



## Supercritical CO<sub>2</sub> extraction of bilberry (*Vaccinium myrtillus* L.) seed oil: Fatty acid composition and antioxidant activity

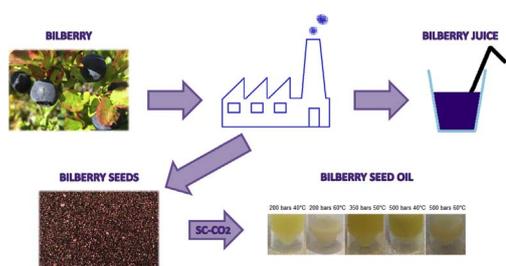


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### GRAPHICAL ABSTRACT



### ARTICLE INFO

#### Keywords:

Supercritical CO<sub>2</sub> extraction  
Bilberry seeds  
Antioxidant activity  
Vitamin E  
Radical scavenging

### ABSTRACT

Bilberry seed oils extracted with supercritical carbon dioxide (SC-CO<sub>2</sub>) for 80 min at 20, 35, and 50 MPa and at 40°, 50° and 60 °C were evaluated to compare the yield, composition, and antioxidant recovery. Analyses of fatty acids, free radical scavenging activity (DPPH), vitamin E and peroxide contents revealed that yield, vitamin E, efficient concentration (EC50), and Peroxide value (PV) varied significantly among the obtained bilberry seed oils, whereas the fatty acid compositions were similar. The oil extracted at 20 MPa and 60 °C had the best recovery of vitamin E and the lowest EC50 and PV.

The high levels of vitamin E and polyunsaturated fatty acids, as well as the low ω6/ω3 ratios (< 1) and the low PVs in all the extracts suggest bilberry seed oil is a valuable source of bioactive compounds and high potential for use of bilberry by-product extracts in added value foods and nutraceutical products.

### 1. Introduction

Bilberries (*Vaccinium myrtillus* L.), which grow wild in northern Europe and in North America, contain many bioactive compounds, such as anthocyanins, flavonols, and tocopherols. The consumption of these naturally occurring bioactive compounds has been associated with a reduced risk of developing several chronic diseases, including cardiovascular and inflammatory disorders, diabetes, and arteriosclerosis [1]. For this reason, bilberries are considered to be an attractive ingredient in different food products and are often processed into juice [2]. The

processing of fruits and vegetables typically results in by-products, such as leaves, seeds, and peels [3], which are currently underutilized, even though they are often rich in bioactive compounds.

Bilberry seeds constitute around 2.9% (f.w.) of the whole berry, and approximately 30.5% (d.w.) of the seed is oil [4]. Bilberry seed oil is a good source of polyunsaturated fatty acids (PUFAs) and contains high levels of antioxidants, such as vitamin E [5]. Consequently, this oil could be used in value-added products, dietary supplements, and cosmetic preparations or it could be stabilized using microencapsulation technologies for use in the food industry [6].

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Solvent extraction and cold pressing are traditionally used to extract oil from seeds. However, solvent extraction has the disadvantage that it uses organic solvents that are toxic for humans and the environment, while cold pressing often results in low yields [5]. Another drawback of conventional solvent extraction methods is the requirement to use high temperatures, which leads to the thermal degradation of bioactive compounds, necessitating additional steps to remove solvent residues from the extracts [7]. An alternative approach is to use supercritical fluid extraction (SFE) to replace these traditional extraction technologies. SFE recovers unaltered biological compounds, and it can use carbon dioxide as a solvent. Carbon dioxide is ideal as a supercritical solvent for the food industry because it is non-toxic and can be easily removed from extracts [8]. Furthermore, carbon dioxide reaches its critical point under relatively mild conditions (31 °C, 74 bar), and the extraction is carried out in the absence of oxygen, which is beneficial for the preservation of bioactive compounds [9]. SFE also allows for the fractionation of compounds through a process in which the pressure and temperature are varied so as to optimize the solubilities of specific compounds with desirable properties [10].

Bilberry seeds and their fruits contain high concentrations of fatty acids, vitamins, and phenolic compounds, and recent studies have shown the potential of using SFE and subcritical CO<sub>2</sub> for the extraction of valuable compounds from bilberries [11,12]. While information on the composition and stability of bilberry lipids following SFE is limited, recent reports on the effects of processing conditions on yield, composition, and storage stability are promising [12,13]. However, information about the recovery of vitamin E and the antioxidant activity of the oil extracted from bilberry seeds remains scarce.

The objective of this study was to evaluate the impacts of pressure (at 20, 35, and 50 MPa) and temperature (at 40°, 50° and 60 °C) during supercritical CO<sub>2</sub> extraction for 80 min on the extraction yield, fatty acid composition, and levels of recovery of antioxidant compounds of bilberry seed oils.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Bilberry seeds

Bilberry seeds (*Vaccinium myrtillus* L.), which are a by-product of juice production, were obtained from Svantes Vilt and Bär (Harads, Sweden) and were stored at –20 °C until the extractions. The seeds were oven-dried (Garomat 142 Electrolux, Stockholm, Sweden) at 40 °C for 16 h. The final moisture content of the seeds was  $2.7 \pm 0.3\%$ . The seeds were ground in a coffee mill (2393 OBH Nordica, Stockholm, Sweden) for 30 s just before each extraction. The particle sizes of the ground samples were determined in triplicate using a sieve shaker (Fritsch, Idar-Oberstein, Germany). The weight percentages of the different size ranges were as follows: < 250 µm, 0.34%; 250–500 µm, 6.08%; 500–710 µm, 75.04%; > 710 µm, 18.54%.

