PAPER

EFFECT OF HARVESTING TIME ON HEMP (*CANNABIS SATIVA* L.) SEED OIL LIPID COMPOSITION

S. MARZOCCHI* and M.F. CABONI
Department of Agricultural and Food Sciences and Technologies, University of Bologna,
Piazza Goidanich 60, 47521 Cesena, FC, Italy
*Corresponding author: silvia.marzocchi4@unibo.it

ABSTRACT

The most common food using hemp (*Cannabis sativa* L.) is hempseed oil (HSO) because it is a rich source of nutrients with nutritional and functional beneficial effects for human body. Harvesting time can affect the quality of HSO, consequently the aim of this study was to evaluate the composition of lipid fraction, fatty acids, tocopherols and sterols, during ripening. Two cultivars, *Futura 75* and *Carmagnola*, were collected at three ripening stages during August and September 2015 and their lipid composition was determined by analytical techniques. Among the fatty acid identified, the linoleic acid was the preponderant, followed by oleic, α-linolenic and palmitic acid. Linoleic:α-linolenic acid and polyunsaturated:saturated fatty acid ratios decreased and increased, respectively, in both varieties with ripening. γ-tocopherol was the preponderant tocopherol identified, *Futura 75* showed the highest content in the middle of maturation while *Carmagnola* at the beginning. β-sitosterol was the predominant sterol identified in both varieties, followed by campesterol, Δ_5-avenasterol, stigmasterol and Δ_7-stigmasterol. Total sterol content increased and decreased with ripening in *Futura 75* and *Carmagnola*, respectively. The study confirms that ripening stage affects the quality of hempseed oil, important parameter to consider for hemp seed producers.

Keywords: *Cannabis sativa L.*, hempseed oil, fatty acids, tocopherols, sterols

1. INTRODUCTION

Cannabis sativa L., belonged to Cannabaceae family, is an annual plant known by its long, thin flower and spiky leaves (MONTSERRAT-DE LA PAZ *et al*., 2014). *Cannabis sativa* subsp. *sativa* is characterized by a low content of THC (Δ^9-tetrahydrocannabinol), it must be lower than 0.2% on dry basis to be cultivated in most European countries (Official Journal of European Union, 2008). Industrial hemp with a low THC content has no psychoactive effects (SIUDEM *et al*., 2019). The most common food using hemp is hempseed oil (HSO); it is a rich source of nutrients that provide nutritional and functional support for humans (CRESCENTE *et al*., 2018). HSO represents the 25-35% of hemp seed (CALLAWAY, 2004; MONTSERRAT-DE LA PAZ *et al*., 2014; CRIMALDI *et al*., 2017) and it contains more than 80% of polyunsaturated fatty acids (PUFAs) (PETROVIĆ *et al*., 2015; SIUDEM *et al*., 2019) including essential fatty acids usually not contained in oils used for human diet (PETROVIĆ *et al*., 2015); in particular ω-6 linoleic acid (LA) and ω-3 α-linolenic acid (ALA). In addition, LA:ALA ratio is 3:1 which agrees with European Food Safety Agency recommendations (EFSA, 2009). HSO is composed of 1.5-2% unsaponifiable fraction, a source of interesting minor compounds like tocopherols, fat-soluble vitamin D and E (SIUDEM *et al*., 2019) and phytosterols (MONTSERRAT-DE LA PAZ *et al*., 2014). Therefore, HSO has a lot of beneficial effects: cancer and cardiovascular disease prevention, cholesterol level normalization, blood pressure lowering (DEVI *et al*., 2019) and rheumatoid arthritis and dermatitis treatment (OOMAH *et al*., 2002; CHOW, 2008). For all these reasons industries are attracted by HSO for drugs, cosmetics, body care products and dietary supplement production (CALLAWAY, 2004; KOLODZIEJCZYK *et al*., 2012).

To the best of our knowledge, in literature are reported only two studies about the effect of maturation on yield, quality of fiber and oil of industrial hemp. HÖPPNER *et al*. (2007) studied the yield and the quality of fiber and oil of different hemp cultivars in Germany at two different harvest times ("intensive flowering" and "initial seed maturity"). The different harvest stages did not have effect on stem diameter, fiber content and yield; on the other hand, seed yield and seed oil content increased with maturation while the γ-linolenic acid content decreased. BURCZYK *et al*. (2009) studied the effect of sowing density and date of harvest (beginning of panicle forming, full bloom and full seed maturity) on industrial hemp yields. They observed that the maximum yields of biomass, cellulose and fiber can be obtained at 30 kg/ha sowing density at full bloom; while considering hemp for seed or panicles the highest yields were obtained at 10-20 kg/ha of density at full maturity of panicles.

The aim of this study was the characterization of lipid fraction from seeds of two different cultivars of *Cannabis sativa* L., *Futura 75* and *Carmagnola*, harvested at three different harvest stages; in order to investigate the quality of hempseed oil during the maturation, which was never investigated before. To this end, fatty acids, tocopherols and sterols content were determined using fast chromatographic techniques, HPLC and GC equipped with different detectors.

2. MATERIALS AND METHODS

2.1. Fruit harvest and sample preparation

Cannabis sativa L. seeds from two different varieties, *Futura 75* and *Carmagnola*, were picked in Ancona area in Italy approximately 43° 30' 34286" N, 13°15' 33052" E. Fruits were harvested at three different harvesting stages during August and September 2015. At first harvest, *Futura 75* were collected on August 26th (F1) and *Carmagnola* on September 3rd (C1); at the second harvest, *Futura 75* were collected on September 3rd (F2) and *Carmagnola* on September 15th (C2); and at third harvest, *Futura 75* were collected on September 15th (F3) and *Carmagnola* on September 23rd (C3). At each sampling time 20 g of seeds were picked from different field's area; all of them were grounded before analyses.

