Liquid chromatographic/electrospray ionization quadrupole/time of flight tandem mass spectrometric study of polyphenolic composition

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RESEARCH PAPER

Liquid chromatographic/electrospray ionization quadrupole/time of flight tandem mass spectrometric study of polyphenolic composition of different *Vaccinium* berry species and their comparative evaluation

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Abstract Ultra-high-performance liquid chromatography coupled with high-resolution quadrupole-time of flight mass spectrometry with both negative and positive ionization was used for comprehensively investigating the phenolic and polyphenolic compounds in berries from three spontaneous or cultivated *Vaccinium* species (i.e., *Vaccinium myrtillus*, *Vaccinium uliginosum* subsp. *gaultherioides*, and *Vaccinium corymbosum*). More than 200 analytes, among phenolic and polyphenolic compounds belonging to the classes of anthocyanins, monomeric and oligomeric flavonols, flavanols, dihydrochalcones, phenolic acids, together with other polyphenolic compounds of mixed structural characteristics, were identified. Some of the polyphenols herein investigated, such as anthocyanidin glucuronides and malvidin-feruloyl-hexosides in *V. myrtillus*, or anthocyanindin aldopentosides and coumaroyl-hexosides in *V. uliginosum* subsp. *gaultherioides* and a large number of proanthocyanidins with high molecular weight in all species, were described for the first time. Principal component analysis applied on original LC-TOF data, acquired in survey scan mode, successfully discriminated the three *Vaccinium* species investigated, on the basis of their polyphenolic composition, underlying one more time the fundamental role of mass spectrometry for food characterization.

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Introduction

The consumption of berries (e.g., blackberry, bilberry, blueberry, and cranberry) is considered an important contribution to healthy diets, owing to the various classes of phenolic compounds contained in large quantities in these fruits [1]. In fact, the class of phenolic compounds comprises a very high and increasing number of bioactive compounds [2], which are suggested to provide important health-protecting attributes such as anti-inflammatory, antihypertensive, antimicrobial, and anticancer properties [3].

Among the different berry species, *Vaccinium myrtillus* is the wild bilberry native to mountain areas of Northern and Central Europe, widely diffused also in Italian Alps and Apennines. In these zones, the increasing presence of a different spontaneous *Vaccinium* species, recently identified through genetic analyses as the *Vaccinium uliginosum* subsp. *gaultherioides* (locally named "false bilberry"), has been recently observed [4]. The cultivation and commercialization of *Vaccinium corymbosum* berries (i.e., the blueberry) is also widespread in the same area.

V. myrtillus is one of the richest fruit in polyphenols, with particular regard to anthocyanins [5] and is therefore considered a "functional food" [6]. Accordingly, *V. myrtillus* berries are largely consumed both as fresh fruits and processed products, such as juices and dietary supplements.

Many researches focusing on the determination of selected anthocyanins were carried out on bilberries from different European areas [7–11]. Interestingly, the composition of the

most abundant anthocyanins (i.e., glucosides, galactosides, and arabinosides of cyanidin, delphinidin, petunidin, peonidin, and malvidin) of *V. myrtillus* berries has been found different from the ones of blueberry [7], suggesting the potential use of polyphenolic profiles for the discrimination of transformed products from these fruit species. This aspect is very important since *V. myrtillus* is supposed to be a food with a higher nutraceutical value than *V. corymbosum* [6].

Furthermore, the feasibility of using the anthocyanin profile as a species fingerprint becomes noteworthy for discerning *V. myrtillus* from *V. uliginosum* L. subsp. *gaultherioides*. In fact, the phenotype of this latter berry is very similar to the *V. myrtillus* one, and the two fruits might be confused by the harvesters involved in the production chain of transformed bilberry. Italian *V. uliginosum* L. subsp. *gaultherioides* fruits were recently analyzed for the first time by our team, evidencing a lower content of total soluble polyphenols and total monomeric anthocyanins, as well as smaller antioxidant and antiradical activities, compared to *V. myrtillus* ones [4]. Hence, from this point of view, a lower nutraceutical value of "false bilberry," compared to bilberry, can be assumed. Concentrations of individual anthocyanins found in "false bilberry" were in most cases lower than those of bilberry, as well. Moreover, the relative abundance of the predominant anthocyanins of *V. uliginosum* L. subsp. *gaultherioides* berries was found very different from that of *V. myrtillus* fruits [4] and, interestingly, rather similar to the profile of *V. corymbosum*, being for instance both "false bilberry" and blueberry characterized by the predominance of malvidin derivatives [4, 7].

The analysis of further classes of polyphenols, such as flavonols, flavanols, and phenolic acids, which might be also important for discriminating one *Vaccinium* species from another, has been performed only occasionally in *V. myrtillus* [12, 13] and *V. corymbosum* berries [14]. Data concerning some phenolic compounds have been recently reported also for *V. uliginosum* L. subsp. *gaultherioides* berries [4].

Nevertheless, in the current literature, there is a lack of in-depth studies dealing with the simultaneous investigation of the different polyphenolic classes in *V. myrtillus*, *V. corymbosum*, and *V. uliginosum* L. subsp. *gaultherioides* berries.

In order to carry out such a kind of studies, complex analytical approaches, involving nontarget metabolomic investigations, are required. These investigations are commonly performed using liquid chromatography (LC) coupled with mass spectrometry (MS) [15, 16], employing in some cases also ultraviolet detection [17, 18] and occasionally nuclear magnetic resonance, as well [19]. Actually, LC-MS is one of the most powerful analytical technique for polyphenol analysis. In fact, atmospheric pressure ionization sources provide a soft ionization of target analytes, which is particularly recommended for structure elucidation of polar, nonvolatile, and thermally labile compounds, such as flavonoids. Moreover, the use of tandem mass spectrometry (MS/MS) enables to obtain important structurally related information through the fragmentation of parent molecules. In this context, the adoption of high-resolution mass spectrometry (e.g., time-of-flight-based instruments) allows for obtaining accurate mass readout, thus facilitating the assignment of an elemental formula to the parent molecule and/or to the fragments and its fragmentation characteristics [20].

Based on the above-reported considerations, this study aimed at comprehensively investigating the polyphenolic profiles of *V. myrtillus*, *V. corymbosum*, and *V. uliginosum* L. subsp. *gaultherioides* berries through a nontarget LC-MS/MS approach, using a quadrupole/time of flight mass spectrometry (Q/TOF).

Material and methods

Reagents and standards

Polyphenol standards were supplied as follows: cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, delphinidin-3-glucoside, delphinidin-3-galactoside, malvidin-3-glucoside, and malvidin-3-galactoside by Extrasynthese (Genay, France); peonidin-3-glucoside, peonidin-3-galactoside, peonidin-3-arabinoside, and petunidin-3-glucoside by Polyphenols Laboratories AS (Sandnes, Norway); and (+)-catechin, epicatechin, procyanidin B1, procyanidin B2, procyanidin A2, quercetin-3-galactoside, quercetin-3-glucoside, querectin-3-rutinoside, quercetin-3-rhamnoside, quercetin-3-glucuronide, quercetin, myricetin, keampferol-7-neohesperidoside, gallic acid, caffeic acid, *p*-coumaric acid, ferulic acid, chlorogenic acid, neochlorogenic acid, cryptochlorogenic acid, 1,5-dicaffeoylquinic acid, esculetin, scopoletin, and phloridzin by Sigma-Aldrich (St. Louis, MO, USA).

LC-MS grade methanol and water were obtained from J.T. Baker (Deventer, the Netherlands). HPLC grade methanol and formic acid eluent additive for LC-MS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium fluoride was obtained by Merck (Darmstadt, Germany). Ultrapure water was taken from a Milli-Q system supplied by Millipore (Billerica, MA, USA). Nylon membranes (porosity 0.2 μm) for the filtration of the bilberry extracts before HPLC analysis were obtained from VWR™ International (Radnor, PA, USA).

Fruit sampling and postharvest treatment

V. myrtillus and *V. uliginosum L.* subsp. *gaultherioides* samples analyzed in the present study consisted of blends of berries collected in 15 different zones of Tuscan Apennines in August 2014 (see Table S1 of the Electronic supplementary material). Hence, representative samples of the two wild berry

species were obtained for the whole investigated area. In these samples, the variations in polyphenolic composition within the species, due to different genotypes and/or environmental conditions (such as altitude and solar exposure of collection areas), should be therefore minimized.

In order to confirm the attribution to *V. uliginosum* subsp. *gaultherioides* of the "false bilberry" plants included in the present study, a genetic analysis was carried out following the specifications reported by Ancillotti and coworkers [4].

The *V. corymbosum* sample was a mixture of berries of the genotypes "Duke," "Berkely," and "Bluecrop," cultivated in a site included in the area of Tuscan Apennines selected for the harvest of wild species. These cultivars were chosen on the basis of their wide diffusion in the Italian market [21].

After the sampling, all berries were immediately frozen in liquid nitrogen, freeze-dried, and finally ground in order to obtain a homogeneous powder. All samples were stored at −20 °C until analyses were performed.

Sample extraction

Three representative aliquots from each berry sample were extracted according to a procedure previously developed for *Fragaria vesca* berries [22] and successively verified for bilberry and "false bilberry" [4]. Briefly, about 500 mg dry weight (d.w.) berry aliquots were homogenized in an ice bath under magnetic stirring with 15 mL of a methanol/water solution 8/2 (*v*/*v*), containing NaF 10 mM to inactivate polyphenol oxidase; the mixture was centrifuged at 1800×*g* for 5 min and the supernatant recovered. This procedure was repeated three times and the resulting extracts were combined. The organic solvent was removed by vacuum evaporation, acidified with formic acid up to pH = 2.0 ± 0.1 (volume of formic acid 170–190 μL), and filtrated at 0.2 μm with nylon membranes, before LC-MS/MS analysis. A final extract volume of approximately 9.2 mL was therefore obtained.

LC-TOF and LC-Q/TOF analysis

LC analysis was performed on an Agilent Infinity 1290 system equipped with an Acquity BEH C18 column (15 cm × 2.1 cm i.d., particle size 1.7 μm) and a guard column containing the same stationary phase (Waters, Milford, MA, USA). Column temperature was set at 50 °C. Water/formic acid 95:5 *v*/*v* (eluent A) and methanol/formic acid 95:5 *v*/*v* (eluent B) were used for the analyte elution, according to the following gradients: 0–2 min isocratic 2% B, 2–30 min linear gradient 2–30% B, 30–35 min linear gradient 30–95% B, and 35–37 min isocratic 95% B. The flow rate was 450 μL/min and the injection volume was 2 μL.

The LC system was coupled with a SCIEX (Framingham, MA, USA) TripleTOF® 5600 hybrid Q/TOF mass analyzer by the DuoSpray™ Source for MS and MS/MS analysis and the following source parameters were kept constant during the whole acquisition: heater temperature 400 °C, Curtain Gas™ 25, nebulizing gas 45, heating gas 45, and spray voltage +5300 and −4500 V for positive and negative polarity, respectively.

Each sample was analyzed, both under positive and negative ionization, using two different mass acquisition methods for each ionization mode. The first one consisted of a high-resolution TOF MS survey scan (from 100 to 2000 Da, cycle time 250 ms). The second acquisition method was a TOF survey scan experiment from 100 to 2000 Da (accumulation time 250 ms), followed by the selection of the top 10 candidate ions collected within each cycle, by the Information Dependent Acquisition (IDA) software. Q/TOF MS/MS spectra of the ions selected in each cycle were then acquired from 100 to 2000 Da, each one with an accumulation time of 75 ms, using a collision energy of 35 eV and a collision energy spread of ±15 eV (whole cycle time 1050 ms). In order to enhance the general quality of MS/MS spectra of peaks with low signal intensity, Q/TOF MS/MS analysis was also performed using narrower mass ranges, typically from 100 to 1250 Da (accumulation time of 50 ms and whole cycle time 800 ms).

Automated calibration was performed using an external calibrant delivery system (CDS) which infuses proper calibration solution prior to sample introduction.

Data processing and metabolite identification

The high number of information derived from the 5600 TripleTOF® analysis of investigated samples, both in negative and positive ionization, needs to be processed with specific software. PeakView® 2.2 and MasterView® 1.1 software were used for the compound identification based on the TOF accurate mass and isotope pattern determinations, as well as on the Q/TOF fragmentation spectra of parent ions.

The following identification criteria were adopted in this study.

- TOF accuracy of the pseudo-molecular ion: <5 ppm
- Isotope ratio difference compared to the theoretical isotope profile: <20%
- Purity score of the MS/MS spectra compared to the one of available standards: ≥80%

In this manuscript, we used the words "identification/identified," sometimes stressed by the words "undoubted/undoubtedly," "unequivocal/unequivocally" when an authentic reference standard was available. Conversely, the terms "putative/putatively" or "tentative/tentatively" were used in the sentence when the reference standard was not available.

Then, in order to compare the polyphenolic compositions of the three investigated species and to highlight the polyphenols that mainly contributed to their differentiation, principal

component analysis (PCA) of molecular or quasi-molecular ions of identified and putatively assigned compounds was performed using MarkerView 1.2.1 software. This approach was carried out separately for negative and positive ionization modes. Quality control (QC) of PCA was performed, using a QC sample, consisting of a mixture of equal aliquots of each berry extract. QC evaluation was carried out by verifying if PCA object scores obtained by replicated injections of the QC sample were close to the origin of PCA coordinates.

Results and discussion

Compound identification by LC-ESI-TOF and LC-ESI-Q/TOF analysis

The polyphenols found in berries of the investigated *Vaccinium* species were identified according to their chromatographic behavior, their TOF MS and Q/TOF MS/MS spectra, also in comparison with standard reference compounds, when available. Both positive and negative ionization modes were used for compound attribution.

Molecules that were unequivocally or putatively identified belonged to the compound classes of anthocyanins, flavonols, flavanols, and phenolic acids; other polyphenolic compounds belonging to miscellaneous classes (e.g., coumarins and dihydrochalcones) were also tentatively recognized.

Compound identification within each class is detailed below and summarized in Tables 1, 2, 3, and 4, which show retention time (Rt, min), mass (Da) found by the TOF survey scan experiment and main MS/MS fragments (Da) obtained by the Q/TOF experiment, proposed formula and corresponding exact mass (Da), mass accuracy (Δ, ppm), and putative identification of the peaks considered. Peaks reported in these tables were also shown in Figs. S1–S4 of the Electronic supplementary material (ESM).

Anthocyanins

Anthocyanins are characterized by a positive charge at pH < 3 and therefore are typically determined in the form of molecular ion $[M]^+$ [23]; accordingly, these polyphenols were identified under positive ionization (Table 1). Moreover, their attribution was also confirmed under negative ionization, by monitoring the quasi-molecular ion $[M-2H]^-$, according to the mass spectrometric behavior observed for these polyphenols by Sun and colleagues [24]. However, for this latter ionization mode, a less complete profile of the anthocyanin fraction was obtained, owing to its generally lower sensitivity that prevented in several cases the signal detection (data not shown).

It should also be remarked that when the anthocyanin has molecular weight 1 Da higher than that of a flavonol (i.e., delphinidin vs. quercetin, cyanidin vs. kaempferol, and petunidin vs. isorhamnetin derivatives of the same sugar), the $[M]^+$ or the $[M-2H]^-$ ions of the former and the $[M+H]^+$ or the $[M-H]^-$ ions of the latter are isobars, thus making relevant for their discrimination the chromatographic behavior.

As widely reported elsewhere [15, 18, 25], also in this study, MS/MS fragmentation of anthocyanins produced only the loss of the sugar units (e.g., 162 Da for a hexose and 132 Da for a pentose) and the corresponding detection of the aglycone fragment (i.e., 287.06 Da for cyanidin, 303.05 Da for delphinidin, 317.07 Da for petunidin, 301.07 Da for peonidin, and 331.08 Da for malvidin) (Table 1).

