3	5.11 b	3.07 b	1.39 c	0.67 c	1.74 c	11.99 c
N-treatment						
C	3.50 a	2.29 a	0.87 a	0.42 a	0.97 a	8.04 a
N1	3.99 a	2.28 a	0.99 a	0.41 a	1.06 a	8.72 a
N2	4.47 a	2.95 a	1.10 a	0.53 a	1.22 a	11.27 a
total						
mean	3.97	2.5	0.98	0.45	1.08	8.99
%	38.0	27.9	10.9	5.0	12.0	100.0
			2006			
sampling date						
1	1.19 a	1.43 a	0.34 a	0.18 a	0.24 a	3.87 a
2	2.58 c	2.42 c	0.87 c	0.49 c	0.90 b	7.81 c
3	1.97 b	1.98 b	0.72 b	0.44 b	0.79 b	6.43 b
N-treatment						
C	1.91 a	1.94 a	0.66 a	0.38 a	0.67 a	6.11 a
N1	2.03 a	2.11 a	0.70 a	0.42 a	0.73 a	6.53 a
N2	1.97 a	1.89 a	0.63 a	0.35 a	0.62 a	5.95 a
total						
mean	1.97	1.98	0.67	0.38	0.67	6.20
%	31.8	31.9	10.8	6.1	10.8	100.0
			2007			
sampling date						
1	5.27 a	5.06 a	1.29 a	0.47 a	0.89 a	13.39 a
2	9.04 b	7.62 b	2.55 b	0.95 b	2.22 b	22.83 b
3	11.32 b	9.87 c	3.61 c	1.68 c	3.95 c	30.85 c
N-treatment						
C	7.95 a	7.26 a	2.29 a	0.98 a	2.11 a	20.98 a
N1	9.40 a	7.32 a	2.70 a	0.97 a	2.54 a	23.36 a
N2	8.43 a	8.34 a	2.55 a	1.22 a	2.53 a	23.51 a
total						
mean	8.69	7.62	2.54	1.05	2.41	22.73
%	38.2	33.5	11.2	4.6	10.6	100.0

[a] Different letters (a,b,c) represent significant differences at the $P < 0.05$ probability level between sampling dates and between treatments.

why only berries with a uniform blue skin color were analyzed in this study). For instance, several studies have shown that concentrations of various phenolic acids in blackberries and strawberries decline as they mature (*26*). In contrast, anthocyanins in small red fruits have been found to accumulate at the end of their maturation phase (*27*). In addition, Prior et al. (*6*) found that anthocyanin and total phenolic contents in mature blueberries were higher in the later of two sets of samples collected 49 days apart. Our results from 2005 and 2007 are consistent with this observation. However, the anthocyanidin levels had decreased by the third sampling date in 2006, indicating that the rate of degradation was greater than the rate of synthesis of anthocyanins between sampling dates 2 and 3 during this year. Prior et al. (*6*) did not analyze berries they considered overmature, and we believe that a decline in anthocyanidin concentrations may also have been observed in 2005 and 2007 if berries had been collected at a later date. This hypothesis is supported by the fact that the thermal sum from the first of May to the last sampling date in 2006 (21st of August) was not reached until the third of September in either 2005 or 2007, 13 days later than in 2006 (**Figure 3**). The higher temperatures and lower relative air humidity in 2006 compared with that in 2005 and 2007 (**Figure 2**) might have accelerated the bilberry maturation process in 2006. This may have been due to late-maturation degradation processes and/or active processes in which carbon is transferred to the seeds and/or sugars incorporated into plant vegetative tissues as part of the

