Effect of ultrasound pre-treatment of hemp (*Cannabis sativa* L.) seed on supercritical CO₂ extraction of oil

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Abstract Ultrasound pre-treatment of intact hemp seeds without any solvent assistance was carried out for 10, 20 and 40 min prior to SCCO₂ extraction at 40 °C, 300 bar and 45 kg CO₂/kg feed. Sonication time effect on SC-CO₂ extraction was investigated by the extraction kinetics. The maximum extraction yield was estimated to be 24.03 (% w/w) after 10 min of ultrasonic pre-treatment. The fatty acid compositions of the oils extracted by SC-CO₂ without and with ultrasound pre-treatments was analyzed using gas chromatography. It was shown that the content of linoleic, α -linolenic and oleic acids (the most abundant unsaturated fatty acids) of the hemp seed oils were not affected significantly by the application of ultrasound. UV spectroscopy indices (K_{232} and K_{268}) and antiradical capacity were used to follow the quality of oils. Significant were the changes in their antiradical capacity due to ultrasound treatment. A comparison with the oil extracted by Soxhlet was also given.

Keywords Hemp seed · Oil · Ultrasound · Supercritical CO₂ extraction

Introduction

Hemp (*Cannabis sativa* L.) seed oil is considered to be one of the best nutritional oil for health (Deferne and Pate 1996; Callaway et al. 1996; Oomah et al. 2002) because it contains two polyunsaturated essential fatty acids (EFAs) - linoleic acid (LA), from the “omega-6” family, and α -linolenic acid (ALA), from the “omega-3” family - which usually account for approximately 50–70 % and 15–25 % respectively, of the total seed fatty acid content. No other vegetable oil offers EFAs, in such 3:1 desirable omega-6/omega-3 ratio. Western

diets typically have omega-6/omega-3 ratios of 10:1 or more, which is far too rich in omega-6 and correspondingly too deficient in omega-3. Recent clinical research continues to identify this imbalance as a co-factor in a wide range of common illnesses, including cardiovascular diseases, arthritis, diabetes, and skin disorders (Simopoulos 2002; Oh et al. 2005; Harris 2006). Hemp oil also provides significant amounts of γ -linolenic acid (GLA) which appears to alleviate the symptoms of atopic dermatitis and other skin diseases (Callaway et al. 2005). Hemp oil typically contains less than 10 % saturated fatty acids, and no trans-fatty acids, which are particularly detrimental to blood cholesterol balance. Within hemp seed oil, γ -tocopherol is present in significantly higher amount than α -tocopherol (Deferne and Pate 1996; Oomah et al. 2002). Both these tocopherols are important fat-soluble antioxidants.

Currently, the oil is mainly extracted from hemp seed by press extraction and solvent extraction. However, press extraction is often associated with lower yield, and solvent extraction involves longer extraction time and is associated with the presence of toxic residue. In a previous paper we applied supercritical CO₂ extraction to hemp seed for oil recovery (Da Porto et al. 2012). With this technology, it is possible to extract heat sensitive, easily oxidized compounds such as polyunsaturated fatty acids (PUFAs), and to avoid using any toxic solvent like *n*-hexane, leaving residues in the product, since CO₂ is non-toxic, recyclable, cheap, relatively inert, non-inflammable and easily separated from the extract (Balaban and Chen 1992; Lang and Wai 2001).

The benefit of using ultrasound in plant extraction has already been demonstrated for bioactive substances (Vinatoru et al. 1999). The ultrasonic enhancement of extraction is attributed to disruption of cell walls, particle size reduction and enhanced mass transfer of the cell content via cavitation bubble collapse (Vinatoru et al. 1999; Romdhane and Gourdan 2002). The collapse of acoustic cavitation bubbles generates transient hot spots with extremely high local

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temperature and pressure. Many factors govern the action of ultrasound: ultrasound frequency, pressure, temperature, sonication time, etc. (Romdhane and Gourdan 2002).

The aim of this study was to evaluate the effects of ultrasound pre-treatment of hemp seeds on the SCCO₂ oil extraction in terms of yield, fatty acid composition, UV indices and antiradical capacity. Therefore, ultrasound pre-treatment was carried out: a) on intact seeds, ground only prior to SCCO₂ extraction, to reduce oxidation and/or non-enzymatic browning reactions potentially promoted by transient hot spots and to avoid that raw material becomes a slurry not suitable to the extractor of the supercritical apparatus; b) without any solvent assistance to prevent the extraction of compounds during pre-treatment prior to SCCO₂ extraction. The results were compared with those obtained using solvent extraction.