Using the IDA TOF-Q/TOF workflow and, when possible, by comparing the retention time and mass spectra of unknowns with those of authentic standards, the undoubted or at least the tentative identification of 64 anthocyanins was achieved. TOF MS $[M]^+$ molecular ions matched the proposed formulae with very high mass accuracy, being Δ absolute values ≤1 ppm in about 80% of the cases, and included in the range of 1.1–2.2 ppm for the remaining compounds (Table 1). Among the 64 anthocyanins identified, the presence of the 3-*O*-glucoside derivatives of delphinidin, cyanidin, petunidin, peonidin, and malvidin (peaks 9, 16, 22, 28, and 34); 3-*O*-galactoside derivatives of delphinidin, cyanidin, peonidin, and malvidin (peaks 6, 12, 25, and 29); and 3-*O*-arabinoside derivatives of cyanidin and peonidin (peaks 18 and 32) was confirmed by spiking the extracts with authentic reference standards (Table 1). Peaks 11, 17, 26, and 36 were putatively annotated to delphinidin-3-*O*-arabinoside, petunidin-3-*O*-galactoside, petunidin-3-*O*-arabinoside, and malvidin-3-*O*-arabinoside on the basis of their (i) TOF MS accuracy, isotope ratio difference, and MS/MS data (Y_0 cleavage of the sugar and formation of the aglycone ion), as well as (ii) relative chromatographic retention, the latter being in agreement with the retention observed by various authors under reversed-phase conditions for different glycosides with the same aglycone (i.e., increasing retention in the order galactoside < glucoside < arabinoside) and for different anthocyanins glycosylated with the same sugar (i.e., increasing retention in the order delphinidin < cyanidin < petunidin < peonidin < malvidin) [4, 7, 23]. The 15 anthocyanins reported above were detected in all the investigated species and resulted in all cases among the most abundant anthocyanidin derivatives (signal intensities approximately included in between 1×10^5 and 1×10^6 counts), as widely reported elsewhere [4, 7, 8, 26].

Among the first eluting analytes (peaks 1–5, Rt = 9.7–11.4 min), which were detected only in the *V. myrtillus* berry extracts and with very low signal intensity (i.e., 1000–1800 counts), peaks 1, 3, and 5 exhibited an $[M]^+$ ion at 627.15 Da

t1.1 **Table 1** Retention times (Rt, min), $[M]^+$ molecular ions (TOF MS, Da), mass fragments (Q/TOF MS/MS, Da), proposed formula, corresponding exact mass (Da), and accuracy (Δ, ppm) of peaks tentatively identified as anthocyanins in *V. myrtillus* (M), *V. uliginosum* L. subsp. *gaultherioides* (G), and *V. corymbosum* (C) under positive ionization. Symbols "+" and "–" mean detected and not detected

t1.2	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative identification
t1.3	1	9.7	627.1565	465.1078; 303.0485	$C_{27}H_{31}O_{17}$	627.1556	1.4	+	–	–	Delphinidin-dihexoside (I)[a]
t1.4	2	10.2	611.1677	449.1052; 287.0551	$C_{27}H_{31}O_{16}$	611.1607	0.9	+	–	–	Cyanidin-dihexoside (I)[a]
t1.5	3	10.5	627.1549	465.1078; 303.0485	$C_{27}H_{31}O_{17}$	627.1556	−1.0	+	–	–	Delphinidin-dihexoside (II)[a]
t1.6	4	11.0	611.1609	449.1118; 287.0559	$C_{27}H_{31}O_{16}$	611.1607	0.3	+	–	–	Cyanidin-dihexoside (II)[a]
t1.7	5	11.4	627.1551	465.1007; 303.0502	$C_{27}H_{31}O_{17}$	627.1556	−0.8	+	–	–	Delphinidin-dihexoside (III)[a]
t1.8	6	12.6	465.1031	303.0501	$C_{21}H_{21}O_{12}$	465.1027	0.8	+	+	+	Delphinidin-3-*O*-galactoside[b]
t1.9	7	13.0	479.0818	303.0495	$C_{21}H_{19}O_{13}$	479.0820	−0.1	+	–	–	Delphinidin-glucuronide
t1.10	8	13.2	597.1447	303.0499	$C_{26}H_{29}O_{16}$	597.1450	−0.6	+	–	–	Delphinidin-aldopentose-hexoside (I)[c]
t1.11	9	13.8	465.1032	303.0510	$C_{21}H_{21}O_{12}$	465.1027	0.9	+	+	+	Delphinidin-3-*O*-glucoside[b]
t1.12	10	14.0	597.1447	303.0502	$C_{26}H_{29}O_{16}$	597.1450	−0.6	+	–	–	Delphinidin-aldopentose-hexoside (II)[c]
t1.13	11	14.8	435.0926	303.0505	$C_{20}H_{19}O_{11}$	435.0922	0.9	+	+	+	Delphinidin-3-*O*-arabinoside
t1.14	12	14.8	449.1081	287.0554	$C_{21}H_{21}O_{11}$	449.1078	0.7	+	+	+	Cyanidin-3-*O*-galactoside[b]
t1.15	13	15.4	611.1598	287.0551	$C_{27}H_{31}O_{16}$	611.1607	−1.5	+	–	–	Cyanidin-dihexoside (III)[c]
t1.16	14	15.7	581.1502	287.0551	$C_{26}H_{29}O_{15}$	581.1501	0.3	+	–	–	Cyanidin-aldopentose-hexoside (I)[c]
t1.17	15	15.8	463.0867	287.0543	$C_{21}H_{19}O_{12}$	463.0871	−0.9	+	–	–	Cyanidin-glucuronide
t1.18	16	16.3	449.1080	287.0555	$C_{21}H_{21}O_{11}$	449.1078	0.3	+	+	+	Cyanidin-3-*O*-glucoside[b]
t1.19	17	16.9	479.1188	317.0659	$C_{22}H_{23}O_{12}$	479.1184	0.8	+	+	+	Petunidin-3-*O*-galactoside
t1.20	18	17.0	419.0976	287.0558	$C_{20}H_{19}O_{10}$	419.0978	0.9	+	+	+	Cyanidin-3-*O*-arabinoside[b]
t1.21	19	17.2	581.1503	287.0555	$C_{26}H_{29}O_{15}$	581.1501	0.3	+	–	–	Cyanidin-aldopentose-hexoside (II)[c]
t1.22	20	17.6	493.0973	317.0652	$C_{22}H_{21}O_{13}$	493.0977	−0.8	+	–	–	Petunidin-glucuronide
t1.23	21	17.9	611.1603	317.0650	$C_{27}H_{31}O_{16}$	611.1607	−0.6	+	–	–	Petunidin-aldopentose-hexoside[c]
t1.24	22	18.1	479.1188	317.0657	$C_{22}H_{23}O_{12}$	479.1184	0.9	+	+	+	Petunidin-3-*O*-glucoside[b]
t1.25	23	18.6	551.1392	287.0546	$C_{25}H_{27}O_{14}$	551.1395	−0.5	+	–	–	Cyanidin-aldodipentoside[c]
t1.26	24	18.7	435.0932	303.0492	$C_{20}H_{19}O_{11}$	435.0922	2.2	+	+	+	Delphinidin-3-*O*-xyloside
t1.27	25	18.9	463.1235	301.0713	$C_{22}H_{23}O_{11}$	463.1235	0.1	+	+	+	Peonidin-3-*O*-galactoside[b]
t1.28	26	19.0	449.1081	317.0660	$C_{21}H_{21}O_{11}$	449.1078	0.5	+	+	+	Petunidin-3-*O*-arabinoside
t1.29	27	19.6	595.1652	301.0715	$C_{27}H_{31}O_{15}$	595.1657	−0.9	+	–	–	Peonidin-aldopentose-hexoside (I)[c]
t1.30	28	20.2	463.1238	301.0714	$C_{22}H_{23}O_{11}$	463.1235	0.7	+	+	+	Peonidin-3-*O*-glucoside[b]
t1.31	29	20.3	493.1342	331.0816	$C_{23}H_{25}O_{12}$	493.1340	0.4	+	+	+	Malvidin-3-*O*-galactoside[b]
t1.32	30	20.5	419.0971	287.0552	$C_{20}H_{19}O_{10}$	419.0973	−0.4	+	+	+	Cyanidin-aldopentoside
t1.33	31	20.8	595.1661	301.0708	$C_{27}H_{31}O_{15}$	595.1657	0.6	+	–	–	Peonidin-aldopentose-hexoside (II)[c]
t1.34	32	21.0	433.1131	301.0711	$C_{21}H_{21}O_{10}$	433.1129	0.5	+	+	+	Peonidin-3-*O*-arabinoside[b]
t1.35	33	21.1	507.1137	303.0502	$C_{23}H_{23}O_{13}$	507.1133	0.7	–	–	+	Delphinidin-acetyl-hexoside (I)
t1.36	34	21.3	493.1345	331.0818	$C_{23}H_{25}O_{12}$	493.1340	0.8	+	+	+	Malvidin-3-*O*-glucoside[b]
t1.37	35	21.4	419.0976	287.0540	$C_{20}H_{19}O_{10}$	419.0973	0.7	+	+	+	Cyanidin-3-*O*-xyloside
t1.38	36	22.3	463.1237	331.0822	$C_{22}H_{23}O_{11}$	463.1235	0.4	+	+	+	Malvidin-3-*O*-arabinoside
t1.39	37	23.1	449.1086	317.0659	$C_{21}H_{21}O_{11}$	449.1078	1.8	+	+	+	Petunidin-3-*O*-xyloside
t1.40	38	23.2	639.1924	331.0807	$C_{29}H_{35}O_{16}$	639.1919	0.7	–	–	+	Malvidin-deoxyhexose-hexoside
t1.41	39	23.5	535.1076	287.0556	$C_{24}H_{23}O_{14}$	535.1082	−1.1	–	–	+	Cyanidin-malonyl-hexoside
t1.42	40	23.7	491.1186	287.0550	$C_{23}H_{23}O_{12}$	491.1184	0.4	+	–	+	Cyanidin-acetyl-hexoside (I)
t1.43	41	24.5	507.1136	303.0405	$C_{23}H_{23}O_{13}$	507.1133	0.6	–	–	+	Delphinidin-acetyl-hexoside (II)
t1.44	42	25.2	579.1339	331.0819	$C_{26}H_{27}O_{15}$	579.1344	−0.9	–	–	+	Malvidin-malonyl-hexoside (I)
t1.45	43	25.4	521.1290	317.0645	$C_{24}H_{25}O_{13}$	521.1290	0.1	+	–	+	Petunidin-acetyl-hexoside (I)
t1.46	44	25.4	433.1138	301.0714	$C_{21}H_{21}O_{10}$	433.1129	2.1	+	+	+	Peonidin-3-*O*-xyloside
t1.47	45	26.2	463.1240	331.0820	$C_{22}H_{23}O_{11}$	463.1235	1.2	+	+	+	Malvidin-3-*O*-xyloside
t1.48	46	27.2	505.1343	301.0703	$C_{24}H_{25}O_{12}$	505.1340	0.5	+	–	+	Peonidin-acetyl-hexoside (I)
t1.49	47	27.2	491.1189	287.0552	$C_{23}H_{23}O_{12}$	491.1184	1.0	+	–	+	Cyanidin-acetyl-hexoside (II)

t1.50 **Table 1** (continued)

	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative identification
t1.51	48	27.5	579.1349	331.0825	$C_{26}H_{27}O_{15}$	579.1344	0.9	−	−	+	Malvidin-malonyl-hexoside (II)
t1.52	49	27.9	535.1455	331.0813	$C_{25}H_{27}O_{13}$	535.1446	1.7	+	−	+	Malvidin-acetyl-hexoside (I)
t1.53	50	28.2	521.1293	317.0662	$C_{24}H_{25}O_{13}$	521.1290	0.7	+	−	+	Petunidin-acetyl-hexoside (II)
t1.54	51	29.3	611.1401	303.0503	$C_{30}H_{27}O_{14}$	611.1395	1.0	+	−	−	Delphinidin-coumaroyl-hexoside (I)
t1.55	52	30.5	505.1345	301.0702	$C_{24}H_{25}O_{12}$	505.1340	0.8	+	−	+	Peonidin-acetyl-hexoside (II)
t1.56	53	30.7	535.1456	331.0814	$C_{25}H_{27}O_{13}$	535.1446	1.9	+	−	+	Malvidin-acetyl-hexoside (II)
t1.57	54	31.3	595.1451	287.0549	$C_{30}H_{27}O_{13}$	595.1446	0.8	+	+	−	Cyanidin-coumaroyl-hexoside (I)
t1.58	55	31.5	611.1395	303.0496	$C_{30}H_{27}O_{14}$	611.1395	0.0	+	−	−	Delphinidin-coumaroyl-hexoside (II)
t1.59	56	31.7	625.1551	317.0652	$C_{31}H_{29}O_{14}$	625.1552	−0.1	+	−	+	Petunidin-coumaroyl-hexoside (I)
t1.60	57	32.1	595.1457	287.0556	$C_{30}H_{27}O_{13}$	595.1446	1.9	+	+	−	Cyanidin-coumaroyl-hexoside (II)
t1.61	58	32.1	609.1609	301.0712	$C_{31}H_{29}O_{13}$	609.1603	1.1	+	−	−	Peonidin-coumaroyl-hexoside (I)
t1.62	59	32.2	625.1557	317.0656	$C_{31}H_{29}O_{14}$	625.1552	0.8	+	−	+	Petunidin-coumaroyl-hexoside (II)
t1.63	60	32.2	639.1717	331.0811	$C_{32}H_{31}O_{14}$	639.1708	1.4	+	−	+	Malvidin-coumaroyl-hexoside (I)
t1.64	61	32.3	669.1821	331.0812	$C_{33}H_{33}O_{15}$	669.1814	1.0	+	−	−	Malvidin-feruloyl-hexoside (I)
t1.65	62	32.4	609.1611	301.0712	$C_{31}H_{29}O_{13}$	609.1603	1.3	+	−	−	Peonidin-coumaroyl-hexoside (II)
t1.66	63	32.4	639.1719	331.0824	$C_{32}H_{31}O_{14}$	639.1708	1.8	+	+	+	Malvidin-coumaroyl-hexoside (II)
t1.67	64	32.5	669.1819	331.0810	$C_{33}H_{33}O_{15}$	669.1814	0.8	+	−	−	Malvidin-feruloyl-hexoside (II)

[a] Hexoses separately linked to the aglycone

[b] Confirmed by spiking the extracts with authentic standards

[c] Hexoses linked as disaccharides

and a fragmentation pattern with ions at *m/z* 465.10 (loss of 162 Da, hexose residue) and *m/z* 303.05 (further loss of a hexose unit), the latter corresponding to the delphinidin aglycone. Similarly, peaks 2 and 4 ($[M]^+$ ion at *m/z* 611.16) gave rise to two independent losses of 162 Da, producing the fragments at *m/z* 449.11 (i.e., cyanidin monoglycoside) and *m/z* 287.06 (i.e., cyanidin). In order to correctly interpret these findings, it should be noted that the ESI-MS/MS fragmentation of anthocyanin derivatives with two sugars linked to different hydroxyls of the aglycone actually produces the aglycone monoglycoside as a consequence of the Y_0 cleavage of one sugar [27]. Conversely, in the case of disaccharide derivatives of anthocyanins, only the molecular ion and the aglycone fragment have been reported [28]. On the basis of the aforementioned considerations, peaks 1–5 can be putatively ascribed to dihexoside derivatives of delphinidin and cyanidin, the two hexoses being separately linked to the aglycone. Remarkably, only one anthocyanidin derivative with two hexose units linked in different aglycone positions (i.e., cyanidin) was elsewhere reported [25].

