JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

pubs.acs.org/JAFC

Article

NMR Metabolite Profiling and Antioxidant Properties of Spartan, Jewels, Misty, and Camelia Blueberries

Mattia Spano, Giacomo Di Matteo, Simone Carradori, Marcello Locatelli, Gokhan Zengin, Luisa Mannina,* and Anatoly Petrovich Sobolev

Cite This: https://doi.org/10.1021/acs.jafc.4c01442

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: The health-promoting properties of blueberries are widely recognized and are mainly attributed to anthocyanins. However, fruit's chemical composition includes also other components and strongly depends on varieties and climatic conditions. Here, ^{1}H NMR metabolite profiling and biological activity of four blueberry cultivars (Spartan, Jewels, Misty, Camelia) grown in Central Italy over two years were reported. Untargeted and targeted NMR analyses allowed the quantification of sugars, organic acids, amino acids, anthocyanins, lipids, and other compounds. Spectrophotometric assays evaluated total phenolic and flavonoid content, antioxidant activity, and enzyme inhibitory activity toward cholinesterase, α-amylase, α-glucosidase, and tyrosinase. Statistical analysis showed a correlation between chemical composition and biological activity, revealing markers specific to blueberry cultivars (quinic acid, quercitrin, *myo*-inositol, myrtillin, and petunidin-3-*O*-glucoside). Almost all antioxidant assays were correlated with the chlorogenic acid levels. A strong effect of harvesting on chemical composition and biological activities was observed, with Misty cultivar having the highest antioxidant activity.

KEYWORDS: *blueberry cultivars, NMR, antioxidant properties, anthocyanins, metabolomics, enzyme inhibition*

1. INTRODUCTION

The blueberry plant belongs to the Ericaceae family, genus *Vaccinium*, which includes about 130 species. The varieties of blueberry cultivated today are mainly those derived from the giant blueberry. Blueberry fruits are sweet, nutritious, and widely popular. In the past decade, their consumption has rapidly increased since blueberry consumption has been associated with significant health benefits, conferring blueberries the title of "functional food".[1]

In particular, blueberry consumption has been associated with a reduced risk of obesity, diabetes, and heart diseases[2−4] maintaining healthy bones, lowering blood pressure, and preventing cancer.[5,6] Furthermore, blueberry fruits are characterized by a high antioxidant capacity, one of the highest among commonly consumed fruits and vegetables. The antioxidant capacity has been attributed to specific compounds mainly anthocyanins but also proanthocyanidins, phenolic acids, and stilbenes. Indeed, anthocyanins and other bioactive components (ascorbic acid, phenolics, cinnamic acids, and other flavonoids) from blueberries have been shown to reduce oxidative stress and modulate important enzymes.[7,8]

Despite a large variability among these compounds, some polyphenols and anthocyanins can permeate the blood−brain barrier (BBB) and have been found in the central nervous system after administration *in vivo*; thus, their effect on enzymes also located centrally can be evaluated.[9] Actually, chlorogenic acid was shown to exert important health-promoting effects in this biological context[10] as well as anthocyanins from blueberry.[11] In this last work, the authors suggested a main role for the antityrosinase, anti-α-glucosidase, and antioxidant activities exerted by anthocyanins and polyphenols detected in huge amounts in blueberry. Conversely, no effect was registered on acetylcholinesterase.[11,12]

Acetylcholinesterase hydrolyzes acetylcholine in the synaptic gaps between neuronal cells. In Alzheimer's patients, acetylcholine levels are lower than in healthy people and therefore inhibition of acetylcholinesterase can increase acetylcholine levels and improve memory function in Alzheimer's patients.[13]

So far, numerous studies focused on the chemical composition of blueberries have dealt with a limited number of constituents namely total polyphenol and flavonoid content, total sugars, and organic acids,[14] or specifically with the polyphenolic fraction,[15,16] thus ignoring other components or a more detailed chemical characterization.

Nevertheless, targeted methods do not provide a complete picture of the chemical composition of a given sample. Besides polyphenols, blueberries contain many other metabolites important as nutrients that need to be determined. NMR spectroscopy is one of the most suitable methodologies employed for untargeted studies[17] providing a comprehensive metabolite profile of the sample owing to "high-throughput" spectroscopic/structural information on a wide range of metabolites with high analytical precision. Using NMR, the

Received: February 16, 2024
Revised: July 17, 2024
Accepted: July 18, 2024

metabolic profile of several fruits and plants such as acerola,[18] table grapes,[19] kiwifruits,[20] hemp,[21,22] mango,[23] melon,[24] goji fruits,[25] and other species[26] has been determined and it has been possible to obtain information on geographical origin, varieties, quality, adulteration, nutritional properties, processing, shelf life, and the proper harvesting time.[17]

The first ^{1}H NMR-based characterization of blueberry metabolite profile reported in 2014[27] has offered an ample picture of the fruit's chemical composition that included identification of sugars, organic acids, amino acids, anthocyanins, and other polyphenols, as well as different classes of lipids. Considering the general tendency to correlate chemical composition with antioxidant and other blueberries' biological properties,[11,28,29] and the clear evidence that fruits' chemical composition depends on genetic, environmental, and other factors,[14,30] the application of untargeted metabolite profiling in this context is indispensable, although is still scarce. Furthermore, so far only a few examples of studies combining untargeted metabolic profiling of blueberry fruits with biological activity measurements are reported in the literature.[16,31] The results of these studies can be formulated as a hypothesis that biological activity can depend not only on phenolic fraction composition but also on the entire phytocomplex. For example, the multivariate statistical analysis of untargeted metabolite profiling data obtained by UPLC-TOF-MS analysis of different blueberry cultivars showed rather unexpected positive higher correlations between the levels of quinic acid, methyl succinic acid, chlorogenic acid, oxoadipic acid, and malic acid and all the tested anticancer activities.[16]

In this paper, for the first time to our knowledge, the untargeted NMR metabolite profiling of four different highbush blueberry cultivars, named Spartan, Jewels, Misty, and Camelia, harvested in two different years was investigated to reveal how genotype and climatic conditions can influence the chemical profile. The targeted NMR characterization of anthocyanins using solid-phase extraction was also carried out. The total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity were also evaluated by means of *in vitro* spectrophotometric assays[32] to correlate differences in the chemical composition to different biological/nutraceutical properties. Moreover, the inhibitory effects of blueberry extracts on key enzymes involved in different human diseases (cholinesterases for neurodegenerative disorders, tyrosinase for skin pigmentation dysfunctions, α-amylase and α-glucosidase for reduction of postprandial glycemia) were also tested *in vitro*.

2. MATERIALS AND METHODS

2.1. Chemicals and Solvents. Deuterated water (D_2O) 99.97 atom % of deuterium, 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP), EDTA-d_{16} 98.0 atom % of deuterium, deuterated methanol (CD_3OD) 99.80 atom % of deuterium, deuterated chloroform (CDCl3) 99.80 atom % of deuterium, and sodium 4,4-dimethyl-4-silapentane-1-sulfonate-d_6 (DSS) were purchased from Euriso-Top (Saclay, France). Methanol (HPLC-grade), chloroform (HPLC-grade), and distilled water were obtained from Carlo Erba Reagenti (Milan, Italy). Sodium carbonate (Na_2CO_3; 99.999% purity), Folin-Ciocalteu's phenol reagent, tannic acid (Ph. Eur. purity) and aluminum chloride hexahydrate ($AlCl_3 \times 6H_2O$; Ph. Eur. purity), tetramethylsilane (TMS), potassium phosphate monobasic (KH_2PO_4), and potassium phosphate dibasic (K_2HPO_4) were purchased from Merck (Darmstadt, Germany). Commercial standards of malvidin-3-*O*-glucoside chloride (oenin, >95%), and delphinidin-3-*O*-glucoside chloride (myrtillin, >95%) were purchased from Extrasynthese (Lyon, France). Malvidin-3-*O*-galactoside chloride (primulin, >95%) and delphinidin-3-*O*-galactoside chloride (empetrin, >95%) were supplied by PhytoLab (Vestenbergsgreuth, Germany). Chlorogenic acid (>95%) was purchased from Sigma-Aldrich (Milan, Italy).

2.2. Sampling. Four different blueberry cultivars, namely Spartan, Jewels, Misty, and Camelia, were hand-harvested in the same experimental field (1000 m^2) located in Ciampino (41°48′N 12°36′E), Lazio, Italy. The harvest was performed from June to August for each year (2021 and 2022) considering, for each cultivar, ten plants differentially distributed in the experimental field. Means of minimum and maximum air temperatures, total rainfall, average humidity, and potential evapotranspiration during each month in the two consecutive years were recorded by local weather stations[33] and reported in Table S1. To avoid environmental interference, only fruits at completed maturation were analyzed. Considering that these berries are not cultivated for human consumption nor do they require to be stored long-term, the widespread practice of harvesting fruit at very early stages of ripeness was avoided; conversely, the berries were selected according to their biological maturity as judged by color and firmness.

