Identification of Polyphenolic Compounds in Berries from Different *Vaccinium* Species using Liquid Chromatography High Resolution Mass Spectrometry (LC-HR-MS)

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Overview

Extracts of different blueberries (*Vaccinium myrtillus*, *V. gaultherioides*, and *V. corymbosum*) were analyzed using LC-HR-MS on a SCIEX TripleTOF® 4600 system. Non-target statistical data analysis was performed to obtain comprehensive information on profiles of polyphenolic composition. The three species exhibited very different profiles of phenolic substances and could be discriminated according to their polyphenolic compositions using principal components analysis (PCA).

Introduction

Berries of different *Vaccinium* species are widely considered important sources of polyphenolic compounds in the human diet.[1]

V. myrtillus is a wild berry native to mountain areas of Northern and Central Europe, largely used in transformed products, as well as dietary supplements. The interest in these fruits is due to its high content of phenolic compounds, well-known for their health-protecting attributes, as anti-inflammatory, anti-hypertensive, anti-microbial and anti-cancer agents.[2] Accordingly, research focusing on the characterization of selected phenolics in *V. myrtillus* berries has been recently published, evidencing that the most abundant class of polyphenols in these fruits are anthocyanins.[3]

The anthocyanidine composition of *V. myrtillus* berries has been found different from the ones of other *Vaccinium* species, such as the widely commercialized *V. corymbosum*[4], suggesting the potential use of polyphenolic profiles for the chemotaxonomic discrimination of *V. myrtillus* fruits from berries of other cultivated and wild species. This aspect is very important since *V. myrtillus* shows a nutraceutical value much higher than that of *V. corymbosum* and the two species are not well-distinguished by consumers, due to their quite similar look (see Figures 1A and 1C).

Nevertheless, to the best of our knowledge, no comprehensive investigation of the polyphenolic profiles of these *Vaccinium* species has been published to date.

Moreover, the presence of a different *Vaccinium* species – namely *V. gaultherioides*, for which no data regarding the primary and secondary metabolic profiles are reported in literature, has been recently observed in the zones traditionally populated by *V. myrtillus*, such as Tuscan Apennines. In this regard, it should be underlined that the phenotype of *V. gaultherioides* berry is very similar to the one of *V. myrtillus* (see Figures 1A and 1B) and the two berries can be confused by the harvesters involved in the production chain of transformed bilberry.

For this reason, the polyphenolic profiles of *V. myrtillus*, *V. corymbosum* and *V. gaultherioides* berries have been investigated using LC-HR-MS with the SCIEX TripleTOF® 4600 system) to obtain comprehensive information on the profiles of polyphenolic fractions.

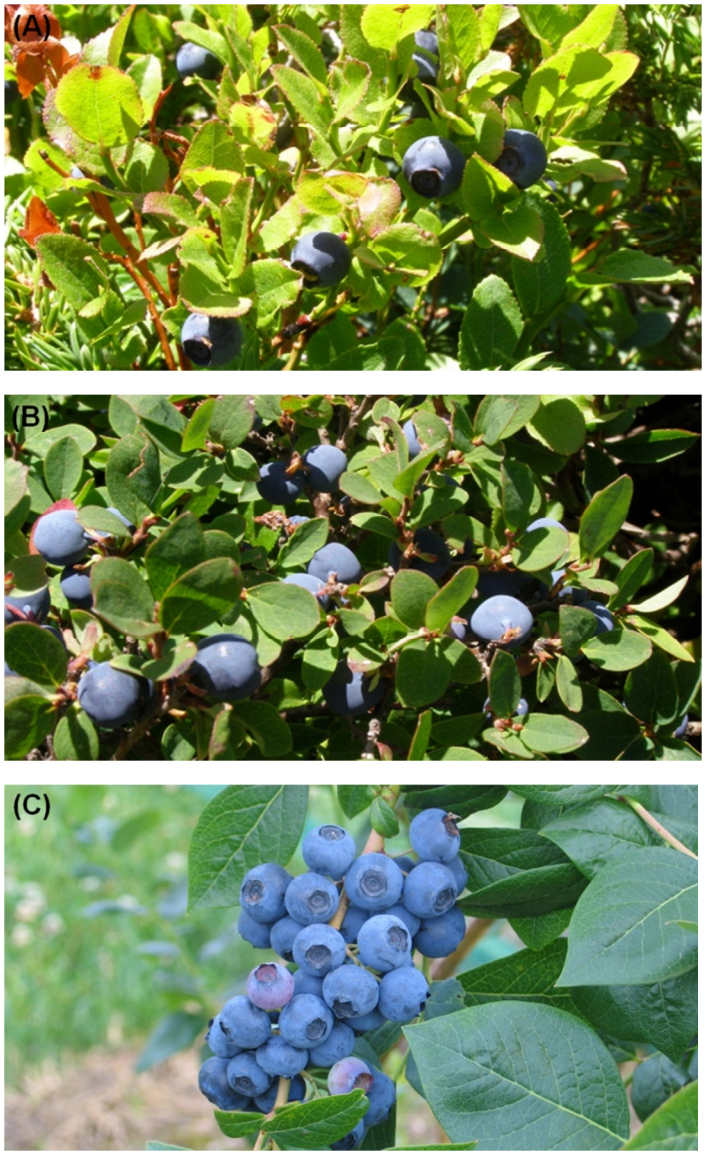

Figure 1. Berries of wild *V. myrtillus* (A), *wild V. gaultherioides* (B) and cultivated *V. corymbosum* (C)[5] species

