Anthocyanin profile in berries of wild and cultivated *Vaccinium* spp. along altitudinal gradients in Alps.

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Abstract

Vaccinium spp. berries provide one of the best natural sources of anthocyanins. In the wild bilberry (*V. myrtillus* L.), a clear increasing trend in anthocyanin biosynthesis has been reported towards northern latitudes of Europe, but studies related to altitude have given contradictory results. The present study focused on the anthocyanin composition in wild bilberries and highbush blueberry (*V. corymbosum* L., cv. Brigitta Blue) growing along altitudinal gradients in the Alps of Northern Italy. Our results indicate an increasing accumulation of anthocyanins in bilberries along an altitudinal gradient of about 650 m. The accumulation was due to a significant increase in delphinidin and malvidin-glycosides, whereas the accumulation of cyanidin and peonidin-glycosides was not affected by the altitude. Seasonal differences, especially temperature, had a major influence on the accumulation of anthocyanins in blueberries.

Keywords: alpine environment, altitudinal gradient, anthocyanins, bilberry, blueberry, light, temperature, *Vaccinium*

Introduction

In recent years, the health benefits of *Vaccinium* berries, e.g. blueberries, cranberries and bilberries, have gained visibility for the highest antioxidant activity linked with the high content of phenolic compounds, especially anthocyanins (ACs), compared with other fruits and vegetables[1]. These compounds have been reported to have various health beneficial activities including antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative and antimicrobial effects[2].

Wild berries belonging to the genus *Vaccinium* such as bilberry, *V. myrtillus* L. and lingonberry, *V. vitis ideae* L. are a valuable part of the European nature and tradition, especially in the Northern and Eastern parts of Europe where they grow abundantly and have economic importance. However, both bilberry and lingonberry are also distributed within alpine environments of Southern Europe, covering large areas of the Italian mountains, which are characterized by acidic soils[3]. Bilberry, particularly, establishes on a wide range of environmental conditions, from hilly areas to high altitudes above tree-line, although, its optimal range is between 1500 m and 2000 m above sea level (a.s.l.), where pure bilberry formations may occur both in open habitats and in the understory of conifer-dominated forests[4].

In Italy, bilberry is collected in natural environments of the Apennines and Alps. Studies on the nutritional quality have been performed on bilberries of Central Apennines[5], from Western[6] to Eastern Alps[3], and information is available also for some neighboring alpine countries[7,8]. However, due to the difficulties in handling and storability of bilberries, the economic interest on these berries has been low in South Europe where major interest has been focused on soft berry fruit cultivation (e.g. blueberry, *V. corymbosum* L. and raspberry, *Rubus* spp.). Cultivation of these berries fits well to alpine climate and soil conditions, and takes place up to 1000 m of altitude.

Several studies have been specifically performed to evaluate the natural variation of ACs in bilberry ecotypes from different geographical areas and in blueberry varieties, including comparison between populations subjected to different environmental conditions[1,8-11]. There is a huge variety of ACs spread in nature, consisting of differences in the number of hydroxylated groups, the nature and the

number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar residues and the position of these bonds[12]. According to the number and position of hydroxyl and methoxyl groups on the flavonoid molecule, six most common anthocyanidin aglycons are classified: pelargonidin (Pg), the mono-hydroxylated cyanidin (Cy) and peonidin (Pn), and the tri-hydroxylated delphinidin (Dp), petunidin (Pt) and malvidin (Mv)[13]. The most common ACs found in *Vaccinium* berries are monoarabinosides (ara), monoglucosides (glu) and monogalactosides (gal) of Cy, Pt, Pn, Dp and Mv, though several other phenolic compounds, and their glycosides, have been described (e.g. catechin, epicatechin, myricetin, kaempferol, quercetin, myricetrin and caffeic, p-coumaric and ferulic acids)[1,8,14-17]. Dp and Mv derivatives are described as the majority of ACs found in blueberries, constituting about 70% of total ACs[18]. Acylated ACs are also found in blueberries and bilberries but they account as a small portion of total amount[18,19].

Latitude appears to influence the accumulation of ACs in bilberries, as a clear increasing trend in AC production towards north has been reported in high latitudes. Higher AC content was reported in northern (63-70°N) latitudes of Northern Europe, compared to southern latitudes (54-62°N)[10,11]. The berries of the northernmost clones have been shown to contain higher total content of ACs and the higher proportion of the more hydroxylated anthocyanidins, Dps and Mvs, whereas Cys accumulated more in the more southern North European populations (latitudes 54-62°N). Studies have also been performed in relation to different altitudes, although they have given contradictory results. Spinardi et al.[20] reported higher levels of ACs and ascorbic acid in blueberries grown at 600 m a.s.l. compared with the same cultivar grown at 450 m a.s.l. in Valtellina (Northern Italy). In a two year study in Austria[7], decreasing AC contents were found in bilberry fruits along with increasing altitude (between 800 m a.s.l. and 1500 m a.s.l.). In studies performed in the areas of Northern Europe, where altitudinal differences are less pronounced, no clear relationship with elevation and AC concentration have been found[11,21].

In the present study, we investigated the effect of the altitude on the accumulation of ACs in the berries of two *Vaccinium* species (*V. myrtillus* L., bilberry and *V. corymbosum* L., cv. Brigitta Blue)

85 growing in Northern Italy over two growing seasons. The aim was to investigate the effect of
86 temperature and light conditions on the accumulation of ACs. The AC profile of six wild bilberry
87 populations growing in the Alps of Northern Italy along an altitudinal gradient of about 650 m was
88 analyzed and compared with the AC profile of one of the most popular variety of highbush blueberry
89 (cv. Brigitta Blue), which is cultivated in a range of about 550 m. Furthermore, temperature and light
90 conditions monitored along the altitudinal gradient were compared with AC profiles of berries. The
91 study provides detailed information about the AC composition of *Vaccinium* berries which is valuable
92 for food quality control in the berry industry. With specific regard to the wild species, the metabolic
93 profile of individuals within each bilberry population may also allow selection of specific genotypes
94 for cultivation and breeding purposes.
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Materials and method

Plant material and altitudinal gradients. The field trials were established along altitudinal gradients in the region Trentino (Italy). Permissions for field work were granted by the municipalities of Grumes, Valda and Zambana and the Province of Trento (Italy). Six sites were identified for bilberry (*V. myrtillus* L., Vm) experimental fields. The gradient was established between Val di Cembra (46.22°N, 11.26°E) and Monte Paganella (46.16°N, 11.04°E) at 1166 m, 1261 m, 1406 m, 1520 m, 1620 m and 1829 m a.s.l. (Table 1). In each site 10-25 individual bilberry plants within an area of about 20 m^2 were considered for AC analysis of the berries.

Blueberries (*V. corymbosum* L., Vc) of cv. Brigitta Blue, were collected from plants cultivated by farmers of Valsugana valley (46.08°N, 11.50°E). Three sites, located in Telve (495 m a.s.l.), Spera (749 m a.s.l.) and Pinè (1034 m a.s.l.) were considered for the altitudinal gradient (Table 1). Plants were cultivated according to conventional farming methods. Fruits were collected from five plants chosen randomly on five different rows of plants in the field in an area of 100 m^2.

Fruit harvesting. The study was conducted in two consecutive growing seasons (years 2013 and 2014). Bilberries were harvested when fruits reached the full development in size and color, and total soluble content (TSSC) was higher than 6.0 °Brix. In 2013, bilberry fruits from Val di Cembra were collected between July 17 (in lowest location - site Vm1) and July 24 (in middle high locations - sites Vm2 and Vm3), while on Monte Paganella, on sites Vm4 and Vm5 bilberries were collected on August 21 while bilberries from the highest location (site Vm6) on September 3. In 2014, the growing season was anticipated in Val di Cembra, as the berry harvesting started on June 20 in the lowest site (Vm1). The harvest continued on July 3 on sites Vm2 and Vm3, on August 1 for Vm4 and August 28 on sites Vm5 and Vm6 (Table 1).

Blueberry fruits were manually picked at the commercial ripening stage, when berries were fully developed in size and color, with TSSC content higher than 10 °Brix, and at least the 80% of berries on the plant reached ripeness. Fruit collection started on July 22 2013 in sites of Telve (Vc1) and Spera (Vc2), and ended on August 12, 2013 in the highest location Pinè (Vc3). In the following year,

2014, fruits were harvested on the same plants, starting from Telve on July 7 and finished on August 12 in Spera and Pinè (Table 1).

Berries were placed in plastic tubes and stored on ice to prevent sample degradation during the transport from the field to the laboratories, where they were stored at -80 °C. Metabolic analyses were performed within three months from the collection.

Total soluble solid content (TSSC) analysis. TSSC analyses were conducted on mature fruits, as homogenous as possible for size and colour. TSSC were measured with a DBR35 refractometer.

ACs extraction. Frozen ripe berries were ground into a fine powder with a cryomill (Retsch, Haan, Germany). The homogenized samples (1 g fresh weight, FW, out of at least 10 g of fresh berries) were extracted twice in 1,5 mL of methanol 80% under shaking for 1 h. Samples were centrifuged at 12000 g for 2 min. Supernatants were joined and brought to a volume of 5 mL. Samples were filtered (0.22 µm PVDF filters), transferred to glass vials and randomized before analysis by UPLC-MS/MS[22].

