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INSTRUMENTAL AND SENSORY ANALYSES FOR THE CHARACTERIZATION OF FOOD, PHYTOTHERAPEUTIC OR PHARMACEUTICAL HEMP PRODUCTS

Presentata da: Matilde Tura

Coordinatore Dottorato

Prof. Massimiliano Petracci

Supervisore

Prof.ssa Tullia Gallina Toschi

Co-Supervisori

Dott. Enrico Valli
P.Chim. Mara Mandrioli

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Abstract

This Ph.D. project aimed at assessing food, phytotherapeutic, and pharmaceutical hemp products from an instrumental and sensory point of view. The Italian and the European regulatory frameworks classify two types of *Cannabis sativa* L., depending on the content of Δ_9- tetrahydrocannabinol (Δ9-THC). Consequently, there is a request to develop cost-effective and easy to use qualitative and quantitative methods for the determination of the cannabinoids, the principal and unique bioactive compounds in hemp.

Two different studies were performed concerning cannabinoids and the first one regarded development and *in-house validation* of an HPLC-UV method for rapid, easy and cost-efficient determination of the 10 main cannabinoids in hemp inflorescences. This method, applied to cannabis inflorescences, involves extraction in methanol/chloroform, drying of the extract, taking it up in acetonitrile and injection into an HPLC. It allows the quantitative determination of the 10 cannabinoids using a single wavelength (220 nm) in 8 min. Moreover, it has the sensitivity and accuracy to discriminate samples with amounts of Δ_9- and Δ_8-THC below the limit of 0.2%, from those that are subjected to legal restrictions in many EU countries, with a total THC content above 0.6%, which cannot be classified as hemp.

The second study was focused on evaluating the potential antioxidant activity of cannabidiol (CBD) in two oily matrices in comparison to the antioxidant activity of α-tocopherol. The higher scavenging activity (determined by DPPH$^{\bullet}$) of CBD compared to the α-tocopherol does not seem to be related with a greater oxidative stability of the oily model system when the CBD is added to it. This evidence could be useful to predict and possibly adjust (e.g. with the addition of antioxidants) the stability of oily solutions, largely diffuse in the market, containing CBD and to correctly formulate oily foods, medicines, or supplements containing CBD.

Regarding food hemp products, the research activities dealt with the characterization and the quality control of cold-pressed hemp seed oils (HSOs). In fact, although the market interest in HSOs has increased, nowadays there is no univocal legislation that gives indications on evaluation of the quality and authenticity of this specific product, not even at the European level. Only the limit values reported for cold-pressed oils by the Codex Alimentarius for free acidity and peroxides can be used as basic parameters to evaluate the qualities of HSOs. For this reason, several studies were carried out to assess the quality, composition, and characteristics of HSOs from instrumental and sensory perspectives.

Firstly, the quality parameters and the composition of 13 commercial HSOs were assessed. Considerable variability in terms of oxidative state, the content of minor compounds (e.g. carotenes,

chlorophylls and tocopherols), and volatile profile were recorded, confirming the need to establish quality parameters, purity, and authenticity of this product to better guarantee the consumer.

Next, a sensory evaluation of HSOs was carried out. In particular, following the ISO 13299:2010, a panel was trained, a specific sensory wheel for HSOs was developed, and a sensory testing sheet was established. Moreover, a focus group with 8 participants was used to investigate consumers' attitudes, toward HSOs. Several interesting aspects emerged, such as the fact that participants would like to see information about the production process and the color of HSOs on the label, because they are convinced that a high-quality hemp seed oil has to be cold-pressed, not filtered, and green. Furthermore, sensory evaluation of HSOs performed by the trained panel at home (remotely) and in the sensory laboratory was conducted. This experimentation was carried out during spring-summer 2020, i.e. the pandemic period caused by Covid-19. The aim was to evaluate the possibility of performing descriptive sensory tests at home, and for this reason, the same assessors tested the same 4 HSOs both in the sensory booth and remotely. Results, as scores given to describe the intensities of the attributes, were elaborated in terms of panel performances as well as sample evaluation. The panel showed a good level of alignment, discriminating ability and repeatability, both in the laboratory and remotely. Regarding the evaluation of samples, the main issues were in the scoring of "yellow". In fact, light (type, intensity) is a factor to which attention must be paid because if non-standardized, it may influence the evaluation of appearance.

Then, the evaluation of stability during the storage of HSOs was investigated. To this aim, one cold-pressed HSO was produced using a screw press, packaged in amber glass bottles, and stored for three months to mimic supermarket conditions (12 hours light and 12 hours dark, LED lighting with an intensity of 270 lux). The results showed that photo-oxidation, which can partially act when amber glass bottles are used, did not seem to significantly affect the quality of the oil during the first 3 months of storage.

Finally, a study about the evolution of the volatile profile of 9 HSOs under accelerated oxidation conditions (60°C for 18 days, with analyses every 3 days) (Schaal oven test) was performed to identify volatile markers of oxidation and freshness of HSOs. This experimentation was carried out at the Universidad de Navarra (Pamplona, Spain), Department of Nutrition, Food Science and Physiology (School of Pharmacy), under the supervision of Dr. Icíar Astiasarán and Dr. Diana Ansorena during a period abroad (3 months) that was financially supported by the Marco Polo program.

All the activities of this Ph.D. project were developed in the context of the Ph.D. scholarship related to the research topic "Harmonized analytical protocols of medical, herbal, food and industrial cannabis: development and validation of cannabinoids quality control methods and preparation of

derivatives from the plant raw material" ("Metodiche armonizzate di analisi della cannabis ad uso medico, fitoterapico-alimentare ed industriale: messa a punto e validazione di metodi per il controllo di qualità della materia prima vegetale e sviluppo di tecniche estrattive e preparative di derivati, secondo destinazione merceologica"), funded by Enecta S.r.l.

Sommario

Questo progetto di dottorato è stato focalizzato sulla valutazione di prodotti alimentari, fitoterapici o farmaceutici di canapa, dal punto di vista strumentale e sensoriale. A tale scopo, sono state svolte diverse attività di ricerca.

Sono stati condotti due diversi studi sui cannabinoidi, composti bioattivi e peculiari della canapa.

Il primo è stato lo sviluppo e la validazione di un metodo HPLC-UV, come soluzione analitica rapida, facile ed economica per la determinazione dei 10 principali cannabinoidi in infiorescenze di canapa. Infatti, il quadro normativo italiano ed europeo prevede la classificazione della *Cannabis sativa* L. a seconda del contenuto di Δ_9-tetraidrocannabinolo (Δ_9-THC) in "canapa da fibra" (Δ_9-THC<0,2%) o "canapa da droga" (Δ_9-THC>0,6%) e per questo motivo vi è la crescente richiesta, anche da parte di piccoli laboratori, di sviluppo di metodi rapidi, di facile utilizzo e poco costosi per la determinazione dei cannabinoidi. Il metodo, messo a punto nel contesto del dottorato, è stato applicato su infiorescenze di canapa e prevede una estrazione in metanolo/cloroformio, successiva evaporazione del solvente, raccolta dell'estratto in acetonitrile ed iniezione in HPLC. Tale determinazione consente l'identificazione e la quantificazione di 10 cannabinoidi, alla lunghezza d'onda di 220 nm, in soli 8 minuti e permette la discriminazione di campioni con una quantità di THC totale inferiore al limite dello 0,2% da quelli sottoposti alle restrizioni legali in molti Paesi dell'UE, caratterizzati da un contenuto totale di THC superiore allo 0,6%.

Il secondo studio si è concentrato sulla valutazione della potenziale attività antiossidante del cannabidiolo (CBD) in due matrici oleose, in confronto con quella dell'α-tocoferolo. La più elevata attività di *scavenging* (determinata mediante metodo DPPH$^{\bullet}$) del CBD rispetto all'α-tocoferolo non sembra essere correlata ad una maggiore stabilità ossidativa del sistema modello oleoso addizionato con CBD. Questo risultato potrebbe essere utile sia per stabilire la stabilità di soluzioni oleose contenenti CBD, largamente diffuse sul mercato, ma anche per formulare correttamente alimenti oleosi, medicinali o integratori contenenti CBD.

In merito alla canapa alimentare, le attività di ricerca si sono concentrate sugli oli di semi di canapa spremuti a freddo. Infatti, nonostante l'interesse del mercato per gli oli di semi di canapa sia aumentato, al momento non esiste una normativa univoca che dia indicazioni riguardo alla valutazione della qualità e della genuinità di questo prodotto, né a livello nazionale né europeo. Attualmente, a livello internazionale, i valori riportati dal Codex Alimentarius per l'acidità libera ed i perossidi in riferimento agli oli vegetali spremuti a freddo sono quelli che vengono generalmente presi in considerazione per la valutazione qualitativa degli oli di semi di canapa ottenuti mediante pressatura a freddo. Su queste premesse, sono stati effettuati diversi studi al fine di valutare la qualità,

la composizione e le caratteristiche degli oli di semi di canapa, sia da un punto di vista strumentale che sensoriale.

In primo luogo, sono stati valutati i parametri di qualità e la composizione di 13 oli di semi di canapa commerciali. È stata verificata una grande variabilità in termini di stato ossidativo, contenuto di composti minori (es. caroteni, clorofille e tocoferoli) e profilo in composti volatili. Tale risultato conferma la necessità di stabilire parametri di qualità, autenticità e purezza per questo prodotto che possano dare garanzie al consumatore.

Successivamente, è stata effettuata anche una descrizione sensoriale di 15 oli di semi di canapa. Sono emersi diversi spunti interessanti, per esempio gli intervistati vorrebbero che il processo produttivo ed il colore dell'olio di semi di canapa fossero riportati in etichetta, poiché rappresentano per loro dei *driver* di scelta. Infatti, sono convinti che un olio di semi di canapa di alta qualità debba essere spremuto a freddo, non filtrato e di colore verde. Inoltre, durante il periodo di pandemia causata da Covid-19, è stata effettuata una valutazione sensoriale di 4 differenti oli di semi di canapa a distanza, ovvero il panel addestrato ha assaggiato i medesimi campioni sia in sala sensoriale che a casa. L'obiettivo era valutare la possibilità di eseguire test sensoriali descrittivi da casa, per questo motivo gli stessi assaggiatori hanno testato gli stessi 4 campioni sia in cabina sensoriale che a distanza. I risultati sono stati elaborati in termini di prestazioni del panel e di descrizione dei campioni, ovvero valutando i punteggi di intensità assegnati a ciascun attributo individuato. In termini di prestazioni, il panel ha mostrato un buon livello di allineamento, capacità discriminante e ripetibilità sia in presenza che a distanza. Per quanto riguarda la valutazione dei campioni, le problematiche principali sono state riscontrate nella valutazione dell'intensità di "giallo". La luce, infatti (tipo, intensità), è un fattore a cui bisogna prestare attenzione perché se non standardizzata può influenzare l'esame visivo di questo prodotto.

Successivamente, è stata esaminata la stabilità durante lo stoccaggio dell'olio di semi di canapa. A tale scopo è stato prodotto un olio di semi di canapa spremuto a freddo utilizzando una pressa a vite, confezionato in bottiglie di vetro ambrato e conservato per tre mesi, imitando le condizioni ambientali del supermercato (12 ore di luce e 12 ore di buio, illuminazione a LED con un'intensità pari a 270 lux). I risultati hanno mostrato come la fotossidazione, che può agire parzialmente anche quando vengono utilizzate bottiglie di vetro ambrato, non sembri influenzare significativamente la qualità dell'olio di semi di canapa spremuto a freddo nei primi 3 mesi di conservazione.

Infine, è stato eseguito uno studio sull'evoluzione del profilo in composti volatili in 9 oli di semi di canapa pressati a freddo e sottoposti a test di ossidazione accelerata (60°C per 18 giorni, con analisi ogni 3 giorni) (*Schaal oven* test), al fine di identificare eventuali marcatori volatili di ossidazione e freschezza per gli oli di semi di canapa. Questa sperimentazione è stata realizzata presso l'Universidad

de Navarra (Pamplona, Spagna), Dipartimento di Nutrizione, Scienza dell'Alimentazione e Fisiologia (Scuola di Farmacia), sotto la supervisione della Dr. Iciar Astiasarán e della Dr. Diana Ansorena durante un periodo all'estero (3 mesi) sostenuto finanziariamente dal programma Marco Polo.

Tutte le attività di questo progetto di dottorato sono state sviluppate nel contesto della borsa di dottorato dedicata al tema di ricerca "Metodiche armonizzate di analisi della cannabis ad uso medico, fitoterapico-alimentare ed industriale: messa a punto e validazione di metodi per il controllo di qualità della materia prima vegetale e sviluppo di tecniche estrattive e preparative di derivati, secondo destinazione merceologica" finanziata da Enecta S.r.l.

Table of content

Chapter 1 Aim of the work

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Over the past decades the authorities of many countries have banned cultivation of all varieties of *Cannabis*, without making a distinction based on the THC content, to counteract its use as a recreational drug. However, during the last years several countries have recognized the economic and potential value of industrial hemp and have promoted its cultivation through *ad hoc* legislations. For example, the European Union established a legal threshold for THC concentrations in dry plant material of 0.2% (Cerino et al., 2021). Thus, in recent years the interest in investigating its potential uses as food or nutraceuticals has been growing (Rupasinghe, Zheljazkov, Davis, Kumar, & Murray, 2020). In particular, hemp seeds have gained popularity among consumers and it is estimated that the hemp market has around 25,000 different products belonging to several industrial sectors (e.g. textile, food, pharmaceutical, cosmetic) (Rupasinghe et al., 2020). Δ_9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD) and cannabinol (CBN) are the most studied cannabinoids. Δ9-THC and CBN, albeit to a significantly lesser extent, have psychoactive effects, while CBD is not considered psychoactive (Kladar, Čonić, Božin, & Torović, 2021). Basically, the maximum allowed concentration of cannabinoids in hemp food products is oriented towards the amount of Δ_9-THC. However, there are no uniform legislations in this regard, either globally nor in European countries. This is due to the lack of data relating to the consumption patterns of hemp-based food products and to some disagreements about the relevant instrumental methods for determination of cannabinoids in hemp-based foods (Kladar et al., 2021). Namely, cannabinoids in plant materials are present in their acidic forms, i.e. tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabinolic acid (CBNA). Heating leads to decarboxylation to their corresponding neutral forms (i.e. THC, CBD and CBN). For this reason, the analytical methods based on the use of gas chromatography techniques without previous derivatization of the sample determine the sum of the acidic and neutral forms of cannabinoids, also for Δ_9-THC, caused by the high temperature in the GC inlet (Bakro et al., 2020; Christinat, Savoy, & Mottier, 2020). Derivatization may help overcome this problem, but requires additional sample preparation (Cardenia, Gallina Toschi, Scappini, Rubino, & Rodriguez-Estrada, 2018). HPLC is a good alternative to GC because it does not require lengthy derivatization procedures and avoids decarboxylation problems (Leghissa, Hildenbrand, & Schug, 2018). The most suitable detection techniques depend on the analytes of interest. Namely, for matrices with relatively high levels of cannabinoids such as hemp plant material, UV detection can be applied. Mass spectrometry and tandem mass spectrometry (MS/MS) have higher sensitivity and specificity, which may be of interest for cannabinoid analysis of hemp derivatives or hemp-based products (Kladar et al., 2021). The growing popularity and presence of hemp-based foods on the market has underlined the need for

control in terms of health safety and improvement of the regulatory framework. Moreover, CBD oils, which can be easily found on the market, are not currently authorized by either the European Commission or the FDA (Kladar et al., 2021). In addition, regarding one of the main hemp food products, i.e. hemp seed oil, a specific regulation concerning the analytical parameters for the evaluation of the quality of hemp seed oils is still lacking (Spano et al., 2020). Hemp seed oils are sold and labelled as foods. In this context, the final product must ensure the safety standard for consumers, but relevant information, such as the plant variety, chemical characterization in terms of unsaturated fatty acids, and secondary metabolites, are not mandatory, and thus are not indicated on the labels (Spano et al., 2020). At present, there are no specific quality controls for marketed hemp seed oils. Free acidity, peroxide value, absorbance characteristics through K_{232} and K_{270} measurements, and composition, are the parameters commonly used to qualify several edible oils (e.g. virgin olive oil) as being suitable for marketing. The quality of hemp seed oil should be guaranteed and monitored by more restrictive parameters which consider its complex composition and other factors, such as the variety or methods used to obtain it (e.g. cold-pressing or solvent extraction), as well as the refining and bleaching processes (Izzo et al., 2020).

In this context, this Ph.D. project assessed food, phytotherapeutic, and pharmaceutical hemp products from an instrumental and sensory points of view.

Concerning determination of cannabinoids in hemp plant material, two different studies were performed. The first was development and *in-house validation* of a HPLC-UV method for rapid, easy, and cost-efficient determination of the 10 main cannabinoids in hemp inflorescences. The second focused on evaluation of the potential antioxidant activity of cannabidiol (CBD) in two oily matrices in comparison with the antioxidant activity of α-tocopherol. Regarding the sensory and instrumental evaluation of hemp seed oils, five experiments were carried out. In particular:

- Instrumental assessment of 13 commercial hemp seed oils;
- Sensory evaluation of 15 hemp seed oils from the market and preliminary investigation of the consumer perspective on hemp seed oil;
- Sensory evaluation of 4 commercial hemp seed oils by a trained panel, both at home (due to the Coivd-19 pandemic) and in a sensory laboratory;
- Assessment of changes of hemp seed oil during three months of storage by simulating supermarket conditions (12 hours light and 12 dark, LED light at 270 lux);
- Evaluation of changes in the volatile profile of 9 hemp seed oils during an accelerated storage test (60°C for 18 days, analyses every 3 days) to identify possible volatile oxidation and freshness markers.

All the activities of this Ph.D. project were developed in the context of a Ph.D. scholarship related to the research topic "Metodiche armonizzate di analisi della cannabis ad uso medico, fitoterapico-alimentare ed industriale: messa a punto e validazione di metodi per il controllo di qualità della materia prima vegetale e sviluppo di tecniche estrattive e preparative di derivati, secondo destinazione merceologica", which was funded by Enecta S.r.l.

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Chapter 2. Thesis structure

The dissertation reports the research activities conducted during the Ph.D. project entitled "*Instrumental and sensory analyses for the characterization of food, phytotherapeutic or pharmaceutical hemp products*" funded by Enects Srl.

Several research activities were followed for the development of the Ph.D. project. After describing the work's purpose (**Chapter 1**), a brief introduction is reported (**Chapter 3**) to give an overview that is helpful in reading the following chapters. Next, the experimentations carried out and results will be presented (**Chapters 4, 5, 6 and 7**).

In particular, two experiments were carried out relating to cannabinoids, which are the typical compounds naturally present in *Cannabis sativa* L. (**Chapter 4 and 5**). After this, the research activities were addressed to the evaluation of the composition and characteristics of hemp seed oils by instrumental and sensory approaches (**Chapter 6**) and by evaluating changes under different storage conditions (**Chapter 7**). When available, the relative publications in peer-reviewed journals are reported.