2.2. Oil extraction and moisture content determination

The lipid content was obtained by grounding the samples of *Cannabis sativa* L. seeds (5 g) and the oil was extracted with *n*-hexane in a Soxhlet apparatus according to ISO method 659:1998. The remaining solvent was removed under vacuum and the oil was taken up with *n*-hexane/isopropanol (4:1 v/v) solution and stored at −18°C until use. Each extraction was carried out two times for each cultivar.
Moisture content (%) was evaluated on hemp seeds samples in an oven at 105°C until constant weight was receached. For each samples, 3 replicates of 3 g weighted for each extraction were dried (n=6) (AOAC, 1995).

2.3. Fatty acid analysis

The fatty acid composition was determined as fatty acid methyl esters (FAMEs) by capillary gas chromatography analysis after alkaline treatment (CHRISTIE, 1982). Methyl tridecanoate (C13:0, 2 mg/mL) was used as internal standard and FAMEs were measured on a GC 2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector (FID) and an AOC-20s auto sampler (Shimadzu Corporation), at the same conditions reported in MARZOCCHI *et al.* (2018). Peak identification was accomplished by comparing peak retention time with GLC-463 standard mixture from Nu-Check (Elysian, MN, USA) and FAME 189-19 standard mixtures from Sigma-Aldrich Chemicals (St. Louis, MO, USA) and expressed as weight percentage of total FAMEs. FAMEs composition was measured in 3 replicates for each lipid extract (n = 6) and each analysis lasted 7 minutes.

2.4. Tocopherols analysis

Approximately 50 mg of hempseed oil was dissolved in 0.5 ml of *n*-hexane. After homogenization, the solution was filtered through a 0.2 μm nylon filter and 2.5 μl was injected in a HPLC 1200 series (Agilent Technologies, Palo Alto, California, USA) equipped with a fluorimeter detector (Agilent, Palo Alto, CA, USA). The excitation wavelength was 290 nm and the emission wavelength was 325 nm. The separation of tocopherols was performed by a HILIC Poroshell 120 column (100 mm × 3 mm and 2.7μm particle size; Agilent Technologies, USA), in isocratic conditions, using an *n*-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) mobile phase. The flow rate was 0.8 ml/min. Tocopherols were identified by co-elution with the respective standards. The calibration

curve used for quantification was constructed with α-tocopherol standard solutions. Tocopherols composition was measured in 3 replicates for each lipid extract (n = 6) and expressed in mg/100g of oil; each analysis lasted 8 minutes.

2.5. Sterols determination

In order to determine the sterols content, 0.5 ml of dihydro-cholesterol (2 mg/mL) was added to 250 mg of oil and saponification was conducted at room temperature (SANDER *et al.*, 1989). After about 20 h, the organic fraction was washed with 10 ml of diethyl ether and 10 ml of water. The unsaponifiable fraction was further extracted twice with 10 ml of diethyl ether, 10 ml of 0.5 N aqueous KOH and 10 ml of distilled water, respectively. The organic solvent was removed under vacuum and the unsaponifiable fraction was used for the sterols analysis. Before injection, samples were silylated according to SWEELEY *et al.* (1963) and the sterol separation was performed by GC/MS (GCMS-QP2010 Plus, Shimadzu, Tokyo, Japan) in the same chromatographic conditions reported by CARDENIA *et al.* (2012). Sterols identification was achieved by comparing peak mass spectra with peaks of standard mixture and by comparing them to the GC–MS data reported by PELILLO *et al.* (2003). An internal standard was used to quantify all the sterols identified in seed samples. Sterols composition was measured in 3 replicates for each lipid extract (n = 6) and expressed in mg/100g of oil.

2.6. Statistical analysis

Relative standard deviation was obtained, where appropriate, for all data collected. One-way analysis of variance (ANOVA) was evaluated using Statistica 10 software (StatSoft, Tulsa, OK, USA). The differences between the means of data for the two different cultivars at the three different harvesting stages were compared at the 5% level of significance (p<0.05) using Tukey honest significant difference (HSD) test.

3. RESULTS AND DISCUSSION

3.1. Oil and moisture content

Table 1 shows oil content of *Futura 75* and *Carmagnola* at three different harvest stages expressed on dry basis. *Futura 75* showed a significantly higher (p<0.05) oil content during the second harvest stage (29.9%) rather than the other two harvestings, 22.9% and 26.4% for the first and the third harvest, respectively. This trend should be related to a non homogeneous maturation, in fact *Cannabis sativa* L. shows a maturation from the bottom to the top of the plant and for this reason is not suitable wait the complete seeds maturation because they tend to fall from the plant. This can be solved operating a constant plant selection to make a more uniform maturation and by adopting appropriate cultivation techniques. In addition, also climatic conditions should be responsible of this trend, in fact *Cannabis sativa* L. shows higher yield in raining condition (MADIA et al., 1998). On the other hand, *Carmagnola* did not show any significant differences (p<0.05) in oil content during maturation, in fact it was 24.5%, 24.8% and 24.9% at first, second and third harvest, respectively. Our results are in line, or slightly lower, with literature, LATIF *et al.* (2009), DA PORTO *et al.*, (2012) and KOSTIĆ *et al.* (2014) that reported an oil content of 26-32%, 30% and 25-29%, respectively. The lower oil content may be attributed to cultivation