UPLC analysis. Analytical separation of ACs was performed in an Acquity ultraperformance liquid chromatographic (UPLC) system (Waters, UK) operating under MassLynx XS software. The system was coupled to a mass spectrometer detector Waters Xevo TQ MS (Milford, MA, USA) equipped with an electrospray (ESI) source. All samples were analyzed on a reverse phase Acquity UPLC BEH C18, 1.7 µm, 2.1 x 150 mm column (Waters), protected with Acquity UPLC BEH C18, 1.7 um, 2.1 x 5 mm precolumn (Waters) at 40 °C and under mobile phase flow rate of 0.4 mL/min. Water was used as weak eluting solvent (A) and methanol as strong elution solvent (B); formic acid 5% v/v was used as additive in both eluents. The multistep linear gradient used was as follows: from 95 to 60% of a for the first 4 min, from 60 to 45% A from 4 to 9 min, from 45 to 5% A from 9 to 11 min and isocratic hold for 3 min to clean the column. The equilibration time was 4 min, and the injection volume was 2 µl. 4-hydroxy-stilbene was used as internal standard (2.5 ppm) to check that the sensibility of the machine was kept constant during the analysis. A quality control standard mixture

151 was injected periodically to check that the sensibility of the machine was kept constant during the
152 analysis.
153 **Identification and quantification (MS/MS).** ACs were detected by multiple reaction monitoring
154 (MRM), by screening the MS/MS transitions and using the parameters earlier optimized for grape
155 wine[22]. The method was slightly modified to allow the detection of gal and ara anthocyanidins that
156 have earlier been described for bilberry. For some of the compounds, there were no standards
157 available, but they could be tentatively identified on the basis of their MRM transitions and the
158 relative retention time in respect to known compounds and considering previous results (Suppl. Table
159 1)[19].
160 For quantification, external calibration curves were prepared by injecting authentic standards of each
161 compound at different concentrations. In case the authentic standard was not available, the ACs were
162 quantified relative to Mv-3-*O*-glu, using the Mv-3-*O*-glu calibration curve (Suppl. Table 1). Data
163 processing was done using Waters MassLynx 4.1 and TargetLynx software. The amount of
164 anthocyanidin classes (Dp, Cy, Mv, Pn and Pt) and total ACs was calculated by summing up the
165 amounts of single AC compounds identified.
166 **Air temperature measurement.** The air temperature was measured along the altitudinal gradient,
167 between 410 m and 2125 m a.s.l., at the meteorological stations of the Protezione Civile of Trento
168 (Italy). Among all the stations present on the Province of Trento, the ones chosen were the nearest to
169 the experimental fields. In detail, stations were located in Telve (T0392, 46.06°N, 11.47°E, 410 m
170 a.s.l.), Bieno (T0015, 46.08°N, 11.56°E, 843 m a.s.l.), Lavarone (T0032, 45.94°N, 11.25°E, 1155 m
171 a.s.l.), Monte Bondone (T0368, 46.01°N, 11.05°E, 1490 m a.s.l.) and Monte Paganella (T0099,
172 46.14°N, 11.04°E, 2125 m a.s.l.). Maximum daily temperatures were recorded in 2013 and 2014
173 during berry growing season (June 1 – August 31). Data are available online at www.meteotrentino.it.

174 **Solar radiation and light quality measurements.** Light at top of canopies was measured with a
175 USB 2000 Spectrometer (Ocean Optics, US). Measurements were recorded during fruit development

and ripening at noon on sunny days under clear sky, within a range of altitude between 495 m and 1404 m a.s.l. (in locations Vc1, Vc2, Vc3, Vm1, Vm2 and Vm3). The quality of light reaching the plants was measured as Photosynthetic Active Radiation (PAR) by integrating the area between 200-850 nm for full sunlight spectra, 200-380 nm for UV light, 380-750 nm for visible light, 380-495 nm for blue, 590-710 nm for red and 710-750 nm for far-red components[23]. Spectra and integration of light were measured from five consecutive scans of the sunlight, after calibration of the instrument according to the manufacturer's instructions.

Soil pH. For bilberry, in every location, five points were spotted out of the edges of the bilberry matrix frame for soil sampling. In these spots, about 1 kg of soil between 20 and 50 cm underground was collected. The samples were first cleaned from plant roots, leaves and small stones and then pooled together. Pooled sample of 80 g was dissolved in 200 ml distilled water[24], stirred overnight, and the next day pH was measured with Crison PH 25+ (Hach Lange Srl, Spain).

Statistics. All data were analyzed using STATISTICA 9 software (StatSoft Inc., Tulsa, USA). Two-way ANOVA followed by pair-wise comparison using Fisher's Least Significant Difference (LSD) test were made between the variables: metabolites vs. altitude and year of collection, light conditions vs. altitude, temperature vs. altitude. Differences were considered significant when $p<0.05$.

PCA (Principal Component Analysis) was performed on bilberries' AC composition to investigate the variation within bilberry populations and to discriminate geographic areas of collection. Regression analyses were made on the full set of bilberry samples, in order to measure the coefficient of determination r^2 and p value between the anthocyanidin classes (Dp, Cy, Pn, Pt, Mv), total ACs and altitude.

Results and discussion

AC profile of bilberry populations. AC profiles of bilberry fruits as well as AC variation among native bilberry populations in Alps of Northern Italy were systematically analyzed. The ACs identified were Dp, Cy, Pn, Pt and Mv conjugated with glu, gal and ara, and their acylated forms, including acetylated and *p*-coumaroylated ACs. In small amounts, also Cy 3 sambubioside and Pg were identified (Suppl. Table 2). The amounts of Dp glycosides were higher than of Cy glycosides on average, similarly to the profiles detected in other Southern European bilberries, e.g. from Austria[7], Slovenia[8] and Montenegro[25] but also in Northern European bilberries, e.g. from Finland[10,19]. However, the comparison between bilberries from Finland[19] and Italy (presented in this study) which were analyzed with the same analytical method[22], revealed differences in the sugar moiety. In bilberries growing in Northern Europe over 60% of the ACs was conjugated with glu, whereas the rest 40% was shared equally between gal and ara[19]. In the present study, ACs were equally conjugated with the 3 sugars (about 30% each) which is in line with the results of the study on Slovenian bilberries[8]. In the present study, also climate differences affected accumulation of acetylated compounds, which were more abundant in 2013 than in 2014 (Suppl. Table 2).

The AC variability among bilberry populations, estimated through PCA analysis, was minimal as the first axis explained the 98.5% of the diversity, but grouped all samples in one single cluster (Fig. 1a). However, the second and third axis, which respectively explained the 0.9% and 0.3% of the diversity, separated the populations from sites Vm1, Vm3 and Vm5 (Fig. 1b), indicating that the AC profile may be used as a discriminative factor of bilberry populations from close geographical sites.

AC profile of blueberries (cv. Brigitta Blue). The AC profile was typical for the cv. Brigitta blue[26], with Dp and Mv as predominant anthocyanidins, followed by Pt and in lowest proportion Cy and Pn (Suppl. Table 3). AC compounds were conjugated with glu, gal and ara sugars, but were not uniformly distributed among the anthocyanidins. Dp was equally conjugated with glu and ara, whereas very little amount of Dp 3 gal was detected. On the contrary, Mv and Pt (which are methylated forms of

Dps[27]), and the mono-hydroxylated Cy and Pn were equally conjugated with gal and ara and to a small extent with glu. No acylated forms were identified in blueberries (Suppl. Table 3).

AC accumulation in wild and cultivated *Vaccinium* spp. along altitudinal gradients. The two *Vaccinium* species, bilberry and blueberry, showed important differences in the accumulation of ACs along the altitudinal gradient. Our results indicated an increase in the accumulation of ACs in bilberries along a gradient of about 650 m, which was consistent over the two growing seasons (Fig. 2). Indeed, regression analysis of total AC concentration gave a significant positive correlation between altitude and total AC accumulation in berries ($p = 0.001$ in 2013 and $p = 0.002$ in 2014) in both years (Fig. 2). The trend was due to a significant increase in the tri-hydroxylated anthocyanidins such as Dp ($p = 0.0003$ in 2013, $p = 0.0002$ in 2014) and Mv ($p = 0.0002$ in 2013, $p = 0.0026$ in 2014). To a small extent also Pt increased with increasing altitude ($p = 0.001$ in 2013). The accumulation of the mono-hydroxylated Cy and Pn glycosides, instead, was not affected by the altitude (Fig. 2).

The results of the present study are in line with Jovancevic et al.[25], who showed that bilberries collected in sunny locations contained higher amounts of ACs with increasing altitude. In particular, an increase in Dp-type ACs was observed in locations above 1500 m a.s.l.[25]. Contrasting results were reported by Rieger et al.[7] in bilberries collected at different altitudes in Austria, where decreasing amounts of ACs were observed with increasing altitude. However, results by Rieger et al.[7] are also in contrast with the same analysis performed within the same study on other plant species (*Calluna vulgaris* L. and *Sambucus nigra* L.). Moreover, no information about the exposition and the climatic conditions of the bilberry collection sites was presented. The content and composition of ACs in bilberries is highly affected by environmental factors such as light intensity, light quality and temperature[19,28-30], which needs to be taken into consideration to explain the accumulation of metabolites in fruits growing in natural environments.

In blueberry, an opposite trend was observed during the two years in the present study; in 2013 the accumulation of total AC was positively increasing towards higher altitudes ($p = 0.0006$), whereas in

2014 accumulation was negatively correlated with altitude ($p = 0.0000$; Fig. 3). In the case of blueberry, only the cultivar (Brigitta Blue) was used in the study and, therefore, the seasonal differences in the AC content were considered to be connected to environmental effects.

Environmental effect on AC accumulation. In all types of fruits, environmental factors affect the quantitative and qualitative composition of ACs in the ripening fruit[31]. In general, the genetic background of the species/variety determines the AC content in fruit, yet the environmental factors can affect the concentration of diverse ACs in different ways. Temperature, solar radiation and soil are considered the major environmental factors affecting the accumulation of ACs in fruits[32]. All these factors may vary markedly along latitudinal gradients[33], where changes in phenolic content of bilberries were recorded with changing environments from southern to northern latitudes[10,11,21].

In the present study we observed that the same factors vary markedly also along an altitudinal gradient. Maximum daily temperatures decreased progressively with increasing altitude (Fig. 4). Moreover, differences in temperatures were recorded throughout the two growing seasons, which significantly affected the accumulation of ACs in berries ($p < 0.05$; Suppl. Table 2). Higher temperatures were recorded during the year 2013, when also higher accumulation of AC was found in bilberries, with higher proportion of acylated forms of ACs (Suppl. Table 2) in line with findings in grape berries (cv. Merlot)[34] and in our previous study on bilberry[30].

Light conditions were also measured in six locations along the gradient, within 495 m and 1404 m a.s.l.; Fig. 5). Sunlight PAR (full spectra) increased constantly towards increasing altitudes, and significant changes ($p < 0.001$) were recorded between locations at 495 m a.s.l., at 756 m a.s.l. and locations higher than 1034 m a.s.l. The increase was due to a progressive increase of visible light along with altitude, although the blue and red components were not significantly affected with increasing altitude. The UV radiation counted only for the 1.3-3.8% of the total radiation, and was not significantly affected by altitude. The soil pH ranged between 4.3 and 5.1 (Table 1), which is optimal for the growth of bilberry[36], but did not show any particular trend connected with altitude.