In particular:

- **Chapter 4** focuses on developing in-house validation of an HPLC-UV method to determine the 10 main cannabinoids in hemp inflorescences to explore the possibility of using a quick, economic and easy to use method for rapid quality control that small laboratories could also utilize.
- **Chapter 5** presents the tests related to evaluation of the antioxidant activity of cannabidiol (CBD) compared to the antioxidant activity of α-tocopherol, which is added in refined olive oil and sunflower oil. To do this, several analyses were performed, including peroxide value, oxidative stability index, electron spin resonance (ESR) forced oxidation and DPPH$^{\bullet}$ assays.
- **Chapter 6** reports the three main experimentations conducted during the Ph.D. project on evaluation of the characteristics of hemp seed oils from instrumental and sensory points of view. In particular, the first paragraph (**Paragraph 6.1**) focuses on the instrumental characterization of 13 hemp seed oils from the Italian market. The second (**Paragraph 6.2**) presents the sensory evaluation of hemp seed oil made by a trained panel (by following the ISO 13299:2010) and consumers (made by a focus group). Finally, the third (**Paragraph 6.3**) reports the sensory evaluation of 4 hemp seed oils by the same trained assessors in the sensory booths (laboratory condition) and at home (remote condition). In fact, due to Covid-19 pandemic period, it was difficult to conduct tastings in person, and therefore the possibility of conducting remote tasting remotely was evaluated. The results obtained in presence and remotely were compared in terms of sensory profile and the main training parameters of the judges (assessor, product and replicate effects).

- **Chapter 7** is about the evaluation of the changes in hemp seed oils under different storage conditions by an instrumental approach. This chapter is divided into three different paragraphs, which report three experiments. The first (**Paragraph 7.1**) focuses on the evaluation of the modifications which can occur during three months of storage of hemp seed oil at room temperature under controlled light conditions (i.e. 12 hours of light at 270 lux and 12 hours of dark). The second (**Paragraph 7.2**) presents the evaluation of changes in 9 different cold-pressed hemp seed oils subjected to an accelerated oxidation test (60°C for 18 days). In particular the experimentation focused on the evolution of the volatile profile of the oils, in order to study oxidation and freshness volatile markers.

Chapter 8 presents the conclusions of this Ph.D. project.

Chapter 3. Introduction

3.1 *Cannabis sativa* L.

Hemp (*Cannabis sativa* L.) is an erect and herbaceous plant with an annual cycle, dicotyledonous, belonging to the *Cannabaceae* family and native to Central Asia (Donà Dalle Rose, 1938; Bonini et al., 2018). The sturdy and fibrous stem has a more pronounced medullary cavity in the median part of the plant. High-density crops have a more significant longitudinal development, while in lower density crops they can easily branch from the base. The very variable height can even reach 5-6 m. The leaves are opposite, palmate, with 5-7-9 lanceolate and serrated divisions. Since the plant is generally a dioecious species, the flowers differ in male and female plants that are only occasionally monoecious (i.e. male and female flowers occur on the same plant) (Donà Dalle Rose, 1938; Chandra et al., 2017; Bonini et al., 2018). The male flowers consist of cluster inflorescences that develop on the axillary twigs; the female ones, less numerous, are grouped in pairs at the axil of the upper leaves. Anemophilous pollination has a considerable range of action, reaching 500 m (Donà Dalle Rose, 1938). The cultivation of Cannabis was originated in China as a fiber crop. Its cultivation later spread worldwide due to its multifunctional uses, such as the production of biomaterials (textile, paper, building, and insulation materials), food use (oil and seeds), cosmetics, and personal care products in the pharmaceutical industry (Salentijn et al., 2015). Moreover, several new applications have been explored for Cannabis in more recent years, which involve bioethanol production starting from the plant biomass (Finnan & Styles, 2013; Kuglarz et al., 2016). Starting from the 1930s, hemp began to disappear from international markets for several reasons: lack of industrialization of hemp culture, difficulty in processing techniques, need for capital to invest in the sector, spread of synthetic fibers and promotion of printing on paper are among the main causes (Luginbuhl, 2001). The gradual reintroduction of hemp cultivation in Europe began in the 1990s; in 1994, the hemp planted area covered about 8,000 hectares, up to 16,000 hectares in 2004 (Karus, 2005), and more than 33,000 hectares in 2016 (Carus, 2016). Furthermore, the recent characterization of oil (Leizer et al., 2000; Oomah et al., 2002; Callaway, 2004; Kriese et al., 2004; Latif & Anwar, 2009; House et al., 2010; Citti et al., 2018; Izzo et al., 2020; Moczkowska et al., 2020; Singh et al., 2020) and protein isolates (Tang et al., 2006, 2009; Wang et al., 2008; Dapčević-Hadnađev et al., 2018; Wang & Xiong, 2019; Shen et al., 2020) obtained from hemp seeds have shown that not only the fiber, but also the seeds, have attractive commercial potential in the food (Callaway, 2004; Matthäus & Brühl, 2008; Crescente et al., 2018; Leonard et al., 2020) and cosmetics (Vogl et al., 2004; Sapino et al., 2005; Huang et al., 2020) fields; as well as in animal feed (Hessle et al., 2008; Goldberg et al., 2012; Bailoni et al., 2021; Xu et al., 2021).

3.1.1 The legislative landscape

Legislative restrictions on hemp have been reduced starting from 1997 when the European Union established the reintroduction of hemp for industrial use. EC Regulation n. 2860/2000 allowed hemp cultivation from seeds authorized by the government, with a tetrahydrocannabinol (THC) content lower than 0.03% in absolute value. In 2007, to incentivize the textile sector and the other possible utilizations of hemp, such as cosmetics and foods, EC Regulation n° 1234/2007 included in the Common Organisation of Markets not only hemp fiber and shives, but also hemp seeds.

Subsequently, the EU Regulation n. 1307/2013 introduced hemp among the crops authorized to receive incentives from the Common Agricultural Policy, provided that the seeds are of certified varieties with a THC content of less than 0.2% as required by European legislation, and following the indications of the *Common Catalog of Varieties of Agricultural Plant Species*.

The current Italian legislation regarding the cultivation of hemp refers to law no. 242 of 2 December 2016 (Provisions for promoting the cultivation and agro-industrial chain of hemp), published in the GU General Series n.204 of 30-12-2016, and entered into force on 14-01-2017. Article 2 established that the cultivation of hemp varieties registered in the *Common Catalog of Varieties of Agricultural Plant Species* is allowed without authorization. It is also established that from hemp, it is possible to obtain different derivatives, such as:

a) Foods and cosmetics;
b) Semi-finished products, such as fiber, shives, powders, wood chips, oils or fuels, for supplies to industries and craft activities in various sectors, including energy;
c) Material intended for the practice of green manure;
d) Organic material intended for bioengineering or useful products for green building;
e) Material aimed at phytoremediation for the remediation of polluted sites;
f) Crops dedicated to teaching activities as well as research by public or private institutes;
g) Crops intended for horticulture.

The same law also established the maximum content of THC, which is the only cannabinoid with a psychotropic action present in some hemp varieties, allowing for cultivation of this plant. The limit is equal to 0.2%; if the total THC content of the crop is higher than this value, but less than 0.6%, the farmer will be exempt from penalties. Subsequently, the circular of the Mipaaf n. 5059 of 22 May 2018 notified some clarifications regarding the Law n. 242 of 2016. In particular, the maximum THC content must be calculated as a weight/weight ratio.

According to EU Delegated Regulation n° 1155/2017, the THC content has to be quantitatively determined by gas chromatography, equipped with a flame ionization detector and a split/splitless injector after extraction with a suitable solvent.

The Decree of 4 November 2019 indicates the Italian limits of THC in foods, particularly considering the sum of the concentration of this compound and its acidic and non-psychoactive precursor, which is tetrahydrocannabinolic acid (THCA). Table 3.1.1.1 reports the Italian limits for THC+THCA in foodstuff. Following the Commission Recommendation (EU) n° 2016/2115, the determination of Δ9-THC in foods should be conduced by a chromatographic separation coupled with mass spectrometry detector (GC-MS or LC-MS), after an extraction (SPE) or cleaning (liquid-liquid) step. In any case, a chromatographic technique should be used that allows the separation of Δ9-THC and its precursor as well as the other cannabinoids present in the food.

Table 3.1.1.1 Maximum value of total THC according to the Italian legislation (Decree of 4 November 2019)

Food	Limit (mg/kg)
Hemp seeds (*), flour obtained from hemp seeds	2.0
Hemp seed oil	5.0
Supplements containing foods derived from hemp	2.0

* including shredded, chopped, ground ones other than flour

These limits have been set based on scientific opinion of the Istituto Superiore di Sanità (ISS). Moreover, the same decree also established the maximum limits to foods containing ingredients derived from hemp, according to the criterion reported in Regulation n° 1881/2006/EC and considering their dilution factor in the final product (Pisciottano et al., 2021).

3.1.2 Cannabis classification

The debate regarding taxonomy classification is still open (McPartland & Guy, 2017; Bonini et al., 2018). Several authors described the three species (*Cannabis sativa Linnaeus*, *Cannabis indica Lamarck* and *Cannabis ruderalis Janisch*), which basically differ in terms of the height of plants and content of psychoactive molecules (Bonini et al., 2018). On the other hand, the nomenclature does not align with the botanical-based on Linnaeus' and Lamarck's protologues. Moreover, the classification "*sativa*" and "*indica*" became almost impossible due to the breeding done in the past 40 years (Brighenti et al., 2017; McPartland, 2017). The generally recognized botanical taxonomy classification of Cannabis, made by Small & Cronquist (1976), identified two subspecies: *C. sativa*

subsp. *sativa*, and *C. sativa* subsp. *indica*, while other botanists considered them two different species (*C. sativa* and *C. indica*) (McPartland, 2017). At present, the scientific consensus is that Cannabis is monotypic (only *C. sativa* L.) (Brighenti et al., 2017; Ryu et al., 2021); however, it should be preferable to identify and classify plants of Cannabis according to their chemical fingerprint instead of "*sativa*-dominat" or "*indica*-dominant" (McPartland, 2017).

Currently, the following classification, based on the major cannabinoids content, has been proposed (Aizpurua-Olaizola et al., 2016):

- Chemotype I: drug-type, THCA/CBDA ratio >> 1;
- Chemotype II: intermediate ratio (0.5-2.0) of THCA/CBDA;
- Chemotype III: fiber-plants, low ratio of THCA/CBDA (<<1);
- Chemotype IV: plants that contain CBGA as the main cannabinoid;
- Chemotype V: fiber-plants that contain almost no cannabinoids.

Also, a classification based on the THC/CBD ratio is accepted for *C. sativa* L. (Borroto Fernandez et al., 2020):

- THC-predominant type (CBD/THC ratio 0.00–0.05);
- CBD-predominant type (CBD/THC ratio 15–25);
- intermediate phenotype (CBD/THC ratio 0.5–3).

Even if the dialogue about the classification of Cannabis is still active, it is interesting to highlight that several laboratories moved "*from cultivar to chemovar*", identifying plants by their cannabinoids and terpenoids (McPartland, 2017).

3.2 Cannabinoids

Cannabis sativa L. is characterized by a chemically complex composition (Pellati et al., 2018): more than 525 compounds have been identified (Ryu et al., 2021), including specific compounds of this plant, namely cannabinoids (Pellati et al., 2018). Cannabinoids are a unique class of terpenophenolic (meroterpenoids C_{21} and C_{22}), synthesized in glandular trichomes and more abundant in female inflorescences. Around 120 cannabinoids have been isolated and classified into 11 different classes (Δ9-THC type, Δ8-THC type, CBG type, CBC type, CBD type, CBND type, CBE type, CBL type, CBN type, CBT type and Miscellaneous-type) (Brighenti et al., 2017; Radwan et al., 2017; Pellati et al., 2018). The neutral cannabinoid is formed starting from an acidic precursor by a non-enzymatic decarboxylation, which occurs due to heating and time (Ryu et al., 2021). Generally, the cannabinoids most present in the drug-type cannabis plants are tetrahydrocannabinolic acid (Δ9-THCA) and Δ9-tetrahydrocannabinol (Δ9-THC); while in the fiber-type plants they are cannabinoic acids, such as cannabidiolic acid (CBDA) and cannabigerolic acid (CBGA), followed by their decarboxylated

forms, namely, cannabidiol (CBD) and cannabigerol (CBG). Other minor cannabinoids include cannabicromenic acid (CBCA) and cannabichromene (CBC), but also two products of the oxidative degradation of THCA and THC, namely cannabinolic acid (CBNA) and cannabinol (CBN), respectively (Brighenti et al., 2017; Pellati et al., 2018).

From a pharmaceutical point of view, CBD is one of the most interesting and valuable cannabinoids (Brighenti et al., 2017) because it is a non-psychoactive (Atalay et al., 2020) cannabinoid and has anti-oxidant, anti-inflammatory, anxiolytic, anticonvulsant, antidepressant and antipsychotic proprieties (Mechoulam et al., 2007; Brighenti et al., 2017; Atalay et al., 2020; Ryu et al., 2021). For these reasons, there are more than 1000 CBD based products on the market (Ryu et al., 2021), with an estimated value equal to USD 13.1 billion in 2028 in the United States (Cerino et al., 2021). Moreover, CBD has been used to treat epilepsy with interesting results (Tzadok et al., 2016; Lattanzi et al., 2018). In addition, several other cannabinoids, such as tetrahydrocannabivarin (THCV) and CBC showed biological effects (Izzo et al., 2012; McPartland et al., 2015; Cardenia et al., 2018).

For these reasons, there is a growing interest and the need to develop analytical methods for determining cannabinoids, considering the differences in cannabinoid composition, their biological properties and commercial value (Cardenia et al., 2018).

3.2.1 Determination of cannabinoids

One of the most commonly applied techniques for the quantitative determination of cannabinoids is gas chromatography (GC) (Lazarjani et al., 2020). Usually, the GC analysis of cannabinoids involves low polarity stationary phases (mainly 5% diphenyl, 95% dimethyl polysiloxane). Moreover, the cannabinoids elute at temperatures <300°C in less than 20 minutes (Leghissa et al., 2018). It is essential to highlight that due to the high temperature required by GC, the acid forms of cannabinoids can undergo decarboxylation during the analysis (Citti et al., 2018; Baranauskaite et al., 2020; Lazarjani et al., 2020). For this reason, it is necessary a derivatization step before GC analysis in order to determine both the acid and neutral forms (Leghissa et al., 2018). In order to avoid the decarboxylation in GC, several derivatization methods have been performed in latest years (Fodor & Molnár-Perl, 2017; Cardenia et al., 2018; Micalizzi et al., 2021). Moreover, the derivatization increases the volatilization of cannabinoids, determining a better peak shape (Lazarjani et al., 2020). Various methods are reported in the literature regarding the determination of cannabinoids by GC, using different detectors (Ciolino et al., 2018; Cardenia et al., 2018; Fiorini et al., 2019; Franchina et al., 2020); Micalizzi et al., 2021). In particular, mass spectrometry (MS) and flame ionization detectors (FID) are employed (Lazarjani et al., 2020).

In addition, high-performance liquid chromatography (HPLC) is often used for determination of cannabinoids. In this case, the column is usually a C18 stationary phase (Citti et al., 2018; Leghissa et al., 2018). The mobile phase, generally methanol or water, is added with formic acid to improve the peak shape and resolution (Citti et al., 2016). In particular, the use of HPLC coupled with a diode array detector (DAD) or ultraviolet detector (UV) has been widely applied (De Backer et al., 2009; Citti et al., 2016; Leghissa et al., 2018, Mandrioli et al., 2019; Nahar et al., 2020) because they represent an alternative to the use of GC, also permitting the determination of the acid forms of cannabinoids without the need for derivatization (Lazarjani et al., 2020). The determination by using HPLC-UV or HPLC-DAD is are cost-effective compared to the use of GC (Mandrioli et al., 2019), but these techniques lack specificity and sensitivity, and thus the use of HPLC-MS is preferred (Lazarjani et al., 2020).

3.3 Main uses of hemp

First, it is necessary to highlight that hemp cultivation presents many benefits in weed control, pest and disease resistance, pesticide elimination without disadvantages, and soil improvement through crop rotation (Ranalli & Venturi, 2004). Hemp is an attractive industrial crop, which presents many industrial opportunities (e.g. production of paper for cigarettes, books or tea bags). Starting from hemp, it is possible to obtain many derivatives such as fabrics, paper, plastic paints, fuels, edible oil, etc., giving the possibility of having perfect substitutes for what is used today with lower consumption of energy and production of pollutants (Pergamo et al., 2018). Moreover, hemp seeds have many nutritional advantages as food; they are rich in fiber, essential amino acids and polyunsaturated fatty acids (in particular ω3 and ω6), and carbohydrates, vitamins, and minerals (Leizer et al., 2000).

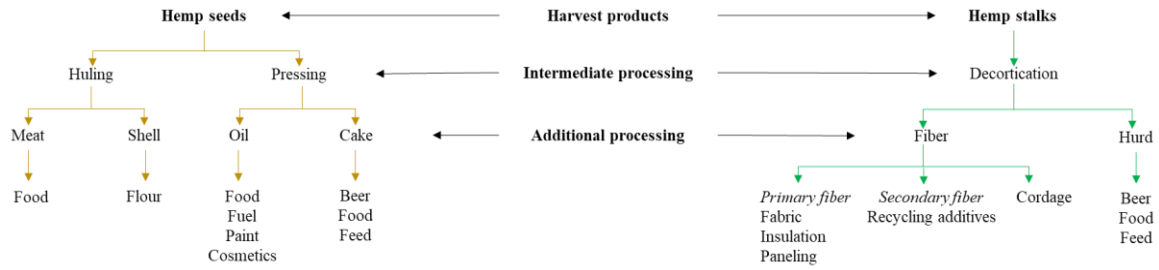

Figure 3.1.6.1 Potential industrial uses of hemp (Fike, 2016).

For animal food, it is possible to use hemp seeds, even if considering the value of the hemp seed oil (see paragraph 3.4.2), animal feeding with hemp is usually limited to the cake, which is a by-product produced during oil extrusion (Small & Marcus, 2002; Gibb et al., 2005; Fike, 2016). The protein component of hemp seeds is the focus numerous research. It has been observed that hemp flour

(characterized by a nutty flavor), as well as soy flour, contains functional proteins. Its use in feeding chickens has confirmed that hemp seed is an excellent source of nutrition for laying hens, which is capable of positively influencing the characteristics of the egg (Silversides & Lefrancois, 2005; Gakhar et al., 2012; Goldberg et al., 2012).

Furthermore, hemp seed oil is applicable in the cosmetic industry because it is considered a valuable source for 'green' cosmetics due to its natural emollient and moisturizing properties. In fact, it is used as an ingredient in the production of body care products, such as shampoo, creams, and soap (Crini et al., 2020). Regarding the main applications of hemp fiber, hemp was once generally used in textiles, fabrics, and furniture production because hemp fibers are more robust than other natural fibers (e.g. cotton and flax). Hemp fabrics have also advantages such as strength, durability, hypoallergenicity, and biodegradability (Crini et al., 2020). Fiber also represents a sustainable alternative in building constructions, given the characteristics of this material such as durability, lightness, impermeability, transpiration, resistance to humidity and parasites, and is able to retain heat in winter as well as coolness in summer and resist earthquakes (Shahzad, 2012; Crini et al., 2020). Finally, hemp may also be used for animal bedding due to its high absorbency (Fike, 2016).