Peak 13 (Rt = 15.4 min, signal intensity 1200 counts) showed a $[M]^+$ at *m/z* 611.16 and only one fragment at *m/z* 287.05. Accordingly, it was tentatively assigned to a cyanidin hexose-hexose disaccharide. In this regard, it should be noted that such an attribution is in agreement with the reversed-phase chromatographic behavior of anthocyanidins diglycosides, since, for example, 3,5-diglycoside derivatives have been reported to elute before the corresponding 3-diglycosides [23, 29]. Interestingly, the occurrence of cyanidin disaccharides was not previously reported in bilberry.

Peaks 7, 15, and 20, which had moderate intensity (i.e., 4000–11,000 counts) were peculiar of the *V. myrtillus* extract. These peaks were ascribed to glucuronide derivatives of delphinidin, cyanidin, and petunidin, respectively. In fact, peaks 7, 15, and 20 were characterized by the loss in common of 176 Da, consistent with glucopyranuronic acid, and the consequent formation of fragments at *m/z* 303.05, *m/z* 287.05, and *m/z* 317.07, respectively, attributable to delphinidin, cyanidin, and petunidin. Relative chromatographic retention of these peaks in respect to the corresponding glucoside derivatives was also in accordance with their putative identification as glucuronide derivatives [30, 31]. It should be underlined that this study is the first one reporting the identification of delphinidin, cyanidin, and petunidin glucuronides in *V. myrtillus* fruits.

Peaks 8, 10, 14, 19, 21, 27, and 31, which were detected only in the *V. myrtillus* extract, exhibited the communal loss of 294 Da, attributable to an aldopentose-hexose residue, and MS/MS resulting fragments at *m/z* 303.05 (delphinidin), *m/z* 287.05 (cyanidin), *m/z* 317.07 (petunidin), and *m/z* 301.07 (peonidin). In this regard, it is notable that the chromatographic retention order of the aforementioned peaks was in agreement with the proposed aglycone attribution. It should also be noted that peaks 8 (i.e., delphinidin disaccharide), 14 (i.e.,

Q1

Q2 t2.1 **Table 2** Retention times (Rt, min), $[M-H]^-$ quasi-molecular ions (TOF MS, Da), main mass fragments (Q/TOF MS/MS, Da), proposed formula, corresponding exact mass (Da), and accuracy (Δ, ppm) of peaks tentatively identified as flavonols in *V. myrtillus* (M), *V. uliginosum* L. subsp. *gaultherioides* (G), and *V. corymbosum* (C) under negative ionization. Mass fragments in italics refer to the most intense signals. Symbols "+" and "−" mean detected and not detected

t2.2	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative identification
t2.3	65	19.6	493.0617	*317.0297*; 178.9989; 151.0036	$C_{21}H_{18}O_{14}$	493.0624	−1.3	+	+	+	Myricetin-glucuronide
t2.4	66	19.9	479.0826	317.0299; *316.0225*; 271.0229	$C_{21}H_{20}O_{13}$	479.0831	−1.1	+	+	+	Myricetin-3-*O*-galactoside
t2.5	67	20.6	479.0830	317.0299; *316.0216*; 271.0236	$C_{21}H_{20}O_{13}$	479.0831	−0.1	+	+	+	Myricetin-3-*O*-glucoside
t2.6	68	20.9	449.0713	317.0288; *316.0207*; 271.0236	$C_{20}H_{18}O_{12}$	449.0725	−2.8	+	+	−	Myricetin-3-*O*-aldopentoside (I)
t2.7	69	22.5	449.0714	317.0281; *316.0212*; 271.0230	$C_{20}H_{18}O_{12}$	449.0725	−2.6	−	+	+	Myricetin-3-*O*-aldopentoside (II)
t2.8	70	22.9	449.0715	317.0240; *316.0211*; 271.0251	$C_{20}H_{18}O_{12}$	449.0725	−2.4	+	+	+	Myricetin-3-*O*-aldopentoside (III)
t2.9	71	24.1	463.0876	301.0346; *300.0271*; 271.0248	$C_{21}H_{20}O_{12}$	463.0882	−1.4	+	+	+	Quercetin-3-*O*-galactoside[a]
t2.10	72	24.2	521.0926	317.0278; *316.0216*; 271.0246	$C_{23}H_{22}O_{14}$	521.0937	−2.1	−	−	+	Myricetin-acetyl-hexoside (I)
t2.11	73	24.4	477.0661	*301.0352*; 178.9979; 151.0028	$C_{21}H_{18}O_{13}$	477.0675	−2.8	+	+	+	Quercetin-glucuronide[a]
t2.12	74	24.6	609.1452	301.0335; *300.0262*; 271.0235	$C_{27}H_{30}O_{16}$	609.1461	−1.5	+	−	+	Quercetin-3-*O*-deoxyhexose-hexoside
t2.13	75	25.0	463.0879	301.0346; *300.0267*; 271.0248	$C_{21}H_{20}O_{12}$	463.0882	−0.7	+	+	+	Quercetin-3-*O*-glucoside[a]
t2.14	76	25.6	609.1454	301.0335; *300.0255*	$C_{27}H_{30}O_{16}$	609.1461	−1.2	−	−	+	Quercetin-3-*O*-rutinoside[a]
t2.15	77	25.7	433.0762	301.0341; *300.2630*; 271.0230	$C_{20}H_{18}O_{11}$	433.0776	−3.3	+	+	+	Quercetin-3-*O*-aldopentoside (I)
t2.16	78	26.1	493.0983	331.0450; *330.0369*; 315.1320	$C_{22}H_{22}O_{13}$	493.0988	−1.0	+	+	+	Laricitrin-3-*O*-galactoside
t2.17	79	26.4	433.0768	301.0341; *300.0267*; 271.0237	$C_{20}H_{18}O_{11}$	433.0776	−1.9	+	+	+	Quercetin-3-*O*-aldopentoside (II)
t2.18	80	26.4	521.0941	317.0271; *316.0202*; 271.0225	$C_{23}H_{22}O_{14}$	521.0937	0.7	−	−	+	Myricetin-acetyl-hexoside (II)
t2.19	81	26.5	317.0294	178.9983; 165.0192; *151.0031*; 137.0237	$C_{15}H_{10}O_8$	317.0303	−2.8	+	+	+	Myricetin[a]
t2.20	82	26.6	493.0989	331.0461; *330.0383*; 315.0151	$C_{22}H_{22}O_{13}$	493.0988	0.3	+	+	+	Laricitrin-3-*O*-glucoside
t2.21	83	26.7	507.0777	*331.0445*; 316.0210; 178.9978	$C_{22}H_{20}O_{14}$	507.0780	−0.7	+	+	+	Laricitrin-glucuronide
t2.22	84	26.7	549.0876	505.0983; 463.0870; 301.0333; *300.0263*	$C_{24}H_{22}O_{15}$	549.0886	−1.8	−	−	+	Quercetin-malonyl-hexoside (I)
t2.23	85	27.0	549.0877	505.0997; 463.0892; 301.0350; *300.0271*	$C_{24}H_{22}O_{15}$	549.0886	−1.6	−	−	+	Quercetin-malonyl-hexoside (II)
t2.24	86	27.2	447.0912	285.0395; *284.0308*; 255.0280; 227.0335	$C_{21}H_{20}O_{11}$	447.0933	−3.2	+	+	+	Kaempferol-3-*O*-galactoside
t2.25	87	27.5	433.0768	301.0343; *300.0270*; 271.0236	$C_{20}H_{18}O_{11}$	433.0776	−2.0	−	+	+	Quercetin-3-*O*-aldopentoside (III)
t2.26	88	28.0	463.0862	331.0456; *330.0361*; 315.0149	$C_{21}H_{20}O_{12}$	463.0882	−4.4	+	+	+	Laricitrin-3-*O*-xyloside
t2.27	89	28.2	505.0972	301.0338; *300.0273*; 271.0237	$C_{23}H_{22}O_{13}$	505.0988	−3.0	−	−	+	Quercetin-acetyl-hexoside (I)
t2.28	90	28.4	461.0721	*285.0390*; 257.0438; 229.0491	$C_{21}H_{18}O_{12}$	461.0725	−0.9	+	+	+	Kaempferol-glucuronide
t2.29	91	28.5	463.0869	331.0431; *330.0378*; 315.0140	$C_{21}H_{20}O_{12}$	463.0882	−2.9	+	+	+	Laricitrin-3-*O*-aldopentoside (I)
t2.30	92	28.8	447.0923	301.0350; *300.0271*; 271.0238	$C_{21}H_{20}O_{11}$	447.0933	−2.1	+	−	+	Quercetin-3-*O*-rhamnoside[a]
t2.31	93	28.9	447.0923	285.0397; *284.0325*; 255.0293; 227.0348	$C_{21}H_{20}O_{11}$	447.0933	−2.1	−	+	−	Kaempferol-3-*O*-glucoside
t2.32	94	29.3	417.0823	*284.0316*; 255.0291; 227.0343	$C_{20}H_{18}O_{10}$	417.0827	−0.9	+	+	+	Kaempferol-3-*O*-aldopentoside
t2.33	95	29.3	505.0979	301.0352; *300.0272*; 271.0237	$C_{23}H_{22}O_{13}$	505.0988	−1.6	−	−	+	Quercetin-acetyl-hexoside (II)
t2.34	96	29.5	593.1504	*285.0398*; 284.0326	$C_{27}H_{30}O_{15}$	593.1512	−1.3	−	−	+	Kaempferol-7-*O*-neohesperidoside[a]
t2.35	97	29.6	477.1032	*314.0419*; 271.0232; 243.0290	$C_{22}H_{22}O_{12}$	477.1038	−1.4	+	+	+	Isorhamnetin-3-*O*-galactoside
t2.36	98	29.7	505.0977	301.0337; *300.0268*; 255.0293	$C_{23}H_{22}O_{13}$	505.0988	−2.1	−	−	+	Quercetin-acetyl-hexoside (III)
t2.37	99	30.0	463.0870	*330.0355*; 331.0455; 315.0133	$C_{21}H_{20}O_{12}$	463.0882	−2.5	−	+	+	Laricitrin-3-*O*-aldopentoside (II)
t2.38	100	30.0	579.1350	301.0351; *300.0262*; 271.0235	$C_{26}H_{28}O_{15}$	579.1355	−0.9	+	−	−	Quercetin-3-*O*-deoxyhexose-pentoside
t2.39	101	30.2	623.1613	315.0496; *314.0405*; 299.0174	$C_{28}H_{32}O_{16}$	623.1618	−0.8	−	−	+	Isorhamnetin-3-*O*-deoxyhexose-hexoside
t2.40	102	30.2	505.0983	301.0335; *300.0255*; 271.0226	$C_{23}H_{22}O_{13}$	505.0988	−0.9	−	−	+	Quercetin-acetyl-hexoside (IV)
t2.41	103	30.4	477.1029	*314.0426*; 271.0258; 243.0299	$C_{22}H_{22}O_{12}$	477.1038	−2.0	+	+	−	Isorhamnetin-3-*O*-glucoside
t2.42	104	30.4	477.1029	*331.0438*; 330.0362; 315.0135	$C_{22}H_{22}O_{12}$	477.1038	−2.0	−	−	+	Laricitrin-3-*O*-rhamnoside
t2.43	105	30.6	535.1078	*330.0362*; 315.097	$C_{24}H_{24}O_{14}$	535.1093	−2.9	−	−	+	Laricitrin-acetyl-hexoside
t2.44	106	30.7	491.0833	*315.0509*; 300.0255; 271.0239	$C_{22}H_{20}O_{13}$	491.0831	0.4	+	+	+	Isorhamnetin-glucuronide
t2.45	107	30.8	507.1141	345.0616; *344.0530*; 301.0353	$C_{23}H_{24}O_{13}$	507.1144	−0.7	+	+	+	Syringetin-3-*O*-galactoside

t2.46 **Table 2** (continued)

	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative identification
t2.47	108	30.9	623.1611	*315.0492*; 314.0419; 299.0203	$C_{28}H_{32}O_{16}$	623.1618	−1.0	−	−	+	Isorhamnetin-7-*O*-deoxyhexose-hexoside
t2.48	109	31.3	507.1147	345.0609; *344.0533*; 301.0343	$C_{23}H_{24}O_{13}$	507.1144	0.6	+	+	+	Syringetin-3-*O*-glucoside
t2.49	110	31.4	417.0814	285.0405; *284.0323*; 255.0299	$C_{20}H_{18}O_{10}$	417.0827	−3.1	−	−	+	Kaempferol-3-*O*-aldopentoside
t2.50	111	31.5	521.0932	*345.0600*; 330.0363; 315.0129	$C_{23}H_{22}O_{14}$	521.0937	1.9	+	+	+	Syringetin-glucuronide
t2.51	112	31.6	447.0931	*314.0426*; 271.0238; 243.0289	$C_{21}H_{20}O_{11}$	447.0933	−0.3	+	+	+	Isorhamnetin-3-*O*-aldopentoside (I)
t2.52	113	31.8	447.0919	*314.0413*; 299.0251	$C_{21}H_{20}O_{11}$	447.0933	−3.2	+	+	+	Isorhamnetin-3-*O*-aldopentoside (II)
t2.53	114	32.0	477.1025	*344.0544*; 301.0341; 273.0390	$C_{22}H_{22}O_{12}$	477.1038	−2.8	+	+	+	Syringetin-3-*O*-aldopentoside (I)
t2.54	115	32.1	301.0347	178.9980; *151.0033*; 149.0219; 121.0282	$C_{15}H_{10}O_7$	301.0354	−2.4	+	+	+	Quercetin[a]
t2.55	116	32.2	447.0920	*314.0411*; 271.0218; 243.0283	$C_{21}H_{20}O_{11}$	447.0933	−2.8	−	+	+	Isorhamnetin-3-*O*-aldopentoside (III)
t2.56	117	32.2	477.1026	*344.0537*; 301.0354; 273.0382	$C_{22}H_{22}O_{12}$	477.1038	−2.5	+	+	+	Syringetin-3-*O*-aldopentoside (II)
t2.57	118	32.6	331.0458	*316.0196*; 178.9976; 151.0062	$C_{16}H_{12}O_8$	331.0459	−0.4	+	+	+	Laricitrin
t2.58	119	33.3	315.0504	*300.0269*; 271.0227; 151.0026	$C_{16}H_{12}O_7$	315.0510	−1.9	+	+	+	Isorhamnetin

[a] Confirmed by spiking the extracts with authentic standards

cyanidin disaccharide), 21 (i.e., petunidin disaccharide), and 27 (peonidin disaccharide) eluted between galactoside and glucoside derivatives of the corresponding aglycone, thus suggesting their putative identification as sambubioside derivatives [23]. This hypothesis is in agreement with the findings of Du and coworkers, who reported the occurrence of delphinidin-3-sambubioside and cyanidin-3-sambubioside in bilberry [32].

Peak 23 showed a neutral loss of 264 Da, corresponding to an aldopentose disaccharide, and the resulting formation of a fragment at *m*/*z* 287.05 (i.e., cyanidin). This peak was found only in *V. myrtillus* extract, in agreement with the results previously reported by Latti and coworkers [25].

Peaks 24, 30, 35, 37, 44, and 45 were detected in berries from all the investigated *Vaccinium* species (Table 1) and can be ascribed to aldopentoside derivatives of delphinidin (peak 24), cyanidin (peaks 30 and 35), petunidin (peak 37), peonidin (peak 44), and malvidin (peak 45), based on the neutral loss of 132 Da (i.e., aldopentose) and the formation of the corresponding aglycone fragment. These peaks occurred in the three investigated berry species with very different intensities, being those determined in *V. uliginosum* subsp. *gaultherioides* characterized by much higher signals than the others. It should also be underlined that peak 30 differentiate itself from the others, owing to a much lower signal intensity. It is remarkable that this study is the first one putatively identifying anthocyanidin aldopentosides in blueberry and aldopentose derivatives of delphinidin, cyanidin, and peonidin in bilberry, whereas the occurrence of petunidin and malvidin xylosides was previously reported in this latter species [25, 33]. Aldopentose derivatives were elsewhere detected in *V. uliginosum* berries and identified as xylosides of the five anthocyanidin mentioned above [34]. Accordingly, for delphinidin, petunidin, peonidin, and malvidin aldopentose herein detected, the attribution to xyloside derivatives can be proposed. This putative attribution is also confirmed by the earlier elution of arabinosides compared to the compounds tentatively identified as xylosides [23], being in our study the difference in retention included in the range 3.9–4.4 min. Moreover, considering peaks 30 and 35, retention time was found to be 3.5 and 4.4 min higher than cyanidin arabinoside (peak 18, Table 1), respectively. Accordingly, peak 35 (Rt = 21.4 min) should be putatively ascribed to cyanidin-3-*O*-xyloside, whereas peak 30 (Rt = 20.5 min) must be attributed to another cyanidin-aldopentoside, such as cyanidin-7-*O*-arabinoside, which is characterized by a lesser retention under reversed-phase chromatographic conditions and was found in other fruits [29].

