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(Article begins on next page)

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# Extraction of bilberry (*Vaccinium myrtillus*) antioxidants using supercritical/subcritical $CO_2$ and ethanol as co-solvent

Oxana Babova[a], Andrea Occhipinti[a, b], Andrea Capuzzo[b], Massimo E. Maffei[a, b, ,]

## Highlights

•Bilberry antioxidant compounds are selectively extracted by subcritical $CO_2$.
•Anthocyanins, flavonoids, phenolics and proanthocyanidins show antioxidant properties.
•Cyanidin-3-O-glucoside and cyanidin-3-O-arabinoside are selectively extracted.

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## Abstract

Anthocyanins and other phenolic compounds of bilberry (*Vaccinium myrtillus*) are known for their antioxidant properties. Supercritical (SC) and subcritical (SubC) $CO_2$ extractions have been used to improve extraction and selectivity of plant bioactive compounds. Bilberry was extracted by SC $CO_2$ followed by SubC $CO_2$ with 10% v/v ethanol as co-solvent. Total phenolic compounds, anthocyanins and proanthocyanidins were quantified and chemically characterized by HPLC-DAD-ESI-MS/MS. Antioxidant activity was tested by reducing power assay, free radical scavenging activity (DPPH) and ABTS radical cation assay. SubC $CO_2$ selectively extracted cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside. Delphinidin-3-*O*-glucoside, ellagic acid pentoside, feruloyl hexoside and several quercetin glycosides were also extracted. SubC $CO_2$ extracts showed a high antioxidant activity [DPPH $IC_{50}$ = 102.66 (±2.64); ABTS $IC_{50}$ = 8.49 (±0.41) and reducing power activity $IC_{50}$ = 10.30 (±0.10)]. SubC-$CO_2$ extraction of bilberry is an efficient method to recover selectively compounds with a high antioxidant activity and a high potential for pharmacological applications.

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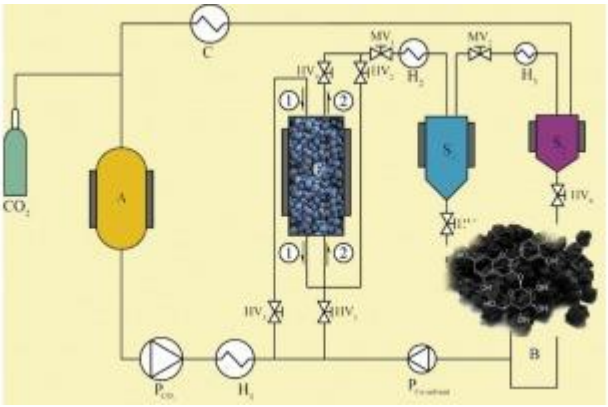
## Graphical abstract

Supercritical and subcritical $CO_2$ extraction of bilberry allowed to recover selectively cyanidin-3-*O*-glucoside and other compounds with a high antioxidant activity.



# Abbreviations

- ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid);
- DMAC, 4-dimethylamino-cinnamaldehyde;
- DPPH**.**, 1,1-diphenyl-2-picryl-hydrazil stable radical;
- SC, supercritical;
- SubC, subcritical;
- Trolox®, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid


# Keywords

- Anthocyanins;
- Antioxidant activity;
- Phenolic compounds;
- Proanthocyanidins;
- Supercritical $CO_2$ extraction;
- Subcritical $CO_2$ extraction;
- *Vaccinium myrtillus*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# 1. Introduction

The dietary intake of natural antioxidants such as vitamins, polyphenols and carotenoids might help to reduce the risk of degenerative diseases such as cardiovascular disorders, ageing-induced oxidative stress and inflammatory responses. In the last few years, the market of dietary supplements exploited the natural antioxidants of *Vaccinium* berry extracts [1]. The biological activity of these extracts is correlated to the high content of anthocyanins, a large group of water-soluble flavonoids that provide the characteristic blue/red colour to fruits, flowers and vegetables [2]. Bilberry (*Vaccinium myrtillus* L.) is one of the richest natural sources of this class of polyphenols. Bilberry contains five of the six common natural anthocyanidins: cyanidin, delphinidin, malvidin, peonidin, and petunidin, which accumulate in both peel and flesh and occur mainly as glycosides [1] and [3]. Besides anthocyanins, bilberry also contains other phenolic compounds with health promoting activities [4].

Bilberry extraction techniques can significantly influence the anthocyanin concentration. In the last decade, anthocyanin extraction has been widely investigated by using green technologies such as ultrasound assisted extraction [5] and supercritical fluid extraction [6] as alternatives methods to conventional solvent extraction. Supercritical $CO_2$ (SC $CO_2$) extraction uses carbon dioxide above its critical point and exhibits liquid-like properties (solvent power, negligible surface tension) as well as gas-like proprieties (matrix penetration and transport) [7]. SC $CO_2$ is widely used for the extraction of natural compounds since it is a colourless, odourless, nontoxic, non-flammable, safe, highly pure and cost-effective solvent. Moreover, the $CO_2$ critical point is at relatively low pressures and near room temperature (Pc = 7.38 MPa and Tc = 31.1 °C) and the use of mild extraction parameters is known to limit thermal and oxidative degradation of bioactive compounds [8], [9], [10], [11] and [12]. However, the lipophilic properties of SC $CO_2$ make this solvent poorly suitable for extraction of polar compounds such as glycosylated phenolic compounds. Nevertheless, the addition of small amounts of co-solvents such as water or ethanol (<5% w/w) can increase the polarity of SC $CO_2$ by enhancing the solubility of more polar compounds; in these conditions, the system moves in a two-phases subcritical state [8] and [9]. The effectiveness of subcritical

extraction of anthocyanins and other polyphenols has been investigated in the food industry [13], [14] and [15].