Experimental

Sample Preparation

A representative berry sample of each *Vaccinium* species was extracted according to a previously developed procedure.[5]

About 500 mg aliquots of dry weight berries were homogenized in an ice bath under magnetic stirring with 15 mL of a methanol/water solution 80/20 (v/v) containing 10 mM NaF to inactivate polyphenol oxidase. The mixture was centrifuged at 1800xg for 5 min and the supernatant recovered. This procedure was repeated twice and the extracts were combined. Quality control (QC) samples were also prepared by mixing equal aliquots of each sample. The LC-HR-MS analysis was carried out on the extracts after removal of the organic solvent by vacuum evaporation, followed by acidification with formic acid up to pH = 2.0 and filtration using 0.2 µm nylon membranes.

LC Separation

LC separation was achieved using an Agilent Infinity 1290 system with an Acquity BEH C18 (150 × 2.1 mm, 1.7 µm) column at a flow rate of 450 µL/min. A gradient of water and methanol with 5% formic acid in both phases was used (Table 1). The column temperature was 50ºC and the injection volume was 2 µL.

Table 1. LC gradient

Step	Time (min)	A (%)	B (%)
0	0.0	98	2
1	2.0	98	2
2	30.0	70	30
3	35.0	5	95
4	37.0	5	95
5	37.1	98	2
6	45.0	98	2

HR-MS and MS/MS Detection

A SCIEX TripleTOF® 4600 system equipped with DuoSpray™ source was used for HR-MS and analysis and the following source parameters were kept constant during the acquisition:

- Heater temperature (TEM) 400ºC
- Curtain Gas™ (CUR) 25 psi
- Nebulizing gas (GS1) 45 psi
- Heater gas (GS2) 45 psi
- Electrospray voltage (ISVF) of +5300 V and - 4500 V, respectively, for positive and negative polarity

Each sample was analyzed, in positive and negative polarity, using two different acquisition methods for each ionization mode.

The first acquisition method consisted of a high resolution TOF-MS scan only (100 to 2000 Da) aiming at multivariate analysis in the investigated polarity. The second acquisition method was an information dependent acquisition (IDA) method containing a TOF-MS survey scan (100 to 2000 Da with an accumulation time of 250 msec) and 10 dependent MS/MS scans (100 to 2000 Da