#### 2.1.2. Solvents and reagents

Carbon dioxide (> 99.99% purity), was supplied by AGA Gas AB (Växjö, Sweden). DL  $\alpha$ -tocopherol ( $\geq 97\%$  purity) and 1,1-diphenyl-2-picrylhydrazil (DPPH) were purchased from Alfa Aesar (Karlsruhe, Germany). The standards  $\alpha$ -tocotrienol (97%) and (R)- $\gamma$ -tocotrienol ( $\geq 97.0\%$ ) were obtained from Fluka, Sigma–Aldrich (Stockholm, Sweden).  $\delta$ -Tocopherol (95.5%) was from Supelco (Bellefonte, PA, USA). The external standard of the FAME mixture GLC 463 and the internal standard heptanoic (margaric) acid C 17:0 ( $\geq 99\%$ ) were purchased from Nu-Chek Prep (Elysian, MN, USA). Butylated hydroxytoluene (BHT) and acetyl chloride ( $\geq 99.0\%$ ) were obtained from Fluka, Sigma–Aldrich (Stockholm, Sweden). Methanol (LC–MS grade,  $\geq 99.9\%$ ), chloroform (HPLC grade,  $\geq 99.8\%$ ), toluene (HPLC grade, 99.9%), cyclohexane (HPLC grade,  $\geq 99.7\%$ ), petroleum ether (ACS reagent,  $\geq 95.0\%$ ), barium chloride dehydrate ( $\geq 99.0\%$ ), ammonium

thiocyanate, and cumene hydroperoxide (~80%) were all from Sigma–Aldrich (Stockholm, Sweden). 2,2,4-Trimethylpentane (HPLC grade, 99.5%) was purchased from Lab-Scan (Dublin, Ireland). Iron (II) sulfate heptahydrate ( $\geq 99.5\%$ ) was obtained from Merck (Darmstadt, Germany). HCl (M = 36.46) was purchased from Merck (Darmstadt, Germany). Ethanol absolute (AnalaR NORMAPUR) was from VWR International (Spånga, Sweden).

### 2.2. Methods

#### 2.2.1. Supercritical CO<sub>2</sub> extraction

Supercritical CO<sub>2</sub> extractions were carried out using the laboratory-scale supercritical fluid system WATERS SFE-500M1-2-C50 (Waters Inc., Pittsburgh, PA, USA). The unit consisted of a CO<sub>2</sub> pump connected to a cooling bath (F32-HD; Julabo GmbH Seelbach, Germany), an automated back-pressure regulator to control the pressure, a 500 mL extractor vessel surrounded by a heating jacket, and one separation vessel (500 mL), maintained at 10 bar and 25 °C. Ground seeds ( $50 \pm 1$  g) were loaded into the extraction vessel. Glass wool (2 g) was used to protect the vessel filters and to fill up the empty space in the vessel. The SC-CO<sub>2</sub> flow rate was 40 g min<sup>-1</sup>. After extraction, the oils were stored at –80 °C for 1 month until analysis. The experiments were performed based on a factorial design (Table 1), and the mid-point was selected based on data obtained from previous studies [5]. The remaining conditions were chosen based on the variation of the densities of SC-CO<sub>2</sub> under the final conditions (Table 1). Density is one of the most important properties of a supercritical fluid, since the solvent capacity is density-dependent and allows fine-tuning for the selective separation of specific compounds [14]. Preliminary trials were performed with the chosen conditions, to select the optimal extraction time. Samples were collected every 10 min for 120 min, to create an extraction curve for each condition. The extraction time chosen was 80 min, as the yield was scarcely improved by extending the extraction time beyond this time-point (up to 120 min).

The SFEs were performed in duplicate, except for the mid-point sample (Sample 3) that was extracted six times. Duplicate analyses were performed for all the samples.