In this study, we investigate the use of supercritical and subcritical fluids for the selective extraction of antioxidant bioactive compounds from *V. myrtillus*. To our knowledge, the use of subcritical $CO_2$ (SubC-$CO_2$) by has never been tested on bilberry.

# 2. Materials and methods

## 2.1. Chemicals

Ethanol (95%), potassium ferricyanide (99.8%), 1,1-diphenyl-2-picryl-hydrazil stable radical (DPPH$^{\bullet}$) (>98%) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid) (98%) were purchased from Sigma–Aldrich, USA; procyanidin A2 (>99%) and cyanidin-3-*O*-glucoside (96%), were purchased from Extrasynthese, France; trichloracetic acid (99%) was purchased from Carlo Erba, Italy, Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (98%) and DMAC (4-dimethylamino-cinnamaldehyde) (99%) were purchased from TCI-Europe, Belgium; acetonitrile was purchased from Panreac, Spain.

## 2.2. Plant material

Dried bilberry fruits (*V. myrtillus* L.) marketed by Herbamea S.r.l. (Italy) were ground in a blender and stored in closed containers at 4 °C in the dark until use.

## 2.3. Supercritical and subcritical $CO_2$ extraction

A multistage supercritical/subcritical extraction of ground bilberries was performed with a pilot plant kindly provided by Separeco S.r.l. (Piscina – Turin, Italy) with the aim to extract polar phenols from dried ground bilberries. To improve the solubility of target compounds, the extraction of anthocyanins and phenols was performed with the use of aqueous ethanol as co-solvent. Berries were air dried to 5% moisture and were directly ground with a blender and passed through a sieve with a 4 mm diameter. The system was equipped with a 5 L extractor, a gravimetric and a cyclonic separator (1.2 L). The gravimetric and the cyclonic separators were kept at $P$ = 2.5 MPa and $T$ = 40 °C, respectively (Fig. 1). Dried bilberry (430 g) extraction was carried out in three steps at $P$ = 25 MPa, $T$ = 45 °C: (1) SC-$CO_2$ extraction (flow-rate 8 kg $h^{-1}$ $CO_2$) with 6% w/w of co-solvent (30% distilled water, 70% ethanol, Sigma–Aldrich, USA); (2) Subcritical $CO_2$ extraction (SubC-$CO_2$) (flow-rate 6 kg $h^{-1}$ $CO_2$) with 6% w/w of co-solvent (50% distilled water, 50% ethanol) at 6 ml $min^{-1}$; and (3) SubC-$CO_2$ extraction (flow-rate 6 kg $h^{-1}$ $CO_2$) with 9% w/w of co-solvent (90% distilled water, 10% ethanol). During the SubC-$CO_2$, the solvent flow through the extraction vessel was reversed from the previous bottom-up to a top-down direction. The first and second extraction steps were carried out for 1 hour, the last step for 3 h. Extracts were collected from both gravimetric and cyclonic separators. The system was equipped with a cyclonic separator to assure the complete recovery of extract and to avoid the reflux of extract into the $CO_2$ storage tank. Three extractions were performed.

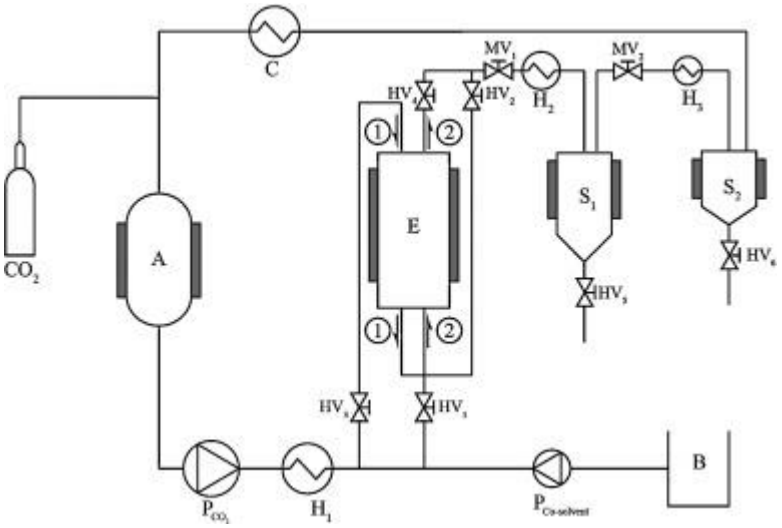


Fig. 1.

Schematic representation of the supercritical fluid extractor used in this work. A $CO_2$ tank provides the $CO_2$ used for extraction. (1) Fluid circulation during supercritical extraction; (2) fluid circulation during sub-critical extraction; (A) $CO_2$ buffer storage tank; (B) co-solvent storage tank; (E) extraction vessel; ($S_1$) gravimetric separator; ($S_2$) cyclonic separator; (P) diaphragm pumps; ($H_1$–$H_3$) heat exchangers; (C) condenser; ($HV_1$–$HV_6$) hand valves; ($MV_1$, $MV_2$) membrane valves.

Courtesy of Separeco S.r.l. (Italy).

## 2.4. Determination of total phenols, anthocyanin and proanthocyanidin content

The total phenolic content of berry extracts was determined according to the Folin–Ciocalteu method [16] and the data were expressed as mg $g^{-1}$ of dry weight (d.wt).

Total anthocyanin content was determined according to the pH differential method by Giusti and Wrolstad [17]. Briefly, samples were properly diluted in either 1 M KCl buffer at pH 1.0 or 0.4 M ammonium acetate buffer at pH 4.5. Absorbance was read at 510 and 700 nm at room temperature using a Pharmacia Biotech Ultrospec 3000 UV-Visible spectrophotometer. Quantification of total anthocyanins was calculated based on the cyanidin-3-*O*-glucoside molar extinction coefficient ($\varepsilon$ 26,900) and molecular weight (449.2 Da) using the following formula:

$$C(mg/l)=A*MW*DF*1000\varepsilon*l$$

where $A=[(A_{\lambda max}-A_{700})_{pH1.0}-(A_{\lambda max}-A_{700})_{pH4.5}]A=(A\lambda max-A700)pH1.0-(A\lambda max-A700)pH4.5$, *MW* is the molecular weight of cyanidin-3-*O*-glucoside; *DF* is the dilution factor; $\varepsilon$ is the molar extinction coefficient of cyanidin-3-*O*-glucoside and *l* is the cuvette path length (1 cm). Total anthocyanins were quantified by using a cyanidin-3-O-glucoside calibration standard curve ($R$ = 0.997).