3.3.1 Food uses of hemp

After the legalization of the cultivation of hemp, the food sector of this product is also growing; in recent decades, hemp-based food products have gradually spread (Carus & Sarmento, 2016), mainly due to their nutraceutical properties (Citti et al., 2019).

The most widely used part of this plant for food production is seeds (Mikulcovà et al., 2017). The protein component of the seed is about 20%, while approximately 75% is made up of essential fatty acids (Leizer et al., 2000) (see paragraph 3.4 on hemp seeds for more information). Hemp seed oil is the principal food derived from hemp, which is useful for cooking and provides nutritional benefits (Rupasinghe et al., 2020) (hemp seed oil will be addressed in paragraph 3.4.2).

Hemp seed flour, obtained from oil production waste, can be used to produce gluten-free baked goods such as bread, sweets, and biscuits. The bread obtained from this type of flour is characterized by a high protein content that varies between 13.38 and 19.29 g/100 g, compared with the bread obtained from white flour, which contains about 11.02 g/100 g (Mikulec et al., 2019).

Mikulec et al., 2019 have also shown that the addition of hemp flour contributes to an increase in polyphenols from 256.43 to 673.59 mg GAE/kg (Gallic Acid Equivalents).

In addition, hemp seeds have been used as a source of vegetable protein and dietary fiber, and can be incorporated into food products such as energy bars, flavored yoghurt, baked goods, etc. (Rupasinghe et al., 2020).

A problem related to hemp-based foods, particularly hemp seed oil, is the susceptibility to oxidation due to the high presence of PUFAs (Johnson, 1999; Gao & Birch, 2016). Hemp seed oils have a much shorter shelf-life than other cold-pressed oils, which have a lower degree of unsaturation (Johnson, 1999). Therefore, the use and enhancement of hemp seed oil are more complex also because many consumers do not like the typical sensory characteristics of this product (Johnson, 1999).
A further difficulty is due to the reduced availability of hemp varieties with high seed production on the market. In fact, in recent decades, breeding has been carried out exclusively to increase the production of fiber without considering the possible use of other parts of the plant (Lachenmeier & Walch, 2005).

3.4 Hemp seeds

Hemp seeds have been used in various fields (Carvalho et al., 2006; House et al., 2010), and have been considered as food products for many centuries (Montserrat-de la Paz et al., 2014). In fact, their role as an edible resource is well documented since ancient times, when it was consumed raw, cooked, or roasted, and hemp seed oil was used as a food and in medicine (De Padua et al., 1999); for example, it has been used in China for food and healing purposes for at least 3000 years (Montserrat-de la Paz et al., 2014). Currently, the consumption of raw or lightly toasted seeds is recommended in order to not reduce the high nutritional value of polyunsaturated fatty acids (PUFAs), which are altered by high temperatures due to the deterioration of the unsaturated bonds of fatty acids (Carvalho et al., 2006; House et al., 2010). Monoecious varieties are preferred as they are characterized by a higher yield and a lower vegetative development, making them easier to manage when harvested. In fact, dioecious species produce the seed only on female plants, grow immeasurably in height, and show a considerable delay compared to cultivars specialized in seed production or with dual aptitude (seed and fiber). It should be remembered that in some experiments aimed exclusively at the production of seeds for food use, very early dioecious varieties were used, which, reaching a reduced height at harvesting, also made it easier to chop crop residues. In other experiments, the topping technique has allowed increasing the branches to increase the inflorescences and lower the final size of the crops. Depending on the earliness of the variety, the harvest can be carried out from the end of July to mid-September. During the operation, the combine harvesters must be adjusted to limit seed losses and not damage the seminal integuments (Amaducci et al., 2019). Once harvested, the seed must be immediately dried at low temperatures (below 38°C), carefully heating the whole mass. Depending on the plant used and the initial humidity of the seeds, a drying interval ranging from 12 to 96 hours is needed. Drying can also take place at room temperature, but strictly in environments protected from light and ventilated to reduce the rancidity of the seeds and the spread of harmful microorganisms

(Amaducci et al., 2019). The moisture content of seeds can vary from one variety to another (Callawy, 2004; Da Porto et al., 2012a), but the important thing is that it remains below 10% to prevent germination of seeds (Deferne & Pate, 1996). Finally, it is possible to sift and blow the seeds to eliminate inert material and broken kernels (Amaducci et al., 2019).

The hemp seed is an achene. It is a small single-seeded fruit with a hard shell (Bender, 2006; Garcia, 2017) (Figure 3.4.1).

Figure 3.4.1 Hemp seed variety Futura 75.

Hemp seeds consist of 20-25% protein, 20-30% carbohydrates, 10-15% insoluble fiber and the remaining 25-35% oil (Leizer et al., 2000; Matthaus & Bruhl, 2008; Montserrat-de la Paz et al., 2014). Following removal of the shell, however, the edible portion of the seeds contains, on average, 46.7% oil and 35.9% protein (Wang & Xiong, 2019). Furthermore, they contain many minerals, notably phosphorus, potassium, magnesium, sulfur, calcium and even modest amounts of iron and zinc (Leizer et al., 2000; Matthaus & Bruhl, 2008; Montserrat-de la Paz et al., 2014). Moreover, hemp seed is rich in vitamin E (Rodriguez-Leyva & Pierce, 2010). The two main proteins found in hemp seed are edestin and albumin. Both are easily digestible proteins with a significant essential amino acid content, and therefore with high nutritional value. In addition, hemp seeds have exceptionally high levels of the amino acid arginine (Callaway, 2004). The amount of oil present in seeds is between 28 and 35 g/100 g and varies according to the variety, year of cultivation, climatic conditions, and place of cultivation. An extraction yield of oil ranging from 60 to 80% is obtained with cold pressing (Matthaus & Bruhl, 2008). Therefore, seeds are considered an important source of protein of high biological value since they contain all the essential amino acids, PUFA, and fiber (Leizer et al., 2000). The lipid component consists mainly of linoleic acid (LA) and linolenic acid (ALA), both of which are polyunsaturated essential fatty acids (EFAs), respectively omega-6 and omega-3 (Oomah et al., 2002; Carvalho et al. al., 2006), which are typically present in a nutritionally favorable 3: 1 ratio (Deferne & Pate, 1996; Callaway, 2004; Vonapartis et al., 2015; Mikulcovà et al., 2017). These fatty

acids, called EFA (Essential Fatty Acids), perform several essential functions, including regulation of cholesterol. Furthermore, by representing an indispensable element in the structure of biological membranes, they are considered fundamental for the human body (Cappelli & Vannucchi, 2005; Karimi & Hayatghaibi, 2006; Prociuk et al., 2008). Among the monounsaturated fatty acids, the main are palmitic acid (C16: 0) and stearic (C18: 0), while among the monounsaturated fatty acids, oleic acid (C18: 1) (Da Porto et al., 2012a) is notable. Hemp seeds also contain (to a lesser extent) their respective biological metabolites: gamma-linolenic acid (GLA) and stearidonic acid (SDA) (Callaway, 2004; Farinon et al., 2020). Fiber, consisting mainly of cellulose, hemicellulose, and lignin, is found primarily on the pericarp of the seed and affects the digestibility of proteins (Vonapartis et al., 2015). Ripe hemp seeds also contain chlorophyll, which gives hemp seed oil its natural dark green color, but which accelerates self-oxidation of the oil when exposed to light (Callaway, 2004; Teh & Birch, 2013). Cannabidiol (CBD) was also found in the oil, which generally should not be present, but is considered a contaminant of inadequately washed seeds, which therefore have flower or resin residues (Deferne & Pate, 1996; Leizer et al., 2000). Finally, seeds contain anti-nutritional factors such as tannins, phytic acid and trypsin inhibitors, which can reduce the availability of proteins by precipitation or inhibition of digestive enzymes, or limit the absorption of vitamins and minerals by chelation, but are found in low concentrations (Russo & Reggiano, 2013; Mattila et al., 2018).

3.4.1 The hemp seed market

In recent years, the agricultural market has pushed producers to search for alternative crops that offer more solid development prospects (Hart, 2020). This is accompanied by the significant growth of organic products in Europe, which requires foods and products based on renewable resources grown organically for non-food uses, thus expanding research in the hemp field (Vogl et al., 2004). Thanks to the possibility of cultivating several industrial hemp varieties, its application has been relaunched in recent years (Moscariello et al., 2021). Hemp, as a very versatile natural resource, can satisfy part of this demand. The return to this crop is mainly due to its wide range of applications (Vogl et al., 2004) and to the lower impact of its cultivation on the environment, in line with the new production objectives aimed at the sustainability of agricultural systems (Ranalli, 2020). Traditionally used as a fiber plant, it can be exploited to manufacture paper, oil, panels, and hemp composites for green building, and for the manufacture of bioplastics and therapeutic applications (Ranalli, 2020). In particular, there is great commercial value for seeds (Bouloc, 2013; Moscariello et al., 2021) and the secondary metabolites of hemp used in the pharmaceutical and cosmetic industries (Moscariello et al., 2021). Worldwide, the leading countries for hemp production are Europe, China, South Korea,

and Russia. According to FAO data, the hemp market in Europe is the largest globally, being remarkably active, with good production in most member countries, such as France, the Netherlands, Lithuania, and Romania (Johnson, 2018). Data reported by FAO in 2019 showed that the global hemp production between 1961 and 2019 has undergone many variations. In particular, the global production of hemp seed has increased in the last 60 years, with hemp seed production of more than 100,000 tons, while the harvested area decreased, thus indicating the improvement of agronomic practices (Moscariello et al., 2021). This is due to the rapid increase of the hemp seed market every year, as is the demand for hemp products (Alonso-Esteban et al., 2020).

3.4.2 Hemp seed oil

3.4.2.1 Hemp seed oil production processes

Vegetable oils can be extracted from the matrix through the use of mechanical, chemical systems, or a combination of them (Çakaloğluet al., 2018); the yield of extraction, the quality and characteristics of the oil vary according to the different extraction conditions (Devi & Khanam, 2019a). If the hemp seed oil is obtained by mechanical systems, the residue of the extraction is defined as "*cake*" (Pojić et al., 2015). It has been shown that the use of enzymes such as cellulase, α-amylase, and pectinase during pre-treatment can increase the final extraction yield and, in addition, hemp seed oil pre-treated with enzymes has a higher content in tocopherols and better oxidative stability compared to the untreated oil (Latif & Anwar, 2009). On the other hand, extraction using solvents is a procedure that is a high-yield method, and, for this reason, it is often used for raw materials with an oil content of less than 20%. However, it has some disadvantages. In particular, residual solvents can contaminate the final product (Pavlovic et al., 2018), and it is often necessary to apply heat, which can compromise the nutritional characteristics of the final product (Çakaloğluet al., 2018). A valid alternative to the extraction of vegetable oils employing organic solvents is using supercritical fluids (Da Porto et al., 2012b). Supercritical fluids are characterized by high mass transport properties and a density comparable to the liquid state and variable selectivity; by modifying the temperature and pressure of the system, it is possible to alter the selectivity of the supercritical fluid, being able to extract predetermined compounds at specific concentrations (Aladic et al., 2015). The use of supercritical CO_2 is due to the peculiar properties of this compound: it is fireproof and easy to remove (Wejnerowska & Ciaciuch, 2018), and free from toxicity and relatively inert (Da Porto et al., 2012). Also, due to the low critical temperature of CO_2, of about 31.1°C, the extraction processes take place at temperatures close to environmental ones, minimizing the need to apply heat which could lead to thermal degradation of bioactive compounds (Da Porto et al., 2012b). A problem is related to the poor polarity of CO_2, which can be overcome by using a polar co-solvent; one of the most used co-solvents

is ethanol, as it has low toxicity to humans (Wejnerowska & Ciaciuch, 2018). Grijó et al. (2019) have shown that the use of pressurized *n*-propane as an extraction solvent can reduce the costs associated with the need to operate at high pressures. Moreover, hemp seed oil obtained was characterized by a higher concentration in antioxidant compounds than that obtained by supercritical CO_2 (Grijó et al., 2019). However, cold-pressing is now employed to produce specialty oils, such as hemp seed oil (Faugno et al., 2019). The primary method of extracting hemp seed oil is cold-pressing (Figure 3.4.2.1.1); this process allows the extraction of a large number of minor components such as tocopherols, polyphenols and phytosterols, which represent the unsaponifiable fraction of the product (approximately 1.5-2% of the total) (Liang et al., 2015).

Figure 3.4.2.1.1 Extraction of hemp seed oil by cold-pressing.

The definition of "cold-pressed oil" is not unique. For example, in the United Kingdom, oil is defined as "*cold-pressed*" if obtained by maintaining the temperature below 50°C (Zheng et al., 2003). In Italy, however, the definition of "*cold-pressed oil*" refers to that given by the Codex Alimentarius, according to which there are no maximum temperature limits at the exit from the press, but the application of heat during extraction is prohibited (Codex Stan 210-1999). It is necessary to follow specific provisions to produce cold-pressed seed oils: the seeds must be clean, homogeneous and free of contamination, and the temperature and relative humidity must be controlled (Jian et al., 2019, Güneşeret al., 2017). Moreover, the extraction must take place without the application of heat, solvents, or other chemical compounds (Güneşeret al., 2017). The cold pressing method does not guarantee the extraction of all the oil contained in the matrix (Faugno et al., 2019), but a correct calibration of the pressing parameters, as well as the application of some pre-treatment techniques (for example, skinning, grinding and drying), can, however, increase the final yield (Zheng et al., 2003).

3.4.2.1 Hemp seed oil composition

Hemp seed oil, like other vegetable oils, is almost entirely made up of triglycerides and partial glycerides of fatty acids (Leizer et al., 2000), and is characterized by a high concentration of essential polyunsaturated fatty acids (PUFAs), about 76-80% of the total (Matthäus & Brühl, 2008; Montserrat-de la Paz et al., 2014). In particular, α-linoleic acid (ALA) and linolenic acid (LA) constitute about 80% of the total fatty acids (Matthäus & Brühl, 2008). The concentration of LA varies from 52% to 62%, and a concentration of ALA between 12% and 23% of the total fatty acids (Leizer et al., 2000; Da Porto et al., 2012b; Porto et al., 2015; Alonso-Esteban et al., 2020). In fact, hemp seed oil presents a unique ratio of ω6:ω3 fatty acids, equal to 3:1 (Calzolari et al., 2021). The percentage content of monounsaturated fatty acids (MUFAs), of the total fatty acids, corresponds to values between 7% and 16% (Matthaus & Bruhl, 2008; Montserrat-de la Paz et al., 2014), while the saturated fatty acids (SAs) are between 9% and 11% (Sapino et al., 2005). Among the SAs, the highest concentrations are palmitic acid and stearic acid (Matthaus & Bruhl, 2008). The palmitic acid content is approximately 5% of the total fatty acids (Da Porto et al., 2012b). Another interesting aspect is that hemp seed oil contains from 1% to 4% of γ-linolenic acid (GLA, 18:3 n-6) and between 0.5% to 2% of stearidonic acid (18:4 n-3) (Mikulcová et al., 2017; Calzolari et al., 2021).

The free fatty acids present in the oil act as pro-oxidants that can accelerate the oxidative process (Frega et al., 1999; Liang et al., 2015). Therefore, they are usually taken as a reference in establishing the quality of the oil, resulting in alterations in taste, odor, and other properties (Liang et al., 2015).

Hemp seed oil mainly contains free fatty acids such as α-linolenic acid and ɣ-linolenic acid, which in the free form are particularly susceptible to oxidation, especially when exposed to high temperatures, light, and oxygen (Liang et al., 2015). On the other hand, ω-3 are related to lowering blood pressure and serum cholesterol, cause decrease in insulin dependence in diabetic subjects, normalise fat metabolism, increase the metabolic rate and membrane fluidity, and have anti-inflammatory properties (Leizer et al., 2000). The essential role of LA and ALA in the human diet is linked to both intermediate and final products of various biochemical pathways (Leizer et al., 2000). LA and ALA are introduced with the diet, and starting from them, some essential PUFAs are produced thanks to enzymes such as elongase and desaturase (Rodriguez-Leyva & Pierce, 2010). The enzyme Δ-6-desaturase is responsible for the biosynthesis of the ω6 and ω3 fatty acid families starting from LA and ALA, respectively (Rodriguez-Leyva & Pierce, 2010). LA is metabolized into GLA and, subsequently, into arachidonic acid. On the other hand, ALA is metabolized into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The body metabolizes EPA and arachidonic acid into eicosanoids. The prostaglandins, which influence different functions, such as blood coagulation,

inflammation response, and immunoregulation, are metabolised from those compounds (Leizer et al., 2000).

The fatty acid profile is a fundamental criterion for evaluating hemp seed oil, as they determine the product's suitability for specific uses, explain its tendency to oxidize, and, therefore, define the shelf-life (Matthäus & Brühl, 2008). Thus, presenting a high PUFA content, it can be used in different industrial sectors. In particular, it is used as a condiment (food). However, the low smoke point (165°C) and the low stability make it unsuitable for frying (Leizer et al., 2000). In addition, it can also be used for the production of "*drying oils*", which are applied in the production of paints, varnishes, sealants, bio-plastics (Callaway & Pate, 2009), printer ink, and wood preservatives (Oomah et al., 2002). In addition to this, hemp seed oil is used in body care products, particularly soaps and shampoos (Callaway & Pate, 2008).

The nutritional value and health benefits of hemp seed oil are attributable to the high content of PUFAs and minor components, such as tocopherols and polyphenols, which exhibit significant antioxidant properties (Liang et al., 2015). On the other hand, some of the minor components naturally extracted and present in hemp seed oil, such as free fatty acids and chlorophyll, are undesirable and can alter the product, causing changes in color, nutritional values, flavor, and oxidative stability of the oil (Liang et al., 2015).

Tocopherols, which are minor components of a fat-soluble nature, appear to be naturally present in hemp seed oil (Matthaus & Bruhl, 2008). In nature, there are eight substances grouped under the term of vitamin E: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol (Sen et al., 2006). Four isomers of tocopherols can be found in hemp seed oil: α, β, γ and δ-tocopherol, and their content decreases during storage due to oxidative deterioration. Due to their ability to bind free radicals, they are known as important natural antioxidants. Tocopherols are potent antioxidants and reduce cardiovascular, neoplastic, and age-related macular degeneration risk (Matthaus & Bruhl, 2008). Among tocopherols, ɣ-tocopherol is the one most present in hemp seed oil. It constitutes about 85-91% of total tocopherols (Montserrat-de la Paz et al., 2014; Liang et al., 2015). The average tocopherol content in hemp seed oil varies between 80 and 150 mg/100g of oil (Sapino et al., 2005; Matthaus & Bruhl, 2008; Liang et al., 2015). In fact, the tocopherol content varies according to the cultivar used, conditions and agronomic techniques of cultivation, and processing and conservation methods (Liang et al., 2015).