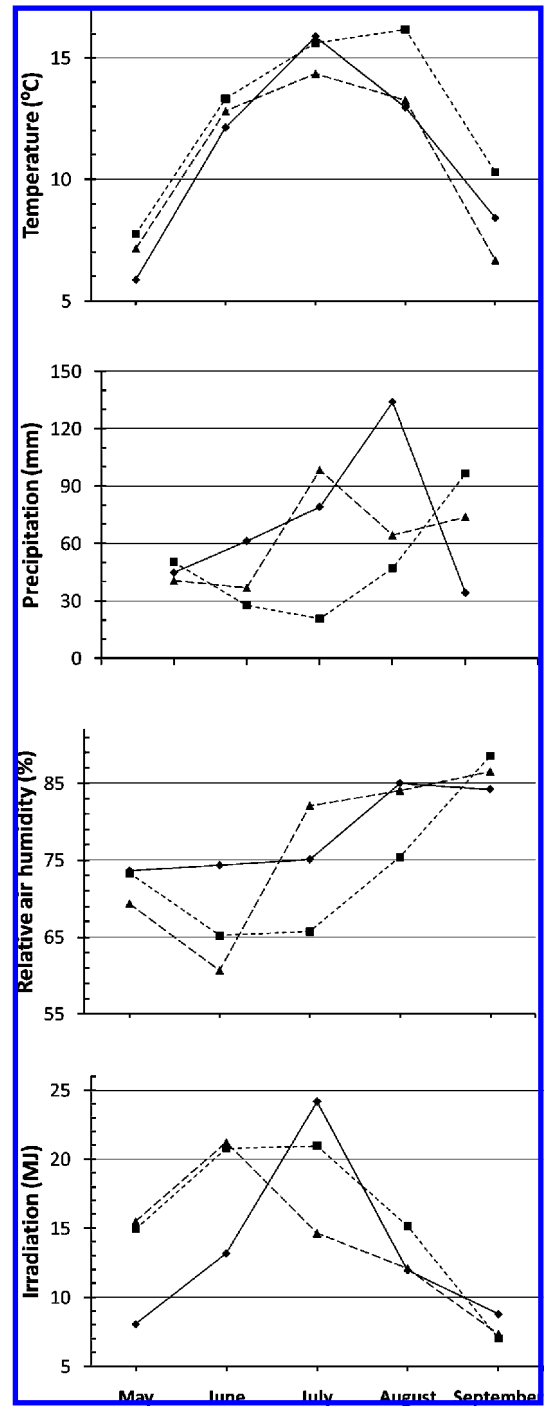

Figure 2. Monthly means of air temperature (°C), precipitation (mm), relative air humidity (%), and irradiation (MJ) over the vegetative growth periods (May−September) in 2005 (-◆-), 2006 (-■-), and 2007 (-▲-). Data obtained from Svartberget research station, Vindeln, Sweden.

plants' frost protection mechanisms. In such a situation, it would be impossible to judge the degree of bilberry maturity with the naked eye. It also suggests that the state of maturity varies sufficiently to be reflected in the results, even within the relatively short time period when berries have a uniform blue color. In addition, the strong influence of the sampling date highlights the need to be very careful when comparing anthocyanin concentrations between and within species on unspecified dates, especially if fruits are sampled at various sites, or no climate data are available to support the results.

The N content in the vegetative tissues of *V. myrtillus* has been shown to increase in response to N addition in the studied

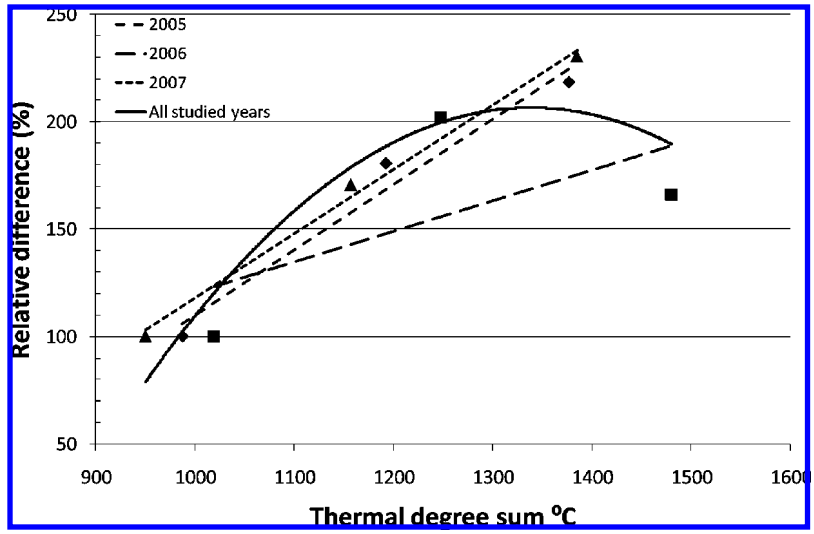

Figure 3. Linear regressions of the anthocyanidin content yearly mean at specific sampling dates (% difference from first sampling date) on the thermal degree sum (calculated as the accumulated daily mean temperature above 5 °C from 1st of May) for 2005 (◆), 2006 (■), and 2007 (▲). Quadratic regression line based on data from all studied years (—) is also shown.

