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Original article

Effects of supercritical and liquid carbon dioxide extraction on hemp (*Cannabis sativa* L.) seed oil

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Summary The increase in the popularity of hemp-based products is mainly linked to the presence of non-psychoactive cannabinoids that provide relief from aches, pain and anxiety. In this study, hemp seed oils were produced by two innovative and environmentally friendly extraction techniques: supercritical and liquid carbon dioxide (CO_2) extraction. The chemical composition of the two oils was analysed and compared with that of a control oil obtained by solvent (*n*-hexane) extraction. Both oils obtained by liquid and supercritical CO_2 extraction presented interesting compositions: they contained large amounts of cannabinoids, polyphenols and tocopherols and were less oxidised than the control and contained a large amount of hexanal, which provided a pleasant aroma. The maximum cannabinoid content was found in the oil obtained by liquid CO_2 extraction (71.51 mg of cannabidiol per kg of oil and 113.92 mg of cannabinol per kg of oil). Carotenoids were prevalent in the oil obtained by supercritical CO_2 extraction (61.00 mg kg^{-1} of oil).

Keywords Cannabinoids, hemp seed oil, liquid carbon dioxide, polyphenols, supercritical carbon dioxide, volatile organic compounds.

Introduction

Hemp (*Cannabis sativa* L.) seeds contain 25–35% oil, depending on the variety and climatic conditions, that can be extracted to produce edible oil. The oil contains large amounts of polyunsaturated fatty acids, which represent approximately 70–80% of the total fatty acids and have beneficial effects on human health (Da Porto *et al.*, 2012). However, as a result of its high polyunsaturated fatty acid content, this oil is very unstable and susceptible to oxidation. Hemp seed oil contains phytosterols in concentrations from 3.6 to 6.7 g kg^{-1}, with β-sitosterol as the main component, representing approximately 70% of the total phytosterols (Matthäus & Brühl, 2008). The minor compounds are tocopherols (approximately 800 mg kg^{-1} of oil), in particular γ-tocopherol, accounting for approximately 85% of the total tocopherols (Oomah *et al.*, 2002). Hemp seed oil contains very large amounts of chlorophylls (with the content depending on the type of extraction), which give the oil its characteristic dark green colour and increase its susceptibility to oxidation because they are photosensitizers. Cannabinoids can be found in hemp seed oil as a result of contamination of the seeds by resins in the inflorescences. Cannabinoids are a group of terpenophenolic compounds, among which Δ^9-tetrahydrocannabinol (THC) is the psychoactive component. Other major constituents are cannabidiol (CBD) and cannabinol (CBN), which do not activate cannabinoid receptors and thus do not have psychoactive effects (Hampson *et al.*, 1998). EC regulation no. 73/2009 made the use of only certified seeds included in the common catalogue of varieties for agricultural use with a maximum certified THC content ≤ 0.2% obligatory. Several studies have suggested that CBD is effective in treating epilepsy and other neuropsychiatric disorders, including anxiety and schizophrenia. CBD may also be effective in treating post-traumatic stress disorder and may have anxiolytic, anti-psychotic, anti-emetic and anti-inflammatory properties (Pavlovic *et al.*, 2018). These possibilities have resulted in high consumer interest in the utilisation of cannabinoid oils obtained from non-psychoactive hemp varieties.

Hemp seed oil is conventionally extracted by solvents or cold pressure. Compared with solvent extraction, cold pressure extraction allows a higher-quality

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oil to be obtained but produces a lower yield. By the cold pressure method, 60–80% of the oil can be extracted from the seed material, depending on the settings of the screw press (Matthäus & Brühl, 2008). Solvent extraction is efficient and relatively inexpensive but requires significant refinement, and solvent residues can remain in the final product. Carbon dioxide (CO_2) extractions, both supercritical and liquid, are innovative and environmentally friendly extraction techniques. They replace the organic solvents used in traditional extraction with CO_2, which is a non-toxic, non-flammable, recyclable and inexpensive solvent that is easy to remove from the extracts (Da Porto *et al.*, 2012). Upon returning to room temperature and pressure conditions, supercritical CO_2 leaves the system and leaves no solvent residue in the extract (Romano *et al.*, 2014). By selecting the appropriate parameters, with this extraction method, it is possible to extract more than 90% of the oil present in the seeds (Tomita *et al.*, 2013). Studies on the use of liquid CO_2 to produce oil are very scarce. Romano *et al.* (2014) found that oils extracted from olive paste with liquid CO_2 exhibited limited hydrolysis and oxidative reactions, higher concentrations of biophenols than oils obtained with classic methods and more aromatic compounds such as limonene than such oils. To the best of our knowledge, there are no previous reports on the use of liquid CO_2 to produce hemp seed oil and very few works have been published on the analysis of CBD and CBN in hemp seed oil (Citti *et al.*, 2018).

