agronomy

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Chemical Profiling and Antioxidant Potential of Berries from Six Blueberry Genotypes Harvested in the Italian Alps in 2020: A Comparative Biochemical Pilot Study

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Abstract: This pilot study investigates the chemical profiling and antioxidant potential of six blueberry genotypes: three tetraploids from the highbush blueberry species and three hexaploids from the rabbiteye blueberry species. The goal was to characterise the biochemical composition of these genotypes, grown under identical pedoclimatic conditions, and to evaluate the variation in bioactive compounds associated with antioxidant activity. Metabolomic and ionomic analyses were employed to identify and relatively quantify these compounds. Multivariate analyses clustered the genotypes based on phytochemical profiles, pinpointing key genotype-clustering metabolites. Significant variations among genotypes were observed. Ochlockonee and Overtime had the highest anthocyanins, phenols, ascorbates, and antioxidant activity levels. Over thirty metabolites (organic acids, sugars, and flavonoid glycosides) drove the genotype clustering. Although Last Call and Legacy had more metabolites, their antioxidant properties were lower, suggesting that phenolic quality influences antioxidant activity more than quantity. Overtime had the highest phenolic content, followed by Last Call in anthocyanins. Liberty and Legacy showed the lowest anthocyanin and ascorbate levels, while Overtime and Ochlockonee were characterised by an increase in ascorbate content. Rabbiteye blueberries showed higher antioxidant potential than highbush, indicating that antioxidant efficacy depends more on the composition and quality of the bioactive compounds than on their total concentration.

Keywords: *Vaccinium* species; antioxidant properties; bioactive compounds; genotype variations; ionomic profiling; metabolomic analysis; phenolic content

Academic Editor: Kirsten Brandt

Received: 10 December 2024
Revised: 13 January 2025
Accepted: 20 January 2025
Published: 22 January 2025

Citation: Araniti, F.; Baron, G.; Ferrario, G.; Pesenti, M.; Della Vedova, L.; Prinsi, B.; Sacchi, G.A.; Aldini, G.; Espen, L. Chemical Profiling and Antioxidant Potential of Berries from Six Blueberry Genotypes Harvested in the Italian Alps in 2020: A Comparative Biochemical Pilot Study. *Agronomy* **2025**, *15*, 262. https://doi.org/10.3390/agronomy15020262

1. Introduction

The interest in the health benefits of small fruits, particularly those rich in antioxidants, has been steadily increasing. Small fruits such as berries are known for their rich content of bioactive compounds, including phenolics, anthocyanins, and ascorbic acid [1–6]. These compounds are widely studied for their biochemical properties, which contribute to the functional characteristics of small fruits and their derived products [6,7].

Among the commercially important small fruit species are those belonging to the *Vaccinium* genus, including *Vaccinium corymbosum* L. (northern highbush blueberry) and *Vaccinium virgatum* (Aiton, rabbiteye blueberry) [8]. These species are characterised by

different ploidy, which is crucial for understanding the genetic variations and potential breeding strategies within these species. Ploidy levels, which refer to the number of sets of chromosomes in a cell, play a significant role in determining the characteristics and traits of plants. *V. corymbosum* is a tetraploid species [9,10], whereas *V. virgatum* is hexaploid [10,11]. These species have been studied for their antioxidant capacity, which is influenced by factors such as total phenolic and anthocyanin contents, maturity, and variety [12]. *V. corymbosum* is a cultivated species rich in phenolic compounds, making it a potential source of antioxidants [13]. On the other hand, *V. virgatum* has been found to contain compounds, like resveratrol, which are associated with its biochemical properties [14]. Additionally, studies have explored *V. virgatum* for its potential antioxidant, anti-inflammatory, and neuroprotective characteristics [15].

Highbush blueberries are an important fruit crop cultivated in Southern Chile, Peru, and the United States [16]. *V. virgatum* is extensively cultivated in the southeastern United States coastal flatwoods region, which serves as the primary production area for rabbiteye blueberries, and in Brazil in regions with mild winter climates [11,17,18]. In recent years, the cultivation of these species has increased significantly alongside the rise in global consumption of their fruits. This trend is particularly notable in Europe, where the demand for blueberries has surged, as evidenced by the considerable increase in blueberry production in Poland [19].

Similar to other small fruits, blueberries have gained attention for their rich content of bioactive compounds, particularly anthocyanins, which contribute to their functional and biochemical properties [20–24]. These compounds have been studied for their potential roles in various physiological processes, including cardiovascular function and cellular protection [25–27]. Furthermore, blueberries are investigated for their effects on metabolic processes, including endothelial function and fat metabolism [28–31]. The antioxidant and anti-inflammatory activities of blueberries are largely attributed to their polyphenolic compounds, particularly anthocyanins [29,30,32]. Additionally, studies suggest that blueberries may influence oxidative stress, inflammation, endothelial function, cholesterol metabolism, and gut microbiota, which are key areas of ongoing research [33–35].

The significance of blueberries in Europe is also reflected in several studies regarding cultivation and adaptation to environmental conditions. Studies have investigated the adaptation of blueberry species to the environmental conditions of northern Spain, particularly in their flowering and ripening seasons, to identify the types best suited for this geographical area [36]. Moreover, the potential of the European wild blueberry (*Vaccinium myrtillus*, L.) for cultivation and industrial exploitation in Norway underscores the agricultural and commercial value of blueberries in Europe [37]. According to this trend, the area of Italy devoted to the cultivation of blueberries has increased constantly, from 1042 to 1390 ha from 2018 to 2021, respectively [38].

This work aimed to comprehensively investigate the chemical composition and antioxidant properties of various blueberry varieties. This included three tetraploid varieties of highbush blueberry (*Vaccinium corymbosum* L., i.e., Last Call, Legacy, and Liberty) and three hexaploid varieties of rabbiteye blueberry (*Vaccinium virgatum*, Aiton, i.e., Ochlockonee, Overtime, and Titan). The study focused on identifying and profiling the bioactive compounds potentially responsible for their antioxidant capacities. Importantly, the different blueberry varieties were cropped in the same area. This is crucial for several reasons. First, it allows us to understand how genetic differences influence the berries' chemical composition and antioxidant properties when grown under identical environmental conditions. In addition, this research can guide farmers from the interested area, or from areas with similar pedoclimatic characteristics, in selecting the most nutritionally beneficial blueberry varieties, ultimately enhancing the crops' health benefits and market value.