Material and methods

Raw material

Hemp seed (*Cannabis sativa* L.) samples with a moisture content of 9.8 (± 0.3) % (w/w) were collected from experimental cultivation of hemp Felina cultivar (THC ≤ 0.2 %) (Regulation EC n. 2860/2000) carried out at Prato Carnico (Udine, Italy).

Solvents and reagents

The carbon dioxide used (purity >99.99 %) was supplied by Rivoira Spa (Udine, Italy). All other solvents and reagents used in analytical determinations were Sigma–Aldrich Co. (Milan, Italy), pro analysis type. The chemicals used were of analytical reagent grade that include *n*-hexane, isooctane, ethyl acetate, potassium hydroxide, methanol, 1,1-diphenyl-2-picrylhydrazyl (DPPH–90 % purity, Sigma–Aldrich Co., Milano, Italy) and (+)- α -tocopherol (Sigma–Aldrich Co., Milano, Italy) and individual standard for FAME analysis (approximately 99 % pure purchased from Sigma Aldrich, Milan, Italy).

Solvent extraction

Thirty grams of ground hemp seeds were transferred into a filter paper extraction thimble and extracted with 240 ml *n*-hexane for 8 h at a maximum temperature of 70 °C in a Soxhlet apparatus. After extraction was completed, *n*-hexane was removed at 50 °C under reduced pressure using a rotary evaporator (Rotavapor R210, Buchi, Flawil, Switzerland). Subsequently, the flask was placed into a desiccator chamber for 1 h. The oil obtained was weighed and the yield was calculated. Determination was done in triplicate.

Ultrasound pre-treatment and supercritical CO₂ extraction

Aliquots of 50 g of intact hemp seeds were put in 250 mL Erlenmeyer flask without any solvent assistance and were sonicated by an ultrasonic probe (Elettrofor Sonoplus model HD2200 with TT13FZ probe, Bandelin, Berlin; 20 KHz working frequency; 200 W-amplitude setting displayed in % on the scale of 10–100) operating at 100 % of the scale for 10, 20 and 40 min. During ultrasound irradiation, the temperature was maintained at the desired level 25 (± 2) °C by placing the flask into a beaker containing ice-water.

Supercritical CO₂ extractions were carried out in a laboratory-scale plant (M-LAB-SFE100; Tecno-process srl, Roma, Italy) equipped with a 100 cm³ extraction vessel (Fig. 1). Hemp seeds were ground in a stainless steel blender for different time (s) to obtain approximatively the same grain size. The hemp seeds particles were characterized by size classification in a standard sifter with several mesh sizes (<0.25, 0.25–0.5, 0.8–1.0, 1.0–1.25, 1.25–1.50, 1.50–1.75, 1.75–2.0, > 2.0 mm). An average particle diameter was adopted $d_p = 0.83$ (± 0.05) mm, being calculated by Sauter's equation (Povh et al. 2001) to a set of fractions within the previous mesh sized:

$$d_p = m_t / S_{i=1}^k m_i / d_{pi}$$

where m_i is the mass of particles retained below mesh size d_{pi} , m_t is the total mass of milled seeds and k is the number of mesh sized.

In supercritical fluid extraction (SFE) the substrate is generally considered as consisting of particles having a single dimension, which is chosen as being representative of all the particles. This average particle dimension is normally the Sauter mean diameter, which preserves the interfacial exchange area between the phases involved in the process.

The amount of ground hemp seeds placed in the extractor was 15 g. Pressure within the extraction vessel was built up with a constant carbon dioxide flow rate at 8×10^{-5} kg/s. SCCO₂ extractions were performed at 300 bar, 40 °C (Da Porto et al. 2012) and the total extraction time was fixed at 4 h, corresponding to 80 Q (kg CO₂/kg feed). The extractor was operated discontinuously, for intervals of about 30 min, to assess several data points to determine the overall extraction curves (OECs). The hemp seed extracts were collected during extractions in volumetric flask and weighted.