2.3. Sample Preparation. The weight of each berry can vary from one to two grams. About 100 g of berries, randomly selected from different plants representative of the whole seasonal harvest, were mildly cleaned to remove impurities and green parts, then frozen in liquid nitrogen, and finely powdered, keeping the temperature low and not allowing the pulp to thaw during grinding. Two grams of ground berries were used for extraction and each sample was replicated 5 times. The extraction was performed according to the procedure from our previous study[27] with methanol/chloroform/water in a 2:2:1 volumetric ratio to obtain both the hydroalcoholic and the organic liposoluble extracts. Samples were kept at 4 °C during extraction and centrifugation. Both fractions were dried under a N_2 flow at room temperature until the solvent was completely evaporated. The dried phases were then stored at −80 °C until the NMR and biological analyses.

The liposoluble extract was dissolved in 700 μL of a 2:1 *v*/*v* $CDCl_3/CD_3OD$ mixture before NMR analysis. The hydroalcoholic phase was dissolved in 1.0 mL D_2O. One part of the solution (200 μL) was mixed with 500 μL of D_2O phosphate buffer (400 mM, pH = 7.0, 0.3 mM EDTA) containing 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP, 1.40 mM) and this solution was analyzed by NMR. Another aliquot of the D_2O solution (500 μL) was used for the isolation of the phenolic compounds carried out by solid phase extraction (SPE).

Initially, C18 SPE columns were equilibrated with 0.1% trifluoroacetic acid (TFA) in double-distilled H_2O after column washing with 0.1% TFA in HPLC-grade methanol according to the method originally developed for black raspberries.[34] Successively, an aliquot (500 μL) of the hydroalcoholic phase, acidified by adding 0.1% TFA (*v*/*v*), was loaded onto the C18 column (CHROMABOND C18 polypropylene column, 45 μm particle size, Avantor, Milan, Italy) followed by washing three times with acidified H_2O. Anthocyanins and other phenolic compounds were eluted with methanol containing 0.1% TFA. Methanol was removed from the eluted materials under N_2 flow, and then water was removed by lyophilization. The dry residue was dissolved in 700 μL of CD_3OD containing 5.0% (vol) of TFA-d_1 and sodium trimethylsilylpropanesulfonate (DSS, 2 mM) as an internal standard for NMR analysis.

2.4. NMR Measurements. The ^{1}H NMR spectra of blueberry extracts were recorded at 27 °C on a Bruker AVANCE 600 NMR spectrometer operating at a proton frequency of 600.13 MHz equipped with a Bruker multinuclear z-gradient 5 mm probe. All NMR acquisition and processing parameters are reported in Table S2.

2.4.1. Quantitative Analysis. For the quantification of metabolites, the selected ^{1}H NMR signals listed in Tables S3−S5 were integrated using the Bruker TOPSPIN 1.3 software, and the integrals were normalized with respect to the integral of the internal standard signal at 0.0 ppm (TSP or DSS for aqueous or SPE methanol extracts, respectively) and the number of equivalent protons. In the case of

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

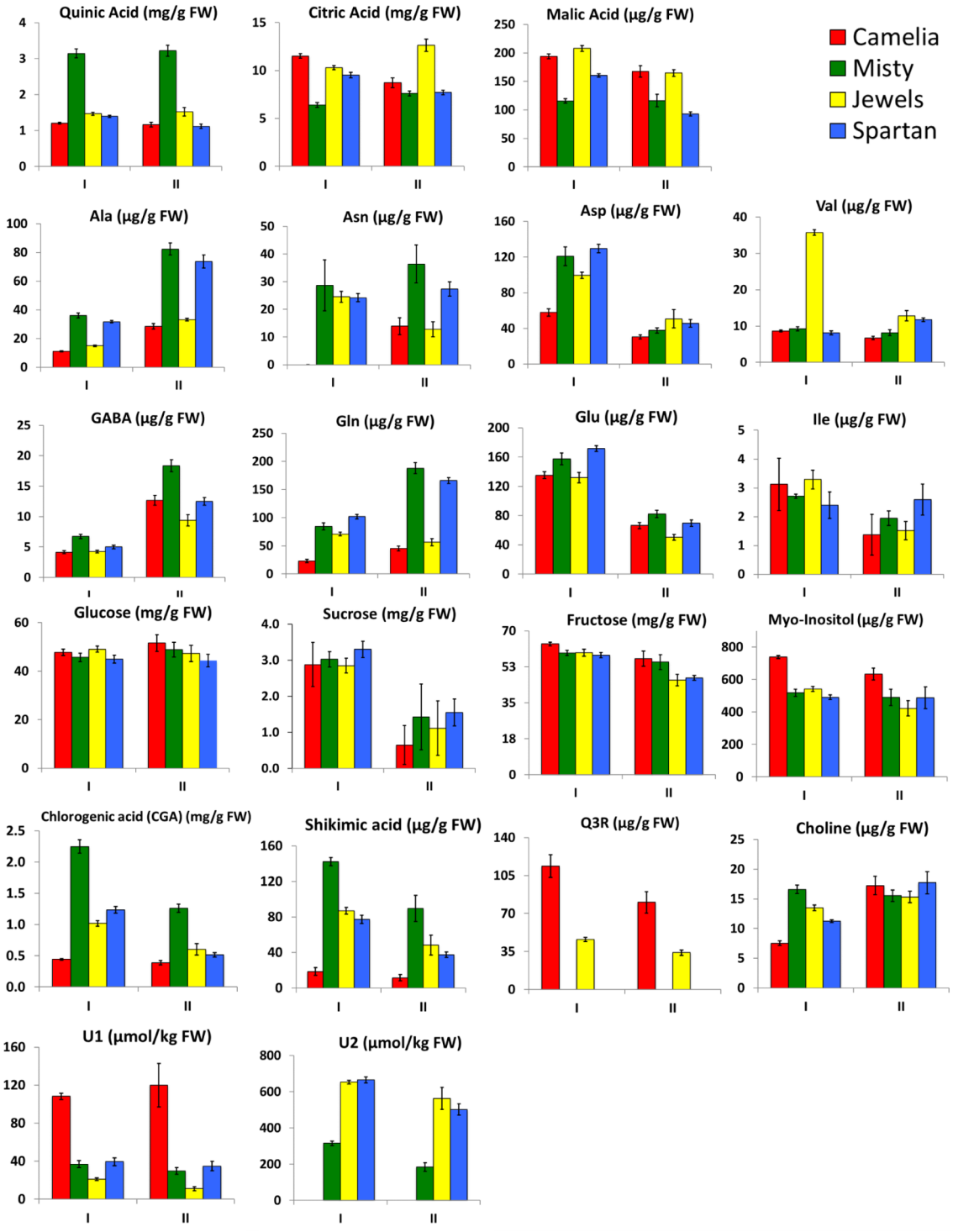

Figure 1. Histograms report mean values and standard deviations (n = 5) of water-soluble metabolites in the fruits of four blueberry cultivars (Camelia, red bars; Misty, green bars; Jewels, yellow bars; Spartan, blue bars) in the first (I) and the second (II) year of harvest. U1: unknown 1, putative primary aliphatic amine; U2: unknown 2, putative β-sugar.

liposoluble metabolites, the integrals of ten selected signals in organic extract ^{1}H NMR spectra (Table S8) were normalized with respect to the sum of integrals in the 2.21−2.35 ppm range due to α-CH_2 signals of free and esterified fatty acids, set to 100. The molar % values of fatty acids, sterols, diacylglycerophosphocholine, and digalactosyldiacylglycerol have been calculated considering the number of equivalent protons (see Table S5 for the corresponding equations).

Data obtained for Bligh−Dyer hydroalcoholic extracts were expressed as μg or mg/g FW ± SD (n = 5).

Data obtained for Bligh−Dyer organic extracts were expressed as molar % ± SD (n = 5).

2.5. Total Phenolic Content (TPC) and Total Flavonoid Content (TFC). The Folin−Ciocalteu and $AlCl_3$ assays, respectively, were utilized to determine the total phenolic and flavonoid contents, and the procedures are reported in our earlier paper.[35] Gallic acid and rutin were used to explain the obtained results (mg of GAE/g of DW of extract; mg of RE/g of DW).