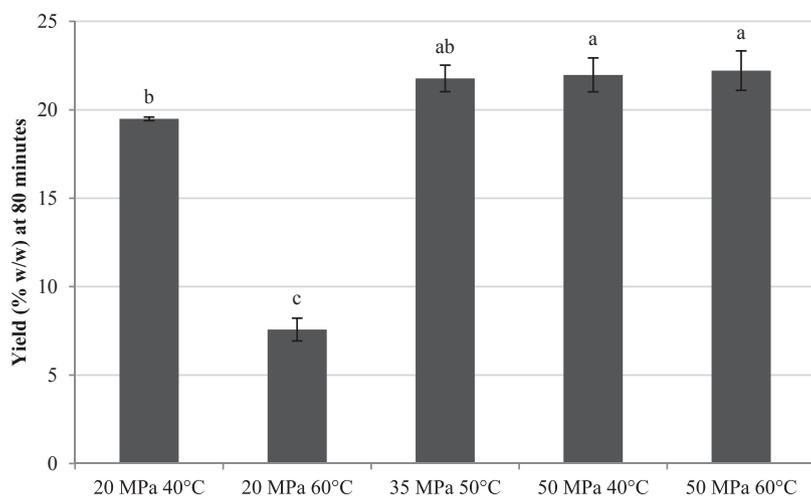
#### 2.2.2. Analysis of fatty acid composition

The methylation of fatty acids derived from bilberry seed oil was based on the method of Cavonius and co-workers [15]. Approximately  $25 \pm 5$  mg of extract were diluted in 1 mL of toluene and mixed with 1 mL of freshly prepared 10% (v/v) acetyl chloride in methanol. The tubes were incubated for 2 h at 70 °C. After cooling, 1 mL of Milli-Q water was added. The methyl esters of fatty acids (FAMES) were extracted by mixing the sample with 2 mL of petroleum ether, centrifuging for 5 min (2500 × g) and collecting the upper organic phase in a new tube. The FAMES were evaporated under N<sub>2</sub> at 40 °C and re-dissolved in 1 mL of 2,2,4-trimethylpentane. Analysis of the FAMES was performed in an Agilent 7890 A GC system equipped with a J&W DB-wax column (30 m × 0.250 mm × 0.25 µm), interfaced with an Agilent 5975C triple-axis mass spectrometric (MS) detector in electron impact mode. The injection volume was 1 µL, the split ratio used was 1:10, and the inlet temperature was 250 °C. Helium was used as the carrier gas

**Table 1**

Experimental conditions for supercritical CO<sub>2</sub> extraction of bilberry seeds, density of SC-CO<sub>2</sub> and SC-CO<sub>2</sub>/Extract.

Sample	Pressure (MPa)	Temperature (°C)	Density (kg/m <sup>3</sup> )	SC-CO <sub>2</sub> /Extract (g/g)
1	20	40	840	328
2	20	60	724	847
3	35	50	899	294
4	50	40	991	291
5	50	60	934	288



**Fig. 1.** Extraction yield (% w/w) of bilberry seed oils extracted at different conditions. Results are mean values  $\pm$  standard deviation of duplicate extractions for all samples except 35 MPa at 50 °C, where 6 replicate extractions were performed. Different letters above error bars denote significant difference ( $p < 0.05$ ) according to ANOVA followed by Tukey's post-hoc test.

with a fixed flow rate of  $1 \text{ mL} \cdot \text{min}^{-1}$  during the temperature program, which consisted of: 100 °C for the first 4 min; 100–205 °C at  $4 \text{ }^\circ\text{C} \cdot \text{min}^{-1}$ ; 205–230 °C at  $1 \text{ }^\circ\text{C} \cdot \text{min}^{-1}$ ; and 230 °C for the final 5 min. An external standard of a mixture of FAMES was used to identify the different peaks of the bilberry seed oil samples. An internal standard (C 17:0) was used for the quantification of fatty acids, expressed as grams of FA per 100 g of bilberry seed oil.

### 2.2.3. Analysis of vitamin E

Samples were prepared by weighing  $40 \pm 5 \text{ mg}$  of bilberry seed oil in a glass tube, adding 2 mL of methanol, and vortexing. Thereafter, the tubes were placed in an ultrasonic bath for 15 min. The upper phase (1 mL) was collected and transferred to a vial. The analyses of tocopherols and tocotrienols were carried out in the JASCO LC-Net II ADC liquid chromatography system (JASCO International Ltd., Tokyo, Japan) equipped with a quaternary pump (JASCO PU-2089), Intelligent Autosampler (JASCO AS-2057), and a fluorescence detector (model RF-551). A reverse-phase C18 Kromasil column ( $150 \text{ mm} \times 2.1 \text{ mm}$  i.d., particle size  $5 \mu\text{m}$ ; EKA Nobel, Bohus, Sweden) was used. The mobile phase was a mixture of methanol and water (95:5 v/v). The flow rate of the mobile phase was  $0.6 \text{ mL} \cdot \text{min}^{-1}$  and the injection volume was  $10 \mu\text{L}$ . The excitation and emission wavelengths were 295 nm and 330 nm, respectively. Standard solutions of  $\delta$ -tocopherol and  $\alpha$ - and  $\gamma$ -tocotrienols were freshly prepared and run in parallel with the samples. The tocopherols and tocotrienols were identified by comparing their retention times with those of authentic standards. For quantification, calibration curves in the range of  $1\text{--}5 \mu\text{g} \cdot \text{mL}^{-1}$  for  $\delta$ -tocopherol,  $0.4\text{--}2 \mu\text{g} \cdot \text{mL}^{-1}$  for  $\alpha$ -tocotrienol, and  $4\text{--}20 \mu\text{g} \cdot \text{mL}^{-1}$  for  $\gamma$ -tocotrienol were used by plotting the peak area of each compound against the respective concentration. The values shown are in mg of vitamin E  $100 \text{ g}^{-1}$  of bilberry seed oil.