Total proanthocyanidin (PACs) content was quantified by BL-DMAC method [18]. The spectrophotometric assay was performed in a final volume of 1.12 ml with 0.28 ml of diluted sample and 0.84 ml of DMAC (4-dimethylamino-cinnamaldehyde) solution. Total PACs were quantified by using a Procyanidin A2 calibration standard curve ($R$ = 0.998).

## 2.5. HPLC-DAD-ESI-MS/MS of anthocyanins, proanthocyanidins and phenolic compounds

Samples were analyzed by liquid chromatography (1200 HPLC, Agilent Technologies, USA) equipped with a Luna reverse phase C18 (3.00 μm, 150 mm × 3.0 mm, Phenomenex, USA) column. The binary solvent system for identification and quantification of anthocyanins was: (A) MilliQ $H_2O$ (Millipore, U.S.A.) with 10% v/v of formic acid and (B), methanol/water/formic acid 50/40/10 v/v with 10% v/v of formic acid. The chromatographic separation was carried out at constant flow rate (200 μl $min^{-1}$) with the following conditions: linear gradient from 15% to 45% of B in 15 min, at 35 min 70% of B and at 55 min 100% of B. The initial mobile phase was re-established for 11 min before the next injection. To analyze phenolic compounds, the solvent system was: (A) MilliQ $H_2O$ with 0.1% v/v of formic acid and (B) acetonitrile with 0.1% v/v of formic acid. The separation was performed at constant flow rate (200 μl $min^{-1}$) with the following conditions: isocratic elution with 3% of B for 5 min, from 3% to 10% in 5 min, at 43 min 25% of B, 50% of B at 47 min, and 90% of B at 50 min. Mass spectrometry analyses were performed with a 6330 Series Ion Trap LC–MS System (Agilent Technologies, U.S.A.) equipped with an electrospray ionization source (ESI) operating in positive mode for anthocyanins and negative mode for phenolic compounds. Qualitative analyses were performed in scan mode (500–850 m/z) while quantitative analyses were obtained by Multiple Reaction Monitoring (MRM). To analyze proanthocyanidins, the solvent system was: (A) MilliQ H2O (Millipore, U.S.A.) with 0.1% v/v of formic acid and (B), acetonitrile (VWR International, USA) with 0.1% v/v of formic acid. The chromatographic separation was carried out at constant flow rate (200 μl $min^{-1}$) with the following conditions: linear gradient from 5% to 30% of B in 10 min, isocratic elution for 5 min, at 20 min 50% of B and 90% of B at 24 min. The initial mobile phase was re-established for 10 min before the next injection. The initial mobile phase was re-established for 10 min before the next injection. Tandem mass spectrometry analyses were performed with a 6330 Series Ion Trap LC–MS System (Agilent Technologies, USA) equipped with an electrospray ionization source (ESI) operating in negative mode. Identification of PACs was performed by Multiple Reaction Monitoring (MRM).

## 2.6. Determination of antioxidant activity

The reducing power assay was performed by mixing a proper dilution of samples (100 μl) with 250 μl of phosphate buffer (0.2 M, pH 6.6) and 250 μl of 1% w/v potassium ferricyanide [19]. The mixture was incubated at 50 °C for 20 min. At the end of incubation, 250 μl of 10% w/v of trichloracetic acid were added and the mixture and centrifuged at 1000 × *g* for 2 min. The supernatant (500 μl) was mixed with distilled water (500 μl) and 0.1% w/v iron (III) chloride (100 μl). The absorbance was measured at 700 nm against a blank sample. Trolox® (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as reference antioxidant compound.

The free radical scavenging activity was measured using 1,1-diphenyl-2-picryl-hydrazil stable radical (DPPH•) [19]. Five hundred microliter of DPPH working solution (0.1 mM in 95% v/v ethanol) were added to different volumes (10–500 μl) of diluted samples (50% v/v ethanol). The reaction mixture was left to stand for 30 min in the dark at room temperature and periodically shaken. The absorbance was measured at 517 nm against a blank sample. Trolox® was used as a reference antioxidant compound. For each extract, the antioxidant activity of samples was calculated using the following equation: % = $[(A_{blank} - A_{sample})/A_{blank}] * 100$; where $A_{blank}$ is the absorbance of blank, and $A_{sample}$ the absorbance of sample at 517 nm.

ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) was dissolved in MilliQ water at the final concentration of 7 mM and stored at −80 °C until use [19]. The ABTS radical cation ($ABTS^{\bullet+}$) was obtained by mixing the ABTS stock solution (7 mM) with 2.45 mM potassium persulfate. The

reaction was left in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with 95% v/v ethanol to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C before use. The samples were properly diluted with 50% v/v ethanol before the assay. For the colorimetric reaction, 450 μl of $ABTS^{\bullet+}$ solution were mixed with increasing volumes of samples (2.2–50 μl). The absorbance was read exactly after 2 min at 734 nm. Trolox® was used as a reference antioxidant compound. The antioxidant activity of samples was calculated using the following equation: % = $[(A_{blank} - A_{sample})/A_{blank}] * 100$; where $A_{blank}$ is the absorbance of blank, and $A_{sample}$ the absorbance of sample at 734 nm.