Another class of compounds detected in the unsaponifiable fraction of hemp seed oil is represented by polyphenols, hydrophilic antioxidants present in most vegetable oils, which significantly impact the stability and nutritional and sensory characteristics of this product. Due to the significant amount of PUFAs, which are highly susceptible to oxidation, the presence of polyphenols is helpful to slow

down the oxidative deterioration processes of lipids (Liang et al., 2015). Their content in hemp seed oil varies between 44 and 188 mg/100 g of gallic acid equivalent (Liang et al., 2015).

Moreover, phytosterols are found in hemp seed oil. They represent an important class of compounds as they have been shown to reduce levels of LDL cholesterol in the blood (Matthaus & Bruhl, 2008). Some studies have shown that the concentration of phytosterols in hemp seed oil is between 2000 and 3000 mg/kg of oil (Montserrat-de la Paz et al., 2014; Leonard et al., 2020), and about 70% of the total phytosterols consists of β-sitosterols. Other quantitatively relevant phytosterols are campesterol, 5-avenasterol, and stigmasterol (Matthaus & Bruhl, 2008; Siano et al., 2019).

Chlorophylls are fat-soluble pigments present in many oils of vegetable origin; together with their derivatives, such as phaeophytes and pyrophytophytins, they constitute a powerful pro-oxidant that can negatively affect the quality of the oil and its shelf-life (Liang et al., 2015). The presence of these pigments in hemp seed oil contributes to numerous adverse effects; since chlorophylls are extremely sensitive to photo-oxidation and color changes, and their presence can increase the instability of the PUFAs present in the oil, thus accelerating the rancidity process (Liang et al., 2015). The main isomers in hemp seed oil are chlorophyll-a and chlorophyll-b; both have a maximum absorbance between 640 and 670 nm. Therefore, the quantity of these isomers can be quickly determined by spectrophotometric analysis (Liang et al., 2015). The intense green color typical of hemp seed oil is mainly due to the high total chlorophyll content. Liang et al. (2015) found an amount of chlorophylls equal to 98.6 μg/g in hemp seed oil, and in particular 59.22 μg/g of chlorophyll-a and 39.4 μg/g of chlorophyll-b. Liang et al. (2018) reported a total content of chlorophylls equal to 53.6 μg/g, while Izzo et al. (2020) detected a value ranged from 0.41 to 2.64 μg/g. Furthermore, carotenes are also present in hemp seed oil, and their content is between 4.3 and 66.6 μg/g depending on the methods used for pressing (Devi & Khanam, 2019b).

Several studies have shown that the use of new technologies can increase the efficiency of oil extraction methods and oxidative stability of the product. For example, Liang et al. (2018) reported that through bleaching treatment using ultrasound, a large amount of chlorophyll can be removed from cold-pressed hemp seed oil, obtaining a lighter oil with a less intense color; this treatment also reduces the formation of primary oxidation products, thus extending the shelf-life of the product. Rezvankhah et al. (2019) highlighted that microwave-assisted extraction of hemp seed oil gave rise to higher oxidation stability compared to conventional extraction.

Moreover, cannabinoids are found in hemp seed oil, even if hemp seeds do not contain cannabinoids. In fact, their presence is due to the contact of hemp seed with the resin located on flowers, leaves, or bracts, and are considered as “impurities” or “contaminants” of hemp seed oil (Citti et al., 2018). In particular, the presence of CBDA and CBD is interesting because the ratio between those two

cannabinoids is considered a valuable indicator for monitoring hemp seed oil storage (Citti et al., 2018; Singh et al., 2020). Moreover, among the contaminants of hemp seed oil, it is possible to find terpenes, which are not contained in the seeds of the cannabis plant but in the glandular structures (Leizer et al., 2000). The presence of β-caryophyllene with anti-inflammatory and cytoprotective action and myrcene with antioxidant action gives additional beneficial value to the oil (Leizer et al., 2000).

Regarding the evaluation of the quality and genuineness of hemp seed oil, there is not yet a Regulation nor standard methods (Rapa et al., 2019). The Codex Alimentarius reports several qualitative parameters for cold-pressed vegetable oils. In particular, it reports (Codex Stan 210-1999):

- a maximum value for peroxides equal to 15 $mEqO_2$/kg of oil;
- a maximum value for free acidity equal to 4 mgKOH/g of oil.

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Chapter 4

Development and in-house validation of an RP-HPLC-UV method for the determination of the main cannabinoids in Cannabis sativa L

4.1 Details of the publication based on Chapter 4

Title: Fast Detection of 10 Cannabinoids by RP-HPLC-UV Method in *Cannabis sativa* L.

Authors: Mara Mandrioli[a], Matilde Tura[a], Stefano Scotti[b], Tullia Gallina Toschi[a]

[a] Department of Agricultural and Food Sciences, Alma Mater Studiorum-University of Bologna, Viale Fanin 40, 40127 Bologna, Italy
[b] Shimadzu Italia, Via G. B. Cassinis 7, 20139 Milano, Italy

Corresponding author: Tullia Gallina Toschi -tullia.gallinatoschi@unibo.it

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Abstract

Cannabis has regained much attention as a result of updated legislation authorizing many different uses and can be classified on the basis of the content of tetrahydrocannabinol (THC), a psychotropic substance for which there are legal limitations in many countries. For this purpose, accurate qualitative and quantitative determination is essential. The relationship between THC and cannabidiol (CBD) is also significant as the latter substance is endowed with many specific and non-psychoactive proprieties. For these reasons, it becomes increasingly important and urgent to utilize fast, easy, validated, and harmonized procedures for determination of cannabinoids. The procedure described herein allows rapid determination of 10 cannabinoids from the inflorescences of *Cannabis sativa* L. by extraction with organic solvents. Separation and subsequent detection are by RP-HPLC-UV. Quantification is performed by an external standard method through the construction of calibration curves using pure standard chromatographic reference compounds. The main cannabinoids dosed (g/100 g) in actual samples were cannabidiolic acid (CBDA), CBD, and Δ9-THC (Sample L11 CBDA 0.88 ± 0.04, CBD 0.48 ± 0.02, Δ9-THC 0.06 ± 0.00; Sample L5 CBDA 0.93 ± 0.06, CBD 0.45 ± 0.03, Δ9-THC 0.06 ± 0.00). The present validated RP-HPLC-UV method allows determination of the main cannabinoids in *Cannabis sativa* L. inflorescences and appropriate legal classification as hemp or drug-type.

Keywords: cannabinoids; *Cannabis sativa* L.; HPLC; validation

4.2 Introduction

Cannabis is classified into the family of Cannabaceae and initially encompassed three main species: *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis* (Montserrat-de la Paz et al., 2014). Nowadays, *Cannabis* has only one species due to continuous crossbreeding of the three species to generate hybrids. In fact, all plants are categorized as belonging to *Cannabis sativa* and classified into chemotypes based on the concentration of the main cannabinoids. Depending on the THCA/CBDA ratio, some chemotypes have been distinguished. In particular, chemotype I or "drug-plants" have a TCHA/CBDA ratio >1.0, plants that exhibit an intermediate ratio are classified as chemotype II, chemotype III or "fiber-plants" have a THCA/CBDA ratio <1.0, plants that contain cannabigerolic acid (CBGA) as the main cannabinoid are classified as chemotype IV, and plants that contain almost no cannabinoids are classified as chemotype V (Appendino et al., 2011; Andre et al., 2016; Aizpurua-Olaizola et al., 2016; Brighenti et al., 2017). Recently, in Italy the interest in *Cannabis sativa* L. has increased mainly due to the latest legislation (Legge n. 242 del 2 dicembre 2016). As a consequence, there is a request to develop cost-effective and easy-to-use quantitative and qualitative methods for analysis of cannabinoids. The Italian regulatory framework has classified two types of *Cannabis sativa* L. depending on the content of Δ9-THC. In particular, fiber-type plants of *Cannabis sativa* L., also called "hemp", are characterized by a low content of Δ9-THC (<0.2% w/w). If the content of Δ9-THC is >0.6% w/w, it is considered as drug-type, also called "therapeutic" or "marijuana". Industrial hemp is used in several sectors, such as in the pharmaceutical, cosmetic, food, and textile industries, as well as in energy production and building. In general, fiber-type plants are less used in the pharmaceutical field, where drug-type plants are more often employed (Brighenti et al., 2017). However, there is also an increased interest in hemp varieties containing non-psychoactive compounds. In fact, the European Union has approved 69 varieties of *Cannabis sativa* L. for commercial use (European Union Common Catalogue of Varieties of Agricultural Plant Species, Plant Variety Database). Hemp has a complex chemical composition that includes terpenoids, sugars, alkaloids, stilbenoids, quinones, and the characteristic compounds of this plant, namely cannabinoids. *Cannabis sativa* L. has several chemotypes, each of which is characterized by a different qualitative and quantitative chemical profile (Brighenti et al., 2017). The cannabinoids, terpenes, and phenolic compounds in hemp are formed through secondary metabolism (Andre et al., 2016; Pisanti et al., 2017). The term "cannabinoid" indicates terpenophenols derived from *Cannabis*. More than 90 cannabinoids are known, and some are derived from breakdown reactions (Pisanti et al., 2017). Mechoulam and Gaoni (1967) were the first to define cannabinoids "as a group of C21 compounds typical of and present in *Cannabis sativa*, their carboxylic acids, analogs, and transformation products". Currently, cannabinoids have been classified according to their chemical structure, mainly

seven types of cannabigerol (CBG); five types of cannabichromene (CBC); seven types of cannabidiol (CBD); the main psychoactive cannabinoid D9-tetrahydrocannabinol (Δ9-THC) in nine different forms including its acid precursor (Δ9-tetrahydrocannabinolic acid, Δ9-THCA); D8-tetrahydrocannabinol (Δ8-THC), which is a more stable isomer of Δ9-THC but 20% less active; three types of cannabicyclol (CBL); five different forms of cannabielsoin (CBE); seven types of Cannabinol (CBN), which is the oxidation artifact of Δ9-THC; cannabitriol (CBT); cannabivarin (CBDV); and tetrahydrocannabivarin (THCV) (Radwan et al., 2017; Leghissa et al., 2018). THC, CBD, CBG, CBN, and CBC are not biosynthesized in *Cannabis sativa*, and the plant produces the carboxylic acid forms of these cannabinoids (THCA, CBDA, CBGA, CBNA, and CBCA). Cannabinoid acids undergo a chemical decarboxylation reaction triggered by different factors, mainly temperature. This decarboxylation reaction leads to the formation of the respective neutral cannabinoids (THC, CBD, CBG, CBN, and CBC) (Citti et al., 2018a; Citti et al., 2018b). There are several methods to quantify cannabinoids (Rodrigues et al., 2018; Cardenia et al., 2018; Leghissa et al., 2018; Patel et al., 2017; Burnier et al., 2019; Ciolino et al., 2018; Fekete et al., 2018), some of which require expensive mass spectrometry detectors (Purschke et al., 2016; Pacifici et al., 2017; Casiraghi et al., 2018; Lin et al., 2018). Furthermore, there is a great deal of uncertainty around the use of gas chromatography (GC) for the titration of cannabinoids due to the high temperature of the injector and detector that can lead to the decarboxylation of cannabinoid acids if not derivatized correctly (Mudge et al., 2017). Moreover, recent studies have reported that cannabinoid acid decarboxylation is only partial, and as result the actual value is underestimated. An HPLC system allows for determination of the actual cannabinoid composition, both neutral and acid forms, without the necessity of the derivatization step (Citti et al., 2018a). It is necessary, in addition to honed methods, to develop new procedures with a view to discriminate different *Cannabis* varieties in order to identify and titrate cannabinoids in a simple way. These methods should ideally be fast, easy, robust, and cost-effcient as they can be used not only by research laboratories but also by small companies with a view on quality control. This study focuses on the development, validation, and step-by-step explanation of a rapid and simple HPLC-UV method for identification and quantification of the main cannabinoids in hemp inflorescences that can be easily reproduced and applied. The method described is focused on the quantification of CBD but can also be applied to check the levels of THC.

4.3 Materials and Methods

4.3.1 Chemicals, Standards and Apparatus

All chemicals used were of analytical grade. Methanol p.a CAS 67-56-1, chloroform p.a CAS 67-66 3, acetonitrile CAS 75-05-8, water CAS 7732-18-5, and orthophosphoric acid CAS 7664-38-2 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen, pure gas for analysis CAS 7727-37 9 was purchased from SIAD Spa (Bergamo, Italy). Standard mixture of phytocannabinoids 0.1% in acetonitrile: Cannabidiolic acid (0.01%) CAS 1244-58-2, cannabigerolic acid (0.01%) CAS 25555 57-1, cannabigerol (0.01%) CAS 25654-31-3, cannabidiol (0.01%) CAS 13956-29-1, tetrahydrocannabivarin (0.01%) CAS 31262-37-0, cannabinol (0.01%) CAS 521-35-7, tetrahydrocannabinolic acid (0.01%) CAS 23978-85-0, Δ-9-tetrahydrocannabinol (0.01%) CAS 1972-08-3, Δ-8-tetrahydrocannabinol (0.01%) CAS 5957-75-5, cannabichromene (0.01%) CAS Number 20675-51-8, were purchased from Cayman Chemical Company, (Ann Arbor, MI, USA). Cannabidiol 1.0 mg/mL in methanol CAS 13956-29-1: LGC Standards S.r.l., (Milan, Italy). Analytical mill, IKA A11 Basic (IKA® Werke GMBH & Co. KG, Germany). Analytical balance with precision of 0.1 mg, mod. E42, (Gibertini, Italy). Vortex vibrating shaker, mod. ST5, (Janke & Kunkel, Germania). Centrifuge mod. ALC, PK 120 (Thermo Electron Corporation, Massachusetts, USA). Termoblock heating block, mod. A120, (Falc, Italy). Natural ventilation stove. Sieve with 1 mm meshes. Tilting shaker. Ultrasound bath Branson 2150, (Danbury-CT, USA). Volumetric flasks of 1, 2, 10 and 25 mL. SOVIREL-type tubes with screw cap. Glass syringes with luer lock attachment, 0.45 µm nylon membrane filters. Microsyringes from 1 to 1000 µL. HPLC Cannabis Analyzer for Potency Prominence-i LC-2030C equipped with a reverse phase C18 column, Nex-Leaf CBX Potency 150 x 4.6 mm, 2.7 µm with a guard column Nex-Leaf CBX 5 x 4.6 mm, 2.7, UV detector and acquisition software LabSolutions version 5.84 (Shimazu, Kyoto, Japan).

4.3.2 Sampling

The samples were supplied by a company that produces industrial hemp. In particular, two samples (L11 and L5) of inflorescences of Cannabis sativa L. Futura 75 were analyzed, having come from the same land and harvested in August 2017, and supplied by Enecta Srl. Sampling of material was carried out on a population of hemp plants, according to a systematic path, so that the sample taken was representative of the particle, excluding the edges, taking the upper third of the selected plant as indicated in Reg. (EU) No 1155/2017. The sample was dried in an oven at 35°C±1 to constant weight, and gross wood parts and seeds with a length of more than 2 mm were removed. The samples were then subjected to grinding and subsequent sieving through a sieve with 1 mm meshes. The sieved

material was transferred into polypropylene containers and stored under nitrogen atmosphere, protected from light at a temperature of -20°C until extraction. Three independent replicates were performed for each sample, and three HPLC injections were performed for each replication.

4.3.3 Cannabinoid Extraction

To extract cannabinoids, an aliquot of powder sample, about 25 mg, was weighed using an analytical balance; 10 mL of methanol-chloroform extraction solvent 9:1 (v/v) was added as reported by De Backer et al. (2009), Jin et al. (2017), and was placed first for 10 min on an oscillating oscillator set at 350 oscillations per minute and then for 10 min in an ultrasonic bath. The sample was centrifuged for 10 min at 1125 g, and the supernatant was removed. The extraction was performed twice. The two fractions containing cannabinoids were collected in a 25 mL volumetric flask and were brought to volume with methanol/chloroform (9:1, v/v). The samples were filtered with a 45 µm nylon filter. Two mL of the filtered extract was transferred to a glass tube. The solvent was removed, leading to dryness with the help of a weak nitrogen flow, and recovered with 500 µL acetonitrile. The solution was injected into an HPLC-UV.

4.3.4 Preparation of Standard Solution

Appropriate aliquots of a standard mixture of cannabinoids are diluted with acetonitrile to obtain solutions of known concentration, in particular eight points in a concentration range between 0.05 and 100 µg/mL (0.05, 0.50, 4.17, 8.33, 16.70, 25.00, 50.00, 100.00 µg/mL). The standard solutions were prepared to construct calibration curves for the 10 cannabinoids considered: CBDA, CBGA, CBG, CBD, THCV, CBN, D9-THC, D8-THC, CBC, and THCA. The standard solutions were stored away from light at a temperature of -20°C. The stability of standard solutions stored at -20°C was evaluated every week for 3 months with the HPLC-UV system, and no degradation of cannabinoids was found.

4.3.5 HPLC conditions

For the RP-HPLC analysis, the column was thermostated at 35°C, and the autosampler was thermostated to 4°C. Sample concentration was 4 mg/mL, and injection volume was 5.0 µL. UV detection was used at 220 nm, and gradient elution was used at flow rate of 1.6 mL/min according to the following procedure. Eluent mixture: Water + 0.085% phosphoric acid (A), acetonitrile + 0.085% phosphoric acid (B). Gradient elution: 70% of B up to 3 min, 85% of B to 7 min, 95% of B to 7.01

up to 8.00 min, and 70% of B up to 10 min. The eluent mixture was previously filtered with a Millipore system equipped with a 0.2 µm nylon filter.

4.3.6 Validation parameters

Precision

Precision is the closeness of agreement among independent test results, obtained with stipulated conditions and usually in terms of standard deviation or relative standard deviation (Thompson et al., 2002). Precision was calculated with the following formula: CV% = [(SD/x) x 100], where SD is the estimate of the standard deviation and x is the average of the replications made.

Repeatability, R

The repeatability (intraday) of the method was evaluated by analyzing three replicates of the same sample, injected three times on the same day, performed by the same operator with the same method and instrument. The result corresponds to the arithmetic mean of the three determinations made considering the estimate of the standard deviation (SD) calculated on the three replicates performed. The repeatability (interday) of the method was evaluated by performing three replicates of the same sample, injected three times on three different days, performed by the same operator with the same method and instrument. The result corresponds to the arithmetic mean of the three determinations made considering the estimate of the standard deviation (SD) calculated on the three replicates performed.

Reproducibility, R

Reproducibility was evaluated by the agreement between the results obtained on the same sample with the same procedure carried out by different operators in the laboratory and was measured with the coefficient of variation.

Recovery

Recovery is the fraction of analyte that was added to the sample being tested. Recovery was expressed as a percentage (R (%)) according to the following formula: R (%) = [(Cf - C)/Cc] x 100, where Cf is the endogenous amount of the cannabinoid in the sample plus the amount of standard added to the analyte under examination. C is the endogenous amount present in the sample not added with the standard. Cc is the amount of the standard analyte added to the sample.