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

2.6. Antioxidant and Enzyme Inhibitory Assays. To appraise the antioxidant potential of the extracts, six distinct spectrophotometric tests were conducted. This consisted of the ABTS and DPPH assays, which assess the antioxidants' aptitude to neutralize free radicals. Through the FRAP and CUPRAC tests, the reduction potential of the extract was assessed. Phosphomolybdenum (PMo) and ferrozine (CHEL) assays also measured the total antioxidant ability and metal chelating potential, respectively. Apart from CHEL, each of these assays was evaluated with a Trolox standard (TE). As for CHEL, its comparison was made according to EDTA equivalents per gram of extract (EDTAE). All procedures are detailed in our previous works.[35] To investigate the inhibitory effects of the tested extracts on various enzymes, we applied acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, α-amylase, and α-glucosidase. Our earlier work provides information on the experimental procedures.[35,36] We measured AChE and BChE inhibition in terms of milligrams of galantamine equivalents (GALAE) per gram of extract, tyrosinase inhibition expressed as milligrams of kojic acid equivalents (KAE) per gram of extract, and α-amylase and α-glucosidase inhibition quantified as millimoles of acarbose equivalents (ACAE) per gram of extract. The results were also described in terms of percent inhibition in Tables S6 and S7.

2.7. Statistical Analysis. The statistical analysis of all data was performed using Unscrambledr 9.8 software (CAMO Software, Oso, Norway). In order to analyze the multivariate structure of the data, principal component analysis (PCA) was applied. The data were organized in matrices: the means of each column were set to 0, whereas their standard deviations were set to 1. This procedure, applied to the data matrix prior to PCA analyses, allowed comparison of the covariations of the signals independent of their numerical size while keeping intact the factorial structure. *In vitro* antioxidants and enzymatic assays were performed in triplicate, and differences between the extracts were compared using an ANOVA and Tukey's test. Graph Pad Prism (version 9.2) was used for the analysis.

3. RESULTS AND DISCUSSION

3.1. NMR-Based Metabolite Profiling. *3.1.1. Water-Soluble Metabolites.* The previously developed NMR blueberries analysis[27] applied in the present study allowed us to obtain a comprehensive picture of the most abundant water-soluble and liposoluble metabolites including sugars, organic acids, amino acids, anthocyanins and other polyphenols, fatty acids, lipids, and sterols.

In the case of water-soluble metabolites, four sugars and polyols (glucose, fructose, sucrose and *myo*-inositol), five organic acids (citric, malic, quinic, shikimic, and chlorogenic acids), eight amino acids (alanine, aspartic acid, asparagine, isoleucine, gamma-aminobutyric acid, glutamine, glutamic acid, and valine), choline and 3-*O*-α-L-rhamnopyranosyl quercetin (Q3R, or quercitrin) were identified and quantified (see Table S3, Figure 1, and Figure S1).

Sugar fraction was the most abundant, reaching up to 10−11% of fruit weight (100−110 mg/g fresh weight). The main components were fructose (60−50 mg/g) and glucose (about 50 mg/g), whereas sucrose (3−1 mg/g) and *myo*-inositol levels (0.8−0.5 mg/g) were substantially lower. The content of fructose, glucose, and sucrose perfectly agrees with food database data.[37]

The second most abundant metabolite fraction included organic acids, namely citric acid (CA, 12−6 mg/g), quinic acid (QA, 3−1.5 mg/g), malic acid (MA, 0.2−0.1 mg/g) and shikimic acid (SHA, 150−10 μg/g). The relative content of main organic acids in the order citric > quinic > malic > shikimic corresponds to those reported for blueberry juice.[28] The wide ranges of content indicated above evidence the variability owing to the year and the cultivar and will be discussed later.

Chlorogenic acid was the most abundant phenolic compound in blueberry and its level (50−200 mg/100 g fresh weight) also corresponds to literature (65−208 mg/100 g fresh weight)[38] as well as the content of Q3R (0−10 mg/100 g fresh weight).[38] It is noteworthy that the Q3R level was extremely variable; in particular, the Q3R level was below the detection limit for Misty and Spartan fruits.

The metabolite profile was influenced by both the harvest year and cultivar. For example, in the case of Gln, its highest level (about 188 μg/g, Misty, second year) was about eight times higher than the lowest level (about 23 μg/g, Camelia, first year). Glu and Gln were the most abundant amino acids in almost all cases, followed by Asp. Ile level was the lowest, followed by Val and GABA.

Additionally, the characteristic ^{1}H NMR signals from two unidentified compounds were included in the analysis as potential cultivar markers according to preliminary data. These signals are doublets at 0.93 ppm (J^1_{H-H} = 6.6 Hz), and 4.49 ppm (J^1_{H-H} = 7.8 Hz), labeled subsequently as U1 and U2. Considering the chemical shift and multiplicity, the U1 signal is probably a methyl group bound to a methine (CH_3-CH). In fact, in the ^{1}H−^{1}H TOCSY experiment (Figure S2) the signal at 0.93 ppm was correlated with three other signals at 1.52, 1.64, and 3.00 ppm. The signal of the CH group can be one of them (at 1.52 or 1.64 ppm), whereas the signal at 3.00 ppm is typical for a CH_2-NH_2 or $CH_2-NH_3^+$ group, indicating that U1 is a primary aliphatic amine. Tentatively, based only on the mentioned TOCSY correlations U1 can be assigned to 3-methylbutan-1-amine. The ^{1}H chemical shifts of four proton signals of U1 practically coincide with those for $(CH_3)_2CHCH_2NH_3^+Cl^-$ (hydrochloride salt of 3-methylbutan-1-amine) dissolved in deuterated water.[39]

U2 signal probably belongs to an anomeric CH group of a β-sugar considering chemical shift, *J*-coupling constant values, and TOCSY correlations to ^{1}H NMR signals at 3.37, 3.45, and 3.52 ppm typical for protons in a sugar ring (Figure S2). Only the molar content (μmol/kg fresh weight) of U1 and U2 was determined because their molecular weights were not available; anyway, the relative levels of these components were cultivar dependent.

3.1.2. Anthocyanins. Anthocyanins are also present in the water-soluble metabolite fraction of blueberries, but in neutral D_2O solution, ^{1}H NMR signals of the anthocyanidin moiety are too broad to be efficiently observed. According to our experience,[27] the combination of SPE extraction with the use of methanol and TFA as a solvent seems to be the best approach to the NMR-based anthocyanin characterization; therefore, this method has been also chosen for the present study. The five most abundant components of anthocyanin fraction have been previously identified in the ^{1}H NMR spectra of blueberries.[27] They are malvidin-3-*O*-glucoside (M3Glp), malvidin-3-*O*-galactoside (M3Gal), delfinidin-3-*O*-glucoside (D3Glp) known as myrtillin, delfinidin-3-*O*-galactoside (D3Gal), and petunidin-3-*O*-glucoside (P3Glp). In our study, the content of these compounds in blueberries was measured together with the content of other eight anthocyanins (AN1−AN8) still not identified but observable in the NMR spectra (see Table S4 for ^{1}H NMR parameters). At least 13 anthocyanins were clearly observable in the 8.90−9.10 spectral region of blueberry SPE extract, see the ^{1}H spectrum in Figure 2B. As previously reported,[27] in this

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

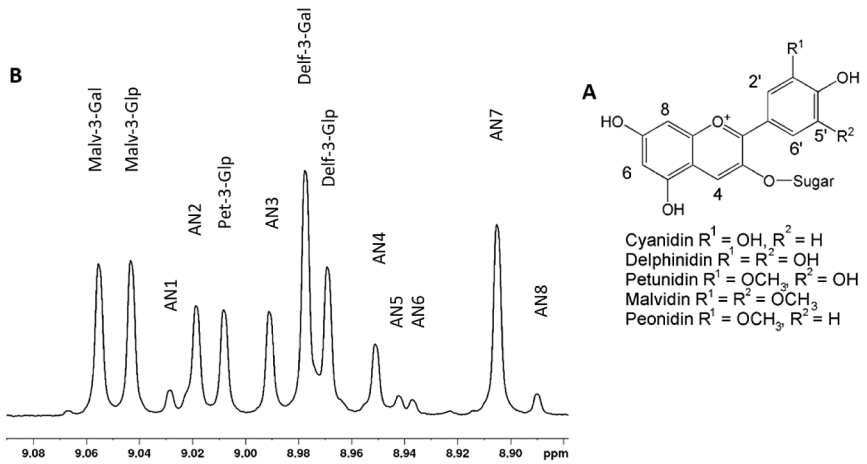

Figure 2. (A) General structure of anthocyanins. (B) A selected part of ^{1}H NMR spectrum at 600.13 MHz of anthocyanin SPE extract from Jewels blueberry cultivar dissolved in CD_3OD/TFA-d_1 (95:5 *v*/*v*).

