

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

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16 **Abstract**

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

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

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33 **Introduction**

34 In recent years, the health benefits of *Vaccinium* berries, e.g. blueberries, cranberries and bilberries,
35 have gained visibility for the highest antioxidant activity linked with the high content of phenolic
36 compounds, especially anthocyanins (ACs), compared with other fruits and vegetables¹. These
37 compounds have been reported to have various health beneficial activities including antioxidant,
38 antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative and antimicrobial effects².

39 Wild berries belonging to the genus *Vaccinium* such as bilberry, *V. myrtillus* L. and lingonberry, *V.*
40 *vitis idaeae* L. are a valuable part of the European nature and tradition, especially in the Northern and
41 Eastern parts of Europe where they grow abundantly and have economic importance. However, both
42 bilberry and lingonberry are also distributed within alpine environments of Southern Europe, covering
43 large areas of the Italian mountains, which are characterized by acidic soils³. Bilberry, particularly,
44 establishes on a wide range of environmental conditions, from hilly areas to high altitudes above tree-
45 line, although, its optimal range is between 1500 m and 2000 m above sea level (a.s.l.), where pure
46 bilberry formations may occur both in open habitats and in the understory of conifer-dominated
47 forests⁴.

48 In Italy, bilberry is collected in natural environments of the Apennines and Alps. Studies on the
49 nutritional quality have been performed on bilberries of Central Apennines⁵, from Western⁶ to Eastern
50 Alps³, and information is available also for some neighboring alpine countries^{7,8}. However, due to
51 the difficulties in handling and storability of bilberries, the economic interest on these berries has
52 been low in South Europe where major interest has been focused on soft berry fruit cultivation (e.g.
53 blueberry, *V. corymbosum* L. and raspberry, *Rubus* spp.). Cultivation of these berries fits well to
54 alpine climate and soil conditions, and takes place up to 1000 m of altitude.

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

59 number of bonded sugars to their structure, the aliphatic or aromatic carboxylates bonded to the sugar
60 residues and the position of these bonds¹². According to the number and position of hydroxyl and
61 methoxyl groups on the flavonoid molecule, six most common anthocyanidin aglycons are classified:
62 pelargonidin (Pg), the mono-hydroxylated cyanidin (Cy) and peonidin (Pn), and the tri-hydroxylated
63 delphinidin (Dp), petunidin (Pt) and malvidin (Mv)¹³. The most common ACs found in *Vaccinium*
64 berries are monoarabinosides (ara), monoglucosides (glu) and monogalactosides (gal) of Cy, Pt, Pn,
65 Dp and Mv, though several other phenolic compounds, and their glycosides, have been described
66 (e.g. catechin, epicatechin, myricetin, kaempferol, quercetin, myricetrin and caffeic, p-coumaric and
67 ferulic acids)^{1,8,14-17}. Dp and Mv derivatives are described as the majority of ACs found in blueberries,
68 constituting about 70% of total ACs¹⁸. Acylated ACs are also found in blueberries and bilberries but
69 they account as a small portion of total amount^{18,19}.

70 Latitude appears to influence the accumulation of ACs in bilberries, as a clear increasing trend in AC
71 production towards north has been reported in high latitudes. Higher AC content was reported in
72 northern (63-70°N) latitudes of Northern Europe, compared to southern latitudes (54-62°N)^{10,11}. The
73 berries of the northernmost clones have been shown to contain higher total content of ACs and the
74 higher proportion of the more hydroxylated anthocyanidins, Dps and Mvs, whereas Cys accumulated
75 more in the more southern North European populations (latitudes 54-62°N). Studies have also been
76 performed in relation to different altitudes, although they have given contradictory results. Spinardi
77 et al.²⁰ reported higher levels of ACs and ascorbic acid in blueberries grown at 600 m a.s.l. compared
78 with the same cultivar grown at 450 m a.s.l. in Valtellina (Northern Italy). In a two year study in
79 Austria⁷, decreasing AC contents were found in bilberry fruits along with increasing altitude (between
80 800 m a.s.l. and 1500 m a.s.l.). In studies performed in the areas of Northern Europe, where altitudinal
81 differences are less pronounced, no clear relationship with elevation and AC concentration have been
82 found^{11,21}.

83 In the present study, we investigated the effect of the altitude on the accumulation of ACs in the
84 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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100 **Materials and method**

101 **Plant material and altitudinal gradients.** The field trials were established along altitudinal
102 gradients in the region Trentino (Italy). Permissions for field work were granted by the municipalities
103 of Grumes, Valda and Zambana and the Province of Trento (Italy). Six sites were identified for
104 bilberry (*V. myrtillus* L., Vm) experimental fields. The gradient was established between Val di
105 Cembra (46.22°N, 11.26°E) and Monte Paganella (46.16°N, 11.04°E) at 1166 m, 1261 m, 1406 m,
106 1520 m, 1620 m and 1829 m a.s.l. (Table 1). In each site 10-25 individual bilberry plants within an
107 area of about 20 m² were considered for AC analysis of the berries.

108 Blueberries (*V. corymbosum* L., Vc) of cv. Brigitta Blue, were collected from plants cultivated by
109 farmers of Valsugana valley (46.08°N, 11.50°E). Three sites, located in Telve (495 m a.s.l.), Spera
110 (749 m a.s.l.) and Pinè (1034 m a.s.l.) were considered for the altitudinal gradient (Table 1). Plants
111 were cultivated according to conventional farming methods. Fruits were collected from five plants
112 chosen randomly on five different rows of plants in the field in an area of 100 m².