Therefore, in this research, the composition of hemp seed oil obtained by supercritical and liquid CO_2 extraction was compared with that of hemp seed oil extracted by the solvent method. This work is significant because it provides insight into the techniques used to obtain economically important hemp products such as hemp seed oil.

Materials and methods

Materials

Hemp seeds (*Cannabis sativa* L.) of the USO31 cultivar containing $\leq 0.2\%$ tetrahydrocannabinol (THC) were procured from the Campania district and were stored under a vacuum at room temperature until analysis. Subsequently, seeds were milled in a knife mill (Grindomix M200, Retsch Italia, Verdere Scientific Srl, Bergamo, Italy) and sieved to obtain two sets of particles with different diameters ($\varnothing \leq 1$ mm; $1 < \varnothing < 2$ mm) for subsequent extractions. The moisture content of the seeds was determined by using the standard hot air oven method at 105 ± 1 °C for 24 h (AOAC Official Method 925, 40 2000).

Chemicals

The carbon dioxide (CO_2) (assay purity 99.9%) used was provided by SOL Spa (Naples, Italy). All solvents and reagents used in determinations were purchased from Sigma-Aldrich Co. (Milano, Italy).

Organic solvent extraction

Approximately 3 g of ground seeds ($\varnothing \leq 1$ mm) was added to 15 mL of *n*-hexane, stirred for 1 min and centrifuged at 4000 ***g*** for 10 min in a multispeed centrifuge (PK 131, ALC International Srl, Milano, Italy). The supernatant hexane phase was filtered by using filter paper with anhydrous sodium sulphate and collected in an evaporator flask. The extraction was repeated five times in the same matrix until the supernatant hexane phase was clear and free of fat, and the hexane extracts were combined. Subsequently, the solvent was removed under vacuum using a Rotavapor Laborota 4000-Efficient instrument (Heidolph Instrument, Schwabach, Germany). The oil obtained was used as a control. The extraction was performed in triplicate.

Supercritical and liquid carbon dioxide extraction

The experiments were carried out using an SFC 4000 extractor (JASCO International Co., Ltd., Tokyo, Japan) equipped with a 50-mL-volume extractor vessel. Approximately 18 g of ground hemp seeds (particles with $\varnothing \leq 1$ mm and $1 < \varnothing < 2$ mm) were loaded into the extraction vessel for each experimental extraction.

The supercritical CO_2 extraction parameters were as follows: $T = 40$ °C, $P = 300$ bar and flow = 10 mL min^{-1} carbon dioxide; these conditions were reported to give the best yield by Da Porto *et al.* (2012). The extraction time was 195 min.

The liquid CO_2 extraction parameters were as follows: $T = 20$ °C, $P = 150$ bar, flow = 10 mL min^{-1} and extraction time = 195 min. The extraction was repeated twice in the same matrix.

The extracts were collected in previously weighed 100 mL glass vials. Each extraction was performed in triplicate. The oils obtained from seeds with $\varnothing \leq 1$ mm were submitted to subsequent chemical analysis.

Determination of the peroxide value and spectrophotometric indices

Determination of the peroxide value (PV) and spectrophotometric indices was carried out according to AOAC Official Method 965, 33 (1990). The PV was determined by an iodometric assay, and the results were expressed as meq of O_2 per kg of oil. For the

spectrophotometric indices, absorbance was measured at 232 and 270 nm.

Determination of chlorophyll and carotenoid contents

This determination was performed with the spectrophotometric method described by Aladić *et al.* (2014), with some modifications. Approximately 100 mg of oil was dissolved in 5 mL of ethyl ether in a tube, placed in an ultrasonic bath for 1 min and vortexed for 30 s. The solution was placed in a quartz cuvette, and absorbance measurements were performed by using a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) at wavelengths of 400–700 nm. The results were expressed as mg kg^{-1} of oil.

Determination of total polyphenol content

The total polyphenol content was determined by the colorimetric method (Folin-Ciocalteau) reported by Singleton & Rossi (1965), with some modifications. The extraction of phenols from the samples was carried out as follows: 1 g of oil was weighed in a 15 mL tube, to which 1 mL of hexane was added and the mixture was vortexed for 30 s. Then, 5 mL of a 60:40 methanol:water solution was added, and the solution was vortexed for 1 min and centrifuged at 6000 ***g*** for 10 min. The extraction was repeated twice in the same matrix. For spectrophotometric analysis, standards and samples were prepared in the same way: 400 μL of solution containing phenols was supplemented with 400 μL of bidistilled water, 3.2 mL of a 5% sodium carbonate solution and 400 μL of Folin–Ciocalteu reagent. Gallic acid was used as a standard for the calibration curve (15.62, 31.25, 62.5 and 125 ppm). The results were expressed as mg of gallic acid equivalents (GAE) per kg of oil.