Detection Limit, LOD

The detection limit is the smallest amount or concentration of analyte in the sample that can be reliably distinguished from zero (Thompson et al., 2002). It can be calculated using the following formula: LOD = (3.3 x σ)/m, where: σ represents the residual standard deviation of the calibration

curve and m represents the slope of the calibration curve. Furthermore, the LOD of the method from the signal (S)/noise (N) ratio can be determined as LOD: S/N = 3.

Quantification limit, LOQ

The quantification limit is the concentration of analyte below which it is determinable with a level of precision that is too low with inaccurate results. The LOQ can be determined according to the following formula: LOQ = (10 x σ)/m, where σ represents the residual standard deviation of the calibration curve and m represents the slope of the calibration curve. The LOQ of the method can also be determined by the signal-to-noise ratio (S/N): LOQ: S/N = 10.

Linearity

Linearity can be tested by examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set (Thompson et al., 2002). In order to quantify the analytes of interest, the equation of the calibration curve obtained for each standard is used. The equation is: y = ax + b, where y = area of the analyte obtained by HPLC/UV analysis, a = slope of the calibration curve, x = unknown concentration (µ\g/mL) of analyte in the sample, b = intercept of the calibration curve.

4.4 Results and Discussion

4.4.1 Method Development

The aim of this work was to develop a new analytical method for determination of the main cannabinoids in hemp samples. In fact, the method described below can be used as a routine quality control procedure and can be applied by the pharmaceutical industry, small laboratories, or even small pharmacies.

A crucial aspect for accurate identification and quantification of analytes is optimization of separation conditions, and therefore various preliminary tests were carried out (e.g., mobile phase, detection wavelength). Different mobile phases were tested, and trials were performed with different compositions and gradient elution to optimize the separation of all 10 target compounds considered (Appendix A). The greatest difficulty was that of separating CBD and THCV, which in many cases co-eluted. It was also difficult to separate the isomers Δ9-THC and Δ8-THC. The best resolution of cannabinoids was obtained using a chromatographic column and, as an eluent mixture, water with 0.085% phosphoric acid and acetonitrile with 0.085% phosphoric acid.

The quantification of cannabinoids was made at 220 nm after testing different wavelengths (Appendix A). This wavelength represents the best compromise for all the cannabinoids considered and was selected to detect and integrate all compounds of interest within the dedicated concentration range.

As far as chromatographic analysis is concerned, before using the instrument, the system was conditioned for 20 min by fluxing the eluent mixture in the instrument under the same conditions as the method, and then a chromatographic run was performed by injecting 5 µL of acetonitrile to verify that the chromatographic system was adequately cleaned. Simultaneously with the analysis of the sample, standard solutions were injected at different concentrations for the construction of calibration curves and to evaluate the separation and identification of each compound. The identification of cannabinoids was performed by comparing their retention times with those obtained by the injection of pure standards and by an enhancing procedure. Figure 4.4.1.1 shows a chromatogram of a standard mixture of cannabinoids and Figure 4.4.1.2 shows a chromatogram of a sample of hemp.

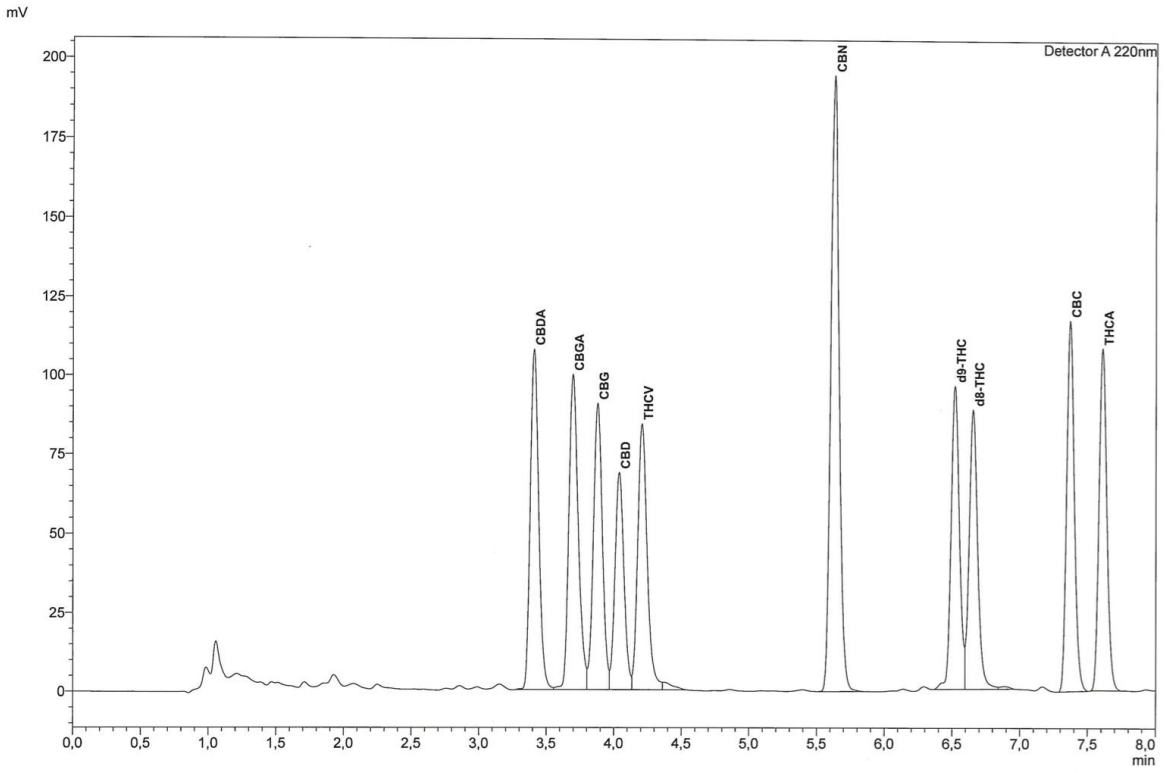

Figure 4.4.1.1 Chromatographic trace of a standard cannabinoid mixture analyzed by RP-HPLC-UV equipped with reverse phase C18 column.

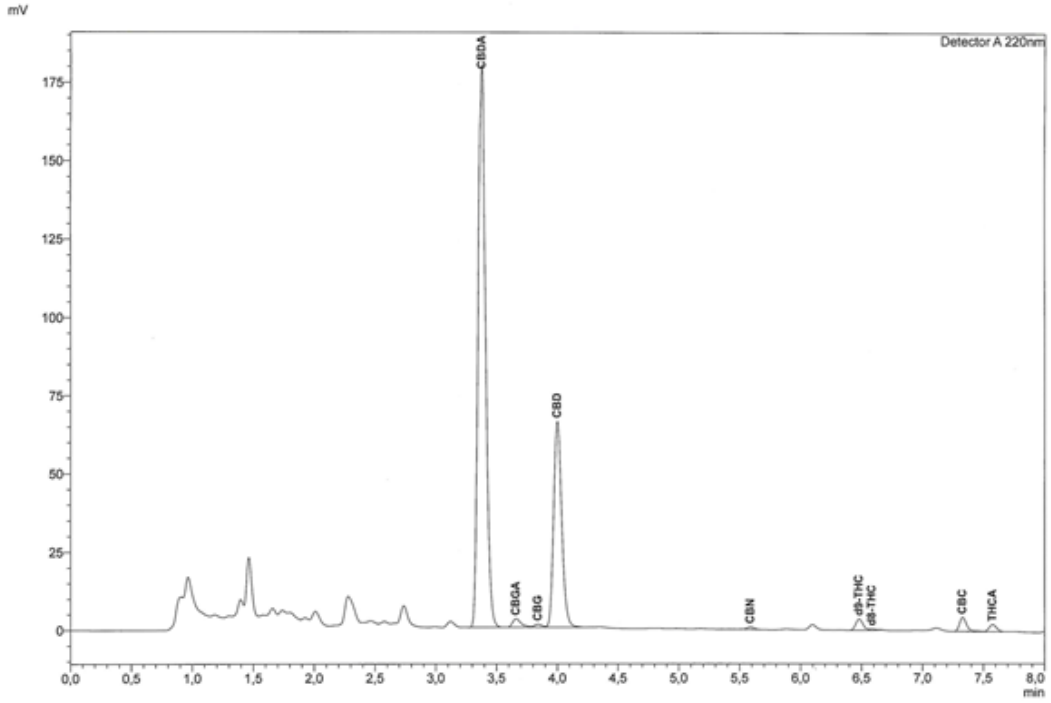

Figure 4.1.1.2 Chromatographic trace of *Cannabis sativa* L. inflorescence extract analyzed by RP-HPLC-UV equipped with a reverse phase C18 column.

Cannabinoids in different varieties of *Cannabis sativa* L. can be present in very different concentrations. In order to obtain good chromatographic separation and correct quantification, it may be necessary to dilute or concentrate the extract, performing two different injections. For example, in the case of high levels of CBDA or CBD it will be necessary to dilute the extract. For THC, it is often found at low concentration in hemp inflorescences, so it may be necessary to concentrate the extract before injection. In our case, 2 mL of filtered extract was dried using a weak nitrogen flow, and the dry extract was recovered in 500 μL of acetonitrile.

4.4.2 Validation

Precision

The precision of the method was measured by the expression of repeatability (r) and reproducibility (R). Precision was expressed through coefficient of variation (CV%).

Repeatability, R

Table 4.4.2.1 shows data on the intraday and interday repeatability, evaluated as reported in Section 3.6, which demonstrates very high repeatability. In fact, the relative standard deviation (RSD) varied from 2.59 to 5.65 for intraday repeatability and from 2.83 to 5.05 for interday repeatability. In both cases, the highest RSD was found for CBDA, which is probably due its higher concentration compared to the other cannabinoids.

Table 4.4.2.1 Validation parameters of RP-HPLC-UV method.

Compound	R^2	[1]LOD (µg/mL)	[2]LOQ (µg/mL)	[3]LOD (µg/mL)	[4]LOQ (µg/mL)	Intraday (Repeatability) RSD	Interday (Repeatability) RSD	Reproducibility RSD	Recovery (%)
CBDA	0.9999	0.34	1.05	0.11	0.37	5.65	5.05	0.09	96.06
CBGA	0.9999	0.32	0.98	0.12	0.40	4.71	4.34	2.13	93.90
CBG	0.9995	0.62	1.87	0.13	0.45	3.34	2.83	0.91	94.60
CBD	0.9995	0.63	1.91	0.17	0.58	4.89	4.44	0.70	84.92
THCV	0.9989	0.95	2.87	0.15	0.49	-	-	nd	nd
CBN	0.9999	0.28	0.84	0.06	0.21	2.59	2.95	0.81	97.08
Δ9-THC	0.9981	1.25	3.79	0.15	0.50	3.05	3.22	0.13	99.69
Δ8-THC	0.9987	1.02	3.10	0.17	0.56	3.81	3.64	0.74	100
CBC	0.9999	0.29	0.88	0.11	0.36	5.3	4.78	0.89	98.68
THCA	0.9998	0.43	1.29	0.11	0.37	5.55	5.01	1.91	95.27

[1] Limit of detection (LOD) determined by the calibration curves (Instrumental LOD = (3.3 × σ)/m). [2] Limit of quantification (LOQ) determined by the calibration curves (Instrumental LOQ = (10 × σ)/m). [3] LOD determined by the signal-to-noise ratio (Instrumental LOD: S/N = 3). [4] LOQ determined by the signal-to-noise ratio (Instrumental LOQ: S/N = 10). * Not detectable.

Reproducibility, R

The RSDs obtained in the reproducibility studies are shown in Table 4.4.2.1. The maximum RSD value was 2.13 for CBGA. The other cannabinoids show RSD values lower than 1.91, and the lowest of the RSDs was 0.09 for CBDA, which is probably due to the higher concentration of this cannabinoid.

Recovery

The tests were performed by using three different concentrations to test the recovery values in the linearity range of the method.

Quantities of CBD (4, 8, and 24 µg/mL) were added, thus assessing concentrations similar to, higher, and lower than those found in samples.

Recovery was determined according to this modality for CBD and was 84.92%.

An evaluation of recovery on all the compounds present in the sample was carried out by proceeding with a further extraction with 10 mL of methanol-chloroform on the sample residue after the usual extraction; in this extract, some cannabinoids were present, and indirectly the percentage of recovery was determined.

The percentage of recovery values, as shown in Table 4.4.2.1, were higher than 84.92% and can be considered very satisfactory. In fact, considering CBD, the percentages are higher than those previously reported in the literature (Brighenti et al., 2017).

Detection Limit, LOD

The instrumental limit of detection was determined by the calibration curve, according to the formulas expressed in Materials and methods section. The instrumental limit of detection (LOD) values

obtained for CBDA and CBGA (Table 4.4.2.1) were lower, while those of CBG and CBD were comparable with similar methods described in literature (Brighenti et al., 2017; Gul et al., 2015). Low LOD values were found also for the other cannabinoids (THCV, CBN, Δ-9 THC, Δ-8 THC, CBC, THCA), indicating that the method is sensitive.

Quantification Limit, LOQ

The instrumental limit of quantification was determined by a calibration curve, considering that the signal-to-noise method is particularly useful to quantify the cannabinoids present at lower concentrations, such as THC. As reported for the LODs, the instrumental limit of quantification (LOQ) values obtained for CBDA and CBGA (Table 4.4.2.1) were also lower than those reported in the literature, while those for CBG and CBD were comparable with those of other methods described for similar procedures (Brighenti et al., 2017; Gul et al., 2015). In addition, the other cannabinoids (THCV, CBN, Δ-9 THC, Δ-8 THC, CBC, THCA) showed low LOQs. The instrumental noise was registered in μV, by performing 3 blank injections with the ASTM method (ASTM E685-93, 2013) given by the instrument, and a maximum CV% of 3.49% was calculated for all individual compounds to determine the single LOD and LOQ, which was considered acceptable.

Linearity

In order to evaluate the linearity of the method, eight different points of standard mixture solutions were analyzed in triplicate by HPLC-UV.

The following equations are related to the calibration curves in a concentration range between 0.01–100 μg/mL: CBDA, y = 18955x − 1612.6 (r^2=0.9999); CBGA, y = 19796x − 3475.7 (r^2=0.9999); CBG, y = 18094x − 9195.3 (r^2=0.9995); CBD, y = 13703x − 6009.5 (r^2=0.9995); THCV, y = 18534x − 15213 (r2 = 0.9989); CBN, y = 34148x − 7943.1 (r^2=0.9999); Δ9 − THC, y = 19893x − 31896 (r^2=0.9981); Δ8-THC, y = 17526x − 18267 (r^2=0.9987); CBC, y = 18590x − 4777.1 (r^2=0.9999); THCA, y = 18239x − 8969.3 (r^2=0.9998) (Table 4.4.2.1).

With the aid of the equation obtained from the calibration curve, the quantity of each cannabinoid was calculated.

To express the data relative to the content of the individual cannabinoid as a percentage (%, p/p) referred to the dried material, it is necessary to refer to the weight of the sample considering the dilution factor. The linearity in the concentration range analyzed was good for cannabinoid standards, being r^2>0.998, as reported before.

4.4.3 Cannabinoids in Hemp Samples

The method developed in this study was applied to quali-quantitative analysis of main cannabinoids in two samples of hemp inflorescences. The samples analyzed, belonging to the same variety of

Cannabis sativa L., did not show a significant difference in the concentration of the target compounds. As shown in Table 4.4.3.1, CBDA is the only cannabinoid for which a different concentration was determined. The other cannabinoids had a similar or the same concentration (e.g., CBGA, CBG, CBN, Δ-9-THC, and Δ-8-THC) in both samples. THCV was not found in the hemp inflorescence samples analyzed, as shown in Figure 4.1.1.2 and Table 4.4.3.1. Δ-9-THC and Δ-8-THC were found at a low concentration, below the legal limit. Under the current legislation regarding *Cannabis sativa* L. cultivation (Reg. EU n°1307/2013; Legge n.242, 2016), in fact, the total content of THC must not be higher than 0.2% and in any case within 0.6%. Indeed, only the hemp varieties reported in the Common catalogue of varieties of agricultural plant species can be cultivated without authorization (Legge 2 Dicembre 2016, n.242; European Union Common Catalogue of Varieties of Agricultural Plant Species, Plant Variety Database). These kinds of results confirmed that the analyzed samples were correctly classified as hemp, since the quantity of Δ8-THC and Δ9-THC was found to be lower than the limits established by the legislation. According to what is indicated in literature (del M. Contreras et al., 2018), in the hemp variety considered (Futura 75), the most present compound was CBDA, followed by CBD; all the other compounds were in very low amounts ranging from 0.01 to 0.06%. CBGA is the compound from which all other cannabinoids are biosynthesized (Brighenti et al., 2017), which is probably why it was found at a low concentration in both samples examined.

Table 4.4.3.1 Number of cannabinoids in hemp samples.

	Cannabinoids									
Sample	CBDA (%)	CBGA (%)	CBG (%)	CBD (%)	THCV (%)	CBN (%)	Δ9-THC (%)	Δ8-THC (%)	CBC (%)	THCA (%)
L11	0.88±0.04	0.02±0.00	0.02±0.00	0.48±0.02	Nd*	0.01±0.00	0.06±0.00	0.03±0.00	0.03±0.00	0.03±0.00
CV%	5.05	4.34	2.83	4.44		2.95	3.22	3.64	4.78	5.10
L5	0.93±0.06	0.02±0.00	0.02±0.00	0.45±0.03	Nd*	0.01±0.00	0.06±0.00	0.03±0.00	0.02±0.00	0.04±0.00
CV%	6.48	1.28	1.73	6.28		1.49	0.21	2.20	2.98	7.17

*Not detectable

The number of cannabinoids in hemp samples is reported in Table 4.4.3.1.

4.5 Conclusions

One of the most relevant problems in analytical determinations for quality control, especially when there are legal problems related with quantitation, such as for cannabis, relates to the proficiency of laboratories. Therefore, detailed and validated procedures that are freely available are essential for the full understanding of any analytical step and its careful application. This is also true for “daily”

methods that can be easily applied for quality control, carried out using traditional RP-HPLC and UV-Vis detectors, with less efficient performance than diode-array detectors but with lower costs, rendering them affordable even for small laboratories.

The validated method described herein allows the quantitative determination of the 10 most relevant cannabinoids using a single wavelength (220 nm) in 8 min. A full separation is obtained, even in the elution sequence of a difficult resolution, of the group of peaks related to CBGA, CBG, CBD, and THCV (from 3.5 to 4.5 min).

The method is applied to cannabis inflorescences and involves extraction in methanol/chloroform, drying of the extract, taking it up in acetonitrile and injection into an HPLC. The method has sensitivity and accuracy to discriminate samples with amounts of Δ-9- and Δ-8-THC (total THC content) that are below the limit of 0.2% from those that are subjected to legal restrictions in many EU countries, with a total THC content above 0.6%, which cannot be classified as hemp. Due to its simplicity and rapidity, it can be used to check raw material or crops during the harvesting period.