### 2.7. Statistical analysis

All experiments were carried out in triplicate and the results are expressed as mean ± standard deviation (SD). Statistic differences were estimated by ANOVA followed by the post hoc Bonferroni test. Values were considered statistically significant at $P < 0.05$. Regarding DPPH and ABTS antioxidant assays, the sample concentration that reduces by 50% the initial radical concentration ($IC_{50}$) was calculated by interpolation of experimental points. Regarding the reducing power assay, $IC_{50}$ values were expressed as the concentration at which absorbance was 0.5. All statistical analyses were carried out using SYSTAT 10.0.

## 3. Results and discussion

### 3.1. Subcritical $CO_2$ extraction of bilberry phenolics

Bilberry is a rich source of anthocyanins and other phenolic compounds and several methods have been used to improve their extraction. In the present work, SC $CO_2$ was applied for the pre-treatment of bilberry to improve extraction and was followed by a subsequent SubC $CO_2$ extraction with the use of a polar solvent. As expected, SC $CO_2$ was too lipophilic to extract phenolics and the extraction of anthocyanins and other phenolic compounds occurred only during SubC $CO_2$, using 10% v/v ethanol as co-solvent. Fig. 2 shows the kinetics of anthocyanin extraction; after 0.5 h the system was switched from supercritical to subcritical conditions and the anthocyanin recovery peaked about 30 min after the beginning of SubC $CO_2$, using 10% v/v ethanol as co-solvent. The yield of extraction was 0.62 (±0.05) mg $g^{-1}$ d.wt anthocyanins. In grape pomace, the subcritical extraction achieved the efficient recovery of anthocyanins with the use of 50% and 70% v/v of ethanol/water mixtures [15]. Our results are in agreement with the use of co-solvents for the efficient extraction of phenolics and anthocyanins from dried berries [20] and [21].

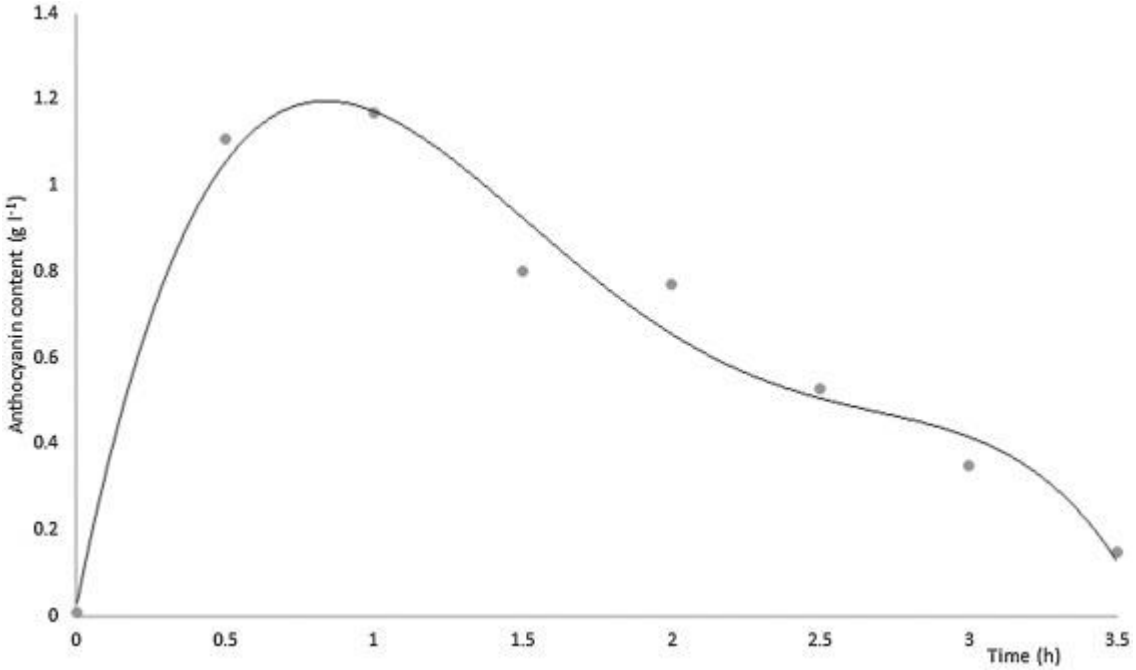


Fig. 2.

Kinetics of anthocyanin extraction. The extraction starts in supercritical $CO_2$ conditions and after 30 min the system is switched to SubC $CO_2$, using 10% v/v ethanol as co-solvent. The highest recovery of anthocyanins occurs after 30 min of subcritical $CO_2$ extraction.

The total content of phenols with SubC-$CO_2$ + 10% v/v ethanol was 72.18 (±1.13) mg $g^{-1}$ d.wt., which is in line with the literature data [1], [3] and [22]. The BL-DMAC assay allows the specific quantification of flavan-3-ols and proanthocyanidins at 640 nm excluding the anthocyanin interferences [18]. The quantification of total PACs performed by BL-DMAC method by SubC-$CO_2$ was 2.77 mg $g^{-1}$ d.wt.

## 3.2. Chemical composition of bilberry SubC-$CO_2$ extracts

The chemical composition of bilberry anthocyanins extracted by SubC-$CO_2$ + 10% v/v ethanol was characterized by the presence of delphinidin, cyanidin, petunidin and peonidin glycosides, whereas the presence of malvidin glycosides was not detected. In particular, subcritical $CO_2$ selectively extracted cyanidin-3-*O*-glucoside and cyanidin-3-*O*-arabinoside, which account for 60 and 24% of total detected anthocyanins, respectively. Delphinidin-3-*O*-glucoside was the most extracted among delphinidin glycosides, whereas the extraction of petunidin and peonidin glycosides was very low ( Table 1). The qualitative profile and the relative percentage of anthocyanins are consistent with the reported literature data [1] and [23].

Table 1.