### 2.2.4. Free radical scavenging activity (DPPH)

Radical scavenging activity was evaluated using the 1,1-diphenyl-2-picrylhydrazil (DPPH) method adapted from Brand-Williams et al. [16]. The solutions of DPPH radicals and  $\alpha$ -tocopherol were prepared in ethanol. The DPPH radical stock solution was prepared daily at a concentration of  $100 \mu\text{M}$  and stored at 4 °C in the dark. Bilberry seed oils were diluted in ethanol to concentrations of 20, 16, 12, 8, and  $4 \text{ mg} \cdot \text{mL}^{-1}$ , and 1 mL of each dilution was mixed with 1 mL of freshly prepared DPPH solution. Solutions of  $\alpha$ -tocopherol at concentrations of 0.045, 0.035, 0.025, 0.015, and 0.005 mM were used as positive controls [17]. The mixtures were incubated in the dark for 40 min at room temperature. At 40 min, all the reactions had reached the steady state. The absorbance values were measured at 517 nm in a spectrophotometer (Ultrospec 1000; Pharmacia Biotech (Biochrom) Ltd, Cambridge, England). The efficient concentration ( $\text{EC}_{50}$ ) was calculated

using linear regression analysis.

### 2.2.5. Determination of the peroxide value

Determination of the peroxide value (PV) was based on the method described by Undeland et al. [18] for oil samples. Bilberry seed oil (approximately 0.05 g) was diluted in 2 mL of cold  $\text{CHCl}_3\text{:MeOH}$  (1:1 v/v) plus 0.05% BHT. Then, 1.33 mL of  $\text{CHCl}_3\text{:MeOH}$  (1:1 v/v) + 0.05% BHT were added and the mixture was vortexed. Ammonium thiocyanate (4.38 M) and iron(II) chloride (9 mM) were added ( $33.4 \mu\text{L}$  of each), with rapid vortexing between the additions of each solution. The samples were incubated for exactly 20 min at room temperature, and the absorbance at 500 nm was read. A standard curve of cumene hydroperoxide was used for quantification and the results are expressed as milliequivalents (mEq) of peroxide per kilogram (kg) of oil.

### 2.2.6. Statistical analysis

Each extract was analyzed in duplicate. The tests for statistical significance of differences between treatments were performed by analyzing variance (ANOVA) at a significance level of  $p = 0.05$ , followed by a Tukey post-hoc test ( $\alpha = 0.05$ ).

## 3. Results and discussion

### 3.1. Extraction yield

The effects of pressure and temperature on SFE of bilberry seeds were investigated for pressures of 20, 35, and 50 MPa and temperatures of 40°, 50° and 60 °C. Fig. 1 shows that the different extraction conditions influenced the obtained yields (expressed as g oil/100 g seeds). The yields ranged from 7.6% to 22.2% (w/w), depending on the pressure and temperature applied. The lowest yield was obtained at 20 MPa and 60 °C, and the highest yield was at 50 MPa and 60 °C. The level of oil recovery was significantly higher at the higher pressures (35 MPa and 50 MPa), independently of the temperature used. This outcome has been attributed to the facts that: solvent density is increased at higher pressures; and the solubility of oil is higher in  $\text{CO}_2$  [19,20]. The temperature, as well as the pressure, influenced the yield. An increase in temperature at 20 MPa decreased the extraction yield. However, extraction involving an increase in temperature at a pressure 50 MPa did not result in a reduction of the yield. The difference in yields observed with increases in the temperature of the process, in combination with a change in pressure, can be explained by the opposite effects exerted by temperature on oil solubility [19]. Increasing the temperature leads to a reduction in solvent density, i.e., decreases the solvation power, while at the same time increasing the vapor pressure of the solutes. This leads to an increase in the solubility of oil in  $\text{CO}_2$  [19]. The pressure at which the temperature effect on the oil yield is reversed is called the 'crossover