113 **Fruit harvesting.** The study was conducted in two consecutive growing seasons (years 2013 and
114 2014). Bilberries were harvested when fruits reached the full development in size and color, and total
115 soluble content (TSSC) was higher than 6.0 °Brix. In 2013, bilberry fruits from Val di Cembra were
116 collected between July 17 (in lowest location - site Vm1) and July 24 (in middle high locations - sites
117 Vm2 and Vm3), while on Monte Paganella, on sites Vm4 and Vm5 bilberries were collected on
118 August 21 while bilberries from the highest location (site Vm6) on September 3. In 2014, the growing
119 season was anticipated in Val di Cembra, as the berry harvesting started on June 20 in the lowest site
120 (Vm1). The harvest continued on July 3 on sites Vm2 and Vm3, on August 1 for Vm4 and August 28
121 on sites Vm5 and Vm6 (Table 1).

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

126 2014, fruits were harvested on the same plants, starting from Telve on July 7 and finished on August
127 12 in Spera and Pinè (Table 1).

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

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

133 **ACs extraction.** Frozen ripe berries were ground into a fine powder with a cryomill (Retsch, Haan,
134 Germany). The homogenized samples (1 g fresh weight, FW, out of at least 10 g of fresh berries)
135 were extracted twice in 1,5 mL of methanol 80% under shaking for 1 h. Samples were centrifuged at
136 12000 g for 2 min. Supernatants were joined and brought to a volume of 5 mL. Samples were filtered
137 (0.22 µm PVDF filters), transferred to glass vials and randomized before analysis by UPLC-
138 MS/MS²².

139 **UPLC analysis.** Analytical separation of ACs was performed in an Acquity ultraperformance liquid
140 chromatographic (UPLC) system (Waters, UK) operating under MassLynx XS software. The system
141 was coupled to a mass spectrometer detector Waters Xevo TQ MS (Milford, MA, USA) equipped
142 with an electrospray (ESI) source. All samples were analyzed on a reverse phase Acquity UPLC BEH
143 C18, 1.7 µm, 2.1 x 150 mm column (Waters), protected with Acquity UPLC BEH C18, 1.7 µm, 2.1
144 x 5 mm precolumn (Waters) at 40 °C and under mobile phase flow rate of 0.4 mL/min. Water was
145 used as weak eluting solvent (A) and methanol as strong elution solvent (B); formic acid 5% v/v was
146 used as additive in both eluents. The multistep linear gradient used was as follows: from 95 to 60%
147 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
148 isocratic hold for 3 min to clean the column. The equilibration time was 4 min, and the injection
149 volume was 2 µL. 4-hydroxy-stilbene was used as internal standard (2.5 ppm) to check that the
150 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 wine²². 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

176 and ripening at noon on sunny days under clear sky, within a range of altitude between 495 m and
177 1404 m a.s.l. (in locations Vc1, Vc2, Vc3, Vm1, Vm2 and Vm3). The quality of light reaching the
178 plants was measured as Photosynthetic Active Radiation (PAR) by integrating the area between 200-
179 850 nm for full sunlight spectra, 200-380 nm for UV light, 380-750 nm for visible light, 380-495 nm
180 for blue, 590-710 nm for red and 710-750 nm for far-red components²³. Spectra and integration of
181 light were measured from five consecutive scans of the sunlight, after calibration of the instrument
182 according to the manufacturer's instructions.

183 **Soil pH.** For bilberry, in every location, five points were spotted out of the edges of the bilberry
184 matrix frame for soil sampling. In these spots, about 1 kg of soil between 20 and 50 cm underground
185 was collected. The samples were first cleaned from plant roots, leaves and small stones and then
186 pooled together. Pooled sample of 80 g was dissolved in 200 ml distilled water²⁴, stirred overnight,
187 and the next day pH was measured with Crison PH 25+ (Hach Lange Srl, Spain).

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

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

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199 **Results and discussion**

200 **AC profile of bilberry populations.** AC profiles of bilberry fruits as well as AC variation among
201 native bilberry populations in Alps of Northern Italy were systematically analyzed. The ACs
202 identified were Dp, Cy, Pn, Pt and Mv conjugated with glu, gal and ara, and their acylated forms,
203 including acetylated and *p*-coumaroylated ACs. In small amounts, also Cy 3 sambubioside and Pg
204 were identified (Suppl. Table 2). The amounts of Dp glycosides were higher than of Cy glycosides
205 on average, similarly to the profiles detected in other Southern European bilberries, e.g. from Austria⁷,
206 Slovenia⁸ and Montenegro²⁵ but also in Northern European bilberries, e.g. from Finland^{10,19}.
207 However, the comparison between bilberries from Finland¹⁹ and Italy (presented in this study) which
208 were analyzed with the same analytical method²², revealed differences in the sugar moiety. In
209 bilberries growing in Northern Europe over 60% of the ACs was conjugated with glu, whereas the
210 rest 40% was shared equally between gal and ara¹⁹. In the present study, ACs were equally conjugated
211 with the 3 sugars (about 30% each) which is in line with the results of the study on Slovenian
212 bilberries⁸. In the present study, also climate differences affected accumulation of acetylated
213 compounds, which were more abundant in 2013 than in 2014 (Suppl. Table 2).

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

219 **AC profile of blueberries (cv. Brigitta Blue).** The AC profile was typical for the cv. Brigitta blue²⁶,
220 with Dp and Mv as predominant anthocyanidins, followed by Pt and in lowest proportion Cy and Pn
221 (Suppl. Table 3). AC compounds were conjugated with glu, gal and ara sugars, but were not uniformly
222 distributed among the anthocyanidins. Dp was equally conjugated with glu and ara, whereas very
223 little amount of Dp 3 gal was detected. On the contrary, Mv and Pt (which are methylated forms of

224 Dps²⁷), and the mono-hydroxylated Cy and Pn were equally conjugated with gal and ara and to a
225 small extent with glu. No acylated forms were identified in blueberries (Suppl. Table 3).