2. Materials and Methods

2.1. Plant Materials

The fruits of the six blueberry commercial genotypes Last Call, Legacy, Liberty (*V. corymbosum* L.) and Ochlockonee, Overtime, and Titan (*V. virgatum*, Aiton) were collected in the Alpes Agia farm, Albosaggia (550 m above sea level), Sondrio (Italy), in the summer of 2020.

Ochlockonee and Titan™ are two protected rabbiteye blueberry cultivars developed by Dr. D. Scott NeSmith at the University of Georgia. Ochlockonee, released in 2002 by the University of Georgia College of Agricultural and Environmental Sciences and the U.S. Department of Agriculture's Agricultural Research Service, is a late-season variety with propagation rights controlled by the University of Georgia Research Foundation. Titan™, introduced in 2010, is the result of crossing T-460 and FL 80-11. Its propagation and sale are restricted to individuals licensed by the University of Georgia Research Foundation (UGARF—University of Georgia, Athens, GA, USA) and Georgia Seed Development (GSD) (2420 S. Milledge Ave Athens, GA, USA). A U.S. plant patent application has also been filed for Titan™.

The variety Overtime is a rabbiteye blueberry intended for the hand-harvest fresh market; Overtime is a patented variety owned by Fall Creek Farm and Nursery, Inc. (39318 Jasper Lowell Rd, Lowell, OR, USA). It ripens about one week earlier than Ochlockonee and has comparable yields.

The variety Last Call is a very late-season Northern Highbush blueberry that is exclusive to Fall Creek® Genetics.

Liberty is a late-season blueberry licensed by Fall Creek®, whereas Legacy is a public variety.

All the genotypes were cropped with a planting layout of 3 m by 1.10 m and grown using the same fertilisation and irrigation regime. The fields, each one cultivated with one different genotype, were divided into three plots, and from each plot, one biological replicate consisting of 500 g of fresh fruits was collected. For each replicate, approximately 500 g of fully ripe fruits were frozen during the collection in liquid nitrogen, and then powdered in laboratory using liquid nitrogen and the high-speed blender Blixer 4 (Robot-Coupe, Montceau-en-Bourgogne, France). Samples were collected at complete maturation, which was evaluated in the field using a portable refractometer to measure the sugar content (°Brix) in fruit juice. After collection, the samples were stored at −80 °C until use. Aliquots of each biological replicate (N = 3) were used for the following analyses.

2.2. Determination of Total Anthocyanins, Polyphenol, and Ascorbate Contents

The total anthocyanin and total polyphenol contents were determined as described by Baron et al. [39] and Giusti and Wrolstad [40].

The total anthocyanin content was assessed using UV-Vis spectroscopy following the procedure outlined by Giusti and Wrolstad [40]. Quantification was conducted in triplicate for extracts obtained from the powders of the different genotypes. Stock solutions (40 mg/mL) were prepared and diluted using a pH 1 buffer (0.025 M potassium chloride adjusted to pH 1 with 12 M HCl) and a pH 4.5 buffer (0.4 M sodium acetate adjusted to pH 4.5 with 12 M HCl) to reach a final concentration of 0.5 mg/mL. Each sample was analysed in technical triplicate. Absorbance measurements were taken at 520 nm (peak absorption wavelength) and 700 nm using a Shimadzu UV 1900 spectrophotometer (Shimadzu, Milan, Italy).

The anthocyanin concentration in the stock solution was determined in mg/L using the formula reported by Giusti and Wrolstad [40].

The total phenolic content was determined using an adapted version of the Folin–Ciocalteu colorimetric assay. Extracts were prepared at a concentration of 100 µg/mL to ensure absorbance readings fell within the standard curve's linear range (0.0–100.0 µg/mL of gallic acid). To each 200 µL extract sample, 100 µL of the Folin–Ciocalteu reagent (FCR) and 1 mL of distilled water were added. After a 5 min incubation, 700 µL of a 7% sodium carbonate solution was introduced. The samples were left at room temperature for 90 min before measuring the absorbance at 760 nm using a plate reader (BioTek's PowerWave HT, Winooski, VT, USA) in a 96-well plate. Both total anthocyanin and phenolic contents were expressed as mg/100 g of frozen fruit.

For the determination of the contents of reduced, oxidised, and total ascorbate, 0.5 g of sample powder was extracted by 2.5 mL of 6% (*w/v*) trichloroacetic acid (TCA); after that, the homogenate was centrifuged at 12,000× *g* for 15 min at 4 °C. The supernatant was then purified through a Solid Phase Extraction using a silica gel Superclean™ LC-4 cartridge (Merk Life Science S.r.l. Via Monte Rosa, 93, Milan, Italy) that permitted the removal of anthocyanins and other interfering compounds. The column was conditioned with 1 mL of methanol and 6 mL of 6% (*w/v*) TCA; after that, 1 mL of the sample was eluted, adding 2 mL of 6% (*w/v*) TCA, recovering 3 mL of the purified sample at the end. The determination of reduced, oxidised, and total ascorbate was then performed as previously described by Gillespie and Ainsworth [40,41].

2.3. ICP-MS Analysis

The ion contents were determined as described by Álvarez-Rodríguez et al. [42]. Briefly, to 200 mg of dried fruit sample was added 10 mL of 65% (*v/v*) HNO_3; after that, it was digested by a microwave digestion system (MULTIWAVE-ECO, Anton Paar Italia Sfl., Rivoli, Italy). The digestion process involved two sequential power ramping steps: Step 1 involved heating to 500 W over 10 min and holding for 5 min, while Step 2 increased the power to 1200 W over 10 min and maintained it for 15 min. After cooling for 20 min, the digested samples were transferred to polypropylene tubes and diluted 1:20 with Milli-Q water. Elemental concentrations (Na, Mg, Al, K, Ca, Cr, Mn, Fe, Zn, Se, Cd, and P) were determined using inductively coupled plasma–mass spectrometry (ICP-MS; Bruker AURORA M90 ICP-MS, Bruker Daltonik GmbH, Leipzig, Germany). A 2 mg/L internal standard solution containing isotopes (^{72}Ge, ^{89}Y, and ^{159}Tb) was added to the samples and both multi-element and single-element calibration standards (for As and P) to achieve a final concentration of 20 µg/L. A collision-reaction interface (CRI) with a hydrogen flow rate of 70 mL/min was employed to mitigate potential polyatomic interferences.