experimental area (*28*). However, anthocyanin concentrations of the berries were not significantly affected by N fertilization in our study. This might indicate a chain of priority since an increase in available N may not result in additional anthocyanin biosynthesis in reproductive tissues if a plant already has sufficient contents of these compounds. Instead, the extra N may, for example, be used in biomass production or stored for the following year's growth as indicated in a study from 2002 by Strengbom et al. (*28*). The lack of an N effect may also be caused by restrictions in water availability due to reductions in microbial activity (*29*) and/or soil nutrient mobility (*30*). For instance, the *V. myrtillus* plants may not have been able to take up as much N in 2006 as in 2005 and 2007. However, this does not explain the lack of N treatment effect on anthocyanidins in 2005 and 2007, which have to be considered as wet years. Overall, these considerations emphasize the importance of controlled growth conditions, especially if bilberry anthocyanin levels are to be optimized in agricultural production.

The biosynthetic pathways of phenolic compounds in *V. myrtillus* are quite well understood (*31*). It is also known that the biosynthesis and accumulation of secondary compounds are affected by both endogenous and environmental factors (such as CO_2 concentrations, light, and temperature), which influence various responses such as stomatal opening and closure (*32*). In C3 plants, stomatal closure causes the light reactions that form ATP and reduce $NADP^+$ to cease; hence, no sugars can be synthesized that could be used for the biosynthesis of secondary metabolites. It is possible that this cost of producing secondary metabolites mediates endogen-dependent fluctuations in the abundance of these substances in plants. In 2008, Wilber and Williamson (*33*) detected between-year variations in the yield and vegetative growth of two highbush blueberries in a fertilizer treatment experiment that could not be explained by variations in climate factors. Thus, the between-year differences we observed in the mean bilberry anthocyanidin concentrations in this study are likely to be natural fluctuations. The abundance of *V. myrtillus* in Swedish forests has been shown to be negatively correlated with N deposition (*17*). Furthermore, it has been reported that N addition to forest soils leads to the accumulation of N-based metabolites in the vegetative tissues of *V. myrtillus* (*17*, *28*). In contrast, Glynn and Herms (*34*) found reduced concentrations of phenolic compounds in forest trees after N addition. The hydrolyzed bilberries in the present study had characteristic anthocyanidin profiles, with five components in similar relative proportions to previous findings (*25*). Previously, anthocyanin contents of between 200 and 600 mg/100 g fresh weight (fw) (*6*, *25*) and anthocyanidin contents of 360 mg/100 g fw (*25*) have been reported in bilberries. Anthocyanidin concentrations were quantified on a dry weight basis in this study, and after conversion to fresh weight equivalents, the results for 2005 and 2006 were slightly lower than the cited values (188.8 and 130.4 mg/100 g fw for 2005 and 2006, respectively), while the values for 2007 were in the upper level of previous measurements (500.0 mg/100 g fw). Bilberry water contents of 80−85% have also been reported (*29*), which are in the same range as those found in this study. Since the method used in this study for determining bilberry anthocyanin concentrations has been shown to provide reliable quantitative results, the low total anthocyanidin levels measured during 2005 and 2006 are unlikely to be underestimates due to the use of inappropriate techniques. Instead, the observed low values from 2005 and 2006, and the high values from 2007 are more likely due to naturally occurring annual fluctuations of anthocyanin concentrations in combination with the effects of different weather conditions.

In conclusion, this study demonstrated that time (sampling date) and climatic conditions strongly influence anthocyanin concentrations of mature bilberries, while N fertilization has no significant effect (within the range of studied doses). Further studies in controlled environments and the field will provide valuable information on the influence of temperature and light on the antioxidant concentrations in mature bilberries.

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