LWT - Food Science and Technology 194 (2024) 115807

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

New insights on phytocannabinoids role in antioxidant activity of hemp inflorescences extract in high-oleic sunflower oil

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ARTICLE INFO

Keywords:
Hemp
Cannabis
Lipid oxidation
Phytosterol oxidation products
Cannabinoids

ABSTRACT

Hemp (*Cannabis sativa* L.) inflorescences contain bioactive compounds potentially able to counteract lipid oxidation in vegetable oils. In this study, two hemp inflorescences extracts with (E) and without (wE) phytocannabinoids were tested at 0.02, 1.0, and 2.0 g/100 g of high-oleic sunflower oil (HOSO) exposed to thermo-oxidative conditions (90 °C; pO_2, 600 KPa). Immediately at the end of induction time, the oxidative stability index (OSI), peroxide value (PV), main lipid classes, phytosterols, phytosterols oxidation products (POPs) content, and volatile organic compounds were determined. E extract added at 2.0 g/100 g proved to be more effective than wE extract, extending the OSI by almost 30 % with respect to control. The lowest PV ($p < 0.01$) was observed in the oil containing E (2.0 g/100 g), which also proved to significantly reduce the POPs content ($p < 0.05$). Both extracts had no effect on lipolysis during the entire experiment. The principal component analysis highlighted different antioxidant activity related to both type of extract and their concentration. These results suggest that hemp inflorescences could be a source of natural preservatives for vegetable oils even under severe oxidative conditions and confirm that phytocannabinoids play a key role in the modulation of antioxidant activity.

1. Introduction

Lipid oxidation represents the main degradative process of vegetable oils, developing rancidity, unpleasant odors and different toxic components. This process leads to changes in the nutritional composition and the production of volatile and/or nonvolatile (Hammouda, Triki, Matthäus, & Bouaziz, 2018; Tura, Ansorena, Astiasar, Mandrioli, & Gallina Toschi, 2022), and potentially toxic secondary compounds in the final products (Pizzimenti, Bernazzani, Duce, Tinè, & Bonaduce, 2023).

High-oleic sunflower oil (HOSO), known for its high oleic acid content (up to 80 % of the total fatty acids), owns a great content of phytosterols, mainly β-sitosterol, campesterol, stigmasterol, able to lower blood cholesterol levels, particularly low-density lipoproteins (Xu, Zhang, et al., 2022). Again, due to its high content in monounsaturated fatty acids, the use of HOSO is advantageous for heating processes in cooking (Hammouda et al., 2018; Tura et al., 2022). Moreover, recently, attention has been focused on phytosterols (PS) and their oxidation (Dragoun et al., 2022). Like cholesterol, PS can incur in oxidation reactions with consequent formation of compounds known as phytosterol oxidation products (POPs) (Bortolomeazzi, Cordaro, Pizzale, & Conte, 2003; Kmiecik et al., 2021). However, knowledge about POPs is still limited and confusing with numerous gaps in the literature regarding the biological effects, mechanisms of formation and analysis for their monitoring. Kasprzak, Rudzińska, Juzwa, and Olejnik (2023) reported that thermo-oxidized stigmasterol and stigmasteryl linoleate had harmful effects in intestinal cells. Wang et al., 2023 demonstrated that 7-ketophytosterols absorption and transport was faster than phytosterols and cholesterol in Caco-2 cell model; while proved the involvement of POPs in aortic atherosclerosis due to marked alteration in fibroblast and B cells of $ApoE^{-/-}$ mice. Moreover, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) fixed a safe level of POPs exposure equal to 0.64 mg POP/kg body weight per day (Turck et al., 2020).

Antioxidant can prevent lipid oxidation but there are concerns about safety of synthetic type (e.g., butylated hydroxytoluene, BHT)

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https://doi.org/10.1016/j.lwt.2024.115807
Received 3 November 2023; Received in revised form 25 January 2024; Accepted 26 January 2024
Available online 29 January 2024

Abbreviations

E	hemp inflorescences ethanolic extracts with phytocannabinoids
wE	hemp inflorescences ethanolic extracts without phytocannabinoids
C	control
HOSO	high-oleic sunflower oil
PUFAs	polyunsaturated fatty acids
OSI	oxidative stability index
IP	induction period
PV	peroxide value
VOCs	volatile organic compounds
HS-SPME-GC/MS	headspace solid-phase microextraction gas chromatography/mass spectrometry
PS	phytosterols
POPs	phytosterols oxidation products
FFA	free fatty acids
FS	free sterols
ES	esterified sterols
MG	monoacylglycerols
DG	diacylglycerols
TG	triacylglycerols
TPCs	total polar compounds
7α-OHS	7α-hydroxysitosterol
7β-OHS	7β-hydroxysitosterol
7α-OHC	7α-hydroxycampesterol
7β-OHC	7β-hydroxycampesterol
7-KS	7-ketositosterol
7-KC	7-ketocampesterol
α-ES	α-epoxysitosterol
β-ES	β-epoxysitosterol
α-EC	α-epoxycampesterol
β-EC	β-epoxycampesterol
PCA	principal component analysis
CPCA	categorical principal component analysis

(Eskandani, Hamishehkar & Dolatabadi, 2014; Oikawa et al., 1998). As a result, there is growing interest in natural antioxidants, which can improve the stability of vegetable oils, but also enhance their nutritional value with a positive effect on health.

Hemp is an annual plant widespread worldwide for its many applications, climatic adaptability, and spatial versatility (Spano et al., 2021). Based on the content of the psychotropic (Δ9-tetrahydrocannabinol, THC) and non-psychotropic (cannabidiol, CBD) cannabinoids, cannabis is classified in drug type (THC/CBD ratio >1; psychoactive) and fiber type (THC/CBD ratio <1; non psychoactive) (Kopustinskiene, Masteikova, Lazauskas, & Bernatoniene, 2022), whose cultivation has been allowed since 2001 by the European Union with constraint of 0.2 % THC limit on dried biomass (Piluzza, Delogu, Cabras, Marceddu, & Bullitta, 2013). Hemp inflorescences due to their bioactive compounds such as cannabinoids, terpenoids, and flavonoids (Bonini, et al., 2018) find many applications in pharmaceutical and cosmetic fields; however, the application in the foods is still scarce due to national regulations, consumer acceptability and technological challenges (Chen & Pan, 2021). However, the co-presence of these molecules plays a key role on the positive effects of hemp. In fact, it is referred as the “entourage effect” the synergistic activity between terpenes and cannabinoids ("inter-entourage") or between cannabinoids ("intra-entourage") that makes a greater positive contribution with respect to the individual molecules (Koltai & Namdar, 2020). Several authors reported high antioxidant activity of hemp, both *in vivo* (Jastrząb et al., 2021) and *in vitro* (Cantele et al., 2020; Frassinetti et al., 2018), mainly resulting from the ability to scavenge free radicals and chelate metal ions.

In consideration of our preliminary study (Cantele et al., 2020), this research aims to test the effectiveness of two hemp extracts (with and without cannabinoids) to retard lipid oxidation in vegetable oil under thermo oxidative stress conditions (high temperature and oxygen overpressure). Different concentrations of hemp extracts and the potential synergistic effect of terpenes with cannabinoids were tested. The knowledge of the behavior of these extracts is essential to hypothesize their use as natural additives in food processing.