2.4. Radical Scavenging Activity

Radical scavenging activity was tested by DPPH assay [43]. For each sample, an aliquot of 100 µL was mixed with 1 mL of acetate buffer (100 mM, pH 5.5) and 500 µL of DPPH solution (500 µM in ethanol), and the mixtures were maintained in the dark for 90 min before reading the absorbance at 517 nm with a UV reader Shimadzu™ UV 1900 (Shimadzu, Milan, Italy). Results were expressed as IC_{50}, and the inhibition percentage was calculated as follows:

$$\% \text{ I} = (\text{Abs (blank)} - \text{Abs (sample)})/(\text{Abs (blank)}) \times 100$$

2.5. Anti-Inflammatory Activity

The in vitro anti-inflammatory activity was tested against TNFα on R3/1-Nf-κB cell model as described by Della Vedova et al. [44]. In summary, R3/1-Nf-κB cells were plated at a density of 5000 cells per well in a white 96-well plate (BRANDplates®, cell grade, Otto-Schott- Strasse, 25, Wertheim, Germany). The cells were pre-incubated for

18 h in complete medium (DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% Penicillin/Streptomycin) with varying concentrations of the extracts (ranging from 1 to 250 μg/mL). Afterward, the cells were stimulated for 6 h with 10 ng/mL TNFα. To prevent interference from assay components during luciferase activity measurements, the cells were washed once with 100 μL of warm PBS, followed by the addition of 100 μL of DMEM. Next, 100 μL of ONE-Glo™ Luciferase Assay Substrate (Promega Corporation, Madison, WI, USA) was directly added to each well. Luciferase activity was then measured using a luminometer (Wallac Victor2 1420, Perkin-Elmer™ Life Science, Monza, Italy).

The cell viability for all the concentrations tested in the anti-inflammatory assay was verified by MTT assay on R3/1-Nf-κB cells, and the test was performed successively for all the genotype extracts at the same concentration. The results were expressed as a percentage reduction in inflammation in relation to the inflamed condition.

2.6. LC-MS Analyses

2.6.1. Sample Preparation

The frozen powder was weighed, and the extraction phase CH_3OH—0.1% HCOOH (80/20, %*v*/*v*) was added in the proportion 500 mg: 2.5 mL. The suspension was mixed and then placed in an ultrasonic bath for 30 min to extract polyphenols. The extraction was repeated twice, and the total volume was then filtered on 0.45 μm filters and brought to dryness under vacuum for 12 h. The dry residue was taken up in ethanol, vortexed, and again brought to dryness to remove residual water. The procedure was repeated until the weight stability of the residue was reached. The extracts were finally solubilised in EtOH: H_2O (70/30, %*v*/*v*).

2.6.2. LC-MS Conditions for Anthocyanins Analysis

Stock solutions were diluted 1:20 in mobile phase A (H_2O/HCOOH, 100/2 %*v*/*v*) and added with naringenin-7-O-glucoside as internal standard (IS) at a final 0.01 mg/mL concentration. Each sample was analysed in triplicate using the method described by Baron et al. [45], with mild modifications. An aliquot of 20 μL was injected into an RP Zorbax SB-C18 (150 mm, i.d. 2.1 mm, particle size 3.5 μm, CPS analytical, Milan, Italy) protected by an Agilent Zorbax precolumn and maintained at 50 °C by an HPLC UltiMate 3000 (Dionex, Thermo Fisher Scientific, Waltham, MA, USA) equipped with an autosampler and maintained at 8 °C. The chromatographic separation was obtained using a multistep gradient of mobile phase A (H_2O/HCOOH 100/2, %*v*/*v*) and mobile phase B (CH_3CN/CH_3OH/HCOOH 50/50/2, %*v*/*v*) as previously described. The qualitative profile of anthocyanins contained in the genotypes was obtained with an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with an ESI source set to acquire in positive ion mode with the following parameters: capillary temperature of 300 °C, capillary voltage of 30 V, sheath gas 45 a.u. (arbitrary unit), auxiliary gas 10 a.u., spray voltage of 4.5 kV, tube lens offset of 110 V. The instrument worked in data-dependent acquisition mode, acquiring both full MS and MS/MS spectra. The FT analyser acquired full MS spectra in a scan range of m/z 100–1500, using an AGC target of 5×10^4 and a resolution of 30,000 (FWHM at m/z 400). MS/MS spectra of the three most intense ions (counts > 1×10^4) of the full MS scan were registered with the linear ion trap (LTQ) and with the following parameters: centroid mode, AGC target of 5×10^5, collision energy 40 eV, precursor isolation window of m/z 3. Moreover, dynamic exclusion was used to avoid redundant spectra acquisition: maximum 2 repeat counts, 20 s of repeat duration, and 30 s of exclusion duration. Compound identification was carried out by using the exact mass (mass tolerance 5 ppm) and fragmentation pattern of known anthocyanins present in *Vaccinium corymbosum* L. Quantitative analysis was performed with the LTQ

analyser working in full MS scan mode (scan range of m/z 100–1500) with the same ESI parameters mentioned above. The ion current relative to the ion (m/z) of each anthocyanin was extracted, and the AUC (area under the curve) of the relative peak was calculated using the Genesis algorithm. The equations of the straight lines were obtained by linear regression using the least squares method: on the x-axis, the nominal concentrations, and on the y-axis, the AUC ratio. The galactoside and arabinoside derivatives were quantified through the calibration curve of the relative glucoside, while petunidin-derivatives content was assessed using the cyanidin-3-O-glucoside equation. Results were expressed as mg anthocyanin/100 g of frozen fruit. The software programs used for instrument control and data analysis were Chromeleon Xpress 6.80 and Xcalibur 4.0.