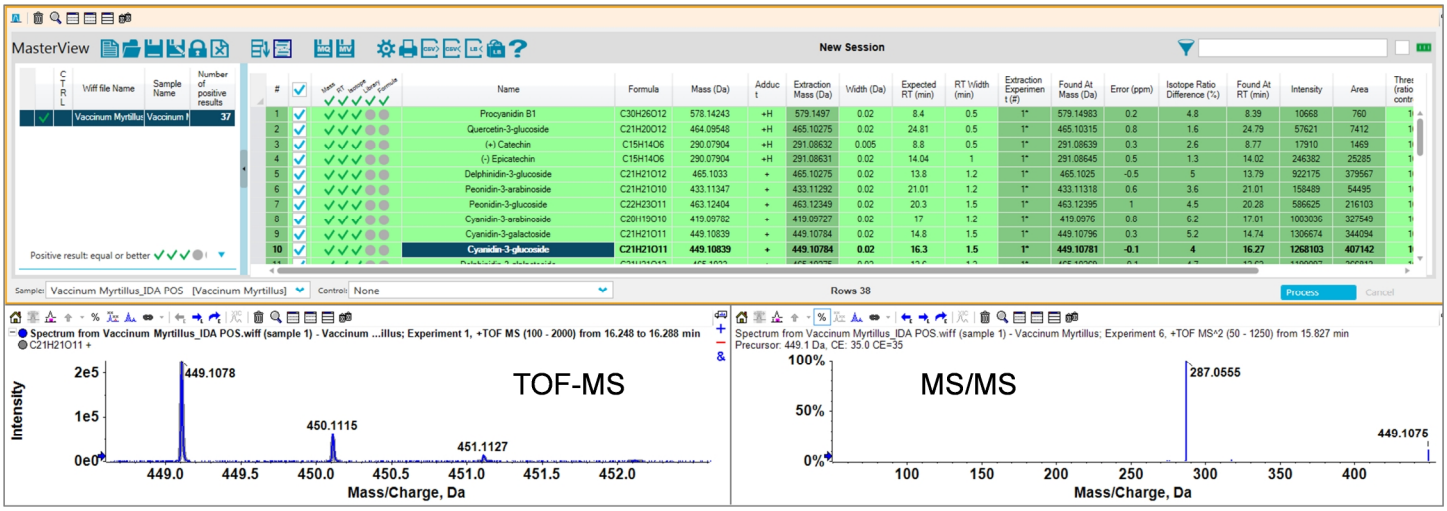

Figure 2. An example screenshot of MasterView™ software showing the list of the identified compounds associated with their isotope pattern and MS/MS spectrum

with an accumulation time of 75 msec). The collision energy was set to 35 V with a collision energy spread of ±15 V.

Automated calibration was performed using the calibrant delivery system (CDS) which infuses calibration solution between sample analysis.

Data Processing

The information rich chromatograms deriving from the LC-HR-MS and MS/MS analysis of investigated samples, both in negative and positive ionization, need to be processed with powerful software tools.

PeakView® software version 2.2 with MasterView™ software version 1.1 were used for compound identification based on the accurate mass, the isotope pattern, and the MS/MS spectra of detected ions.

PCA was performed using MarkerView™ software version 1.2.1.

Results and Discussion

Table 2 shows the pseudo-molecular ions and main mass fragments of the most relevant polyphenolic compounds found in the extracts of the investigated *Vaccinium* species, in positive and negative polarity. All compounds were identified by evaluating the accurate mass of the quasi-molecular ion (mass accuracy less than 3 ppm in both polarities), the isotope pattern and the MS/MS spectrum, as illustrated in the screenshot of MasterView™ software (Figure 2).

Among the most abundant identified compounds, besides anthocyanin hexosides and pentosides, which are well-known predominant metabolites in *V. myrtillus* and *V. corymbosum* berries, the presence of other classes of phenolic compounds was highlighted. More in detail, 3 flavanols ((+)-catechin, (-)-epicatechin and gallocatechin), 3 procyanidin (B1, B2 and A2), 19 flavonol glycosides (derivatives of myricetin, quercetin, laricitrin, isorhamnetin and syringetin), 2 coumaroyl iridoid

Table 2. Polyphenolic compounds identified in extracts of different *Vaccinium* species in positive and negative polarity with retention time (RT) and relevant MS and MS/MS information. The identification of compounds labeled with star (*) was confirmed by the comparison with a pure standard.

			Positive Polarity					Negative Polarity				
Identification	**Formula**	**RT (min)**	**Adduct**	**Extracted Mass (Da)**	**Found at Mass (Da)**	**Dm (ppm)**	**Fragment Ions**	**Adduct**	**Extracted Mass (Da)**	**Found at Mass (Da)**	**Dm (ppm)**	**Fragment Ions**
*Procyanidin B1**	C30H26O12	8.4	[M+H]+	579.1497	579.15006	0.6	127.0389 139.039 275.0540 409.0905	[M-H]-	577.13515	577.13525	0.2	289.0711 407.0766
Gallocatechin	C15H14O7	8.7	[M+H]+	307.08123	307.0814	0.6	139.0391 163.0394	[M-H]-	305.06668	305.06694	0.9	125.0241 137.0240 165.0190 179.0349

Table 2. cont.

