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Metabolite profiling of polyphenols in *Vaccinium* berries and determination of their chemopreventive properties

Francesco Pio Prencipe[a], Renato Bruni[b], Alessandra Guerrini[c], Damiano Rossi[c], Stefania Benvenuti[a], Federica Pellati[a,*]

[a] Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 183, 41125 Modena, Italy
[b] Department of Food Science-LS9 Interlab Group, University of Parma, Via G.B. Usberti 95/a, 43134 Parma, Italy
[c] Department of Life Sciences and Biotechnologies-LT Terra&Acqua Tech UR7, University of Ferrara, Corso Ercole I d'Este 32, 44121 Ferrara, Italy

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ABSTRACT

A detailed investigation on the chemical composition and chemopreventive activity of *Vaccinium floribundum* Kunth berries was carried out in comparison with *Vaccinium myrtillus* L. Berry polyphenols were extracted by using two sequential dynamic maceration steps, which enabled to maximize the yields of secondary metabolites. In particular, phenolic acids and flavonols were extracted from berries using ethyl acetate (EtOAc), whereas anthocyanins were extracted from the residue with 0.6 M HCl in methanol (MeOH).

The analysis of secondary metabolites in berry extracts was performed by means of two specific HPLC methods. Phenolic acids and flavonols were analyzed on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 μm), with a gradient mobile phase composed of 0.1 M HCOOH in H_2O and ACN. Anthocyanin analysis was carried out on a Zorbax SB-C_{18} column (150 mm × 4.6 mm I.D., 5 μm), with a gradient mobile phase composed of H_2O–HCOOH (9:1, v/v) and MeOH–H_2O–HCOOH (5:4:1, v/v/v). Detection was performed by UV/DAD, MS and MS^2.

The polyphenol composition of *V. floribundum* and *V. myrtillus* was studied in detail. The samples of *V. floribundum* analyzed in this study had a much higher content of both phenolic acids and flavonols in comparison with *V. myrtillus* (mean value 41.6 ± 10.2 and 13.7 ± 0.2 mg/100 g FW, respectively), while *V. myrtillus* showed a higher amount of anthocyanins if compared with *V. floribundum* (568.8 ± 8.8 and mean value 376.2 ± 49.9 mg/100 g FW, respectively).

The extracts gave negative results in antimutagenic assays against carcinogens 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 4-nitroquinoline-1-oxide (4-NQO), while they performed similarly in both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ antioxidant assays.

1. Introduction

The research on phenolic compounds in fruit and on their health-related properties has grown enormously in the last twenty years [1]. In this ambit, berries and their derived products have demonstrated to possess several positive effects on human health, due to the high content of polyphenols and to the chemopreventive activity against a wide range of degenerative diseases, involving oxidative stress, inflammation and carcinogenesis [2]. From a phytochemical point of view, berry phenolic compounds are a diverse and composite group, including anthocyanins, phenolic acids and flavonoids [3]. Berry polyphenols have been identified as strong antioxidant, especially anthocyanins, with the potential to prevent oxidative damage caused by reactive oxygen species [4].

Among berry fruits, *Vaccinium myrtillus* L. (bilberry), which is a spontaneous species native to Europe, has received a large attention, because of its traditional use in folk medicine and diet. Several studies have demonstrated the benefit of bilberry in the inhibition of cancer cell growth [5], in management of visual disorders [6] and in the prevention of the onset of various pathologies, including metabolic and degenerative diseases [5]. Moreover, bilberry extracts have demonstrated both atheroprotective and hypoglycemic properties [7,8].

Vaccinium floribundum Kunth, commonly known as Mortiño, is a wild shrub native to South America, whose berries are widely consumed in Ecuador as fresh fruit or processed products, like juice and jam [9]. In addition to the nutritional value, local communities use the extracts of this plant to treat various medical conditions, including diabetes and inflammation [9]. Despite the wide local use of this

Abbreviations: $ABTS^{\bullet+}$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline; 4-NQO, 4-nitroquinoline-1-oxide.

* Corresponding author. Tel.: +39 059 205 5144; fax: +39 059 205 5131.
E-mail address: federica.pellati@unimore.it (F. Pellati).

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berry, only two studies have been described in the literature on *V. floribundum* fruit [10,11] and one on derived products [12]. Even if the chemical composition of *V. floribundum* has been described [10,11], the detailed polyphenol characterization and the biological properties of its berry extracts have not been deeply investigated. Considering the significant health-promoting properties of plant polyphenols, a study on *V. floribundum* extracts can be helpful both to suggest their role in human diet and also to gather information on their potential use in food supplements and phytotherapy. At the same time and due to their marketplace value, *Vaccinium* berries have been frequently the object of adulteration and sophistication, thus highlighting the need for proper and reliable fingerprinting protocols enforceable in quality control [13].

In this study, a detailed metabolite profiling of polyphenols in *V. floribundum* berry samples from Ecuador was carried out for the first time by means of HPLC-UV/DAD, HPLC-ESI-MS and MS^2, using an ion trap mass analyzer. As a comparison, *V. myrtillus* berries from the Italian Northern Apennines were analyzed in parallel, due to the wide use of this species in the pharmaceutical and nutraceutical fields. A sequential selective extraction procedure was developed to maximize the yields of berry secondary metabolites, using ethyl acetate (EtOAc) for phenolic acids and flavonols, followed by acidified methanol (MeOH) for anthocyanins.

Two specific HPLC methods were developed for each extract in order to study *V. floribundum* phenolic compounds. The flavonoid composition of *V. floribundum* berries was characterized in detail in this study for the first time. After the MS and MS^2 characterization of secondary metabolites, phenolic acids, flavonols and anthocyanins were quantified using validated methods based on HPLC-UV/DAD, which is widely used for qualitative and quantitative analysis of polyphenols in plant extracts [14,15].

The chemopreventive activity of *V. floribundum* and *V. myrtillus* berry extracts was evaluated by the determination of their antioxidant and radical-scavenging activity using a panel of *in vitro* tests, including spectrophotometric and HPTLC bioautographic assays based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), while their antimutagenic properties were assessed against dietary mutagens 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ) and 4-nitroquinoline-1-oxide (4-NQO) in the *Salmonella* and SOS chromotest assays, respectively. The results of these biological assays were compared with the chemical composition of the two investigated *Vaccinium* species to establish the potential application of *V. floribundum* as a source of phytochemicals in the nutraceutical and functional food industry.

2. Materials and methods

2.1. Chemicals and solvents

Chlorogenic acid, caffeic acid, quercetin-3-*O*-galactoside, ABTS diammonium salt, $DPPH^{\bullet}$ and 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich-Fluka (Milan, Italy). Quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside and cyanidin-3-*O*-glucoside were from Extrasynthese (Genay, France). Delphinidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside were from Sequoia Research Products Ltd. (Pangbourne, United Kingdom). All compounds were of chromatographic grade. Quercetin-3-*O*-arabinopyranoside and quercetin-3-*O*-arabinofuranoside were kindly gifted by Prof. Shoei-Sheng Lee, School of Pharmacy, National Taiwan University.

Formic acid (HCOOH), hydrochloric acid (37%) (HCl), HPLC-grade acetonitrile (ACN), MeOH, EtOAc and dimethylsulfoxide (DMSO) were from Sigma-Aldrich-Fluka (Milan, Italy). Water (H_2O) was purified using a Milli-Q Plus185 system from Millipore (Milford, MA, USA).

Escherichia coli PQ37 strain was purchased from Ecotox inside the SOS chromotest kit (Environmental Bio-Detection Products Inc., Ontario, Canada). Broth ingredients were purchased from Oxoid and Sigma-Aldrich; MeIQ was supplied by Toronto Research Chemicals Inc. (Toronto, Canada). All the microbial culture media were from Oxoid Italia (Garbagnate, Italy). Lyophilized post-mitochondrial supernatant S9 fraction was purchased from Molecular Toxicology Inc. (Boone, NC, USA).