**Table 2**  
Fatty acid composition of bilberry seed oils extracted at different conditions.

Experimental conditions	Fatty acids% (w/w)					$\omega 6/\omega 3$ ratio
	16:0	18:0	18:1 n-9	18:2 n-6	18:3 n-3	
20 MPa 40 °C	5.5 ± 0.1 <sup>a</sup>	1.5 ± 0.1 <sup>a</sup>	23.2 ± 0.0 <sup>a</sup>	33.6 ± 0.1 <sup>a</sup>	36.2 ± 0.1 <sup>b</sup>	0.93 ± 0.00 <sup>a</sup>
20 MPa 60 °C	6.1 ± 0.1 <sup>b</sup>	1.4 ± 0.1 <sup>a</sup>	23.3 ± 0.0 <sup>b</sup>	33.7 ± 0.2 <sup>a</sup>	35.5 ± 0.0 <sup>a</sup>	0.95 ± 0.01 <sup>b</sup>
35 MPa 50 °C	5.3 ± 0.1 <sup>a</sup>	1.5 ± 0.0 <sup>a</sup>	23.3 ± 0.0 <sup>b</sup>	33.6 ± 0.0 <sup>a</sup>	36.3 ± 0.1 <sup>b</sup>	0.93 ± 0.00 <sup>a</sup>
50 MPa 40 °C	5.3 ± 0.1 <sup>a</sup>	1.5 ± 0.0 <sup>a</sup>	23.4 ± 0.0 <sup>c</sup>	33.6 ± 0.0 <sup>a</sup>	36.1 ± 0.0 <sup>b</sup>	0.93 ± 0.00 <sup>a</sup>
50 MPa 60 °C	5.3 ± 0.1 <sup>a</sup>	1.6 ± 0.1 <sup>a</sup>	23.4 ± 0.0 <sup>b</sup>	33.6 ± 0.1 <sup>a</sup>	36.2 ± 0.0 <sup>b</sup>	0.93 ± 0.00 <sup>a</sup>

Results are mean values ± standard deviation of duplicates and different letters within the same column indicate significant difference ( $p < 0.05$ ) according to ANOVA followed by Tukey's post-hoc test.

pressure'. Similar findings have been reported for the SFE of sea buckthorn oil and the Colombian blueberry [21,22]. In the present study, at 20 MPa and 60 °C, the effect on the solvent density predominated while at 50 MPa and 60 °C the effect of vapor pressure was stronger.

### 3.2. Fatty acid composition

Berry seed oils are rich in lipids and generally have high contents of PUFAs. Table 2 presents the fatty acid composition and the  $\omega 6/\omega 3$  ratio of the bilberry seed oil. The PUFAs linoleic acid (18:2 n-6) and  $\alpha$ -linolenic acid (18:3 n-3) and the MUFA (monounsaturated fatty acid) oleic acid (18:1 n-9) comprised the major fatty acids derived from the bilberry seed oils. As the PUFAs represent more than 69% of the total fatty acids, extraction of bilberry seeds provides a rich source of essential fatty acids. The  $\omega 6/\omega 3$  ratios were  $\leq 1$  for all the extracts, indicating the bilberry seed oils have a high content of n-3 PUFAs, which when consumed are associated with health effects such as decreased risk of CVD [23]. These results are in agreement with the results reported by Yang and coworkers [5], who showed that bilberry seed oil has a high content of essential FAs, i.e., linoleic (18:2 n-6) and  $\alpha$ -linolenic (18:3 n-3) acids.

The extraction conditions investigated in the present study had only minor impacts on the fatty acid compositions of the extracted bilberry seed oils. However, some significant differences ( $p < 0.05$ ) were observed. The largest differences were found for the extracts obtained at 20 MPa and 60 °C, which had a higher content of palmitic acid (16:0) and a lower content of  $\alpha$ -linolenic acid (18:3 n-3). Berry seed oils are complex mixtures of different lipid classes, such as free fatty acids, and mono-, di- (DAG), and tri-acylglycerols (TAG), with TAG as one of the major constituents [4]. As the intermolecular interactions of these oils are also complex, their solubilities are different for those of the corresponding pure compounds [24]. The yields of unsaturated fatty acids of *Pistacia terebinthus* berries, extracted at different conditions, have also been reported to increase slightly with increasing pressure [25]. Those authors concluded that the effect of pressure was the most significant parameter, and that the increase in yield was potentially due to increased solvent density at higher pressures [25].

Western diets are often deficient in  $\omega 3$  and have excessive levels of

$\omega 6$ , giving a  $\omega 6/\omega 3$  ratio of around 20. An imbalance in  $\omega 6/\omega 3$  ratio is associated with greater risks for depression, inflammatory diseases, and death from cardiovascular disease whereas low  $\omega 6/\omega 3$  ratios are fundamental to maintaining homeostasis, so that from a health perspective, a  $\omega 6/\omega 3$  ratio in the range of 1–2 is recommended [26,27]. As can be shown in Table 2, all the analyzed bilberry seed extracts had  $\omega 6/\omega 3$  ratios close to 1. The low ratios indicate an opportunity to use these extracts as food ingredients or food supplements to increase  $\omega 3$  consumption and obtain a  $\omega 6/\omega 3$  ratio closer to 1. Besides the importance of low  $\omega 6/\omega 3$  ratios, it is also important to distinguish between the different classes of PUFAs, since their functions and biochemical properties are different [23]. Low  $\omega 6/\omega 3$  ratios for long-chain PUFAs (LC-PUFA) are associated with protection against obesity and diabetes and the prevention of inflammation, while low LA/ALA (linoleic/ $\alpha$ -linolenic acid) ratios are associated with lowering of the LDL cholesterol levels, as well as the high blood pressure levels seen in CVD [28,29]. The selectivity of SFE could be used to target n-3 FA, thereby lowering the  $\omega 6/\omega 3$  ratios of the final products.