Analytical methods

GC analysis of fatty acids

GC analysis of fatty acids was performed following the method described by International Olive Council (2001). The fatty

Fig. 1 Scheme of the SC-CO₂ laboratory unit for supercritical fluid extraction: (1) solvent cooler; (2) pump; (3) heater; (4) extractor; (5) separator

acid methyl esters (FAME) were prepared by trans-esterification of oil with 2 N KOH in methanol and *n*-hexane. Gas chromatographic (GC) analysis of FAME were performed in a Varian 3400 gas chromatograph equipped with a SP-2380 fused-silica column (Supelco, Bellafonte, PA) (30 m×0.32 mm i.d., film thickness 0.20 µm), a split injector at 250 °C; flame ionization detector at 260 °C. Helium was used as carrier gas and the split ratio was used 1:50. The programmed temperature was: 2 min at 50 °C, 50 °C to 250 °C at 4 °C/min. The fatty acid methyl esters peaks were identified comparing their retention times with individual standard FAME (approximately 99 % pure purchased from Sigma Aldrich, Milan, Italy) of palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), γ-linolenic (C18:3), α-linolenic (C18:3), eicosenoic (C20:1), behenic (C22:0) acids. The relative percentage of the fatty acid was calculated on the basis of the peak area of a fatty acid species to the total peak area of all the fatty acids in the oil sample. Fatty acid methyl esters peak identification was confirmed by GC–MS (NIST 2002 database) operating under similar conditions as used for the GC–FID. Each result presents the mean and the standard deviation for a minimum of three analyses.

Spectroscopic indices (K_{232} , K_{268})

The spectroscopic indices, K_{232} , K_{268} , in the UV region, were determined with a spectrophotometer (Shimadzu UV 1650, Italia) according to the standard (ISO 3656 2002) and the oil was diluted with isooctane.

Antiradical capacity of oils

It was evaluated following the methodology described by Espin et al. (2000) with slight modification. In brief, 10 µl of ethyl acetate sample solution at different concentrations was added with 1990 µl of fresh ethyl acetate DPPH solution (93 µM). Then the mixture was shaken vigorously and left in dark for 60 min. Finally, the absorbance of the mixture was measured against pure ethyl acetate (blank) at 515 nm using a

UV-Visible spectrophotometer (Shimadzu UV 1650, Italia). The total free radical scavenger capacity (RSC) is the variation of the concentration of DPPH[•] free radical previously dissolved in ethyl acetate, after 60 min of reaction with the samples. It was expressed in terms of α-tocopherol equivalents, i.e., the concentration of α-tocopherol solution which gives rise to the same RSC. A calibration curve was built using a series of tocopherols standard solutions in ethyl acetate ($R^2=0.98$). All determinations were done in triplicate.

Statistical analysis

Statistica 7.0 software (StatSoft Italia srl) was used for statistical analysis by one-way analysis of variance (ANOVA, with Tukey's HSD multiple comparison) with the level of significance set up at $p \leq 0.05$

Results and discussion

Overall extraction curves of hemp seed

Figure 2 shows the overall extraction curves (OECs) (yield % vs. Q) carried out at 300 bar, 40 °C to evaluate the effect of ultrasound pretreatments on oil yield. The curves SCCO₂ (no US pre-treatment) US10-SCCO₂ and US20-SCCO₂ exhibit three periods: (1) a constant-extraction rate period (CER); (2) a falling-extraction rate period (FER); and (3) a diffusion-controlled period (DC) (Sovová 2005). The initial linear period (~20 Q) corresponds about the 65 % of the final yield (calculated as yield at 20 Q/yield at 80 Q*100) where the slopes of the lines were not significantly different because they were related to the extract solubility, which only depends on pressure and temperature. However, up to 20 Q the extraction curves start to diverge. From 20 to 50 Q there is a transition period where both phase equilibrium and mass transfer control the extractions. Up to 50 Q there is a third smooth asymptotic period where diffusion phenomena appear

Fig. 2 Yield (wt.%) of oil extracted by supercritical CO₂ from untreated (SCCO₂) and ultrasound pre-treated hemp seeds (US10-SCCO₂, US20-SCCO₂, US40-SCCO₂)

and where the slopes depend on particle size and solvent flow-rate. Such trends corroborates the hypothesis of the broken plus intact cells model proposed by Sovová (2005). About the 10 % of the final extracted phenols are deposited inside the hemp seed and diffusion to the particle surface is slow. The curve US40-SCCO₂ exhibit two periods: (1) a constant-extraction rate period (CER) and (2) a falling-extraction rate period (FER). The extraction curves for SCCO₂, US10-SCCO₂, US20-SCCO₂, US40-SCCO₂ resulted in extraction yields of 21.20, 24.50, 20.70 and 13.70 (% w/w) respectively (Table 1). The results show that 10 min of ultrasonic irradiation of seeds caused an increase of penetration rate of SCCO₂ solvent into tissue, enhanced the mass transfer in comparison to control (SCCO₂). In fact, under these conditions the maximal extraction yield (24.50 % w/w) was achieved. This could be due to the fact that sonication time of 10 min altered the stability of the oil bodies that separate the oil from the rest of the cell content. Oil bodies consist of a triglyceride matrix surrounded by a monolayer of phospholipids linked together with proteins, termed oleosins and caleosin. They completely cover oil bodies' surface stabilizing the oil bodies (Capuano et al. 2007). The action of ultrasound for 10 min could promote, by coagulation of oleosins and caleosin, the disruption of the oil bodies and facilitate the oil extraction.