Table 1. Total Bioactive Compounds and Antioxidant Activity of Camelia, Jewels, Misty, and Spartan Blueberry Cultivars at the Two Harvesting Years (Values Are Reported as Mean ± SD of Three Replicates)[a]

	total bioactive compounds		total antioxidant activity	metal chelating activity	radical scavenging activity		reducing power activity	
sample	total phenolic (mg GAEs/g extract)	total flavonoid (mg REs/g extract)	phosphomolybdenum assay (mmol TEs/g extract)	chelating effect (mg EDTAEs/g extract)	DPPH radical (mg TEs/g extract)	ABTS radical cation (mg TEs/g extract)	CUPRAC (mg TEs/g extract)	FRAP (mg TEs/g extract)
1st year								
Camelia	12.26 ± 0.28	4.20 ± 0.37	0.85 ± 0.17	1.48 ± 0.18	22.03 ± 1.55	31.66 ± 0.85	48.19 ± 0.73	37.69 ± 0.47
Jewels	18.38 ± 0.07	4.46 ± 0.07	1.17 ± 0.04	4.31 ± 0.08	36.77 ± 0.83	57.47 ± 1.42	56.14 ± 0.26	59.51 ± 0.58
Misty	34.73 ± 1.10	5.45 ± 0.61	1.47 ± 0.32	1.15 ± 0.07	52.14 ± 0.29	106.91 ± 4.14	71.77 ± 1.87	93.77 ± 3.51
Spartan	19.48 ± 0.25	3.98 ± 0.04	1.07 ± 0.07	1.67 ± 0.19	32.49 ± 1.59	58.99 ± 0.81	53.10 ± 1.85	52.94 ± 0.52
2nd year								
Camelia	9.90 ± 0.07	1.48 ± 0.01	0.26 ± 0.03	1.05 ± 0.01	14.44 ± 0.84	25.51 ± 1.13	36.81 ± 0.18	30.31 ± 2.52
Jewels	10.22 ± 0.04	1.36 ± 0.08	0.26 ± 0.01	0.14 ± 0.01	14.33 ± 0.70	26.40 ± 2.41	38.18 ± 0.85	31.35 ± 0.96
Misty	18.05 ± 0.11	1.53 ± 0.14	0.36 ± 0.02	na	29.43 ± 0.50	72.01 ± 2.61	70.81 ± 0.62	52.31 ± 0.68
Spartan	14.91 ± 0.08	1.75 ± 0.02	0.36 ± 0.03	0.71 ± 0.02	24.88 ± 0.05	53.00 ± 3.09	58.73 ± 1.54	44.52 ± 1.01

[a]GAEs: gallic acid equivalents; REs: rutin equivalents; TEs: Trolox equivalents; EDTAEs: ethylenediamine tetraacetate equivalents; na: sample with no activity in the test.

spectral region only the singlet signals attributed to the H-4 proton of anthocyanidins are present (Figure 2B); therefore, each signal corresponds to a specific compound. Unfortunately, it was not possible to assign and identify all of the signals considering the elevated number of different anthocyanins that potentially can be present and the limited number of commercially available pure standards. Nevertheless, considering the abundance and the relative position of the H-4 signal of AN7 (0.07 ppm upfield with respect to the H-4 signal of D3-Gal,[40] it can belong to delphinidin-3-*O*-arabinoside, relatively abundant in blueberries.[38] The total content of the five identified anthocyanins was about 50%. Generally, D3Gal, M3Gal, and AN7 were the most abundant components of the anthocyanin fraction (see Figure S3). As in the case of other water-soluble metabolites, substantial variations in some anthocyanins' levels (such as P3Glp, M3Glp, D3Glp, and AN8) due to both harvest year and cultivar were observed; anyway, the mean values averaged over four cultivars and two years were in the perfect agreement with the literature (see Table S5).

3.1.3. Liposoluble Metabolites. The content of liposoluble metabolites previously identified[27] was also measured by ^{1}H NMR analysis (see Table S8 and Figure S4). In the case of different types of lipids (fatty acids, triacylglycerols (TG), diacylglycerophosphocholine (PC), digalactosyldiacylglycerols (DG)) the quantification of different classes (as a sum of all components belonging to a specific class) was performed. For example, saturated, mono-, di-, and triunsaturated fatty acid chains (free and esterified altogether) were quantified without separation according to chain length. The total content of free fatty acid chains was also determined. Two sterols namely *β*-sitosterol, and a nonidentified one were also determined. Among liposoluble metabolites saturated (SFA), monounsaturated (MUFA), and diunsaturated linoleic fatty acids were the most abundant (Figure S5). Total TG was the most abundant class of lipids followed by PC and DG, moreover, 15−20% of fatty acids were not esterified. *β*-Sitosterol was the predominant component of the sterol fraction that seems to be cultivar-dependent.

3.2. Total Phenolic Content and Flavonoid Content, Antioxidant Activities, Enzymes Inhibitory Effects. Six different antioxidant assays carried out in parallel were chosen in order to cover different possible mechanisms of antioxidant activity, such as free radical scavenging capacity (ABTS, DPPH), transition metal ion chelation (CHEL) (preventing them from participating in the free radical generation process),

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Fe^{3+} (FRAP) and Cu^{2+} (CUPRAC) reduction (electron transfer capacity), and total antioxidant activity (phosphomolybdenum assay, PMo).

Table 1 reports the TPC and TFC and antioxidant activities of the four cultivars harvested in 2021 (first harvesting year). The results of all six antioxidant activity assays were similar to those for pomegranate juice measured using the same methods.[35] Misty turned out to be the cultivar with the highest values of TPC (up to 3-fold) and TFC. The highest antioxidant potential (except for the metal chelating ability) was detected in Misty among the samples (Table 1). Misty was also very active against α-glucosidase and tyrosinase and promising toward AChE and α-amylase (Table S7). Conversely, Camelia was the cultivar with the lowest TPC and antioxidant potential and with a low TFC and chelating activity. Jewels was the cultivar with the best metal chelating ability. Collectively, the four cultivars had no inhibitory activity against BChE.

In the second harvesting year, the results confirmed that Misty was the cultivar with the highest values of TPC, TFC, and antioxidant potential (except for the metal chelating ability) (Table 1), as well as potent inhibitory activity against α-glucosidase, α-amylase, and tyrosinase (Table S7). As in the previous year, Camelia was characterized by a weak capacity to counteract the oxidative stress, probably due to low values of TPC and TFC. The general impact on α-amylase and acetylcholinesterase was weak, whereas no activity was registered against butyrylcholinesterase. More in detail, the second harvesting year (2022) furnished extracts characterized by a lower amount of total phenolics and flavonoids, antioxidant potential, and, concurrently, less evident inhibitory effects against the panel of enzymes except tyrosinase and α-amylase. This could be due to specific compounds or to additive/synergistic interactions of compounds present in these extracts. Collectively, the four cultivars had no inhibitory activity against BChE. It is noteworthy that TPC, TFC, and almost all antioxidant and enzyme−inhibitory activities were lower for samples harvested in the second year compared to the first year of harvest.

In all, the gathered chemical-biological data strongly suggest that there were differences in the metabolite content and the corresponding biological activities between the two harvesting years, with trends proposing the Misty cultivar as the best extractable matrix.

3.3. Multivariate Statistical Analysis. *3.3.1. Principal Component Analysis (PCA) Highlight.* PCA was applied to have a comprehensive picture of the main factors influencing the metabolite profile and biological activity of the blueberry extracts. Principal component analysis has been frequently applied in previous studies of blueberry metabolite profiling to distinguish Highbush and Rabbiteye species,[15] cultivar comparison,[15] to correlate flavor and taste with composition and physicochemical data,[28] and to highlight cultivars- and developmental stages-variations.[16]

The first three principal components account for 78.2% of the variability. The combinations of score and loading plots (PC2 vs PC1 and PC2 vs PC3) are reported in Figure 3. For the sake of simplicity, only the mean values of the scores are reported. The samples along PC1 are separated according to the year, whereas the distribution along PC3 is predominantly due to a genetic factor (different cultivars). It is clear that year-related variations in metabolite profiling are the most important and influence a large number of metabolites.