Anthocyanin chemical composition of bilberry SubC-$CO_2$ (10% v/v ethanol) extract. Values are expressed as percentage of total anthocyanins [0.62 (±0.05) mg $g^{-1}$ d.wt] (standard deviation).

| Compound | Molecular ion $[M]^+$ (*m*/*z*) | Fragment ion (*m*/*z*) | Percentage |
|---|---|---|---|
| **Delphinidin-3-*O*-galactoside** | 465 | 303 | 0.62 (0.09) |
| **Delphinidin-3-*O*-glucoside** | 465 | 303 | 8.90 (0.72) |
| **Delphinidin-3-*O*-arabinoside** | 435 | 303 | 0.63 (0.04) |
| **Cyanidin-3-*O*-galactoside** | 449 | 287 | 4.94 (0.61) |
| **Cyanidin-3-*O*-glucoside** | 449 | 287 | 60.26 (1.15) |
| **Cyanidin-3-*O*-arabinoside** | 419 | 287 | 23.81 (1.14) |
| **Petunidin-3-O-galactoside** | 479 | 317 | 0.42 (0.07) |
| **Petunidin-3-*O*-glucoside** | 479 | 317 | 0.10 (0.03) |
| **Petunidin-3-*O*-arabinoside** | 449 | 317 | 0.02 (0.003) |
| **Peonidin-3-*O*-galactoside** | 463 | 301 | 0.22 (0.04) |
| **Peonidin-3-*O*-glucoside** | 463 | 301 | 0.03 (0.001) |
| **Peonidin-3-*O*-arabinoside** | 433 | 301 | 0.01 (0.002) |

HPLC-ESI-$MS_2$ analyses showed also the presence of several phenolic compounds including benzoic acid derivatives, phenyl propanoids and flavonoids in accordance to literature data [22] and [24]. The most abundant compound of this fraction was ellagic acid pentoside, followed by relatively high amounts of feruloyl hexoside (Table 2). Several quercetin glycosides were also present, with particular reference to quercetin-3-*O*-galactoside and quercetin-3-*O*-glucoside. Very low amounts of myricetin-3-*O*-galactoside were also present. Among other compounds, ellagic acid, chlorogenic acid, and dihydroxiybenzoic acid hexoside were also detected ( Table 2).

Table 2.

Chemical composition of other phenolic compounds present in the bilberry SubC-$CO_2$ (10% v/v ethanol) extract (standard deviation).

| Compound | Molecular ion $[M]^+$ (*m*/*z*) | Fragment ions (*m*/*z*) | Content (mg $g^{-1}$ of dry extract) |
|---|---|---|---|
| **Caffeic acid** | 179 | 135 | 0.028 (0.01) |
| **Ferulic acid** | 193 | 134 | 0.014 (0.003) |
| **Ellagic acid** | 301 | 257, 229 | 0.242 (0.02) |
| **Dihydroxybenzoic acid hexoside** | 315 | 153, 162 | 0.274 (0.01) |
| **Vanilic acid hesoside** | 329 | 167 | 0.010 (0.002) |
| **Caffeoyl hexoside** | 341 | 179, 135 | 0.014 (0.01) |
| **Chlorogenic acid** | 353 | 191, 179 | 0.712 (0.07) |
| **Feruloyl hexoside** | 355 | 193, 134 | 1.201 (0.06) |
| **Unknown quinic acid derivative** | 383 | 191 | 0.081 (0.01) |
| **Ellagic acid pentoside** | 433 | 301 | 2.609 (0.88) |
| **Quercetin-3-*O*-galactoside** | 463 | 301 | 0.812 (0.05) |
| **Quercetin-3-*O*-glucoside** | 463 | 301 | 0.752 (0.07) |
| **Quercetin-3-*O*-rutinoside** | 609 | 301 | 0.129 (0.03) |
| **Quercetin-3-*O*-glucuronide** | 477 | 301 | 0.018 (0.002) |
| **Quercetin-3-*O*-xyloside** | 433 | 301 | 0.023 (0.004) |
| **Quercetin-3-*O*-arabinoside** | 433 | 301 | 0.099 (0.01) |
| **Myricetin-3-*O*-galactocside** | 479 | 317 | 0.005 (0.001) |
| **Kaempferol-3-*O*-galactoside** | 447 | 285 | 0.005 (0.001) |
| **Kaemferol-3-*O*-glucoside** | 447 | 285 | 0.017 (0.002) |
| **Kaempferol-3-*O*-(-6-coumaroyl)-glucoside** | 593 | 285 | 0.014 (0.002) |

Dimeric and trimeric proanthocyanidins were already reported in *V. myrtillus*, although they were tentatively quantified by depolymerisation methods of fresh berry [25] and [26]. From a qualitative point of view, only dimeric B-type proanthocyanidins were detected in HPLC-ESI-MS/MS analyses and only one peak was detected at *m*/*z* 577 with base fragment ions *m*/*z* 289 and 407 (Supplementary Fig. S1). The presence of proanthocyanidins can also significantly affect the antioxidant activity of extracts [27]. Fig. 3 depicts the structure formulae of some identified phenolic compounds.