2.2. Berry samples

Five samples of *V. floribundum* Kunth berries (250 g each), harvested around Ilinizas, a pair of volcanic mountains in the south of Quito (Ecuador), were kindly provided by Dr. Paolo Grasso, University of Modena and Reggio Emilia (Italy). These samples were indicated in the text as VF-1/VF-5.

For comparison purpose, one sample of *V. myrtillus* L. berries (350 g) was collected from the Italian Northern Apennines.

Fruit samples were collected from both species during the berry season in 2012, frozen and stored at -20 °C until analysis.

2.3. Extraction of phenolic compounds

Phenolic acids and flavonols were extracted from a weighted amount of frozen berries (2.2 g) by dynamic maceration with 20 ml of EtOAc at room temperature for 20 min. The extract was then paper filtered and the extraction procedure was repeated three times on the residue. The extracts were combined, concentrated at 40 °C under vacuum and brought to 25 ml in a volumetric flask. An aliquot of 10 ml of the extract was further concentrated to 1 ml for HPLC analysis.

Anthocyanins were sequentially extracted from the berry residue of the procedure described above, using dynamic maceration with 0.6 M HCl in MeOH, using the same solvent volume and time as previously described. Finally, the four extracts were pooled, concentrated at 40 °C under vacuum and brought to 25 ml in a volumetric flask.

The extraction procedure was repeated twice for each sample. The extracts were filtered using a 0.45 μm PTFE filter into a HPLC vial and injected into the HPLC system.

2.4. HPLC-UV/DAD analysis

HPLC analyses were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostated column compartment and a diode array detector (DAD). The chromatograms were recorded using an Agilent Chemstation for LC and LC–MS systems (Rev. B.01.03).

The HPLC analysis of phenolic acids and flavonols was carried out on an Ascentis C_{18} column (250 mm × 4.6 mm I.D., 5 μm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of (A) 0.1 M HCOOH in H_2O and (B) ACN. The gradient elution was modified as follows: 0–15 min from 10% to 20% B, 15–35 min from 20% to 30% B, 35–45 min from 30% to 50% B. The post-running time was 5 min. The flow rate was 1.0 ml/min. The column temperature was set at 25 °C. The sample injection volume was 5 μl. The UV/DAD acquisitions were carried out in the range 190–550 nm and chromatograms were integrated at 328 nm (for phenolic acids) and 352 nm (for flavonols). Three injections were performed for each sample.

Regarding anthocyanins, a Zorbax SB-C_{18} column (150 mm × 4.6 mm I.D., 5 μm, Agilent Technologies) was used for HPLC analysis. The mobile phase was composed of (A)

H_2O–HCOOH (9:1, v/v) and (B) MeOH–H_2O–HCOOH (5:4:1, v/v/v). The gradient elution was set as follows: 0–20 min from 10% to 60% B, 20–25 min from 60% to 80% B. The post-running time was 5 min. The flow rate was 1.0 ml/min. The column temperature was set at 25 °C. The injection volume was 5 μl. The UV/DAD acquisitions were carried out in the range 190–650 nm and chromatograms were integrated at 520 nm. Three injections were performed for each sample.

2.5. HPLC-ESI-MS and MS^2 analysis

HPLC-ESI-MS and MS^2 analyses were carried out using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, a thermostated autosampler, a thermostated column compartment and a 6310A ion trap mass analyzer with an ESI ion source. The HPLC column and the applied chromatographic conditions were the same as reported for the HPLC-UV/DAD system. The flow rate was split 5:1 before the ESI source.

The HPLC-ESI-MS system was operated both in the positive and in the negative ion modes. For the positive ion mode (used for phenolic acids, flavonols and anthocyanins), the experimental parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer (N_2) pressure was 32 psi, the drying gas temperature was 350 °C, the drying gas flow was 10 l/min and the skimmer voltage was 40 V. For the negative ion mode (used for phenolic acids and flavonols), the conditions were set as follows: the capillary voltage was 4.0 kV, the nebulizer (N_2) pressure was 35 psi, the drying gas temperature was 350 °C, the drying gas flow was 11 l/min and the skimmer voltage was 40 V.

Data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The mass spectrometer was operated in the full-scan mode in the m/z range 100–1000. MS^2 spectra were automatically performed with helium as the collision gas in the m/z range 50–1000 with the SmartFrag function.

2.6. HPLC-UV/DAD method validation

The validation of the HPLC-UV/DAD method was performed to show compliance with international requirements for analytical techniques for the quality control of pharmaceuticals (ICH guidelines) [16].

Regarding linearity, the stock standard solution of each compound (chlorogenic acid, caffeic acid, quercetin-3-*O*-galactoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and malvidin-3-*O*-glucoside) was prepared as follows: an accurately weighed amount of pure reference standard was placed into a volumetric flask (2.5 mg in 10 ml for phenolic acids; 2.2 mg in 5 ml for quercetin-3-*O*-galactoside; 0.3–0.7 mg in 1 ml for anthocyanins); the solvent (EtOAC for phenolic acids and flavonols; 0.6 M HCl in MeOH for anthocyanins) was added and the solution was diluted to volume. The external standard calibration curve was generated using five data points. 5 μl aliquots of each standard solution were used for HPLC analysis. Injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level *versus* the concentration of the sample. The amount of polyphenols in berry samples was determined by using these calibration curves, when the standard was available. All the other constituents identified in berry samples were quantified with the calibration curve of the reference standard with the same chromophore and the amounts were corrected by using the molecular weight ratio. In particular, neochlorogenic acid and caffeoylshikimic acid isomers were quantified using the calibration curve of chlorogenic acid; the caffeic acid derivative with caffeic acid; quercetin glycosides with quercetin-3-*O*-galactoside. Anthocyanin galactosides and arabinosides were quantified with the calibration curves of the corresponding glucosides.

For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were experimentally verified by HPLC analysis of serial dilutions of a standard solution to reach a signal-to-noise (S/N) ratio of 3 and 10, respectively.

The accuracy of the analytical procedure was evaluated using the recovery test. This involved the addition of a known quantity of standard compound to half the sample weight of *V. floribundum* berries (sample VF-2) to reach 100% of the test concentration. The fortified samples were then extracted and analyzed with the proposed method.

The precision of the extraction technique was validated by repeating six times the extraction procedure on the same sample of *V. floribundum* berries (sample VF-2). An aliquot of each extract was then injected and quantified. The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of berry extracts and then checking the %RSD of retention times and peak areas. Six injections were performed each day for three consecutive days.

Specificity was evaluated by the analysis of both EtOAc and acidified MeOH extracts of *V. floribundum* and *V. myrtillus* berries using both HPLC methods to verify that the secondary metabolites were not present in both solvent fractions.

Stability was tested using berry extracts stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h for 72 h.

2.7. Antioxidant activity

Measurements and evaluations of both spectrophotometric and HPTLC bioautographic $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays were performed as previously reported [17,18]. The IC_{50} values (μg/ml) of berry extracts were calculated starting from a stock solution at 0.54 mg/ml in DMSO, which was further diluted 1:2, 1:5 and 1:10 (v/v). Trolox solution was prepared at 1 mg/ml in DMSO and further diluted 1:5, 1:10, 1:50, 1:100 and 1:500 (v/v).