techniques and climatic conditions; in addition, seeds were completely covered by a green skin that certainly influenced the oil content, it took up space which was reflected in the overall oil content.
As regard moisture (Table 1), an expected reduction in percentage of moisture was recorded in both varieties with the increasing of maturation; it is well known that immature seeds contain a higher water content rather than mature seeds (MATTHÄUS *et al.*, 2006). *Futura 75* starting from a moisture value of 50% at F1, that decreased significantly (p<0.05) at F2 (32%) reaching a final value of 20% at full maturity. Moisture content in *Carmagnola* had the same trend, starting from a value of 31% at C1 and decreasing significantly (p<0.05) at C2 (17%) and finally at C3 (10%). Moisture value reported in various studies, OOMAH *et al.* (2002), TANG *et al.*, (2006) and DA PORTO *et al.* (2012), are much lower than ours; in fact, they reported values of 7.7, 6.7 and 7.8%, respectively. This could be due to the green skin, as previously mentioned, that covered the seeds that certainly had a high impact on moisture content. SACILIK *et al.* (2006) conducted a study on physical properties of hemp seeds (size, density and porosity) as a function of moisture content and found out values more similar to ours (ranging from 8.6 to 20.9%).

Table 1. Oil (%) and moisture content (%) of the two different cultivars of hemp at three harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
Oil content	22.9±0.6 ^{c}	29.9±0.6 ^{a}	26.4±0.4 ^{b}	24.5±0.2 ^{a}	24.8±0.2 ^{a}	24.9±1.1 ^{a}
Moisture	50.0±1.0 ^{a}	32.0±0.4 ^{b}	20.7±1.3 ^{c}	31.1±2,1 ^{a}	17.5±0.2 ^{b}	10.0±0.1 ^{c}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05, lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

3.2. Fatty acid profile

A total of 17 and 15 fatty acids in *Futura 75* and in *Carmagnola*, respectively, were identified and quantified by fast GC-FID analysis, within a run time less than 7 minutes. As showed in Table 2, the predominant fatty acid in all samples was linoleic acid (C18:2, ω-6), ranging from about 49 to 54%. Oleic acid (C18:1 *cis9*) was the second major fatty acid detected (~15-16%), followed by α-linolenic acid (C18:3, ω-3, ~ 12-15%), palmitic acid (C16:0, ~ 7-9 %), stearic acid (C18:0, ~ 2-3%) and γ-linolenic acid (C18:3, ω-6, ~2-3%).
In *Futura 75*, the saturated fatty acids (SFA) were present in significantly (p<0.05) greater amount at the first harvesting time (16.7%), whereas mono-unsaturated fatty acids (MUFA) did not showed significant differences among the three different harvest stages (18.7%, 18.3% and 18.1% for F1, F2 and F3, respectively). Polyunsaturated fatty acids (PUFA), on the other hand, increased significantly (p<0.05) their concentration with hemp maturation, accounting for 64.6%, 69.6% and 69.6% for F1, F2 and F3, respectively. These trends reflect those of the main fatty acid: in fact, PUFA was the most abundant class because of linoleic acid concentration. In *Carmagnola*, SFA had a significantly higher (p<0.05) concentration at first harvest stage (12.2%), than the other two (11.8% and 11.6%

in C2 and C3, respectively); while MUFA did not show significant differences between first and third harvest stages (18.2% and 18.0%, respectively), with a slightly decrease in the middle stage (16.7%). PUFA, on the contrary, showed a significant increase (p<0.05) at the second harvest stage (71.6%), between first and third harvestings (69.7% and 70.5%). Comparing our results with literature, SFA content is generally higher than data already reported for hemp native from Italy (7%, DA PORTO *et al.*, 2012) and from other countries like Croatia (9-11%, PETROVIĆ *et al.*, 2015), Spain (11%, MONTSERRAT-DE LA PAZ *et al.*, 2014) and Turkey (9-10%, KIRALAN *et al.*, 2010); this because of palmitic and stearic acid content that is higher than reported literature. Only in a study conducted by DEVI *et al.* (2019) is reported a similar concentration of palmitic acid compared to our results, 10% referred to hemp cultivated in India. MUFA content was higher in our study than literature; 12-16% (KIRALAN *et al.*, 2010), 11% (DA PORTO *et al.*, 2012), 13% (MONTSERRAT-DE LA PAZ *et al.*, 2014) and 10-14% (PETROVIĆ *et al.*, 2015). This because of oleic acid, that represent almost the entire MUFA content. On the contrary, PUFA determined in this study was lower than the already cited literature; in fact, KIRALAN *et al.* (2010) showed a 73-78%; DA PORTO *et al.* (2012) a 80-81%, MONTSERRAT-DE LA PAZ *et al.* (2014) a 75% and Petrović *et al.* (2015) a 74-80%. For this reason, the ratio between unsaturated and saturated fatty acids reported in our study, 3-5 in *Futura 75* and 5-6 in *Carmagnola* is slightly lower than that reported in literature (6,7 showed by MONTSERRAT-DE LA PAZ *et al.*, 2014). In general, this characteristic high ratio between unsaturated and saturated fatty acids can reduce serum cholesterol and atherosclerosis; and prevent heart diseases (REENA *et al.*, 2007). On the other hand, because of its high unsaturation level and susceptibility to oxidation, hemp seeds oil has a short shelf life (KIRALAN *et al.*, 2010) and it is not suitable for hot uses (DA PORTO *et al.*, 2012).