Determination of α- and γ-tocopherol contents

Tocopherols were determined by high-performance liquid chromatography (HPLC) as reported by Grilo *et al.* (2014), with some modifications. A total of 100 μL of hemp oil was dissolved in 1.9 mL of isopropanol and vortexed for 1 min. Subsequently, 20 μL of this solution was injected into an Agilent 1100 series HPLC instrument equipped with a quaternary pump, a G4225A degasser, DAD G1315B and FLD G1221A detectors and a Spherisorb ODS2 column (5 μm, 4.6 mm × 250 mm; Agilent Technologies, Santa Clara, CA, USA). The mobile phase used was 100% methanol with a flow rate of 0.8 mL min^{-1} under isocratic conditions. The detector wavelength was set at 292 nm. Two calibration curves for γ-tocopherol (5, 10 and 50 ppm) and α-tocopherol (1, 5, 10 and 50 ppm) were constructed with standard methanolic solutions. The range of linearity and the square of the correlation coefficient (R^2) were 5–50 ppm and 0.9990 for γ-tocopherol and 1–50 ppm and 0.9980 for α-tocopherol, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were calculated to be 0.5 and 1 ppm, respectively, for both compounds. The results were expressed as mg kg^{-1} of oil.

Determination of cannabidiol and cannabinol content

Determination of cannabidiol (CBD) and cannabinol (CBN) was performed by HPLC as reported by Citti *et al.* (2018) with some modifications. A total of 100 μL of oil was dissolved in 0.9 mL of isopropanol. A total of 20 μL of this solution was injected into a ZORBAX Eclipse XDB-C18 reverse-phase column (50 μm, 150 mm × 4.6 mm, Agilent Technologies). An Agilent Technologies model 1100 series HPLC instrument equipped with a vacuum degasser, a quaternary pump and a diode array detector (DAD) was used. The mobile phase was composed of 0.1% formic acid in water (A) and 0.05% formic acid in acetonitrile (B). The chromatographic conditions were set as follows: 0.0–1.0 min, 50% B; 1.1 min, 80% B; 8.1 min, 95% B; and 8.6 min, 50% B. The flow rate was set at 1 mL min^{-1}. Chromatograms were acquired at 210 nm. The compounds were identified by comparing the retention times of the peaks in the sample with those of the peaks of solutions obtained with CBD and CBN standards. For quantification, two calibration curves were built for CBD and CBN in methanol using a range of concentrations (1, 5 and 10 ppm) that covered the ranges expected for the samples. The range of linearity was 1–10 ppm for both compounds and the R^2 was 0.9979 for CBD and 0.9997 for CBN. The LOD and LOQ were calculated to be 0.5 and 1 ppm, respectively, for both compounds. The results were expressed as mg kg^{-1} of oil.

Determination of fatty acid composition

Determination of the acidic fraction was performed by analysing the fatty acid methyl esters (FAMEs) obtained after trans-esterification (EEC, 1991). A 1% solution of oil in hexane was prepared, and 300 μL of 2 N KOH in methanol was added to 1 mL of this solution. After vortexing, phase separation was achieved. A total of 1 μL of the upper layer, containing the FAMEs, was injected into an Agilent Technologies 6890N gas chromatograph equipped with a capillary column (100 m × 0.25 mm inner diameter, film thickness of 0.20 μm) with a poly stationary phase (90% biscyanopropyl/10% cyanopropylphenyl siloxane) (Supelco, Bellafonte, PA, USA), hydrogen flame ionisation detector (FID) and programmed

temperature vaporiser (PTV). The carrier gas used was helium with a flow rate of 1 mL min^{-1}. The oven temperature programme was as follows: 70 °C for 1 min, 20 °C min^{-1} ramp to 140 °C for 5 min, and then 7 °C min^{-1} ramp to 240 °C for 10 min. The chromatogram peaks were identified using an external 37-component standard (Supelco TM 37 component FAME mix) by comparing the retention times of the standards with those of the samples under the same operating conditions. The results were expressed as % w/w.

Determination of triglyceride composition

Determination of triglycerides present in the hemp oil was carried out according to the method described by Romano *et al.* (2011), with some modifications. A solution of 0.25% oil in hexane was prepared and vortexed for a few minutes. A total of 1 μL of the solution was injected into an Agilent Technologies 6890N gas chromatograph (Agilent Technologies) equipped with PTV, an FID and a Restek RTX-65TG capillary column with a stationary phase of 65% diphenyl and 35% dimethylpolysiloxane, dimensions of 30 m × 0.25 mm ID and a film thickness of 0.10 μm. The carrier gas used was helium with a flow rate of 2 mL min^{-1}. The oven temperature programme was as follows: 80 °C for 1 min, 30 °C min^{-1} ramp to 240 °C for 0.10 min and then 4 °C min^{-1} ramp to 360 °C for 5 min. For the identification of peaks, the retention times of triglycerides were compared with those of an external certified standard, BCR-519. The results were expressed as % w/w.