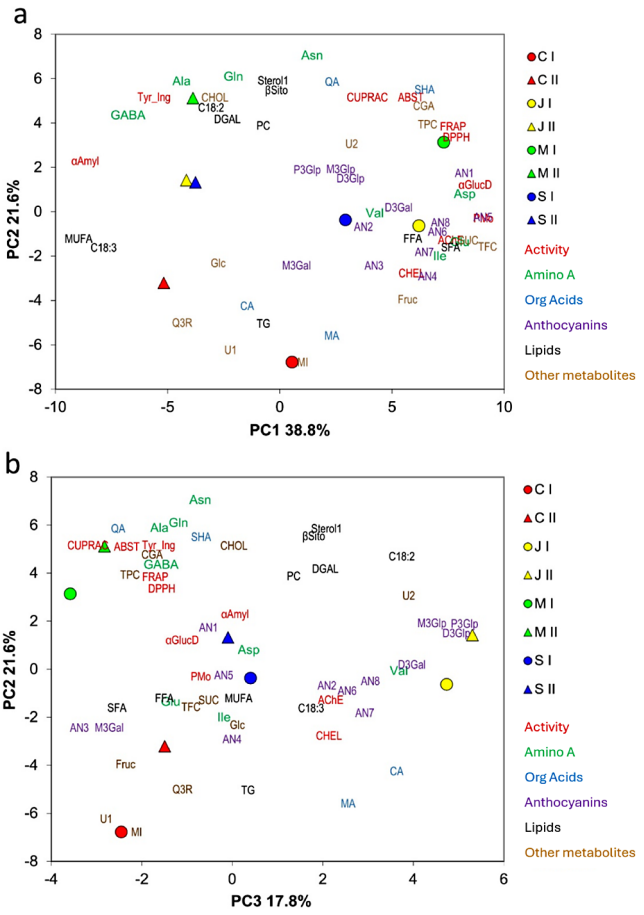

Figure 3. Combined PCA scores and loadings plots. (a) PC2 vs PC1, (b) PC2 vs PC3. The colored circles and triangles correspond to samples of the first and the second year, respectively. Abbreviations: C, Camelia; J, Jewels; M, Misty; S, Spartan; C18:2, linoleic acid; C18:3, linolenic acid; CA, citric acid; CGA, chlorogenic acid; FFA, free (nonesterified) fatty acids; Fruc, fructose; Glc, glucose; MA, malic acid; MI, *myo*-inositol; QA, quinic acid; SHA, shikimic acid; SUC, sucrose.

Positive and negative PC1 scores correspond to the first and second years, respectively. The highest/lowest PC1 loadings indicate the variables (metabolite levels and biological activities) substantially influenced by the year of cultivation. Amino acids (Ile, Glu, and Asp), anthocyanins (AN1, AN4, AN5, AN6, AN7, and AN8), lipids (FFA and SFA), and other metabolites (fructose, sucrose, shikimic acid, and CGA) were the most abundant in the first year, whereas MUFA, linolenic acid, and GABA were the most abundant in the second year. PCA loadings also evidenced the highest biological activities shown by the first-year samples, except for α-amylase and tyrosinase inhibition values.

The annual variation can be accounted for by the effect of different pedoclimatic conditions as also previously reported for other berries.[41] In the experimental design, blueberries were harvested in the same period of the year (June−August) and cultivated in the same field. In addition, the two harvesting years were selected, because the meteorological data indicated significant variations. Comparing the two years, the average temperature in 2022 was higher compared to that in 2021. Regarding total rainfall for the month, the high levels were registered in June (33.6 mm) for 2021 and in July/August (70.0 mm) for 2022. This could have influenced or modulated

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

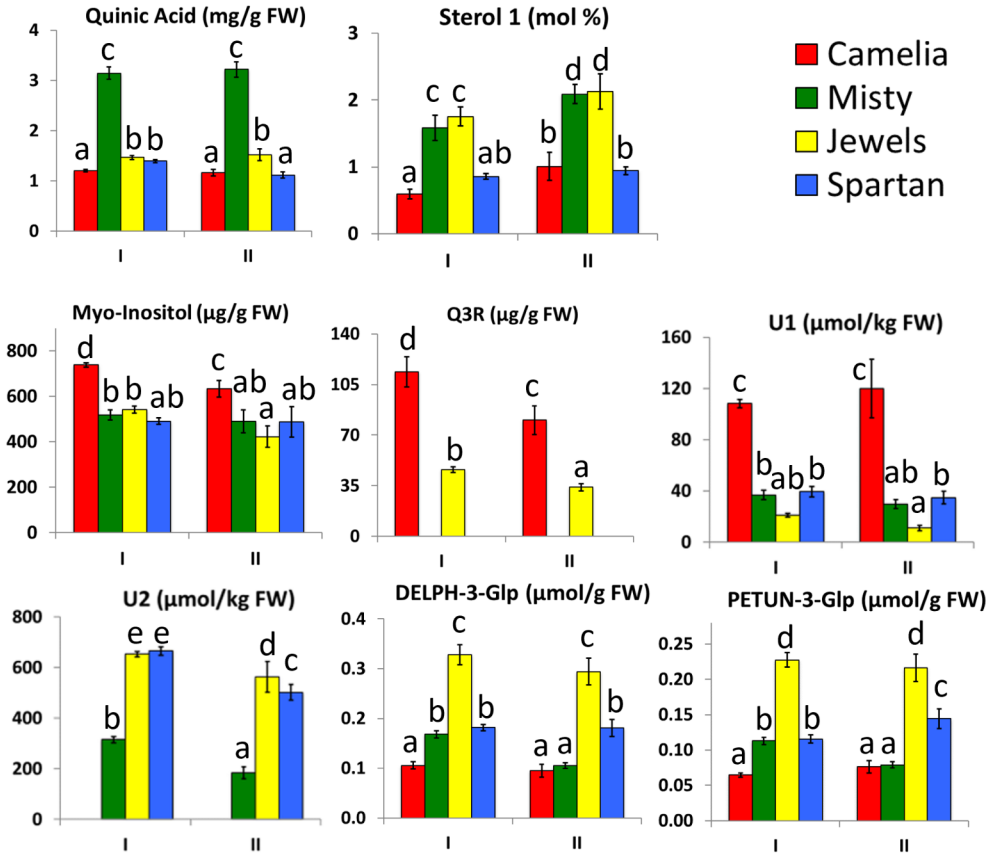

Figure 4. Histograms (mean values ± S.D., $n = 5$) of selected markers. The different letters above boxes indicate the significant difference between mean values ($p < 0.05$) according to ANOVA. Camelia, red bars; Misty, green bars; Jewels, yellow bars; Spartan, blue bars; (I) and (II) indicate the first (I) and the second (II) year of harvest.

the content of specific bioactive compounds and the healthy properties of this "superfruit". In the literature, the effect of harvesting year and/or different pedoclimatic conditions on blueberry fruit composition is scarcely investigated. For example, in a 3-year study of quality parameters that included blueberry among other 14 tree fruit and berry species, only FRAP assay showed a significant annual variation, whereas significant interactions between species and harvesting year have been observed for dry matter, soluble solids, pH, ascorbic acid and FRAP.[30] It is worth mentioning that these quality parameters did not include metabolite profiling and therefore cannot be used as a reference for the present study. Another limitation of this analysis was the lack of information regarding berries' exposure to the sun for the time period considered.

In the case of the PC2 vs PC3 scores plot, the influence of year is quite negligible, especially for PC3 scores; therefore, this plot can be used for discovering the relationships between metabolite profile and cultivar that persist in spite of year-associated variability. The distribution of samples along PC3 allows one to distinguish Jewels samples from all other cultivars owing to the highest loadings from three anthocyanins (P3-Glp, M3-Glp, and D3-Glp) corresponding to the highest level of these metabolites in Jewels extracts. Misty and Camelia cultivar scores along PC3 are similar, but their PC2 scores are completely different, indicating that the metabolites responsible for the differences between these two cultivars can be found on the combined scores and loadings plot as follows: quinic acid for Misty, and MI, U1, and Q3R for Camelia. In fact, Misty has shown the highest level of quinic acid among all other samples, whereas MI, U1, and Q3R levels were the highest in the case of Camelia. No specific markers for the Spartan cultivar have been found among the analyzed metabolites. Besides, the PC2 vs PC3 loading plot shows that the highest CUPRAC, ABTS, TPC, Tyr_Ing, FRAP, and DPPH values were associated with the Misty cultivar, and the lowest ones were found for extracts from Camelia.

The obtained results suggested that environmentally induced variations of the chemical profile and biological activities have to be taken into consideration when a comparison between different berry cultivars or species is performed. The ideal experimental design should include samples with different genetic backgrounds under the same environmental conditions, and repetition of the entire experimental set after natural or controlled variation in environmental conditions is mandatory.

3.3.2. ANOVA and Markers of Cultivars. The results of quantitative analysis and PCA indicated that the level of some metabolites was strictly related to the cultivar, despite year-associated variability due to the natural variation of climatic conditions. To confirm these results, a multifactorial analysis of variance (ANOVA) was carried out considering the harvesting year and cultivar as two independent factors and taking into account the interaction between them. The results of ANOVA for three different metabolite fractions are reported in Table S8. Two key parameters (F-value and p-level) were considered for each metabolite and the data were grouped and sorted according to the cultivar separation (beginning from the highest/lowest F-value/p-level, respectively (see Table S8). A

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

substantial and statistically significant year-cultivar interaction is present for a major part of the variables, as indicated by high F-values in the Interaction column. The combination of relatively low F-values in the columns Year and Interaction, as well as high F-values in the column Cultivar, is therefore an indicator of year-independent cultivar markers. For example, valine seems to show one of the highest F-values in the Cultivar column but contemporarily has high F-values in the Interaction and Year columns; therefore, it cannot be considered a cultivar marker. In fact, the valine level in Jewels was considerably higher than in the other three cultivars in the first year, but drastically dropped in the second year and became comparable with those in the Spartan cultivar (see Figure 1).