2.6.3. LC-MS Conditions for Polyphenols Analysis

For the polyphenolic profile analysis, the stock solutions were diluted 1:20 in H_2O/HCOOH, 100/0.1 %*v*/*v* (mobile phase A) and added with Trolox as IS at a final concentration of 50 µM. Each sample (20 µL) was analysed in triplicate by LC-HRMS as described by Baron et al. [39]. A targeted data analysis was performed after building a database containing compounds (N = 95) found in the literature in *Vaccinium* spp. [46–56]: the identification was performed using the exact mass (mass tolerance of 5 ppm) and isotopic and fragmentation patterns.

An untargeted data analysis was also carried out for the most intense peak not identified with the targeted analysis. Two approaches were used for the annotation based on the accurate mass, the isotopic pattern, and the MS/MS spectrum. In the first approach, possible diagnostic loss of neutral fragments such as sugars (such as −162 for galactoside or −146 for rhamnoside) was evaluated, and the remaining ion, usually corresponding to the aglycone, was calculated to see if it belonged to a particular compound. Next, the hypothetical structure was drawn, and the correspondence of the calculated m/z with that of the experimental one was evaluated and accepted when a tolerance of 5 ppm was found. The second approach was initially focused on calculating the molecular formula performed by Xcalibur 4.0 with the following settings: mass tolerance 5 ppm; C, H, O, N, P, S as elements in use; charge −1. The top 10 formulae were searched in databases such as MassBank and PubChem to achieve a list of candidates. The MS/MS spectrum of the candidates was searched in MS/MS databases (e.g., HMDB) and compared with the experimental one. If no spectrum was found in databases, CFM-ID Peak Assignment tool (version 3.0) was used to compare the in silico-generated spectrum of the candidate with the experimental one.

2.7. GC-MS-Driven Untargeted Metabolomic Analysis

2.7.1. Samples Extraction and Derivatisation

Fruit samples were gathered during the midpoint of the light period and promptly flash-frozen in liquid nitrogen to halt endogenous metabolic processes. Each biological sample, consisting of berries from different genotypes and their replicates, was homogenised to obtain fresh plant material (100 mg), which was then placed in 2 mL microcentrifuge round bottom vials. For extraction, 1400 µL of methanol (at −20 °C) was added, and after introducing 60 µL of ribitol (0.2 mg/mL stock in ddH_2O) as an internal standard for the polar phase, the mixture was vortexed for 10 s. The samples were then transferred to a thermomixer set at 70 °C, shaken for 10 min (950 rpm), and centrifuged for 10 min at 11,000× *g*. The resulting supernatants were collected and transferred to glass vials. To these, 750 µL $CHCl_3$ (−20 °C) and 1500 µL ddH_2O (4 °C) were sequentially added. After vortexing for 10 s, the samples were centrifuged for an additional 15 min at 2200× *g*. Each

replicate's upper polar phase (150 µL) was collected, transferred to a 1.5 mL tube, and dried in a vacuum concentrator without heating.

Before freezing and storing at −80 °C, the tubes were filled with argon and placed in a plastic bag with silica beads to prevent moisture and hydration during short-term storage. Before derivatisation, the stored samples underwent a 30 min treatment in a vacuum concentrator to remove any trace of humidity. Subsequently, 40 µL of methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the dried samples, which were then incubated for 2 h in a Thermomixer (950 rpm) at 37 °C.

Following methoxyamination, silylation was carried out by adding 70 µL of MSTFA to the aliquots with further shaking for 30 min at 37 °C. The derivatised samples (110 µL) were then transferred into glass vials suitable for the GC/MS autosampler for the subsequent analysis.

2.7.2. GC/MS Analysis

The derivatised samples underwent injection into a MEGA-5MS capillary column (30 m × 0.25 mm × 0.25 µm equipped with 10 m of precolumn) using a gas chromatograph apparatus (Agilent 7890A GC, Agilent Technologies, Santa Clara, CA, USA) equipped with a single quadrupole mass spectrometer (Agilent 5975C, Agilent Technologies, Santa Clara, CA, USA). The injector and source were established at 250 °C and 260 °C, respectively.

A precise method was employed for sample injection, with a one-microliter volume introduced in splitless mode utilizing a helium flow of 1 mL/min. The temperature programming involved an initial isothermal period of 5 min at 70 °C, followed by a ramp of 5 °C per minute up to 350 °C, and a final heating phase of 5 min at 330 °C.

Mass spectra were captured in electronic impact (EI) mode at 70 eV, conducting scans across the 40–600 m/z range with a scan time of 0.2 s and a solvent delay of 9 min. To ensure the reliability of the instrumental performance, tentative identification, and monitoring of shifts in retention indices (RI), pooled samples designated as quality controls (QCs), n-alkane standards, and blank solvents (pyridine) were systematically injected at scheduled intervals.

2.7.3. MS-DIAL Data Analysis

Data processing was carried out using MS-DIAL ver. 2.90, which utilises an open-source EI spectra library. The process included raw peak extraction, baseline filtering, baseline calibration, peak alignment, deconvolution, peak identification, and integration of peak heights, following the methodology outlined in reference [57]. Peak detection employed an average peak width of 20 scans and a minimum peak height of 1000 amplitudes. Deconvolution was implemented with a sigma window value of 0.5 and an EI spectra cut-off of 5000 amplitudes.

For peak identification, a retention time tolerance of 0.2 min, an m/z tolerance of 0.5 Da, an EI similarity cut-off of 60%, and an identification score cut-off of 80% were applied. During the alignment parameters setup, a retention time tolerance of 0.5 min and a retention time factor of 0.5 were utilised.

To enhance the accuracy of metabolite annotation, publicly available spectral libraries were employed, including the NIST Mass Spectral Reference Library (NIST14/2014, 100 Bureau Drive, Gaithersburg, MD, USA) and the MSRI spectral libraries from Golm Metabolome Database, MassBank, and MoNA. These libraries were essential for compound identification, comparing mass spectral patterns, and referencing EI spectral data from sources such as the National Institute of Standards and Technology (100 Bureau Drive, Gaithersburg, MD, USA) and the Max Planck Institute for Plant Physiology (Am Mühlenberg 1, Potsdam, Germany) [58,59].