### 3.3. Vitamin E

The general terms 'vitamin E' and 'tocol' are often used to describe the naturally occurring compounds tocopherols and tocotrienols [30]. Table 3 presents the distributions and total concentrations of tocopherols and tocotrienols in the extracted bilberry seed oils. For all the extracts, the constituent with the highest concentration was  $\gamma$ -tocotrienol, followed by  $\delta$ -tocopherol and  $\alpha$ -tocotrienol. The  $\gamma$ -tocotrienol concentrations were in the range of 45.7–100.2 mg 100 g<sup>-1</sup> oil, which is higher than the values reported for palm oil (32.3 mg 100 g<sup>-1</sup>) but lower than the levels found in annatto oil (3300 mg 100 g<sup>-1</sup>), which is known to be an excellent source of  $\gamma$ -tocotrienol [31,32].

The vitamin E content was significantly influenced by the extraction conditions applied. The oil that was extracted at 20 MPa and 60 °C had the highest concentration of vitamin E (129.2 ± 5.0 mg 100 g<sup>-1</sup> oil), while the oil that was extracted at 50 MPa and 40 °C had the lowest concentration of vitamin E (58.0 ± 1.4 mg 100 g<sup>-1</sup> oil). It is noteworthy that the oil with the lowest yield (7.6 ± 0.6%) had the highest concentration of vitamin E. An increase in temperature at 20 MPa was associated with a significant increase in the recovery of vitamin E.

**Table 3**  
Content of tocopherols (T) and tocotrienols (Tr) in bilberry seed oils extracted at different conditions.

Experimental conditions	mg 100 g <sup>-1</sup> oil				mg 100 g <sup>-1</sup> dry seeds			
	$\delta$ -T	$\alpha$ -Tr	$\gamma$ -Tr	Total	$\delta$ -T	$\alpha$ -Tr	$\gamma$ -Tr	Total
20 MPa 40 °C	11.4 ± 0.2 <sup>b</sup>	2.5 ± 0.1 <sup>bc</sup>	56.8 ± 0.3 <sup>b</sup>	70.0 ± 0.6 <sup>b</sup>	2.2 ± 0.0 <sup>a</sup>	0.5 ± 0.0 <sup>b</sup>	11.0 ± 0.0 <sup>ab</sup>	13.6 ± 0.1 <sup>ab</sup>
20 MPa 60 °C	24.5 ± 0.2 <sup>a</sup>	4.5 ± 0.2 <sup>a</sup>	100.2 ± 5.0 <sup>a</sup>	129.2 ± 5.0 <sup>a</sup>	1.9 ± 0.0 <sup>b</sup>	0.3 ± 0.0 <sup>c</sup>	7.6 ± 0.4 <sup>c</sup>	9.8 ± 0.4 <sup>c</sup>
35 MPa 50 °C	11.0 ± 0.4 <sup>bc</sup>	2.2 ± 0.1 <sup>c</sup>	49.3 ± 1.9 <sup>cd</sup>	62.5 ± 1.8 <sup>cd</sup>	2.4 ± 0.1 <sup>a</sup>	0.5 ± 0.0 <sup>b</sup>	10.7 ± 0.4 <sup>ab</sup>	13.6 ± 0.4 <sup>ab</sup>
50 MPa 40 °C	10.7 ± 0.2 <sup>bc</sup>	2.7 ± 0.0 <sup>b</sup>	52.9 ± 0.6 <sup>bc</sup>	66.2 ± 0.3 <sup>bc</sup>	2.4 ± 0.1 <sup>a</sup>	0.6 ± 0.0 <sup>a</sup>	11.6 ± 0.1 <sup>a</sup>	14.5 ± 0.1 <sup>a</sup>
50 MPa 60 °C	10.0 ± 0.3 <sup>c</sup>	2.3 ± 0.1 <sup>c</sup>	45.7 ± 1.0 <sup>d</sup>	58.0 ± 1.4 <sup>d</sup>	2.2 ± 0.1 <sup>a</sup>	0.5 ± 0.0 <sup>b</sup>	10.2 ± 0.2 <sup>b</sup>	12.9 ± 0.3 <sup>b</sup>

Results are mean values ± standard deviation of duplicates and different letters within the same column indicate significant difference ( $p < 0.05$ ) according to ANOVA followed by Tukey's post-hoc test.