Taking into consideration ANOVA, PCA and quantitative analysis, selected metabolites reported in Figure 4 can be considered robust markers of the investigated blueberry cultivars. In the case of Camelia, the levels of Q3R, *myo*-inositol, and U1 were always significantly higher with respect to other cultivars. The highest level of quinic acid characterized the Misty cultivar, whereas the highest levels of P3Glp and D3Glp were characteristic of the Jewels cultivar. The absence or a low level of a particular metabolite can also be a marker of the cultivar as in the case of Q3R, absent or under the detection limit of the method, in Misty and Spartan cultivars, or U2 absent or under the detection limit of the method in Camelia and significantly lower in Misty with respect to Jewels and Spartan cultivars. Finally, "group" markers also can be considered as, for example, the Sterol1 level significantly higher in both Misty and Jewels with respect to Camelia and Spartan, or U2 level in Jewels and Spartan blueberry cultivars (see Figure 4).

3.3.3. Pearson's Correlations. Pearson correlations between metabolite levels and antioxidant and enzyme inhibitory effects are reported in the heat map in Figure 5. Moreover, the correlations among different indicators of biological activities are also shown. It is clearly seen that all four indicators of antioxidant activity (DPPH, ABTS, CUPRAC, and FRAP) are strongly correlated among themselves and with TPC, whereas TFC is correlated with the PMo value. The correlation between ABTS, DPPH, and TPC was earlier observed for three different species of blueberries (*Vaccinium* spp.).[31] As expected, TPC is tightly correlated to chlorogenic acid (the most abundant phenolic compound in blueberry extract) and shikimic acid (a known precursor of aromatic compounds). TFC and PMo show significant correlations with Asp, Glu, sucrose, and two anthocyanins (AN1 and AN5). DPPH, ABTS, and FRAP antioxidant activities are correlated to chlorogenic and shikimic acids and AN1 levels. Metal-chelating activity showed a correlation with components of the anthocyanin fraction (AN6, AN7, and AN8) and Val. Alpha-glucosidase enzyme inhibitory activity is strongly correlated with TPC, TFC, PMo, DPPH, and FRAP and the following metabolite levels: Asp, Glu, shikimic acid, chlorogenic acid, AN1, and AN5. Acetylcholinesterase inhibition (AChE) showed correlations with Ile, AN6, AN7, and AN8 levels. Finally, α-amylase and tyrosinase inhibitory activities were negatively correlated with a major part of metabolites, except a few amino acids, especially in the case of GABA and Ala positively correlated with these parameters.

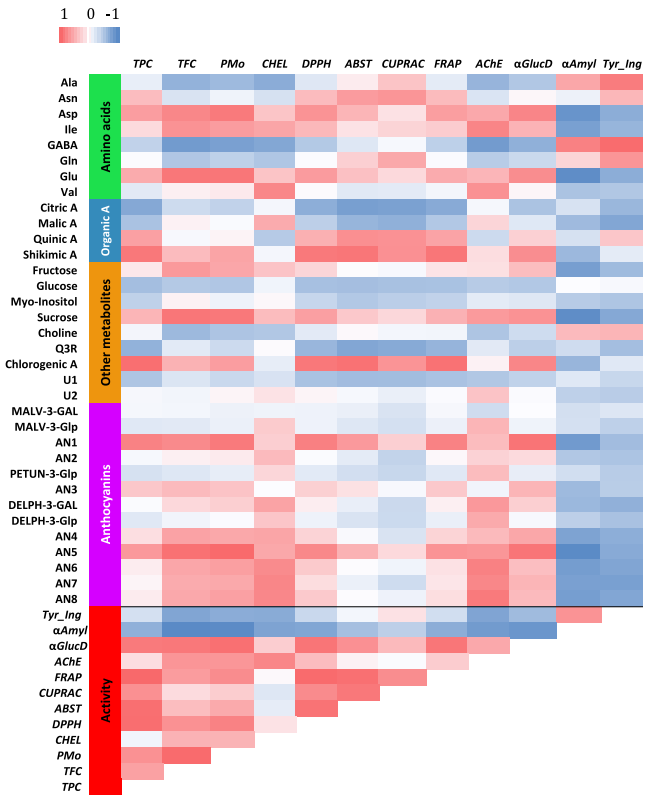

Figure 5. Heat map of Pearson's correlation.Total phenolic content (TPC); total flavonoid content (TFC); 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt (TSP); phosphomolybdenum assay (PMo); ferrozine assay (CHEL); 2,2-diphenyl-1-picrylhydrazyl assay (DPPH); 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid assay (ABTS); cupric ion reducing antioxidant capacity (CUPRAC); ferric reducing antioxidant power (FRAP); acetylcholinesterase inhibition assay (AChE); α-glucosidase inhibition assay (αGlucD); α-amylase inhibition assay (αAmyl); tyrosinase inhibition assay (Tyr_Ing); alanine (Ala); asparagine (Asn); aspartic acid (Asp); isoleucine (Ile); γ-aminobutyric acid (GABA); glutamine (Gln); glutamic acid (Glu); valine (Val); 3-*O*-α-L-rhamnopyranosyl quercetin (Q3R); citric acid (CA); quinic acid (QA); malic acid (MA); shikimic acid (SHA); unknown compound 1 (U1); unknown compound 2 (U2); malvidin-3-*O*-galactoside (MALV-3-GAL); malvidin-3-*O*-glucoside (MALV-3-Glp); anthocyanin (AN); petunidin-3-*O*-glucoside (PETUN-3-Glp); delfinidin-3-*O*-galactoside (DELPH-3-GAL); delfinidin-3-*O*-glucoside (DELPH-3-Glp).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c01442.

Table S1: means of minimum (T Min) and maximum (T Max) air temperatures, total rainfall, average humidity (AH) and potential evapotranspiration (ET0) throughout the periods of study from June to August in 2021 and 2022 cropping season; Table S2: proton NMR spectral acquisition and processing parameters; Table S3: selected ^{1}H NMR signals for the integration and quantification of metabolites in water-soluble blueberry fruit extracts; Table S4: selected ^{1}H NMR signals for the integration and quantification of anthocyanins in water-soluble blueberry fruit extracts; Table S5: the average content of anthocyanins in blueberry fruits according to NMR analysis and the

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

literature data;[38] Table S6: radical scavenging ability of the tested samples (%); Table S7: enzyme inhibition ability of the tested samples (%); Table S8: selected ^{1}H NMR signals for the integration and formulas for quantification of liposoluble metabolites in blueberry fruit extracts; Table S9: enzyme inhibitory effects of the four blueberry cultivars; Table S10: multifactorial ANOVA; Figure S1: proton NMR spectrum at 600.13 MHz of blueberry aqueous extract from Camelia blueberry cultivar dissolved in D_2O and 400 mM phosphate buffer (pH = 7.0); Figure S2: selected regions of ^{1}H−^{1}H TOCSY NMR spectrum of blueberry aqueous extract from Spartan blueberry cultivar; Figure S3: histograms report mean values and standard deviations for anthocyanins in the fruits of four blueberry cultivars (Camelia, red bars; Misty, green bars; Jewels, yellow bars; Spartan, blue bars) in the first (I) and the second (II) year of harvest; Figure S4: proton NMR spectrum at 600.13 MHz of blueberry organic extract from Camelia blueberry cultivar dissolved in $CDCl_3/CD_3OD$ 2:1 v/v; Figure S5: histograms report mean values and standard deviations for liposoluble metabolites in the fruits of four blueberry cultivars (Camelia, red bars; Misty, green bars; Jewels, yellow bars; Spartan, blue bars) in the first (I) and the second (II) year of harvest (PDF)

AUTHOR INFORMATION

Corresponding Author

Luisa Mannina − *Food Chemistry Lab, Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome 00185, Italy;* orcid.org/0000-0001-8659-5890; Email: luisa.mannina@uniroma1.it

Authors

Mattia Spano − *Food Chemistry Lab, Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome 00185, Italy;* orcid.org/0000-0001-5035-0971

Giacomo Di Matteo − *Food Chemistry Lab, Department of Chemistry and Technology of Drugs, Sapienza University of Rome, Rome 00185, Italy*

Simone Carradori − *Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy;* orcid.org/0000-0002-8698-9440

Marcello Locatelli − *Department of Pharmacy, "G. d'Annunzio" University of Chieti-Pescara, Chieti 66100, Italy*

Gokhan Zengin − *Department of Biology, Science Faculty, Selcuk University, Konya 42130, Turkey*

Anatoly Petrovich Sobolev − *Magnetic Resonance Laboratory "Segre-Capitani", Institute for Biological Systems, Monterotondo 00015, Italy*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.4c01442

Funding

This project was partially funded under the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.3—Call for proposals No. 341 of 15 March 2022 of the Italian Ministry of University and Research funded by the European Union—NextGenerationEU. Award Number: Project code PE00000003, Concession Decree No. 1550 of 11 October 2022 adopted by the Italian Ministry of University and Research, CUP D93C22000890001, Project title "ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security—Working ON Foods". Ministero Italiano della Salute, "Mediterranean Diet for Human Health Lab" project (MeDiHealthLab)—No.T5-AN-07 (POS5) CUP: B83C22005580006.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the contribution of Simone Circi in the processing of NMR data.