A detailed standard operating procedure (SOP), as a supplementary information file, is also available, so that any operator with basic knowledge of HPLC can easily apply it and make all the elution and calibration control checks using commercially available mixtures of standards, which are more affordable and sustainable than single cannabinoid standards in terms of costs and solvents used for calibration.

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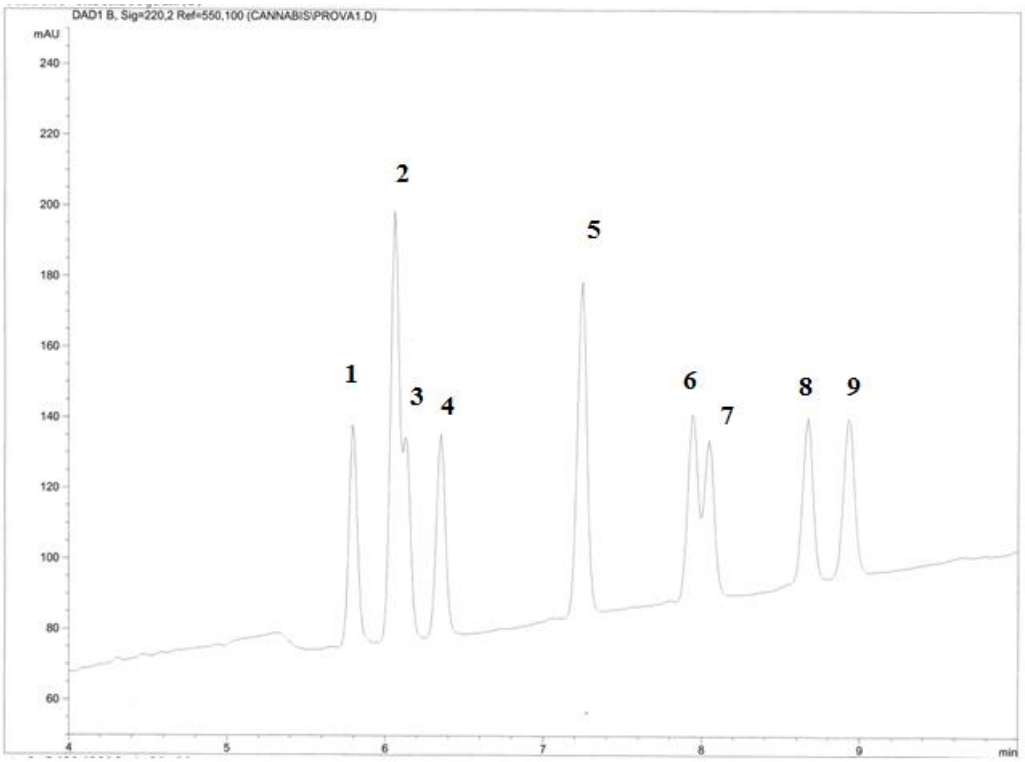
4.6 Appendix A

Preliminary tests carried out for the development of the analytical procedure by RP-HPLC-UV:
The following are some of the preliminary analysis that have been performed by testing different eluent mixtures, columns and chromatographic conditions.

TEST 1

HPLC system consisted of a Series 1260 chromatograph coupled to a Series 1100 autosampler and Diode Array Detector (DAD, Agilent Tecnologies, Palo Alto, CA) and Software HPLC ChemStation (Rev.A.08.03 Agilent Technologies, USA).
Eluent mixture: A water + 0.1% formic acid, B methanol-acetonitrile 75:25 + 0.1% formic acid.
Isocratic elution with Sphereclone 5µ ODS(2) 80 Å at a flow rate of 1.5 mL/min.
With these chromatographic conditions there is no acceptable separation of CBGA, CBG and there is the co-elution of CBD and THCV, as shown in figure below.



1-CBDA, 2-CBGA, 3-CBG, 4-CBD+THCV, 5-CBN, 6- Δ9- THC, 7- Δ8-THC, 8-CBC, 9THCA.

TEST 2

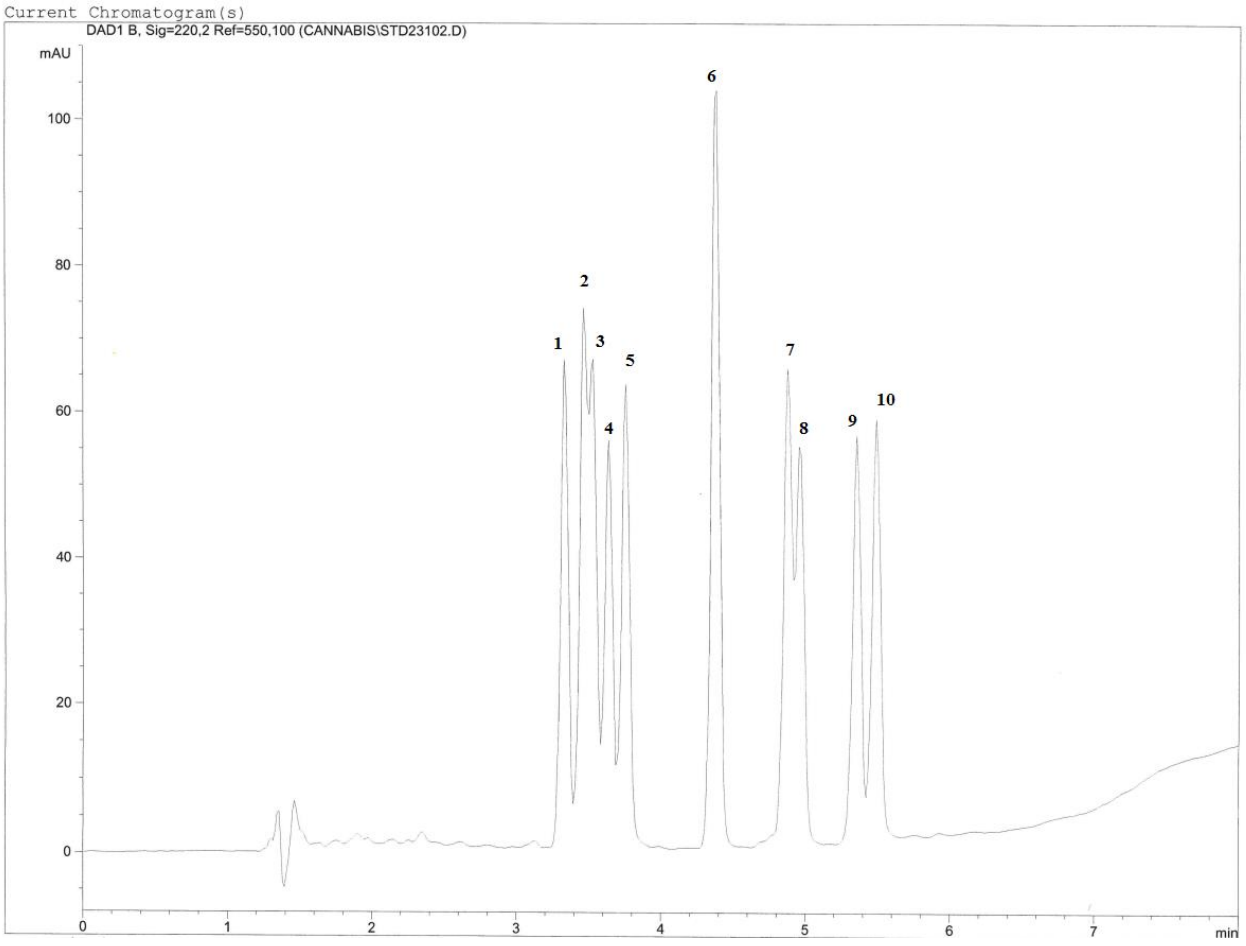
HPLC system consisted of a Series 1260 chromatograph coupled to a Series 1100 autosampler and Diode Array Detector (DAD, Agilent Tecnologies, Palo Alto, CA) and Software HPLC ChemStation (Rev.A.08.03 Agilent Technologies, USA).

Gradient elutions was applied with a flow rate of 1.0 mL/min., according to the following procedure. Eluent mixture: A water + 0.1% phosphoric acid, B acetonitrile + 0.1% phosphoric acid.

Gradient elution with a Kinetex C18 150 x 4,6 mm., 2,6 µm 100 Å (Phenomenex) thermostatically at 50 °C, flow rate of 1.8 mL/min.

Gradient elution: 75% of B up to 6.0 min. 100% of B to 6.01, 75% of B maintained for 4 minutes.

With these chromatographic conditions all compounds are separated but there is no good separation of CBGA, CBG and Δ9- THC, Δ8-THC, as shown in the figure below.



1-CBDA, 2-CBGA, 3-CBG, 4-CBD, 5THCV, 6-CBN, 7- Δ9- THC, 8- Δ8-THC, 9-CBC, 10-THCA

TEST 3

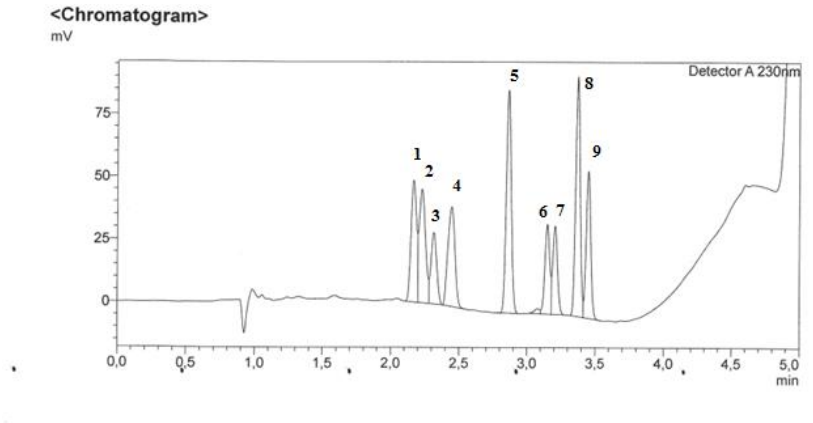
HPLC system consisted of a Cannabis Analyzer for Potency Prominence-i LC-2030C equipped with a reverse phase C18 column, Nex-Leaf CBX Potency 150 x 4.6 mm, 2.7 µm with a guard column Nex-Leaf CBX 5 x 4.6 mm, 2.7, UV detector and an acquisition software LabSolutions version 5.84 (Shimazu, Kyoto, Japan).

Gradient elution was used at flow rate of 1.5 mL/min., according to the following procedure.

Eluent mixture: A water + 0.1% phosphoric acid, B acetonitrile + 0.1% phosphoric acid.

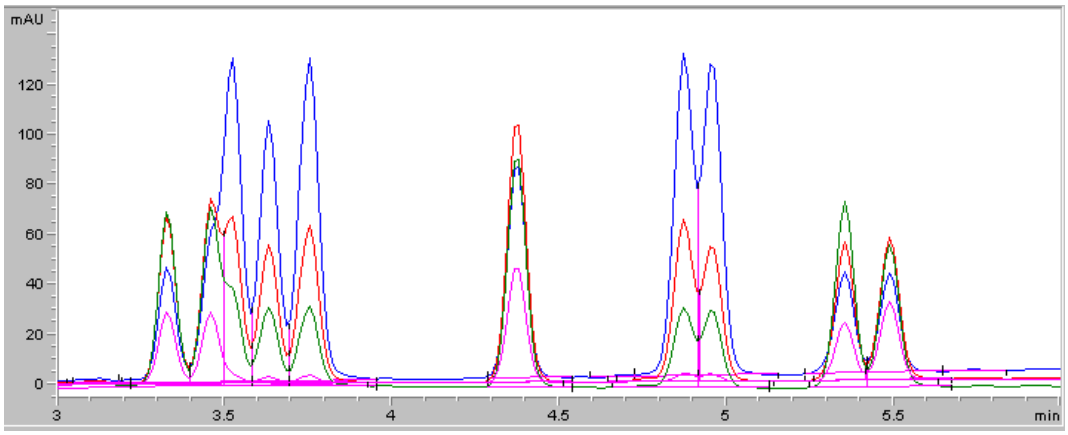
Gradient elution: 75% of B up to 0.70 min. 85% of B to 2 min. 100% of B to 3.00 until 3.50 min. up to 3.60 min. and 75% of B up to 5 minutes.

With these chromatographic conditions there is no acceptable separation of CBDA, CBGA, CBG and there is the co-elution of CBD and THCV, as shown in figure below.



1-CBDA, 2-CBGA, 3-CBG, 4-CBD+THCV, 5-CBN, 6- Δ9- THC, 7- Δ8-THC, 8-CBC, 9-THCA.

Evaluation of the DAD detector response at different wavelengths 210, 220, 228, 273 nm.



DAD1 A, Sig=210,2 Ref=550,100

DAD1 B, Sig=220,2 Ref=550,100

DAD1 C, Sig=228,2 Ref=550,100

DAD1 D, Sig=273,2 Ref=550,100

Chapter 5

Evaluation of the antioxidant activity of cannabidiol in comparison with α-tocopherol added to refined olive and sunflower oils

5.1 Details of the publication based on Chapter 5

Title: Preliminary study: comparison of antioxidant activity of cannabidiol (CBD) and α-tocopherol Added to refined olive and sunflower oils

Authors: Matilde Tura[a], Mara Mandrioli[a], Tullia Gallina Toschi[a]

[a] Department of Agricultural and Food Sciences, Alma Mater Studiorum-University of Bologna, Viale Fanin 40, 40127 Bologna, Italy

Corresponding author: Tullia Gallina Toschi -tullia.gallinatoschi@unibo.it

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Abstract

This study evaluates the antioxidant activity of cannabidiol (CBD), added to model systems of refined olive (ROO) and sunflower (SO) oils, by measuring the peroxide value, oxidative stability index (OSI), electron spin resonance (ESR) forced oxidation, and DPPH• assays. Free acidity, a parameter of hydrolytic rancidity, was also examined. CBD was compared using the same analytical scheme with α-tocopherol. CBD, compared to α-tocopherol, showed a higher scavenging capacity, measured by DPPH• assay, but not better oxidative stability (OSI) of the oily systems considered. In particular, α-tocopherol (0.5%) showed an antioxidant activity only in SO, registered by an increase of more than 30% of the OSI (from 4.15±0.07 to 6.28±0.11 h). By ESR-forced oxidation assay, the concentration of free radicals (μM) in ROO decreased from 83.33±4.56 to 11.23±0.28 and in SO from 19.21±1.39 to 6.90±0.53 by adding 0.5% α-tocopherol. On the contrary, the addition of 0.5% CBD caused a worsening of the oxidative stability of ROO (from 23.58 ± 0.32 to 17.28 ± 0.18 h) and SO (from 4.93±0.04 to 3.98±0.04 h). Furthermore, 0.5% of CBD did not lower dramatically the concentration of free radicals (μM) as for α-tocopherol, which passed from 76.94±9.04 to 72.25±4.13 in ROO and from 17.91±0.95 to 16.84±0.25 in SO.

Keywords: cannabidiol (CBD); lipid oxidation; α-tocopherol; antioxidant activity; oxidative stability; free radicals.

5.2 Introduction

Cannabidiol (CBD) is a non-psychoactive cannabinoid present in *Cannabis sativa* L., and unlike tetrahydrocannabinol (THC), CBD has a very low affinity for the cannabinoid CB1 and CB2 receptors (Fouad et al., 2013). From the chemical structure of CBD (Figure 5.2.1), it is easy to recognize the presence of two hydroxyl groups that can endow it with antioxidant activity (Iuvone et al., 2009). CBD is a cyclohexene that is substituted in position 1 by a methyl group, in position 3 by a 2,6-dihydroxy-4-pentylphenyl group, and in position 4 with a prop-1-en-2-yl group (Silvestro et al., 2019). According to Borges et al. (2013), CBD has potential antioxidant activity because of the fact that the cation free radicals show several resonance structures in which the unpaired electrons are mainly distributed on the ether and alkyl groups, as well as on the benzene ring (Borges et al., 2013). One of the most common antioxidants is α-tocopherol, which is a key component in biological systems as it integrates into the cell membranes to protect their constituents (Beddows et al., 2000).

Tocopherols are natural antioxidants and liposoluble vitamins with antioxidant action both in vivo and in vitro. They can be classified as four derivatives (Muhammad et al., 2012), depending on the position and the number of methyl groups on the chromanol ring (Zaunschirm et al., 2018), called alpha (Figure 5.2.1), beta, gamma, and delta tocopherol. These tocopherol isomers differ in their antioxidant activities, with the highest antioxidant activity found for α-tocopherol (Muhammad et al., 2012). In fact, when α-tocopherol becomes a free radical the resulting α-tocopheroxyl species is able to delocalize the free electron, thus forming a more stable and less reactive intermediate (Beddows et al., 2000).

The human body cannot synthetize tocopherols, hence they must be included in the diet (Muhammad et al., 2012). According to Muhammad et al. (2012), the amount of total tocopherol present in crude sunflower oil is between 447 and 900 mg/g of oil, with extreme values between 389 and 1873 mg/g of oil. Up to 90% of the total content of tocopherols in sunflower oil is represented by α-tocopherol (Muhammad et al., 2012). If sunflower oil is refined, the total content of these compounds is lower, and in fact, for vegetable oils, up to 32% of native tocopherols are removed during the refining process (Zaunschirm et al., 2018). Regarding the content of α-tocopherol in refined olive oil, Schwartz et al. 2008 (Schwartz et al., 2008) reported a content of α-tocopherol equivalents equal to 17 mg/100 g.

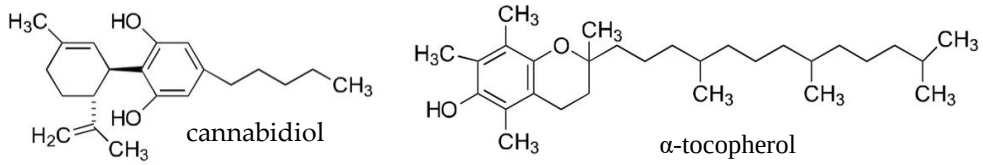

Figure 5.2.1. Chemical structures of cannabidiol and α-tocopherol.

Several assays can be used to evaluate the in vitro antioxidant capacity of extracts, such as lipid peroxidation inhibition assay, ferrous-ion chelating activity, inhibition of DPPH•(2,2-diphenyl-1-picrylhydrazyl), inhibition of 2,2′-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid)—ABTS•+ (Skenderidis et al., 2019), superoxide dismutase mimetic activity, and many others. All these assays have their pros and cons, but when the antioxidant activity is evaluated it is essential to consider that these methods have specificity in relation to the mechanism of action, target, pH, time, and temperature; furthermore, different standards are used to construct the calibration curves that produce quantitative results in terms of antioxidant activity. Therefore, no single in vitro "antioxidant activity" assay will reflect the total antioxidant capacity (Granato et al., 2018; Apak et al., 2013), but all possible measures have a partial meaning that is strictly related to the method of measurement and the setting (e.g., biological, food system, pure oil). Moreover, antioxidants may respond in a different way to various radical or oxidant sources (Gülcin, 2012). In fact, the activity of antioxidants depends not only on their structural features, but also on many other factors, such as concentration, temperature, type of substrate, and physical state of the system, as well as on the numerous micro-components acting as pro-oxidants or synergists (Gülcin, 2012).