2.8. Mutagenicity/antimutagenicity

Dried extracts of *V. floribundum* and *V. myrtillus* were dissolved in DMSO at different concentrations (1–1000 μg/plate) and tested using plate incorporation assay on MeIQ (10^{-8} mol/plate) with *S. typhimurium* strains TA98 and TA100 (100 μl/plate of a fresh overnight culture), with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic system, as previously described [19].

2.9. SOS chromotest assay

Genotoxicity and antigenotoxicity assays were performed in accordance with Quillardet and Hofnung [20]. An aliquot of 500 μl of a bacterial culture of *E. coli* PQ37 was added to 5 ml of fresh LB medium containing 20 μg/ml ampicillin, left to grow overnight and shaken constantly at 37 °C. 1 ml of the precedent culture was added to 5 ml of fresh LB medium and was grown at 37 °C for 3.5 h. At this point, the bacterial concentration was determined as 2×10^8 UFC/ml. This solution was diluted 1:10 (v/v) with fresh LB medium and 0.6 ml were distributed into test tubes containing 20 μl of genotoxic agent (4-NQO); 20 μl of a solution of tested material at different concentrations and 2-hydroxy-4-methacryloyloxybenzaldehyde (positive control) without 4-NQO were added to the experiment (as controls) to show the possible cytotoxic or genotoxic effect of the tested phytocomplex and pure molecules. After 2 h of incubation at 37 °C, the evaluation of the genotoxic/antigenotoxic activity (β-galactosidase) and the cell viability (alkaline phosphatase) was performed.

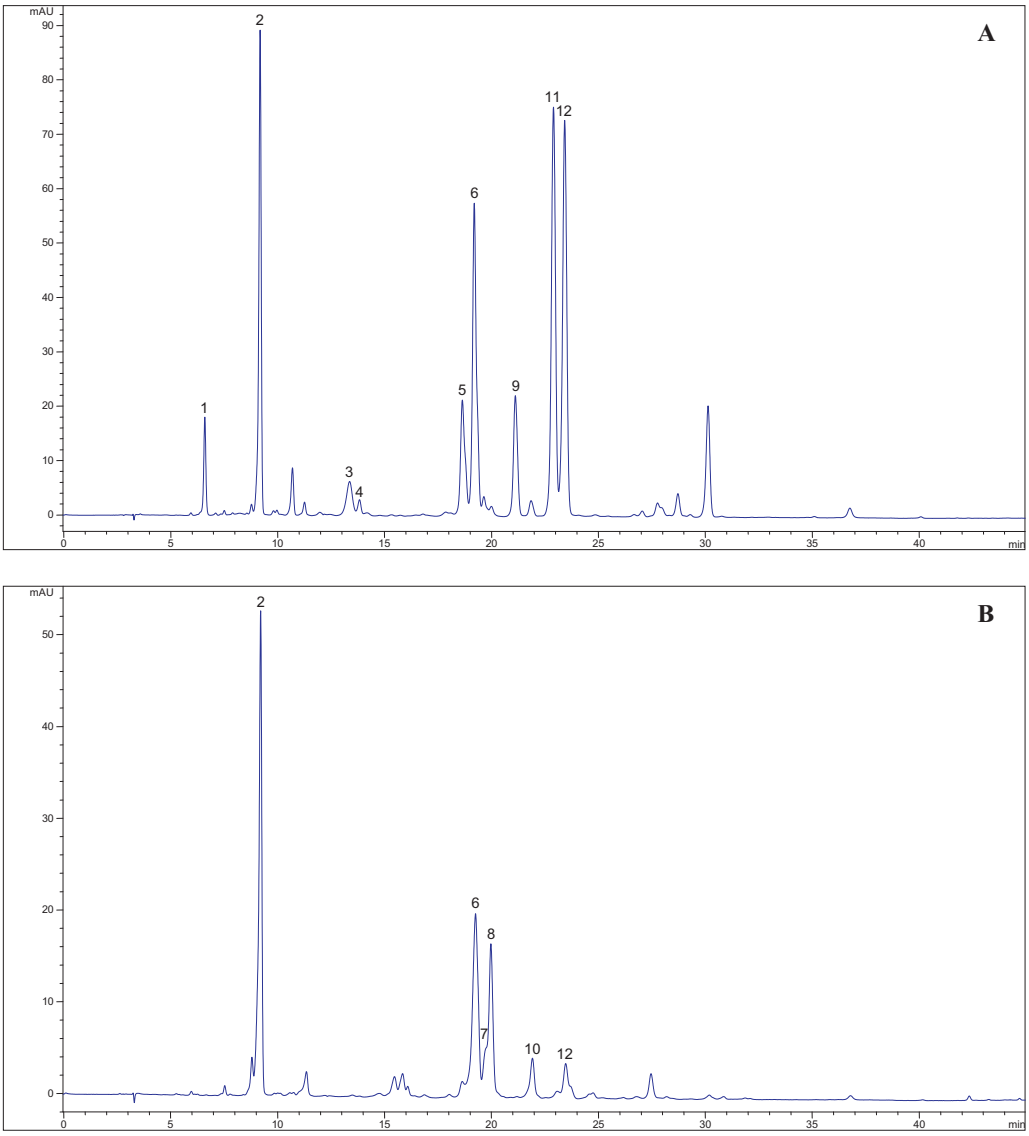

Fig. 1. Chromatograms obtained by HPLC-UV/DAD analysis of EtOAc berry extracts of *V. floribundum* (sample VF-2) (A) and *V. myrtillus* (B) at 352 nm. Experimental conditions as in Section 2.4. For peak identification, see Table 1.

The antigenotoxic assay (for β-galactosidase) was performed using 0.3 ml of the last obtained bacterial solution, which were added to 2.7 ml of B buffer. After a period of incubation of 10 min at 37 °C, 0.6 ml of a 0.4% solution of *o*-nitrophenyl-β-D-galactopyranoside were added. After other 60 min of incubation, the addition of 2 ml of 1 M sodium carbonate stopped the reaction. The absorbance of the mixture was recorded with a spectrophotometer at 420 nm.

The viability assay (for alkaline phosphatase) was carried out at the same time of the β-galactosidase assay, using 0.3 ml of bacterial solution which were added to 2.7 ml of P buffer. In this case, after a period of incubation of 10 min at 37 °C, 0.6 ml of a 0.4% solution of *p*-nitrophenyl phosphate were added. After other 60 min of incubation, the addition of 1 ml of 2.5 M HCl stopped the reaction and caused the color disappearance. Five min later, the addition of 1 ml of 2 M tris(hydroxymethyl)aminomethane changed the pH, restoring the color. The absorbance of the mixture was measured at 420 nm.

3. Results and discussion

3.1. Optimization of extraction conditions

Due to the variety of chemical structures and polarity of polyphenols, it is difficult to select a single method suitable for the extraction of these substances from a plant material [21,22]. Anthocyanins are the most abundant phenolic compounds in berries [1] and, in view of their high polarity, they can be easily extracted with acidified polar solvents, including H_2O, acetone, ethanol (EtOH), MeOH or mixtures of hydrophilic solvents [22]. MeOH is indeed the most common and effective solvent for extracting anthocyanins [15,21,22] and it is usually acidified with HCl or HCOOH to extract these compounds in the flavylium form [15,23].