226 **AC accumulation in wild and cultivated *Vaccinium* spp. along altitudinal gradients.** The two
227 *Vaccinium* species, bilberry and blueberry, showed important differences in the accumulation of ACs
228 along the altitudinal gradient. Our results indicated an increase in the accumulation of ACs in
229 bilberries along a gradient of about 650 m, which was consistent over the two growing seasons (Fig.
230 2). Indeed, regression analysis of total AC concentration gave a significant positive correlation
231 between altitude and total AC accumulation in berries ($p = 0.001$ in 2013 and $p = 0.002$ in 2014) in
232 both years (Fig. 2). The trend was due to a significant increase in the tri-hydroxylated anthocyanidins
233 such as Dp ($p = 0.0003$ in 2013, $p = 0.0002$ in 2014) and Mv ($p = 0.0002$ in 2013, $p = 0.0026$ in
234 2014). To a small extent also Pt increased with increasing altitude ($p = 0.001$ in 2013). The
235 accumulation of the mono-hydroxylated Cy and Pn glycosides, instead, was not affected by the
236 altitude (Fig. 2).

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

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

250 2014 accumulation was negatively correlated with altitude ($p = 0.0000$; Fig. 3). In the case of
251 blueberry, only the cultivar (Brigitta Blue) was used in the study and, therefore, the seasonal
252 differences in the AC content were considered to be connected to environmental effects.

253 **Environmental effect on AC accumulation.** In all types of fruits, environmental factors affect the
254 quantitative and qualitative composition of ACs in the ripening fruit³¹. In general, the genetic
255 background of the species/variety determines the AC content in fruit, yet the environmental factors
256 can affect the concentration of diverse ACs in different ways. Temperature, solar radiation and soil
257 are considered the major environmental factors affecting the accumulation of ACs in fruits³². All
258 these factors may vary markedly along latitudinal gradients³³, where changes in phenolic content of
259 bilberries were recorded with changing environments from southern to northern latitudes^{10,11,21}.

260 In the present study we observed that the same factors vary markedly also along an altitudinal
261 gradient. Maximum daily temperatures decreased progressively with increasing altitude (Fig. 4).
262 Moreover, differences in temperatures were recorded throughout the two growing seasons, which
263 significantly affected the accumulation of ACs in berries ($p < 0.05$; Suppl. Table 2). Higher
264 temperatures were recorded during the year 2013, when also higher accumulation of AC was found
265 in bilberries, with higher proportion of acylated forms of ACs (Suppl. Table 2) in line with findings
266 in grape berries (cv. Merlot)³⁴ and in our previous study on bilberry³⁰.

267 Light conditions were also measured in six locations along the gradient, within 495 m and 1404 m
268 a.s.l.; Fig. 5). Sunlight PAR (full spectra) increased constantly towards increasing altitudes, and
269 significant changes ($p < 0.001$) were recorded between locations at 495 m a.s.l., at 756 m a.s.l. and
270 locations higher than 1034 m a.s.l. The increase was due to a progressive increase of visible light
271 along with altitude, although the blue and red components were not significantly affected with
272 increasing altitude. The UV radiation counted only for the 1.3-3.8% of the total radiation, and was
273 not significantly affected by altitude. The soil pH ranged between 4.3 and 5.1 (Table 1), which is
274 optimal for the growth of bilberry³⁶, but did not show any particular trend connected with altitude.

275 Our results suggest that lower temperatures have a major effect on the accumulation of ACs in
276 bilberry. Decreasing temperatures observed with the increasing altitude, positively influenced the
277 accumulation of ACs in bilberries being in line with the results of Uleberg et al.²⁸, who showed that
278 higher amounts of Dp derivatives are produced at low temperatures (12°C) compared to higher
279 temperatures (18 °C). Recently, we also observed that when plants from the same bilberry populations
280 were moved from higher to lower altitude (485 m a.s.l.), in open-field conditions with higher
281 temperature, berries accumulated lower amounts of ACs, confirming the role of temperature in the
282 determination of the final AC profile of berries³⁰. Thus, the difference in the average daily
283 temperature in the original locations (Vm1-Vm6) was at least 5 °C lower than in the test field at 485
284 m a.s.l. and the AC accumulation was almost two fold higher in the original site. Excessive light
285 stress appeared to inhibit AC accumulation³⁰, but considering the present results, where light intensity
286 is higher at altitudes above 1034 m a.s.l. compared to fields at altitude 495 m a.s.l. (Fig. 5), light
287 intensity appears to have less influence than temperature on AC accumulation in bilberry fruits
288 growing in natural environments. Supporting this assumption, in the locations above 1034 m a.s.l.,
289 where bilberry populations were growing, no significant difference in the intensity of the light
290 reaching the plants was detected (Fig. 5). Therefore, we hypothesized that bilberry plants grown at
291 higher altitudes may have developed genetic adaptation mechanisms which respond to low
292 temperatures with an increased production in ACs, in particular of the Dp-type.

293 The biosynthesis of Dp-type ACs is driven by the flavanone 3'5'-hydroxylase (*F3'5'H*) gene, which
294 is responsible of the hydroxylation at the 3'5' positions of the B-ring of the precursor
295 dihydrokaempferol into Dp and the methylated derivatives Mv and Pt, and which also shifts the
296 biosynthesis from the Cy and Pn branch towards the branch of the flavonoid pathway producing Dp,
297 Mv and Pt as final core structures of ACs²⁷. Studies conducted on blueberry²⁷, bog bilberry (*V.*
298 *uliginosum* L.)³⁷ and grape berries (*Vitis vinifera* L.)³⁸ showed that the *F3'5'H* gene is weakly
299 expressed during the earliest ripening stages and is abundant only during the late ripening stages,
300 closely paralleling the appearance of ACs. In blueberry, as the fruit ripens and the exocarp colour

301 changes from mostly green to partially pink, blue-purple Dp-type ACs begin to accumulate. The
302 appearance of the tri-hydroxylated anthocyanidin Dp and its derivatives Mv and Pt is coordinated
303 with the abundance of *VcF3'5'H* transcripts at developmental stage S5 (at the initiation of ripening)²⁷.
304 Based on conservation of the flavonoid pathway in diverse species, the *VmF3'5'H* gene expression
305 is expected to increase at the late ripening stages of bilberry as well as when exposed to low
306 temperatures as found in the present study and as suggested for grapevine³⁹.