Our results suggest that lower temperatures have a major effect on the accumulation of ACs in bilberry. Decreasing temperatures observed with the increasing altitude, positively influenced the accumulation of ACs in bilberries being in line with the results of Uleberg et al.[28], who showed that higher amounts of Dp derivatives are produced at low temperatures (12°C) compared to higher temperatures (18 °C). Recently, we also observed that when plants from the same bilberry populations were moved from higher to lower altitude (485 m a.s.l.), in open-field conditions with higher temperature, berries accumulated lower amounts of ACs, confirming the role of temperature in the determination of the final AC profile of berries[30]. Thus, the difference in the average daily temperature in the original locations (Vm1-Vm6) was at least 5 °C lower than in the test field at 485 m a.s.l. and the AC accumulation was almost two fold higher in the original site. Excessive light stress appeared to inhibit AC accumulation[30], but considering the present results, where light intensity is higher at altitudes above 1034 m a.s.l. compared to fields at altitude 495 m a.s.l. (Fig. 5), light intensity appears to have less influence than temperature on AC accumulation in bilberry fruits growing in natural environments. Supporting this assumption, in the locations above 1034 m a.s.l., where bilberry populations were growing, no significant difference in the intensity of the light reaching the plants was detected (Fig. 5). Therefore, we hypothesized that bilberry plants grown at higher altitudes may have developed genetic adaptation mechanisms which respond to low temperatures with an increased production in ACs, in particular of the Dp-type.

The biosynthesis of Dp-type ACs is driven by the flavanone 3’5’-hydroxylase (*F3’5’H*) gene, which is responsible of the hydroxylation at the 3’5’ positions of the B-ring of the precursor dihydrokaempferol into Dp and the methylated derivatives Mv and Pt, and which also shifts the biosynthesis from the Cy and Pn branch towards the branch of the flavonoid pathway producing Dp, Mv and Pt as final core structures of ACs[27]. Studies conducted on blueberry[27], bog bilberry (*V. uliginosum* L.)[37] and grape berries (*Vitis vinifera* L.)[38] showed that the *F3’5’H* gene is weakly expressed during the earliest ripening stages and is abundant only during the late ripening stages, closely paralleling the appearance of ACs. In blueberry, as the fruit ripens and the exocarp colour

changes from mostly green to partially pink, blue-purple Dp-type ACs begin to accumulate. The appearance of the tri-hydroxylated anthocyanidin Dp and its derivatives Mv and Pt is coordinated with the abundance of *VcF3′5′H* transcripts at developmental stage S5 (at the initiation of ripening)[27]. Based on conservation of the flavonoid pathway in diverse species, the *VmF3'5'H* gene expression is expected to increase at the late ripening stages of bilberry as well as when exposed to low temperatures as found in the present study and as suggested for grapevine[39].

In blueberry, marked differences in AC content were recorded in the same locations during the two seasons. The accumulation of ACs in blueberries is developmentally regulated and occurs during the pink and the purple-blue (full ripe) stages of berry development[27]. At the lowest location Vc1, the berries started in 2013 to visibly turn color and to accumulate ACs on June 30 and they reached full ripeness on July 22 whereas in 2014 the berries started to turn color on June 10 and reached ripeness already on July 7. In the central location Vc2, blueberries started to turn color on June 30 in 2013 and reached ripeness on July 22 (as in location Vc1), whereas in 2014 the ripening was delayed of almost 3 weeks as berries started AC accumulation on August 1 and ripened on August 12. In the highest location Vc3, the ripening of berries was more uniform during the two seasons and the berries started to turn color around August 1 in both years and were ready for picking on August 12.

The accumulation of AC in blueberries is regulated also by environmental factors[30]. To our knowledge, very little information is known about the effect of temperature on AC biosynthesis in blueberries. Therefore, in the present study, the cultivation of blueberries at different altitudes allowed us to investigate the effect of natural differences in temperature on the AC composition of the berries at harvest. The meteorological station present in location Vc1 (Telve, Fig. 4) recorded maximum daily temperatures ranging from 26 °C at the beginning of the pink stage and progressively increased up to 32.5 °C at full ripeness in 2013. In 2014, during the pink stage, temperature peaked up to 33.5 °C and later decreased fluctuating around an average of 26 °C until ripening (Fig. 4). In correspondence of the temperature flow recorded in 2014 (i.e. high temperatures during the pink stage followed by lower temperatures during the last ripening phase), blueberries ripened faster and

accumulated the highest content of AC (385 ± 59 mg/100 g FW), which was doubled compared to the amount recorded in 2013 (168 ± 22 mg/100 g FW; Fig. 3f, Suppl. Table 3). Meteorological stations were not present on the site for the other two localities (Vc2 and Vc3) and therefore information from the closest meteorological stations (Bieno, 6 km far from Vc2 and Lavarone, 43 km far from Vc3, respectively) was used. In location Vc2, temperatures recorded were almost constant throughout the pink and purple stages of berry development in both years (Fig. 4). Consistently, the difference in the content of ACs in berries between the two seasons was not as marked as in the other two localities (270 ± 28 mg/100 g FW, in 2013 and 210 ± 11 mg/100 g FW in 2014; Fig. 3f, Suppl. Table 3). In location Vc3, a difference of 5-10°C between the two seasons markedly affected the accumulation of AC in blueberries. In 2013, temperatures ranged between 20 °C and 28 °C during the berry ripening (Fig. 4) and the AC content of blueberries was 260 ± 13 mg/100 g FW. In 2014, accumulation of total ACs was only 91 ± 27 mg/100 g FW due to the lower temperatures (between 14°C and 25 °C) during the berry ripening stage. The optimum temperatures for blueberry fruit set, size and ripening are 20-26 °C during the day and 16 °C during the night[40]. However, the temperature conditions registered in location Vc1 during 2014 appeared to be optimal for the AC accumulation in blueberries (Fig. 3f, Suppl. Table 3). The present results are comparable with studies conducted on grape berries, in which the highest concentration of ACs was recorded in berries grown in temperature regimes with maximum daily temperatures of 25 °C[41]. The AC content instead dramatically dropped when the maximum daily temperatures reached 35 °C[41].

The present study suggests that temperature is the major environmental factor affecting the AC concentration and composition in berries of the *Vaccinium* species under examination. This information will be helpful when considering the effects of climate change on the species under examination. If the scenarios of global warming to be continued in the future become true, the distribution of bilberry populations may change both in Nordic countries and Alpine environments, and the nutritional value of berries may change consequently. Furthermore, the proportions of specific ACs such as Dp and Mv-glycosides in bilberry along increasing altitude followed the same trend

along increasing latitude, indicating that temperature conditions are related to adaptation of bilberry to the environment[10,11]. The present study indicates that for production of high-quality berries with regards to AC production, bilberry plants located at high latitudes or altitude should be preferred for propagation. However, it is important to take environmental conditions and suitable bilberry genotypes into consideration during the establishment of the field propagation. For this purpose, the metabolic profile of individuals within each bilberry population may allow selection of potential genotypes for cultivation and breeding purposes. This aspect is also important to blueberry growers, who may have to compromise the agro-economical needs to offer berries with high nutritional value to consumers.

Abbreviations Used

a.s.l., above sea level; AC, anthocyanin; ara, monoarabinoside; Cy, cyanidin; Dp, delphinidin; FW, fresh weight; Mv, malvidin; gal, monogalactoside; glu, monoglucoside; PCA, Principal Component Analysis; Pg, pelargonidin; Pn, peonidin; Pt, petunidin; TSSC, Total Soluble Solid Content; Vm, *Vaccinium myrtillus*; Vc, *Vaccinium corymbosum*.

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Supporting information description

Suppl. Table 1. UPLC-MS/MS data for anthocyanin quantification. Dp = Delphinidin, Cy = Cyanidin, Pt = Petunidin, Pn = Peonidin, Mv = Malvidin, Pg = Pelargonidin, glu = glucose, gal = galactose, ara = arabinose, Std = quantification by authentic standard. In case of two MRM transitions for a given compound, the first was used as quantifier and the second as qualifier. RT = retention time, CV = cone voltage, CE = collision energy.

Suppl. Table 2. Average amount of AC content (± SD; mg/100 g FW) in bilberries (*V. myrtillus* L.) collected at six different altitudes (Vm1-Vm6) in Alps of Northern Italy during years 2013 and 2014.

Suppl. Table 3. Average amount of AC content (± SD; mg/100 g FW) in blueberries (*V. corymbosum* L., cv Brigitta Blue) cultivated at three different altitudes (Vc1-Vc3) in Alps of Northern Italy during years 2013 and 2014.

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References

1. Prior, R. L.; Cao, G. H.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M., Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *Journal of Agricultural and Food Chemistry* 1998, *46* (7), 2686-2693.
2. Landete, J. M. Updated knowledge about polyphenols: functions, bioavailability, metabolism, and health. *Critical Reviews in Food Science and Nutrition* 2012, *52* (10), 936-948.
3. Barizza, E.; Guzzo F.; Fanton, P.; Lucchini, G.; Sacchi, G. A.; Lo Schiavo, F.; Nascimbene, J. Nutritional profile and productivity of bilberry (*Vaccinium myrtillus* L.) in different habitats of a protected area of the Eastern Italian Alps. *Journal of Food Science* 2013, *78* (13), 673-678.
4. Woodward, F. I. Ecophysiological studies on the shrub *Vaccinium myrtillus* L. taken from a wide altitudinal range. *Oecologia* 1986, *70* (4), 580-586.
5. Giovanelli, G.; Buratti, S., Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chemistry* 2009, *112* (4), 903-908.
6. Beccaro, G.; Mellano, M. G.; Botta, R.; Chiabrando, V.; Bounous, G. Phenolic and anthocyanin content and antioxidant activity in fruits of bilberry (*Vaccinium myrtillus* L.) and of highbush blueberry (*V. corymbosum* L.) cultivars in North Western Italy. *Proceedings of the 8th International Symposium on Vaccinium Culture* 2006, 715, 553-557.
7. Rieger, G.; Muller, M.; Guttenberger, H.; Bucar, F. Influence of altitudinal variation on the content of phenolic compounds in wild populations of *Calluna vulgaris*, *Sambucus nigra*, and *Vaccinium myrtillus*. *Journal of Agricultural and Food Chemistry* 2008, *56* (19), 9080-9086.
8. Moze, S.; Polak, T.; Gasperlin, L.; Koron, D.; Vanzo, A.; Poklar Ulrih, N.; Abram, V., Phenolics in Slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.). *Journal of Agricultural and Food Chemistry* 2011, *59* (13), 6998-7004.
9. Connor, A. M.; Luby, J. J.; Tong, C. B. S.; Finn, C. E.; Hancock, J. F. Genotypic and environmental variation in antioxidant activity, total phenolic content, and anthocyanin content among blueberry cultivars. *Journal of the American Society for Horticultural Science* 2002, *127* (1), 89-97.
10. Lätti, A. K.; Riihinen, K. R.; Kainulainen, P. S. Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland. *Journal of Agricultural and Food Chemistry* 2008, *56* (1), 190-196.
11. Åkerstrom, A.; Jaakola, L.; Bang, U.; Jäderlund, A. Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *Vaccinium myrtillus* L. (bilberries). *Journal of Agricultural and Food Chemistry* 2010, *58* (22), 11939-11945.
12. Kong, J. M.; Chia, L. S.; Goh, N. K.; Chia, T. F.; Brouillard, R. Analysis and biological activities of anthocyanins. *Phytochemistry* 2003, *64* (5), 923-933.
13. Castaneda-Ovando, A.; Pacheco-Hernandez, M. D.; Paez-Hernandez, M. E.; Rodriguez, J. A.; Galan-Vidal, C. A. Chemical studies of anthocyanins: a review. *Food Chemistry* 2009, *113* (4), 859-871.
14. Kalt, W.; McDonald, J. E.; Ricker, R. D.; Lu, X. Anthocyanin content and profile within and among blueberry species. *Canadian Journal of Plant Science* 1999, *79* (4), 617-623.
15. Määtta-Riihinen, K. R.; Kähkonen, M. P.; Törrönen, A. R.; Heinonen, I. M. Catechins and procyanidins in berries of *Vaccinium* species and their antioxidant activity. *Journal of Agricultural and Food Chemistry* 2005, *53* (22), 8485-8491.
16. Mikulic-Petkovsek, M.; Slatnar, A.; Stampar, F.; Veberic, R. HPLC-MSn identification and quantification of flavonol glycosides in 28 wild and cultivated berry species. *Food Chemistry* 2012, *135* (4), 2138-46.