In this study, an initial extraction of anthocyanins from *V. floribundum* berries was performed, based on dynamic maceration with 0.6 M HCl in MeOH, followed by liquid–liquid extraction with EtOAc from the methanolic extract to recover phenolic acids and

flavonoids [24,25]. Using this method, good extraction yields were obtained for both anthocyanins and flavonoids. However, a high amount of quercetin was observed in the EtOAc fraction. In the literature, the major flavonoids of *Vaccinium* fruits have been described as flavonol glycosides, especially quercetin glycosides [26]. The high amount of quercetin observed in the *V. floribundum* EtOAc extract was therefore attributed to the acidic conditions of the first extraction, which led to a partial hydrolysis of the glycosidic bond [27]. The second shortcoming of this extraction method was the presence of a series of methyl quinates of chlorogenic acid in the EtOAc fraction, which were identified by means of UV, MS and MS^2 data. These artifacts had the same UV spectrum of chlorogenic acid and a molecular weight of 368, corresponding to chlorogenic acid derivatives with a methyl group bound to the quinic acid moiety. These methyl quinates of chlorogenic acid have been actually attributed to the use of MeOH as the extraction solvent [28].

To avoid the drawbacks of the method previously described, a more suitable extraction procedure was developed to efficiently extracts polyphenols from *Vaccinium* berries, without any alteration. In particular, a first extraction was carried out on *V. floribundum* berries using EtOAc to extract phenolic acids and flavonols, followed by a second extraction of the berry residue with 0.6 M HCl in MeOH, allowing a good recovery of anthocyanins [29]. The absence of quercetin and methyl quinates of chlorogenic acid in the EtOAc fraction confirmed that they were derived from the previous extraction procedure. Anthocyanins were also found to be stable under the applied extraction conditions.

3.2. Optimization of chromatographic conditions

Two specific HPLC methods were developed for the analysis of *Vaccinium* berry extracts. As regards the EtOAc fraction, which included phenolic acids and flavonols, a RP-HPLC analysis was carried out on an Ascentis C_{18} column, using an acidified mobile phase composed of 0.1 M HCOOH in H_2O and ACN, under gradient elution. Fig. 1 shows a representative HPLC-UV/DAD chromatogram for the separation of phenolic acids and flavonols in a *V. floribundum* EtOAc berry extract in comparison with *V. myrtillus*.

Anthocyanins, for their particular chemical structure, which presents various pH-dependent equilibria, are usually analyzed by RP-HPLC using a mobile phase at pH 1–2 [30]. The strong acidic media allow the complete displacement of the equilibria to the flavylium cation, thus resulting in a better resolution and increased absorbance between 515 and 540 nm [30]. In the light of this, the analysis of anthocyanins in the acidified MeOH berry extracts was performed on a Zorbax SB-C_{18} column, with a gradient mobile phase composed of a high percentage of HCOOH to reach the desired pH value. A representative chromatogram of *V. floribundum* anthocyanins in comparison with *V. myrtillus* is shown in Fig. 2.

3.3. Identification of polyphenols in berry extracts

In this study, a reliable identification of polyphenols in *V. floribundum* and *V. myrtillus* berry extracts was carried out by means of UV/Vis, MS and MS^2 data, which were compared with the literature [14,26,31–35] and with reference standards commercially available.

The phenolic acids and flavonols identified in the EtOAc extracts of both berries are shown in Table 1. As described in the literature, the negative ion mode provided higher level of sensitivity for phenolic acids if compared with the positive one [36,37]. In the berry extracts investigated, the most representative component of this class was chlorogenic acid (3-*O*-caffeoylquinic acid), which was confirmed by comparison with a commercial standard, by its UV spectrum and MS fragmentation (Fig. 3) in both

Table 1
Phenolic acids and flavonols identified in EtOAc extracts of *Vaccinium* berries by HPLC-UV/DAD, HPLC-ESI-MS and MS^2.[a]

Peak number	Compound	t_R (min)	UV λ_{max} (nm)	$[M+H]^+$ (*m/z*)	MS^2 (*m/z*)		$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	
					Base peak	Secondary peaks		Base peak	Secondary peaks
1	Neochlorogenic acid	6.6	240, 300, 326	355	163	–	353	191	–
2	Chlorogenic acid[b]	9.2	240, 300, 326	355	163	–	353	191	–
3	Caffeoylshikimic acid isomer	13.4	244, 300, 328	337	163	–	335	179	–
4	Caffeoylshikimic acid isomer	13.8	244, 300, 328	337	163	–	335	179	–
5	Caffeic acid derivative	18.6	296, 328	325	307	163 (49)	433	323	161 (89)
6	Quercetin-3-*O*-galactoside[b]	19.2	256, 355	465	303	–	463	300/301	271 (20), 151 (6)
7	Quercetin-3-*O*-glucoside[b]	19.7	256, 355	465	303	–	463	300/301	271 (3)
8	Quercetin-3-*O*-glucuronide	20.0	256, 355	479	303	–	477	300/301	–
9	Quercetin-3-*O*-xyloside	21.1	256, 355	435	303	–	433	300/301	–
10	Quercetin-3-*O*-arabinopyranoside[b]	21.9	256, 355	435	303	–	433	300/301	–
11	Quercetin-3-*O*-arabinofuranoside[b]	22.9	256, 353	435	303	–	433	300/301	–
12	Quercetin-3-*O*-rhamnoside[b]	23.4	256, 350	449	303	–	447	300/301	151 (21)

[a] Experimental conditions as in Sections 2.4 and 2.5.
[b] Confirmed with standard compound.