*(+) Catechin**	C15H14O6	8.8	[M+H]+	291.08632	291.08658	0.9	139.0392 123.0441 147.0441 161.0598	[M-H]-	289.07176	289.07186	0.4	123.0448 203.0712 245.0811 109.0244
Caffeic acid-hexoside	C15H18O9	10.5	-	-	-	-	-	[M-H]-	341.08781	341.08777	-0.1	135.0450 179.0348
*Chlorogenic acid**	C16H18O9	10.8	[M+H]+	355.10236	355.10249	0.4	163.0391 139.0390 291.0862 409.0912	[M-H]-	353.08781	353.08793	0.4	191.0561
*Procyanidin B2**	C30H26O12	11.9	[M+H]+	579.1497	579.15008	0.7	127.0390 409.0912 291.0862 139.0390	[M-H]-	577.13515	577.13551	0.6	407.0773 289.0714 425.0877 125.0247
*Delphinidin-3-galactoside**	C21H21O12	12.6	[M]+	465.10275	465.10304	0.6	303.0501	[M-2H]-	463.0871	463.08838	2.7	300.0270 301.0348
Ferulic acid-hexoside	C16H20O9	13.7	-	-	-	-	-	[M-H]-	355.10346	355.10316	-0.8	175.0393 160.0162 193.0503
*Delphinidin-3-glucoside**	C21H21O12	13.8	[M]+	465.10275	465.10306	0.7	303.0510	[M-2H]-	463.0871	463.08762	1.1	300.0244 301.0325
*(-) Epicatechin**	C15H14O6	14.0	[M+H]+	291.08631	291.08659	0.9	139.0388 123.0438 147.0440 161.0599	[M-H]-	289.07176	289.07191	0.5	203.0697 245.0809 137.0241 123.0448
*Delphinidin-3-arabinoside**	C20H19O11	14.8	[M]+	435.09219	435.09257	0.9	303.0505	[M-2H]-	433.07654	433.07708	1.2	300.0266 301.0344
*Cyanidin-3-galactoside**	C21H21O11	14.8	[M]+	449.10784	449.10815	0.7	287.0554	[M-2H]-	447.09219	447.09276	1.3	284.0316 285.0392
Chlorogenic acid isomer	C16H18O9	15.3	-	-	-	-	-	[M-H]-	353.08781	353.08791	0.3	191.0562
*Cyanidin-3-glucoside**	C21H21O11	16.3	[M]+	449.10784	449.10825	0.9	287.0555	[M-2H]-	447.09219	447.09293	1.6	284.0325 285.0399
*Petunidin-3-galactoside**	C22H23O12	16.9	[M]+	479.1184	479.11862	0.4	317.0659 302.0422	[M-2H]-	477.10275	477.10371	2.0	314.0422 315.0500 299.0185
*Cyanidin-3-arabinoside**	C20H19O10	17.0	[M]+	419.09727	419.0973	0.1	287.0558	[M-2H]-	417.08162	417.08241	1.9	284.0318 285.0401
*Petunidin-3-glucoside**	C22H23O12	18.1	[M]+	479.1184	479.11877	0.8	317.0657 302.0422	[M-2H]-	477.10275	477.10364	1.9	314.0418 315.0498 299.0190 300.0266
*Peonidin-3-galactoside**	C22H23O11	18.9	[M]+	463.12349	463.12395	1.0	301.0713 286.0476	-	-	-	-	-
*Petunidin-3-arabinoside**	C21H21O11	19.0	[M]+	449.10784	479.10812	0.6	317.0660 302.0421	[M-2H]-	447.09219	447.09299	1.8	314.0420 315.0493 299.0184
Myricetin-hexoside I	C21H20O13	19.7	[M+H]+	481.09767	481.09799	0.7	319.0447	[M-H]-	479.08311	479.08333	0.5	316.0214 317.0283
*Malvidin-3-galactoside**	C23H25O12	20.1	[M]+	493.13405	493.13447	0.8	331.0816	[M-2H]-	491.1184	491.11881	0.8	328.0582 329.0659 313.0341

Table 2. cont.

*Peonidin-3-glucoside**	C22H23O11	20.3	[M]+	463.12349	463.1238	0.7	301.0714 286.0470	[M-2H]-	461.10784	461.10829	1.0	299.0562 298.0477 283.0241 284.0308
Myricetin-hexoside II	C21H20O13	20.4	-	-	-	-	-	[M-H]-	479.08311	479.08358	1.0	316.0216 317.0299
*Procyanidin A2**	C30H24O12	20.9	[M+H]+	577.13405	577.13448	0.7	287.0554 425.0860 437.0874	[M-H]-	575.1195	575.11897	-0.9	289.0706 449.0856 423.0718
*Peonidin-3-arabinoside**	C21H21O10	21.0	[M]+	433.11292	433.11286	-0.1	301.0711 286.0477	-	-	-	-	-
Caffeic acid derivative	C17H20O9	21.3	-	-	-	-	-	[M-H]-	367.10346	367.10311	-0.9	179.0342 135.0446 134.0357
*Malvidin-3-glucoside**	C23H25O12	21.4	[M]+	493.13405	493.13395	-0.2	331.0818	[M-2H]-	491.1184	491.11952	2.3	329.0642 328.0562
*Malvidin-3-arabinoside**	C22H23O11	22.3	[M]+	463.12349	463.12389	0.9	331.0822	[M-2H]-	461.10784	461.10845	1.3	328.0589 329.0664 313.0348
Myricetin-pentoside	C20H18O12	22.6	[M+H]+	451.0871	451.08743	0.7	319.0456	[M-H]-	449.07255	449.07224	-0.7	316.0235 317.0302 271.0251
*Quercetin-3-galactoside**	C21H20O12	23.7	[M+H]+	465.10275	465.10316	0.9	303.0511	[M-H]-	463.0882	463.08841	0.5	300.0271 301.0346
Quercetin-glucuronide	C21H18O13	24.1	[M+H]+	479.08202	479.08213	0.2	303.0516	[M-H]-	477.06746	477.06743	-0.1	301.0352 178.0979 151.0028
Coumaroyl iridoid isomer I	C25H28O13	24.2	[M-H2O+H]+	519.1497	519.15011	0.8	147.0444 175.0392 193.0499 119.0491	[M-H]-	535.14571	535.1458	0.2	163.0398 147.0450 91.0346 119.0501
*Quercetin-3-glucoside**	C21H20O12	24.8	[M+H]+	465.10275	465.10316	0.9	303.0497	[M-H]-	463.0882	463.08828	0.2	300.0267 301.0346
Coumaroyl iridoid isomer II	C25H28O13	25.4	[M-H2O+H]+	519.1497	519.15014	0.8	147.0444 175.0391 193.0499 119.0491	[M-H]-	535.14571	535.14584	0.2	163.0396 147.0443 191.0337 119.0500
Laricitrin-hexoside	C22H22O13	25.8	[M+H]+	495.11332	495.11356	0.5	333.0603 318.0388	[M-H]-	493.09876	493.09867	-0.2	330.0373 331.0445 315.0133
Quercetin-pentoside I	C20H18O11	26.1	[M+H]+	435.09219	435.0926	0.9	303.0509	[M-H]-	433.07764	433.07761	-0.1	300.0267 301.0343
Dicaffeoylquinic acid	C25H24O12	26.1	-	-	-	-	-	[M-H]-	515.1195	515.1210	2.9	353.0867 191.0555 179.0345 173.0452
Malvidin-pentoside	C22H23O11	26.2	[M]+	463.12349	463.12391	0.9	331.0812	-	-	-	-	-
Myricetin	C15H10O8	26.3	-	-	-	-	-	[M-H]-	317.03029	317.03054	0.8	151.0030 137.0235 178.9981 109.0289