Determination of phytosterol composition

Determination of phytosterols was carried out according to Nota *et al.* (1995). A solution of 5% chloroform oil was prepared. The solution was stirred by vortexing for approximately 1 min, 400 μL of 2 N KOH in methanol was added, and the mixture was stirred. A total of 800 μL of a 1 N HCl solution was then added. The solution was vortexed for approximately 1 min and centrifuged at 1800 ***g*** for 5 min. From the lower layer, 1 μL was taken and injected into an Agilent Technologies 6890N gas chromatograph (Agilent Technologies) equipped with a PTV, an FID and a Restek RTX-65TG capillary column with a stationary phase of 65% diphenyl and 35% dimethylpolysiloxane, dimensions of 30 m × 0.25 mm ID and a film thickness of 0.10 μm. The carrier gas used was helium with a flow rate of 2 mL min^{-1}. The oven temperature programme was as follows: 80 °C for 0.1 min and 50 °C min^{-1} ramp to 280 °C for 15 min. For the identification of the peaks, the retention times of the sample were compared with those of external standards of β-sitosterol, stigmasterol and campesterol (5% in chloroform). The results were expressed as % w/w.

Determination of volatile organic compounds

Analysis of volatile organic compounds (VOCs) in the hemp oil was performed by using the solid-phase microextraction (SPME) technique, as reported by Ilias *et al.* (2006), with some modifications. Approximately 300 mg of eugenol (1.03 mg g^{-1}) was added as an internal standard to a 500 mg oil sample and weighed in a vial for headspace analysis. The vial was sealed, and the sample with the standard was subjected to continuous agitation for 10 min at 50 °C and then another 30 min at 50 °C while exposing a divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB/CAR/PDMS) (Supelco) to the headspace. Then, the fibre was extracted and inserted into an Agilent 19091S-433 gas chromatograph equipped with an HP-5MS capillary column (30 m × 0.25 mm ID × 0.25 μm, Agilent Technologies). The results were expressed as mg kg^{-1} of oil.

Statistical analysis

All experiments and determinations were performed in triplicate, and the reported results are the average values (±standard deviations) of the three replicates. One-way analysis of variance (ANOVA) and Tukey's multiple-range test ($P \leq 0.05$) were conducted on the data using XLSTAT software (Addinsoft, New York, NY, USA).

Results and discussion

Extraction yields

The average moisture content was found to be 6.1 ± 0.3%. The initial oil content in the hemp seeds was determined by hexane extraction (sample A) and was 33.24 g oil per 100 g of seeds (Table 1). The yield obtained from ground seeds with a diameter ≤ 1 mm by the use of supercritical CO_2 (sample B) was 30.98 g oil per 100 g of seeds, representing a recovery of 93%, very close to the yield of sample A obtained by solvent extraction (Table 1). Sample C, obtained by liquid CO_2 extraction from the same ground seeds, showed a yield (22.71 g oil per 100 g of seeds) significantly ($P < 0.05$) lower than that of sample B, with a recovery of 68%. This is due to the lower ability of liquid CO_2 to penetrate inside the matrix compared with the other two solvents. Furthermore, the extraction yields decreased as the extraction pressure decreased because the oil solubility decreased due to the decrease in density of CO_2 (Da Porto *et al.*, 2012). The oil recovery using ground seeds with diameter ≤ 1 mm was 93.19%

Table 1 Yield and recovery of hemp seed oil with different extraction methods

Samples	Seed particle diameter (mm)	Extraction method	Yield (g of oil per 100 g of seeds)	Recovery (% total oil)
A (control)	ø ≤ 1	Solvent (hexane)	33.24 ± 0.28[a]	100
B	ø ≤ 1	Supercritical CO_2	30.98 ± 1.02[a]	93.19 ± 3.08[a]
C	ø ≤ 1	Liquid CO_2	22.71 ± 0.41[b]	68.31 ± 1.23[b]
D	1 < ø < 2	Supercritical CO_2	19.05 ± 0.2[b]	57.31 ± 0.63[b]
E	1 < ø < 2	Liquid CO_2	9.90 ± 1.44[c]	32.39 ± 8.64[c]

Different letters in the same column indicate statistically significant differences ($P < 0.05$).

and 68.31% (w/w) in supercritical and liquid CO_2 extraction, respectively. As expected, by increasing the particle size of the seeds to a diameter between 1 and 2 mm, the extraction yield decreased to 19.05 and 9.90 g oil per 100 g of seeds in supercritical and liquid CO_2 extraction, respectively, due to the smaller exchange surface between the matrix and the solvent (Table 1); therefore, only samples A, B and C were submitted to subsequent analysis.