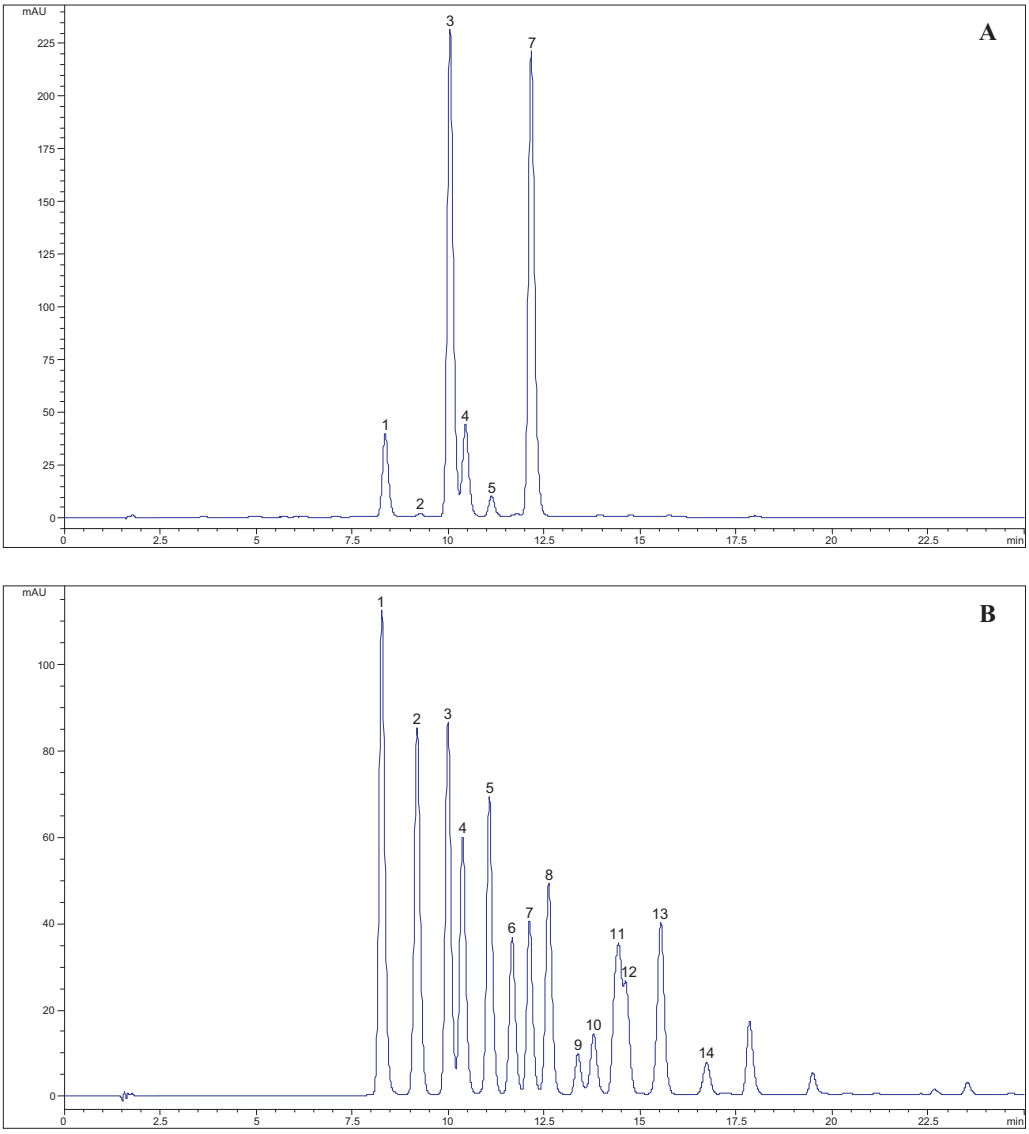

Fig. 2. Chromatograms obtained by HPLC-UV/DAD analysis of acidified MeOH berry extracts of *V. floribundum* (sample VF-2) (A) and *V. myrtillus* (B) at 520 nm. Experimental conditions as in Section 2.4. For peak identification, see Table 2.

V. floribundum and *V. myrtillus* berry extracts. According to Clifford et al. [31], in addition to chlorogenic acid peak, other two major isomers at *m*/*z* 353 may occur in a plant extract, including neochlorogenic acid (5-*O*-caffeoylquinic acid) and cryptochlorogenic acid (4-*O*-caffeoylquinic acid). In particular, a negative product ion at *m*/*z* 191 has been described as the base peak in MS^2 spectra when the caffeoyl group is linked to the 3- or 5-OH groups, while a negative product ion at *m*/*z* 173 has been reported as the base peak when the caffeoyl group is linked to the 4-OH group [31]. This allowed to assign the first peak of *V. floribundum* chromatogram to neochlorogenic acid (Fig. 3); cryptochlorogenic acid was not detected in the berry extracts analyzed in this study.

In the ambit of phenolic acids, two caffeoylshikimic acids at *m*/*z* 335 were also identified in *V. floribundum*. The MS^2 spectra of these compounds (Fig. 3) showed an intense product ion at *m*/*z* 179, corresponding to the loss of the shikimoyl moiety. The main regioisomers of caffeoylshikimic acids previously described in the literature include the position 3-, 4- and 5-; however, the absence of the product ion *m*/*z* 161 in the MS^2 spectra of these peaks allowed to certainly exclude 4-caffeoylshikimic acid, as previously described [35]. Therefore, both 3- and 5-caffeoylshikimic acids were supposed to occur in *V. floribundum* berries, but the exact peak assignment was uncertain, due to the lack of reference standards.

A caffeic acid derivative was also identified in the EtOAc extract of *V. floribundum* berries (Fig. 3). Garzón et al. [34] have described a precursor ion of a caffeic acid derivative at *m*/*z* 433 in *V. meridionale* berries, which is probably the same constituent, since the chemical profile of *V. meridionale* is close to that of *V. floribundum*. This compound showed a characteristic product ion at *m*/*z* 161 in the MS^2 spectrum, which has been previously assigned to a dehydration fragment of the deprotonated molecular ion of caffeic acid [34].

The second group of secondary metabolites identified in the EtOAc extracts of *Vaccinium* berries was characterized by flavonol glycosides, in particular quercetin derivatives. For this class of constituents, which is very frequent and abundant in berries [26], both the positive and negative ion modes were applied for structural characterization. In the MS^2 spectra of these compounds recorded in the positive ion mode, the cleavage of the glycosidic bond led to the elimination of the sugar residue, resulting in a strong fragment at *m*/*z* 303, corresponding to quercetin. In the negative ion mode, all quercetin glycosides generated the

corresponding aglycone at m/z 300 (homolytic cleavage) and m/z 301 (heterolytic cleavage), suggesting that the glycosylation site was probably located at the 3-position [37]. However, a reliable identification of these compounds in *Vaccinium* berry extracts was performed by comparing their chromatographic data with authentic reference standards analyzed under the same conditions, because their t_R depends on the sugar moiety bound to the aglycone. The most challenging identification involved peaks 9, 10 and 11 of *Vaccinium* berry extracts, which were attributed to three quercetin-3-*O*-pentosides. These peaks were identified as quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinopyranoside and quercetin-3-*O*-arabinofuranoside, respectively, by comparison with reference standards and literature data [14,26]. A further confirmation was obtained by a direct comparison with the same compounds previously identified in *V. macrocarpon* [39]. Finally, seven quercetin glycosides, including quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinopyranoside, quercetin-3-*O*-arabinofuranoside, quercetin-3-*O*-rhamnoside, were identified in the *Vaccinium* species analyzed in this study.

The comparison of *V. floribundum* and *V. myrtillus* EtOAc extracts revealed the presence of neochlorogenic acid, caffeoylshikimic acid, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-rhamnoside in *V. floribundum* only, thus leading