Following the Metabolomics Standards Initiative (MSI) guidelines for metabolite identification [60], a two-tiered approach was adopted. Level 2 identification was used based on spectral database matches with a factor exceeding 80%. Additionally, Level 3 annotation was applied, where only compound groups were known, such as specific ions and retention time regions of metabolites. This comprehensive methodology ensured rigorous and standardised metabolite identification and annotation.

2.8. Statistical Analysis

All the experiments were carried out using a completely randomised design with three replications. Data were analysed through multivariate and univariate analysis using the open-source software Metaboanalyst 6.0. Before the statistical analysis, the GC-MS metabolomic data were first normalised by reference feature (ribitol). Then, all the data from the different analyses were evaluated for missing values (replaced by 1/5 of the minimum positive value of each variable) and then combined, log-transformed (Ln10), and Pareto scaled. Data were previously analysed through one-way ANOVA using the LSD (least significant difference) test as post hoc ($p \leq 0.05$). A clustered heatmap further represented the significant features resulting from the analyses. The cluster analysis used the Spearman distance and Ward's algorithm to cluster the groups.

Data were further classified through multivariate unsupervised Principal Component Analysis (PCA) by virtue of the first two components (PC1 vs. PC2). The output comprised the score plots to visualise the contrast between the different blueberry genotypes and loading plots to explain the cluster separation. Partial least squares discriminant analysis (PLS-DA) was further used to highlight differences among the different blueberry genotypes and identify the metabolites/parameters mainly involved in group separation and their changes in concentration. The PLS-DA model was previously validated through cross-validation (CV), using the 5-fold CV method and the Q2 as the performance measure, and successively through a permutation test using the separation distance as a statistic test and a permutation number equal to 20. The model was considered valid if the empirical p value was $\leq$0.05.

3. Results and Discussion

3.1. Phytochemical and Nutraceutical Profiles of Blueberry Genotypes

Different blueberry genotypes grown in the same pedoclimatic conditions exhibit distinct phytochemical and nutraceutical profiles, primarily due to genetic variation [60]. These differences influence the concentration and composition of bioactive compounds like anthocyanins, other flavonoids, and phenolic acids, which are key to the berries' antioxidant capacity and health benefits [20,61,62]. Consequently, blueberries' nutritional value and health-promoting properties can vary significantly among genotypes.

This study investigated six blueberry genotypes belonging to two different species (highbush and rabbiteye blueberry) using a comprehensive metabolomic and ionomic approach; moreover, the blueberry extracts' antioxidant and anti-inflammatory activities were also evaluated. All the experiments were carried out using different quantification approaches. For some of them, an absolute quantification was achieved, whereas for others, a relative quantification approach was used (Supplementary Table S1). Then, to harmonise the data and to allow a comprehensive comparison among varieties, all data were subjected to statistical normalisation, transformation, and scaling (Figures 1 and 2 and Supplementary Table S1). Successively, data were analysed through univariate and multivariate methods to identify which genotypes of the two species were characterised by the higher nutraceutical profiles when cropped in identical conditions. The dataset, analysed through a cluster analysis (Figure 1a), revealed two main clusters reflecting the genotypic differences between

the two species. Ochlockonee, Overtime, and Titan genotypes formed one major cluster. In contrast, Legacy, Last Call, and Liberty genotypes formed another (Figure 1a).

3.2. Multivariate Analysis and Genotypic Clustering

To confirm the clustering and to investigate the main parameters involved in cluster formation, the data were further analysed through the multivariate unsupervised Principal Component Analysis, which was built by virtue of the first two PCs (PC1 vs. PC2) that explained 69.9% of the total variability (61.3% PC1 and 8.6% PC2) (Figure 1b). The PCA scores plot effectively distinguished between the six blueberry genotypes based on their profiles, with each genotype forming a distinct and tightly grouped cluster. The plot indicated that the profiles of these genotypes are significantly different, allowing for clear differentiation in a two-dimensional space (Figure 1b). In addition to the clear separation, the PCA confirmed the presence of the two major groups previously reported (Figure 1a,b).

After data exploration and dimensionality reduction through the PCA, without any prior assumptions, data were further analysed through PLS-DA to specifically focus on and enhance class separation using the class labels [63]. This sequential approach allowed for a comprehensive analysis that maximised data exploration and class discrimination (Figure 1c). After validation (Supplementary Table S1—reported in the sheet plsda_vip), the model highlighted a clear separation among all the genotypes. Moreover, through the PLS-DA analysis, it was possible to identify the VIP scores, indicating which specific metabolites are most important in distinguishing between the genotypes (Figure 1d). A common threshold for significance is a VIP score greater than 1.0 [64]. All the metabolites shown in Figure 1d exceeded this threshold, indicating that they are all important for the model. In particular, the data highlighted that more than thirty metabolites were characterised by a VIP score > 1.4. Those compounds were mainly represented by organic acids and sugars (Figure 1d). In particular, the genotypes Last Call, Legacy, and, to a lesser extent, Liberty (highbush blueberries) were characterised by the higher amounts of these compounds. On the contrary, the rabbiteye genotypes were characterised by lower amounts (Figure 1d).

It should be noted that none of the features resulting from the PLS-DA analysis with a high VIP score were ions, suggesting that the ionomic profile of the six genotypes did not contribute to the highbush and rabbiteye discrimination, probably due to the identical growth environmental conditions. Indeed, previous studies have shown that the mineral composition of blueberry fruits depends on many factors, such as the growth substrate composition and pedoclimatic conditions [19,65–67]. Moreover, differences in mineral composition have also been related to the changes in plant physiological parameters and the content of important blueberry fruit components such as anthocyanins [65,67]. Nevertheless, as highlighted by our analyses, different plant genotypes can exhibit similar ionomic profiles in fruits when grown under the same pedoclimatic conditions. Although further work is required to investigate the impact of pedoclimatic conditions on mineral nutrient composition, it is interesting to observe that the differences in metabolic composition among the genotypes in the present study could be more closely attributed to peculiar genetic traits than to environmental factors.

3.3. Key Metabolites Driving Genotype Distinction

The metabolites identified in the VIP score are integral to the blueberries' quality, impacting their taste, preservation, and health benefits [68]. Key metabolites such as citric acid, isorhamnetin-3-O-rutinoside, and 2-hydroxycinnamic acid play significant roles in these areas [69,70].