2. Materials and methods

2.1. Chemicals

Ethyl ether, potassium hydroxide, citric acid, pyridine, hexamethyldisilazane, trimethylchlorosilane, 3,5-di-tert-4-butylhydroxytoluene (BHT), 5α-cholestane, 5α-cholestan-3β-ol, triolein, tripalmitin, tristearin, 1,3-diolein, 1,2(3)-dipalmitin, cholesteryl palmitate, 1-oleoyl-rac-glycerol, methyl tridecanoate, cholesterol, stigmasterol, and β-sitosterol were obtained from Sigma-Aldrich (Milan, Italy). The standard mixtures of fatty acid methyl esters (GLC 463) and free fatty acids (GLC 406) were purchased from Nu-Chek (Elysian, MN, USA). 19-Hydroxycholesterol was supplied by Cayman Chemical Company (Ann Arbor, Michigan, USA). Methanol, *n*-hexane, acetone, ethanol (anhydrous), propan-2-ol and ethyl acetate were purchased from Carlo Erba (Milan, Italy). Strata NH_2 solid-phase extraction (SPE) cartridges (500 mg/3 mL) from Phenomenex (Bologna, Italy) were utilized for POPs purification.

2.2. Preparation of hemp inflorescences extracts and oil samples

Hemp dried inflorescences (Carmagnola variety; fiber-type) without seeds were ground and dissolved in absolute ethanol (ratio of 1:10 (*w/v*)) to obtain the ethanolic extract (E) (Bakro et al., 2020). The solution was stirred for 15 min (350 oscillations/min), sonicated for 20 min and centrifuged (8000×*g* at 10 °C for 10 min). The supernatant was collected, filtered (PTFE, 0.45 μm) and ethanol removed with nitrogen. To obtain an extract without phytocannabinoids (wE), before ethanolic extraction, the ground inflorescences were washed with *n*-hexane (ratio of 1:10 (*w/v*)), sonicated for 20 min and centrifuged (8000×*g* at 10 °C for 10 min). The solvent was discharged, and the solid material was washed twice until the content of phytocannabinoids was not detected according to our published analytical method (Cardenia, Gallina Toschi, Scappini, Rubino, & Rodriguez-Estrada, 2018). Then, the remaining solid material was dried flushing nitrogen and the ethanolic extraction was performed as reported above. The final concentrations of the two ethanolic extract were 24.33 mg/mL and 9.67 mg/mL for, respectively, E and wE.

2.3. Sampling

Based on our preliminary studies (data not shown), 0.002 g, 0.1 g, and 0.2 g of the hemp inflorescence extracts with (E) and without phytocannabinoids (wE) were added to 10 g of HOSO (after removal of the solvent), which correspond, respectively, to 0.02-E/wE, 1-E/wE, and 2-E/wE. In brief, accurate aliquots of the ethanolic extract were volumetrically transferred into 15 mL test tubes to achieve the desired concentration within the oil. Subsequently, the solvent was evaporated using nitrogen gas, and HOSO was added. Samples were accurately sonicated (10 min) and stirred (20 min) to reach complete homogenization, confirmed by the absence of precipitates or suspended material (visual observation). A control sample (C) without extracts was also

prepared. Ten grams of oil samples were put in the reactor of an OXITEST (Velp Scientific, Usmate, Italy) to be exposed at high oxygen pressure (pO_2 600 KPa) at 90 °C until the end of induction time. The latter was previously determined using the same instrument under the same conditions (section 2.4). When the end of induction time was reached the samples were immediately cooled and kept at −20 °C until analysis.

2.4. Measurement of the oxidative stability

The OXITEST tracks the oxygen uptake of the sample reactive compounds, measuring over time the absolute pressure changes in the reactors, and is governed by the OXISoftTM software (Velp® Scientifica, Usmate, Italy) that automatically determine the oxidative stability index (OSI), expressed as induction period (IP) (hours) using the two tangents method on the graphically obtained oxidation curve (Tinello et al., 2018).

2.5. Determination of peroxide value

The peroxide value (PV) was determined by iodometric titration (NGD Method C35, 1976) in the oxidized samples, and results were reported as milliequivalents of oxygen per kilogram of oil ($meqO_2$/kg oil).

2.6. Determination of main lipid classes

Main lipid classes were determined according to Gallina Toschi, Cardenia, Bonaga, Mandrioli, and Rodriguez-Estrada (2014). Oxidized oil (20 mg) were dissolved in 1 mL of *n*-hexane:propan-2-ol (3:2, *v/v*) containing 1.05 mg of 5-α-cholestane (IS), and 1 μL was injected (split 1:50) into a Shimadzu QP 2010 Plus gas chromatograph coupled with flame ionization detector (GC-FID) (Shimadzu, Kyoto, Japan) equipped with a silica capillary column (Restek RTX-5, 20 m × 0.10 mm i.d. × 0.10 μm film thickness; Bellafonte, PA, USA). The oven temperature ramped from 100 °C to 350 °C at 5 °C/min and then maintained at 350 °C for 20 min. The temperature of injector and FID were set at 348 °C and 350 °C, respectively. Helium was used as carrier gas with a linear velocity of 47 cm/s. The different lipid classes (free fatty acids (FFA), free (FS) and esterified (ES) sterols, monoacylglycerols (MG), diacylglycerols (DG) and triacylglycerols (TG)) were identified by comparing the retention times of commercial standards injected under the same chromatographic conditions. The internal standard approach was used to ascertain the quantity of each lipid class by response factor of each main lipid class determined by injection of appropriate commercial standards. Results were expressed as grams per 100 g of oil (%).

2.7. Determination of volatile organic compounds

Volatile organic compounds (VOCs) were determined by headspace solid-phase microextraction (HS-SPME) and analyzed by gas chromatography coupled with mass spectrometry (GC/MS). Into a 20 mL HS-SPME vial were placed 2.000 ± 0.001 g and conditioned for 10 min at 40 °C under agitation to reach equilibrium of VOCs in the headspace. Then, a Restek divinylbenzene/carboxen/polydimethylsiloxane coated fiber (DVB/CAR/PDMS) (d_f 50/30 μm; 1 cm) was exposed to the headspace for 40 min in continuous heating and desorbed into the GC/MS injector at 250 °C in split mode (1:30) for 1 min. A Restek RXi-5ms fused silica capillary column (10 m, 0.10 mm i.d., thickness 0.10 μm) was used, with helium as a carrier gas, and maintaining the oven temperature at 65 °C for 5 min and then rising it to 240 °C at 4 °C/min with a final holding time of 10 min. Transfer line and ion source were at 230 °C and 200 °C, respectively. Mass between 33 and 350 *m/z* were acquired with a 1166 amu/s scan velocity. Mass spectra contained in the NIST08s (National Institute of Standards and Technology, Gaithersburg) library were used for the identification of the VOCs. VOCs content is reported as percentage (%) of single peak area on total VOCs area.