Peroxide value and spectrophotometric indices

Peroxide, K232 and K270 measurements are the most common methods used to evaluate the degree of oil oxidation. Primary oxidation compounds, that is hydroperoxides, are typically evaluated by the peroxide value and the specific extinction coefficient K232. The latter is also associated with conjugated dienes, the intermediate stages of oil oxidation. The literature also states that K270 is related to carbonyl compounds, the secondary stages of oil oxidation (Muik *et al.*, 2005; Guillen & Goicoechea, 2009; Guzmán *et al.*, 2011). Samples B and C had peroxide, K232 and K270 values lower than 19.50 meq O_2 kg^{-1}, 6.32 and 1.25, respectively (the values for the oil obtained by solvent extraction (Table 2), indicating that samples obtained by CO_2 were less oxidised than the control. In fact, exposure to temperature, light and air during the extraction process used to obtain sample A lead to substantial and rapid degradation of the oil, with a high concentration of primary and secondary oxidation compounds. The oxidation of oil is also related to the level of chlorophyll, which greatly promotes photo-oxidation (Bradley & Min, 1992; Tekaya & Hassouna, 2007).

Oil pigments

Cert *et al.* (2000) reported that the dark green colour of hemp oil is due to the most important pigments in hemp oil: chlorophylls *a* and *b*. Furthermore, carotenoids are also present in hemp oil. Food scientists

Table 2 Peroxide value, K232, K270, pigments, polyphenols, tocopherols and cannabidiols in hemp seed oils obtained with different extraction methods

	Samples		
Compounds	**A (hexane)**	**B (supercritical CO_2)**	**C (liquid CO_2)**
Peroxides value (meq O_2 kg^{-1} oil)	19.50 ± 0.50[a]	5.50 ± 1.00[c]	8.90 ± 0.20[b]
K232	6.32 ± 0.01[a]	3.81 ± 0.00[c]	4.15 ± 0.01[b]
K270	1.25 ± 0.01[a]	1.20 ± 0.02[b]	1.13 ± 0.01[c]
Pigment content (mg kg^{-1} oil)			
Chlorophyll *a*	125.51 ± 3.53[a]	107.23 ± 2.80[b]	12.80 ± 1.99[c]
Chlorophyll *b*	17.86 ± 1.21[a]	23.29 ± 3.99[a]	14.55 ± 0.83[a]
Chlorophyll *a* + *b*	143.37 ± 2.31[a]	130.52 ± 6.79[a]	27.35 ± 0.83[b]
Tot. carotenoids	60.65 ± 2.61[a]	61.00 ± 1.04[a]	9.60 ± 1.16[b]
Polyphenols (mg GAE per kg oil)	33.59 ± 2.17[b]	51.42 ± 0.31[a]	47.18 ± 0.75[a]
Tocopherols (mg kg^{-1} oil)			
α-tocopherol	49.31 ± 0.89[a]	39.57 ± 0.72[a]	39.19 ± 5.18[a]
γ-tocopherol	967.05 ± 17.20[a]	770.08 ± 10.75[b]	774.18 ± 9.77[b]
Cannabidiols (mg kg^{-1} oil)			
Cannabidiol (CBD)	41.15 ± 3.82[b]	47.40 ± 0.85[b]	71.51 ± 6.10[a]
Cannabinol (CBN)	68.66 ± 12.31[b]	76.52 ± 1.44[b]	113.92 ± 8.97[a]

Different letters in the same row indicate statistically significant differences ($P < 0.05$).

have considerable interest in chlorophyll and carotenoid effects. The role of carotenoids as protective antioxidants in olive oils has been mentioned by several authors (Bradley & Min, 1992; Velasco & Dobarganes, 2002), but the literature on these pigments in hemp oil is very limited. Chlorophylls *a* and *b* significantly accelerate olive oil photo-oxidation, with chlorophyll *b* having a more important effect (Tekaya & Hassouna, 2007). Sample B showed chlorophyll *a* + *b* and total carotenoid contents (130.52 and 61.00 mg kg^{-1}, respectively), which were very similar to the contents in control sample A (143.37 and 60.65 mg kg^{-1}, respectively), and in both samples, chlorophyll *a* was more abundant than chlorophyll *b*. In contrast, in the oil obtained by liquid CO_2 extraction (sample C), the pigment content was very low: 27.35 mg kg^{-1} of chlorophylls *a* + *b* and 9.60 mg kg^{-1} of carotenoids, and chlorophyll *b* was more abundant than chlorophyll *a* (Table 2). Therefore, hexane and supercritical CO_2 showed greater extractive capacites for these pigments than did liquid CO_2. Hexane is a non-polar solvent that eases the extraction of lipid-soluble pigments, such as chlorophylls and carotenoids, while CO_2 has lower viscosity in the supercritical phase than in the liquid phase (Cavalcanti & Meireles, 2012), such that it has a better ability to penetrate the sample and solubilise the pigments when in the supercritical phase.