ABBREVIATIONS

NMR, nuclear magnetic resonance; BBB, blood-brain barrier; UPLC-TOF-MS, ultrahigh performance liquid chromatography with time-of-flight mass spectrometry; TPC, total phenolic content; TFC, total flavonoid content; TSP, 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid sodium salt; HPLC, high-performance liquid chromatography; Na_2CO_3, sodium carbonate; $AlCl_3 \times 6H_2O$, aluminum chloride hexahydrate; EDTA, ethylenediamine tetraacetic acid; SPE, solid phase extraction; TFA, trifluoroacetic acid; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; TMS, tetramethylsilane; GAE, gallic acid equivalents; RE, rutin equivalents; DW, dried weight; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power; CUPRAC, cupric ion reducing antioxidant capacity; PMo, phosphomolybdenum assay; CHEL, ferrozine assay; TE, Trolox standard; EDTAE, EDTA equivalents; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GALAE, galanthamine equivalents; KAE, kojic acid equivalents; ACAE, acarbose equivalents; FW, fresh weight; PCA, principal component analysis; Q3R, 3-*O*-α-L-rhamnopyranosyl quercetin; CA, citric acid; QA, quinic acid; MA, malic acid; SHA, shikimic acid; Gln, glutamine; Glu, glutamic acid; Asp, aspartic acid; Ile, isoleucine; Val, valine; GABA, γ-aminobutyric acid; Ala, alanine; Asn, asparagine; CGA, chlorogenic acid; U1, unknown compound 1; (U2, unknown compound 2; TOCSY, total correlation spectroscopy; M3Glp, malvidin-3-*O*-glucoside; M3Gal, malvidin-3-*O*-galactoside; D3Glp, delfinidin-3-*O*-glucoside; D3Gal, delfinidin-3-*O*-galactoside; P3Glp, petunidin-3-*O*-glucoside; AN, anthocyanin; TG, triacylglycerols; PC, diacylglycerophosphocholine; DG, digalactosyldiacylglycerol; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; MI, *myo*-inositol; Tyr_Ing, tyrosinase inhibitory activity

REFERENCES

(1) González-Gallego, J.; García-Mediavilla, M. V.; Sánchez-Campos, S.; Tuñó, M. J. Fruit Polyphenols, Immunity and Inflammation. *Br. J. Nutr.* **2010**, *104*, S15−S27.

(2) Basu, A.; Du, M.; Leyva, M. J.; Sanchez, K.; Betts, N. M.; Wu, M.; Aston, C. E.; Lyons, T. J. Blueberries Decrease Cardiovascular Risk Factors in Obese Men and Women with Metabolic Syndrome. *J. Nutr.* **2010**, *140* (9), 1582−1587.

(3) Martineau, L. C.; Couture, A.; Spoor, D.; Benhaddou-Andaloussi, A.; Harris, C.; Meddah, B.; Leduc, C.; Burt, A.; Vuong, T.; Mai Le, P.; et al. Anti-Diabetic Properties of the Canadian Lowbush Blueberry Vaccinium Angustifolium Ait. *Phytomedicine* **2006**, *13* (9−10), 612−623.