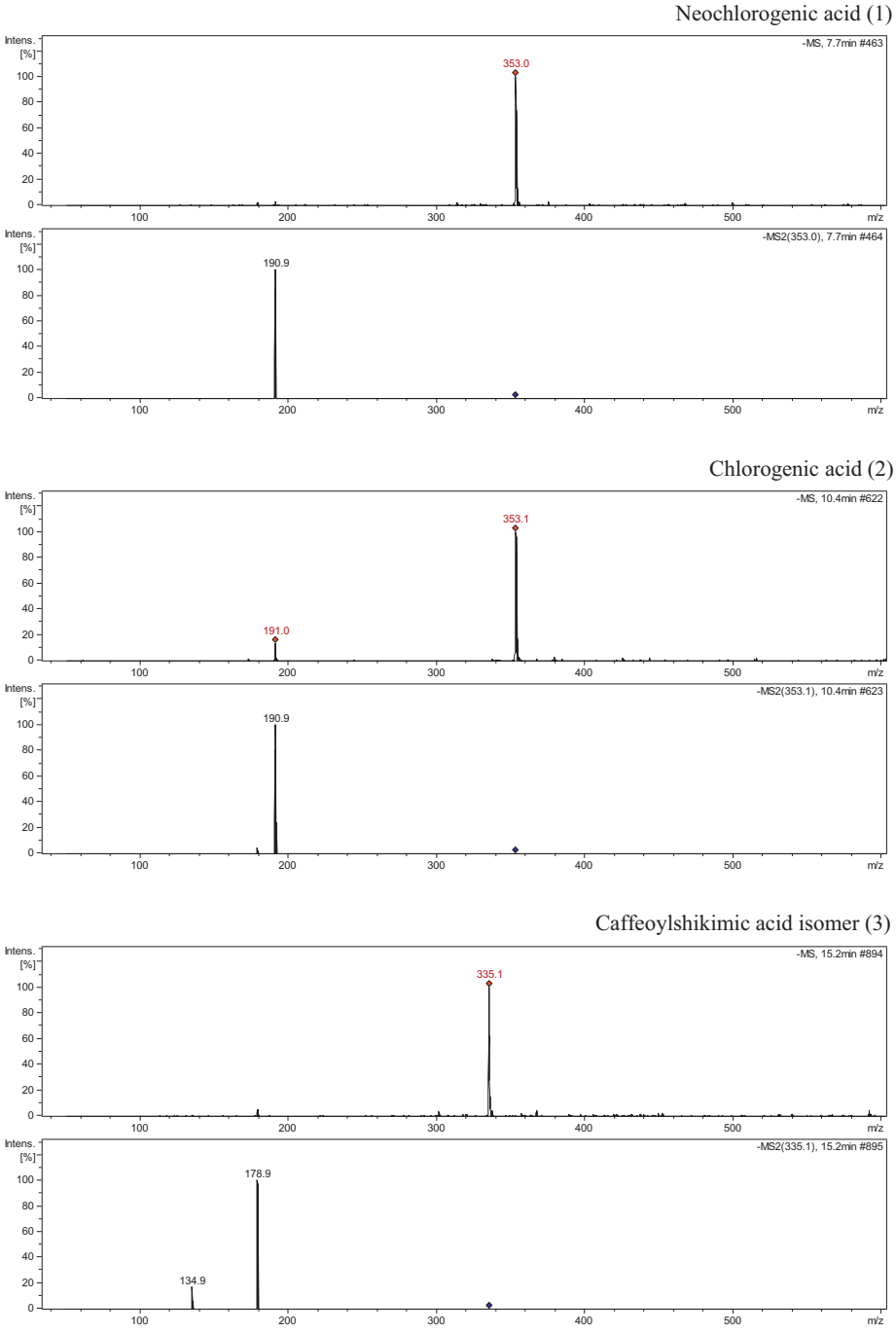

Fig. 3. ESI-MS and MS^2 spectra of neochlorogenic acid (1), chlorogenic acid (2), caffeoylshikimic acid isomers (3 and 4) and caffeic acid derivative (5) in the negative ion mode. Compounds are listed in order of elution time. Experimental conditions as in Section 2.5.

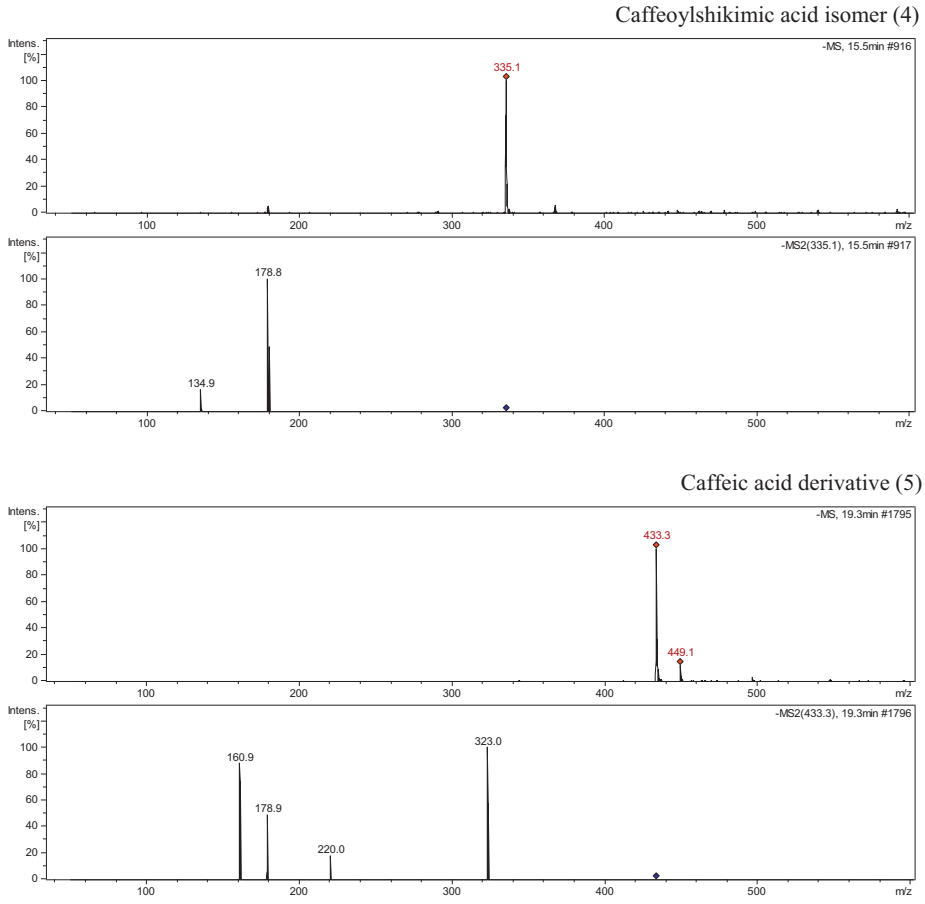

Fig. 3. (*continued*).

to suggest that these substances may represent potential markers to discriminate these berries.

Several anthocyanins were identified in the acidified MeOH extracts obtained from both *Vaccinium* berries. The UV/Vis, MS and MS^2 data of these compounds are shown in Table 2. HPLC-ESI-MS and MS^2 experiments were carried out in the positive ion mode only, as previously described in the literature [38], due to the fact that these compounds are present as flavylium ions at acidic conditions and produce molecular cations M^+ in their ESI mass spectra.

Many studies have been carried out on the composition of *V. myrtillus* anthocyanins [33,40]. The MS^2 spectra of these compounds showed characteristic fragments at *m/z* 303, 287, 317, 301 and 331, corresponding to the $[M-sugar]^+$ product ions of delphinidin, cyanidin, petunidin, peonidin and malvidin, respectively. In particular, fourteen anthocyanins, including 3-*O*-galactosides, 3-*O*-glucosides and 3-*O*-arabinosides of delphinidin, cyanidin, petunidin, peonidin and malvidin, have been reported in *V. myrtillus* extracts by many authors [33,40] and they were confirmed in this study. In contrast, *V. floribundum* was found to contain six anthocyanins only, including delphinidin and cyanidin glycosides, in agreement with the composition described in the literature [10,11].

3.4. Method validation

HPLC-UV/DAD was selected for quantitative analysis of polyphenols in berry samples, because of the wider availability and use of this equipment in the phytochemical analysis of natural products [14,15].

Good linearity was observed for reference compounds used as polyphenol standards over the range tested ($r^2 > 0.998$). The LOD value was 1.9 μg/ml for chlorogenic acid, 4.3 μg/ml for quercetin-3-*O*-galactoside and 1.5–3.4 μg/ml for anthocyanins. The LOQ value was 6.8 μg/ml for chlorogenic acid, 14.3 μg/ml for quercetin-3-*O*-galactoside and 5.1–11.3 μg/ml for anthocyanins. These values indicated that the proposed HPLC-UV/DAD methods had a good sensitivity for the determination of the phenolic composition of berry extracts.

The accuracy of the analytical procedure was evaluated using the recovery test. The percentage recovery values, obtained by comparing the results from samples and fortified samples of *V. floribundum*, were found to be in the range 70–80% and can be considered satisfactory for the compounds studied.

The low intra- and inter-day SD values for *V. floribundum* constituents (≤3 mg/100 g FW for phenolic acids and flavonols and ≤20 mg/100 g FW for anthocyanins), and %RSD for retention times (≤0.4) and peak areas (≤4.3) indicated the high precision of both the extraction procedure and the chromatographic system.

As regards specificity, the HPLC analysis of *V. floribundum* and *V. myrtillus* EtOAc extracts, carried out with the method developed for anthocyanins, indicated that these constituents were not present in these fractions. As far as the HPLC analysis of phenolic acids and flavonols in acidified MeOH extracts of *V. floribundum* and *V. myrtillus* is concerned, they were found to be below the LOD/LOQ values in both cases.