Table 2. cont.

Quercetin-pentoside II	C20H18O11	27.4	-	-	-	-	-	[M-H]-	433.07764	433.07875	2.6	301.0343 300.0270 271.0236
Laricitrin-pentoside	C21H20O12	28.6	-	-	-	-	-	[M-H]-	463.0882	463.08736	-1.8	330.0378 315.0140 331.0454
Quercetin-3-rhamnoside*	C21H20O11	28.8	-	-	-	-	-	[M-H]-	447.09329	447.09343	0.3	300.0271 301.0350 271.0238
Quercetin-acetyl-hexoside	C23H22O13	29.2	-	-	-	-	-	[M-H]-	505.09876	505.09845	-0.6	300.0272 301.0352
Isorhamnetin-hexoside	C22H22O12	29.4	[M+H]+	479.1184	479.11869	0.6	317.0662	[M-H]-	477.10385	477.10378	-0.1	314.0435 315.0511 271.0245
Syringetin-hexoside I	C23H24O13	30.6	[M+H]+	509.12897	509.12903	0.1	347.0759	[M-H]-	507.11441	507.11475	0.7	344.0523 345.0590 301.0353
Malvidin-acetyl-hexoside	C25H27O13	30.7	[M]+	535.14462	535.1448	0.3	331.0814	-	-	-	-	-
Syringetin-hexoside II	C23H24O13	31.0	-	-	-	-	-	[M-H]-	507.11441	507.11492	1.0	344.0533 345.0609 301.0343
Isorhamnetin-pentoside	C21H20O11	31.4	-	-	-	-	-	[M-H]-	447.09329	447.09289	-0.9	314.0426 315.0493 271.0238 243.0289
Syringetin pentoside	C22H22O12	31.9	[M+H]+	479.1184	479.11886	0.9	347.0772	[M-H]-	477.10385	477.10327	-1.2	344.0544 301.0347 273.0400
Quercetin	C15H10O7	32.0	-	-	-	-	-	[M-H]-	301.03538	301.03548	0.3	151.0031 178.9982
Cyanidin-coumaroyl-hexoside	C30H27O13	32.1	[M]+	595.14462	595.145	0.6	287.0556	-	-	-	-	-
Peonidin-coumaroyl-hexoside	C31H29O13	32.4	[M]+	609.16027	609.16112	1.4	301.0712	-	-	-	-	-
Coumaric acid derivative	C20H28O9	32.2	-	-	-	-	-	[M-H]-	411.16606	411.16636	0.7	163.0398 145.0292 119.0498
Caffeic acid derivative II	C22H22O10	32.3	-	-	-	-	-	[M-H]-	445.11402	445.11423	0.5	179.0351 135.0449

isomers, the coumaroyl-hexoside of cyanidin and peonidin, 3 caffeoylquinic acids (chlorogenic acids and dicaffeoylquinic acid) and 5 coumaric, caffeic or ferulic acid derivatives were recognized.

Interestingly, these phenolic compounds were present with very different relative abundance in *V. myrtillus*, *V. gaultherioides* and *V. corymbosum* berries, as evidenced the extracted ion chromatograms of the 25 most abundant phenolic compounds found in the three *Vaccinium* species, in positive polarity are shown in Figure 3 and in negative polarity are shown in Figure 4.