These results are in agreement with the work of Bravi and co-workers [33], who observed an increase in the recovery of vitamin E from grape seeds when the temperature was increased at low pressure. They showed that while extracts obtained at higher extraction temperatures had lower yields, they contained higher concentrations of tocopherol. This phenomenon is likely related to the selectivity of SFE in extracting targeted compounds [10]. In the current study, the solubility of vitamin E increased from 2.04 g/SC-CO<sub>2</sub> under the extraction conditions of 20 MPa and 40 °C to 16.51 g/SC-CO<sub>2</sub> when the extraction was performed at 20 MPa and 60 °C [34]. A possible explanation for the high concentration of vitamin E in the oil with the lowest yield is that, owing to the high solubility of vitamin E, the concentration of this antioxidant was higher in the initial supercritical CO<sub>2</sub> extraction phase. In addition, at low yields of oil, the relative concentration of vitamin E is constant, whereas with increasing yield, the relative vitamin E concentration decreases due to a dilution effect. The mass transfer of tocopherol is also associated with its solubility in the oil and its availability on the sample surface [35]. The extraction rate is high at the beginning of the process, and in the latter stage of the process, the extraction rate is controlled by diffusion of the solute and SC-CO<sub>2</sub> in the sample matrix [36]. This variability of the extraction rate underlines the importance of extraction time. Yang and co-workers [5] reported lower concentrations of vitamin E (40 mg 100 g<sup>-1</sup> oil) for bilberry seed oil extracted at 35 MPa and 50 °C for 2 h. This was possibly due to the longer extraction time used. During the latter part of the extraction process, pressure plays an increasingly important role in solubilizing vitamin E within the particle. The effects of using pressures of 35 MPa and 50 MPa on vitamin E extraction became evident when the normalized data, expressed as mg vitamin E 100 g<sup>-1</sup> of dry seeds, were analyzed (Table 3). At 20 MPa and 60 °C, the amount of vitamin E extracted was significantly lower than under the other tested conditions. An increase in temperature also influenced the recovery of vitamin E at 20 MPa and 50 MPa. At 20 MPa, the increase in temperature increased the recovery rate, while at 50 MPa, the increase in temperature had the opposite effect, i.e., decreased vitamin E recovery.

### 3.4. Antioxidant activity

In Table 4, the EC<sub>50</sub> values of the different oils are listed. The EC<sub>50</sub> value represents the concentration of extract that reduces the DPPH absorbance by 50%, whereby lower values indicate that the extracts have stronger antioxidant activity [37]. The extracts obtained at 20 MPa and 60 °C had lower EC<sub>50</sub> values, indicating stronger antioxidant activity compared with the extracts obtained under the other conditions. An increase in the temperature at 20 MPa had a strong effect on the EC<sub>50</sub>, probably due to the higher content of vitamin E in the oil obtained under these conditions. The EC<sub>50</sub> values for all the conditions were lower than those previously reported values for SFE of sea buckthorn seed oil, indicating that the extracts of bilberry seeds have a higher antioxidant activity [37].

The lowest EC<sub>50</sub> value was obtained at 20 MPa and 60 °C, which were the extraction conditions that yielded the highest concentrations of vitamin E, while higher values of EC<sub>50</sub> were found for the conditions

that gave lower concentrations of vitamin E. The results indicate that pressure and temperature influence the antioxidant activity and the vitamin E content in a similar fashion. The close relationship between vitamin E content and the EC<sub>50</sub> value suggests that the content of vitamin E, and especially  $\gamma$ -tocotrienol as the major constituent, contributes to the antioxidant activity of bilberry seed oils. In general, tocopherols are considered to be stronger antioxidants than tocotrienols. It has previously been shown that the order of antioxidant activity for both tocopherols and tocotrienols is  $\alpha > \beta > \gamma > \delta$  [30]. However, tocotrienols in oil and fat systems have been reported to have higher antioxidant activities than their corresponding tocopherols, especially  $\gamma$ -tocotrienol [38]. Thus, both the content and the composition of the tocol constituents, with  $\gamma$ -tocotrienol as the major constituent, may contribute to the strong antioxidant activity of bilberry seed oils. Moreover, compared to tocopherols,  $\gamma$ -tocotrienol is reported to have superior neuroprotective, anticancer, and radioprotective properties [31].

An additional plausible explanation for the strong antioxidant activity is that the antioxidant activity of bilberry seed oils can reflect the by synergistic effects of different antioxidants. Bilberries contain high levels of carotenoids, of which lutein,  $\beta$ -cryptoxanthin, and  $\beta$ -carotene are the major representatives [1]. Carotenoids are strong antioxidants and may exert synergistic effects when combined with vitamin E [39]. Tocopherols can be in the first line of defense against oxidation, acting as chain-breaking antioxidants, thereby sparing the carotenoids to act as quenchers [40]. However, several factors can contribute to the synergistic effects of the various antioxidants in a biological system. The overall impact is dependent upon the concentrations and combinations of antioxidants, and appears to be partly due to their reducing potential and ability to convert the antioxidant free radicals to their native forms [41]. The presented results support the hypothesis that the antioxidant activities of extracts from berries reflect a complex interplay between the compositions and concentrations of antioxidants and potential synergies between the constituents.