Citric acid is essential for the fruit's taste profile and is critical to its metabolic processes and preservation. It enhances the flavour and plays a central role in the antioxidant system, contributing to its overall quality [71]. Notably, citric acid is the most influential metabolite in distinguishing between the blueberry genotypes in this work (Figure 1d). Citric acid is the most abundant organic acid in blueberry fruits, representing 77 to 87% of the total acids, depending on fruit maturity [72,73]. Its content generally decreases during fruit ripening, whereas quinic acid content, another organic acid with a high VIP value (Figure 1d), generally increases [73]. Quinic acid contributes to blueberries' taste and antioxidant capacity. Moreover, citric acid and quinic acid positively correlate with sweet and strawberry aromas, representing a qualitative trait [72,74].

3.4. Analysis of Antioxidant Activity

Among the compounds with high VIP values were isorhamnetin-3-O-rutinoside and 2-hydroxycinnamic acid, both of which are putatively involved in the defence against oxidative stress, enhancing the berries' nutraceutical value. These metabolites are critical for differentiating the genotypes, with notable variations in their concentrations [75,76], as confirmed by our analyses (Figure 1d). Tartaric acid is another organic acid affecting blueberries' taste, and, with other organic acids, it helps to stabilise the fruit, ensuring a longer shelf life [77].

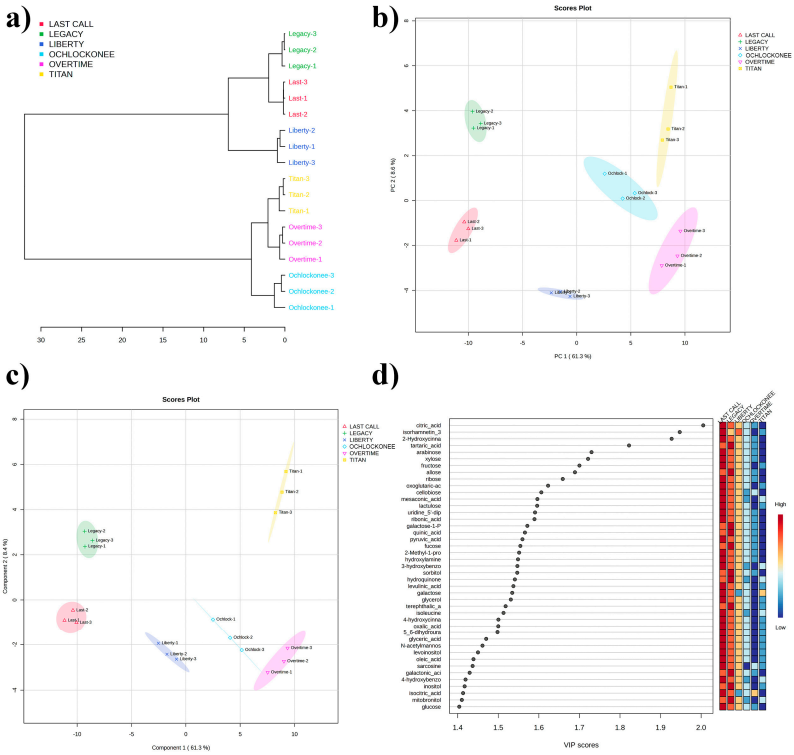

Figure 1. Characterization of six different blueberry genotypes (Last Call, Legacy, Liberty, Ochlockonee, Overtime, and Titan): (**a**) Clustering result shown as a dendrogram (distance measure using Euclidean and clustering algorithm using Ward.D). (**b**) PCA (Principal Component Analysis) scores plot; the explained variances are shown in brackets. (**c**) PLS-DA (Partial Least Squares Discriminant Analysis) scores plot. (**d**) PLS-DA VIP (Variable Importance in Projection). N = 3.

The sugars ribose, fructose, and glucose are important for blueberries' sweetness and overall flavour profile. These sugars are essential energy sources and play significant roles in various metabolic pathways, influencing the fruit's appeal and nutritional content [78,79].

After the multivariate approach, the data were analysed through one-way ANOVA, highlighting that 152 parameters significantly differed among the genotypes (Supplemen-

tary Table S1—reported in the sheet anova_posthoc). The top 60 were reported as a heatmap (Figure 2), which illustrates a comprehensive profile of the 6 blueberry genotypes, showing distinct patterns for each. Even the clusterisation reported on the heatmap, built on the 152 significantly affected parameters, further confirmed the clear separation between the highbush and the rabbiteye species (Supplementary Table S1—heatmap reported in the sheet anova_posthoc).

In particular, Last Call and Legacy were the two genotypes characterised by the higher content of flavonoid glycosides (i.e., quercetin-3-O-arabinoside, petunidin-3-O-galactoside, malvidin-3-O-galactoside, among others), sugars (sucrose, glucose, fructose, rhamnose, cellobiose, arabinose, etc.), and organic acids (citric acid, malic acid, tartaric acid, glyceric acid, etc.); at relatively low levels, Liberty showed a similar trend, whereas the other genotypes pointed out a generally lower content of almost all the metabolites identified (Figure 2). Only Titan was characterised by a significantly high content in flavonoid glycosides such as cyanidin-3-O-galactoside and -arabinoside, quercetin-3-O-glucoside, and peonidin-3-O-glactoside and -arabinoside; and organic acids, polyamines, and amino acid derivatives such as malic, maleic, and succinic acid and putrescine and pyroglutamic acid (Figure 2). Finally, Overtime was the only genotype characterised by a significantly high accumulation of flavonoid glycosides such as delphinidin-3-O-glucoside, petunidin-3-O-glucoside, naringenin-glucoside-galactoside, and cyanidin-3-O-glucoside, among others (Figure 2).

As is widely known, compounds such as flavonoids and, in particular, anthocyanins play a crucial role in the nutritional value of blueberry fruits; generally, their antioxidant potential is also related to these compounds [80]. This is why the significantly higher amounts of normalised identified compounds in the genotypes Last Call and Legacy are particularly noteworthy.