Peak 38, which was detected only in blueberry, at quite low intensity (about 3000 counts) showed a MS/MS spectra characterized by the fragment at *m*/*z* 331.08, thus indicating a malvidin derivative. The neutral loss from the $[M]^+$ ion was 308 Da, which is consistent with a deoxyhexose-hexoside unit, as well as with a coumaroyl-hexoside fragment. However, the $[M]^+$ ion of peak 38 matched the exact mass of a malvidin-deoxyhexose-hexoside with much higher accuracy ($\Delta = 0.7$ ppm) than a malvidin coumaroyl-hexoside ($\Delta = 34$ ppm). Furthermore, peak 38 eluted between the arabinoside and xyloside malvidin derivatives, as elsewhere reported for malvidin-3-*O*-rutinoside [23], the occurrence of which was previously highlighted in *V. corymbosum* berries, by Ramirez and coworkers [18].

Peaks 33, 40, 41, 43, 46, 47, 49, 50, 52, and 53 were characterized by the neutral loss of 204 Da (consistent with an acetyl-hexose unit) and formation of the aglycone fragment, thus suggesting their attribution to acetyl-hexosides of

Q1

t3.1 **Table 3** Retention times (Rt, min), quasi-molecular ions (TOF MS, Da), main mass fragments (Q/TOF MS/MS, Da), proposed formula, corresponding exact mass (Da), and accuracy (Δ, ppm) of peaks tentatively identified as flavanols in *V. myrtillus* (M), *V. uliginosum* L. subsp. *gaultherioides* (G), and *V. corymbosum* (C) under negative ionization. Mass fragments in italics refer to the most intense signals. Symbols "+" and "−" mean detected and not detected

t3.2	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative Identification
t3.3	120	3.6	609.1247[a]	441.0775; *423.0701*; 305.0639; 177.0200; 125.0250	$C_{30}H_{26}O_{14}$	609.1250	−0.4	+	+	−	B-type (E)GC-(E)GC (I)
t3.4	121	4.2	305.0661[a]	219.0667; 167.0345; 165.0182; 139.0391; 137.0245; *125.0239*	$C_{15}H_{14}O_7$	305.0667	−1.7	+	+	+	Gallocatechin
t3.5	122	4.7	609.1237[a]	441.0814; *423.0709*; 305.0645; 177.0187; 125.0226	$C_{30}H_{26}O_{14}$	609.1250	−2.2	+	−	+	B-type (E)GC-(E)GC (II)
t3.6	123	5.8	1167.2377[a]	981.1887; *863.1795*; 711.1422; 573.1045; 411.0698	$C_{60}H_{48}O_{25}$	1167.2412	−3.0	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)GC
t3.7	124	6.5	1151.2463[a]	*863.1837*; 711.1409; 573.1057; 411.0717	$C_{60}H_{48}O_{24}$	1151.2422	−3.6	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)C (I)
t3.8	125	6.7	911.1676[a]	*743.1238*; 483.0904; *427.0650*; 423.0672; *305.0637*; 301.0308	$C_{45}H_{36}O_{21}$	911.1646	−3.3	+	−	−	A/B-type (E)GC-(E)GC-(E)GC
t3.9	126	6.8	451.1229[a]	*289.0719*; 245.0780; 123.0460	$C_{21}H_{24}O_{11}$	451.1246	−3.7	+	+	+	Catechin-hexoside
t3.10	127	7.0	1153.2619[a]	1027.2401; 863.1865; 577.1324; *575.1191*; 287.0543	$C_{60}H_{50}O_{24}$	1153.2588	−2.7	−	−	+	B-type (E)C-(E)C-(E)C-(E)C (I)
t3.11	128	7.1	913.1833[a]	609.1281; 541.0794; *423.0702*; 305.0641	$C_{45}H_{38}O_{21}$	913.1802	−3.4	+	+	−	B-type (E)GC-(E)GC-(E)GC
t3.12	129	7.5	881.1900[a]	713.1523; *695.1375*; 591.1141; 577.1326; 451.1031; 303.0479	$C_{45}H_{38}O_{19}$	881.1935	−3.9	−	−	+	B-type (E)C-(E)C-(E)GC (I)
t3.13	130	7.7	584.1232[b]	577.1389; *289.0701*; 287.0542	$C_{60}H_{50}O_{25}$	584.1248	−2.8	−	−	+	B-type (E)C-(E)C-(E)C-(E)GC (I)
t3.14	131	7.8	727.1469[b]	591.1163; *289.0704*; 125.0236	$C_{75}H_{60}O_{31}$	727.1486	−2.5	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)C-(E)GC
t3.15	132	8.0	895.1704[a]	*727.1298*; 467.0960; *427.0654*; 289.0691; 177.0183	$C_{45}H_{36}O_{20}$	895.1727	−2.6	+	−	−	A/B-type (E)C-(E)GC-(E)GC (I)
t3.16	133	8.5	577.1335[a]	425.0869; *407.0766*; *289.0711*	$C_{30}H_{26}O_{12}$	577.1351	−2.9	+	+	+	Procyanidin B1 [c]
t3.17	134	8.7	895.1705[a]	725.1105; *467.0955*; 427.0676; 305.0661; 125.0238	$C_{45}H_{36}O_{20}$	895.1727	−2.5	+	−	−	A/B-type (E)C-(E)GC-(E)GC (II)
t3.18	135	8.9	305.0663[a]	219.0653; 167.0340; 165.0192; 139.0391; 137.0240; *125.0241*	$C_{15}H_{14}O_7$	305.0667	−1.2	+	+	+	Epigallocatechin
t3.19	136	9.0	289.0711[a]	*245.0811*; 205.0501; *203.0712*; 125.0238; *123.0451*; 109.0294	$C_{15}H_{14}O_6$	289.0718	−2.2	+	+	+	(+)-Catechin [c]
t3.20	137	9.2	719.1489[b]	575.1182; 451.1036; 411.0698; *289.0701*; *287.0550*; *125.0243*	$C_{75}H_{60}O_{30}$	719.1512	−3.2	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)C-(E)C (I)
t3.21	138	9.3	1153.2571[a]	865.2038; *575.1184*; 287.0549	$C_{60}H_{50}O_{24}$	1153.2619	−4.2	−	−	+	B-type (E)C-(E)C-(E)C-(E)C (II)
t3.22	139	9.3	897.1852[a]	711.1469; *593.1353*; 543.0920; *407.0776*; 303.0499; 177.0249	$C_{45}H_{38}O_{20}$	897.1884	−3.5	+	+	−	B-type (E)GC-(E)C-(E)GC (I)
t3.23	140	9.5	865.1957[a]	695.1393; *577.1343*; 407.0755; *287.0546*	$C_{45}H_{38}O_{18}$	865.1985	−3.3	+	+	+	B-type (E)C-(E)C-(E)C (I)
t3.24	141	9.8	577.1336[a]	425.0869; *407.0760*; *289.0713*	$C_{30}H_{26}O_{12}$	577.1351	−2.7	+	+	+	B-type procyanidin (I)
t3.25	142	10.0	879.1757[a]	*727.1312*; 451.1026; *427.0655*; 289.0713	$C_{45}H_{36}O_{19}$	879.1778	−2.4	+	+	−	A/B-type (E)GC-(E)C-(E)C
t3.26	143	10.2	720.1573[b]	407.0819; *289.0701*; 287.0554	$C_{75}H_{62}O_{30}$	720.1590	−2.4	−	−	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (I)
t3.27	144	10.3	865.1965[a]	*577.1344*; *575.1179*; *287.0544*	$C_{45}H_{38}O_{18}$	865.1985	−2.3	−	−	+	B-type (E)C-(E)C-(E)C (II)
t3.28	145	10.4	451.1237[a]	*289.0707*; 245.0802; 125.0247	$C_{21}H_{24}O_{11}$	451.1246	−2.0	+	+	+	Epicatechin-hexoside
t3.29	146	10.7	720.1579[b]	577.1352; 407.0765; *289.0701*; 287.0572; 125.0229	$C_{75}H_{62}O_{30}$	720.1590	−1.5	−	−	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (II)
t3.30	147	10.8	576.1258[b]	*449.0874*; *289.0698*; 287.0563; *125.0259*	$C_{60}H_{50}O_{24}$	576.1273	−2.6	−	−	+	B-type (E)C-(E)C-(E)C-(E)C (III)
t3.31	148	10.9	865.1962[a]	*577.1353*; *575.1171*; 425.0870; *407.0752*; 287.0542	$C_{45}H_{38}O_{18}$	865.1985	−2.7	+	+	+	B-type (E)C-(E)C-(E)C (III)
t3.32	149	11.0	897.1864[a]	*711.1323*; 593.1368; 591.1121; 423.0715	$C_{45}H_{38}O_{20}$	897.1884	−2.2	+	+	−	B-type (E)GC-(E)C-(E)GC (II)
t3.33	150	11.5	720.1568[b]	575.1249; 405.0628; *289.0702*; 243.0290; 125.0244	$C_{75}H_{62}O_{30}$	720.1590	−3.1	−	−	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (III)
t3.34	151	11.7	576.1260[b]	425.0828; *289.0603*; *287.0545*; 245.0436; *125.0229*	$C_{60}H_{50}O_{24}$	576.1273	−2.3	+	+	+	B-type (E)C-(E)C-(E)C-(E)C (IV)
t3.35	152	11.8	879.1755[a]	*727.1377*; 709.1207; 467.0984; *411.0685*; *305.0644*	$C_{45}H_{36}O_{19}$	879.1778	−2.6	+	+	−	A/B-type (E)C-(E)C-(E)GC
t3.36	153	12.2	577.1346[a]	425.0870; *407.0762*; *289.0704*	$C_{30}H_{26}O_{12}$	577.1351	−1.0	+	+	+	Procyanidin B2 [c]

t3.37 **Table 3** (continued)

	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative Identification
t3.38	154	12.6	576.1259[b]	425.0895; 407.0774; *289.0717*; 287.0543; *125.0230*	$C_{60}H_{50}O_{24}$	576.1273	−2.5	+	+	+	B-type (E)C-(E)C-(E)C-(E)C (V)
t3.39	155	13.3	576.1661[b]	407.0732; *289.0706*; 287.0542; 151.0376; *125.0241*	$C_{60}H_{50}O_{24}$	576.1273	−2.1	+	+	+	B-type (E)C-(E)C-(E)C-(E)C (VI)
t3.40	156	13.9	863.1816[a]	711.1353; 693.1265; 573.1040; 451.1048; *411.0716*; 289.0707	$C_{45}H_{36}O_{18}$	863.1829	−1.5	+	+	−	A/B-type (E)C-(E)C-(E)C (I)
t3.41	157	14.0	865.1959[a]	*713.1508*; 695.1377; *577.1352*; 575.1213; 407.0788; 287.0582	$C_{45}H_{38}O_{18}$	865.1985	−3.1	−	−	+	B-type (E)C-(E)C-(E)C (IV)
t3.42	158	14.1	720.1570[b]	289.0710; *125.0235*	$C_{75}H_{62}O_{30}$	720.1590	−3.5	−	−	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (IV)
t3.43	159	14.2	289.0718[a]	*245.0818*; 205.0499; *203.0705*; 125.0233; *123.0448*; 109.0299	$C_{15}H_{14}O_6$	289.0718	0.1	+	+	+	(−)-Epicatechin [c]
t3.44	160	14.3	881.1909[a]	713.1545; *695.1432*; 591.1146; 577.1374; 425.0868; 303.0491	$C_{45}H_{38}O_{19}$	881.1935	−2.9	+	+	+	B-type (E)C-(E)C-(E)GC (II)
t3.45	161	14.5	720.1581[b]	575.1223; *289.0701*; 125.0249	$C_{75}H_{62}O_{30}$	720.1590	−1.3	+	+	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (V)
t3.46	162	15.4	1169.2538[a]	865.2056; 739.1659; 591.1202; 423.0709; 287.0549	$C_{60}H_{50}O_{25}$	1169.2568	−2.6	+	+	+	B-type (E)C-(E)C-(E)C-(E)GC (II)
t3.47	163	15.5	575.1189[b]	411.0730; 407.0722; *289.0697*; 151.0389; *125.0235*	$C_{60}H_{48}O_{24}$	575.1195	−1.0	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)C (II)
t3.48	164	15.5	865.1962[a]	695.1408; *577.1339*; 575.1191; 407.0752; 289.0686; *287.0538*	$C_{45}H_{38}O_{18}$	865.1985	−2.7	+	+	+	B-type (E)C-(E)C-(E)C (V)
t3.49	165	16.2	576.1255[b]	*407.0756*; *289.0713*; 287.0556; *125.0247*	$C_{60}H_{50}O_{24}$	576.1273	−3.2	+	+	+	B-type (E)C-(E)C-(E)C-(E)C (VII)
t3.50	166	16.5	720.1578[b]	289.0707; 125.0238	$C_{75}H_{62}O_{30}$	720.1590	−1.6	+	+	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (VI)
t3.51	167	16.7	720.1583[b]	289.0712; 125.0244	$C_{75}H_{62}O_{30}$	720.1590	−0.9	+	+	+	B-type (E)C-(E)C-(E)C-(E)C-(E)C (VII)
t3.52	168	17.8	863.1826[a]	711.1386; 693.1277; *575.1197*	$C_{45}H_{36}O_{18}$	863.1829	−0.3	−	+	−	A/B-type (E)C-(E)C-(E)C (II)
t3.53	169	18.1	719.1500[b]	411.0728; *289.0684*; 287.0578	$C_{75}H_{60}O_{30}$	719.1512	−1.6	+	+	−	A/B-type (E)C-(E)C-(E)C-(E)C-(E)C (II)
t3.54	170	21.0	575.1191[a]	*449.0856*; 423.0718; *289.0706*; *285.0383*	$C_{30}H_{24}O_{12}$	575.1195	−0.7	+	+	−	Procyanidin A2 [c]
t3.55	171	22.0	863.1812[a]	711.1381; 693.1276; *575.1184*	$C_{45}H_{36}O_{18}$	863.1829	−2.0	−	+	−	A/B-type (E)C-(E)C-(E)C (III)
t3.56	172	22.7	577.1343[a]	425.0873; *407.0759*; *289.0704*	$C_{30}H_{26}O_{12}$	577.1351	−1.4	+	+	+	B-type procyanidin (II)
t3.57	173	27.0	576.1267[b]	407.0749; 287.0517; 151.0388; *125.0233*	$C_{60}H_{50}O_{24}$	576.1273	−1.0	+	+	+	B-type (E)C-(E)C-(E)C-(E)C (VIII)

(E)C (epi)catechin, *(E)GC* (epi)gallocatechin

[a] Mono-charged quasi-molecular ion $[M-H]^-$

[b] Double-charged quasi-molecular ion $[M-2H]^{2-}/2$

[c] Confirmed by spiking the extracts with authentic standards

delphinidin (peaks 33 and 41), cyanidin (peaks 40 and 47), petunidin (peaks 43 and 50), peonidin (peaks 46 and 52), and malvidin (peaks 49 and 53). Interestingly, these acylated anthocyanins showed much higher intensities in blueberry (1.0×10^4–1.4×10^5 counts) than in bilberry (4.0×10^3–1.0×10^4 counts) [29], whereas they were never detected in false bilberry (Table 1), thus representing a potential group of markers for the differentiation of these fruit species.