17. Lätti, A. K.; Jaakola, L.; Riihinen, K. R.; Kainulainen, P. S. Anthocyanin and flavonol variation in bog bilberries (*Vaccinium uliginosum* L.) in Finland. *Journal of Agricultural and Food Chemistry* 2010, *58* (1), 427-433.
18. Norberto, S.; Silva, S.; Meireles, M.; Faria, A.; Pintado, M.; Calhau, C. Blueberry anthocyanins in health promotion: a metabolic overview. *Journal of Functional Foods* 2013, *5* (4), 1518-1528.
19. Zoratti, L.; Sarala, M.; Carvalho, E.; Karppinen, K.; Martens, S.; Giongo, L.; Häggman, H.; Jaakola, L. Monochromatic light increases anthocyanin content during fruit development in bilberry. *BMC Plant Biology* 2014, *14*, 10.
20. Spinardi, A.; Mignani, I.; Folini, L.; Beghi, R. Quality and nutraceutical content of blueberries (*Vaccinium corymbosum*) grown at two different altitudes (450 and 650 m above sea level). *IX International Vaccinium Symposium* 2009, *810*, 817-822.
21. Martz, F.; Jaakola, L.; Julkunen-Tiitto, R.; Stark, S. Phenolic composition and antioxidant capacity of bilberry (*Vaccinium myrtillus*) leaves in Northern Europe following foliar development and along environmental gradients. *Journal of Chemical Ecology* 2010, *36* (9), 1017-1028.
22. Arapitsas, P.; Perenzoni, D.; Nicolini, G.; Mattivi, F. Study of Sangiovese wines pigment profile by UHPLC-MS/MS. *Journal of Agricultural and Food Chemistry* 2012, *60* (42), 10461-10471.
23. Stagnari, F.; Galieni, A.; Cafiero, G.; Pisante, M. Application of photo-selective films to manipulate wavelength of transmitted radiation and photosynthate composition in red beet (*Beta vulgaris* var. conditiva Alef.). *Journal of the Science of Food and Agriculture* 2014, *94* (4), 713-720.
24. Paolo, G.; Paolo, B. *L'interpretazione delle analisi del terreno. Strumento per la sostenibilità ambientale.* ARPAV: 2007.
25. Jovancevic, M.; Balijagic, J.; Menkovic, N.; Savikin, K.; Zdunic, G.; Jankovic, T.; Dekic-Ivankovic, M. Analysis of phenolic compounds in wild populations of bilberry (*Vaccinium myrtillus* L.) from Montenegro. *Journal of Medicinal Plants Research* 2011, *5* (6), 910-914.
26. Rodriguez-Mateos, A.; Cifuentes-Gomez, T.; Tabatabaee, S.; Lecras, C.; Spencer, J. P. E. Procyanidin, anthocyanin, and chlorogenic acid contents of highbush and lowbush blueberries. *Journal of Agricultural and Food Chemistry* 2012, *60* (23), 5772-5778.
27. Zifkin, M.; Jin, A.; Ozga, J. A.; Zaharia, L. I.; Schernthaner, J. P.; Gesell, A.; Abrams, S. R.; Kennedy, J. A.; Constabel, C. P. Gene expression and metabolite profiling of developing highbush blueberry fruit indicates transcriptional regulation of flavonoid metabolism and activation of abscisic acid metabolism. *Plant Physiology* 2012, *158* (1), 200-224.
28. Uleberg, E.; Rohloff, J.; Jaakola, L.; Trost, K.; Junttila, O.; Häggman, H.; Martinussen, I. Effects of temperature and photoperiod on yield and chemical composition of Northern and Southern clones of bilberry (*Vaccinium myrtillus* L.). *Journal of Agricultural and Food Chemistry* 2012, *60* (42), 10406-10414.
29. Jaakola, L.; Määtta-Riihinen, K.; Kärenlampi, S.; Hohtola, A. Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus* L.) leaves. *Planta* 2004, *218* (5), 721-728.
30. Zoratti, L.; Jaakola, L.; Häggman, H.; Giongo, L. Modification of sunlight radiation through colored photo-selective nets affects anthocyanin profile in Vaccinium spp. berries. *PLOS ONE* 2015, DOI: 10.1371/journal.pone.0135935.
31 Jaakola, L., New insights into the regulation of anthocyanin biosynthesis in fruits. *Trends in Plant Science* 2013, *18* (9), 477-483.
32. Carbone, F.; Preuss, A.; De Vos, R. C. H.; D'Amico, E.; Perrotta, G.; Bovy, A. G.; Martens, S.; Rosati, C. Developmental, genetic and environmental factors affect the expression of flavonoid genes, enzymes and metabolites in strawberry fruits. *Plant Cell and Environment* 2009, *32* (8), 1117-1131.

33. Steyn, W. J. Prevalence and functions of anthocyanins in fruits. In: *Anthocyanins: biosynthesis, functions, and applications.*Gould, K., Davies, K., Winefield, C, Eds. Springer: New York, USA, 2009; pp. 85-105.
34. McKown, A. D.; Guy, R. D.; Klapste, J.; Geraldes, A.; Friedmann, M.; Cronk, Q. C. B.; El-Kassaby, Y. A.; Mansfield, S. D.; Douglas, C. J. Geographical and environmental gradients shape phenotypic trait variation and genetic structure in Populus trichocarpa. *New Phytologist* 2014, *201* (4), 1263-1276.
35. Tarara, J. M.; Lee, J. M.; Spayd, S. E.; Scagel, C. F. Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in Merlot grapes. *American Journal of Enology and Viticulture* 2008, *59* (3), 235-247.
36. Primetta, A. K.; Karppinen, K.; Riihinen, K. R.; Jaakola, L. Metabolic and molecular analyses of white mutant *Vaccinium* berries show down-regulation of MYBPA1-type R2R3 MYB regulatory factor. *Planta* 2015, *242* (3), 631-634.
37. Gabriela, O. M.; Gabriela, L.; Emília, O.; Alena, G. *In vitro* propagation of *Vaccinium* species. In *Acta Universitatis Latviensis, Biology*, 2004, 676, 207-212.
38. Castellarin, S. D.; Di Gaspero, G.; Marconi, R.; Nonis, A.; Peterlunger, E.; Paillard, S.; Adam-Blondon, A. F.; Testolin, R. Colour variation in red grapevines (*Vitis vinifera* L.): genomic organisation, expression of flavonoid 3'-hydroxylase, flavonoid 3',5'-hydroxylase genes and related metabolite profiling of red cyanidin-/blue delphinidin-based anthocyanins in berry skin. *BMC Genomics* 2006, *7*.
39. Mori, K.; Goto-Yamamoto, N.; Kitayama, M.; Hashizume, K., Effect of high temperature on anthocyanin composition and transcription of flavonoid hydroxylase genes in 'Pinot noir' grapes (*Vitis vinifera*). *Journal of Horticultural Science & Biotechnology* 2007, *82* (2), 199-206.
40. Gough, E. R. *The highbush blueberry and its management*. The Haworth Press - Food Production Press: New York, 1993; p. 288.
41. Mori, K.; Goto-Yamamoto, N.; Kitayama, M.; Hashizume, K., Loss of anthocyanins in red-wine grape under high temperature. *Journal of Experimental Botany* 2007, *58* (8), 1935-1945.

529 **Figure captions**

530

531 **Figure 1. Genotype variability estimated by PCA analysis on anthocyanin variability among six**
532 **populations in Northern Alps of Italy.** Variability explained by 1st and 2nd PCA components (a) and
533 by 2nd and 3rd PCA components (b).

534

535 **Figure 2. AC accumulation (mg/100 g FW) in bilberries along altitudinal gradient in two**
536 **consecutive seasons (2013-2014).** Regression analysis was performed, and coefficient of
537 determination r^2 and p value were calculated for each class of anthocyanidin (a, Dp; b, Cy, c, Pn; d,
538 Pt; e, Mv) and the total AC content (f). Black squares: samples 2013; white squares: samples 2014;
539 dotted line: regression line of samples 2013; straight line: regression line of samples 2014.

540

541 **Figure 3. AC accumulation (mg/100 g FW) in blueberries (cv Brigitta Blue) along altitudinal**
542 **gradient in two consecutive seasons (2013-2014).** Regression analysis was performed, and
543 coefficient of determination r^2 and p value were calculated for each class of anthocyanidin (a, Dp; b,
544 Cy, c, Pn; d, Pt; e, Mv) and the total AC content (f). Black squares: samples 2013; white squares:
545 samples 2014; dotted line: regression line of samples 2013; straight line: regression line of samples
546 2014.