Furthermore, the comparative analysis of the six blueberry genotypes revealed distinct variations in total anthocyanins, total phenols, and ascorbate content, providing insight into their respective radical scavenging activities. The anti-inflammatory activity did not indicate any significant difference among the six genotypes (Supplementary Materials Table S1—sheet 9 (anova_posthoc)).

Starting with total anthocyanins, which are key contributors to the fruit's colour and antioxidant properties [80,81], the genotypes exhibited a notable range in their content. Contrary to what might be expected by the heatmap clustering, Legacy and Liberty showed the lowest levels of total anthocyanins (Figure 2b). This indicates that these particular genotypes might have less intense pigmentation and potentially lower antioxidant properties derived from anthocyanins. On the other hand, the other genotypes showed a similar content of anthocyanins (Figure 2b), suggesting that they could offer superior health benefits associated with these compounds.

The results depicted a slightly different trend in total phenols, encompassing a broader spectrum of antioxidant compounds beyond anthocyanins. The rabbiteye blueberry species were characterised by the highest amount of these bioactive molecules compared with the highbush species (Figure 2c). Overtime again showed the highest phenolic content, reinforcing its position as a potentially more health-promoting genotype. Ochlockonee followed closely, demonstrating substantial phenolic content, aligning with its overall antioxidant potential (Figure 2d). While not leading, Titan still presented respectable amounts of phenolic compounds, whereas Legacy, Liberty, and Last Call again lagged behind, indicating a weaker presence of these beneficial antioxidants (Figure 2c).

The radical scavenging activity, which measures the ability of the blueberry extracts to neutralise free radicals, further highlighted the differences among the genotypes (Figure 2e). Overtime displayed the most robust radical scavenging activities, corroborating its high

levels of anthocyanins and phenols. This suggests that this genotype is particularly effective at mitigating oxidative stress, a key factor in preventing several chronic diseases. Ochlockonee also showed strong radical scavenging activity, albeit slightly less than Overtime. These two genotypes also showed higher levels of total ascorbic acid (Figure 2d). While not as potent, Legacy maintained a commendable activity level, reflecting its intermediate phenolic content. Last Call, Liberty, and Titan exhibited the least radical scavenging activity, indicating their relatively diminished antioxidant capacities.

In summary, the genotype Overtime emerged as a frontrunner regarding total anthocyanins, total phenols, and radical scavenging activity, closely followed by Ochlockonee and, to a lesser extent, Titan (Figure 2b,c,e). These genotypes, all belonging to the rabbiteye species, offer the highest antioxidant benefits, making them particularly valuable for consumers and breeders aiming to maximise health benefits. Legacy holds a middle ground, providing an intermediate antioxidant property, while Liberty and Last Call fall comparatively short, suggesting that these genotypes might be less effective at contributing to the intake of antioxidant molecules from blueberries. This comprehensive analysis underscores the higher antioxidant potential of rabbiteye species than highbush species.

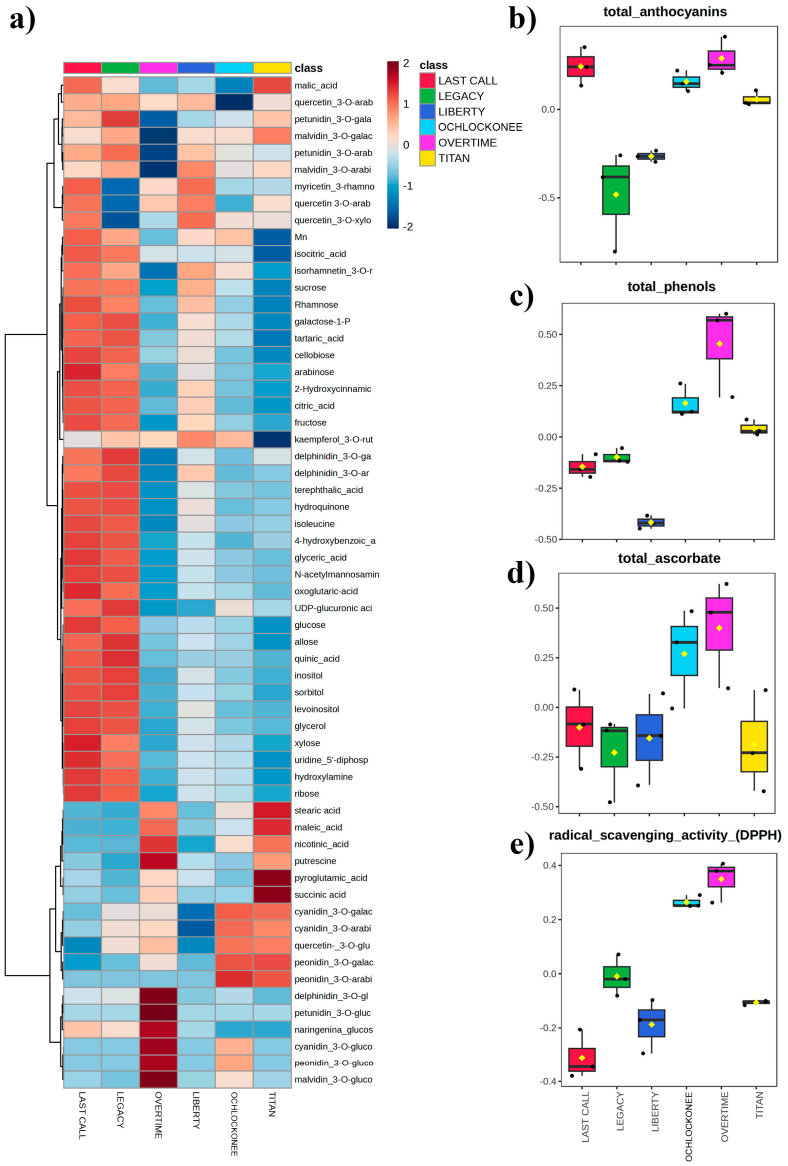

Figure 2. (**a**) The top 60 parameters resulting from the one-way ANOVA are reported as a clusterised heatmap; (**b**) total anthocyanins content; (**c**) total phenols content; (**d**) total ascorbate content; and

(**e**) total radical scavenging activity of the six blueberry genotypes. Data are expressed as mean normalised through Log 10 transformation and Pareto scaled. Normalised data were analysed through one-way ANOVA using the LSD test as post hoc ($p \leq 0.05$). N = 3.