Free radicals act in both food and biological systems; for this reason, dietary antioxidants play an important role in controlling an excess of free radicals (Beddows et al., 2000).

In food systems, free radicals lead to oxidative rancidity whereby polyunsaturated fats break down, causing the formation of off-flavors (Beddows et al., 2000; Lercker et al., 2003).

By leading to the formation of off-flavors, the oxidative process not only makes a product less attractive, but also results in the formation of toxic products, such as oxidized polymers, because of the destruction of fatty acids (Choe & Min, 2006); for this reason, oxidation is a major problem that affects edible oils (Velasco & Dobarganes, 2002). Fats and oils are susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals, metalloproteins, and microorganisms. These catalysts lead to complex processes of oxidation, and in particular autoxidation, photooxidation, and thermal or enzymatic oxidation, most of which involve free radicals and/or other reactive species as the intermediate (Shahidi & Zhong, 2010). Oxidative stability is defined as the resistance to oxidation during processing and storage of oils and is very important to determine the quality of an oil and its shelf life. In fact, it is the period necessary to reach the critical point that determines the sensory changes or a sudden acceleration of the oxidative process (Choe & Min, 2006).

The aim of the present study is to evaluate the antioxidant capacity of cannabidiol (CBD), added to two lipid matrixes, namely refined olive oil (ROO) and refined sunflower oil (SO). Herein, the

antioxidant capacity of cannabidiol was compared with the same oils to which α-tocopherol (AT) was added.

5.3 Materials and methods

5.3.1 Sample Preparation

The antioxidant capacity of cannabidiol (CBD) and α-tocopherol (AT) was evaluated on oily solutions prepared with refined olive oil pharmaceutical grade according European Pharmacopoeia (Ph Eur) and sunflower oil at three different concentrations: 0.01%, 0.1%, and 0.5%. For preparation of these solutions, refined olive oil Ph Eur grade acquired in a pharmacy was used; sunflower oil was from commercial sources; the CBD used was in the form of crystals with 99.8% purity provided by Enecta Srl, Amsterdam (Netherlands), while AT with 97% purity was supplied by Alfa Aesar Thermo Fisher (Erlensbachweg 2, 76870 Kandel, Germania, Germany). In order to obtain complete dissolution of active principles, preparations were placed in an ultrasonic bath, model Branson 2150 (Danbury, CT, USA) for 1 h, divided into four intervals of 15 min, in order to avoid overheating of the solutions. Fifty grams of each solution was prepared and placed in a 100 mL Sovirel bottle. Bottles were stored in the fridge at 4°C until analysis.

5.3.2 Determination of CBD Content

To determine the actual CBD content and ensure the total dissolution of CBD crystals, solutions were analyzed by liquid chromatography. A total of 100 mg of sample was weighed in a 10 mL flask, solubilized, brought to volume with isopropanol, vortexed for 1 min, and placed in an ultrasonic bath (Branson 2150) for 10 min. Next, the solution was filtered through a 0.45 µm nylon filter. CBD determination was performed following the method proposed by Mandrioli et al. (2019); 5 µL was injected into an HPLC-UV, Cannabis Analyzer for Potency Prominence-i LC-2030C chromatographic system, equipped with a Nex-Leaf CBX Potency column 150 × 4.6 mm, 2.7 µm (Shimazu, Kyoto, Japan). The eluent mixture eluted in gradient was water with 0.085% phosphoric acid and acetonitrile with 0.085% phosphoric acid. The signal was acquired at 220 nm and processed with LabSolutions version 5.84 software (Shimazu, Kyoto, Japan). Quantification was carried out using a calibration curve constructed with an external standard, injecting solutions of known concentration in the concentration range 0.05–16.70 µg/mL ($y = 13065x - 1612.7$; $R2 = 0.9989$).

5.3.3 Determination of α-Tocopherol Content

As described above, the oils used to disperse the active ingredients were refined, so that most of the compounds with antioxidant activity such as tocopherols were removed. However, the refining process does not completely remove antioxidant compounds such as tocopherols, and small residual quantities are still present. To evaluate the content of AT in the oily matrix, i.e., natural content plus added content, and to verify the complete dissolution of all active ingredients added, liquid chromatography analysis was performed on prepared oily solutions. The applied method was an internal procedure, briefly described below. An aliquot of 0.5 ± 0.0001 g was solubilized in isopropanol, filtered with 0.45 µm nylon filter, and 20 µL were injected into an RP-HPLC system equipped with a quaternary pump model HP 1260 and fluorimeter detector model HP 1100; the software for data processing was Chemstation for LC3D (Agilent Technologies, Palo Alto, CA, USA). The instrument was also equipped with a column Cosmosil π NAP 150 mm × 4,6 mm Thermo Fisher, 5 µm (Nacalai-Tesque, Kyoto, Japan). The mobile phase was a mixture of: solvent A, methanol–water with 0.2% of H3PO4 (90:10 v/v), and solvent B, acetonitrile (100%), eluted in gradient with a flow rate of 1.0 mL/min. The fluorometric detector was set up at an emission wavelength of 295 nm and an excitation wavelength of 330 nm. Quantification was carried out using a calibration curve constructed with the external standard method, injecting solutions of known concentration in the range of 8.34–83.40 µg/mL ($y = 50.176x - 258.48$; $R2 = 0.9979$).

5.3.4 Peroxide Value

The peroxide value represents a measure of peroxidic compounds, the primary products of lipid oxidation. The determination of peroxide value was carried out according to the NGD C35-1976 method (NGD Method C35), performing an iodometric titration where, following oxide reduction reaction in the presence of starch as an indicator, measures the quantity of peroxide present in the sample expressed as meq of active oxygen per kg of oil.

5.3.5 Free Acidity

The acidity index is defined as mg of KOH needed to neutralize the free acids present in a gram of oil, determined as indicated by the European Pharmacopoeia 9th Edition 2017 (European Pharmacopeia, 9th Ed.). The tested substance, dissolved in a mixture of alcohol and petroleum ether, is titrated with an alkaline hydroxide solution in the presence of phenolphthalein.

5.3.6 Oxidative Stability Index

The oxidative stability index (OSI) was evaluated as the determination of resistance to forced oxidation and was performed using an oxidative stability instrument (OMNION OSI-8 Decatur, IL, USA). A total of 5 g of each sample were weighed in a glass tube and heated to 110 °C in the presence of a continuous flow that reached the end of the path. Through this continuous measurement, the instrument extrapolates the data relative to the induction period from the initial phase of oxidation to that in which it assumes an exponential trend, known as OSI-time (Jebe et al., 1993).

5.3.7 Micro-ESR

The oxidation state of solutions was also investigated using the ESR spectroscopy analysis, a technique that allows evaluation of chemical species with unpaired electrons, such as free radicals. In particular, 1 mL of oily solution was added with 40 µL of 2.5 M N-tert-butyl-α-phenylnitrone (PBN, ≥98% VWR-International, Milan, Italy) in ethanol, vortexed for one minute, and readings were taken with microESR STANDARD V 2.0 (Bruker BioSpin GmbH, Rheinstetten, Germany) heating the sample to 110 °C for 240 min, recording the spectra obtained every 20 min. The standard for the calibration curve used was constructed by TEMPO solutions in mineral oil (Sigma-Aldrich, Milan) at concentrations of 1.0, 2.5, 5.0, 10.0, 20.0, 35.0, and 50.0 µM.

5.3.8 DPPH• Radical Scavenging Activity Assay

The extraction of α-tocopherol and CBD was performed following the procedure reported by Ninfali et al. (2001). A total of 5 g of oily sample were weighed and 5 mL of a mixture of methanol:water 80:20 v/v was added. It was vortexed for two minutes, and the sample was centrifuged at 2500 rpm for 15 min. The supernatant was removed and placed in a 10 mL flask. The extraction phase was repeated with 4.5 mL of the mixture of methanol:water 80:20 v/v and, finally, was brought to volume in a 10 mL flask, with the same mixture.

The antioxidant capacity was investigated by applying the DPPH• radical scavenging activity assay on different extracts. Total of 11.9 mg of DPPH• supplied by Thermo Fisher (Kandel, GmbH, Germany) was weighed in a 50 mL volumetric flask, and brought to volume with methanol, obtaining a solution with a concentration of 0.604 mM. This solution was diluted by taking 5 mL and bringing it to volume, in a volumetric flask, with methanol to 50 mL, obtaining a solution with a concentration equal to 0.0604 mM or 60.4 µM.

The spectrophotometry analysis was performed against methanol at 515 nm using a Jasco dual beam spectrophotometer model V-550 UV-VIS, with the possibility of reading each nanometer. Quartz cuvettes with an optical path of 10 mm, supplied by Lightpath Optical (Cranborne, Churchill,

Axminster EX13 7LZ, UK), were used. A total of 2.9 mL of 60.4 μM DPPH• solution was placed in the cuvette and read; subsequently, 100 μL of methanol:water 80:20 v/v was added in this cuvette, thus obtaining a value relative to blank (A0), and the absorbance was read. Next, in order to have the absorbance value of the control sample (Aj), 2.9 mL of methanol was placed in the cuvette and 0.1 mL of the extract (sample) was added and the absorbance was read (Liu et al., 2008). For each sample, five different concentrations in the range between 5 mg/mL and 50 mg/mL were analyzed at 515 nm, prepared by placing them in 2.9 mL of 60.4 μM DPPH• solution. The solutions were mixed thoroughly and protected from light at room temperature for 30 min, and radical scavenging was estimated by determining the loss of absorbance at 515 nm (Zhang et al., 2019). The solutions were kept in the dark for 30 min; this time was chosen according to the literature (Alma et al., 2003; Szabo et al., 2007; Sharma & Bhat, 2009) and having verified that with it only the oils with CBD at 0.5% reached the plateau of the absorbance value. As all the other solutions reached the plateau within 24 h, 30 min appeared to be the best compromise to compare the results of all DPPH• assays.

The experimental scavenging capacity (ESC) value was calculated according to the following formula (Zhang et al., 2019):

$$ESC = (1 - [Ai - Aj]/A0) \times 100$$

where: Ai = absorbance of the sample; A0 = absorbance of the blank; Aj = absorbance of the control sample.

The effective concentration EC_{50} of DPPH• value was determined for each sample. EC_{50} of DPPH• was defined as the efficient concentration required to decrease the initial DPPH• concentration by 50% (Mishra et al., 2012). The EC_{50} value was extrapolated from the curve obtained by plotting the ESC value against the five concentrations in the range between 5 mg/mL and 50 mg/mL for each sample.

5.3.9 Statistical Analysis

Samples were analyzed in duplicate and the results are shown as mean ± standard deviation and coefficient of variation (RSD%).

Data were statistically analyzed by applying Anova, Fisher's test LSD, $p < 0.05$ using the software XLSTAT Addinsoft (2018.XLSTAT statistical and data analysis solutions. Paris, France. https://www.XLSTAT.com) version 2018.1.1.

5.4 Results

5.4.1 Determination of CBD Content

The determination of CBD was conducted in the oily solutions produced, while, as well-known, refined olive oil and sunflower oil do not present any CBD content. The analysis was carried out using the HPLC-UV method (described in Section 4.2.2) and the amounts of CBD present in the oily solutions were consistent with those added to the oils. The recoveries were calculated with respect to the three concentrations investigated (0.01%, 0.1% and 0.5%), by applying the following formula:

$$R\ (\%) = [(Cf - C)/Cc] \times 100$$

where: Cf is the amount of cannabinoid in the sample added to the analyte under examination. C is the quantity of cannabinoid determined in the sample not added. Cc is the quantity of analyte added to the sample.

The results were between 96.42% and 125.82%, with a mean value of 110.15%; for this reason, CBD can be considered completely solubilized.

5.4.2 Determination of α-Tocopherol Content

The starting concentrations of α-tocopherol were calculated in refined olive oil and in sunflower oil, which were used to prepare solutions with different concentrations of α-tocopherol. The starting concentration of this compound in refined olive oil was 0.027% and in sunflower oil was 0.064%. The total quantities of α-tocopherol found in the oils were consistent with the quantities of alpha tocopherol added to the oil also considering that they are naturally present. Even in this case, as described above, the yield was calculated. Regarding recovery of α-tocopherol added, the mean value was 91.27%.

5.4.3 Peroxide Value

Peroxide value is one of the most frequently used quality parameters, as it measures the amount of total peroxides which are considered as primary oxidation products (Lerma-García et al., 2011). The samples of refined olive oil and sunflower oil used for the preparation of the oils added with the two active ingredients were the same. Given the instability of the peroxides and, as a consequence, the rapid variation of the peroxides value over time, the evaluation of this parameter was carried out on refined olive and sunflower oils whenever samples with CBD or α-tocopherol added were prepared. Therefore, although there is a change in the peroxide value during the storage period (two months in the dark at 4°C), it was possible to compare the antioxidant activity of the sample with the active ingredient (CBD or α-tocopherol) added and the related sample of oil in which the active ingredient was not added.

The initial peroxide values for refined olive oil and sunflower oil, used to prepare solutions with the three different concentrations of CBD (0.01%, 0.1%, and 0.5%), were 1.93 meq O_2/kg of oil and 5.00 meq O_2/kg of oil, respectively (Table 5.4.3.1).

Refined olive oil with CBD added at the three concentrations showed lower values than the sunflower oil with CBD at the same concentrations. In the first case, they were between 2.47 meq O_2/kg of oil and 2.99 meq O_2/kg of oil, while in the second case they were between 6.04 meq O_2/kg of oil and 14.73 meq O_2/kg of oil (Table 5.4.3.1).

Table 5.4.3.1. Results of peroxide value, free acidity, and OSI-time for refined olive oil (ROO), and sunflower oil (SO) with cannabidiol (CBD) added. Data are presented as mean ± standard deviation and coefficient of variation (RSD%).

Sample	Peroxide Value (meq O_2/Kg of oil)	RSD %	Free Acidity (mg KOH/g of oil)	RSD%	OSI-Time (hours)	RSD %
ROO	1.93 ± 0.09 ^{c}	4.66	0.15 ± 0.02 ^{b}	12.86	23.58 ± 0.32 ^{a}	1.35
ROO-CBD 0.01%	2.47 ± 0.04 ^{b}	1.59	0.18±0.02 ^{b}	11.70	23.33 ± 0.18 ^{a}	0.76
ROO-CBD 0.1%	2.98 ± 0.14 ^{a}	4.66	0.18±0.02 ^{b}	10.88	21.90 ± 0.07 ^{b}	0.32
ROO-CBD 0.5%	2.99 ± 0.14 ^{a}	4.68	0.27±0.02 ^{a}	7.30	17.28 ± 0.18 ^{c}	1.02
SO	5.00 ± 0.02 ^{X}	3.00	0.11±0.00 ^{X}	0.00	4.93 ± 0.04 ^{X}	0.72
SO-CBD 0.01%	6.30 ± 0.01 ^{Y}	0.13	0.08±0.00 ^{Y}	0.00	4.80 ± 0.21 ^{Y}	4.42
SO-CBD 0.1%	6.04 ± 0.29 ^{Y}	4.76	0.06±0.00 ^{Z}	0.07	4.90 ± 0.00 ^{Y}	0.00
SO-CBD 0.5%	14.73 ± 0.24 ^{Z}	1.60	0.06±0.00 ^{Z}	0.07	3.98 ± 0.04 ^{Z}	0.89

ROO = refined olive oil. ROO-CBD = refined olive oil with CBD, the percentage indicates the CBD/oil ratio. SO = sunflower oil. SO-CBD = sunflower oil with CBD, the percentage indicates the CBD/oil ratio. Different letters indicate statistically significant differences (Anova, Fisher's test LSD, $p < 0.05$).

The initial peroxide values for refined olive and sunflower oils, used to prepare solutions with three different concentrations of α-tocopherol (0.01%, 0.1%, and 0.5%) were 0.15 meq O_2/kg of oil and 12.31 meq O_2/kg of oil, respectively (Table 5.4.3.2). Refined olive oil with α-tocopherol added at the three different concentrations showed lower values than the sunflower oil added with α-tocopherol at the same concentrations; in particular, it was between 0.52 meq O_2/kg of oil and 0.62 meq O_2/kg of oil for refined olive oil-based samples and between 13.43 meq O_2/kg of oil and 15.08 meq O_2/kg of oil for sunflower oil-based samples (Table 5.4.3.2).

Table 5.4.3.2. Results of peroxide value, free acidity, and OSI-time for refined olive oil (ROO) and sunflower oil (SO) with α-tocopherol (AT) added. Data are presented as mean ± standard deviation and coefficient of variation (RSD%).

Sample	Peroxide Value ($meqO_2$/Kg of oil)	RSD %	Free Acidity (mg KOH/g of oil)	RSD %	OSI-Time (hours)	RSD %
ROO	0.15 ± 0.00^{c}	0.08	0.17 ± 0.00^{b}	0.07	28.35 ± 0.28^{a}	1.00
ROO-AT 0.01%	0.52 ± 0.03^{b}	6.65	0.17 ± 0.00^{b}	0.00	25.30 ± 0.42^{b}	1.68
ROO-AT 0.1%	0.52 ± 0.04^{b}	6.78	0.21 ± 0.02^{a}	9.43	24.88 ± 0.18^{b}	0.71
ROO-AT 0.5%	0.62 ± 0.04^{a}	5.67	0.21 ± 0.02^{a}	9.43	28.23 ± 0.81^{a}	2.88
SO	12.31 ± 0.50^{X}	4.03	0.20 ± 0.00^{X}	0.07	4.15 ± 0.07^{x}	1.70
SO-AT 0.01%	$13.50 \pm 0.09^{X,Y}$	0.64	0.21 ± 0.02^{X}	9.15	4.20 ± 0.07^{x}	1.68
SO-AT 0.1%	$13.43 \pm 0.70^{X,Y}$	5.18	0.17 ± 0.00^{Y}	0.14	5.28 ± 0.04^{y}	0.67
SO-AT 0.5%	15.08 ± 1.16^{Y}	7.68	0.17 ± 0.00^{Y}	0.00	6.28 ± 0.11^{z}	1.69

ROO = refined olive oil. ROO-AT = refined olive oil with α-tocopherol, the percentage indicates the α-tocopherol /oil ratio.SO = sunflower oil**.** SO-AT = sunflower oil with α-tocopherol, the percentage indicates the α-tocopherol /oil ratio. Different letters indicate statistically significant differences (Anova, Fisher's test LSD, $p < 0.05$).

5.4.4 Free Acidity

The free acidity value is related to the degree of lipolysis of triglycerides and is a quality control parameter that needs to be assessed after the production and during shelf-life (Bendini et al., 2007). The initial free acidity value found for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of CBD (0.01%, 0.1%, and 0.5%) was 0.15 mg KOH/g of oil and 0.11 mg KOH/g of oil, respectively (Table 5.4.3.1).