547

548 **Figure 4. Maximum daily temperatures recorded along the altitude gradient during the berry**
549 **ripening process (June, 1 – September, 4) in 2013 (a) and 2014 (b).** The temperatures were
550 recorded at the meteorological stations in Telve (410 m a.s.l., full line), Bieno (843 m a.s.l., dashed
551 line), Lavarone (1155 m a.s.l., double line), Monte Bondone (1490 m a.s.l., full line with dots) and
552 Monte Paganella (2125 m a.s.l., dashed line with dots) in the region of Trentino (Northern Italy). The
553 harvesting date for each location is presented in the squares.

554

555 **Figure 5. Solar radiation PAR measured along the altitudinal gradient.** PAR was measured in
556 six locations between 495 m and 1404 m a.s.l. integrating the spectra between 200-850 nm for full
557 sunlight spectra, 200-380 nm for UV light, 380-750 nm for visible light, 380-495 nm for blue, 590-
558 710 nm for red and 710-750 nm for far-red components.

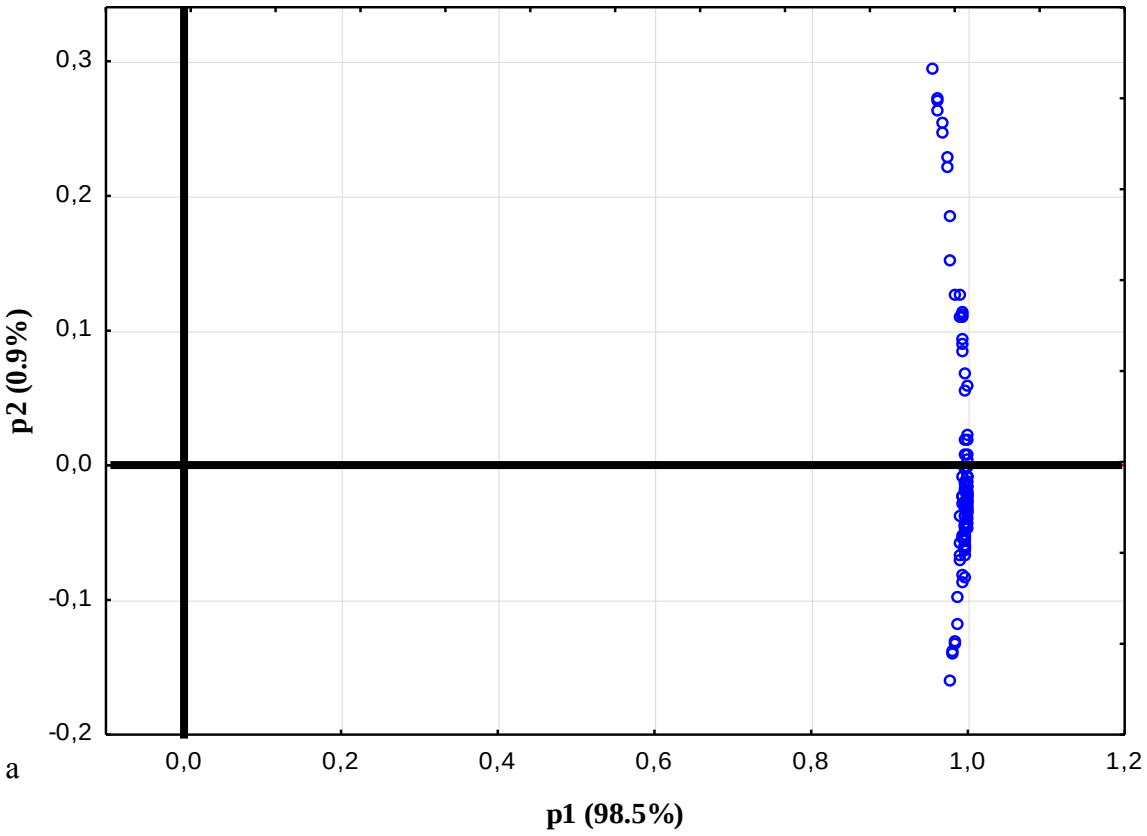
559

Tables

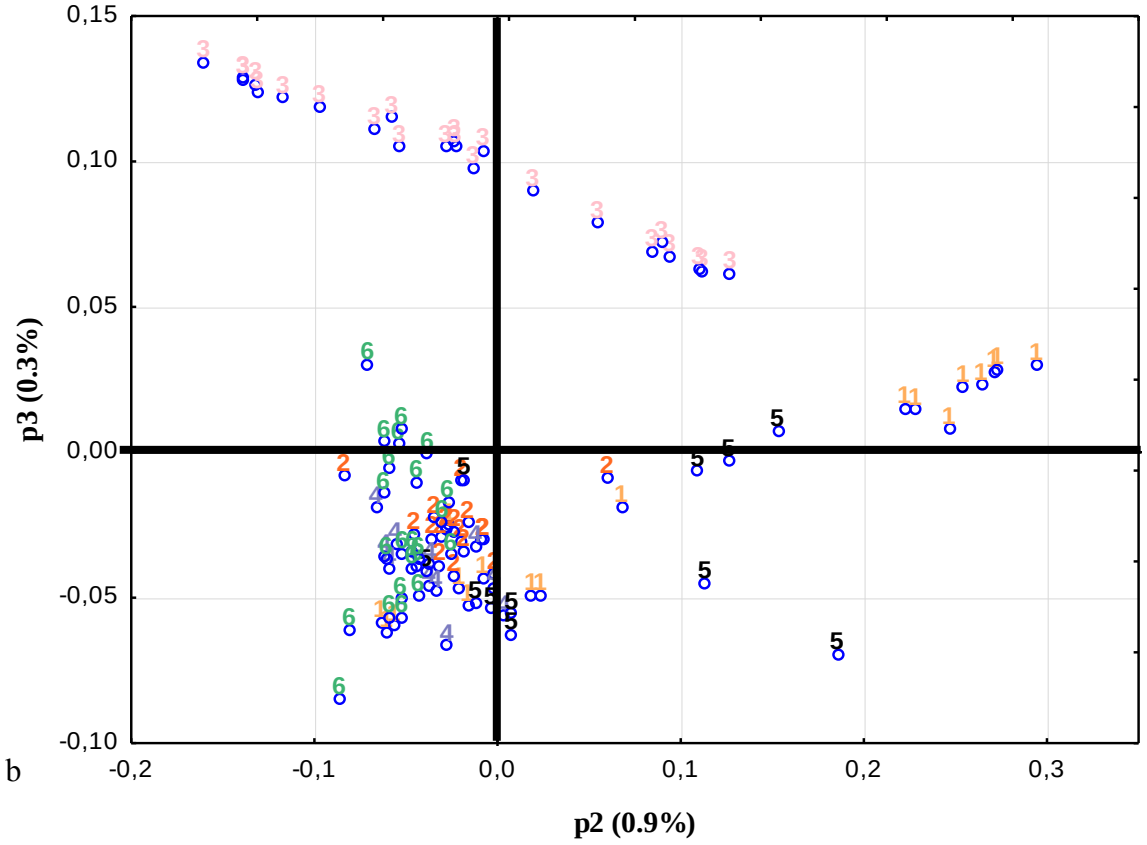
Table 1. Coordinates of locations where fruits of wild bilberry (*Vaccinium myrtillus*, Vm) and cultivated blueberry (*Vaccinium corymbosum*, Vc, cv. Brigitta Blue) were harvested during two consecutive seasons (2013 and 2014). n = number of individuals collected on the site. For bilberry, also pH of soil is reported.

Location	Site	Species	Latitude (°N)	Longitude (°E)	Altitude (m a.s.l.)	Soil pH	n 2013	n 2014	Harvesting date 2013	Harvesting date 2014
Val di Cembra	Vm1	*V. myrtillus*	46.22	11.26	1166 m	5.1	13	18	July, 17	July 3
Val di Cembra	Vm2	*V. myrtillus*	46.22	11.26	1261 m	4.4	7	18	July, 24	July, 3-10
Val di Cembra	Vm3	*V. myrtillus*	46.22	11.24	1404 m	4.8	11	23	July, 24	July, 10
Monte Paganella	Vm4	*V. myrtillus*	46.17	11.04	1520 m	4.7	2	11	August, 21	August, 1
Monte Paganella	Vm5	*V. myrtillus*	46.16	11.04	1617 m	4.8	1	11	August, 21	August, 28
Monte Paganella	Vm6	*V. myrtillus*	46.16	11.03	1829 m	4.3	12	23	September, 3	August, 28
Telve	Vc1	*V. corymbosum*	46.07	11.49	495 m	-	4	4	July, 22	July, 7
Spera	Vc2	*V. corymbosum*	46.08	11.51	749 m	-	4	4	July, 22	August, 12
Pinè	Vc3	*V. corymbosum*	46.03	11.28	1034 m	-	4	4	August, 12	August, 12

Figure 1


0,3
0,2
0,1
0,0
-0,1
-0,2
p2 (0.9%)
0,0
0,2
0,4
0,6
0,8
1,0
1,2
p1 (98.5%)

a


0,15
0,10
0,05
0,00
-0,05
-0,10
p3 (0.3%)
-0,2
-0,1
0,0
0,1
0,2
0,3
p2 (0.9%)