Instead, sonication time of 20 and 40 min significantly decreased the oil extraction yield. Since during sonication pre-treatments no particularly oil exudation was observed, to explain the diminishing yield as a function of sonication time, and the particle size used $0.83 (\pm 0.05)$ mm was quite large to avoid loss of oil during grinding, a reason for the decrease of oil yield after 20 and 40 min of ultrasound pre-treatment could

be due the occurring of degradation reactions (i.e. oxidation, polymerisation and/or non-enzymatic browning reactions) of compounds that make up the structure of the seeds, cell walls or/and oil bodies. It is generally accepted that ultrasonic irradiation affects chemical reactions through acoustic cavitation which generates transient hot spot with extremely high local temperature and pressure (Mason and Lorimer 1988). Too long sonication time exhibits effect such as too high ultrasonic power, decreasing the oil yield (Lou et al. 2010)

Fatty acid composition, UV indices and antiradical capacity of hemp seed oil

The fatty acid composition of hemp seed oils obtained by Soxhlet, SCCO₂ extraction without and with ultrasound pre-treatment is presented in Table 1. Linoleic, α -linolenic and oleic acids are the most abundant unsaturated fatty acids of *Cannabis sativa* L. seed (about 88 % of the total fatty acids). Similar fatty acid profile was reported for the French cultivar Futura-77 by Callaway et al. (1996).

The analytical data (with respect to Soxhlet and SCCO₂ extraction alone) showed that ultrasound pre-treatment prior to SCCO₂ extraction had no significant ($p > 0.05$) influence on the content of linoleic, α -linolenic and oleic acids (Table 1).

Table 2 reports the UV indices and the antiradical capacity of hemp seed oils. The specific extinction coefficient at 232 nm (K_{232}) is related to the degree of primary oxidation of the oil and thus directly correlated to the amount of hydroperoxide (Maskan and Bagci 2003; Ku and Mun 2008). K_{232} is also an indicator of polyunsaturated FA conjugation, whereas K_{268} is related to the secondary oxidation products (α -

Table 1 FAMES composition (%) of oil extracted by supercritical CO₂ from untreated (SCCO₂) and ultrasound pre-treated hemp seeds (US10-SCCO₂, US20-SCCO₂, US40-SCCO₂)

Sample	Soxhlet (<i>n</i> -hexane)	SCCO ₂ extraction	Ultrasound pretreatment + SCCO ₂ extraction		
			US10-SCCO ₂	US20-SCCO ₂	US40-SCCO ₂
Fatty acid composition (%)					
Palmitic acid (C16:0)	5.40 (± 0.24)	5.82 (± 0.02)	5.70 (± 0.21)	5.82(± 0.25)	5.64 (± 0.02)
Stearic acid (C18:0)	1.57 b ^a (± 0.08)	1.46 c (± 0.01)	1.91 a (± 0.09)	1.65 b (± 0.19)	1.65 b (± 0.05)
Oleic acid (C18:1)	11.52 (± 0.21)	10.68 (± 0.16)	11.10 (± 0.21)	11.07 (± 0.00)	11.14±0.02
Linoleic acid (C18:2ω6)	59.06 (± 0.92)	59.01 (± 0.70)	58.86 (± 0.01)	59.22 (± 0.31)	59.13±(0.10)
γ-Linolenic acid (C18:3ω6)	3.53 a (± 0.11)	3.42 a (± 0.10)	3.32 b (± 0.02)	3.30 b (± 0.02)	3.29 b±(0.03)
α-Linolenic acid (C18:3ω3)	17.96 (± 0.34)	18.63 (± 0.67)	18.14 (± 0.15)	18.01 (± 0.12)	18.01 (± 0.14)
Eicosenoic acid (C20:1)	0.19 a (± 0.01)	0.11 c (± 0.11)	0.17 a (± 0.04)	0.15 b (± 0.01)	0.12 c (± 0.01)
Behenic acid (C22:0)	0.76 b (± 0.03)	0.86 a (± 0.07)	0.79 a (± 0.01)	0.78 a (± 0.01)	0.81 a (± 0.00)
EFAs sum	77.02	77.63	77.00	77.23	77.12
ω-6/ω-3 ratio	3.29	3.16	3.43	3.47	3.45
PUFAs sum	80.55	81.06	80.32	80.53	80.42
Monounsaturated	11.71	10.80	11.27	11.22	11.25
Saturated	7.73	8.15	8.41	8.25	8.33
Polyunsaturated/Saturated ratio	10.41	9.95	9.55	9.76	9.66
Yield (g oil/100 g hemp seeds)	30.00 a (± 0.01)	21.20 c (± 0.04)	24.50 b (±0.06)	20.70 c (± 0.04)	13.70 e (± 0.08)