2.8. Determination of total phytosterols content

Total phytosterols (PS) were determined according to Rose-Sallin, Huggett, Bosset, Tabacchi, and Fay (1995) with some modifications. Oil sample (100 mg) was added of 0.7 mg of 5α-cholestane-3β-ol (internal standard; IS_1) and 0.5 mg of 19-hydroxycholesterol (internal standard; IS_2), used to quantify phytosterols and phytosterols oxidation products (POPs), respectively. The oil was then saponified with KOH 4M (with 5 mg/mL BHT) in dark overnight at 25 °C. The unsaponifiable matter was extracted with equal volume of diethyl ether and citric acid (0.1 g/100 mL) and the supernatant was collected after centrifugation (3600×*g* for 15 min, 10 °C). The extraction was repeated at least three times (until neutral pH was reached) and the supernatants combined. The solvent was removed with a rotary evaporator (Buchi R-205, BUCHI Italia S.r.l., Cornaredo, Italia) and the unsaponifiable fraction dissolved in 1 mL of *n*-hexane:propan-2-ol solution (3:2; *v/v*). According to Gallina Toschi et al. (2014), one-to-ten of the solution was silylated adding 100 μL of pyridine and 200 μL of the derivatizing mixture (pyridine:hexamethyldisilazane:trimethylchlorosilane, 5:2:1, *v/v/v*) at 40 °C for 20 min. Solvents were evaporated with nitrogen and the silylated PS dissolved in 100 μL of *n*-hexane. One microliter was injected (split ratio 1:30) into the GC/MS equipped with a RXi-5ms fused silica capillary column. Helium was used as the carrier gas with a linear velocity of 36.0 cm/s. Column oven temperature went from 220 to 330 °C (7 °C/min) and held for 3 min. Injector, ion source, and interface temperatures were 325 °C, 200 °C and 325 °C, respectively. The acquisition was full scan total ion current (TIC) (mass range of 50–600 *m/z*) with a scan speed of 3333 amu/s, while the quantitation was performed by single ion monitoring (SIM) using the most intense ions: 129 *m/z*, for sitosterol-TMS and campesterol-TMS; 83 *m/z* for stigmasterol-TMS; 73 *m/z* for Δ5-avenasterol-TMS and 24-methylenecycloartanol-TMS; 343 *m/z* for Δ7-avenasterol-TMS; and 215 *m/z* for campestanol-TMS.

2.9. Determination of phytosterol oxidation products (POPs)

For POPs determination, according to Gallina Toschi et al. (2014), the nine-to-ten of the unsaponifiable fraction was purified by NH_2 solid-phase extraction (SPE) according to Rose-Sallin et al. (1995). The cartridge was activated with 3 mL of *n*-hexane and then eluted with 6 mL of *n*-hexane:ethylacetate (95:5; *v/v*), 10 mL of *n*-hexane:ethylacetate (9:1; *v/v*) and 10 mL of acetone to collect the fraction comprising POPs. The solvent was evaporated by nitrogen flow and POPs were silylated as described above (see section 2.8) and dissolved in 50 μL of *n*-hexane. One microliter was injected in GC/MS under the same conditions reported for PS analysis (section 2.8). The integration mode was single ion monitoring (SIM), and according to Espinosa, Inchingolo, Alencar, Rodriguez-Estrada & Castro (2015), the characteristic ions were used to identify and quantify their corresponding POPs.

2.10. Statistical analysis

Data are reported as mean ± standard deviation of three independent experiments ($n = 3$). IBM SPSS statistical software (version 28; IBM, Chicago, IL, USA) was used to identify statistical differences among the samples by one-way ANOVA with Tukey's post hoc test (95 % confidence level) and to explore the data variability through principal component analysis (PCA) and categorical principal component analysis (CPCA) on VOCs and all dataset, respectively.

3. Results and discussion

3.1. Oxidative stability

As reported in our preliminary study, a full spectra ethanolic hemp

inflorescences extract was able to counteract lipid oxidation in model system (bulk oil based on PUFAs-rich vegetable oil) (Cantele et al., 2020). However, up to now, the role of phytocannabinoids in the antioxidant activity is not clear, particularly when they are in combination with terpenes.

The induction periods (IP) obtained by stressing the samples are depicted in Fig. 1. The control displayed the shortest IP (45.97 h), whereas when both extracts were added at 1.0 g/100 g and 2.0 g/100 g the IP was significantly extended ($p < 0.01$), reaching the highest value of 59.42 h (2-E) with an IP prolongation by 29.26 %. Instead, both extracts at 0.02 g/100 g did not significantly ($p > 0.05$) increase the IP compared to the control and did not significantly differ from each other. Thus, the presence of phytocannabinoids become evident in terms of oxidative stability only upon specific concentrations. The ability of hemp inflorescence extracts to retard lipid oxidation can be attributed to the bioactive compounds as phytocannabinoids, terpenes and flavonoids. Indeed, it is well demonstrated the capability of these compounds to chelate metal ions and scavenge free radicals (among others), which are responsible for initiating and propagating the chain reactions of lipid oxidation (Cantele et al., 2020; Nuutinen, 2018). In addition, Carmagnola variety inflorescences have been reported to be high in total phenolic (about 20 mg GAE/g) and terpenes (about 60 mg/g) contents, being particularly rich in β-pinene, cedrol, and β-caryophyllene, which possess strong antioxidant activity (Cantele et al., 2020). However, this variety is also rich in phytocannabinoids, especially CBD (about 5 %), whose synergistic effect with terpenoid compounds *in vivo* is well known (Jastrząb et al., 2021). On the other hand, Tura, Mandrioli, and Gallina Toschi (2019) reported that CBD was not able to extend the oxidative stability (OSI) of sunflower oil, whereas in the case of refined olive oil the presence of CBD contributed to reduce the OSI-time.

Based on these results, it is evident the different activity of the extract containing phytocannabinoids (E) and the one deprived (wE), leading to state that the whole spectrum of hemp compounds is more effective, particularly evident with high concentration of extract.

3.2. Peroxide value (PV)

The PV results of the oil after the oxidation are displayed in Fig. 2. The control showed the highest PV (37.00 meqO_2/kg), while all samples with hemp extracts showed lower values ($p \leq 0.001$), ranging 34.11–28.63 meqO_2/kg, with trends that linearly decreased as the concentrations of extracts in both E and wE increased. The E-extract consistently showed the greatest inhibition of peroxide formation, with

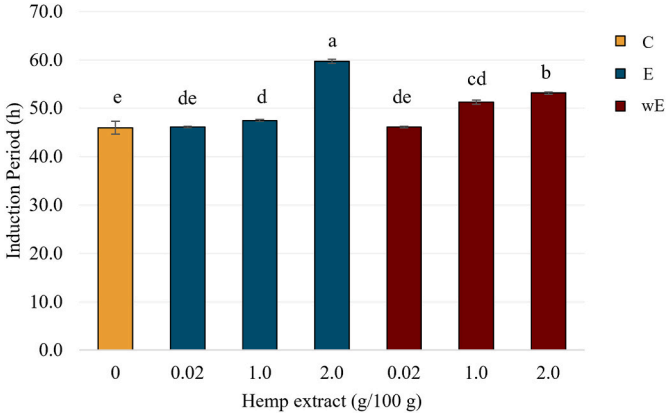

Fig. 1. Induction Period (IP) measured by OXITEST for each type of oil. C, control; E, high oleic sunflower oil (HOSO) added with non-washed hemp inflorescence extract; wE, HOSO added with washed hemp inflorescence extract. Each bar represents the mean ± standard deviation of three independent replicates. Different letters upon the bars indicate significantly different values at $p < 0.001$.