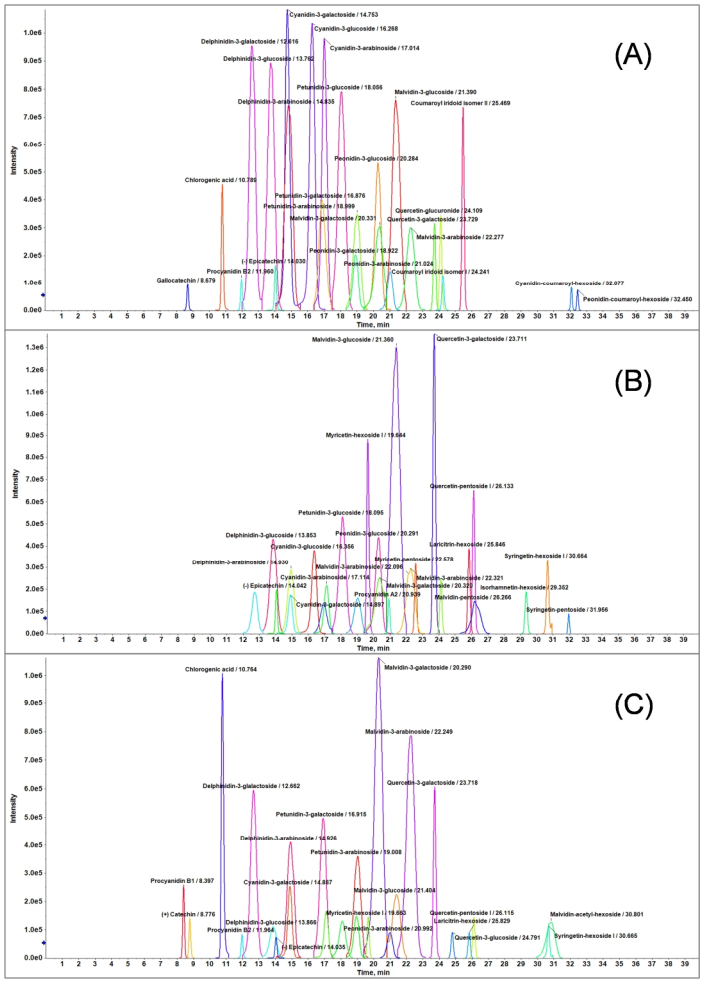

Figure 3. Extracted ion chromatograms (XIC) of the most abundant polyphenolic compounds identified in extracts of *V. myrtillus* (A), *V. gaultherioides* (B) and *V. corymbosum* (C) in positive polarity

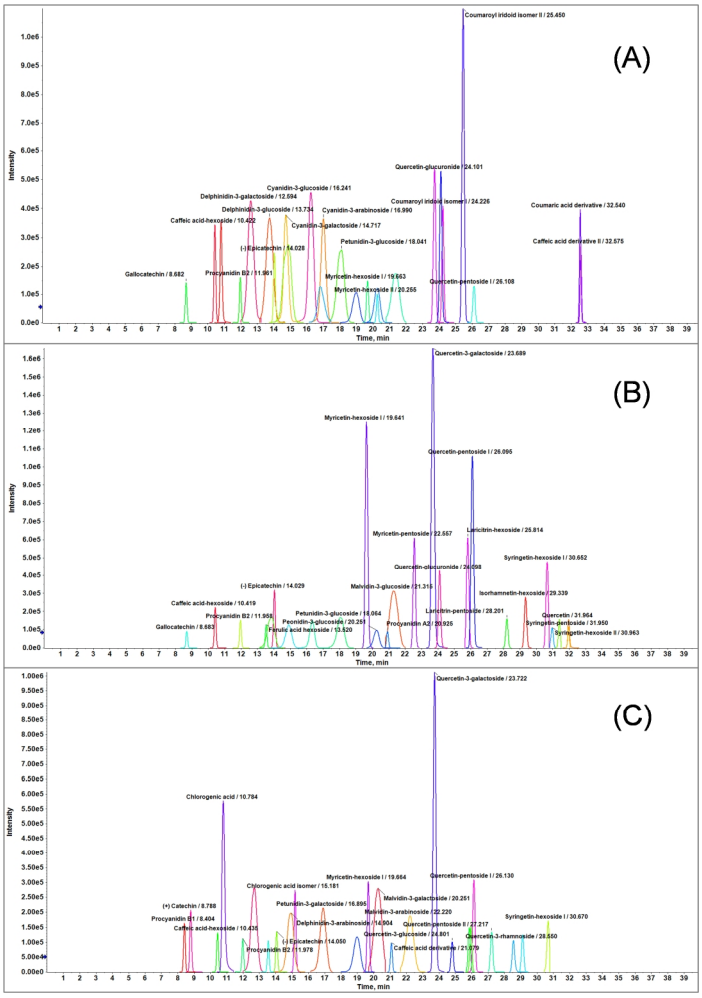

Figure 4. Extracted ion chromatograms (XIC) of the most abundant polyphenolic compounds identified in extracts of *V. myrtillus* (A), *V. gaultherioides* (B) and *V. corymbosum* (C) in negative polarity