As regard to the ratio between the two essential polyunsaturated fatty acids, linoleic and α-linolenic acid, both varieties, *Futura 75* and *Carmagnola*, showed the highest value at the first harvest, 4.05 and 3.90, respectively (Table 2). This even though linoleic and α-linolenic acids did not have the same trend in the two different varieties. In *Futura 75* linoleic acid showed a significant (p<0.05) increase from the beginning to the end of maturation (49.2%, 52.3% and 52.5% for F1, F2 and F3); in *Carmagnola*, instead, its concentration increased significantly (p<0.05) from first (53.7%) to second harvest (54.7%) and then decreased again at the third harvest stage (53.9%). α-linolenic acid, instead, in *Futura 75* increased significantly (p<0.05) between first (12.1%) and second harvest (14.3%), before decreased again at third harvest stage (13.9%); in *Carmagnola* its concentration increased significantly during all the maturation; 13.8%, 14.6% and 14.9% for C1, C2 and C3, respectively. The high quantity of α-linolenic acid improves hemp oil quality for its positive nutritional implications and beneficial effects against coronary disease and cancer (ARSHAD *et al.*, 2011; FRETTS *et al.*, 2013). In general, our results about these two fatty acids are slightly lower than results in literature. In fact linoleic acid content is reported in a range between 48 and 59% and α-linolenic acid in a range between 16 and 26% (MONTSERRAT-DE LA PAZ *et al.*, 2014; ORSANOVA *et al.*, 2015; PETROVIĆ *et al.*, 2015; MIKULCOVÁ *et al.*, 2017; DEVI *et al.*, 2019; SIUDEM *et al.*, 2019).

3.3. Tocopherols

The individual tocopherols identified are showed in Table 3; a total of 49.8, 86.9 and 41.2 mg/100g of oil were quantified in F1, F2 and F3, respectively; while a total of 94.8, 87.7 and 77.8 mg/100 g of oil were quantified in C1, C2 and C3, respectively. It is recognized

that tocopherols are the most important natural antioxidants because of their free radical scavenge activity, involving a tocopherol-tocopheryl semiquinone redox system (MONTSERRAT-DE LA PAZ *et al.*, 2014). Tocopherols, as well as phenols compounds, have shown different beneficial effects, on degenerative diseases such as atherosclerosis, cardiovascular disease, Alzheimer's disease and certain type of cancer (FROMM *et al.*, 2012).

Table 2. Fatty acids composition (mg/100 mg FAMEs) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			**CARMAGNOLA**		
	F1	F2	F3	C1	C2	C3
C14:0	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}	n.d.	n.d.	n.d.
C16:0	9.3±0.8 ^{a}	7.7±0.1 ^{b}	7.5±0.2 ^{b}	7.4±0.1 ^{a}	7.3±0.1 ^{c}	7.3±0.1 ^{b}
C16:1 t	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}	n.d.	n.d.	n.d.
C16:1 c	0.2±0.0 ^{a}	n.d.	n.d.	0.1±0.0 ^{a}	0.1±0.0 ^{a}	0.1±0.0 ^{a}
C17:0	n.d.	0.05±0.0 ^{ab}	0.0±0.1 ^{a}	0.04±0.3 ^{a}	0.06±0.0 ^{a}	0.07±0.0 ^{a}
C18:0	3.4±0.5 ^{a}	2.7±0.1 ^{b}	2.9±0.1 ^{ab}	3.3±0.1 ^{a}	3.0±0.0 ^{b}	3.0±0.2 ^{c}
C18:1 t	n.d.	n.d	0.03±0.0 ^{a}	0.2±0.1 ^{a}	0.1±0.0 ^{a}	0.12±0.0 ^{a}
C18:1 c9	16.7±0.6 ^{a}	16.7±0.3 ^{a}	16.3±0.9 ^{a}	16.5±0.2 ^{a}	15.1±0.1 ^{b}	16.4±0.1 ^{a}
C18:1 c11	1.1±0.1 ^{a}	1.0±0.1 ^{a}	1.0±0.2 ^{a}	0.9±0.1 ^{a}	0.9±0.1 ^{a}	0.8±0.1 ^{a}
C18:2, ω-6	49.2±1.4 ^{b}	52.3±0.2 ^{a}	52.5±0.6 ^{a}	53.7±0.3 ^{b}	54.7±0.1 ^{a}	53.9±0.1 ^{b}
C18:3, ω-6	2.9±0.1 ^{a}	2.9±0.1 ^{a}	3.0±0.1 ^{a}	2.2±0.1 ^{a}	2.2±0.1 ^{a}	1.5±0.1 ^{b}
C18:3, ω-3	12.2±0.4 ^{b}	14.3±0.1 ^{a}	13.9±0.2 ^{a}	13.8±0.1 ^{c}	14.6±0.1 ^{b}	14.9±0.1 ^{a}
C20:0	1.3±0.1 ^{a}	0.9±0.2 ^{b}	1.1±0.1 ^{a}	1.0±0.2 ^{a}	0.9±0.1 ^{b}	0.9±0.1 ^{c}
C20:1 c	0.7±0.3 ^{a}	0.5±0.0 ^{a}	0.7±0.1 ^{a}	0.4±0.1 ^{b}	0.5±0.1 ^{a}	0.4±0.0 ^{b}
C20:2, ω-6	n.d.	0.03±0.0 ^{a}	0.04±0.0 ^{a}	0.02±0.0 ^{a}	0.02±0.0 ^{a}	0.05±0.0 ^{a}
C20:3, ω-6	0.4±0.1 ^{a}	0.1±0.0 ^{b}	0.2±0.0 ^{ab}	0.02±0.0 ^{b}	0.1±0.0 ^{ab}	0.1±0.0 ^{a}
C22:0	1.9±0.7 ^{a}	0.6±0.1 ^{b}	0.7±0.1 ^{b}	0.5±0.0 ^{a}	0.5±0.0 ^{a}	0.4±0.0 ^{b}
SFA	16.7±1.6 ^{a}	12.1±0.2 ^{b}	12.3±0.2 ^{b}	12.2±0.1 ^{a}	11.8±0.1 ^{b}	11.6±0.1 ^{c}
MUFA	18.7±0.5 ^{a}	18.3±0.2 ^{a}	18.1±0.7 ^{a}	18.2±0.3 ^{a}	16.7±0.1 ^{b}	18.0±0.1 ^{a}
PUFA	64.6±2.0 ^{b}	69.6±0.2 ^{a}	69.6±0.8 ^{a}	69.7±0.4 ^{c}	71.6±0.1 ^{a}	70.5±0.1 ^{b}
Omega-6	52.5±2.4	55.3±3.2	55.7±2.9	55.9±1.8	57.0±1.3	55.6±2.4
LA/ALA	4.05	3.66	3.76	3.90	3.75	3.61
PUFA/SFA	3.87	5.66	5.65	5.70	6.08	6.06