Polyphenols and tocopherols

Phenolic compounds and tocopherols are known for their high antioxidant activity and ability to promote the resistance of oils to oxidative deterioration (Velasco & Dobarganes, 2002). Oxidative degeneration is one of the major causes of significant deteriorative changes in the chemical, sensory and nutritional properties of oils. In the presence of oxygen, unstable compounds are generated, leading to changes in the sensory and nutritional characteristics of the oil and, hence, the deterioration of the product. Autoxidation is a slow radical chain process that occurs in three stages: induction, propagation and termination. During the induction period, alkyl radicals are formed and undergo a reaction with oxygen molecules to form hydroperoxides (ROOH) and peroxy radicals during the propagation phase. The termination of chain reactions occurs via the combination of free radicals to form stable adducts (Brand-Williams *et al*., 1995). The total polyphenol content in hemp varies according to the variety and processing conditions. Bibliographic data on the total polyphenol content are available only for oils extracted by cold pressure, revealing a range of 44 to 188 mg of gallic acid per 100 g of oil. Such oils may have applications in the promotion of health and the prevention of oxidative damage caused by radicals (Siger *et al*., 2008). The total polyphenol contents in the oils obtained by supercritical CO_2 (51.42 mg GAE kg^{-1}) and liquid CO_2 treatment (47.18 mg GAE kg^{-1}) did not show statistically significant differences, but these values were significantly ($P < 0.05$) higher than those of the control oil (Table 2). Supercritical and liquid CO_2 therefore showed greater selectivity towards these antioxidant compounds than did the hexane solvent.

The most frequent forms of tocopherols in hemp oil are α and γ tocopherols, followed by the δ and β forms, with a γ:α:δ:β ratio of 90:5:3:2 (Oomah *et al*., 2002). Tocopherols are oil-soluble vitamins and are important for their antioxidative properties. The highest contents of α-and γ-tocopherol contents were found in control sample A and were 49.31 and 967.05 mg kg^{-1} of oil, respectively (Table 2). This is consistent with the results of Callaway & Pate, 2009, who found an average α-tocopherol content of 50 mg kg^{-1} of oil and a γ-tocopherol content of 850 mg kg^{-1} of oil in cold-pressed hemp seed oil. Tocopherol extraction is maximised with the use of an organic solvent such as hexane (sample A) due to the selectivity of such solvents for these lipophilic antioxidants. However, solvent-extracted oils must be subjected to refinement, a process that reduces the tocopherol content by 30–70% (Alpaslan *et al*., 2001). The α-tocopherol contents in samples A, B and C were 49.31, 39.57 and 39.19 mg kg^{-1} of oil, respectively, and did not show statistically significant differences, indicating that this parameter was not influenced by extraction method, temperature or pressure. The γ-tocopherol contents, in contrast, were 967.05, 770.08 and 774.18 mg kg^{-1} of oil in samples A, B and C, respectively, and were significantly ($P < 0.05$) lower in samples B and C than in the control sample (Table 2).

Cannabidiol and cannabinol contents

The quantified cannabinoids were CBD and CBN, which are the major non-psychoactive cannabinoids in *Cannabis sativa* and have beneficial effects on human health, particularly with respect to diseases and neurological disorders. If consumed regularly as part of a diet including raw hemp seed oil, CBD and CBN can, in the long term, have beneficial effects on the body, even at low concentrations (Leizer *et al*., 2000). The highest CBD and CBN contents were 71.51 and 113.92 mg kg^{-1}, respectively, in sample C (Table 2). Sample B showed CBD and CBN contents of 47.40 and 76.52, respectively, which were statistically equal to the contents in sample A. These results are in accordance with the data of Citti *et al*. (2018), who reported CBD and CBN contents in commercially available hemp seed oil varying from 1056 to 1.5 mg kg^{-1} and from 12 to 1 mg kg^{-1}, respectively. Liquid CO_2, which

Table 3 Main fatty acids, triglycerides and phytosterols (% w/w) in hemp seed oils obtained with different extraction methods