The different polyphenolic profiles highlighted for the three *Vaccinium* species were in a very good agreement with the results of PCA, as performed with the whole set of detected ions (Figures 5A-5B and 6A-6B for positive and negative polarity, respectively). In fact, both the PCA score plots evidenced a very good separation of the three *Vaccinium* species giving rise to three well-separated clusters (Figures 5A and 6A).

Furthermore, the PCA performed with the target list of identified marker compounds (Figures 5C-5D and 6C-6D for positive and negative polarity, respectively) produced two significant principal components (PC) accounting for 86.6% and 94.6% of the original variance of data, respectively.

Hence, the selected polyphenols successfully discriminated the three *Vaccinium* species and represent therefore a set of marker compounds useful for the chemotaxonomic discrimination of the investigated *Vaccinium* berries.

It should also be noted that in PCA score plots based on both the compounds ionizing in positive polarity and negative polarity, QC samples (a mix of all three different species) were in proximity of the origin of coordinates, confirming the accuracy and precision of PCA.

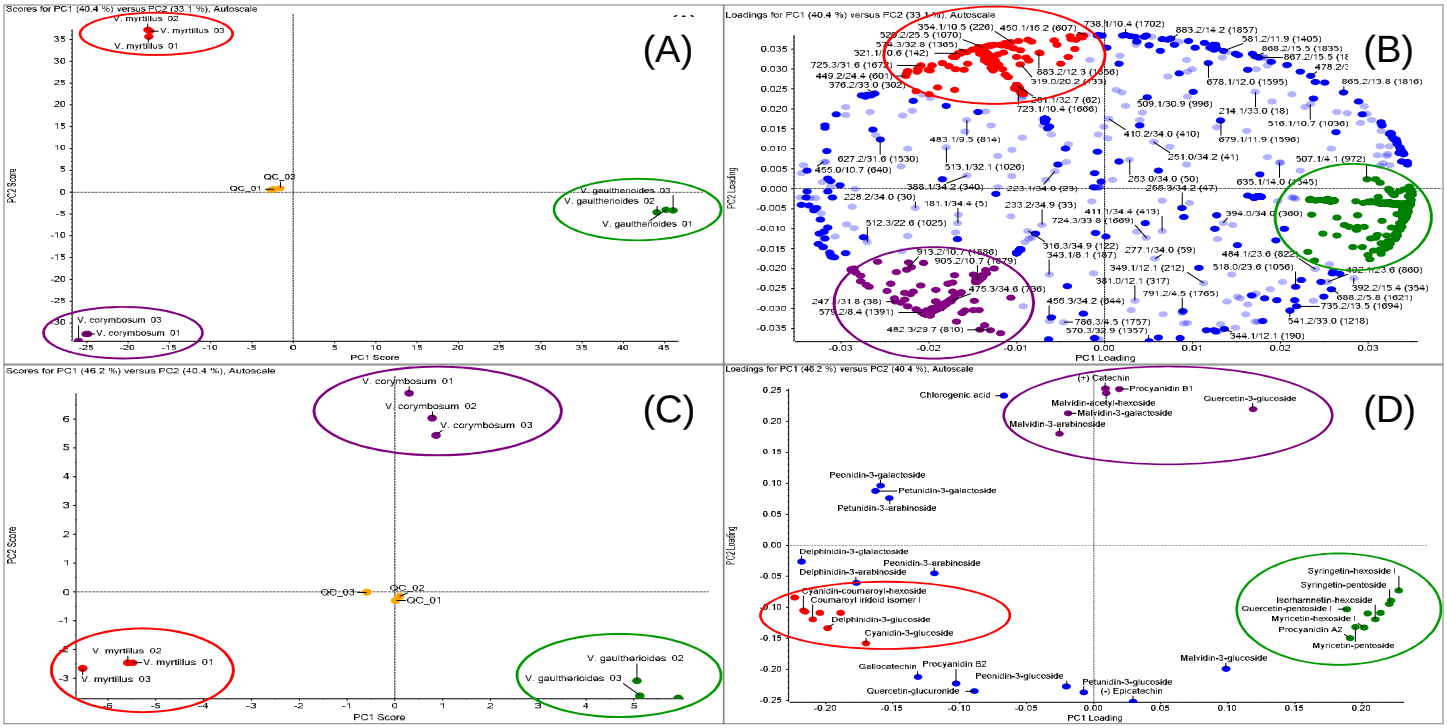

Figure 5. MarkerView™ software scores plot (A and C) and loadings plot (B and D) plots of PCA (PC1 versus PC2) of TOF-MS data acquired in positive polarity. (A) and (B) plots are referred to the PCA performed with the whole set of determined molecular ions, whereas (C) and (D) plots are referred to the PCA performed with the target list of markers identified in MasterView™ software

