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

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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.



## 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 [1,3]. 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 [8–12]. 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 [8,9]. The effectiveness of subcritical extraction of

*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.

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anthocyanins and other polyphenols has been investigated in the food industry [13–15].

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•) (>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.

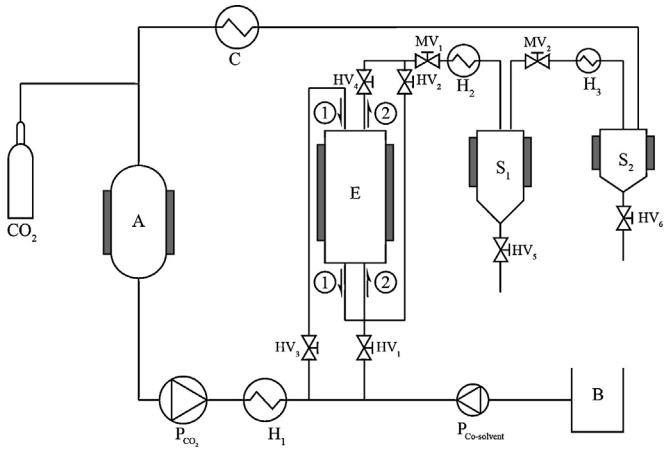


**Fig. 1.** Schematic representation of the supercritical fluid extractor used in this work. A $CO_2$ tank provides the $CO_2$ used for extraction. (1) Fluid circulation during supercritical extraction; (2) fluid circulation during sub-critical extraction; (A) $CO_2$ buffer storage tank; (B) co-solvent 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:

$$C(\mathrm{mg/l}) = \frac{A * MW * DF * 1000}{\varepsilon * l}$$

where $A = \left[(A_{\lambda_{max}} - A_{700})_{pH1.0} - (A_{\lambda_{max}} - A_{700})_{pH4.5}\right]$, $MW$ 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%