Table 2
Anthocyanins identified in acidified MeOH extracts of *Vaccinium* berries by HPLC-UV/DAD, HPLC-ESI-MS and MS^2.[a]

Peak number	Compound	t_R (min)	UV λ_{max} (nm)	M^+ (*m*/*z*)	MS^2 (*m*/*z*)
1	Delphinidin-3-*O*-galactoside	8.4	277, 525	465	303
2	Delphinidin-3-*O*-glucoside[b]	9.3	277, 525	465	303
3	Cyanidin-3-*O*-galactoside	10.1	280, 518	449	287
4	Delphinidin-3-*O*-arabinoside	10.5	277, 525	435	303
5	Cyanidin-3-*O*-glucoside[b]	11.2	280, 518	449	287
6	Petunidin-3-*O*-galactoside	11.8	277, 528	479	317
7	Cyanidin-3-*O*-arabinoside	12.2	280, 518	419	287
8	Petunidin-3-*O*-glucoside[b]	12.7	277, 528	479	317
9	Peonidin-3-*O*-galactoside	13.5	280, 520	463	301
10	Petunidin-3-*O*-arabinoside	13.9	277, 528	449	317
11	Peonidin-3-*O*-glucoside[b]	14.6	280, 520	463	301
12	Malvidin-3-*O*-galactoside	14.8	278, 531	493	331
13	Malvidin-3-*O*-glucoside[b]	15.7	278, 531	493	331
14	Malvidin-3-*O*-arabinoside	16.9	278, 531	463	331

[a] Experimental conditions as in Sections 2.4 and 2.5.
[b] Confirmed with standard compound.

Stability was tested with berry extracts that were stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h. The analytes in solution did not show any appreciable change in the chromatographic profile over 72 h in both EtOAc and acidified MeOH extracts.

The validation data highlighted the suitability of the proposed methods for the quali- and quantitative analysis of phenolic compounds in extracts obtained from *V. floribundum* and *V. myrtillus* berries.

3.5. Quantitative analysis of berry samples

The validated methods were applied to the quantitative analysis of polyphenols in five samples of *V. floribundum* and in one sample of *V. myrtillus* berries. Table 3 shows the amount of phenolic acids and flavonols, while Table 4 shows the content of anthocyanins. Quantitative data were expressed as mg/100 g berry fresh weight (FW).

A significant variability in the content of total phenolics, including phenolic acids, flavonols and anthocyanins, was observed in the *Vaccinium* species analyzed in this study. On the one hand, *V. floribundum* samples had a much higher content of both phenolic acids and flavonols in comparison with *V. myrtillus* (mean value 41.6 ± 10.2 and 13.7 ± 0.2 mg/100 g FW, respectively). On the other hand, *V. myrtillus* had a higher content of anthocyanins in comparison with *V. floribundum* (568.8 ± 8.8 and mean value 376.2 ± 49.9 mg/100 g FW, respectively). The content of *V. floribundum* anthocyanins was in agreement with the literature [10]. Total phenolic acids and flavonols as well as total anthocyanins in *V. myrtillus* fruit were found to be of the same order of magnitude of previous reports [26,33,41]. Regarding phenolic acids and flavonols, the most abundant compounds found in *V. floribundum* berry samples were quercetin-3-*O*-arabinofuranoside (mean value 10.1 ± 1.7 mg/100 g FW), chlorogenic acid (mean value 9.5 ± 2.9 mg/100 g FW) and quercetin-3-*O*-galactoside (mean value 9.2 ± 2.3 mg/100 g FW). Only four anthocyanins were above the LOQ values in the *V. floribundum* extracts and the most abundant ones were cyanidin-3-*O*-galactoside and cyanidin-3-*O*-arabinoside (mean value 130.5 ± 20.1 and mean value 126.1 ± 19.6 mg/100 g FW, respectively). Vasco et al. [10] reported the presence of delphinidin and cyanidin in a *V. floribundum* extract, which could be attributed to hydrolysis products deriving from the extraction.

It should be pointed out that the samples of *V. floribundum* and *V. myrtillus* analyzed in this study were grown in different environmental conditions, because the first one is a species native to South America and the second to Europe. Therefore, the content of their polyphenols is also greatly influenced by the different abiotic and biotic factors at the sites of collection.

3.6. Biological activity

The extracts of *V. floribundum* and *V. myrtillus* berries were evaluated for their antioxidant and radical-scavenging properties and for their capacity to prevent mutation induced by two promutagenic dietary quinolines, namely MeIQ and 4-NQO. In both the modified Ames assay and in the SOS chromotest assay the extracts

Table 3
Content of phenolic acids and flavonols in *V. floribundum* and *V. myrtillus* berries by HPLC-UV/DAD (data are expressed in mg/100 g FW).[a]

Peak number	Compound	*V. floribundum*						*V. myrtillus*[b]
		VF-1[b]	VF-2[b]	VF-3[b]	VF-4[b]	VF-5[b]	Mean	
1	Neochlorogenic acid	1.1 ± 0.9	1.8 ± 0.3	0.9 ± 0.1	0.9 ± 0.6	<LOQ	1.5 ± 0.5	<LOD
2	Chlorogenic acid	9.1 ± 2.8	12.6 ± 1.6	8.6 ± 0.8	9.2 ± 3.2	7.9 ± 2.9	9.5 ± 2.9	6.1 ± 1.8
3	Caffeoylshikimic acid isomer	1.1 ± 0.3	1.8 ± 0.1	1.3 ± 0.2	1.1 ± 0.5	0.9 ± 0.3	1.4 ± 0.4	<LOD
4	Caffeoylshikimic acid isomer	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD
5	Caffeic acid derivative	1.8 ± 0.8	3.4 ± 0.8	2.0 ± 0.3	2.5 ± 0.8	2.0 ± 0.4	2.3 ± 0.9	<LOD
6	Quercetin-3-*O*-galactoside	7.9 ± 1.5	10.6 ± 3.0	11.0 ± 0.9	8.7 ± 1.8	8.0 ± 1.8	9.2 ± 2.3	4.4 ± 0.8
7	Quercetin-3-*O*-glucoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
8	Quercetin-3-*O*-glucuronide	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	3.2 ± 1.1
9	Quercetin-3-*O*-xyloside	2.1 ± 0.5	3.0 ± 0.3	2.6 ± 0.8	2.0 ± 0.5	2.2 ± 0.3	2.4 ± 0.6	<LOD
10	Quercetin-3-*O*-arabinopyranoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
11	Quercetin-3-*O*-arabinofuranoside	9.0 ± 1.3	11.7 ± 1.8	10.6 ± 0.7	10.1 ± 1.5	9.0 ± 1.6	10.1 ± 1.7	<LOD
12	Quercetin-3-*O*-rhamnoside	2.3 ± 0.1	8.8 ± 1.4	7.0 ± 2.3	3.3 ± 1.2	8.9 ± 0.4	6.1 ± 3.2	<LOQ
–	Total phenolic acids and flavonols	34.1 ± 10.8	53.8 ± 7.5	44.0 ± 8.0	37.3 ± 10.8	38.6 ± 10.5	41.6 ± 10.2	13.7 ± 0.2

[a] Experimental conditions as in Section 2.4.
[b] Data are expressed as mean ($n = 6$) ± SD.