307 In blueberry, marked differences in AC content were recorded in the same locations during the two
308 seasons. The accumulation of ACs in blueberries is developmentally regulated and occurs during the
309 pink and the purple-blue (full ripe) stages of berry development²⁷. At the lowest location Vc1, the
310 berries started in 2013 to visibly turn color and to accumulate ACs on June 30 and they reached full
311 ripeness on July 22 whereas in 2014 the berries started to turn color on June 10 and reached ripeness
312 already on July 7. In the central location Vc2, blueberries started to turn color on June 30 in 2013 and
313 reached ripeness on July 22 (as in location Vc1), whereas in 2014 the ripening was delayed of almost
314 3 weeks as berries started AC accumulation on August 1 and ripened on August 12. In the highest
315 location Vc3, the ripening of berries was more uniform during the two seasons and the berries started
316 to turn color around August 1 in both years and were ready for picking on August 12.

317 The accumulation of AC in blueberries is regulated also by environmental factors³⁰. To our
318 knowledge, very little information is known about the effect of temperature on AC biosynthesis in
319 blueberries. Therefore, in the present study, the cultivation of blueberries at different altitudes allowed
320 us to investigate the effect of natural differences in temperature on the AC composition of the berries
321 at harvest. The meteorological station present in location Vc1 (Telve, Fig. 4) recorded maximum
322 daily temperatures ranging from 26 °C at the beginning of the pink stage and progressively increased
323 up to 32.5 °C at full ripeness in 2013. In 2014, during the pink stage, temperature peaked up to 33.5
324 °C and later decreased fluctuating around an average of 26 °C until ripening (Fig. 4). In
325 correspondence of the temperature flow recorded in 2014 (i.e. high temperatures during the pink stage
326 followed by lower temperatures during the last ripening phase), blueberries ripened faster and

327 accumulated the highest content of AC (385 ± 59 mg/100 g FW), which was doubled compared to
328 the amount recorded in 2013 (168 ± 22 mg/100 g FW; Fig. 3f, Suppl. Table 3). Meteorological
329 stations were not present on the site for the other two localities (Vc2 and Vc3) and therefore
330 information from the closest meteorological stations (Bieno, 6 km far from Vc2 and Lavarone, 43 km
331 far from Vc3, respectively) was used. In location Vc2, temperatures recorded were almost constant
332 throughout the pink and purple stages of berry development in both years (Fig. 4). Consistently, the
333 difference in the content of ACs in berries between the two seasons was not as marked as in the other
334 two localities (270 ± 28 mg/100 g FW, in 2013 and 210 ± 11 mg/100 g FW in 2014; Fig. 3f, Suppl.
335 Table 3). In location Vc3, a difference of 5-10°C between the two seasons markedly affected the
336 accumulation of AC in blueberries. In 2013, temperatures ranged between 20 °C and 28 °C during
337 the berry ripening (Fig. 4) and the AC content of blueberries was 260 ± 13 mg/100 g FW. In 2014,
338 accumulation of total ACs was only 91 ± 27 mg/100 g FW due to the lower temperatures (between
339 14°C and 25 °C) during the berry ripening stage. The optimum temperatures for blueberry fruit set,
340 size and ripening are 20-26 °C during the day and 16 °C during the night⁴⁰. However, the temperature
341 conditions registered in location Vc1 during 2014 appeared to be optimal for the AC accumulation in
342 blueberries (Fig. 3f, Suppl. Table 3). The present results are comparable with studies conducted on
343 grape berries, in which the highest concentration of ACs was recorded in berries grown in temperature
344 regimes with maximum daily temperatures of 25 °C⁴¹. The AC content instead dramatically dropped
345 when the maximum daily temperatures reached 35 °C⁴¹.

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

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

362

363 **Abbreviations Used**

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

368

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382

383 **Supporting information description**

384

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

390

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

394

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

398

399 This information is available free of charge via the Internet at <http://pubs.acs.org>

400

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- 527

528

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	<i>V. myrtillus</i>	46.22	11.26	1166 m	5.1	13	18	July, 17	July 3
Val di Cembra	Vm2	<i>V. myrtillus</i>	46.22	11.26	1261 m	4.4	7	18	July, 24	July, 3-10
Val di Cembra	Vm3	<i>V. myrtillus</i>	46.22	11.24	1404 m	4.8	11	23	July, 24	July, 10
Monte Paganella	Vm4	<i>V. myrtillus</i>	46.17	11.04	1520 m	4.7	2	11	August, 21	August, 1
Monte Paganella	Vm5	<i>V. myrtillus</i>	46.16	11.04	1617 m	4.8	1	11	August, 21	August, 28
Monte Paganella	Vm6	<i>V. myrtillus</i>	46.16	11.03	1829 m	4.3	12	23	September, 3	August, 28
Telve	Vc1	<i>V. corymbosum</i>	46.07	11.49	495 m	-	4	4	July, 22	July, 7
Spera	Vc2	<i>V. corymbosum</i>	46.08	11.51	749 m	-	4	4	July, 22	August, 12
Pinè	Vc3	<i>V. corymbosum</i>	46.03	11.28	1034 m	-	4	4	August, 12	August, 12