Peaks 39, 42, and 48 were also characteristic of blueberry, which were absent in bilberries and "false bilberries." The MS/MS spectra evidenced the presence of cyanidin and malvidin aglycone fragments as a consequence of the communal loss of 248 Da, which can be ascribed to a malonyl-hexose group. These peaks were therefore tentatively assigned to cyanidin-malonyl-hexoside (peak 39) and malvidin-malonyl-hexosides (peaks 42 and 48), in partial agreement with the results obtained by Wu and Prior [29], which reported the occurrence of malonyl-glucoside derivatives of delphinidin, cyanidin, and malvidin in blueberry.

Peaks 51, 54–60, 62, and 63 fragmented with a neutral loss of 308 Da and the resulting formation of ions at *m/z* 303.05 (i.e., delphinidin, peaks 51 and 55), *m/z* 287.05 (i.e., cyanidin, peaks 54 and 57), *m/z* 317.07 (i.e., petunidin, peaks 56 and 59), *m/z* 301.07 (i.e., peonidin,

Q1

t4.1 **Table 4** Retention times (Rt, min), $[M-H]^-$ quasi-molecular ions (TOF MS, Da), main mass fragments (Q/TOF MS/MS, Da), proposed formula, corresponding exact mass (Da), and accuracy (Δ, ppm) of peaks tentatively identified as other phenolic compounds in *V. myrtillus* (M), *V. uliginosum* L. subsp. *gaultherioides* (G), and *V. corymbosum* (C) under negative ionization. Mass fragments in italics refer to the most intense signals. Symbols "+" and "−" mean detected and not detected

t4.2	Peak	Rt	TOF MS	Q/TOF MS/MS	Proposed formula	Exact mass	Δ	M	G	C	Tentative identification
t4.3	174	2.0	169.0146	*125.0240*; 124.0244; 79.0189	$C_7H_6O_5$	169.0142	2.0	+	+	+	Gallic acid[a]
t4.4	175	6.8	353.0866	*191.0554*; 179.0345; 135.0446	$C_{16}H_{18}O_9$	353.0878	−3.4	+	+	+	Neochlorogenic acid[a]
t4.5	176	9.2	337.0918	191.0562; *163.0390*; 119.0499	$C_{16}H_{18}O_8$	337.0929	−3.2	−	−	+	Coumaroylquinic acid (I)
t4.6	177	9.4	337.0916	*191.0554*; *163.0387*; 119.0505	$C_{16}H_{18}O_8$	337.0929	−3.7	−	−	+	Coumaroylquinic acid (II)
t4.7	178	10.0	179.0351	*135.0444*; 134.0368	$C_9H_8O_4$	179.0350	0.6	+	+	+	Caffeic acid[a]
t4.8	179	10.6	341.0872	*179.0342*; *135.0446*; 134.0358	$C_{15}H_{18}O_9$	341.0878	−1.7	+	+	+	Caffeic acid hexoside
t4.9	180	11.0	353.0869	*191.0566*	$C_{16}H_{18}O_9$	353.0878	−2.6	+	+	+	Chlorogenic acid[a]
t4.10	181	12.3	325.0920	*163.0395*; *119.0504*	$C_{15}H_{18}O_8$	325.0929	−2.6	+	+	+	Coumaric acid hexoside
t4.11	182	12.4	353.0868	*191.0552*; 179.0348; *173.0451*; 135.0448	$C_{16}H_{18}O_9$	353.0878	−2.8	+	+	+	Cryptochlorogenic acid[a]
t4.12	183	13.7	355.1031	193.0507; *175.0398*; *160.0163*; 132.0210	$C_{16}H_{20}O_9$	355.1035	−1.0	+	+	+	Ferulic acid hexoside
t4.13	184	13.9	739.1657	*587.1205*; 339.0494; 289.0707; 177.0190	$C_{39}H_{32}O_{15}$	739.1668	−1.5	−	−	+	Cinchonain IIx (I)
t4.14	185	14.7	337.0918	*191.0547*; 163.0384	$C_{16}H_{18}O_8$	337.0929	−3.3	+	−	+	Coumaroylquinic acid (III)
t4.15	186	15.4	353.0873	*191.0568*	$C_{16}H_{18}O_9$	353.0878	−1.4	+	−	+	Caffeoylquinic acid
t4.16	187	16.0	739.1658	587.1192; 449.0864; 339.0498; *289.0704*	$C_{39}H_{32}O_{15}$	739.1668	−1.5	−	−	+	Cinchonain IIx (II)
t4.17	188	16.2	335.0767	*179.0343*; 161.0207; *135.0446*; 134.0372	$C_{16}H_{16}O_8$	335.0772	−1.6	+	+	+	Caffeic acid derivative
t4.18	189	16.9	739.1641	*587.1170*; 339.0510; *289.0704*	$C_{39}H_{32}O_{15}$	739.1668	−3.8	+	−	+	Cinchonain IIx (III)
t4.19	190	17.4	191.0349	*176.0109*; *104.0277*	$C_{10}H_8O_4$	191.0350	−0.5	−	+	−	Scopoletin[a]
t4.20	191	17.5	739.1667	*587.1195*; 449.0871; 339.0484; *289.0688*; *177.0174*	$C_{39}H_{32}O_{15}$	739.1668	−0.2	+	−	+	Cinchonain IIx (IV)
t4.21	192	17.6	367.1025	193.0494; *191.0555*; 173.0452; 134.0373	$C_{17}H_{20}O_9$	367.1035	−2.6	+	+	+	Feruloylquinic acid
t4.22	193	17.9	193.0504	*134.0367*; 133.0302	$C_{10}H_{10}O_4$	193.0506	−1.3	−	−	+	Ferulic acid[a]
t4.23	194	21.2	367.1026	*179.0342*; 161.0256 *135.0446*; 134.0357	$C_{17}H_{20}O_9$	367.1035	0.8	+	+	+	Caffeic acid derivative
t4.24	195	24.4	535.1454	371.0980; 329.1038; 191.0346; *163.0398*; *147.0450*; 119.0501	$C_{25}H_{28}O_{13}$	535.1457	−0.5	+	+	−	Coumaroyl iridoid (I)
t4.25	196	25.7	535.1453	371.0987; 329.1025; 191.0337; *163.0396*; *147.0443*; 119.0500	$C_{25}H_{28}O_{13}$	535.1457	−0.7	+	+	−	Coumaroyl iridoid (II)
t4.26	197	26.2	515.1184	*353.0867*; *191.0555*; *179.0345*; 173.0452; 135.0450	$C_{25}H_{24}O_{12}$	515.1195	−2.2	−	−	+	Dicaffeoylquinic acid
t4.27	198	26.8	515.1179	*353.0872*; *191.0550*; 179.0347; 135.0445	$C_{25}H_{24}O_{12}$	515.1195	−3.0	−	−	+	1,5-Dicaffeoylquinic acid[a]
t4.28	199	28.0	435.1291	*273.0751*; 167.0322	$C_{21}H_{24}O_{10}$	435.1297	−1.3	+	+	+	Phloridzin[a]
t4.29	200	32.1	411.1659	163.0402; *145.0290*; 119.0481	$C_{20}H_{28}O_9$	411.1661	1.5	+	+	−	Coumaric acid-malonyl-hexoside (I)
t4.30	201	32.5	411.1659	*163.0398*; *145.0292*; 119.0498	$C_{20}H_{28}O_9$	411.1661	−0.3	+	+	−	Coumaric acid-malonyl-hexoside (II)
t4.31	202	32.6	445.1143	*179.0346*; *135.0441*; 134.0367	$C_{22}H_{22}O_{10}$	445.1040	0.6	+	+	+	Caffeic acid derivative

[a] Confirmed by spiking the extracts with authentic standards

peaks 58 and 62), and *m/z* 331.08 (i.e., malvidin, peaks 60 and 63). The signal intensities of these compounds were much higher in *V. myrtillus* berries (approximately from 1×10^4 to 1×10^5 counts) in respect to *V. uliginosum L.* subsp. *gaultherioides* and *V. corymbosum* ones. Based on the $[M]^+$ TOF accurate mass values determined for these analytes, the putative attribution to coumaroyl-hexoside anthocyanidin derivatives can be proposed ($\Delta \leq 1.9$ ppm), in agreement with the findings obtained under very similar chromatographic conditions by Zoratti and colleagues in *V. myrtillus* fruits collected in the Alps of Northern Italy [35].

Finally, peaks 61 (8.6×10^3 counts) and 64 (1.7×10^5 counts), which were peculiar of *V. myrtillus* fruits, exhibited a single neutral loss of 338 Da (i.e., feruloyl-hexoside) with formation of the fragment at *m/z* 331.08 (i.e., malvidin). Accordingly, these peaks were tentatively identified as malvidin-feruloyl-hexosides. It is remarkable that these compounds, previously found in others fruits (e.g., grape) [36], were herein identified in bilberry for the first time.

Flavonols

According to literature, the detection of flavonols by ESI-MS techniques can be achieved with high sensitivity under negative ionization mode [37]. Hence, in this study, flavonols were identified via negative polarity, by monitoring the quasi-molecular $[M-H]^-$ ion and its fragments (Table 2). Furthermore, peak assignment was confirmed under positive ionization, by monitoring the quasi-molecular $[M+H]^+$ ion, which allowed to pinpoint all the analytes found by negative mode, even though with lower signal intensity.

Using the IDA TOF-Q/TOF workflow and comparisons among retention times and mass spectra of unknown and authentic standards, the unequivocal or at least tentative identification of 55 flavonols was achieved. As illustrated in Table 2, among the identified flavonols, we found 36 glycosides (peaks 66–71, 74–79, 82, 86–88, 91–94, 96–97, 99–101, 103–104, 107–110, 112–114, 116, and 117), 6 glucuronides (peaks 65, 73, 83, 90, 106, and 111), 9 acyl derivatives (peaks 72, 80, 84, 85, 89, 95, 98, 102, and 105), and 4 aglycones (peaks 81, 115, 118, and 119). TOF MS $[M-H]^-$ quasi-molecular ions matched the proposed formulae with very high mass accuracy, being Δ absolute values $\leq$2.5 ppm for 75% of the identified analytes, and included in the range of 2.6–4.4 ppm in the remaining cases (Table 2).

In agreement with literature findings [38], Q/TOF MS/MS spectrum of flavonol glycosides exhibited both the heterolytic and the homolytic cleavage of the glycosidic bond, producing the aglycone fragment ion $[Y_0]^-$ and the radical aglycone ion $[Y_0-H]^{-\bullet}$. Figure S5A of the ESM illustrates as an example the MS/MS spectrum of quercetin-3-*O*-glucoside (peak 75), in which the ions derived from heterolytic (m/z = 301.03) and homolytic (m/z = 300.03) fission of the glycosidic bond are shown. Neutral losses of 18 Da (H_2O), 28 Da (CO), and 30 Da (CH_2O), individual or combined one with the other, have been also observed starting from the $[Y_0]^-$ ion (ESM Fig. S5A), in agreement with characteristic MS/MS behavior of flavonols elsewhere reported [37, 39]. Moreover, the loss of 15 Da ($-CH_3$) from the aglycone was occasionally observed and putatively attributed to methoxylated flavonols (e.g., peak 78, tentatively identified as laricitrin-3-*O*-galactoside, see Fig. S5B of the ESM). Further typical ions, at m/z = 151.00 and m/z = 179.00, originating from different retrocyclization cleavages of the "C" ring and commonly identified as $^{1,3}A^-$ (retro-Diels-Alder) and $^{1,2}A^-$ fragments [40, 41], were observed, even though with low signal intensity (ESM Fig. S5A, B).

A different relative abundance of the aglycone fragment $[Y_0]^-$ and the aglycone radical $[Y_0-H]^{-\bullet}$ ions, resulting from heterolytic and homolytic cleavage of the glycosidic bond, has been elsewhere demonstrated for kaempferol glycosides and suggested also for other flavonol glycosides, on the basis of the linkage position, as well as of the length of the saccharide chain [38]. More in detail, if the sugar is a monosaccharide, the cleavage of the *3-O* position of the aglycone gives rise preferentially to the $[Y_0-H]^{-\bullet}$ than the $[Y_0]^-$, as observed for example in peaks 66–71 (Table 2). Conversely, in this study, the preferential heterolytic cleavage of the monosaccharide glycosidic bond was never observed, thus excluding the presence of 7-*O*-glycoside derivatives of flavonols in the investigated *Vaccinium* species. Based on the aforementioned considerations, peaks 66, 67, 78, 82, 86, 93, 97, 103, 107, and 109 can be ascribed to 3-*O*-monohexoside flavonol derivatives. More in detail, considering the whole mass dataset, as well as the relative peak elution order, 3-*O*-galactoside and 3-*O*-glucoside derivatives of myricetin (peaks 66 and 67), laricitrin (peaks 78 and 82), kaempferol (peaks 86 and 93), isorhamnetin (peaks 97 and 103), and syringetin (peaks 107 and 109) can be putatively identified, whereas peaks 71 and 75 were unequivocally identified as quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside, due to the availability of the authentic reference standards (Table 2). These flavonols were found to be present in all the investigated *Vaccinium* species, with the only exception of the glucoside derivative of kaempferol in bilberry and blueberry, and of the glucoside derivative of isorhamnetin in blueberry. It should also be underlined that flavonol glucosides and especially galactosides occurred with much higher signal intensity in *V. uliginosum* L. subsp. *gaultherioides* berries, suggesting that these analytes could be a typical metabolomic trait of "false bilberry."

Peaks 68–70, 77, 79, 87, 88, 91, 94, 99, 110, 112–114, 116, and 117 showed the neutral loss of 132 Da, indicating the presence of the aldopentoside residue, and were attributed to 3-*O*-aldopentoside derivatives of the aforementioned aglycones, on the basis of exact mass data of pseudo-molecular ions and aglycone fragments. Interestingly, for the most abundant aldopentoside derivatives, a net predominance was observed in "false bilberry," whereas the others generally had higher signal intensities in blueberry.

Peaks 92 and 104 were characterized by the neutral loss of 146 Da (i.e., deoxyhexose unit). The identity of the former peak, which was found in both bilberry and blueberry, was unequivocally attributed to quercetin-3-*O*-rhamnoside, using the reference standard. Peak 104, which was found only in blueberry, was identified as a laricitrin-deoxyhexoside and putatively attributed to laricitrin-3-*O*-rhamnoside.

Data herein obtained for quercetin-3-*O*-rutinoside (peak 76, Fig. S6A of the ESM) and kaempferol-7-*O*-neohesperidoside (peak 96, Fig. S6B of the ESM), which were available as reference standards, suggested, also for flavonol disaccharides, the higher abundance of homolytic or heterolytic cleavages, as diagnostic of the 3-*O*- or 7-*O*-substitution, respectively. However, according to Lu and coworkers [38], a long saccharide chain substituted at the 3-*O* position, could hinder the occurrence of the $[Y_0-H]^{-\bullet}$ ion, resulting in

product ion MS/MS spectra similar to those of flavonol-7-*O*-glycosides. Accordingly, even though the differentiation of 3-*O* and 7-*O*-disaccharides of flavonols is commonly performed on this basis [42, 43], their attribution was considered herein as putative. Following this approach, some 3-*O*-disaccharide (peaks 74, 76, 100, and 101) and 7-*O*-disaccharide (peaks 96 and 108) derivatives of various flavonols were detected (Table 2).

The tentative identification of glucuronide derivatives of myricetin (peak 65), quercetin (peak 73, see Fig. S7 of the ESM), laricitrin (peak 83), kaempferol (peak 90), isorhamnetin (peak 106), and syringetin (peak 111) was associated to the neutral loss of 176 Da (i.e., glucopyranuronic acid) and formation of the $[Y_0]^-$ ion, consequent to the heterolytic cleavage of the glucuronic bond, whereas the homolytic fragmentation was absent. This mass behavior was probably due to the lower electrophilic nature of glucuronic acid compared to glucose. Interestingly, the abovementioned glucuronides showed comparable signal intensities in bilberry and "false bilberry," whereas a much lower occurrence was highlighted in blueberry.