	Samples		
Compounds	**A (hexane)**	**B (supercritical CO_2)**	**C (liquid CO_2)**
Fatty acids			
Palmitic acid (C16:0)	6.50 ± 0.07[a]	6.36 ± 0.05[a]	5.46 ± 0.14[b]
Stearic acid (C18:0)	2.64 ± 0.01[a]	2.68 ± 0.02[a]	1.68 ± 0.07[b]
Oleic acid (C18:1-n9c)	12.56 ± 0.04[a]	12.67 ± 0.03[a]	11.96 ± 0.10[b]
Linoleic acid (C18:2-n6c)	56.85 ± 0.10[b]	57.04 ± 0.02[b]	58.04 ± 0.27[a]
Arachidic acid (C20:0)	0.99 ± 0.00[a]	0.98 ± 0.01[a]	0.39 ± 0.03[b]
γ-Linolenic acid (C18:3-n6)	2.99 ± 0.00[b]	2.99 ± 0.03[b]	4.09 ± 0.04[a]
α-Linolenic acid (C18:3-n3)	15.70 ± 0.02[b]	15.68 ± 0.04[b]	15.86 ± 0.04[a]
Behenic acid (C22:0)	0.45 ± 0.01[a]	0.44 ± 0.02[a]	0.17 ± 0.01[b]
Lignoceric acid (C24:0)	0.20 ± 0.01[a]	0.19 ± 0.02[a]	0.11 ± 0.01[b]
Σ EFAs	72.55 ± 0.12[b]	72.72 ± 0.03[b]	73.90 ± 0.29[a]
ω-6/ω-3	3.81 ± 0.00[b]	3.82 ± 0.01[b]	3.91 ± 0.01[a]
Σ PUFAs	75.66 ± 0.13[b]	75.84 ± 0.06[b]	78.34 ± 0.32[a]
Σ MUFAs	13.39 ± 0.03[a]	13.35 ± 0.05[a]	12.51 ± 0.09[b]
Σ SFAs	10.95 ± 0.10[a]	10.81 ± 0.12[a]	8.23 ± 023[b]
Triglycerides (Carbon atoms)			
C54	72.13 ± 0.48[a]	71.47 ± 0.46[a]	72.13 ± 0.47[a]
C52	26.50 ± 0.63[a]	27.23 ± 0.40[a]	26.47 ± 0.68[a]
C50	1.37 ± 0.15[a]	1.31 ± 0.07[a]	1.41 ± 0.21[a]
Phytosterols			
β-Sitosterol	90.75 ± 0.42[a]	85.83 ± 0.54[b]	80.76 ± 0.35[c]
Campesterol	6.20 ± 0.00[c]	9.69 ± 0.08[b]	14.19 ± 0.63[a]
Stigmasterol	2.88 ± 0.17[c]	4.00 ± 0.06[b]	5.05 ± 0.28[a]

Different letters on the same row indicate statistically significant differences ($P < 0.05$).

is in subcritical conditions, has a greater selectivity towards these components. Although the density of subcritical CO_2 is lower than that of supercritical CO_2, the selectivity is enhanced for cannabinoids since only the most soluble components are efficiently dissolved in the CO_2. This allows selective extraction of the lipophilic cannabinoids by the non-polar CO_2 and implies that although supercritical conditions might improve yields, subcritical conditions provide much higher sensitivity (Galal *et al.*, 2009).

Free fatty acid, triglyceride and phytosterol composition

The hemp seed oil obtained with the three extraction techniques contained very large amount of polyunsaturated fatty acids (Table 3), particularly the essential ω-3 and ω-6 fatty acids, representing more than 70% of the total fatty acids. The ratio between ω-6 and ω-3 fatty acids was optimal and well balanced, with a value varying from 3.8 and 3.9. This ratio is very similar to the ratio found in Mediterranean and Japanese diets; a low occurrence of coronary heart disease has been reported for people who follow these types of diet (Teh & Birch, 2013). In Table 3, only the most abundant fatty acids are given; other acids, such as palmitoleic acid (C16:1) and eicosadienoic acid (C20:2n6), were detected in very small amounts and are therefore not presented in the table. The most abundant acid was linoleic acid (C18:2n6), with percentages >56%, followed by α-linolenic acid (C18:3n3), with percentages >15% and oleic acid (C18:1n9), with percentages >11% (Table 3). These are essential fatty acids and have beneficial effects on human health; in particular, linolenic acid has anti-inflammatory, anti-hypertension, anti-vasoconstrictor, anti-cancer and anti-thrombotic effects. Furthermore, essential fatty acids play an important role in the development of the phospholipid bilayers of cell membranes and organelles (Da Porto *et al.*, 2012). γ-Linolenic acid (C18:3n6), typical of hemp seed oil, was also found with contents varying from 2.99% to 4.09% (Table 3) and has been proven to have a positive effect on patients with rheumatoid arthritis, atopic dermatitis and allergies (Da Porto *et al.*, 2012). Similar results have also been found by other authors (Aladić *et al.*, 2015). The triglycerides found had 54, 52 and 50 carbon atoms, typical of seed oils, consisting of long chain fatty acids. In the data obtained (Table 3), no statistically significant differences were found; therefore, the extraction method did not affect these oil compounds.