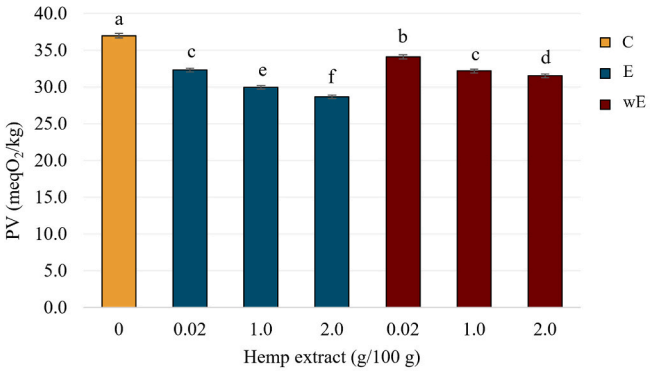

Fig. 2. Peroxide Value (PV) for each type of oil after oxidation. Each bar represents the mean ± standard deviation of three independent replicates. Different letters upon the bars indicate significantly different values at $p < 0.001$. C, control; E, high oleic sunflower oil (HOSO) added with non-washed hemp inflorescence extract; wE, HOSO added with washed hemp inflorescence extract.

the lowest value observed in E−2.0 % ($p \leq 0.001$). Results suggest, again, that the full spectrum of hemp, and the synergistic relationship between its compounds, could have a greater antioxidant effect, which aligns with the findings from the IP values.

3.3. Main lipid classes

Results about the main lipid classes of the oxidized oils are reported in Table 1 (chromatograms of 2-E and 2-wE are presented in the Supplementary Material, Fig. S2). As expected, the main class was triacylglycerols (TAGs; 68.41–74.15 %), followed by diacylglycerols (DAGs; 17.24–19.07 %), free fatty acids (FFAs; 5.83–7.43 %), monoacylglycerols (MAGs; 3.57–4.47 %), esterified sterols (ES; 1.65–1.88 %) and free sterols (FS; <1 %). No significant differences were found between the samples for any lipid class ($p > 0.05$) highlighting that the hemp extracts was not able to reduce the hydrolysis of triacylglycerols, which naturally undergone hydrolytic degradation. However, it is interesting to highlight a tendency ($p = 0.058$) of hemp extracts to prevent lipolysis. In fact, oil with hemp extracts displayed a slightly higher TAGs value with a consequent lower value of DAGs compared to the control. Probably, the high resistance of HOSO to oxidative stress as well as the dispersion of data lead to not observe significant differences on the lipid classes. Moreover, these results agree with our previous work carried out on a PUFAs-rich stripped vegetable oil (Cantele et al., 2020), where the hemp extract reduced lipolysis only on the last day of oil storage (7 days).

3.4. Volatile organic compounds (VOCs)

Fifty different VOCs were identified and grouped into major chemical categories: aldehydes, alcohols, hydrocarbons, ketones, carboxylic acids, esters, furans and other minor compounds (Table 2). As expected, aldehydes (37.59 %–33.86 %) was the main class detected, followed by alcohols (14.27 %–16.67 %), and ketones (from 12.30 % to 14.23 %). Carboxylic acids, furans, hydrocarbons, and esters were <10 %. In general, it was challenging to understand the behavior of hemp extract toward VOCs. As reported by Calvi et al. (2018), hemp inflorescences naturally contain compounds like alcohols, aldehydes, esters, and ketones, which consequently pose interferents in VOC analysis as markers of lipid oxidation. In fact, the content of ketones showed a slight increase in the samples added with the extract. Their presence is correlated with pungency sensations and roasted but, at the same time, also fruity (Xiao et al., 2022). The same trend could be detected for furan compounds; samples 1-wE, 2-wE, and 2-E reported higher values ($p < 0.05$) than

Table 1
Main lipid classes (% on the total oil) determined in the oxidized oils.

	C	0.02-wE	1-wE	2-wE	0.02-E	1-E	2-E	Sig.
FFA	6.32 ± 0.54	6.12 ± 0.09	5.83 ± 0.46	6.01 ± 0.86	6.26 ± 0.05	7.16 ± 0.06	6.43 ± 0.36	n.s.
MG	3.57 ± 0.17	3.74 ± 0.03	3.72 ± 0.75	4.47 ± 0.61	3.66 ± 0.11	4.37 ± 0.05	4.09 ± 0.13	n.s.
DG	19.07 ± 0.40	18.95 ± 0.94	18.99 ± 0.74	17.64 ± 1.50	18.99 ± 0.52	18.65 ± 1.85	17.24 ± 0.12	n.s.
TG	68.41 ± 0.90	69.41 ± 0.50	74.15 ± 1.45	71.13 ± 3.64	69.36 ± 0.75	73.10 ± 1.80	71.00 ± 0.36	n.s.
ES	1.72 ± 0.13	1.75 ± 0.01	1.75 ± 0.01	1.88 ± 0.34	1.73 ± 0.24	1.65 ± 0.01	1.74 ± 0.01	n.s.
FS	0.91 ± 0.15	0.93 ± 0.11	0.85 ± 0.04	0.88 ± 0.06	0.95 ± 0.07	0.96 ± 0.08	0.89 ± 0.06	n.s.

Abbreviations: FFA, free fatty acids; monoacylglycerols, MG; diacylglycerols, DG; TG, triacyclglycerols; ES, esterified sterols; FS, free sterols; C, control; E, HOSO added with non-washed hemp inflorescence extract (0.02-E, 0.02 g/100 g; 1-E, 1.0 g/100g; 2-E, 2.0 g/100g); wE, HOSO added with washed hemp inflorescence extract (0.02-wE, 0.02 g/100 g; 1-wE, 1.0 g/100g; 2-wE, 2.0 g/100g). Sig., statistical significance; n.s., not significant ($p > 0.05$).

Table 2
Percentage composition of Volatile Organic Compounds (VOCs) in the oxidized oils.