Figure 1

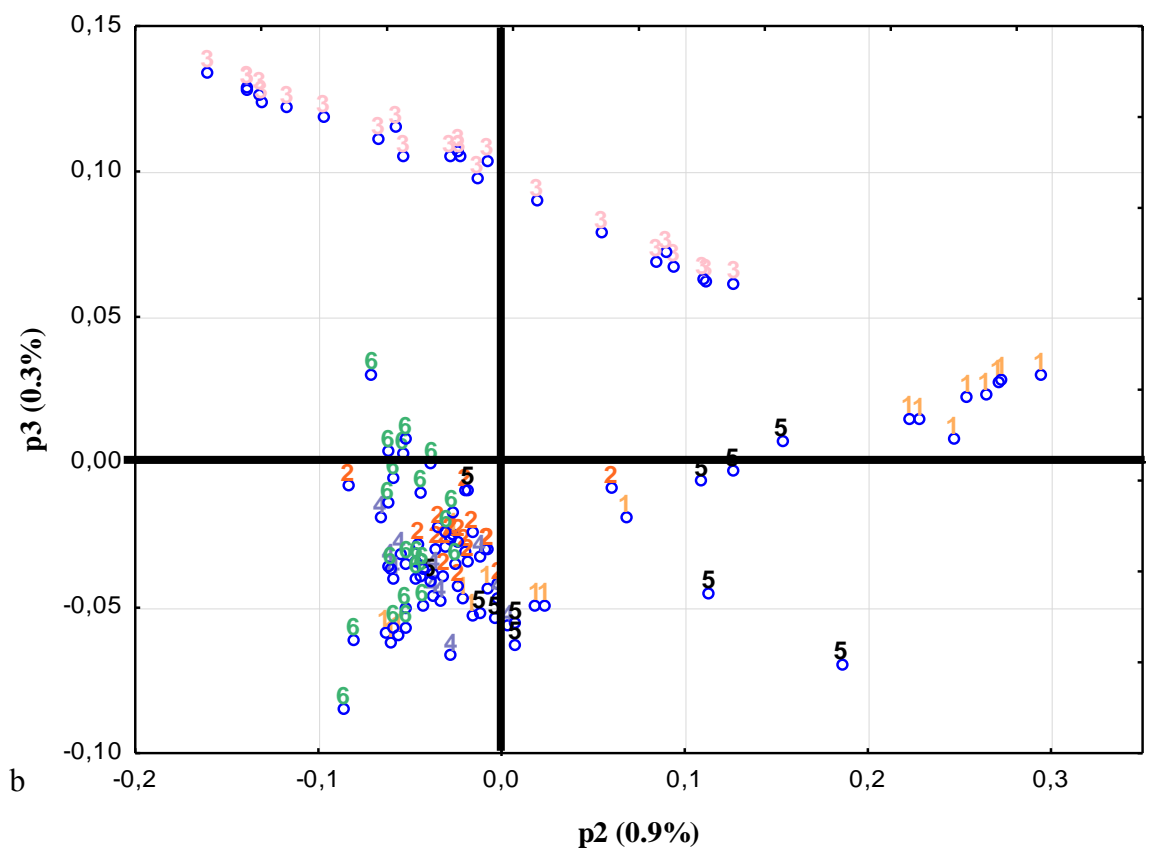
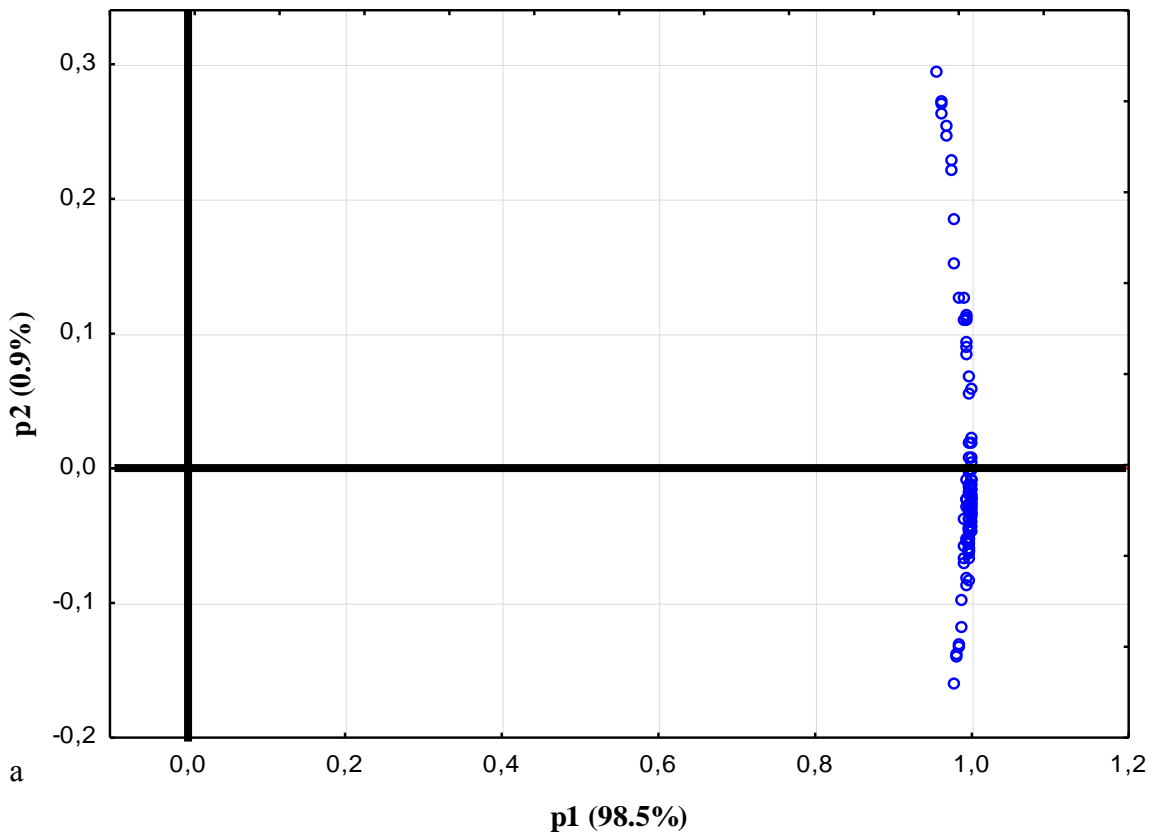