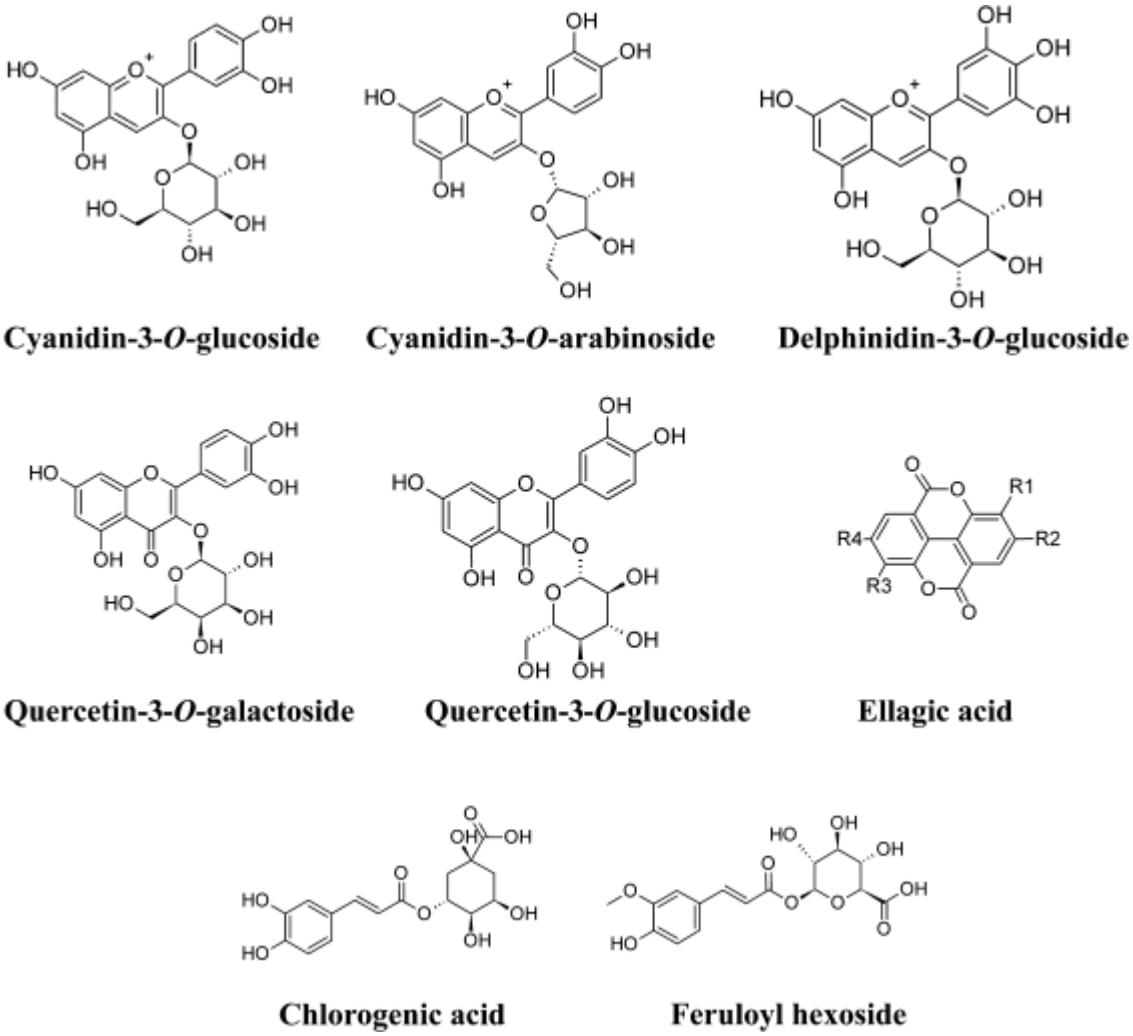


Fig. 3.

Structure formulae of some identified anthocyanins, flavonoids and other phenolic compounds.

## 3.3. Antioxidant activity of bilberry SubC-$CO_2$ extracts

In order to assess the bioactive properties of the SubC-$CO_2$ extract, we evaluated their reducing power by ferric thiocyanate assay and the free radical scavenging activities by ABTS and DPPH radical assays. The antioxidant activity was compared to Trolox® as a reference compound. In general, the extracts were more active as antioxidants when tested by the ABTS assay (Table 3) since the steric accessibility of DPPH nitrogen-centred radical strongly affects the reaction rate of antioxidant compounds [28]. Extracts showed always a high antioxidant activity, with values comparable to those reported for berries containing anthocyanins [5], [26] and [29]. However, SubC-$CO_2$ extract values were always significantly lower when compared to Trolox® (Table 3).

Table 3.

Antioxidant activity of SubC-$CO_2$ (10% v/v ethanol) fraction and comparison with Trolox®. Data are expressed as the $IC_{50}$ in μg of dry extract (standard deviation).

| Assay | SubC-$CO_2$ + ethanol 10% | Trolox® |
|---|---|---|
| DPPH | 102.66 (2.64)[a] | 20.80 (0.26)[b] |
| ABTS | 8.49 (0.41)[a] | 0.82 (0.02)[b] |
| Reducing power | 10.30 (0.10)[a] | 3.00 (0.01)[b] |

In the same row, different letters indicate significant ($P < 0.05$) differences.

The antioxidant activity of the bilberry SubC-$CO_2$ extract correlates with the selective extraction of cyanidin-3-*O*-glucoside. This compound is a potent antioxidant anthocyanin, which has been shown to reduce Reactive Oxygen Species (ROS) formation and possess radical scavenging activity through inhibition of glutamate-induced $Zn^{2+}$ signalling. Cyanidin-3-*O*-glucoside has been involved in protection from oxidation-dependent protein damage [30], interaction with lipid and glucose metabolism [31], protection against the adverse effects of UV-B radiation [32], protection from hepatic damage through antioxidant and anti-inflammatory mechanisms [33], reduction of photoreceptor damage and functionally improve scotopic visual functions [34] and protection against various stressors preventing several pathological conditions [35] and [36].

SubC-$CO_2$ extracts were also rich in delphinidin-3-*O*-glucoside. This compound is another potent antioxidant with antinflammatory properties that prevents hypoxia-induced apoptosis of embryonic cells [37] and has been used to treat diabetes mellitus complications [38], suppress cancer cell invasion [39], with higher activity when the glycoside is hydrolyzed to its aglycon form [40].

In *Vaccinium* species, the antioxidant activity is related not only to anthocyanins but also to chlorogenic acid, caffeic acid derivatives, flavonoids and proanthocyanidins [3], [22] and [24]. Among the several phenolics present in the bilberry SubC-$CO_2$ extract, ellagic acid is known for its antioxidant activity and was recently found to show anti-inflammatory and antiapoptotic properties [41]. The selective extraction of several quercetin glycosides was also of particular interest owing to its anticancer [30] and antioxidant [42] properties.