	C	0.02-wE	1-wE	2-wE	0.02-E	1-E	2-E	Sig.
Aldehydes	37.59 ± 1.37^{a}	35.80 ± 0.65^{ab}	33.86 ± 0.55^{b}	35.66 ± 0.52^{ab}	35.51 ± 0.41^{ab}	34.70 ± 0.29^{b}	35.34 ± 0.49^{ab}	**
Alcohols	15.54 ± 0.39^{ab}	14.27 ± 0.47^{b}	16.26 ± 0.79^{a}	16.67 ± 0.35^{ab}	14.83 ± 0.35^{ab}	15.02 ± 0.07^{ab}	15.27 ± 0.25^{ab}	*
Ketones	12.53 ± 0.32^{cd}	13.90 ± 0.90^{ab}	12.30 ± 0.77^{cd}	14.23 ± 0.30^{a}	13.11 ± 0.60^{ab}	12.77 ± 0.42^{d}	12.88 ± 0.38^{abc}	*
Carboxylic Acids	8.33 ± 0.19	8.75 ± 0.38	7.28 ± 1.33	6.90 ± 0.30	8.69 ± 0.86	8.88 ± 0.13	8.68 ± 0.43	n.s.
Esters	5.30 ± 0.68^{bc}	4.77 ± 0.21^{c}	5.67 ± 0.29^{bc}	5.58 ± 0.11^{bc}	5.88 ± 0.20^{bc}	6.53 ± 0.18^{ab}	7.48 ± 0.27^{a}	**
Furans	7.86 ± 0.18^{c}	7.07 ± 0.47^{c}	10.17 ± 0.05^{a}	9.33 ± 0.28^{b}	7.93 ± 0.33^{bc}	8.14 ± 0.15^{bc}	8.94 ± 0.08^{b}	*
Hydrocarbons	7.83 ± 1.00	7.02 ± 1.03	7.21 ± 0.25	6.74 ± 0.35	7.21 ± 0.71	7.19 ± 0.06	6.81 ± 1.07	n.s.
Others								
Trans-Linalool Oxide	n.d.	n.d.	0.44 ± 0.08^{a}	0.48 ± 0.02^{a}	n.d.	0.24 ± 0.00^{b}	0.36 ± 0.01^{ab}	***
Caryophyllene Oxide	n.d.	n.d.	n.d.	0.13 ± 0.03	n.d.	n.d.	n.d.	

Values are expressed as percentages ± standard deviation of three independent replicates. Aldehydes, alcohols, ketones, carboxylic acids, esters, furans, and hydrocarbons are presented as sum of molecules belonging to each class of compounds. Results of ANOVA with Tukey's post hoc test are reported between samples (row); values followed by different letters within the same row are significantly different at $p < 0.05$. Abbreviation: C, control; E, HOSO added with non-washed hemp inflorescence extract (0.02-E, 0.02 g/100 g; 1-E, 1.0 g/100g; 2-E, 2.0 g/100g); wE, HOSO added with washed hemp inflorescence extract (0.02-wE, 0.02 g/100 g; 1-wE, 1.0 g/100g; 2-wE, 2.0 g/100g). Sig., statistical significance; n.s, not significant; the asterisks denote the level of significance: *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$.

control. Furans belong to the category of tertiary oxidation compounds (Grebenteuch, Kroh, Drusch, & Rohn, 2021), but are also naturally part of the volatile composition of hemp inflorescence (Calvi et al., 2018). Anyway, some differences and trends related to the oxidation phenomenon have been detected. The aldehydes content drastically decreased when both extracts were added at high concentrations, while at 0.02 g/100 g of concentration just a decreasing trend was observed ($p > 0.05$). Aldehydes are considered the most important and closely monitored breakdown products, due to their low perception threshold values and their major role in causing defects, odors, and rancid tastes (Tura et al., 2022).

Significantly higher amount of alcohols were found in 1-wE compared to the other samples, including the control ($p < 0.05$). On the other hand, a greater content of esters was observed in 2-E ($p < 0.01$). Two hemp characteristic oxidized terpenes were detected in sample containing extracts: *trans*-linalool oxide and caryophyllene oxide. Linalool is an acyclic monoterpene that plants naturally produce by an enzymatic pathway and represents a precursor of other molecules such as alcohols and aldehydes, including linalool oxide (Weston-Green, Clunas & Jimenez Narajo, 2021). The *trans*-linalool oxide was found in the oils enriched with both extracts, but was still significantly more abundant ($p < 0.001$) in the wE-samples (0.44 % and 0.48 % ± 0.02 for 1-wE and 2-wE, respectively) than E (0.24 % and 0.36 % for 1-E and 2-E, respectively). This is probably due to the increase in the concentration of the terpene compounds in the wE extract resulting from the exclusion of phytocannabinoids. Moreover, only 2-wE sample contained caryophyllene oxide. β-Caryophyllene, a natural compound, is known for its analgesic, anticancer and neuroprotective properties (Ahmed, Abu Zahra, Ammar, Mohamed & Ibrahim, 2022; Oija, Javed, Azimullah & Haque, 2016), but it is susceptible to oxidation in the presence of oxygen and high temperature (Lee & Ko, 2021). The primary oxidation products of β-caryophyllene are quickly converted to a more stable and less reactive compound called caryophyllene oxide; its absence in the E extract samples suggests that the presence of the full spectrum of hemp would lead to a decrease in ROS levels, implying an activity of the extract to self-protect from oxidation.

Comparing the two extracts at the same concentration, the E extract was found having a greater antioxidant activity than wE. HOSO with 2-E showed a lower amount of ketones than HOSO with 2-wE (12.88 % and 14.23 %, respectively). Since the formation of secondary oxidation products results from the degradation of alkoxy and peroxy radicals (Grebenteuch et al., 2021), these results suggests that the antiradical activity of hemp might be enhanced by the simultaneous presence of phytocannabinoids with terpenes and biphenols. It is also important to consider tertiary oxidation products, which result from the degradation of secondary compounds, and therefore characterize advanced oxidation states (Grebenteuch et al., 2021). Again, furanoids obtained by rapid degradation of aldehydes particularly the unsaturated forms (Grebenteuch et al., 2021) were lower in HOSO with E extract than other samples (8.94 % *vs.* 9.33 %, for 2-E and 2-wE, respectively; $p < 0.01$).

3.5. Phytosterols and phytosterol oxidation products (POPs)

The total phytosterols content in fresh oil was 4937.32 μg/g in agree with literature (Winkler, Warner & Glynn, 2007). After oxidation, the total phytosterols content ranged from 1137.87 μg/g to 1151.57 μg/g oil and no significant differences were found due to treatments ($p > 0.05$). The most abundant phytosterols detected in oxidized oils were β-sitosterol (697.73–634.64 μg/g), campesterol (171.98–167.59 μg/g), stigmasterol (33.06–37.64 μg/g), Δ5-avenasterol (66.19–65.06 μg/g), Δ7-avenasterol (66.38–67.67 μg/g), campestanol (67.00–66.26 μg/g), and 24-methylenecycloartanol (59.94–62.04 μg/g) (Fig. S1, Supplementary Material).

The reduction in phytosterol levels is common in fats that have been heated or exposed to air and light during storage (Kasprzak et al., 2020; Kmiecik et al., 2021). Moreover, the fatty acids composition of vegetable oils as well as the phytosterols form (esterified or free) affect their oxidative behavior. Kmiecik et al. (2021) reported that the presence of

double bonds in phytosterols esters accelerated the oxidative process; in fact, as the number of double bonds in esterified fatty acids increases as the phytosterol oxidation growths. Others reported that PUFAs triacylglycerols could delay the induction time of phytosterols oxidation (Ansorena et al., 2013; Scholz, Guth & Steinberg, 2015). Again, Kmiecik et al., (2015) when oxidized vegetable oils for 4 h at 180 °C found significant differences on phytosterols due to different antioxidant agents.