### 3.5. Peroxide value

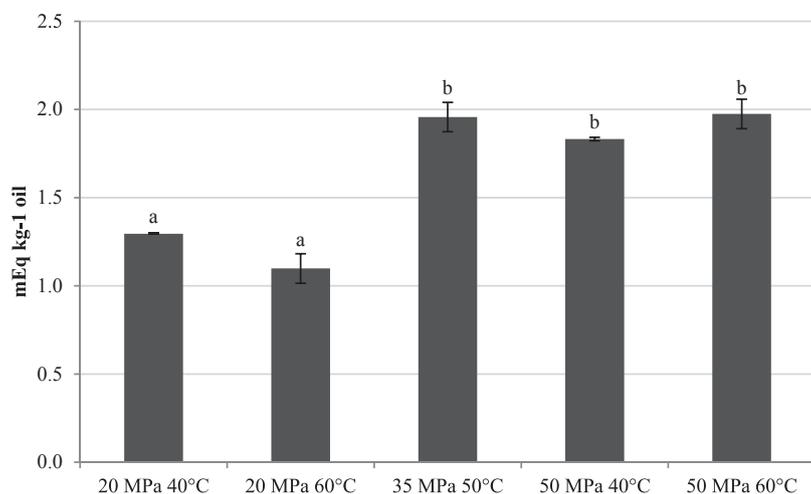
As shown in Fig. 2, the levels of peroxides in the extracted bilberry seed oils ranged from  $1.1 \pm 0.1$  to  $2.0 \pm 0.1$  mEq kg<sup>-1</sup>. The lowest concentration of peroxide occurred after extraction at 20 MPa and 60 °C. The obtained PV for all the analyzed oils was  $\leq 10$  mEq kg<sup>-1</sup>, which is the maximum PV recommended by the Codex Alimentarius Commission for commercial oils [42].

The FA profiles of all the extracts had at least 69% PUFA and 23% MUFA. It is possible that lipid oxidation of PUFA occurred in the seeds even before the compounds were extracted, as well as during the separation process used to obtain the seeds. As indicated in Fig. 2, an increase in pressure had a stronger effect on the PV than an increase in temperature. The observed differences in PVs may be attributed to an increase in the solubility of the peroxide compounds as the pressure increased, which would explain why no difference in PV was observed between the oils extracted at the same pressures. This improvement in solubility is probably a consequence of an increase in the solvent density and, thus, the solvent power [36]. Even though exposure of the oil to 60 °C for 80 min might favor lipid oxidation, during SFE, the samples are not exposed to oxygen, thereby, limiting the initialization of lipid oxidation reactions. Extracts from plant matrices are complex. To comprehend the solubility behaviors of all the compounds during SC-CO<sub>2</sub> extraction, it is necessary to know the physicochemical properties and intermolecular interactions that result from polarity differences for the different compounds, since these interactions may affect the solubility behaviors of the targeted compounds [36]. In addition to the different conditions of pressure and temperature, it would be interesting to investigate further the different extraction times, so as to understand the solubility behavior of each compound of interest.

**Table 4**  
Values of EC<sub>50</sub> of bilberry seed oils extracted at different conditions.

Experimental conditions	EC <sub>50</sub> (mg mL <sup>-1</sup> )
20 MPa 40 °C	9.5 ± 0.1 <sup>a</sup>
20 MPa 60 °C	5.5 ± 0.0 <sup>b</sup>
35 MPa 50 °C	9.3 ± 0.3 <sup>a</sup>
50 MPa 40 °C	8.8 ± 0.1 <sup>a</sup>
50 MPa 60 °C	9.5 ± 0.0 <sup>a</sup>

Results are mean values ± standard deviation of duplicates and different letters within the same column indicate significant difference ( $p < 0.05$ ) according to ANOVA followed by Tukey's post-hoc test.



**Fig. 2.** Levels of milliequivalents of peroxides in bilberry seed oils extracted at different conditions. Results are mean values  $\pm$  standard deviation of duplicates and means with at least one letter in common within the same compound do not have significant difference ( $p < 0.05$ ) according to ANOVA followed by Tukey's post-hoc test.

#### 4. Conclusions

The bilberry seed oils obtained through SFE at 60 °C and 20 MPa had the lowest peroxide values and highest antioxidant activities, and were found to be excellent sources of vitamin E, particularly  $\gamma$ -tocotrienol. However, SFE performed under these conditions resulted in a low yield (7.6%). A higher yield was obtained by performing the extraction at 50 MPa and 60 °C, although the concentration of vitamin E under these conditions was about half that obtained through oil extraction at 20 MPa and 60 °C. The models for the interaction between pressure and temperature in the extraction process showed that, in general, pressure had a stronger effect than temperature. However, at 20 MPa, the temperature affected the yield and, especially, the amount of vitamin E that was extracted. The pressure and temperature used for supercritical carbon dioxide extraction did not have a strong effect on the fatty acid composition, although the selected conditions were very important in terms of the extraction yield, vitamin E content, and, consequently, the level of antioxidant activity. That the extracted bilberry seed oils exhibited high contents of vitamin E and PUFAs and a low  $\omega 6/\omega 3$  ratio indicates a strong potential for their use as ingredients in nutraceuticals or foodstuffs. Further studies are needed to elucidate the solubility behaviors of different compounds during SFE.

#### Acknowledgments

The authors acknowledge the support provided by the Science without Borders program (SwB 1229-13-3), the CAPES Foundation, and the Ministry of Education of Brazil, Brasília, DF 70040-020, Brazil. This study also received financial support from ERA-Net and the SUSFOOD project "Sustainable & Healthy: Development of sustainable processing technologies for converting by-products into healthy, added value ingredients and food products".

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