Figure 2

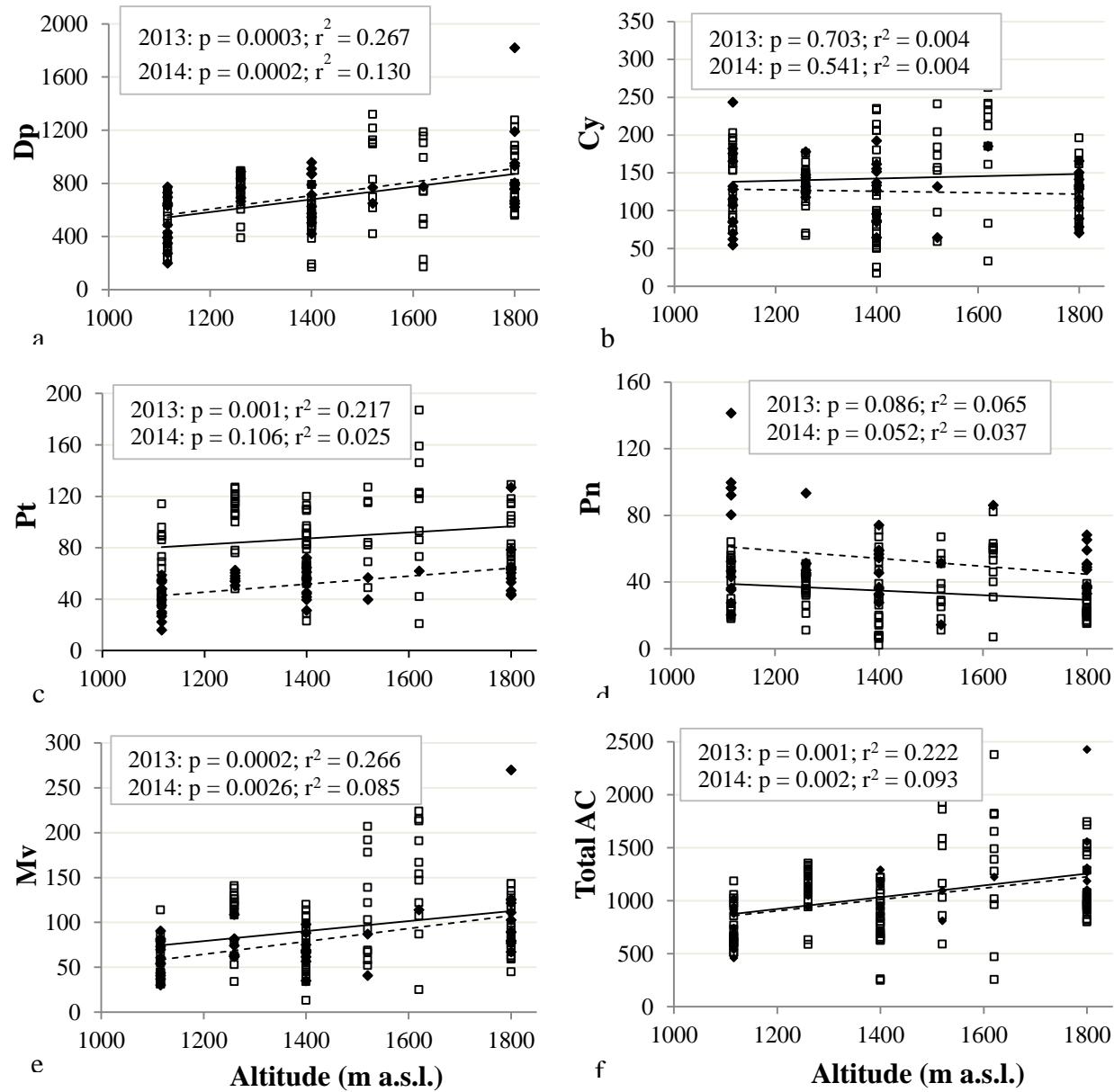


Figure 3

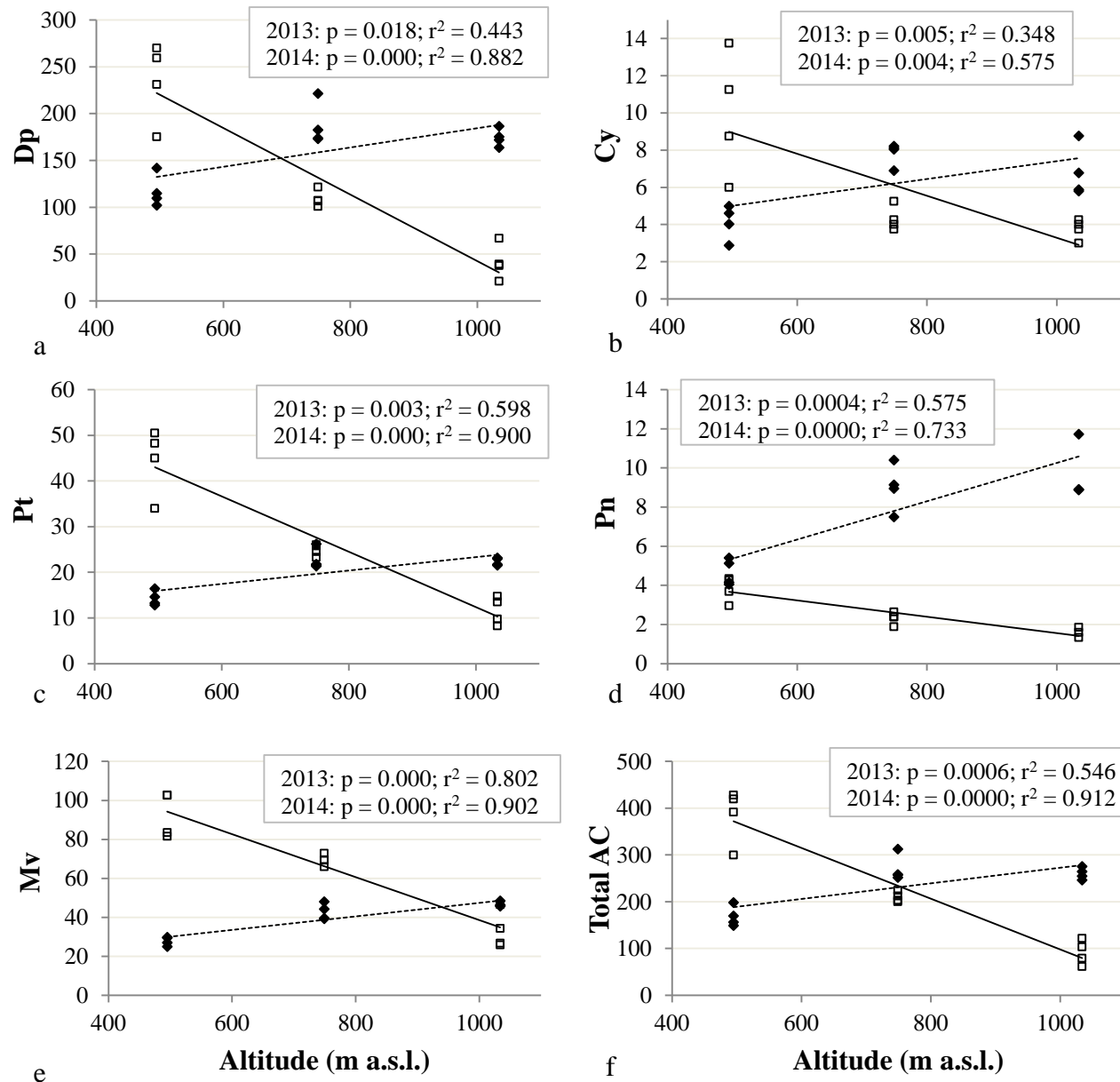


Figure 4

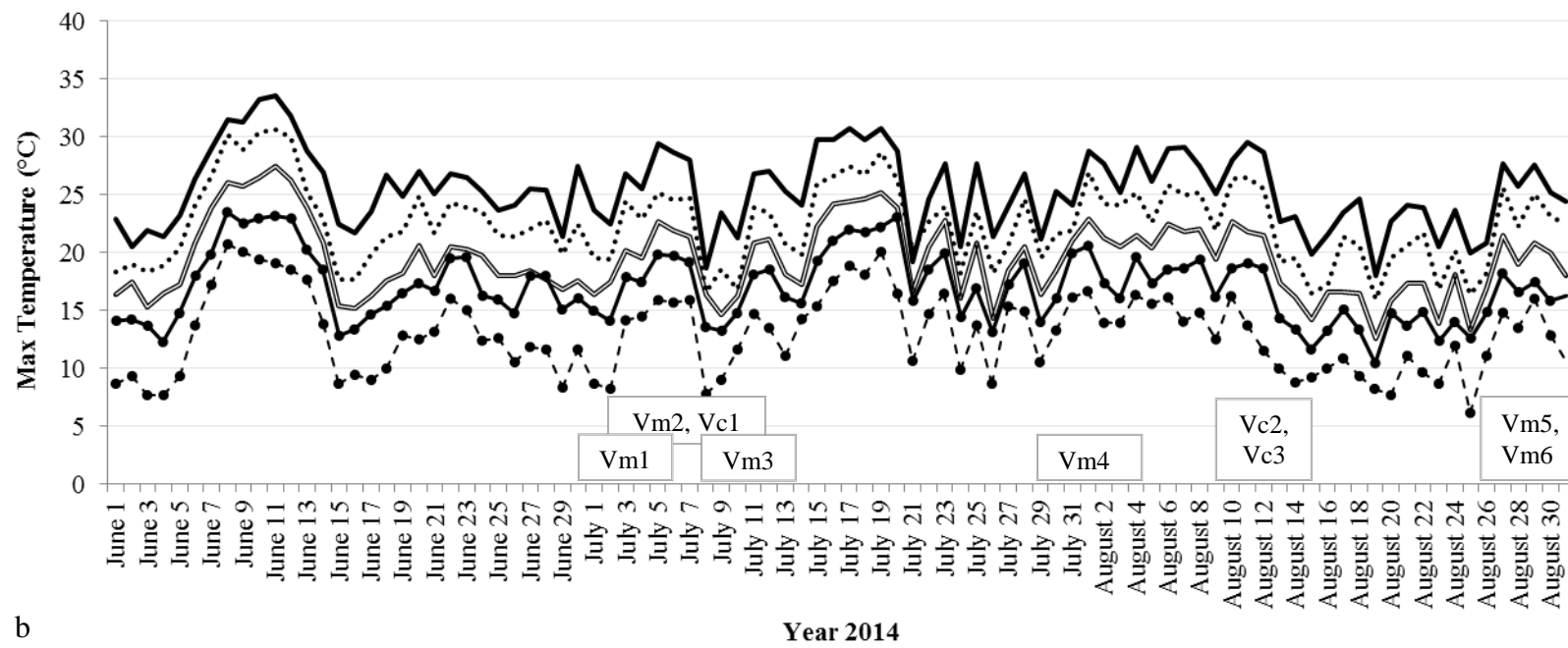