In the present study, the phytosterols content decreased by 77.06 %; however, no significant differences ($p > 0.05$) in the content of phytosterols were observed ascribed to the type of hemp extract (washed or non-washed) and concentrations used. Thus, in order to better evaluate the oxidative behavior of oils the determination of POPs was carried out (chromatograms of 2-E and 2-wE are presented in the Supplementary Material, Fig. S3).

β-Sitosterol and campesterol produced detectable and quantifiable amounts of hydroxy, epoxy and keto isomers (Table 3). Regarding the individual isomers, significant differences between the samples were noted for all the POPs except for 7β-OHC, β-ES and β-EC ($p < 0.05$). In general, the addition of the hemp extracts reduced the isomeric species of POPs, except for the keto forms of β-sitosterol and campesterol, which significantly increased when both hemp extracts were added to HOSO. In all the oxidized oils the dominant POP species was 7β-OHS, except for 1-E and 2-E, where a slight inhibition of its formation was observed. One possible explanation could be ascribed to POPs formation reactions. Although the knowledge about the oxidation pathways of phytosterols is scarce and still under investigation, due to their similarity to cholesterol, it is assumed that phytosterols oxidation could be similar to that of cholesterol (Hovenkamp et al., 2008; Kmiecik et al., 2021). According to Tai, Chen, and Chen (1999), there are two pathways of oxidation of sterols: one characterized by the presence of free radicals and oxygen, which can lead to both the generation of hydroperoxides and epoxides, and one resulting from the direct attack of oxygen in the excited state, which inhibits the formation of epoxides. The antiradical effect of hemp (Cantele et al., 2020) in the early stages of oxidation could lead by direct attack of oxygen to the rapid formation of hydroperoxides, highly unstable species that in the presence of high temperatures generate the 7-hydroxyl isomers. These, in turn, by deprotonation resulted in the accumulation of the stable 7K isomers. In the absence of hemp (control) the generation of epoxides and hydroxides was well promoted. Interestingly, the β-epoxide form showed no variation ($p > 0.05$) among samples; in contrast, the α-EP isomer of sitosterol and campesterol displayed greater variability between samples ($p < 0.01$), suggesting it as a valuable marker for monitoring lipid oxidation. Compared to the control (468.47 μg/g) all samples containing the E extract reported a significant reduction in total POPs content ($p < 0.01$); specifically, a reduction of 20 %, 21 %, and 23 % was quantified in HOSO added with 0.02 g/100 g, 1.0 g/100 g and 2.0 g/100 g, respectively. On the other hand, wE extract accounted a POPs reduction of 15 % only when added at 2.0 g/100 g. These results highlighted the importance of phytocannabinoids presence within the hemp extract, which contributed to improve the antioxidant activity by significant reduction of POPs. These results are comparable with those reported by Kmiecik, Korczak, Rudzińska, Michałowska, and Hęś (2009), where a decrease in POPs content by 30 % and 23 % was obtained in rapeseed oil subjected to high stress conditions in presence of rosemary extract and green tea extract, respectively.

The interest in reducing the formation of POPs during heat treatments can be traced back to the lack of information about the effects on human health. The biological effects following their intake reported in the literature are conflicting and require more in-depth studies. In fact, although their correlation with cytotoxic effects and the onset of cardiovascular disease are widely reported (Fuhrmann et al., 2018; Hovenkamp et al., 2008; Scholz, Guth, Engel, & Steinberg, 2015), positive effects of specific POPs, such as anti-inflammatory, antitumor and antidiabetic activities, have also been found (Hovenkamp et al., 2008). However, the studies conducted so far do not represent actual dietary intakes but were carried out with an excessive amount of POPs, questioning the actual long-term risk. Additionally, the lack of an international analytical validated method for POPs determination and the absence of commercial reference materials (Scholz et al., 2015) make difficult to compare the data present in literature. To better define the extent of oxidation and the action of hemp extracts on counteracting POPs formation, oxidation factors (determined as % POPs/PS) were calculated. The control samples displayed the highest value (F_{ox} 32 %) together with 0.02-wE (F_{ox} 28 %) and 1-wE (F_{ox} 31 %); while 2-E shown the lowest value (25 %) confirming the antioxidant ability of hemp extract through the synergistic action between phytocannabinoids, biphenols and terpenes.

3.6. Principal component analysis (PCA)

Multivariate analysis was used to highlight the correlations between the main oxidative parameters and oxidized oil samples. PCA revealed that the first two principal components accounted for 54.05 % of the total variance (PC1, 31.82 %; PC2, 22.22 %). No clear clusterization of volatile compounds was identified (Fig. 3). However, the VOCs originated through terpenic oxidation (*trans*-linalool oxide and caryophyllene oxide) were well separated from those produced by lipid oxidation. The fatty acid composition of the oil can give a first clue about the volatile oxidation products that will be generated during oxidation. The high presence of oleic acid (C18:1, about 82 % (Roman, Heyd,

Table 3
Phytosterols Oxidation Products (POPs) (μg/g oil) content in the oxidized oils.

	C	0.02-wE	1-wE	2-wE	0.02-E	1-E	2-E	Sig.
7α-OHS	11.13 ± 0.43^{a}	8.77 ± 0.84^{abc}	10.72 ± 0.77^{ab}	7.46 ± 0.12^{cd}	8.83 ± 0.88^{bc}	5.88 ± 0.37^{d}	6.50 ± 0.51^{d}	**
7β-OHS	84.87 ± 0.25^{ab}	58.86 ± 1.92^{b}	87.02 ± 6,95^{a}	68.19 ± 0.10^{bc}	60.86 ± 3.69^{c}	52.28 ± 1.94^{c}	56.54 ± 6.36^{c}	***
7α-OHC	35.77 ± 1.56^{a}	29.38 ± 1.06^{ab}	35.27 ± 1.14^{a}	27.80 ± 0.21^{bc}	28.12 ± 1.00^{b}	22.73 ± 1.01^{d}	23.27 ± 1.54^{cd}	*
7β-OHC	14.72 ± 5.19	11.02 ± 1.99	18.58 ± 0.3	16.13 ± 0.44	12.53 ± 2.05	12.57 ± 0.49	12.86 ± 1.49	n.s.
7-KS	24.97 ± 5.94^{b}	37.91 ± 0.61^{ab}	24.90 ± 3.85^{b}	35.03 ± 1.12^{ab}	45.91 ± 0.14^{a}	37.38 ± 3.31^{ab}	30.14 ± 0.02^{b}	*
7-KC	4.17 ± 0.24^{b}	6.97 ± 1.21^{b}	4.17 ± 0.47^{b}	6.86 ± 0.31^{b}	12.90 ± 1.06^{a}	7.01 ± 2.48^{b}	8.63 ± 0.18^{ab}	**
α-ES	39.42 ± 2.01^{ab}	31.13 ± 3.10^{ab}	44.35 ± 5.58^{a}	22.00 ± 3.76^{bc}	20.13 ± 3.10^{c}	20.52 ± 6.37^{c}	24.02 ± 5.17^{bc}	*
β-ES	78.70 ± 1.00	80.97 ± 3.33	81.82 ± 6.36	76.63 ± 2.29	81.97 ± 4.03	82.42 ± 4.79	80.92 ± 1.46	n.s.
α-EC	62.66 ± 5.44^{a}	37.18 ± 1.97	34.21 ± 1.82^{b}	34.63 ± 4.04^{b}	34.18 ± 2.31^{b}	35.49 ± 2.31^{b}	27.05 ± 1.42^{b}	*
β-EC	20.03 ± 0.05	18.79 ± 3.41	15.65 ± 3.87	16.80 ± 5.11	16.79 ± 4.30	15.20 ± 4.10	11.02 ± 0.06	n.s.
Total	368.47 ± 11.50^{a}	320.98 ± 8.77^{b}	356.72 ± 19.77^{a}	311.54 ± 9.55^{b}	292.22 ± 1.87^{bc}	291.49 ± 18.97^{bc}	281.47 ± 13.17^{c}	***