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05, lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

As expected, considering the literature, in all samples γ-tocopherol was the predominant compound followed by β-tocopherol, α-tocopherol and α-tocotrienol. In *Futura 75* γ-tocopherol showed a significative increase (p<0.05) between first and second harvest (40.8 and 82 mg/100 g of oil in F1 and F2, respectively) and then significantly (p<0.05) decrease

at last maturation time (F3, 37.1 mg/100 g of oil). The other tocopherols were present in very low concentration; in fact, β-tocopherol was present only at the first harvesting stage (9.0 mg/100 g of oil) and α-tocotrienol just in the last one (3.3 mg/100 g of oil). α-tocopherol was not detected at first maturation stage, but only in the other two, with a significative (p<0.05) decrease between them, 4.9 and 0.8 mg/100 g of oil for F2 and F3, respectively. For this reason, the total tocopherols content had the same trend of γ-tocopherol, increasing significantly (p<0.05) the total concentration at the second harvest stage (F2) reaching a value about 87 mg/100 g of oil (Table 3). This trend is related to oil content trend, in fact it increased at the second maturation stage, increasing the total tocopherols content. Also in *Carmagnola* γ-tocopherol was the predominant tocopherol with a significative decrease (p<0.05) during maturation, 90.2, 81.8 and 72.9 mg/100 g of oil for F1, F2 and F3, respectively. β-tocopherol, on the contrary, was not detected during the entire maturation. α-tocopherol was present in every maturity stage with a significant increase (p<0.05) in the middle of maturity; 3.5, 5.9 and 4.9 mg/100 g of oil in C1, C2 and C3, respectively. Finally, α-tocotrienol was present just at the beginning of maturity (C1) with a concentration of 1.1 mg/100 g of oil. Total tocopherol concentration follows γ-tocopherol trend, so a constant significative decrease during maturation was recorded; 94.8, 87.7 and 77.8 mg/100 g of oil in C1, C2 and C3, respectively.
The total tocopherols content showed a different trend in the two cultivars analyzed and considering they were cultivated in the same field this is related to the cultivars different origin and their gene pool.
Our results agree with literature where γ-tocopherol represents the 90% of the total tocopherols content (OOMAH *et al.*, 2002; ANWAR *et al.*, 2006; LATIF *et al.*, 2009; MONTSERRAT-DE LA PAZ *et al.* 2014). In a study conducted by KRIESE *et al.* (2004), where hempseed oil was extracted by supercritical fluids, the general tocopherol content was much lower than ours, so, in addition to botanical characteristics, climatic and cultivation conditions, also the extraction method affects the tocopherols content.

Table 3. Tocopherols content (mg/100 g of oil) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
α-tocopherol	n.d.	4.9±0.9^{a}	0.8±0.1 ^{b}	3.5±1.0 ^{b}	5.9±0.1 ^{a}	4.9±0.2 ^{b}
α-tocotrienol	n.d	n.d	3.3±0.1 ^{a}	1.1±0.1 ^{a}	n.d.	n.d.
β-tocopherol	9.0±0.3	n.d	n.d	n.d	n.d	n.d
γ-tocopherol	40.8±1.8^{b}	82.0±4.9 ^{a}	37.1±8.2 ^{b}	90.2±4.8 ^{a}	81.8±3.8 ^{ab}	72.9±2.0 ^{b}
Total	49.8±0.1 ^{b}	86.9±5.7 ^{a}	41.2±1.0 ^{b}	94.8±6.7 ^{a}	87.7±3.8 ^{ab}	77.8±0.9 ^{b}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05. lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages.

3.4. Sterols

Analysis of the trimethylsilyl derivatives of phytosterols led to identify nine compounds in both varieties of *Cannabis sativa* L. such campesterol, campestanol, stigmasterol,

clerosterol, β-sitosterol, sitostanol, Δ_5- avenasterol, Δ_5-24-stigmastadienol and Δ_7-stigmasterol (Table 4). The total sterols content showed different trend in the two varieties of *Cannabis sativa* L. considered; in fact, it significantly increased (p <0.05) in *Futura 75* during maturation (642.8, 679.1 and 913.4 mg/100 g of oil in F1, F2 and F3, respectively) and, on the other hand, significantly decreased in *Carmagnola* (532.7, 518.7 and 456.1 mg/100 g of oil in C1, C2 and C3, respectively). These trends reflect the β-sitosterol trends, the predominant sterol detected. In fact, it represented the 63% of the total content in both varieties, but its concentration significantly increased and decreased in *Futura 75* and *Carmagnola*, respectively, with maturation (Table 4). In *Futura 75* it had an initial increase about 5% from F1 to F2 and about 36% between F2 and F3; in *Carmagnola*, on the other hand, β-sitosterol decrease about 6% from first to second harvest stage and then, again about 10%, between second and third harvest.
In *Futura 75* campesterol, campestanol, stigmasterol and clerosterol had the same trend reported for β-sitosterol; instead the other sterols detected decreased between the first and the second harvest stage and then increased at full maturity (Table 4). In *Carmagnola*, instead, campesterol and clerosterol decreased significantly (p<0.05) during the maturation; campestanol and stigmasterol did not show significant differences during the three harvesting stages.