b

Figure 2

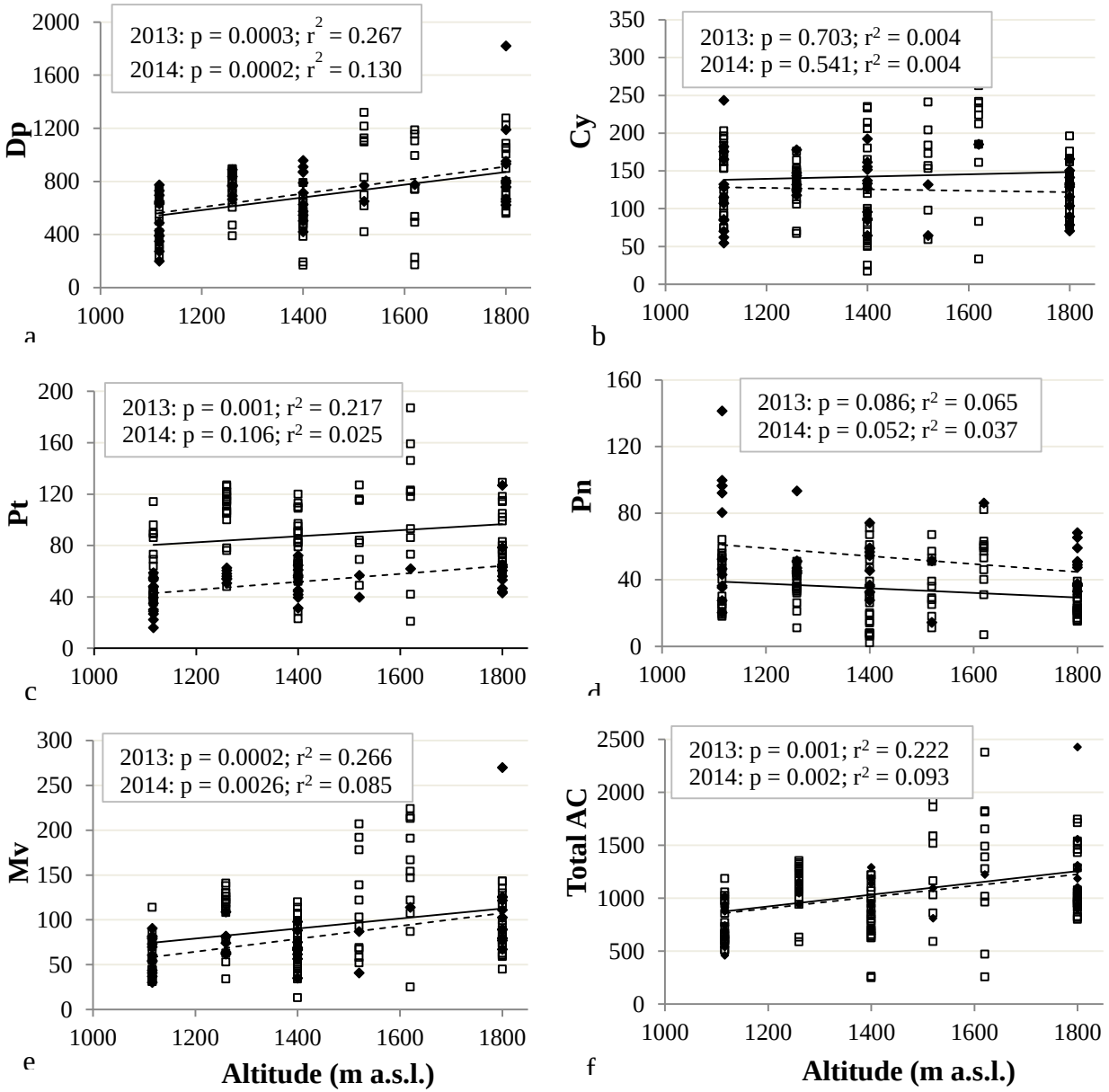

2013: p = 0.0003; r2 = 0.267
2014: p = 0.0002; r2 = 0.130
Dp
a
2013: p = 0.703; r2 = 0.004
2014: p = 0.541; r2 = 0.004
Cy
b
2013: p = 0.001; r2 = 0.217
2014: p = 0.106; r2 = 0.025
Pt
c
2013: p = 0.086; r2 = 0.065
2014: p = 0.052; r2 = 0.037
Pn
d
2013: p = 0.0002; r2 = 0.266
2014: p = 0.0026; r2 = 0.085
Mv
e
2013: p = 0.001; r2 = 0.222
2014: p = 0.002; r2 = 0.093
Total AC
f
Altitude (m a.s.l.)
Altitude (m a.s.l.)

Figure 3

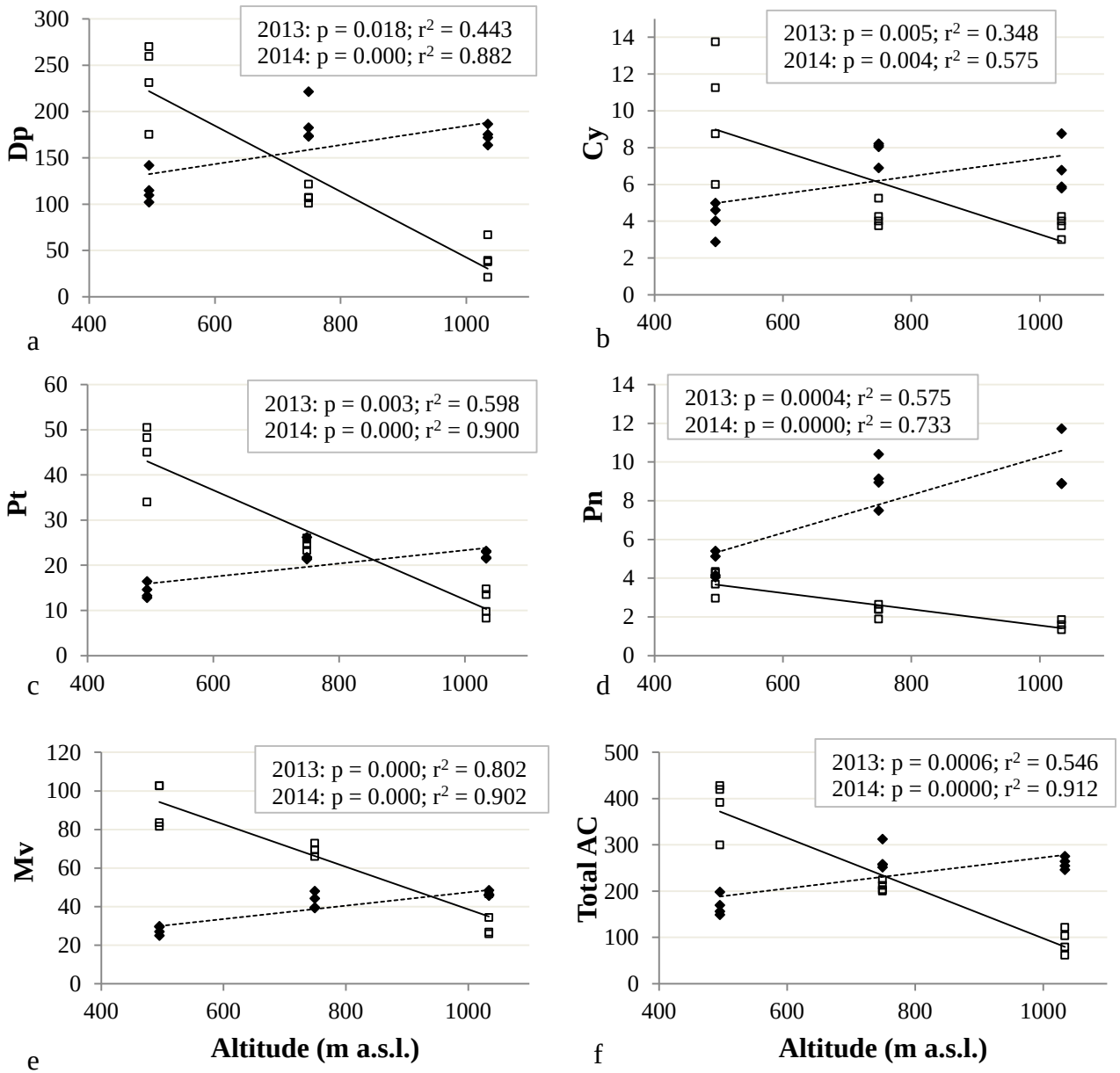
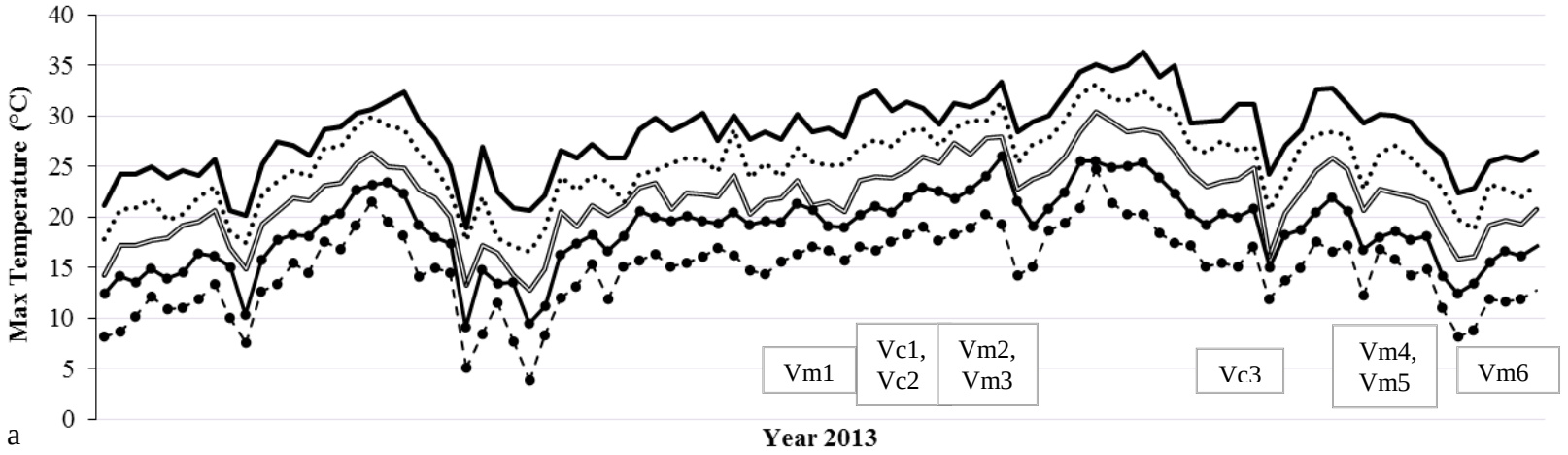
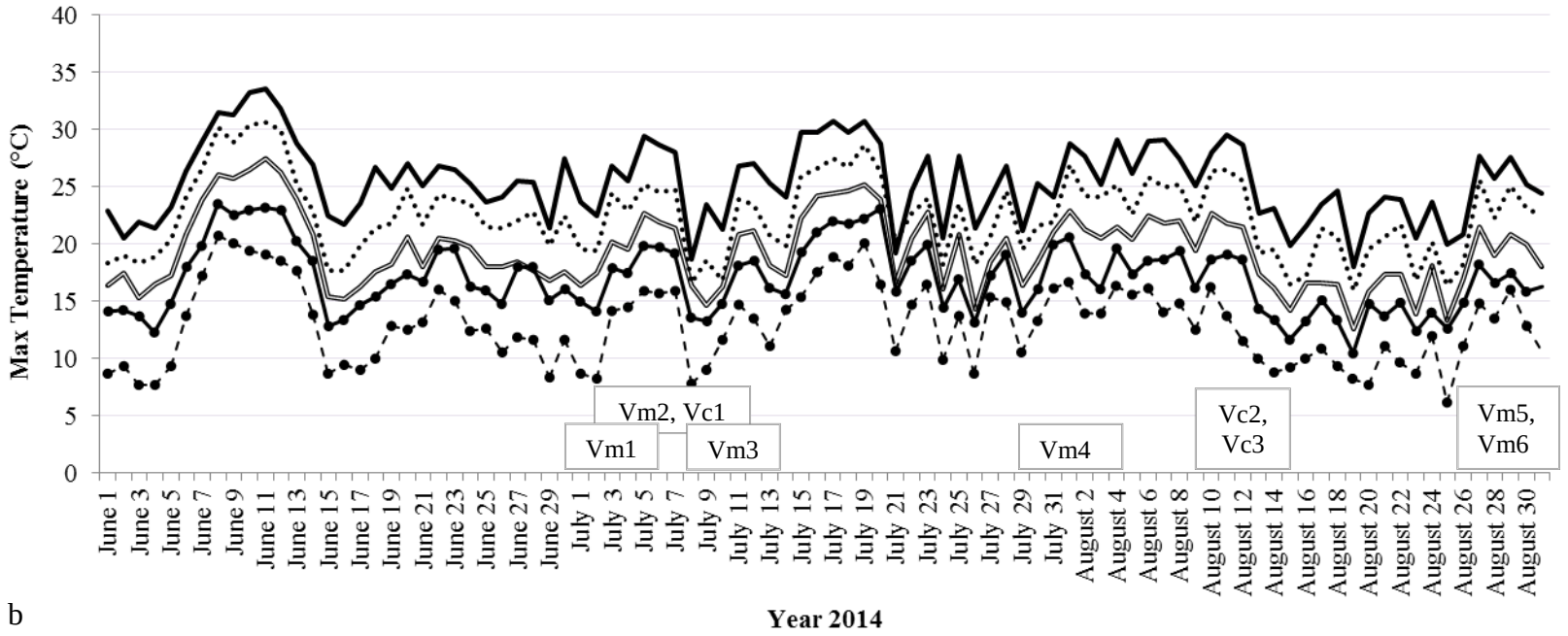