Abbreviations: 7α-OHS, 7α-hydroxysitosterol; 7β-OHS, 7β-hydroxysitosterol; 7α-OHC, 7α-hydroxycampesterol; 7β-OHC, 7β-hydroxycampesterol; 7-KS, 7-ketositosterol; 7-KC, 7-ketocampesterol; α-ES, α-epoxysitosterol; β-ES, β-epoxysitosterol; α-EC, α-epoxycampesterol; β-EC, β-epoxycampesterol; C, control; E, HOSO added with non-washed hemp inflorescence extract (0.02-E, 0.02 g/100 g; 1-E, 1.0 g/100g; 2-E, 2.0 g/100g); wE, HOSO added with washed hemp inflorescence extract (0.02-wE, 0.02 g/100 g; 1-wE, 1.0 g/100g; 2-wE, 2.0 g/100g). Values are expressed as mean ± standard deviation of two independent replicates. Results of ANOVA with Tukey's post hoc test are reported between samples (row); means followed by different letters within the same row are significantly different at $p < 0.05$. Sig., statistical significance; n.s., not significant; the asterisks denote the level of significance: *, $p < 0.05$; **, $p < 0.01$; ***$p < 0.001$.

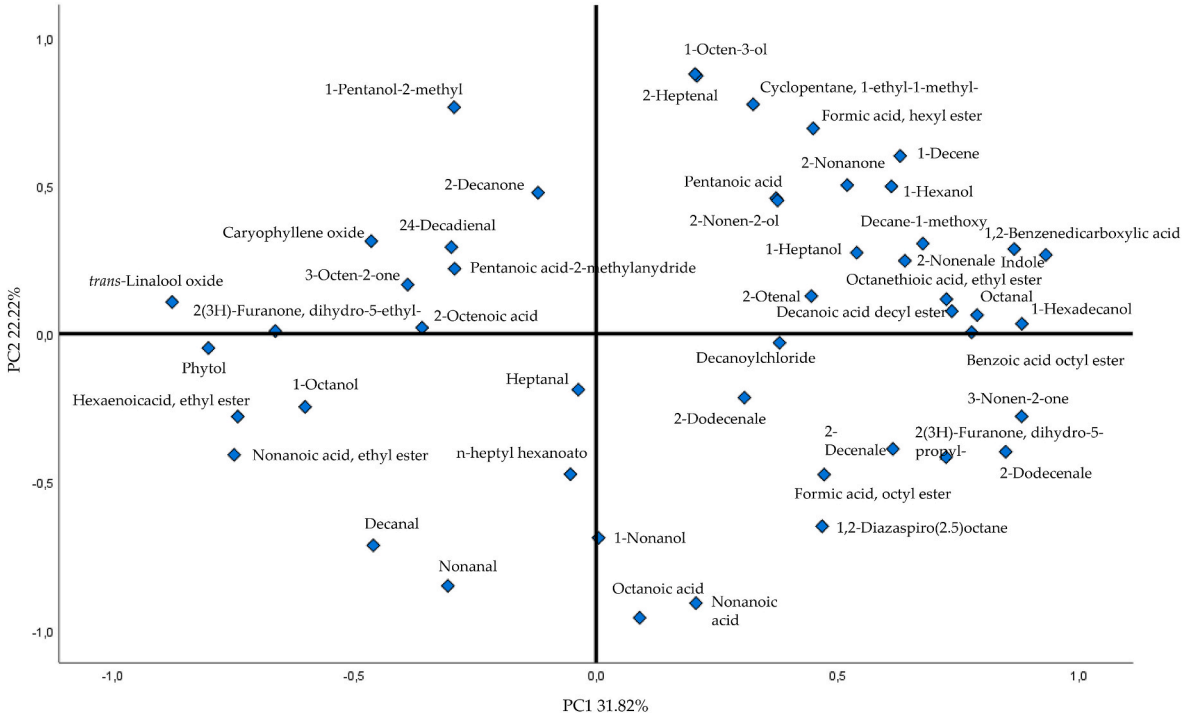

Fig. 3. Principal component analysis plot of volatile organic compounds (VOCs).

Broyart, Castillo, & Maillard, 2013)) explains the presence of nonanal, octanal and decenal, the characteristic carbonyls of oleic acid-rich oils (Cao et al., 2020). In comparison, the second most abundant fatty acid was linoleic acid (C18:2; about 9.5 %), which led to the formation of 2, 4-heptadienal, 2-nonadienal, 1-octen-3-ol, 2-pentyl furan (Chen et al., 2023), 1-pentanol (Zhang, Saleh, Chen, & Shen, 2012) and 3-octe-n-2-one (Wang et al., 2022).

To better understand which was the role of hemp extracts on lipid oxidation in terms of both composition and concentration, a bi-plot PCA analysis was carried out considering both VOCs and POPs (Fig. 4). The explained total variance by the first two principal components reached the 67.28 % of the total variance (PC1, 42.32 %; PC2, 24.96 %). Thus, in order to better clarify the correlation effect only variables with correlation coefficient >0.6 was considered. As reported in Fig. 4, control together with samples containing low concentrations of extracts were separated from those at higher concentration of extracts (1.0 g/100 g and 2.0 % g/100 g). Control samples were characterized by the presence of all POPs except for 7-KC, 7-KS, α-ES and β-ES; in addition, the presence of ketones and carboxylic acids volatiles suggested a pronounced oxidation. Aldehydes resulting from direct degradation of specific fatty acids subjected to long heat treatments can in turn be oxidized generating the corresponding carboxylic acids. Subsequently, during the heating process, the reaction of decarboxylation is favored (Renz, 2005). On the other hand, both extracts (wE and E) added at 0.02 g/100 g of concentrations were not significantly separated and characterized by carboxylic acids and alcohols, which confirm that low dosage of both extracts was useless to retard lipid oxidation. Again, the extracts added at 1.0 g/100 g and 2.0 g/100 g displayed a different behavior, since wE samples resulted clearly separated from E samples. The wE were mainly characterized by β-ES and oxidized terpenes. In fact, it might be highlighted that wE samples did not contain phytocannabinoids, thus, oils with only terpenes were oxidized in different manner from those containing phytocannabinoids.