The sterol composition indicated that β-sitosterol was a principal component in all samples, varying from 90.75% in sample A to 80.76% in sample C. The

other phytosterols identified were campesterol and stigmasterol, which were found prevalently in samples B and C. The campesterol contents were 9.69% and 14.19% and stigmasterol contents were 4.00% and 5.05% in samples B and C, respectively (Table 3).

Volatile organic compounds

In liposoluble matrices, volatile organic compounds (VOCs), particularly aldehydes, are produced through the oxidative degradation of monosaturated fatty acids. These molecules can provide an unpleasant or pleasant odour depending on their concentration and the matrix in which they are contained. The main VOCs in the oil extracted with solvent were hydrocarbons, which were formed as a result of solvent extraction. Sample A, in fact, was not submitted to the refinement process. In the oils extracted with CO_2, mainly aldehydes were found, which provide a characteristic aroma of greens, spices and dried fruit. The results are shown in Table 4. In sample C, only hexanal was found at 254 mg kg^{-1}. Sample B, in contrast, also contained octadienal (10.29 mg kg^{-1}), heptadienal (9.38 mg kg^{-1}), nonanal (8.34 mg kg^{-1}) and nonenal (8.77 mg kg^{-1}). The same aldehydes were reported in the study of Pavlovic *et al*., 2018, who also detected, although at lower concentrations, hexanal (3–10 mg kg^{-1}), heptadienal (1–8 mg kg^{-1}) and nonanal (5 mg kg^{-1}) in commercial hemp seed oils. Rovellini *et al*. (2013) also reported that the main VOCs in the hemp seed oil obtained by cold pressure were hexanal, hexenal and nonenal. In particular, hexenal has an odour of cut greens, leaves, green fruit, almonds, artichokes and flowers; hexanal has a green, fresh odour; and nonenal has a watermelon or old skin odour. Among alcohols, hexanol was detected only in samples B and C, at 30.66 and 53.00 mg kg^{-1}, respectively. This molecule has a pleasant herbaceous and woody odour (Dunford, 2015). In conclusion, oils extracted by CO_2 (both supercritical and liquid) had a pleasant odour due to the presence of aldehydes and hexanol.

Table 4 Volatile organic compound concentrations (mg kg^{-1}) in hemp seed oils obtained with different extraction methods

	Samples		
Compounds	**A (hexane)**	**B (supercritical CO_2)**	**C (liquid CO_2)**
Hydrocarbons			
Nonane	11.17 ± 1.65	nd	nd
Decane	17.13 ± 3.01	nd	nd
Undecane	8.78 ± 0.31	nd	nd
2-Methylundecane	5.01 ± 0.70	nd	nd
Dodecane	112.54 ± 2.06	nd	nd
Tridecane	6.85 ± 1.20	nd	nd
1.3-Di-tert-butylbenzene	46.59 ± 2.25	nd	nd
Tetradecane	68.99 ± 1.40	nd	nd
Hexadecane	6.34 ± 2.35	nd	nd
Aldehydes			
Hexanal	nd	39.57 ± 0.91[b]	254.00 ± 5.66[a]
Octadienal	nd	10.29 ± 3.18	nd
Heptadienal	nd	9.38 ± 1.41	nd
Nonanal	nd	8.34 ± 1.28	nd
Nonenal	nd	8.77 ± 1.27	nd
Alcohols			
Hexanol	nd	30.66 ± 0.95[b]	53.00 ± 4.24[a]

Different letters on the same row indicate statistically significant differences ($P < 0.05$).
nd, Not detected.

Conclusion

Compared with traditional organic solvents, CO_2 allows higher-quality oils to be obtained, with lower energy and solvent costs and without the release of residues in the extract. The product obtained is of food grade and does not require refinement operations that would degrade its bioactive components. In addition supercritical CO_2 extraction allows an oil yield of 30.98 g oil per 100 g of seeds to be obtained, which is very close to the yield of the control oil. Compared with oils obtained by traditional hexane extraction, hemp seed oils obtained by CO_2 extraction are less oxidised and have lower peroxide values and spectrophotometric indices. Moreover, these oils contain large amounts of CBD, CBN and polyphenols, which are well known to have beneficial effects on human health. Analysis of VOCs revealed that the oils obtained by CO_2 extraction prevalently include pleasant odorous molecules such as hexanal and hexanol. The use of CO_2 does not influence the triglyceride content. All types of hemp seed oil contain a large amount of polyunsaturated fatty acids, particularly linoleic and α-linoleic acids, which are essential fatty acids.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

Ethics approval was not required for this research.

Data availability statement

Research data are not shared.

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