Each data represents the mean of three extraction replicates (± standard deviation)

^a Values with different letter within rows indicate significant differences ($p < 0.05$)

EEA linoleic acid + α-linolenic acid; PUFA polyunsaturated fatty acids

unsaturated ketone, α-diketone) (Karleskind 1992). None of the oils extracted by SCCO₂ gave UV values at 232 nm and 268 nm as high as those obtained by Soxhlet extraction (K_{232} 3.66; K_{268} 1.56) indicative of a strong oxidative stress. The relatively high values of K_{232} (1.81, 1.95, 2.08) of oils obtained from ultrasound pretreated seeds indicate that these oils were much oxidized than oil extracted by simple SCCO₂ (K_{232} 1.40). It is interesting to note that significant disappearance of those fatty acids undergoing oxidation, such as linoleic

Table 2 UV indices and antiradical capacity of oil extracted by Soxhlet and by supercritical CO₂ from untreated (SCCO₂) and ultrasound pre-treated hemp seeds (US10-SCCO₂, US20-SCCO₂, US40-SCCO₂)

Sample	UV indices		Antiradical capacity Eq α toc/ml oil
	K_{232}	K_{268}	
SCCO ₂	1.40 (± 0.02) e ^a	0.21 (± 0.01) e	1.87 ± 0.6 a
US10-SCCO ₂	1.81 (± 0.02) d	0.32 (± 0.01) d	0.80 ± 0.4 c
US20-SCCO ₂	1.95 (± 0.02) c	0.36 (± 0.01) c	1.40 ± 0.1 b
US40-SCCO ₂	2.08 (± 0.01) b	0.39 (± 0.01) b	1.38 ± 0.8 b
Soxhlet (<i>n</i> -hexane)	3.66 (± 0.04) a	1.56 (± 0.02) a	0.84 ± 0.2 c

Each data represents the mean of three replicates ± standard deviation

^a Values with different letter within columns indicate significant differences ($p < 0.05$)

and α-linolenic acids, was not detected by ANOVA analysis of FAME's. As reported by Vichi et al. (2003), this could be due to the extent of oxidation, insufficient to reveal fatty acids variations. Instead, K_{232} determination resulted more sensitive. The low values of K_{268} (0.32, 0.36, 0.39) indicate that oils obtained from ultrasound pretreated seeds prior to SCCO₂ extraction contain a low quantity of secondary oxidation products.

Table 2 shows that hemp seed oil extracted by supercritical CO₂ exhibited the highest antiradical capacity, corresponding to 1.87 α-tocopherol equivalents for oil millilitre. This could be explained by the greater selectivity of the supercritical CO₂ in extracting the tocopherols than the oil during the initial extraction phase (Luo et al. 2007; Leo et al. 2005). Ultrasound pre-treatment of hemp seeds for 10 min followed by SCCO₂ extraction produced a decrease of the original antiradical capacity of about two-fold. Such result, similar to that obtained by Soxhlet, could be due to a partial degradation of the original antioxidant compounds (tocopherols) of hemp seeds. However, ultrasound pre-treatment of seeds for 20 and 40 min produced an increase of the oil antiradical capacity. As reported by Hidalgo et al. (2006), Zamora et al. (1997) and (2011) this could be due to the formation in seeds of endogenous antioxidants, which nature and/or level probably depends on sonication time.

Conclusions

Ultrasound pre-treatment of intact hemp seeds without any solvent assistance for 10 min prior to SCCO₂ extraction showed to improve by 3.3 % the oil yield respect to simple SCCO₂ extraction, maintaining unchanged the desirable ratio 3:1 of ω -6 to ω -3. The degree of primary oxidation of the oil as well as its secondary oxidation products resulted slightly higher than those of simple supercritical CO₂ extraction, but much lower than Soxhlet extraction. However, although the ultrasound pre-treatment was carried out on intact seeds, degradation of antioxidant compounds occurred as shown by the low antiradical capacity of oil, similar to that obtained by Soxhlet.

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