Peaks 72, 80, 89, 95, 98, 102, and 105, which were found exclusively in blueberry, were putatively identified as acetyl-hexosides of myricetin, quercetin, and laricitrin. Peaks 84 and 85 were also exclusively present in blueberry and tentatively ascribed to malonyl-hexosides of quercetin (Table 2). These attributions were proposed on the basis of neutral losses of 205/204 Da (homolytic/heterolytic cleavage of the acetyl-hexose unit) or 249/248 Da (homolytic/heterolytic cleavage of the malonyl-hexose group), respectively (Table 2).

Four aglycones were also detected in all the investigated *Vaccinium* species (peaks 81, 115, 118, and 119). These molecules fragmented according to retrocyclization ($^{1,2}A^-$ and $^{1,2}B^-$) and retro-Diels-Alder cleavages ($^{1,3}A^-$ and $^{1,3}B^-$) of the "C" ring and were identified as myricetin, quercetin, laricitrin, and isorhamnetin. Different signal intensities were observed for the four aglycones, with myricetin being the predominant aglycone in bilberry and quercetin the compound more abundant in "false bilberry" and blueberry.

Flavanols

Flavanol ESI-MS detection can be achieved both via positive and negative ionization [44]. Accordingly, in this study, the two ionization modes were evaluated for flavanol identification. The results highlighted a slightly better sensitivity using the negative polarity, notwithstanding the high percentage of formic acid used in the eluents that lowered the ionization efficiency under negative potential. The IDA TOF-Q/TOF workflow applied to berry samples and also to some authentic standards allowed for certainly or putatively identifying 54 flavanols with a very good agreement between TOF MS quasi-molecular ions and proposed formulae (Δ absolute values ≤4.2 ppm). The identification data obtained with the negative ionization are reported in Table 3.

Peaks 121 and 135 were respectively assigned to gallocatechin (GC) and epigallocatechin (EGC), which are stereoisomers not distinguishable by mass spectrometry, but well-discriminated by reversed-phase LC. These peaks showed $[M-H]^-$ quasi-molecular ion at *m/z* 305.07 and the same MS/MS spectra (see Fig. S8A of the ESM) with main fragments at *m/z* 261.04 (loss of 44 Da, CH_2=CHOH), *m/z* 221.05 (cleavage of the "A" ring), *m/z* 219.03 (consecutive losses of 44 and 42 Da), and *m/z* 125, the last being by far the most intense ion of the MS/MS spectrum. The high intensity of this ion can be explained on the basis of its dual origin that is from the fission of the heterocyclic ring or the cleavage of the "B" ring, both characterized by the loss of 180 Da (see fragmentation paths 4 and 8 of Scheme S1 of the ESM). Moreover, in accordance to the findings previously reported for catechin (C) and (EC) [45, 46], fragments at *m/z* 139.04 (probably attributable to the cleavage of the "A" and "C" rings) and *m/z* 137.03 (loss of 168 Da, retro-Diels-Alder reaction) were observed. GC and EGC, herein found in all the investigated *Vaccinium* species, were previously reported only in *V. myrtillus* fruits [44]. When MS/MS spectra of peaks 121 and 135 (ESM Fig. S8A) were compared to the ones of peaks 136 and 159 (ESM Fig. S8B), delta mass of 16 Da was observed in most cases. Spiking procedure of the authentic standards unequivocally confirmed the identification of the latter peaks as C and EC (see also ESM Scheme S1 for detailed fragmentation paths). The predominance of catechin in *V. corymbosum* fruits and of epicatechin in *V. myrtillus* and *V. uliginosum* L. subsp. *gaultherioides* berries has been observed, in agreement with the findings already reported in the literature [4, 7].

MS/MS spectra of peaks 126 and 145 revealed the loss of 162 Da (hexose unit) with formation of the ion at *m/z* 289.07, which is attributable to both C or EC, due to their stereoisomeric nature (following the possible presence of the C or the EC unit is indicated as (E)C). Moreover, the aforementioned typical fragments of (E)C were observed, thus indicating the presence of (E)C-hexosides, never reported in *Vaccinium* species, but previously identified in other berries [15].

Peaks 133 and 153 exhibited the typical fragmentation of B-type (E)C-(E)C dimers, consisting in the retro-Diels-Alder fission of the "C" ring (*m/z* 425.09) and successive loss of water (*m/z* 407.08), as well as the cleavage of the B-type linkage with formation of the (E)C monomer (*m/z* 289.07). These peaks were undoubtedly attributed to procyanidin B1 and procyanidin B2, respectively, on the basis of identity confirmation with authentic standards. Peaks 141 and 172 showed the same MS/MS spectrum and were therefore putatively ascribed to B-type procyanidin isomers, in which the C4→C6 interflavanoid bond, instead of the C4→C8 one, is present between the two (E)C units.

The comparison between MS/MS spectra of B-type procyanidins and peaks 120 and 122 highlighted *m/z* values 16 Da higher in most detected fragments of the latter peaks (i.e., *m/z* 441.08, 423.07, and 305.06, see Fig. 1). Accordingly, peaks 120 and 122 were tentatively identified as B-type (E)GC-(E)GC dimers (Table 3). It is remarkable that for these peaks, the fragment at *m/z* 177.02 has been also observed, in contrast to MS/MS findings of (E)C dimers, in which this ion was absent. It should also be noted that the fragment at *m/z* 177.02 was absent in (E)GC (see ESM Fig. S8A), thus suggesting that it derives from the *m/z* 303.05 ion, as proposed in Scheme 1.

Peak 170 showed the typical fragmentation of A-type procyanidins (e.g., cleavage of the "C" ring, fission of the heterogeneous C2→O interflavanyl linkage and rearrangement with formation of the ion at *m/z* 449.09), which was unequivocally identified as procyanidin A2, based on its authentic standard.

A number of proanthocyanidin trimers, tetramers, and pentamers, characterized by B-type and both A- and B-type (following A/B) interflavanyl linkages, were identified (Table 3) on the basis of the typical mass fragmentation mechanisms of this polyphenol class: retro-Diels-Alder (RDA), quinone methide formation (QM), and heterocyclic ring fissions (HRF) [47].

Most of these proanthocyanidins were trimers (peaks 140, 144, 148, 157, and 164), tetramers (peaks 127, 138, 147, 151, 154, 155, 165, and 173), and pentamers (peaks 143, 146, 150, 158, 161, 166, and 167) consisting of only B-linked (E)C units. More in detail, the MS/MS spectrum of B-type procyanidin trimers was characterized by ions derived from RDA fission (*m/z* 713.15) and successive loss of water (*m/z* 695.14), as well as the typical fragmentation pattern of B-type procyanidin dimers (i.e., *m/z* 425.09 and 407.08) previously discussed. Moreover, the presence of monomer (*m/z* 289.07 and 287.06) and dimer (E)C units (*m/z* 577.13 and 575.12) derived from QM reaction confirmed the identity of B-type procyanidin trimers. B-type procyanidin tetramers were detected both as mono-charged (*m/z* 1153.26) and double-charged (*m/z* 576.13) quasi-molecular ions and produced fragments related to trimeric (*m/z* = 865.20 and 863.19), dimeric (*m/z* = 577.13 and 575.12), and monomeric (*m/z* = 289.07 and 287.07) units, up to the characteristic MS/MS spectra of (E)C. Similarly, B-type procyanidin pentamers showed the typical fragmentations of the lower molecular weight B-type procyanidin oligomers.

A/B-linked proanthocyanidin trimers (peaks 156, 168, and 171), tetramers (peaks 124 and 163), and pentamers (peaks 137 and 169) formed only of (E)C units were also putatively identified. For these compounds, the RDA reaction affecting the B-type-linked (E)C caused the ion at *m/z* 711.14 and, after the loss of water, the ion at *m/z* = 693.13. The QM reaction produced the fragments at *m/z* 573.10 and 289.07, whereas the ions at 451.10 and 411.07 derived from HRF reactions. Similarly, A/B-type procyanidin tetramers and pentamers were identified on the basis of their MS/MS fragments consisting of the abovementioned typical product ions of A/B-type procyanidin trimer (*m/z* = 863.18) and dimer (*m/z* = 575.12).

Interestingly, two compounds exclusively formed by (E)GC units (i.e., peak 125, A/B-type trimer, and peak 128, B-type trimer) were herein identified for the first time in *V. myrtillus* and *V. uliginosum L.* subsp. *gaultherioides* fruits. Peak 125 was identified as A/B-type prodelphinidin trimer on the basis of the characteristic fragments derived from RDA (*m/z* = 743.13) and HRF (*m/z* 483.09 and 427.07) reactions, whereas the MS/MS spectrum of peak 128 exhibited the product ions corresponding to the formation of dimer (*m/z* 609.13) and monomer (*m/z* 305.07) ions. In addition, the ion at *m/z* 541.08, derived from the cleavage of the "B" rings of the trimer, was observed.

Four B-type proanthocyanidin trimers (peaks 129, 139, 149, and 160) and two tetramers (peaks 130 and 162) formed by both (E)C and (E)GC monomers were also identified (Table 3), but no information about the relative position of the different units could be obtained by the MS/MS spectra.

Finally, six proanthocyanidin oligomers were identified as trimers, tetramers, and pentamers consisting of both (E)C and (E)GC units, linked with A/B-type (peaks 123, 131, 132, 134, 142, and 152) bonds. Interestingly, in this case, the fragmentation spectra highlighted in most cases the diagnostic ions that indicated the relative position of a certain monomer and/or the type of linkage (A type or B type) between two monomers. For instance, peaks 142 and 152 were identified as A/B-type trimers constituted by two units of (E)C and one unit of (E)GC. For both peaks, the RDA reaction and the successive loss of water, producing the ions at *m/z* 727.13 and 709.12, were observed. Nevertheless, peak 142 was characterized by fragments at *m/z* 427.07 and 451.10, fully consistent with the occurrence in the molecule of (i) one terminal (E)GC linked to one (E)C by an A-type linkage and (ii) two (E)C units linked by a B-type interflavanyl bond, respectively. Moreover, a high-intensity fragment at *m/z* 289.07 was observed, in accordance with the presence of a B-type terminal (E)C (Fig. 2A). Analogously, the MS/MS spectrum of peak 152 showed fragments at *m/z* 411.07 and 467.10, which are in agreement with the presence of (i) one terminal (E)C linked to the other portion of the molecule by an A-type linkage and (ii) one (E)C and one (E)GC unit linked each other by a B-type bond. Moreover, it should be noted that an intense fragment at *m/z* 305.06, attributable to the (E)GC unit, was also observed (Fig. 2B). Accordingly, peaks 142 and 152 were putatively identified as A/B-type (E)GC-(E)C-(E)C and A/B-type (E)C-(E)C-(E)GC, respectively.

A similar consideration can be done for peaks 132 and 134, which were A/B-type trimer constituted by one unit of (E)C and

Q1

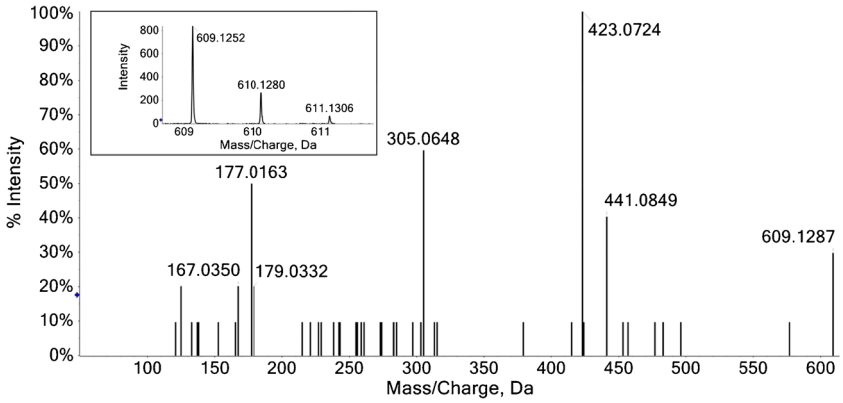
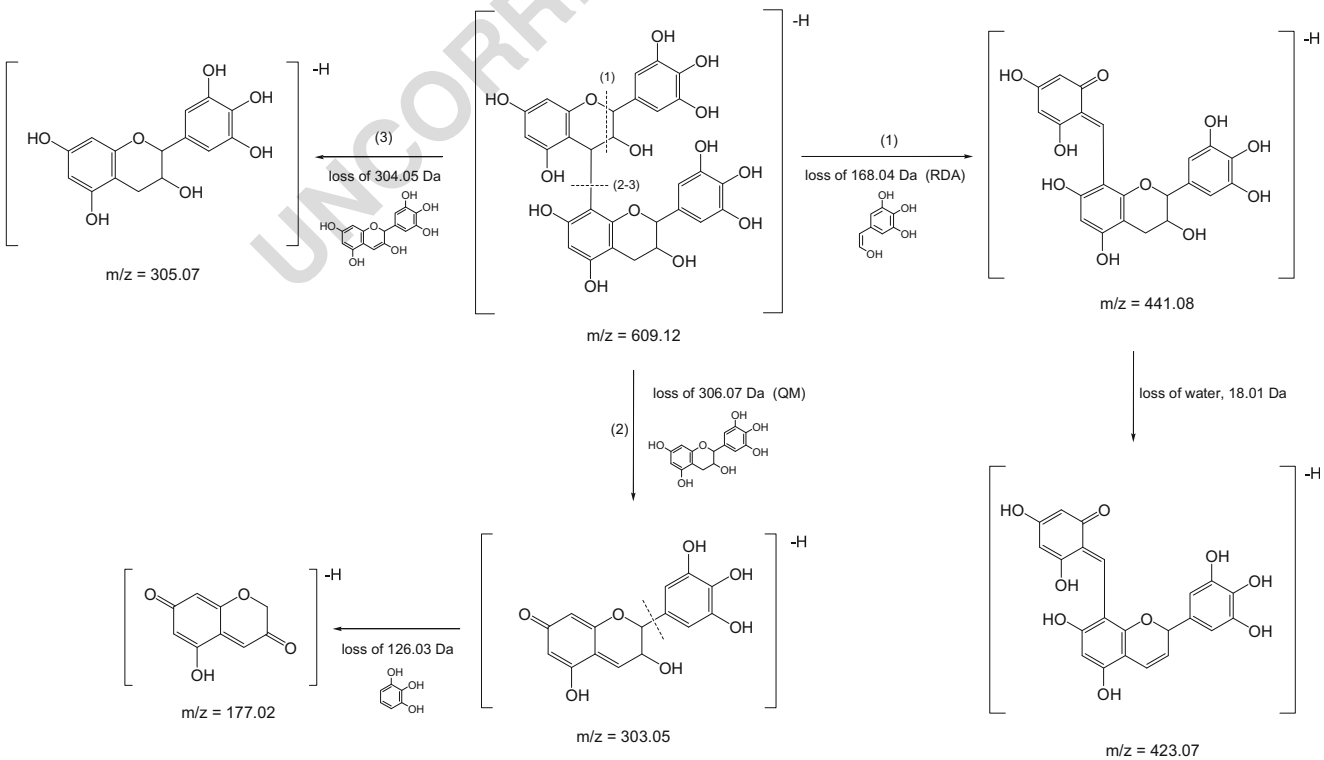


Fig. 1 Q/TOF MS/MS spectrum of peaks 120 and 122, identified as B-type dimer of (epi)gallocatechin

two units of (E)GC. In both these peaks, the ion at *m/z* 427.07 indicated the presence of one terminal (E)GC unit linked with an A-type bond with the rest of the molecule, whereas the ion at *m/z* 467.10 was diagnostic for the occurrence of B-type-linked (E)GC and (E)C. However, peak 132 was also characterized by the presence of an intense fragment at *m/z* 289.07, differently from peak 142 that showed a high-intensity ion at *m/z* 305.07. Therefore, peaks 132 and 134 were putatively identified as A/B-type (E)GC-(E)GC-(E)C and A/B-type (E)GC-(E)C-(E)GC, respectively (see Fig. 3A, B).