(4) Stull, A. J.; Cash, K. C.; Johnson, W. D.; Champagne, C. M.; Cefalu, W. T. Bioactives in Blueberries Improve Insulin Sensitivity in Obese, Insulin-Resistant Men and Women. *J. Nutr.* **2010**, *140* (10), 1764−1768.
(5) Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. The Effect of Fruit and Vegetable Intake on Risk for Coronary Heart Disease. *Ann. Int. Med.* **2001**, *134*, 12.
(6) Seeram, N. P. Berry Fruits for Cancer Prevention: Current Status and Future Prospects. *J. Agric. Food Chem.* **2008**, *56*, 630−635.
(7) Mollica, A.; Locatelli, M.; Macedonio, G.; Carradori, S.; Sobolev, A. P.; De Salvador, R. F.; Monti, S. M.; Buonanno, M.; Zengin, G.; Angeli, A.; et al. Microwave-Assisted Extraction, HPLC Analysis, and Inhibitory Effects on Carbonic Anhydrase I, II, VA, and VII Isoforms of 14 Blueberry Italian Cultivars. *J. Enzyme Inhib. Med. Chem.* **2016**, *31*, 1−6.
(8) Shi, M.; Loftus, H.; McAinch, A. J.; Su, X. Q. Blueberry as a Source of Bioactive Compounds for the Treatment of Obesity, Type 2 Diabetes and Chronic Inflammation. *J. Funct. Foods* **2017**, *30*, 16−29.
(9) Andres-Lacueva, C.; Shukitt-Hale, B.; Galli, R. L.; Jauregui, O.; Lamuela-Raventos, R. M.; Joseph, J. A. Anthocyanins in Aged Blueberry-Fed Rats Are Found Centrally and May Enhance Memory. *Nutr. Neurosci.* **2005**, *8* (2), 111−120.
(10) Naveed, M.; Hejazi, V.; Abbas, M.; Kamboh, A. A.; Khan, G. J.; Shumzaid, M.; Ahmad, F.; Babazadeh, D.; FangFang, X.; Modarresi-Ghazani, F.; et al. Chlorogenic Acid (CGA): A Pharmacological Review and Call for Further Research. *Biomed. Pharmacother.* **2018**, *97*, 67−74.
(11) Cásedas, G.; Les, F.; Gómez-Serranillos, M. P.; Smith, C.; López, V. Anthocyanin profile, antioxidant activity and enzyme inhibiting properties of blueberry and cranberry juices: a comparative study. *Food Funct.* **2017**, *8* (11), 4187−4193.
(12) Hwang, H.; Kim, Y. J.; Shin, Y. Assessment of Physicochemical Quality, Antioxidant Content and Activity, and Inhibition of Cholinesterase between Unripe and Ripe Blueberry Fruit. *Foods* **2020**, *9* (6), 690.
(13) Mishra, P.; Kumar, A.; Panda, G. Anti-Cholinesterase Hybrids as Multi-Target-Directed Ligands against Alzheimer's Disease (1998−2018). *Bioorg. Med. Chem.* **2019**, *27*, 895−930.
(14) Okan, O. T.; Deniz, I.; Yayli, N.; Şat, I. G.; Öz, M.; Serdar, G. H. Antioxidant Activity, Sugar Content and Phenolic Profiling of Blueberries Cultivars: A Comprehensive Comparison. *Not. Bot. Horti Agrobot. Cluj-Napoca* **2018**, *46* (2), 639−652.
(15) Teixeira Barcia, M.; Gómez-Alonso, S.; Teixeira Godoy, H.; Hermosin-Gutierrez, I. Phenolics Profiling by HPLC-DAD-ESI-MSn Aided by Principal Component Analysis to Classify Rabbiteye and Highbush Blueberries. *Food Chem.* **2021**, *340*, 127958.
(16) Das, P. R.; Darwish, A. G.; Ismail, A.; Haikal, A. M.; Gajjar, P.; Balasubramani, S. P.; Sheikh, M. B.; Tsolova, V.; Soliman, K. F. A.; Sherif, S. M.; et al. Diversity in Blueberry Genotypes and Developmental Stages Enables Discrepancy in the Bioactive Compounds, Metabolites, and Cytotoxicity. *Food Chem.* **2022**, *374*, 131632.
(17) Sobolev, A. P.; Ingallina, C.; Spano, M.; Di Matteo, G.; Mannina, L. NMR-Based Approaches in the Study of Foods. *Molecules* **2022**, *27* (12), 7906.
(18) da Franca, L. G.; Alves Filho, E.; Ribeiro, L. B.; Evangelista, J. S. B.; Silva, L. M.; de Souza, P. A.; Moura, C. F. H.; Canuto, K. M.; de Aragão, F. A. S. Metabolomic Profiling of Acerola Clones According to the Ripening Stage. *J. Food Meas. Charact.* **2021**, *15* (1), 416−424.
(19) Gallo, V.; Mastrorilli, P.; Cafagna, I.; Nitti, G. I.; Latronico, M.; Longobardi, F.; Minoja, A. P.; Napoli, C.; Romito, V. A.; Schäfer, H.; et al. Effects of Agronomical Practices on Chemical Composition of Table Grapes Evaluated by NMR Spectroscopy. *J. Food Compos. Anal.* **2014**, *35* (1), 44−52.
(20) Capitani, D.; Mannina, L.; Proietti, N.; Sobolev, A. P.; Tomassini, A.; Miccheli, A.; Di Cocco, M. E.; Capuani, G.; De Salvador, F. R.; Delfini, M. Metabolic Profiling and Outer Pericarp Water State in Zespri, CI.GI, and Hayward Kiwifruits. *J. Agric. Food Chem.* **2013**, *61* (8), 1727−1740.
(21) Spano, M.; Ingallina, C.; Botta, B.; Quaglio, D.; Ghirga, F.; Balducci, S.; Cammarone, S.; Campiglia, E.; Giusti, A. M.; Vinci, G.; et al. A Multimethodological Characterization of Cannabis Sativa l. Inflorescences from Seven Dioecious Cultivars Grown in Italy: The Effect of Different Harvesting Stages. *Molecules* **2021**, *26* (10), 2912.
(22) Spano, M.; Di Matteo, G.; Ingallina, C.; Sobolev, A. P.; Giusti, A. M.; Vinci, G.; Cammarone, S.; Tortora, C.; Lamelza, L.; Prencipe, S. A.; et al. Industrial Hemp (Cannabis Sativa L.) Inflorescences as Novel Food: The Effect of Different Agronomical Practices on Chemical Profile. *Foods* **2022**, *11* (22), 3658.
(23) Gil, A. M.; Duarte, I. F.; Delgadillo, I.; Colquhoun, I. J.; Casuscelli, F.; Humpfer, E.; Spraul, M. Study of the Compositional Changes of Mango during Ripening by Use of Nuclear Magnetic Resonance Spectroscopy. *J. Agric. Food Chem.* **2000**, *48* (5), 1524−1536.
(24) Biais, B.; Allwood, J. W.; Deborde, C.; Xu, Y.; Maucourt, M.; Beauvoit, B.; Dunn, W. B.; Jacob, D.; Goodacre, R.; Rolin, D.; et al. 1H NMR, GC-EI-TOFMS, and Data Set Correlation for Fruit Metabolomics: Application to Spatial Metabolite Analysis in Melon. *Anal. Chem.* **2009**, *81* (8), 2884−2894.
(25) Spano, M.; Maccelli, A.; Matteo, G. D.; Ingallina, C.; Biava, M.; Crestoni, M. E.; Bardaud, J. X.; Giusti, A. M.; Mariano, A.; D'abusco, A. S.; Sobolev, A. P.; Lasalvia, A.; Fornarini, S.; Mannina, L. Metabolomic Profiling of Fresh Goji (Lycium Barbarum l.) Berries from Two Cultivars Grown in Central Italy: A Multi-Methodological Approach. *Molecules* **2021**, *26* (17), 5412.
(26) Roch, L.; Prigent, S.; Klose, H.; Cakpo, C. B.; Beauvoit, B.; Deborde, C.; Fouillen, L.; Van Delft, P.; Jacob, D.; Usadel, B.; et al. Biomass Composition Explains Fruit Relative Growth Rate and Discriminates Climacteric from Non-Climacteric Species. *J. Exp. Bot.* **2020**, *71* (19), 5823−5836.
(27) Capitani, D.; Sobolev, A. P.; Delfini, M.; Vista, S.; Antiochia, R.; Proietti, N.; Bubici, S.; Ferrante, G.; Carradori, S.; Salvador, F. R. D.; et al. NMR Methodologies in the Analysis of Blueberries. *Electrophoresis* **2014**, *35* (11), 1615−1626.
(28) Bett-Garber, K. L.; Lea, J. M.; Watson, M. A.; Grimm, C. C.; Lloyd, S. W.; Beaulieu, J. C.; Stein-Chisholm, R. E.; Andrzejewski, B. P.; Marshall, D. A. Flavor of Fresh Blueberry Juice and the Comparison to Amount of Sugars, Acids, Anthocyanidins, and Physicochemical Measurements. *J. Food Sci.* **2015**, *80* (4), S818−S827.
(29) Silva, S.; Costa, E. M.; Veiga, M.; Morais, R. M.; Calhau, C.; Pintado, M. Health Promoting Properties of Blueberries: A Review. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 181−200.
(30) Skrede, G.; Martinsen, B. K.; Wold, A. B.; Birkeland, S. E.; Aaby, K. Variation in Quality Parameters between and within 14 Nordic Tree Fruit and Berry Species. *Acta Agric. Scand. Sect. B Soil Plant Sci.* **2012**, *62* (3), 193−208.
(31) Lee, S.; Jung, E. S.; Do, S. G.; Jung, G. Y.; Song, G.; Song, J. M.; Lee, C. H. Correlation between Species-Specific Metabolite Profiles and Bioactivities of Blueberries (Vaccinium Spp.). *J. Agric. Food Chem.* **2014**, *62* (9), 2126−2133.
(32) Moon, J. K.; Shibamoto, T. Antioxidant Assays for Plant and Food Components. *J. Agric. Food Chem.* **2009**, *57*, 1655−1666.
(33) SIARL − Servizio Integrato Agrometeorologico della Regione Lazio Servizio Integrato Agronometrico della Regione Lazio http://www.arsial.it/portalearsial/agrometeo/. (accessed 2023 October 17).
(34) Wyzgoski, F. J.; Paudel, L.; Rinaldi, P. L.; Reese, R. N.; Ozgen, M.; Tulio, A. Z.; Miller, A. R.; Scheerens, J. C.; Hardy, J. K. Modeling Relationships among Active Components in Black Raspberry (Rubus Occidentalis L.) Fruit Extracts Using High-Resolution1H Nuclear Magnetic Resonance (NMR) Spectroscopy and Multivariate Statistical Analysis. *J. Agric. Food Chem.* **2010**, *58* (6), 3407−3414.
(35) Balli, D.; Cecchi, L.; Khatib, M.; Bellumori, M.; Cairone, F.; Carradori, S.; Zengin, G.; Cesa, S.; Innocenti, M.; Mulinacci, N. Characterization of Arils Juice and Peel Decoction of Fifteen Varieties

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

of Punica Granatum L.: A Focus on Anthocyanins, Ellagitannins and Polysaccharides. *Antioxidants* **2020**, *9*, 238.
(36) Menghini, L.; Leporini, L.; Vecchiotti, G.; Locatelli, M.; Carradori, S.; Ferrante, C.; Zengin, G.; Recinella, L.; Chiavaroli, A.; Leone, S.; Brunetti, L.; Orlando, G. Crocus sativus L. stigmas and byproducts: Qualitative fingerprint, antioxidant potentials and enzyme inhibitory activities. *Food Res. Int.* **2018**, *109*, 91−98.
(37) *U.S. Department of Agriculture, A. R. S. FoodData* https://fdc.nal.usda.gov/. (accessed 2023 October 10).
(38) *Phenol-Explorer* http://phenol-explorer.eu/. (accessed 2023 October 10).
(39) Jackson, D. M.; Ashley, R. L.; Brownfield, C. B.; Morrison, D. R.; Morrison, R. W. Rapid Conventional and Microwave-Assisted Decarboxylation of L-Histidine and Other Amino Acids via Organocatalysis with R-Carvone under Superheated Conditions. *Synth. Commun.* **2015**, *45* (23), 2691−2700.
(40) Xu, Y.; Xie, L.; Xie, J.; Liu, Y.; Chen, W. Pelargonidin-3-O-Rutinoside as a Novel α-Glucosidase Inhibitor for Improving Postprandial Hyperglycemia. *Chem. Commun.* **2019**, *55* (1), 39−42.
(41) Pereira, G. E.; Gaudillere, J. P.; Pieri, P.; Hilbert, G.; Maucourt, M.; Deborde, C.; Moing, A.; Rolin, D. Microclimate Influence on Mineral and Metabolic Profiles of Grape Berries. *J. Agric. Food Chem.* **2006**, *54* (18), 6765−6775.

https://doi.org/10.1021/acs.jafc.4c01442
J. Agric. Food Chem. XXXX, XXX, XXX−XXX