Based on these results, it seems that E extract succesfully delayed the lipid oxidation; during the initation of oleic acid oxidation, nonanal is the main aldehyde which, as it oxidizes in turn, generated the corresponding carboxylic acid (nonanoic acid) (Xu, Zhang, et al., 2022). Again, the presence of 7-keto isomers could confirm the antioxidant effect of E extract. It has been reported by several authors that the first products of cholesterol oxidation are hydroperoxides (Cardenia, Rodriguez-Estrada, Boselli, & Lercker, 2013; Garcia-Llatas, Mercatante, López-García, & Rodriguez-Estrada, 2021), very unstable species that are rapidly converted to the hydroxyl and ketone forms (7α-OH, 7β-OH, and 7-K), following first-order reaction kinetics (Ansorena et al., 2013). The 7-K isomer has often been used as a marker for the oxidation of sterol molecules and nutritional quality; at the same time, hydroperoxides may also follow a bimolecular reaction pathway in which hydroperoxyesterol interacts with another sterol molecule to form epoxysterol (α-E and β-E) (Cardenia et al., 2013).

4. Conclusions

The antioxidant effect of two hemp inflorescences extracts, with and without phytocannabinoids, were tested in HOSO. Both extracts were not able to protect HOSO from lipolysis, and, when added at 0.02 g/100 g, no effect on lipid oxidation was observed. Nevertheless, when the concentration of the extracts was ≥1 g/100 g lipid oxidation was significantly restrained. In particular, 2-E elongated the induction period by 30 % compared to the control, and the total amount of POPs was drastically reduced. The 7α/β-hydroxy isomers of β-sitosterol and 7α-hydroxycampesterol were the main POPs followed by the epoxy-isomers. However, the POPs differently characterized the samples, the epoxy isomers were concentrated in control samples, while the 7-keto isomers were mainly located in samples less oxidized. E extract was more efficient to counteract lipid oxidation and particularly phytosterol oxidation than wE, demonstrating once again the greater antioxidant activity of the full bioactive spectrum with respect to the individual compounds. Hence, this study showed how even under high oxidative stress conditions hemp inflorescence extract can limit the formation of compounds harmful to human health, such as POPs, being a great advantage for food industries, as hemp would be a source of natural antioxidants capable of extending the shelf life of vegetable oils even in cooking processes requiring very high temperatures. However, a deeper investigation is required in real system to evaluate the contribute of

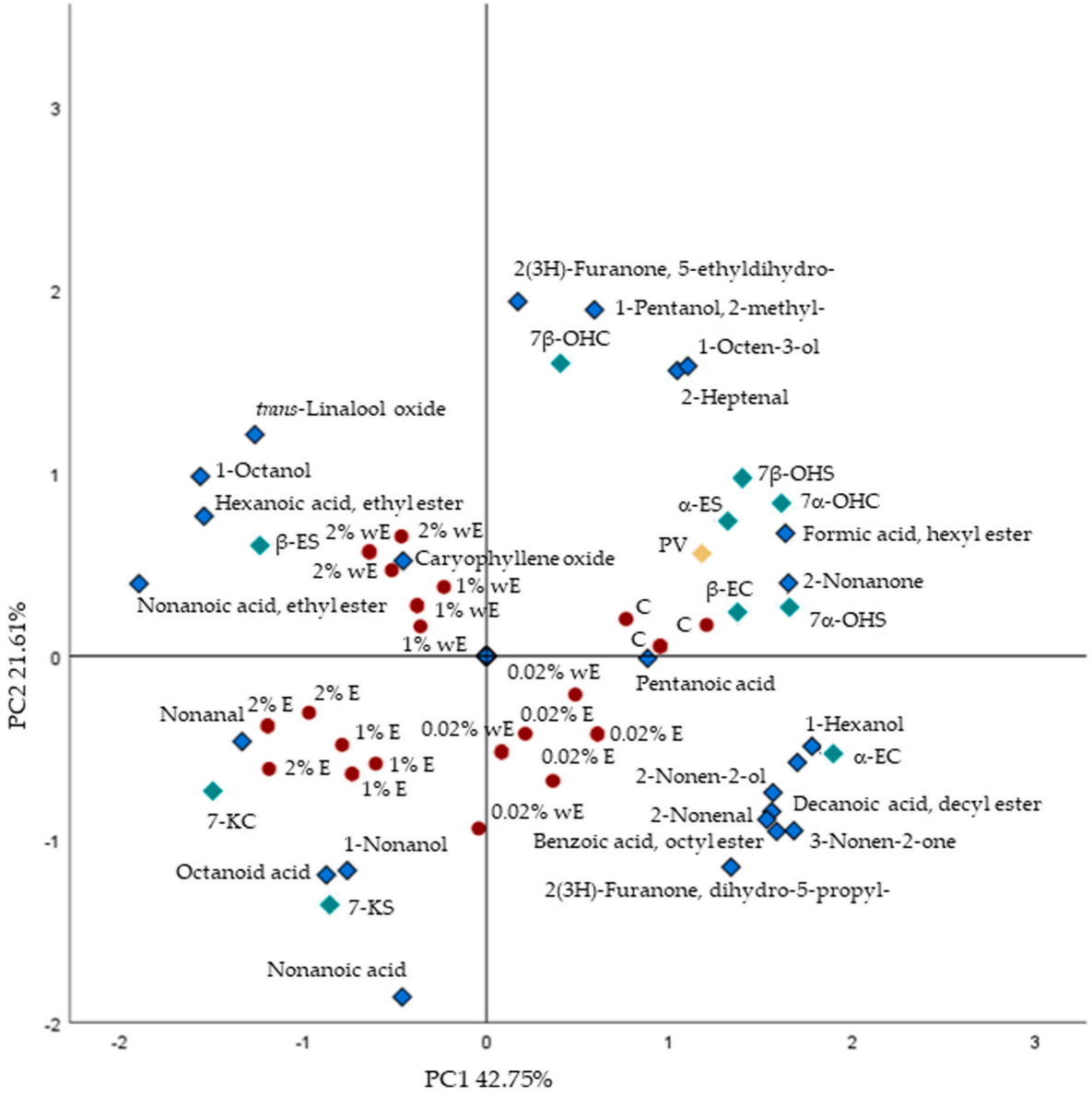

Fig. 4. Principal component analysis Bi-Plot of VOCs and POPs. 7α-OHS, 7α-hydroxysitosterol; 7β-OHS, 7β-hydroxysitosterol; 7α-OHC, 7α-hydroxycampesterol; 7β-OHC, 7β-hydroxycampesterol; 7-KS, 7-ketositosterol; 7-KC, 7-ketocampesterol; α-ES, α-epoxysitosterol; β-ES, β-epoxysitosterol; α-EC, α-epoxycampesterol; β-EC, β-epoxycampesterol.

cooked food and cooking process.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CRediT authorship contribution statement

Ambra Bonciolini: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Carolina Cantele:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation. **Maria Piochi:** Investigation, Formal analysis. **Giuseppe Di Lecce:** Writing – review & editing. **Davide Risso:** Writing – review & editing. **Vladimiro Cardenia:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to thank Società Agricola FACT s.s. for providing hemp inflorescences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

org/10.1016/j.lwt.2024.115807.

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