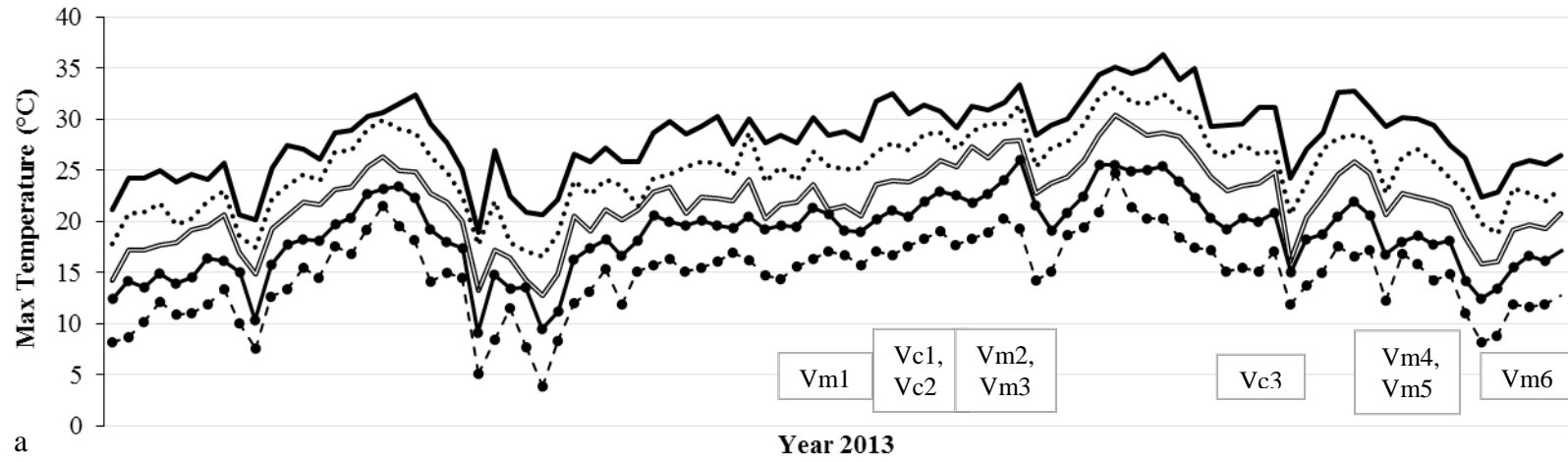
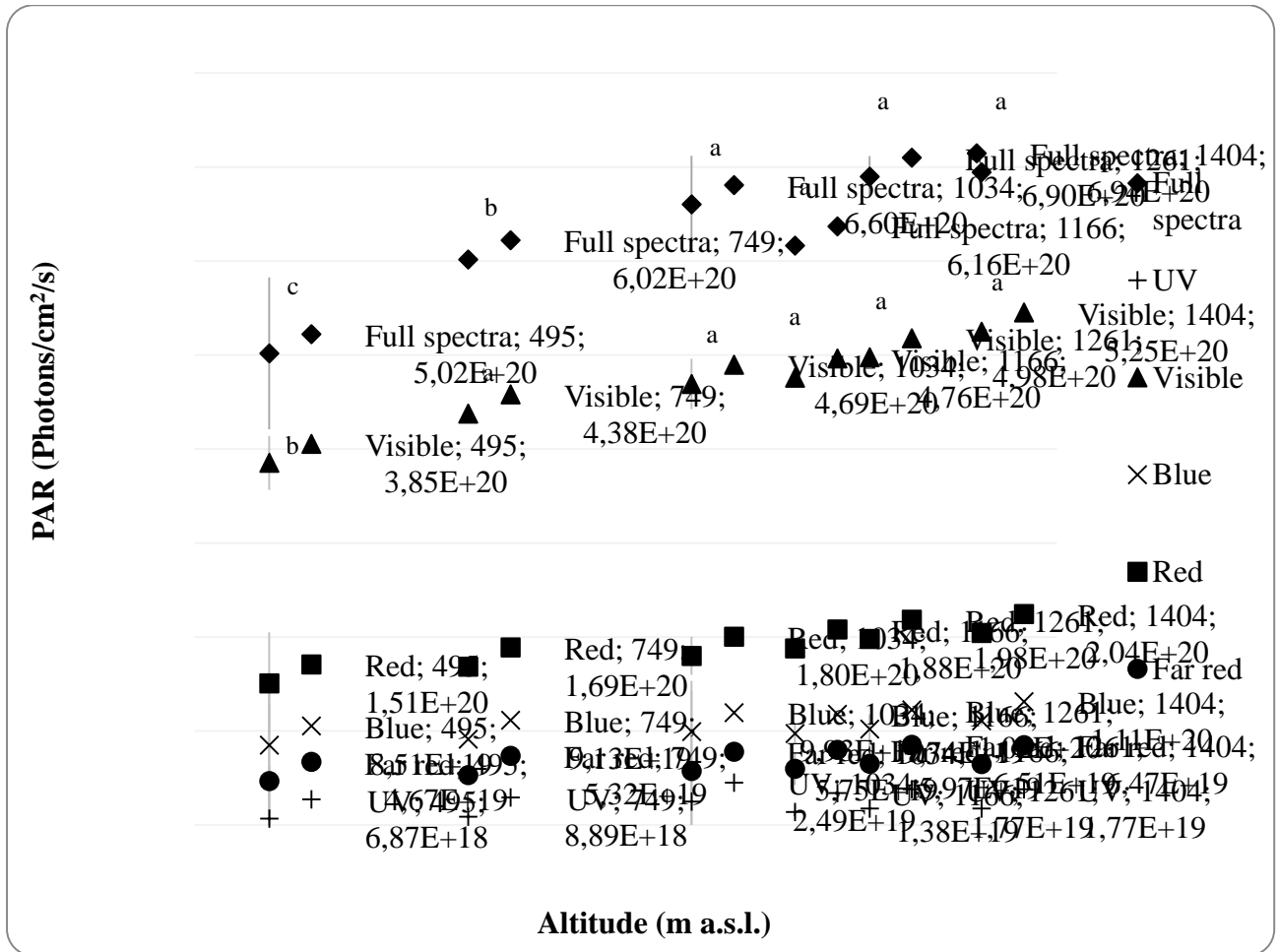


Figure 5



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

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

Location	Year 2013			Year 2014		
	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