# 4. Conclusion

This study investigated the selective recovery of high value molecules from bilberry by a stepwise extraction procedure through supercritical/subcritical $CO_2$ with ethanol as co-solvent. SubC-$CO_2$ selectively extracted a phenolic/anthocyanin rich faction with high antioxidant activity. The preliminary supercritical treatment allowed the selective extraction in subcritical conditions of cyanidin glycosides, ellagic acid and quercetin glycosided, whose biological activity is known and important for pharmaceutical and nutraceutical applications. Possible improvement of the extraction efficiency can be obtained by the use of different drying techniques, as recently reported [43]. The SubC-$CO_2$ extraction of bilberry performed with ethanol as co-solvent may be considered an efficient extraction method to recover fractions with high antioxidant and potential pharmacological activity.

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# References

A.K. Primetta, L. Jaakola, F.A. Ayaz, H. Inceer, K.R. Riihinen Anthocyanin fingerprinting for authenticity studies of bilberry (*Vaccinium myrtillus* L.) Food Control, 30 (2013), pp. 662–667

F. Ishikawa, M. Oishi, T. Shindo, M. Horie, A. Yasui, M. Nakazato Analysis of anthocyanins in dietary supplements containing blueberry extract J. Food Hyg. Soc. Jpn., 49 (2008), pp. 339–346

K. Aaby, S. Grimmer, L. Holtung Extraction of phenolic compounds from bilberry (*Vaccinium myrtillus* L) press residue: Effects on phenolic composition and cell proliferation LWT – Food Sci. Technol., 54 (2013), pp. 257–264

A. Bunea, D. Rugina, A. Pintea, S. Andrei, C. Bunea, R. Pop, C. Bele Carotenoid and fatty acid profiles of bilberries and cultivated blueberries from Romania Chem. Pap., 66 (2012), pp. 935–939

S.h. Zhou, Z.x. Fang, Y. Lue, J.C. Chen, D.h. Liu, X.q. Ye Phenolics and antioxidant properties of bayberry (*Myrica rubra* Sieb. et Zucc.) pomace Food Chem., 112 (2009), pp. 394–399

T. Vatai, M. Skerget, Z. Knez Extraction of phenolic compounds from elder berry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide J. Food Eng., 90 (2009), pp. 246–254

A. Capuzzo, M.E. Maffei, A. Occhipinti Supercritical fluid extraction of plant flavors and fragrances Molecules, 18 (2013), pp. 7194–7238

E. Reverchon, I. De Marco Supercritical fluid extraction and fractionation of natural matter J. Supercrit. Fluids, 38 (2006), pp. 146–166

R. Campardelli, L. Baldino, E. Reverchon Supercritical fluids applications in nanomedicine J. Supercrit. Fluids, 101 (2015), pp. 193–214

A. Hedayati, S.M. Ghoreishi Supercritical carbon dioxide extraction of glycyrrhizic acid from licorice plant root using binary entrainer: experimental optimization via response surface methodology J. Supercrit. Fluids, 100 (2015), pp. 209–217

A.R.M. Costa, L.A.P. Freitas, J. Mendiola, E. Ibanez *Copaifera langsdorffii* supercritical fluid extraction: chemical and functional characterization by LC/MS and in vitro assays J. Supercrit. Fluids, 100 (2015), pp. 86–96

H.T. Celik, M. Guru Extraction of oil and silybin compounds from milk thistle seeds using supercritical carbon dioxide J. Supercrit. Fluids, 100 (2015), pp. 105–109

T.T. Garmus, L.C. Paviani, C.L. Queiroga, F.A. Cabral Extraction of phenolic compounds from pepper-rosmarin (*Lippia sidoides* Cham.) leaves by sequential extraction in fixed bed extractor using supercritical $CO_2$, ethanol and water as solvents J. Supercrit. Fluids, 99 (2015), pp. 68–75

E. Golmakani, A. Mohammadi, T.A. Sani, H. Kamali Phenolic and flavonoid content and antioxidants capacity of pressurized liquid extraction and perculation method from roots of *Scutellaria pinnatifida* A. Hamilt. subsp alpina (Bornm) Rech. F J. Supercrit. Fluids, 95 (2014), pp. 318–324

J.K. Monrad, L.R. Howard, J.W. King, K. Srinivas, A. Mauromoustakos Subcritical solvent extraction of anthocyanins from dried red grape pomace J. Agric. Food Chem., 58 (2010), pp. 2862–2868

V.L. Singleton Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents Agric. Biol. Environ. Sci., 48 (1985) 18-18

M.M. Giusti, R.E. Wrolstad Characterization and measurement of anthocyanins by UV–visible spectroscopy Current Protocols in Food Analytical Chemistry, John Wiley & Sons, Inc. (2001)

R.L. Prior, E. Fan, H. Ji, A. Howell, C. Nio, M.J. Payne, J. Reed Multi-laboratory validation of a standard method for quantifying proanthocyanidins in cranberry powders J. Sci. Food Agric., 90 (2010), pp. 1473–1478

A. Capuzzo, A. Occhipinti, M.E. Maffei Antioxidant and radical scavenging activities of chamazulene Nat. Prod. Res., 28 (2014), pp. 2321–2323

J.W. King Modern supercritical fluid technology for food applications Annu. Rev. Food Sci. Technol., 5 (2014), pp. 215–238

J. Paes, R. Dotta, G.F. Barbero, J. Martinez Extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium myrtillus* L.) residues using supercritical $CO_2$ and pressurized liquids J. Supercrit. Fluids, 95 (2014), pp. 8–16

S. Moze, T. Polak, L. Gasperlin, D. Koron, A. Vanzo, N.P. Ulrih, V. Abram Phenolics in Slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.) J. Agric. Food Chem., 59 (2011), pp. 6998–7004