2013: p = 0.018; r² = 0.443
2014: p = 0.000; r² = 0.882
Dp
a
2013: p = 0.005; r² = 0.348
2014: p = 0.004; r² = 0.575
Cy
b
2013: p = 0.003; r² = 0.598
2014: p = 0.000; r² = 0.900
Pt
c
2013: p = 0.0004; r² = 0.575
2014: p = 0.0000; r² = 0.733
Pn
d
2013: p = 0.000; r² = 0.802
2014: p = 0.000; r² = 0.902
Mv
e
2013: p = 0.0006; r² = 0.546
2014: p = 0.0000; r² = 0.912
Total AC
f
Altitude (m a.s.l.)
Altitude (m a.s.l.)

Figure 4

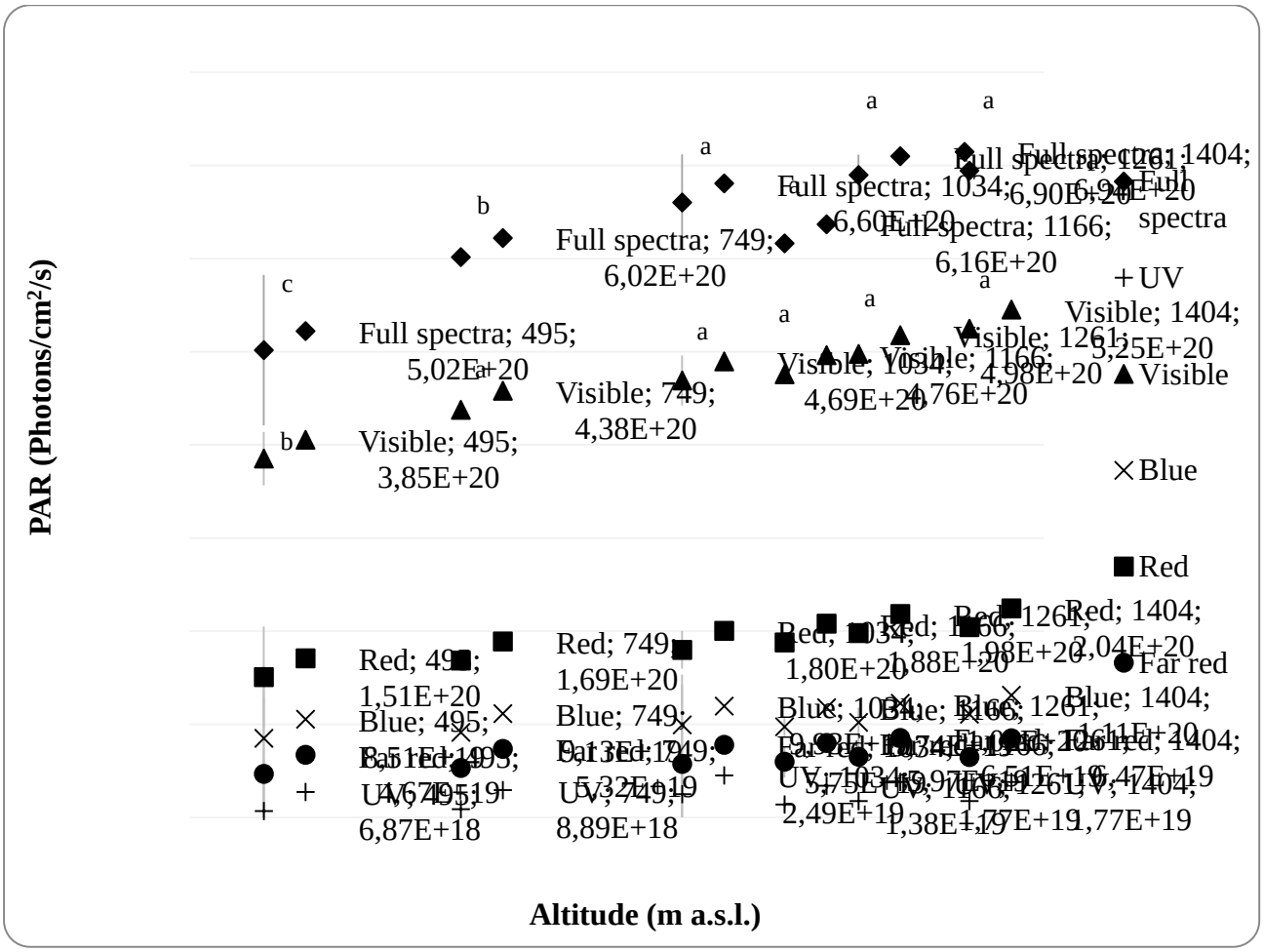

Max Temperature (°C)
Vm1
Vc1, Vc2
Vm2, Vm3
Vc3
Vm4, Vm5
Vm6
Year 2013

a


Max Temperature (°C)
Vm2, Vc1
Vm1
Vm3
Vm4
Vc2, Vc3
Vm5, Vm6
Year 2014

b

Figure 5


PAR (Photons/cm²/s)
Altitude (m a.s.l.)
Full spectra; 495; 5,02E+20
Visible; 495; 3,85E+20
Red; 495; 1,51E+20
Blue; 495; 6,87E+18
Full spectra; 749; 6,02E+20
Visible; 749; 4,38E+20
Red; 749; 1,69E+20
Blue; 749; 8,89E+18
Full spectra; 1034; 6,60E+20
Visible; 1034; 4,69E+20
Red; 1034; 1,80E+20
Blue; 1034; 9,13E+18
Full spectra; 1166; 6,16E+20
Visible; 1166; 4,76E+20
Red; 1166; 1,88E+20
Blue; 1166; 9,97E+18
Full spectra; 1261; 6,90E+20
Visible; 1261; 4,98E+20
Red; 1261; 1,98E+20
Blue; 1404; 1,11E+20
Full spectra; 1404; 6,90E+20
Full spectra + UV
Visible; 1404; 5,25E+20
Visible
Blue
Red
Red; 1404; 2,04E+20
Far red
Far red; 1404; 1,47E+19
UV; 1404; 1,77E+19

Peak	Identification	RT	MRM transitions	CV	CE	Quantified by
1	Dp 3 gal	2.64	465 → 303; 229	20	22; 58	Mv 3 glu
2	Dp 3 glu	2.82	465 → 303; 229	20	22; 58	Std
3	Dp 3 ara	2.90	435 → 303	20	22	Mv 3 glu
4	Cy 3 gal	3.03	449 → 287; 136	28	30; 48	Std
5	Cy 3 sambubioside	3.15	581 → 287; 137	30	28; 66	Mv 3 glu
6	Cy 3 glu	3.19	449 → 287; 136	28	30; 48	Std
7	Pt 3 gal	3.24	479 → 317; 302	28	30; 42	Mv 3 glu
8	Cy 3 ara	3.36	419 → 287; 137	26	24; 52	Std
9	Pg 3 gal	3.36	433 → 271; 121	24	36; 58	Mv 3 glu
10	Pt 3 glu	3.39	479 → 317; 302	28	30; 42	Std
11	Pt 3 ara	3.50	449 → 317	28	30	Mv 3 glu
12	Pg 3 glu	3.51	433 → 271; 121	24	36; 58	Std
13	Pn 3 gal	3.59	463 → 301; 286	28	28; 42	Std
14	Mv 3 gal	3.74	493 → 331; 315	28	24; 34	Mv 3 glu
15	Pn 3 glu	3.76	463 → 301; 286	28	28; 42	Std
16	Mv 3 glu	3.89	493 → 331; 315	28	24; 34	Std
17	Dp acetyl 3 gal	3.91	507 → 303; 229	30	30; 50	Mv 3 glu
18	Pn 3 ara	3.93	433 → 301; 286	26	22; 40	Mv 3 glu
19	Mv 3 ara	4.00	463 → 331	28	24	Mv 3 glu
20	Cy acetyl 3 gal	4.05	491 → 287; 213	28	28; 54	Mv 3 glu