Table 4
Content of anthocyanins in *V. floribundum* and *V. myrtillus* berries by HPLC-UV/DAD (data are expressed as mg/100 g FW).[a]

Peak number	Compound	*V. floribundum*						*V. myrtillus*[b]
		VF-1[b]	VF-2[b]	VF-3[b]	VF-4[b]	VF-5[b]	Mean	
1	Delphinidin-3-*O*-galactoside	35.9 ± 4.9	45.1 ± 5.3	60.8 ± 3.8	80.2 ± 3.7	66.5 ± 12.8	57.7 ± 17.8	92.1 ± 4.7
2	Delphinidin-3-*O*-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	86.6 ± 10.5
3	Cyanidin-3-*O*-galactoside	117.2 ± 6.7	159.0 ± 12.2	108.1 ± 11.4	129.8 ± 1.9	138.5 ± 2.7	130.5 ± 20.1	48.4 ± 6.1
4	Delphinidin-3-*O*-arabinoside	38.9 ± 2.7	50.8 ± 4.4	65.7 ± 3.0	84.9 ± 2.2	69.4 ± 11.3	61.9 ± 17.6	59.1 ± 4.8
5	Cyanidin-3-*O*-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	46.6 ± 2.4
6	Petunidin-3-*O*-galactoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	30.1 ± 3.9
7	Cyanidin-3-*O*-arabinoside	118.2 ± 10.4	156.1 ± 10.1	105.5 ± 11.9	124.4 ± 5.9	126.2 ± 6.1	126.1 ± 19.6	25.0 ± 0.7
8	Petunidin-3-*O*-glucoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	53.4 ± 5.9
9	Peonidin-3-*O*-galactoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
10	Petunidin-3-*O*-arabinoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	13.7 ± 0.8
11	Peonidin-3-*O*-glucoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	41.2 ± 2.3
12	Malvidin-3-*O*-galactoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	21.7 ± 3.9
13	Malvidin-3-*O*-glucoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	50.8 ± 3.3
14	Malvidin-3-*O*-arabinoside	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ
–	Total anthocyanins	310.2 ± 12.3	411.0 ± 41.2	340.1 ± 21.1	419.2 ± 1.6	400.6 ± 39.0	376.2 ± 49.9	568.8 ± 8.8

[a] Experimental conditions as in Section 2.4.
[b] Data are expressed as mean ($n = 6$) ± SD.

Table 5
Antioxidant and radical-scavenging activity of *V. floribundum* and *V. myrtillus* extracts.[a]

	$ABTS^{\bullet+}$ IC_{50} (μg/ml)	$DPPH^{\bullet}$ IC_{50} (μg/ml)
Trolox	0.855	9.595 ± 0.034
V. floribundum		
Acidified MeOH extract	0.339 ± 0.011	0.694 ± 0.028
EtOAc extract	0.185 ± 0.024	–
V. myrtillus		
Acidified MeOH extract	0.422 ± 0.009	0.023 ± 0.002
EtOAc extract	0.130 ± 0.001	–

[a] Experimental conditions as in Section 2.7.

resulted to be inactive at concentrations up to 1000 μg/plate (data not shown). These evidences, despite the previously reported antimutagenic properties of anthocyanin-rich extracts on heterocyclic amines like Trp-P1 [42], seem to diminish the potential use of *Vaccinium* extracts in chemoprevention-oriented food supplements, at least in terms of protection from heterocyclic amines-induced mutagenicity. It must be noticed, however, that heterocyclic amines are known for their different behavior both in terms of mutagenic potency and sensibility to polyphenolic substances and that great differences should be expected [19].

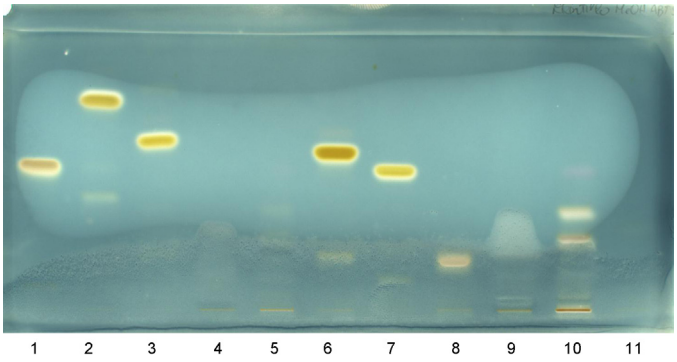

Fig. 4. HPTLC $ABTS^{\bullet+}$ bioautographic assay: 1, chlorogenic acid; 2, quercetin-3-*O*-rhamnoside; 3, quercetin-3-*O*-glucoside; 4, *V. myrtillus* EtOAc extract; 5, *V. floribundum* EtOAc extract (sample VF-2); 6, quercetin-3-*O*-galactoside; 7, quercetin-3-*O*-glucuronide; 8, delphinidin-3-*O*-glucoside; 9, *V. myrtillus* acidified MeOH extract; 10, *V. floribundum* acidified MeOH extract (sample VF-2); 11, cyanidin-3-*O*-glucoside. A brighter spot corresponds to a higher antioxidant activity.

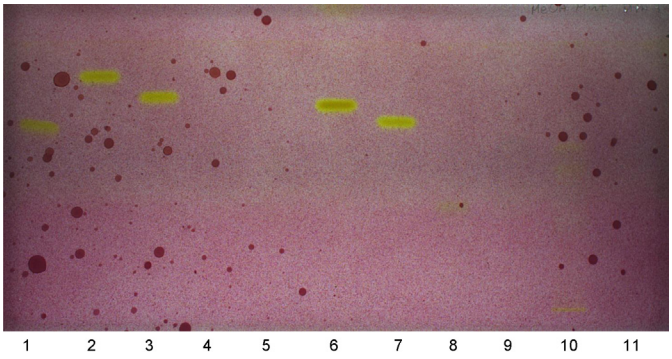

Fig. 5. HPTLC $DPPH^{\bullet}$ bioautographic assay: 1, chlorogenic acid; 2, quercetin-3-*O*-rhamnoside; 3, quercetin-3-*O*-glucoside; 4, *V. myrtillus* EtOAc extract; 5, *V. floribundum* EtOAc extract (sample VF-2); 6, quercetin-3-*O*-galactoside; 7, quercetin-3-*O*-glucuronide; 8, delphinidin-3-*O*-glucoside; 9, *V. myrtillus* acidified MeOH extract; 10, *V. floribundum* acidified MeOH extract (sample VF-2); 11, cyanidin-3-*O*-glucoside. A brighter spot corresponds to a higher radical-scavenging activity.

As regards the antioxidant activity, the extracts of both berries provided a better IC_{50} than Trolox (Table 5). Furthermore, it must be noticed that *V. floribundum* extracts performed slightly better than *V. myrtillus* in the $ABTS^{\bullet+}$ assay. On the contrary, likely as a consequence of the lower amount of anthocyanins, their performance was lower in the $DPPH^{\bullet}$ assay, that emphasizes the behavior of more hydrophylic substances.

This seems to be confirmed by the fact that the flavonol-rich fraction offered always a lower IC_{50} if compared to the anthocyanin-rich one and by the results in the HPTLC bioautographic assay (Figs. 4 and 5), in which the flavonol fraction did not provide an evident activity even if applied without dilution.

These results, along with previous evaluations on the anthocyanin-rich fraction, seem to indicate that its contribution to the radical-scavenging and antioxidant properties of *Vaccinium* berries is the sole to be taken into account [11].

4. Conclusions

The proposed extraction procedures combined with HPLC-UV/DAD, HPLC-ESI-MS and MS^2 analyses provided a reliable phytochemical characterization of *V. floribundum* polyphenols. Both quali- and quantitative analyses were performed in comparison with *V. myrtillus* and may be used in order to authenticate *V.*

floribundum juice or derived products. The results obtained with the samples analyzed in this study indicated that, even if the content of phenolic acids and flavonols was much higher in *V. floribundum*, *V. myrtillus* had a higher amount of anthocyanins.

In terms of overall performance as antioxidant, the results obtained in this study indicated that *V. floribundum* seems to be slightly inferior to *V. myrtillus*; in both cases the proposed dynamic maceration with 0.6 M HCl in MeOH may represent the best protocol in order to obtain an antioxidant extract of *V. floribundum* to be used in food supplements. Further investigations on the antioxidant activity of both species are in progress, using more specific *in vitro* and *in vivo* assays.

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