Table 4. Sterols content (mg/100 mg of oil) of the two different cultivars of hemp at three different harvest stages.

	FUTURA 75			CARMAGNOLA		
	F1	F2	F3	C1	C2	C3
Campesterol	74.5±2.5 ^{c}	88.6±4.5 b	123.6±17.0 ^{a}	66.6±4.6 ^{ab}	69.8±1.3 ^{a}	61.8±0.4 ^{a}
Campestanol	4.5±0.6 ^{b}	6.9±2.9 a	8.2±1.4 ^{a}	2.4±0.8 ^{a}	3.7±0.7 ^{a}	3.3±0.3 ^{a}
Stigmasterol	34.1±1.3 ^{b}	33.7±3.5 b	41.8±1.9 ^{a}	16.1±2.7 ^{a}	16.2±1.5 ^{a}	15.7±1.6 ^{a}
Clerosterol	6.1±0.5^{c}	6.3±0.3 b	8.6±0.4 ^{a}	4.3±0.7 ^{a}	3.9±0.8 ^{a}	3.4±0.3 ^{a}
β-sitosterol	402.1±4.3 ^{c}	423.7±6.6 ^{b}	575.9±6.1 ^{a}	344.1±3.9 ^{a}	324.2±4.7 ^{b}	293.6±1.4 ^{c}
Sitostanol	30.3±1.7 ^{a}	27.5±3.6 a	33.1±5.3 ^{a}	14.8±0.7 ^{a}	11.3±0.4 ^{b}	14.2±0.2 ^{a}
Δ_5-avenasterol	50.6±2.3 ^{c}	54.6±1.2 b	71.8±19.0 ^{a}	47.3±2.1 ^{a}	52.8±8.5 ^{a}	38.8±0.8 ^{b}
Δ_5-24-stigmastadienol	8.7±0.3 ^{a}	8.4±0.6 a	9.0±0.8 ^{a}	7.2±0.7 ^{a}	6.4±0.3 ^{a}	4.8±0.1 ^{b}
Δ_7-stigmasterol	31.9±0.9 ^{b}	29.4±0.3 b	41.5±1.8 ^{a}	29.8±1.3 ^{a}	30.4±6.1 ^{a}	23.1±0.2 ^{b}
Total	642.8±14.4 ^{b}	679.1±21.5 b	913.4±35.7 ^{a}	532.7±17.5 ^{a}	518.7±34.3 ^{a}	456.1±0.8 ^{b}

Abbreviations: F1, *Futura 75* first harvest stage; F2, *Futura 75* second harvest stage; F3, *Futura 75* third harvest stage; C1, *Carmagnola* first harvest stage; C2, *Carmagnola* second harvest stage; C3, *Carmagnola* third harvest stage. Data are reported as mean (n=6)±standard deviation. Results of the analysis of variance by Tukey's test are showed: p<0.05. Lowercase letters on the same row show significantly different values within each cultivar at three different harvest stages

To the best of our knowledge, only MONTSERRAT-DE LA PAZ *et al.* (2014) reported a study of sterols in hemp seed oil. Our results were higher than what is reported by these authors for Spanish hempseed oil (total content of 279.4 mg/100 g of oil) but β-sitosterol (190.5 mg/100 g of oil) and campesterol (50.6 mg/100 g of oil) were the same predominant sterols. Comparing sterols composition of HSO with other oils it is possible to see some differences. Considering other vegetable oils, olive oil, linseed oil and hazelnut oil contain

an higher amount of campesterol, β-sitosterol and Δ_5-avenasterol than HSO. Campesterol reachs values of 40, 50-95, 785 mg/kg; stigmasterol values of 20, 10-18, 343 mg/kg; β-sitosterol values of 750, 1050-1700, 1600 mg/kg and Δ_5-avenasterol value of 40-140, 20-80, 369 mg/kg in olive oil, hazelnut oil (AZADMARD-DAMIRCHI *et al.*, 2015) and linseed oil (MATTHÄUS *et al.*, 2017), respectively. In addition, linseed oil contains cholesterol, brassicasterol and 5,24-stigmasterol that are not present in HSO. On the other hand, HSO is richer in sterols that sunflower oil, in fact campesterol, stigmasterol, β-sitosterol and Δ_5-avenasterol report value of 20, 28, 186 and 20 mg/100g, respectively (YILMAZ *et al.*, 2019). Corn oil has the same stigmasterol content of HSO (about 33 mg/100g); is poorer in β-sitosterol than HSO (266 mg/100 g) and, on the other hand, is richer in campesterol (191 mg/100 g) (YANG *et al.*, 2018).
In general, even if sterols are minor constituents of vegetable oils and are present in the unsaponifiable fraction (GUSAKOVA *et al.*, 1998), it is well known that they have a lot of beneficial effects on human health. In fact, they can reduce the serum level of cholesterol concentration, atherosclerotic risk (NTANIOS *et al.*, 2003; PATEL *et al.*, 2006), low-density lipoprotein cholesterol and they are related to a lower risk of myocardial infarction (KLINGBERG *et al.*, 2013).