The differences in the antioxidant potential among highbush and rabbiteye blueberries could be elucidated through a comprehensive analysis of the various studies focusing on the antioxidant capacities and bioactive compounds in these different *Vaccinium* species. A study by Wang et al. [82] evaluated the antioxidant capacities of different cultivars of rabbiteye and highbush blueberry and found that *V. ashei* (a synonym of *V. virgatum*) exhibited the highest antioxidant capacity among the genotypes studied. This indicates that *V. ashei* has a superior antioxidant potential compared with other *Vaccinium* species like *V. corymbosum*, as also observed in our experiments.

The evidence suggests that in plant tissues, the antioxidant activity depends more on the quality than the quantity of phenolic content. One study, focusing on sweet chestnut cultivars, found that while the total phenolic content is correlated with antioxidant capacity, the specific composition and quality of the phenolic compounds play a crucial role in determining the antioxidant activity [83]. Similarly, an investigation of various leaf infusions from different types of tea showed that, despite similar levels of total phenolic content, the antioxidant activities differed greatly. This variation was attributed to the different phenolic compounds, particularly catechins, which are predominant in green tea and contribute substantially to its high antioxidant activity [84]. Other research, on walnut kernels dried by different methods, revealed that variations in antioxidant activity are not solely correlated with the total phenolic content. Instead, the specific phenolic compounds released or retained during different drying processes significantly impact this property [85]. These studies highlight that the specific phenolic profiles, rather than just the overall phenolic content, could play a crucial role in determining the antioxidant potential of an extract.

4. Conclusions

This study comprehensively analyses the chemical composition and antioxidant properties of six blueberry genotypes, three belonging to the highbush and three to the rabbiteye species, and reveals significant variations in bioactive compound concentrations and antioxidant activities. The Ochlockonee and Overtime genotypes, both belonging to the rabbiteye species, exhibited the highest levels of anthocyanins, phenols, ascorbate, and radical scavenging activity, underscoring their superior health-promoting attributes. Despite lower quantities of specific metabolites, these genotypes outperformed others in antioxidant efficacy, suggesting that the quality of phenolic content is more crucial than the quantity. This research represents a significant advancement by employing, for the first time, a multi-approach analytical framework to compare all six genotypes under identical pedoclimatic conditions. Previous studies have explored individual genotypes for their antioxidant potentials, phenolic contents, and/or bioactive properties. However, this comprehensive and integrative analysis provides deeper insights into genotype-driven biochemical variations and their functional implications.

Cluster and multivariate analyses, including PCA and PLS-DA, confirmed the clustering of the two species based on phytochemical profiles and identified key metabolites, primarily organic acids, sugars, and flavonoid glycosides, that drive genotype categorisation. Notably, Last Call and Legacy had higher amounts of these metabolites (organic acids, sugars, amino acids, and specialised metabolites, among others) but lower antioxidant properties, highlighting the importance of phenolic content quality over quantity. Overtime, with the highest phenolic content, reinforces its potential as a health-promoting genotype.

The findings emphasise the significance of genotype selection in blueberry breeding programs to enhance health-promoting attributes and further confirm the higher antioxidant potential of rabbiteye species compared with highbush, as previously observed by other authors. This study underscores that while some genotypes have higher bioactive compound concentrations, antioxidant efficacy is influenced more by the specific composition and quality. By highlighting the novel integration of multiple approaches and the comparative framework, this study provides a valuable reference for researchers and breeders aiming to optimise functional attributes in blueberries.

Future research should focus on elucidating the specific compositions of bioactive compounds that contribute to higher antioxidant efficacy while considering the potential additive, synergistic, or competitive interactions among these molecules. Such studies would provide a more comprehensive understanding of how bioactive compounds collectively influence antioxidant performance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy15020262/s1, Table S1: In this table, all the raw data from the metabolomic analysis are divided into different Excel sheets. In particular, sheet 1 (data_original) reports the original data obtained from the different analyses; sheet 2 (data_normalized) reports the data after normalisation and transformation; sheet 3 (pca_scores) reports all the scores of the different PCs used to build the unsupervised model and the scree plot of the different PCs combinations; sheet 4 (pca_loadings) reports the loading plots of the different PCs; sheet 5 (plsda_coef) reports the coefficients of the supervised PLS-DA model; sheets 6, 7, and 8 report the PLS-DA loadings, scores, and VIP scores, respectively; sheet 9 (anova_posthoc) reports the results of the one-way ANOVA analysis and the detailed heatmap with all the significant features (LSD, $p \leq 0.05$); sheet 10 (GC-MS MASS DATA) reports the information concerning the metabolites annotation (retention time, quant mass, spectra similarity, S/N ratio, etc.); sheet 11 (LC-MS data) reports the information concerning LC-MS metabolites identification (Rt, m/z calc, m/z exp, Δppm, and fragments); sheet 12 contains the data in which the absolute quantification was reported.

Author Contributions: Conceptualization, F.A., L.E. and B.P.; methodology, F.A., L.E., G.B. and G.A.; formal analysis, F.A., M.P., G.F. and L.E.; investigation, F.A., L.E., G.B., M.P., B.P., G.F. and L.D.V.; data curation, F.A., L.E., G.B., M.P., G.F. and G.A.; writing—original draft preparation, F.A.; writing—review and editing, F.A., L.E., G.B., G.A., B.P., M.P., G.F. and G.A.S.; visualization, F.A.; supervision, G.A.S.; funding acquisition, G.A.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research is part of the project "MIND FoodS HUB (Milano Innovation District Food System Hub): Innovative concept for the eco-intensification of agricultural production and for the promotion of dietary patterns for human health and longevity through the creation in MIND of a digital Food System Hub", cofunded by POR FESR 2014-2020_BANDO Call HUB Ricerca e Innovazione, Regione Lombardia.

Data Availability Statement: Data are contained within the article or Supplementary Materials.

Acknowledgments: The authors would like to thank the Alpes Agia farm, Albosaggia—Sondrio (Italy), for supplying the blueberry fruits utilised in this research study and Giorgio Lucchini for the technical support in the ICP-MS analyses.

Conflicts of Interest: The authors declare no conflicts of interest.

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