Peak 123 was identified as an A/B-type tetramer formed by three units of (E)C and one unit of (E)GC. This peak fragmented originating the ion at *m/z* 863.18, which is consistent with the formation of an (epi)catechin trimer with one A-type and one B-type linkage, together with other characteristic ions (i.e., *m/z* 711.14, 573.10, and 411.07), deriving from the catechin trimer fragmentation. Accordingly, in peak 123, the (E)GC unit should be terminal and linked through a B-type interflavanyl linkage.

Finally, peak 131, which was formed by four units of (E)C and one unit of (E)GC, two of them linked by an A-type interflavanyl bond, showed, among other, a quite intense fragment at *m/z* 591.12. This ion is compatible with the presence of an A-type bond between the (E)GC and one (E)C units.



Scheme 1 Hypothesized structure and fragmentation scheme for peaks 120 and 122 ($[M-H]^-$ = 609.12, putatively attributed to an (epi)gallocatechin dimer. *RDA* retro-Diels-Alder, *QM* quinone methide formation

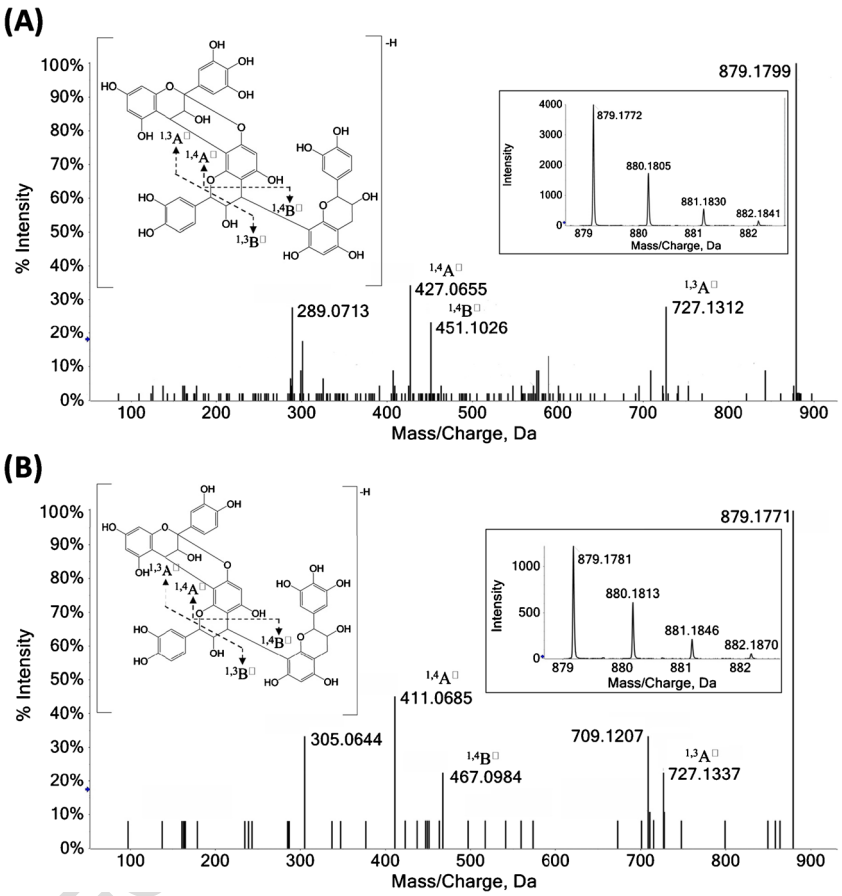


Fig. 2 Q/TOF MS/MS spectrum of peaks (**A**) 142 and (**B**) 152, identified as A/B-type (E)GC-(E)C-(E)C and A/B-type (E)C-(E)C-(E)GC, respectively. *(E)C* (epi)catechin, *(E)GC* (epi)gallocatechin. Note that ion $^{1,3}B^-$ is not evidenced in the mass spectra

Other compounds

Using the IDA TOF-Q/TOF workflow under negative ionization, 29 further phenolic compounds belonging to various classes were putatively or unequivocally identified in berry samples (Table 4). Also in these cases, a very good agreement between TOF MS quasi-molecular ions and proposed formulae was obtained (Δ absolute values ≤3.8 ppm).

Peak 199 was found to be common to the three species and unequivocally identified as phloridzin after comparison with the corresponding authentic standard. The use of the reference standard allowed for certainly identifying also peak 190 as scopoletin, which was detected only in "false bilberry."

Peak 174, which was present at quite similar intensities in all berry species, was unambiguously identified as gallic acid, due to the availability of the authentic reference standard.

Several phenolic acids belonging to the class of the hydroxycinnamic acids (peaks 175–178, 180, 182, 185, 186, 192, 193, 197, and 198) were also putatively or unequivocally identified, depending on the availability of the authentic standards. These compounds were generally found at higher intensity in *V. corymbosum* and in some cases (peaks 176, 177, 193, 197, and 198) detected exclusively in this berry species.

Peak 179 exhibited a quasi-molecular ion at *m/z* 353.08, which fragmented giving rise to a neutral loss of 162 Da and ions attributable to caffeic acid. This peak was therefore putatively attributed to a caffeic acid hexoside. Analogously, peaks 181 and 183 were tentatively identified as coumaric acid and ferulic acid hexosides.

Peaks 188, 194, and 202 showed pseudo-molecular ions at *m/z* 335.08, 367.10, and 445.11, respectively, and shared the typical fragments of caffeic acid (Table 4), thus suggesting their putative attribution as caffeic acid derivatives.

For peaks 201 and 202, the same quasi-molecular ion at *m/z* 411.17 was found. The fragmentation gave rise to a neutral loss that corresponded to a malonyl hexoside (248 Da) with formation of a fragment consistent with coumaric acid $[M-H]^-$ ion (*m/z* 163.04), as well as various fragments typical of coumaric acid, thus suggesting the putative identification of both peaks as coumaric acid-malonyl-hexosides.

Fragmentations of the quasi-molecular $[M-H]^-$ ions of peaks 195 and 196 (*m/z* 535.15) were in full agreement with data reported by Hokkanen et al. [44] for coumaroyl iridoids. These peaks, previously identified in *V. myrtillus* fruits [48], were much more intense in bilberry than in "false bilberry," whereas these were absent in blueberry.

Peaks 184, 187, 189, and 191 were putatively identified as cinchonain II isomers, in agreement with the fragmentation scheme reported by Hokkanen and coworkers [44]; all these compounds have been found in *V. corymbosum* berries,

Q1

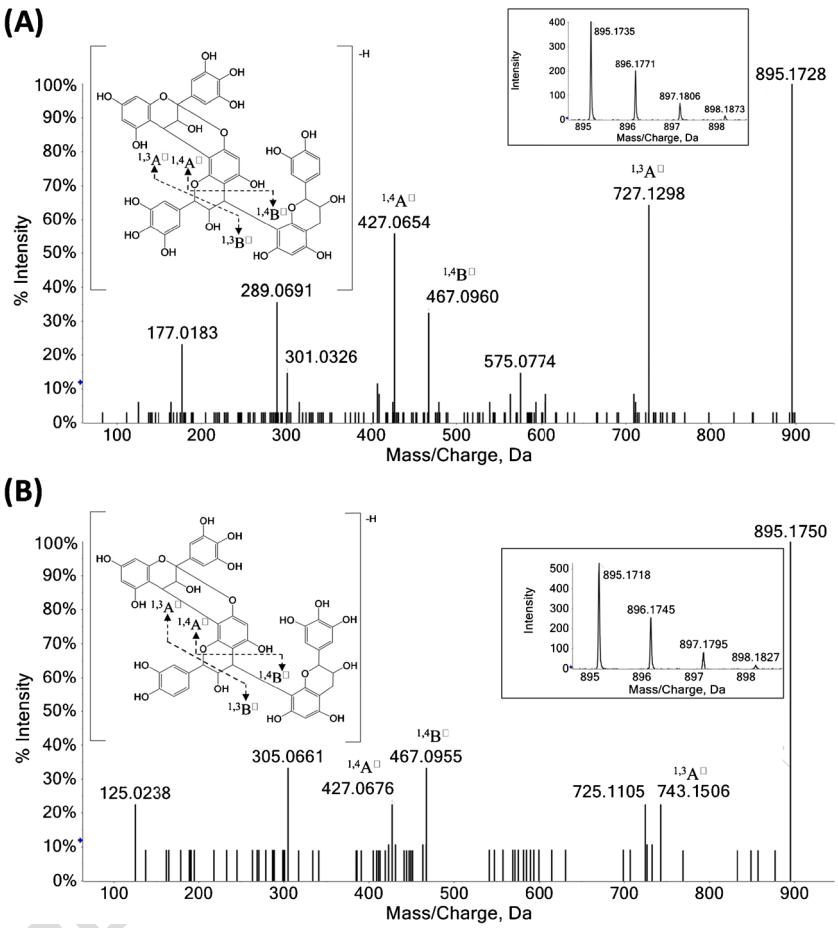


Fig. 3 Q/TOF MS/MS spectrum of peaks (**A**) 132 and (**B**) 134, identified as A/B-type (E)GC-(E)GC-(E)C and A/B-type (E)GC-(E)C-(E)GC, respectively. *(E)C* (epi)catechin, *(E)GC* (epi)gallocatechin. Note that ion $^{1,3}B^-$ is not evidenced in the mass spectra

whereas only the last isomer was detected in *V. myrtillus* fruits. The presence of cinchonain isomers was previously highlighted in other plants [49], as well as in the leaves of various *Vaccinium* plant species [44, 50], but never observed before in berries.

Comparison of polyphenolic compositions by PCA

The polyphenolic compositions of the three investigated berry species, as reported in Tables 1, 2, 3, and 4 and discussed in the previous paragraphs, appear very complex. Therefore, PCAs were separately performed on the LC-ESI-TOF MS data acquired in positive and negative ionization in order to highlight which of the identified polyphenols are the most representative for describing the composition of the three *Vaccinium* species under investigation.

As regards positive ionization, two PCs had eigenvalues higher than 1 and were therefore considered as significant for describing the variance of the original TOF data. These two latent variables explained together 98.6% of the original variance (Fig. 4A). PC1, which accounted for 66.5% of the original variance, was positively and strongly correlated with coumaroyl-hexosides (e.g., peaks 51, 57, 58, and 63) and glucuronides (e.g., peaks 7 and 15), as well as with malvidin-feruloyl-hexosides (peaks 61 and 64) and various anthocyanidin glycosides, such as dihexosides (e.g., peaks 3, 5, and 13) and aldopentose-hexosides (e.g., peaks 21 and 27). An opposite behavior (i.e., strong and negative correlation with PC1) was observed for malonyl (e.g., peak 39) and acetyl (e.g., peaks 49, 50, and 52) derivatives of anthocyanidins, as well as for various malvidin glycosides (e.g., peaks 29 and 36). Conversely, these last metabolites showed high and positive loadings on PC2 (explained variance equal to 32.1%), which was on the other hand negatively correlated with the five xyloside derivatives herein identified (i.e., peaks 24, 35, 37, 44, and 45). A very high and negative loading on PC2 was also observed for malvidin-3-*O*-glucoside (peak 34).

Figure 4B illustrates how the three analyzed samples of each species and the quality control samples (obtained by mixing equal amounts of each extracted sample) were located in the PC1 versus PC2 Cartesian plane. It is remarkable that different samples of each species were very close to the other, generating three well-separated clusters in the PC space. Accordingly, the repeatability of the whole analytical process as well as the robustness of the chemometric approach was demonstrated. It should also be noted that the quality controls were very close to the origin of the PC coordinates, confirming the accuracy and precision of PCA. The clusterization of the three *Vaccinium*

species clearly highlighted their very different whole anthocyanin compositions. More in detail, an important role in the discrimination of *V. myrtillus* samples, which showed very high and positive scores on PC1 and small and positive scores on PC2, was clearly played by the aforementioned coumaroyl-hexosides and glucuronides. Conversely, acetyl and malonyl derivatives were the major responsible for the separation of *V. corymbosum* fruits. Finally, *V. uliginosum* subsp. *gaultherioides* berries, even though generally poorer in the number of identified anthocyanins, as well as in their signal intensity, were interestingly characterized by xyloside derivatives of petunidin (peak 37), peonidin (peak 44), and malvidin (peak 45).

PCA was also applied to the TOF data acquired in negative mode, highlighting two factors with eigenvalues higher than 1, which accounted for a total explained variance of 96.4% (66.3% and 30.1% for PC1 and PC2, respectively). The variable separation on the two PCs was in this case not as good as that obtained for compounds detected under positive ionization, probably also due to the much higher number of analytes detected in negative polarity. In fact, many metabolites were distributed in a very wide range of negative PC1 values, with both positive and negative loadings on PC2. However, some analytes showed very high and positive loadings on PC1 and a very narrow range of PC2 values, thus forming a cluster (see Fig. 5A and the zoomed area). This cluster contained all the identified flavonol acetyl-hexosides (e.g., peaks 89 and 105), some flavonol aldopentosides (e.g., peaks 69, 77, and 87), and a number of B-type proanthocyanidins eluting at relatively low retention times, whereas no A/B-type derivatives were found in this group. Furthermore, some hydroxycinnamic acids, such as neochlorogenic (peak 175), cryptochlorogenic (peak 182), and coumaroylquinic (peak 177), belonged to the cluster. Cinchonains exhibited high loadings on PC1 and low loadings on PC2, as well. Conversely, very high and negative values on the former latent variable were observed for all flavonol glucuronides (e.g., peaks 65, 73, 83, 90, 106, and 111).

The score plot (Fig. 5B) highlighted a very good accuracy and precision of PCA also for data obtained under negative ionization, with quality control samples well centered on the origin of the PC coordinates and evident separations among *V. corymbosum* (high scores on PC1 and close to zero on PC2), *V. myrtillus* (high and negative scores on PC1 and very high and positive on PC2), and *V. uliginosum* subsp. *gaultherioides* (negative scores on both PC1 and PC2) berries. Thus, LC-ESI-MS/MS in negative ionization gave useful and complementary information with respect to the positive mode for the discrimination of the investigated species.