4. CONCLUSION

This study confirms that ripening stage affects the quality of hempseed oil extracted from *Cannabis sativa* L.; oil content was constant during the maturity only in cultivar *Carmagnola* and moisture content decreased constantly for both cultivars; essential fatty acids (LA and ALA) increased with ripening while tocopherols decreased. Sterols showed a different trend in the two varieties considered, in *Futura 75* the total concentration increased and in *Carmagnola* decreased. Knowledge of the influence of ripening stage on hempseed oil quality has important consequences for industrial output, as harvest could be programmed when oil, tocopherols and sterols are most abundant. *Futura 75* seemed to have a higher concentration of oil, sterols and LA/ALA ratio; while *Carmagnola* a higher concentration of tocopherols and PUFA/SFA ratio. The comparison between the two varieties and the results obtained are very important for hemp seeds producers since they can choose the best variety to plant from a production and quality point of view

AKNOWLEDGEMENTS

The authors are grateful to Dr. Itzhak Moreno and the company "Az. Agraria Trionfi Honorati Giuseppe e Giovanna & C." for the seeds samples used in this study.

REFERENCES

Anwar F., Latif S. and Ashraf M. 2006. Analytical characterization of hemp (*Cannabis sativa*) seed oil from different agro-ecological zones of pakistan. Journal of the American Oil Chemists' Society 83:323-329.

AOAC. 1995. Official methods of analysis. 479 Washington. DC. USA: Association of Official Analytical Chemists.

Arshad A., Al-Leswas D., Stephenson J., Metcalfe M. and Denniso, A. 2011. Potential application of fish oil rich in ω-3 fatty acids in the palliative treatment of advanced pancreatic cancer. British Journal of Nutrition 106:795-800.

Azadmard-Damirchi S. and Torbati M. 2015. Adulterations in some edible oils and fats and their detection methods. Journal of Food Quality and Hazards Control 2:38-44.

Burczyk H., Grabowska L., Strybe M. and Konczewicz W. 2009. Effect of sowing density and date of harvest on yields of industrial hemp. Journal of Natural Fibers 6:204-218.

Callaway J.C. 2004. Hempseed as a nutritional resource: an overview. Euphytica 140:65-72.

Cardenia V., Rodriguez-Estrada M.T., Baldacci E., Savioli S. and Lercker G. 2012. Analysis of cholesterol oxidation products by fast gas chromatography/mass spectrometry. Journal of Separation Science 35:424-430.

Chow C.K. 2008. Fatty acids in food and their health implications (3rd ed.) New York: Marcel Dekker.

Christie W.W. 1982. A simple procedure for rapid trans-methylation of glycerolipids and cholesteryl esters. The Journal of Lipid Research 23:1072-1075.

Crescente G., Piccolella S., Esposito A., Scognamiglio M., Fiorentino A. and Pacifico S. 2018. Chemical composition and nutraceutical properties of hempseed: an ancient food with actual functional value. Phytochemistry Reviews 17:733-749.

Crimaldi M., Faugno S., Sannino M. and Ardito L. 2017. Optimization of hemp seeds (*Cannabis sativa* L.) oil mechanical extraction. Chemical Engineering Transactions 58:373-378.

Da Porto C., Decorti D. and Tubaro F. 2012. Fatty acid composition and oxidation stability of hemp (*Cannabis sativa* L.) seed oil extracted by supercritical carbon dioxide. Industrial Crops and Products 36:401-404.

Devi V. and Khanam S. 2019. Study of ω-6 linoleic and ω-3 α-linolenic acids of hemp (*Cannabis sativa*) seed oil extracted by supercritical CO_2 extraction: CCD optimization. Journal of Environmental Chemical Engineering 7:102818.

EFSA. 2009. Scientific opinion of the panel on the dietetic products, nutrition and allergies on a request from European Commission related to labelling reference intake values for n-3 and n-6 polyunsaturated fatty acids. The EFSA Journal 1176:1-11.

Fretts A.M., Mozzafarian D., Siscovick D.S., Heckbert S.R., McKnight B., King I.B., Rimm E.B., Psaty B.M., Sacks F.M., Song X., Spiegelman D. and Lemaitre R.N. 2013. Associations of plasma phospholipid and dietary α-linolenic acid with incident atrial fibrillation in older adults: the cardiovascular health study. Journal of the American Heart Association 2:e003814.

Fromm M., Bayha S., Kammerer D.R. and Carle R. 2012. Identification and quantification of carotenoids and tocopherols in seed oils recovered from different Rosaceae species. Journal of Agricultural and Food Chemistry 60:10733-10742.

Gusakova S.D., Sagdullaev S.S. and Khushbaktova Z. A. 1998. Lipophilic extracts in phytoterapy and phytocosmetics: production and biological properties. Chemistry of Natural Compounds 34(4):411-419.

Höppner F. and Menge-Hartman U. 2007. Yield and quality of fibre and oil of fourteen hemp cultivars in Northern Germany at two harvest dates. Landbauforschung Völkenrode 57(3):219-232.

ISO. 1999. International Standard ISO 659: 1998 Oilseeds Determination of Hexane Extract (or Light Petroleum Extract). Called Oil Content. ISO. Geneva. Switzerland.

Kiralan M., Gül V. and Metin Kara Ş. 2010. Fatty acid composition of hempseed oils from different locations in Turkey. Spanish Journal of Agricultural Research 8(2):385-390.