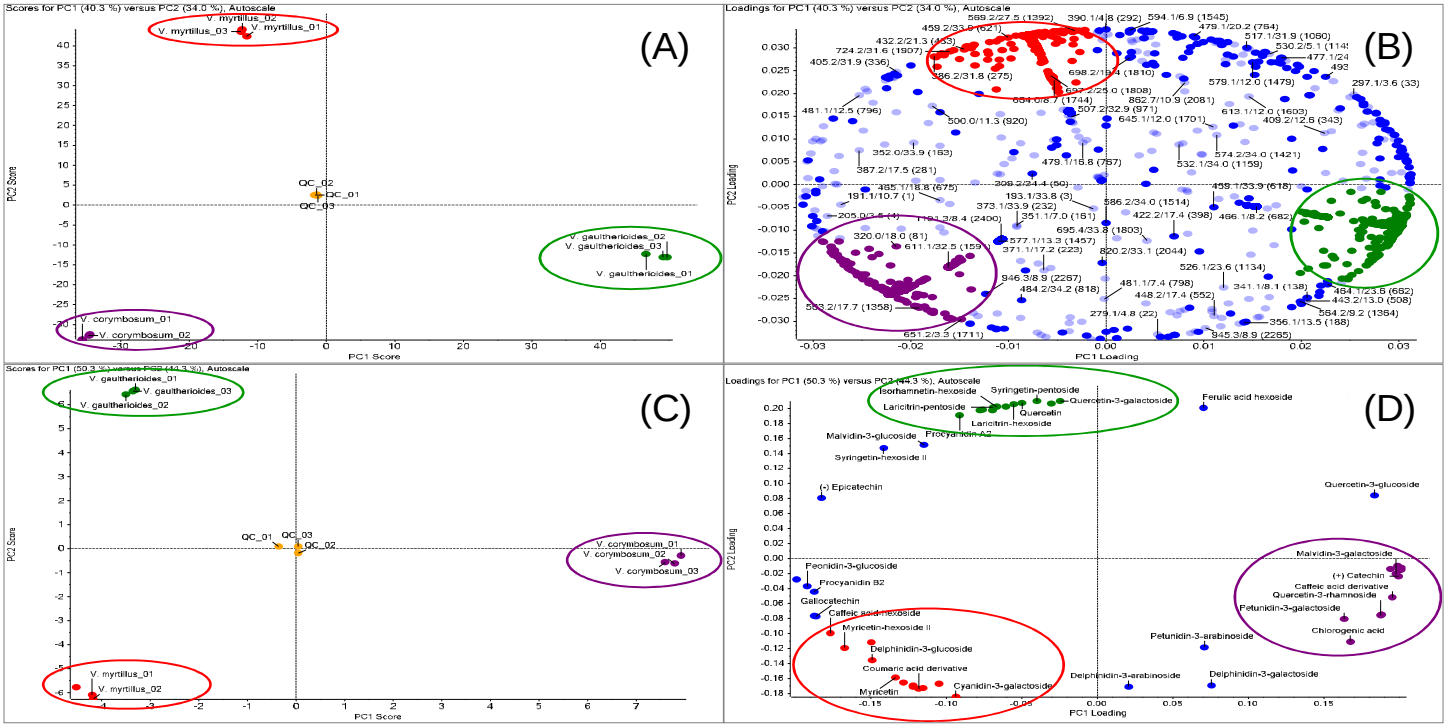

Figure 6. MarkerView™ software scores plot (A and C) and loadings plot (B and D) plots of PCA (PC1 versus PC2) of TOF-MS data acquired in negative polarity. (A) and (B) plots are referred to the PCA performed with the whole set of determined molecular ions, whereas (C) and (D) plots are referred to the PCA performed with the target list of markers identified in MasterView™ software

Summary

In this study, the use of two different LC-HR-MS and MS/MS methods has been successfully applied to the identification of the most abundant phenolic compounds in *V. myrtillus*, *V. gaultherioides*, and *V. corymbosum* by using the accurate mass of quasi-molecular ions, the isotope pattern and the MS/MS spectra.

Important information regarding the characteristic marker compounds of *Vaccinium* berry species were therefore obtained, particularly for *V. gaultherioides* fruits, which have not been previously investigated before.

The PCA analysis allowed for obtaining the clear separation of the samples belonging to the three *Vaccinium* species on the PCA scores plot, thus highlighting that selected phenolic compounds are suitable for the chemotaxonomic discrimination of the investigated species.

Acknowledgements

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