Conclusions

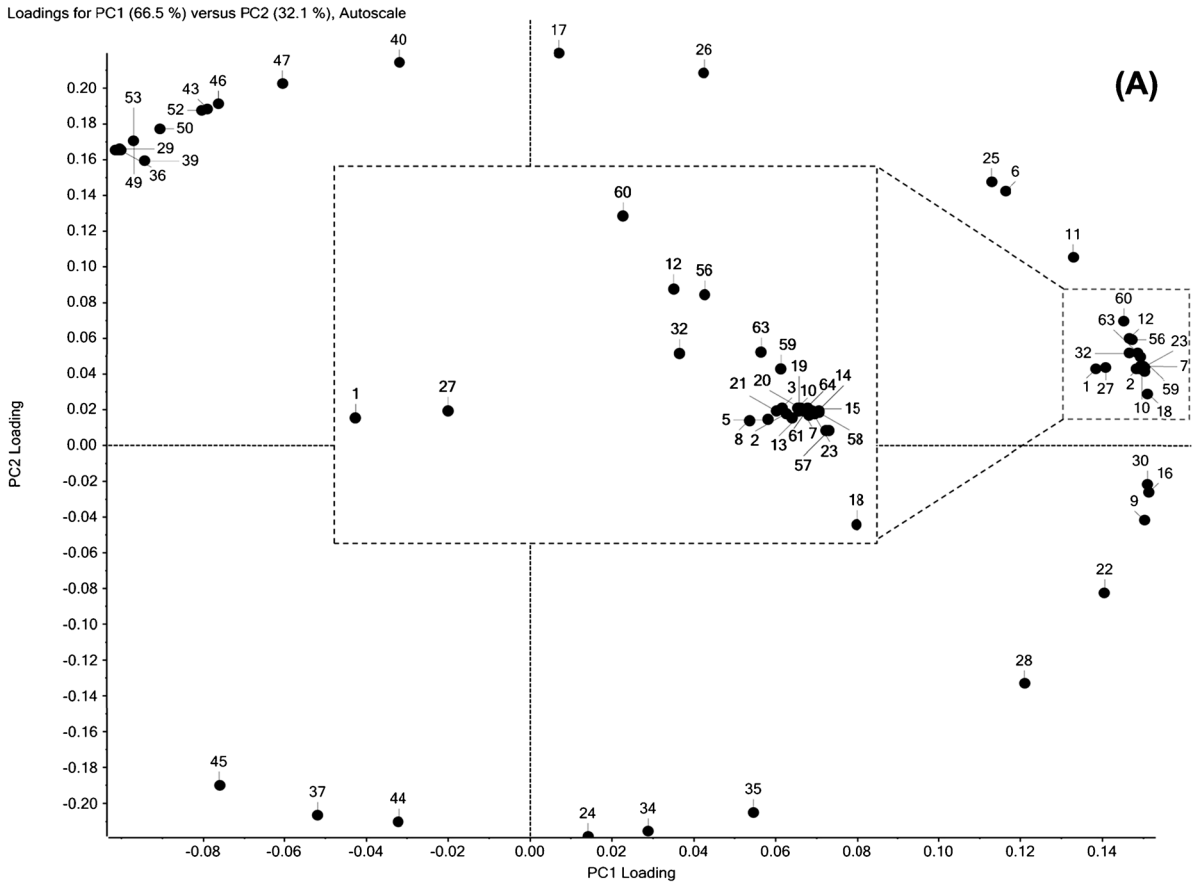
LC-ESI-TOF and LC-ESI-Q/TOF analysis, performed both in positive and negative modes, allowed to obtain a comprehensive picture of the polyphenolic composition of *V. myrtillus*, *V. corymbosum*, and *V. uliginosum* subsp. *gaultherioides* berries.

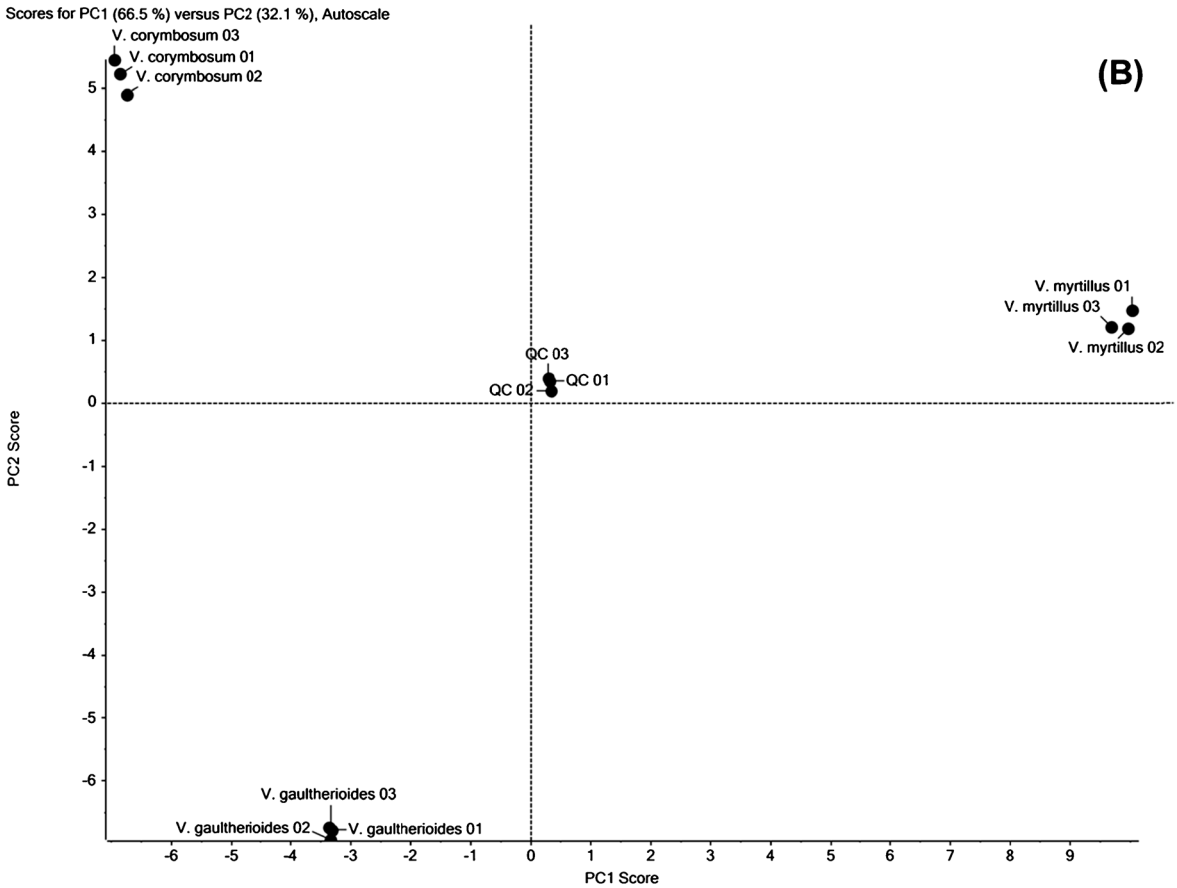
More in detail, 58 of the 64 anthocyanins identified in this study were present in *V. myrtillus*, 39 in *V. corymbosum*, and 24 in *V. uliginosum* L. subsp. *gaultherioides*. As regards this last species, it is remarkable that aldopentosides and coumaroyl-hexosides have been detected herein for the first time. It should also be underlined that this study is the first one reporting the occurrence in *V. myrtillus* berries of anthocyanidin glucuronides and malvidin-feruloyl-hexosides, which represented an intense and characteristic metabolomic trait of this *Vaccinium* species, together with the already reported aldopentose-hexosides and cyanidin-aldodipentoside (Table S2). This study also indicated the exclusive presence of acetyl- and malonyl-hexosides in *V. corymbosum* berries (Table S2), compared to the other two investigated *Vaccinium* species.

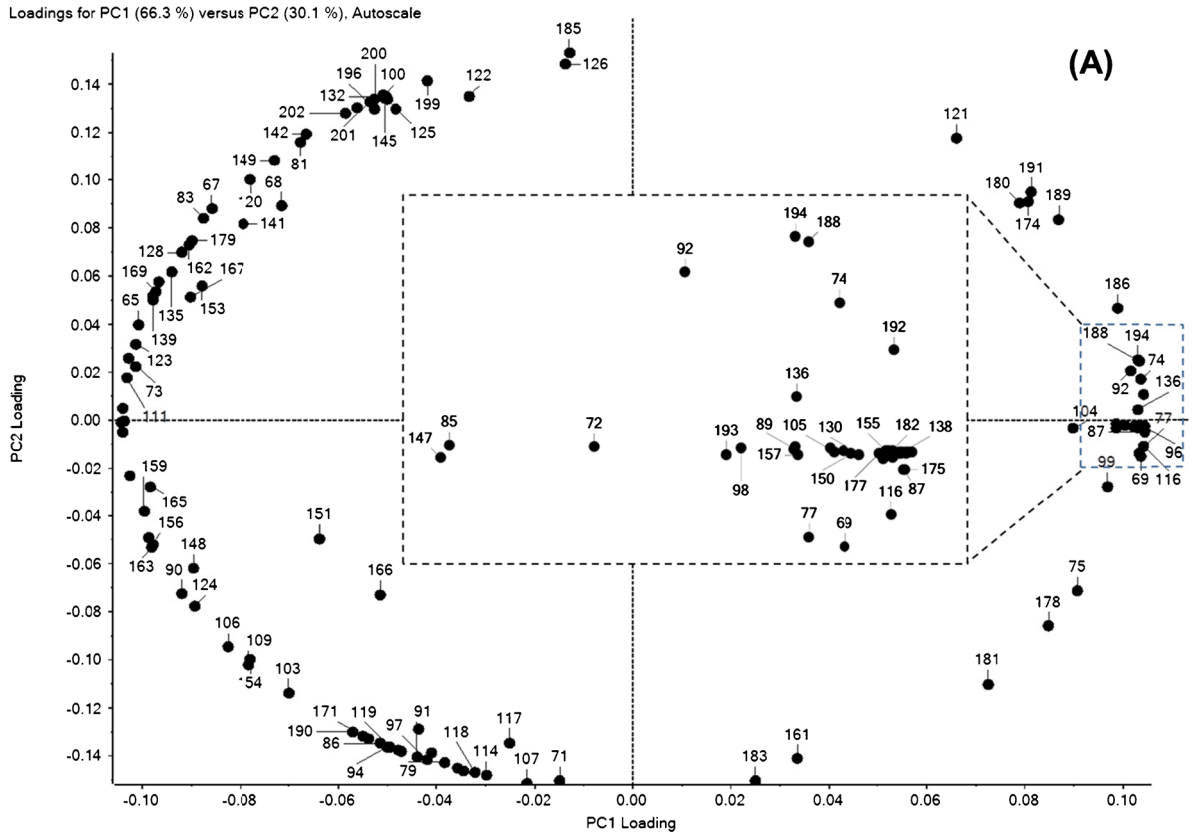
Flavonols resulted generally more abundant in *V. corymbosum*. In fact, 51 of the 55 flavonols identified herein were found to be present in blueberries whereas only 37 in "false bilberry" and 35 in bilberry. Remarkably, in previous works, the flavonol derivatives discussed above were only partially detected in *V. myrtillus* [12, 13, 44] and *V. corymbosum* [24, 51, 52] berries, whereas very few data were elsewhere reported for *V. uliginosum* L. subsp. *gaultherioides* [4]. Hence, this work represents also for flavonol glycosides a more comprehensive study of such metabolites in the investigated *Vaccinium* species.

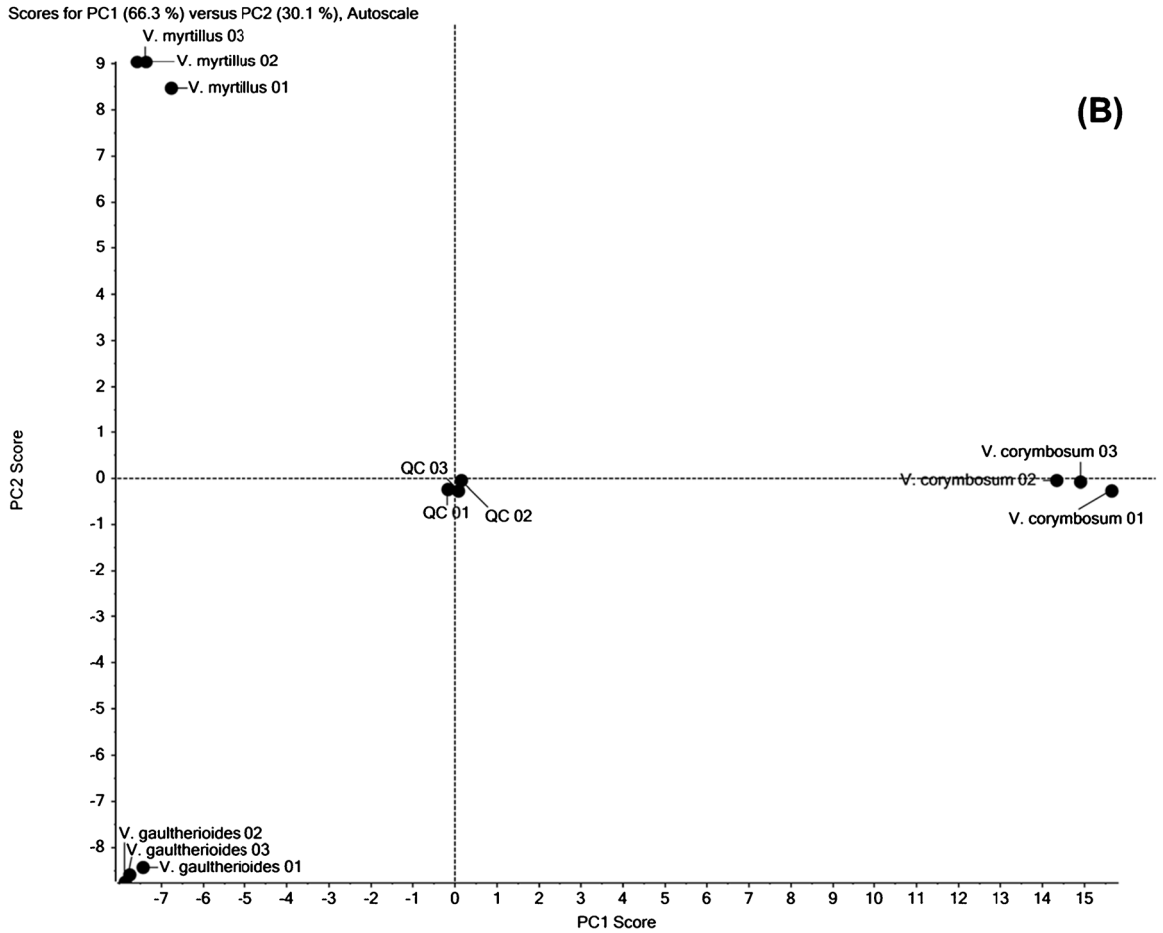
A similar number of flavanols were identified in the three species (i.e., 41, 39, and 35 compounds in bilberry, "false bilberry," and blueberry, respectively), and some of them, including trimers, tetramers, and pentamers, were found to be present in all species. However, some species-specific metabolites were found. For instance, flavanols containing A-type interflavanyl linkages were never observed in *V. corymbosum* (Table S2). Moreover, some B-type trimers (peaks 129, 144, and 157), tetramers (peaks 127, 130, 138, and 147), and pentamers (peaks 143, 146, 150, and 158) were exclusively found in blueberry (see Table 3). Interestingly, these compounds eluted at earlier retention times, compared to the metabolites common to the three species, thus suggesting a greater presence of catechin and gallocatechin, rather than the corresponding epimers, in *V. corymbosum* berries. It should be underlined that, for the first time, this research provides in-depth data on flavanols in *V. uliginosum* subsp. *gaultherioides* and *V. corymbosum* berries. Furthermore, even though data regarding flavanols in *V. myrtillus* fruits have been already

Fig. 4 Loading (**A**) and score (**B**) plots of PC1 versus PC2 (PCA of original LC-ESI-TOF MS data acquired in positive ionization). *Numbers* shown in the loading plot refer to the peak numbers reported in Table 1









◀ **Fig. 5** Loading (**A**) and score (**B**) plots of PC1 versus PC2 (PCA of original LC-ESI-TOF MS data acquired in negative ionization). *Numbers* shown in the loading plot refer to the peak numbers reported in Tables 2, 3, and 4

reported in literature [13, 44], this study provides a much more detailed description of the flavanol composition in these berries, identifying for the first time a large number of proanthocyanidins with high molecular weight.

All the aforementioned LC-MS data were well-integrated using the PCA approach, which demonstrated to be suitable for a clear discrimination of the investigated berry species both in positive and negative ionization modes.

The comprehensive investigation herein illustrated, which evidenced phenolic metabolites exclusively detected in one species or characterized by extremely different intensities in the three berries, can be useful for future developments of methods aiming at evaluating the quality of *Vaccinium* berry transformation products and to avoid frauds. These products, in fact, are not only fruit juices or jams that are not subjected to any particular regulation concerning their phenolic content but also supplements or actual drugs, which must conversely respect what is written in the label, both in terms of plant material used for its preparation and amount of active ingredients contained in the product.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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