21	Dp acetyl 3 glu	4.38	507 → 303; 229	30	30; 50	Mv 3 glu
22	Cy acetyl 3 glu	4.44	491 → 287; 213	28	28; 54	Mv 3 glu
23	Pt acetyl 3 gal	4.45	521 → 317; 302	28	24; 46	Mv 3 glu
24	Dp coumaroyl 3 gal	4.57	611 → 303; 229	34	28; 70	Mv 3 glu
25	Mv acetyl 3 gal	4.67	535 → 331; 315	30	26; 50	Mv 3 glu
26	Pn acetyl 3 gal	4.77	505 → 301; 286	30	28; 50	Mv 3 glu
27	Dp coumaroyl 3 glu	4.83	611 → 303; 229	34	28; 70	Std
28	Pt acetyl 3 glu	4.88	521 → 317; 302	28	24; 46	Mv 3 glu
29	Cy coumaroyl 3 gal	4.89	595 → 287; 137	34	34; 72	Mv 3 glu
30	Pn acetyl 3 glu	4.94	505 → 301; 286	30	28; 50	Mv 3 glu
31	Mv acetyl 3 glu	5.01	535 → 331; 315	30	26; 50	Std
32	Cy coumaroyl 3 glu	5.14	595 → 287; 137	34	34; 72	Std
33	Pn coumaroyl 3 gal	5.29	609 → 301; 286	38	32; 54	Mv 3 glu
34	Mv coumaroyl 3 gal	5.33	639 → 331; 315	38	30; 58	Mv 3 glu
35	Pn coumaroyl 3 glu	5.57	609 → 301; 286	38	32; 54	Std
36	Mv coumaroyl 3 glu	5.58	639 → 331; 315	38	30; 58	Std

	Year 2013						Year 2014					
Location	**Vm1**	**Vm2**	**Vm3**	**Vm4**	**Vm5**	**Vm6**	**Vm1**	**Vm2**	**Vm3**	**Vm4**	**Vm5**	**Vm6**
Dp 3 glu	163.2±56.9	258.8±25.4	206.3±51.8	205.2±68.3	210.1±0.0	224.2±131.8	152.8±62.1	291.3±58.7	221.5±64.9	407.5±166.3	241.8±160.4	236.7±80.6
Dp 3 gal	154.0±54.7	224.0±26.6	212.8±55.6	206.1±35.1	263.5±0.0	230.8±135.6	134.0±59.2	202.2±44.7	53.2±33.8	310.4±138.0	252.9±134.0	213.3±71.0
Dp 3 ara	189.4±73.2	278.6±40.0	258.3±61.2	293.2±22.3	295.5±0.0	311.9±148.1	169.4±64.0	261.2±47.5	242.9±55.3	377.7±151.2	323.2±162.4	350.3±72.2
Dp ac 3 glu	0.1±0.1	0.3±0.1	0.1±0.1	0.1±0.1	0.2±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Dp ac 3 gal	0.2±0.1	0.2±0.1	0.1±0.1	0.2±0.1	0.3±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Dp coum 3 gal	2.1±1.0	3.5±0.5	2.1±0.8	2.5±0.7	3.5±0.0	3.3±3.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Dp coum 3 glu	1.6±0.8	3.1±0.7	1.8±0.6	1.9±1.3	1.8±0.0	2.1±2.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Cy 3 glu	31.9±12.6	36.5±6.4	30.8±7.8	26.6±14.5	49.1±0.0	28.0±13.5	63.4±18.6	65.2±12.2	52.4±29.2	95.5±38.0	76.9±31.6	55.0±15.1
Cy 3 gal	33.7±15.1	33.1±6.6	34.3±10.7	26.6±11.9	60.7±0.0	29.1±14.2	59.4±18.7	65.2±12.2	53.2±33.8	75.0±33.5	89.7±37.1	50.9±14.6
Cy 3 ara	55.4±27.1	66.1±8.0	64.7±20.4	43.2±20.1	71.6±0.0	54.0±14.6	18.2±5.9	16.4±4.2	15.3±7.9	24.5±9.3	28.6±10.7	21.4±4.5
Cy 3 samb	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.3±0.3	0.2±0.2	0.0±0.0
Cy ac 3 glu	0.3±0.2	0.3±0.1	0.2±0.1	0.1±0.1	0.3±0.0	0.2±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Cy coum 3 glu	1.1±0.7	1.4±0.6	0.7±0.4	0.7±0.5	1.4±0.0	0.8±0.6	0.4±0.3	0.9±0.3	0.1±0.1	0.6±0.5	0.2±0.2	0.0±0.0
Cy coum 3 gal	1.1±0.7	1.0±0.4	0.8±0.3	0.6±0.5	2.1±0.0	0.8±0.6	0.0±0.0	0.3±0.3	0.0±0.0	0.5±0.5	0.5±0.5	0.0±0.0
Pt 3 glu	17.0±6.4	26.1±2.9	20.3±5.0	20.9±8.0	25.0±0.0	23.6±14.3	32.0±12.4	59.5±12.6	41.7±13.2	70.5±27.3	52.0±28.1	42.7±12.4
Pt 3 gal	11.2±4.2	15.0±1.3	14.4±3.5	12.9±4.1	19.0±0.0	14.9±7.5	16.4±7.4	25.7±7.2	22.4±8.2	30.0±13.9	31.2±13.8	19.9±5.9
Pt 3 ara	11.4±3.7	15.4±2.0	14.6±2.4	14.3±0.3	18.0±0.0	16.3±7.2	12.9±5.0	18.5±4.7	14.0±4.3	21.1±8.4	23.2±9.4	20.6±4.9
Pt ac 3 glu	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pn 3 glu	44.3±24.0	39.2±13.0	31.9±10.1	24.2±20.2	56.0±0.0	27.6±11.0	31.2±12.6	29.6±7.3	23.5±17.2	31.8±14.5	39.7±15.3	19.3±5.3
Pn 3 gal	19.4±19.0	10.5±3.7	11.9±3.8	5.9±4.9	21.0±0.0	9.2±2.3	6.4±2.7	5.3±1.9	5.0±4.3	4.6±2.6	8.7±4.0	3.2±1.0
Pn 3 ara	4.6±2.8	3.6±0.8	4.0±0.9	2.4±0.8	7.6±0.0	5.7±5.6	1.6±0.5	1.3±0.6	1.0±0.9	1.2±0.6	2.4±1.0	1.3±0.5
Pn coum 3 gal	0.1±0.1	0.1±0.1	0.1±0.0	0.0±0.0	0.2±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pn coum glu	0.4±0.3	0.3±0.2	0.2±0.1	0.1±0.1	0.5±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pn ac 3 gal	0.0±.0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Pn ac 3 glu	0.2±0.2	0.2±0.2	0.1±0.1	0.1±0.1	0.3±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Mv 3 glu	24.4±7.0	30.7±6.6	26.1±5.0	27.8±12.7	45.2±0.0	38.2±21.0	35.4±13.8	74.3±20.0	47.7±18.8	83.9±40.0	93.7±42.8	58.1±19.3
Mv 3 gal	7.8±2.9	8.5±1.1	9.7±2.8	8.3±2.7	16.0±0.0	12.6±6.2	9.2±4.6	17.1±5.5	13.1±6.4	15.8±8.2	26.0±10.2	12.8±4.6
Mv 3 ara	11.6±3.0	11.5±1.1	12.6±2.3	11.3±2.4	22.1±0.0	18.2±9.8	9.6±4.1	17.1±5.4	11.8±5.2	16.6±8.6	30.5±12.6	22.2±8.3
Mv ac 3 glu	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Mv ac 3 gal	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Mv coum 3 glu	12.3±5.3	18.9±7.9	12.0±3.5	12.0±3.5	21.2±0.0	20.5±20.4	0.0±0.0	0.8±0.4	0.0±0.0	0.4±0.4	0.2±0.2	0.0±0.0
Mv coum 3 gal	5.0±2.2	6.6±2.2	5.6±2.0	4.3±2.0	9.5±0.0	8.0±7.7	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.1	0.0±0.0
Pg 3 glu	0.4±0.2	0.6±0.3	0.4±0.1	0.4±0.1	0.4±0.0	0.3±0.3	0.2±0.2	0.7±0.5	0.2±0.2	0.7±0.5	0.6±0.5	0.0±0.0
Pg 3 gal	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.0	0.4±0.0	0.2±0.1	0.1±0.1	0.0±0.0	0.2±0.2	0.3±0.3	0.3±0.3	0.0±0.0
Total AC	686.6±207.2	979.7±84.4	900.7±211.8	879.3±157.9	740.2±0.0	1007.3±509.3	753.7±206.7	1137.7±226.9	819.3±262.1	1569.1±642.8	1323.6±620.9	1128.7±291.9

	Year 2013			Year 2014		
Location	**Vc1**	**Vc2**	**Vc3**	**Vc1**	**Vc2**	**Vc3**
Dp 3 glu	59.4±7.7	90.7±10.5	84.2±6.4	123.3±26.2	52.0±5.4	18.5±8.7
Dp 3 gal	2.5±0.3	4.4±0.7	3.1±1.0	5.0±0.09	1.6±0.3	0.7±0.2
Dp 3 ara	54.8±9.8	92.1±12.2	86.8±4.0	105.5±15.6	55.3±3.4	21.9±10.0
Cy 3 glu	0.2±0.0	0.3±0.1	0.2±0.0	0.4±0.1	0.3±0.0	0.1±0.1
Cy 3 gal	2.3±0.5	4.0±0.3	3.5±0.7	7.6±2.6	3.2±0.6	2.8±0.4
Cy 3 ara	1.5±0.3	3.2±0.2	3.0±0.5	1.9±0.6	0.9±0.1	0.8±0.1
Pt 3 glu	0.5±0.1	0.7±0.2	0.6±0.1	1.4±0.3	0.5±0.0	0.3±0.0
Pt 3 gal	7.6±1.1	12.0±1.3	11.6±0.6	26.5±4.6	13.8±0.8	6.2±1.6
Pt 3 ara	6.1±0.6	10.0±0.8	10.2±0.3	16.6±2.4	10.6±0.5	5.1±1.5
Pn 3 glu	0.2±0.0	0.4±0.1	0.5±0.1	0.3±0.1	0.1±0.0	0.1±0.0
Pn 3 gal	3.1±0.6	5.6±0.8	6.2±0.8	3.1±0.6	1.8±0.2	1.1±0.3
Pn 3 ara	1.3±0.2	3.0±0.3	3.3±0.5	0.5±0.0	0.4±0.1	0.3±0.0
Mv 3 glu	1.3±0.3	1.8±0.4	1.9±0.2	3.8±0.4	2.3±0.2	0.9±0.2
Mv 3 gal	14.9±1.1	22.5±1.9	23.9±0.9	56.4±6.6	38.1±1.8	18.8±5.6
Mv 3 ara	11.5±1.3	18.4±2.0	20.6±0.3	32.4±4.8	29.1±1.1	13.6±3.8
Total AC	168.0±28.6	269.9±28.4	260.1±12.7	384.6±58.8	209.9±11.0	91.3±26.7