Klingberg S., Ellegård L., Johansson I., Jansson J.H., Hallmans G. and Winkvist A. 2013. Dietary intake of naturally occurring plant sterols is related to a lower risk of a first myocardial infarction in men but not in woman in northern Sweden. The Journal of Nutrition 143:1630-163.

Kolodziejczyk P., Ozimek L. and Kozlowska J. 2012. The application of flax and hemp seeds in food, animal feed and cosmetics production. Handbook of Natural Fibres 2:329-366.

Kostić M.D., Nataša M.J., Stamenković O.S., Rajković K.M., Milić P.S. and Veljković V.B. 2014. The kinetics and thermodynamics of hempseed oil extraction by n-hexane. Industrial Crops and Products 52:679-686.

Kriese U., Schumann E., Weber W.E., Beyer M., Brűhl L. and Matthäus B. 2004. Oil content, tocopherol composition and fatty acid pattern of the seeds of 51 Cannabis sativa L. genotypes. Euphytica 137:339-351.

Latif S. and Anwar F. 2009. Physicochemical studies of hemp (*Cannabis sativa*) seed oil using enzyme-assisted cold-pressing. European Journal of Lipid Science and Technology 111:1042-1048.

Madia T. and Tofani C. 1998. La coltivazione della canapa. Una semplice guida per i coltivatori che desiderano coltivare canapa (*Cannabis sativa*), Coordinamento nazionale per la canapicoltura, Ed. Assocanapa.

Marzocchi S., Pasini F., Baldinelli C. and Caboni. M.F. 2018. Value-addition of beef meat by-products: lipid characterization by chromatographic techniques. Journal of Oleo Science 67:143-150.

Matthäus B. and Brühl, L. 2006. Virgin hemp seed oil: an interesting niche product. European Journal of Lipid Science and Technology 110:655-661.

Matthäus B. and Özcan M.M. 2017. Fatty acid composition, tocopherol and sterol contents in linseed (*Linum usitatissimum* L.) varieties. Journal of Chemistry and Chemical Engineering 36(3):147-152.

Mikulcová V., Kašpárková V., Humpolíček P. and Buňková L. 2017. Formulation, characterization, and propertiesof hemp seed oil and its emulsions. Molecules 22:700.

Montserrat-de la Paz S., Marín-Aguillar F., García-Giménez M.D. and Fernández-Arche M.A. 2014. Hemp (*Cannabis sativa* L.) seed oil: analytical and phytochemical characterization of the unsaponifiable fraction. Journal of Agricultural and Food Chemistry 62:1105-1110.

Ntanios F.Y., van de Kooij A.J., de Deckere E.A.M., Duchateau G.S.M.J.E. and Trautwein E.A. 2003. Effects of various amount of dietary plants sterol ester on plasma and hepatic sterol concentration and aortic foam cell formation of cholesterol-fed hamsters. Atherosclerosis 169(1):41-50.

Official Journal of European Union. 2008. Commission Regulation (EC) No. 1124/2008 of 12 November 2008.

Oomah D.B., Busson M., Godfrey D.V. and Drover J.C.G. 2002. Characteristics of hemp (*Cannabis sativa* L.) seed oil. Food Chemistry 76:33-43.

Orsanova J., Misurcova L., Ambrozova J.V. Vicha R. and Mlcek J. 2015. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. International Journal of Molecular Science 16:12871-12890.

Patel M.D. and Thompson P.D. 2006. Phytosterols and vascular disease. Atherosclerosis 186(1):12-19.

Pelillo M., Iafelice G., Marconi E. and Caboni M.F. 2003. Identification of plant sterols in hexaploidy and tetraploid wheats using gas chromatography with mass spectrometry. Rapid Communication in Mass Spectrometry 17:2245-2252.

Petrović M., Debeljak Ž., Kezić N. and Džidara P. 2015. Relationship between cannabinoids content and composition of fatty acids in hempseed oils. Food Chemistry 170:218-225.

Reena M.B. and Lokesh B.R. 2007. Hypolipidemic effects of oil with balanced amounts of fatty acids obtained by blending and interesterification of coconut oil with rice bran oil or sesame oil. Journal of Agricultural and Food Chemistry 55:10461-10469.

Sacilik K., Öztürk R. and Keskin R. 2006. Some physical properties of hemp seed. Biosystems Engineering 86(2):1191-198.

Sander B.D., Addis P.B., Park S.W. and Smith D.E. 1989. Quantification of cholesterol oxidation products in a variety of foods. Journal of Food Protection 2:109-114.

Siudem P., Wawer I. and Paradowska K. 2019. Rapid evaluation of edible hemp oil quality using NMR and FT-IR spectroscopy. Journal of Molecular Structure 1177:204-208.

Sweeley C.C., Bentley R., Makita M. and Wells W.W. 1963. Gas liquid chromatography of trimethylsilyl derivates of sugar and related substances. Journal of the American Oil Chemists' Society 85:2497-2507.

Tang C.E., Ten Z., Wang X.S. and Yang X.Q. 2006. Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. Journal of Agricultural and Food Chemistry 54:8945-8950.

Yang R., Zhang L., Li P., Yu L., Mao J., Wang X. and Zhang Q. 2018. A review of chemical composition and nutritional properties of minor vegetable in China. Trends in Food Science and Technology 74:26-32.

Yilmaz E. and Erden A.K. 2019. Purification of degummed crude sunflower oil with selected metal-organic frameworks as adsorbents. Grasas y Aceites 70(4):e323. DOI: doi.org/10.3989/gya.0930182

Paper Received May 18, 2020 Accepted August 5, 2020