M.N. Stefanut, A. Cata, R. Pop, C. Mosoarca, A.D. Zamfir Anthocyanins HPLC-DAD and MS characterization, total phenolics, and antioxidant activity of some berries extracts Anal. Lett., 44 (2011), pp. 2843–2855

G.A. Garzon, C.E. Narvaez, K.M. Riedl, S.J. Schwartz Chemical composition, anthocyanins, non-anthocyanin phenolics and antioxidant activity of wild bilberry (*Vaccinium meridionale* Swartz) from Colombia Food Chem., 122 (2010), pp. 980–986

L. Jaakola, K. Maatta, A.M. Pirttila, R. Torronen, S. Karenlampi, A. Hohtola Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development Plant Physiol., 130 (2002), pp. 729–739

K.R. Maatta-Riihinen, M.P. Kahkonen, A.R. Torronen, I.M. Heinonen Catechins and procyanidins in berries of *Vaccinium* species and their antioxidant activity J. Agric. Food Chem., 53 (2005), pp. 8485–8491

D. Chatterjee, N.T. Jadhav, P. Bhattacharjee Solvent and supercritical carbon dioxide extraction of color from eggplants: characterization and food applications LWT – Food Sci. Technol., 51 (2013), pp. 319–324

R.L. Prior, X.L. Wu, K. Schaich Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements J. Agric. Food Chem., 53 (2005), pp. 4290–4302

H.G. Duymus, F. Goger, K.H.C. Baser In vitro antioxidant properties and anthocyanin compositions of elderberry extracts Food Chem., 155 (2014), pp. 112–119

K. Sak Site-specific anticancer effects of dietary flavonoid quercetin Nutr. Cancer, 66 (2014), pp. 177–193

S. Park, S. Kang, D.Y. Jeong, S.Y. Jeong, J.J. Park, H.S. Yun Cyanidin and malvidin in aqueous extracts of black carrots fermented with *Aspergillus oryzae* prevent the impairment of energy, lipid and glucose metabolism in estrogen-deficient rats by AMPK activation Genes Nutr., 10 (2015)

P. Pratheeshkumar, Y.O. Son, X. Wang, S.P. Divya, B. Joseph, J.A. Hitron, L. Wang, D. Kim, Y.Q. Yin, R.V. Roy, J. Lu, Z. Zhang, Y.T. Wang, X.L. Shi Cyanidin-3-glucoside inhibits UVB-induced oxidative damage and inflammation by regulating MAP kinase and NF-kappa B signaling pathways in SKH-1 hairless mice skin Toxicol. Appl. Pharmacol., 280 (2014), pp. 127–137

B.O. Cho, C.W. Lee, Y. So, C.H. Jin, S.Y. Kang, D.S. Kim, K. Il Seo, H.S. Yook, I.Y. Jeong Cyanidin-3-glucoside ameliorates $CCl_4$-induced liver injury in mice Food Sci. Biotechnol., 23 (2014), pp. 1313–1319

S.H. Lee, E. Jeong, S.S. Paik, J.H. Jeon, S.W. Jung, H.B. Kim, M. Kim, M.H. Chun, I.B. Kim Cyanidin-3-glucoside extracted from mulberry fruit can reduce N-methyl-N-nitrosourea-induced retinal degeneration in rats Curr. Eye Res., 39 (2014), pp. 79–87

S. Anwar, A. Speciale, D. Fratantonio, M. Cristani, A. Saija, F. Virgili, F. Cimino Cyanidin-3-O-glucoside modulates intracellular redox status and prevents HIF-1 stabilization in endothelial cells in vitro exposed tochronic hypoxia Toxicol. Lett., 226 (2014), pp. 206–213

L.N. Qin, J.L. Zhang, M.L. Qin Protective effect of cyanidin 3-O-glucoside on beta-amyloid peptide-induced cognitive impairment in rats Neurosci. Lett., 534 (2013), pp. 285–288

B.N. Seo, J.M. Ryu, S.P. Yun, J.H. Jeon, S.S. Park, K.B. Oh, J.K. Park, H.J. Han Delphinidin prevents hypoxia-induced mouse embryonic stem cell apoptosis through reduction of intracellular reactive oxygen species-mediated activation of JNK and NF-kappa B, and Akt inhibition Apoptosis, 18 (2013), pp. 811–824

A. Gharib, Z. Faezizadeh, M. Godarzee Treatment of diabetes in the mouse model by delphinidin and cyanidin hydrochloride in free and liposomal forms Planta Med., 79 (2013), pp. 1599–1604

N.K. Im, W.J. Jang, C.H. Jeong, G.S. Jeong Delphinidin suppresses PMA-induced MMP-9 expression by blocking the NF-kappa B activation through MAPK signaling pathways in MCF-7 human breast carcinoma cells J. Med. Food, 17 (2014), pp. 855–861

T. Sogo, T. Kumamoto, H. Ishida, A. Hisanaga, K. Sakao, N. Terahara, K. Wada, D.X. Hou Comparison of the inhibitory effects of delphinidin and its glycosides on cell transformation Planta Med., 81 (2015), pp. 26–31

I. Kilic, Y. Yesiloglu, Y. Bayrak Spectroscopic studies on the antioxidant activity of ellagic acid Spectrochim. Acta A – Mol. Biomol. Spectrosc., 130 (2014), pp. 447–452

P. Duchnowicz, M. Broncel, A. Podsedek, M. Koter-Michalak Hypolipidemic and antioxidant effects of hydroxycinnamic acids, quercetin, and cyanidin 3-glucoside in hypercholesterolemic erythrocytes (in vitro study) Eur. J. Nutr., 51 (2012), pp. 435–443

S. Kerbstadt, L. Eliasson, A. Mustafa, L. Ahrne Effect of novel drying techniques on the extraction of anthocyanins from bilberry press cake using supercritical carbon dioxide Innov. Food Sci. Emerg. Technol., 29 (2015), pp. 209–214