Refined olive oil added with CBD at the three concentrations showed higher values than sunflower oil with CBD at the same concentrations; in the first case they were between 0.18 mg KOH/g and 0.27 mg KOH/g, while in the second case they were between 0.06 mg KOH/g and 0.08 mg KOH/g (Table 5.4.3.1).

The initial free acidity value found for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of α-tocopherol (0.01%, 0.1%, and 0.5%) was 0.17 mg KOH/g of oil and 0.20 mg KOH/g of oil, respectively (Table 5.4.3.2).

Refined olive oil and sunflower oil with α-tocopherol added at the three concentrations showed similar values; in the first case they were between 0.17 mg KOH/g and 0.21 mg KOH/g, while in the second case they were between 0.17 mg KOH/g and 0.21 mg KOH/g (Table 5.4.3.2).

5.4.5 Oxidative Stability Index (OSI)

OSI measures the induction period by plotting conductivity against time (Coppin & Pike, 2001). The initial OSI- time values found for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of CBD (0.01%, 0.1%, and 0.5%) were 23.58 h and 4.93 h, respectively (Table 5.4.3.1).

Refined olive oil with CBD added at the three concentrations showed higher values than the sunflower oil with CBD added at the same concentrations; in the first case they were between 17.28 h and 23.33 h, while in the second case they were between 3.98 h and 4.90 h (Table 5.4.3.1).

The initial OSI-time value found for refined olive and sunflower oils, used to prepare solutions with the three concentrations of α-tocopherol (0.01%, 0.1%, and 0.5%) was 28.35 h and 4.15 h, respectively (Table 5.4.3.2).

Refined olive oil with α-tocopherol added at the 3 concentrations showed higher values than the sunflower oil with α-tocopherol added at the same concentrations; in the first case they were between 24.88 h and 28.23 h, while in the second case they were between 4.20 h and 6.28 h (Table 5.4.3.2).

5.4.6 Electron Spin Resonance

Lipid oxidation is a free radical chain reaction, and for this reason electron spin resonance (ESR) is a valuable tool for detection and quantification of lipid-free radicals. In fact, radicals with unpaired electron have unique magnetic properties (Chen et al., 2017). Studies concerning radicals deriving from lipid oxidation, which are very unstable, are carried out using the spin trap technique and the most used spin trap is n-tert-butyl-α-phenylnitrone (PBN). PBN acts as a trap for temporary radicals and as their scavenger, which is why when there are both antioxidants and PBN in a solution, a competition mechanism occurs and the effect of the interaction among PBN, lipid radicals, and antioxidants depends on the type of oil and antioxidant (Jerzykiewicz et al., 2013). The stable radicals produced by PBN spin-trapping are nitroxides with a spectra characterized by three hyperfine lines for the coupling between the electron spin (S = 1/2) and the nitrogen nuclear spin (I = 1) (Ottaviani et al., 2001).

The results obtained with ESR analysis are expressed as concentration of free radicals (μM) after 240 min of oxidation forced assay (Table 5.4.6.1 and 5.4.6.2) at 110 °C. The analysis was carried out by applying a temperature of 110 °C in order to have the same temperature applied for the OSI analysis. The concentration of free radicals is quantified on the basis of a calibration curve.

Table 5.4.6.1. Results of electron spin resonance (ESR)-forced oxidation assay after 20 min and 240 min for refined olive oil (ROO) and sunflower oil (SO) with cannabidiol (CBD). Data are presented as mean ± standard deviation and coefficient of variation (RSD%).

Sample	Concentration of Free Radicals after 20 min (μM)	RSD %	Concentration of Free Radicals after 240 min (μM)	RSD %
ROO	5.70 ± 0.25^{b}	4.47	76.94 ± 9.04^{a}	11.26
ROO-CBD 0.01%	10.39 ± 0.57^{a}	5.44	83.85 ± 5.45^{a}	6.50
ROO-CBD 0.1%	9.27 ± 0.31^{a}	3.36	70.98 ± 5.62^{a}	7.92
ROO-CBD 0.5%	6.50 ± 0.25^{b}	3.81	72.25 ± 4.13^{a}	5.72
SO	8.35 ± 0.22^{X}	2.66	17.91 ± 0.95^{X}	5.29
SO-CBD 0.01%	9.87 ± 0.00^{Y}	0.00	20.82 ± 0.53^{X}	2.57
SO-CBD 0.1%	$9.00 \pm 0.53^{X,Y}$	5.86	25.37 ± 2.27^{Y}	8.96
SO-CBD 0.5%	5.41 ± 0.24^{Z}	4.51	16.84 ± 0.25^{Z}	1.47

ROO = refined olive oil. ROO-CBD = refined olive oil with CBD, the percentage indicates the CBD/oil ratio. SO = sunflower oil**.** SO-CBD = sunflower oil with CBD, the percentage indicates the CBD/oil ratio. Different letters indicate statistically significant differences (Anova, Fisher's test LSD, $p < 0.05$).

Table 5.4.6.2. Results of ESR-forced oxidation assay after 20 min and 240 min for refined olive oil (ROO) and sunflower oil (SO) with α-tocopherol (AT) added. Data are presented as mean ± standard deviation and coefficient of variation (RSD %).

Sample	Concentration of Free Radicals after 20 min (μM)	RSD %	Concentration of Free Rad icals after 240 min (μM)	RSD %
ROO	3.10 ± 0.15^{a}	4.72	83.33 ± 4.56^{a}	5.48
ROO-AT 0.01%	3.19 ± 0.03^{a}	0.89	81.22 ± 6.04^{a}	7.43
ROO-AT 0.1%	1.27 ± 0.14^{a}	11.14	61.41 ± 5.82^{b}	9.48
ROO-AT 0.5%	0.55 ± 0.03^{b}	5.81	11.23 ± 0.28^{c}	2.52
SO	5.90 ± 0.27^{X}	4.55	19.21 ± 1.39^{X}	7.22
SO-AT 0.01%	6.66 ± 0.23^{Y}	3.43	22.61 ± 0.23^{Y}	1.00
SO-AT 0.1%	3.35 ± 0.28^{Z}	8.24	13.25 ± 0.74^{Z}	5.59
SO-AT 0.5%	2.08 ± 0.20^{W}	9.47	6.90 ± 0.53^{W}	7.64

ROO = refined olive oil. ROO-AT = refined olive oil with α-tocopherol, the percentage indicates the α-tocopherol /oil ratio. SO = sunflower oil**.** SO-AT = sunflower oil with α-tocopherol, the percentage indicates the α-tocopherol/oil ratio. Different letters indicate statistically significant differences (Anova, Fisher's test LSD, $p < 0.05$).

The results were also expressed as concentration of free radicals after 20 min of ESR-forced oxidation assay, since the first measurement still fell within the instrumental noise. The trends are similar to those found after 240 min (Table 5.4.6.1 and 5.4.6.2) with the exception of sunflower oil with 0.5% CBD, which showed a decrease in the concentration of free radicals after 20 min compared to sunflower oil alone; this marked decrease was not evident after 240 min (Table 5.4.6.1). Only the results after 240 min are commented upon in order to better highlight the trends observed.

The initial ESR values found for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of CBD (0.01%, 0.1%, and 0.5%) were 76.94 μM and 17.91 μM, respectively.

Refined olive oil with CBD added at the three concentrations showed higher values than the sunflower oil with CBD at the same concentrations. In the former they were between 70.98 μM and 83.85 μM, while in the latter they were between 16.84 μM and 25.37 μM (Table 5.4.6.1).

For refined olive oil with CBD added, the addition of 0.01% of this cannabinoid resulted in an increase in the concentration of free radicals compared to that obtained for the refined olive oil alone. In contrast, the concentration of free radicals decreased for samples of refined olive oil with 0.1 and 0.5% of CBD (Table 5.4.6.1).

For sunflower oil, the addition of 0.01% and 0.1% of CBD resulted in an increase in the concentration of free radicals compared to sunflower oil alone; the addition of 0.5% CBD led to a decrease in the concentration of free radicals (Table 5.4.6.1).

The initial ESR values for refined olive oil and sunflower oil used to prepare solutions with the three concentrations of α-tocopherol (0.01%, 0.1%, and 0.5%) were 83.30 μM and 19.21 μM, respectively (Table 5.4.6.2).

Refined olive oil with α-tocopherol showed higher values of free radicals than sunflower oil with α-tocopherol at the same concentrations; in the first case they were between 11.23 μM and 81.22 μM, while in the second they were between 6.90 μM and 22.61 μM (Table 5.4.6.2).

In refined olive oil with α-tocopherol, as the concentration of α-tocopherol increased the concentration of free radicals in the sample decreased.

The addition of 0.01% α-tocopherol to sunflower oil caused an increase in the concentration of free radicals, whereas at the other two concentrations the decrease in free radicals was directly proportional to the concentration of α-tocopherol added, compared to that for sunflower oil alone (Table 5.4.6.2).

5.4.7 DPPH• Radical Scavenging Activity Assay

DPPH• was used to evaluate the free radical scavenging effectiveness of antioxidants in different substances. Antioxidants are able to reduce the radical DPPH• to the yellow-colored diphenyl-picrylhydrazine. The method is based on the reduction of DPPH• in an alcoholic solution in the presence of a hydrogen-donating antioxidant because of the formation of the non-radical form DPPH-H in the reaction (Ak & Gülçin, 2008).

The samples analyzed showed some differences, and in particular in oils with α-tocopherol after 30 min. Although they had a lower absorbance than the initial measurement, they did not show a yellow color, but remained purplish. In contrast, samples with cannabidiol at a concentration of 0.5% after 30 min already showed a yellowish color (Figure 5.4.7.1).

The theoretical value of EC50 was calculated by taking into consideration the concentration of the oil, and the concentration of each sample was calculated based on the exact weight of the sample used to carry out the extraction.

The theoretical EC_{50} values for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of CBD, were 1.49 g/mL and 12.86 g/mL, respectively.

For refined olive oil, the addition of 0.01% CBD resulted in a decrease of the scavenging activity and an increase in EC_{50} compared to refined olive oil alone. On the contrary, the scavenging activity increased for samples of refined olive oil with 0.1% and 0.5% CBD (Figure 5.4.7.1).

The addition of CBD in sunflower oil, at the three concentrations, led to an increase in scavenging activity compared to sunflower oil alone; in fact, the decrease in EC_{50} values was directly proportional to the concentration of CBD.

The theoretical EC_{50} values for refined olive oil and sunflower oil, used to prepare solutions with the three concentrations of α-tocopherol, were 3.56 g/mL and 12.86 g/mL, respectively.

Refined olive oil with α-tocopherol showed that the addition of 0.01% α-tocopherol decreased the scavenging activity. However, addition of 0.01% and 0.5% α-tocopherol decreased the EC_{50}, and increased the scavenging activity (Figure 5.4.7.1).

The addition of α-tocopherol to sunflower oil led to an increase in the scavenging activity vs. sunflower oil alone; the decrease in EC_{50} was directly proportional to the concentration of α-tocopherol.

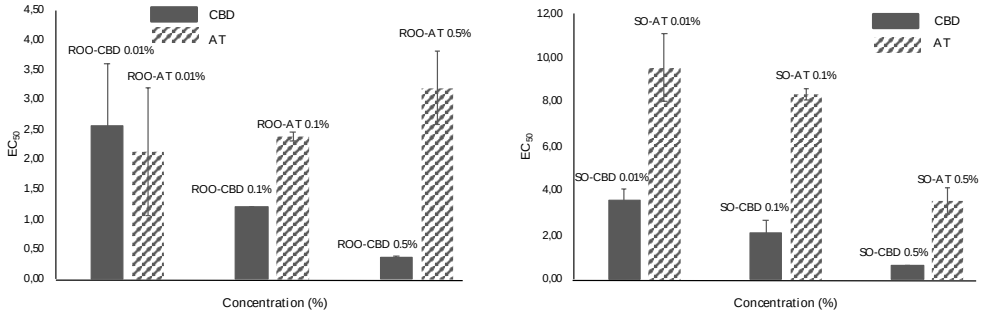

Figure 5.4.7.1. Differences in EC_{50} values of DPPH• obtained by adding CBD or α-tocopherol to refined olive oil (ROO) on the left (a), and to samples obtained by adding CBD and α-tocopherol to sunflower oil (SO) on the right (b). A lower value of EC50 corresponds to higher scavenging activity.

5.5 Discussion

Two refined oils were chosen since the refining process eliminates most of the antioxidant compounds that are naturally present in these matrices. Moreover, it was possible to compare two oils characterized by a different profile in saturated and unsaturated fatty acids: olive oil is mainly characterized by oleic acid, and sunflower by linoleic acid. In order to correctly and reproducibly compare the parameters related to oxidation in the solutions containing the active ingredients, the same parameters were also determined on the oil samples used as a matrix. Conjugated diene and triene systems, molecules related to a more advanced oxidative state (secondary to peroxides), were not evaluated because CBD absorbs at the same wavelengths, and would thus be an interferent in this analysis. Determination of the content of CBD and α-tocopherol and the recovery of these compounds all showed high values, meaning that both these active compounds dissolved in the oils evaluated.

Sunflower oil initially had a higher peroxide content than refined olive oil, which means that it had a more advanced state of oxidation. Moreover, it was noted that at all concentrations investigated, for both CBD and α-tocopherol, an increase in concentration of the active ingredient determined an increase in the peroxides value. The EU Commission Regulation Ec 702/2007 sets a maximum limit equal to 5 meq O_2/kg oil, but refers to refined olive oil and not to pharmaceutical grade refined olive oil (Ph Eur degree). For this reason, we referred to the limits established by European Pharmacopoeia (Ph Eur degree) and by Codex Alimentarius (FAO-WHO, CODEX ALIMENTARIUS) giving both a limit equal to 10 meq O_2/kg oil. The peroxide value of the initial refined olive oil sample, and of the same oil with CBD at 0.01%, 0.1%, and 0.5%, were all below this limit.

The peroxide value of the initial sunflower oil and the same oil with CBD added at 0.01% and 0.1% were lower than the limit fixed by the Codex Alimetarius for refined oils, equal to 10 meq O_2/kg oil, while the value in the sunflower oil sample with 0.5% CBD was above this limit.

Regarding refined olive oil, both Pharmacopoeia and the Codex Alimentarius specify a limit of 10 meq O_2/kg of oil, and the peroxide value of refined oil with and without α-tocopherol added at 0.01%, 0.1%, and 0.5% were all below this limit.

The peroxide value of sunflower oil alone or with α-tocopherol at 0.01%, 0.1%, and 0.5% was higher than the limit established by Codex Alimetarius for refined oils, or 10 meq O_2/kg oil; although this value is above the limit, it was nevertheless used for experimentation since it was preferred to not carry out bleaching treatments, which can remove oxidized polar compounds, in order to not modify the oil native matrix composition.

The values relating to free acidity of sunflower oil and refined olive oil are lower than the limit established by Codex Alimetarius for refined oils of 0.6 mg KOH/g; moreover, the value for refined olive oil is also lower than the limit established by the Pharmacopoeia, namely 0.3 mg KOH/g oil.

The values relative to acidity of sunflower oil with CBD or α-tocopherol and refined olive oil with CBD or α-tocopherol at all the concentrations investigated were lower than the limit of 0.6 mg KOH/g established by Codex Alimetarius for refined oils; moreover, the value for refined olive oil with CBD or α-tocopherol was also lower than the Pharmacopoeia limit of 0.3 mg KOH/g oil.

The OSI-time value found for refined olive oil was much higher than that for sunflower oil, which is well-known and understandable given the different characteristic fatty acid compositions of the two oils. In fact, sunflower oil is an oil with a greater intrinsic presence of unsaturated fatty acids, which are much more sensitive to oxidation.

Regarding the oily matrices with CBD added, the results showed a decrease in OSI-time with the addition of the active ingredient, which was increased at higher concentrations. These data agree with what emerged from the determination of peroxides.

Regarding α-tocopherol added to sunflower oil, the increases in OSI-time values were directly proportional to the concentration. As far as refined olive oil is concerned, we recorded a decrease in OSI-time at the two lowest concentrations of α-tocopherol, while at 0.5% α-tocopherol the OSI-time was very similar to the initial value. In fact, according to the literature, the activity of α-tocopherol is slightly antioxidant and sometimes pro-oxidative (Wagner & Elmadfa, 2000).

The data obtained from the ESR analysis for refined olive oil supplemented with CBD show that this compound may have a slight pro-oxidant effect at the lowest concentration (corresponding to 0.01% of CBD). However, at the other two concentrations investigated, CBD had antioxidant action, causing a decrease in free radicals.

The DPPH• radical scavenging activity assay showed that refined olive oil with CBD at a concentration of 0.01% has a lower scavenging capacity than refined olive oil without additions. Moreover, when increasing the CBD concentration, the DPPH• radical scavenging activity assay showed a marked increase in scavenging capacity, emphasizing its antioxidant power, in agreement with what has been found in the literature, CBD is able to suppress DPPH• (Hayakawa et al., 2007); this trend agrees with what was detected by the ESR assay. The results of the ESR test on sunflower oil with CBD show that only 0.5% CBD had an antioxidant effect. These results differ from what emerged from the DPPH• radical scavenging activity assay, where at each concentration tested there was a greater scavenging activity compared to sunflower oil without additions. It is essential to highlight that the EC50 of the DPPH• value considered is not a kinetic parameter and, therefore, does not express antioxidant or anti-radical activity in a lipidic model system during its oxidation, as also indicated by Foti, 2015. In fact, in the DPPH• radical scavenging activity assay, many compounds react rapidly with DPPH• and more slowly with ROO•, also according to the solvent used (Foti, 2015).

The results of the ESR test on refined olive oil with α-tocopherol showed a marked antioxidant action of this compound, especially at concentrations of 0.1 and 0.5%. At a concentration of 0.01%, although there was a decrease in the concentration of free radicals, the value was not significantly different from those recorded for refined oil, since it falls within the standard deviation. These results are consistent with the findings found in the DPPH• radical scavenging activity assay, with the only difference being that at the highest concentration (0.5% of α-tocopherol) the scavenging activity does not show a significant difference compared to that of refined olive oil.

The ESR data for sunflower oil with α-tocopherol are consistent with the results of the DPPH• radical scavenging activity assay, except for the sample with 0.01% α-tocopherol, wherein a slight increase in scavenging activity was noted compared to sunflower oil without additions. The data from the DPPH• radical scavenging activity assay showed greater scavenging activity of CBD compared to α-tocopherol.

No correlation was found between the peroxide value and antioxidant capacity, as measured by the DPPH• radical scavenging activity assay. In fact, the peroxide value increased with an increase in the concentration of both CBD and α-tocopherol, while a lower theoretical value of EC_{50} was found for refined oil and sunflower oil with CBD, at all concentrations investigated, and for sunflower oil with α-tocopherol at all concentrations tested. On the other hand, regarding refined oil added with α-tocopherol, the theoretical EC_{50} value remained stable at α-tocopherol concentrations of 0.01% and 0.1%, while it underwent a slight increase at an α-tocopherol concentration of 0.5%.

The lack of a clear and definite correlation between these two assays for some oily matrices has also been reported in previous studies (Sielicka et al., 2014; Kemerli-Kalbaran & Ozdemir, 2019).

Moreover, the decrease in the induction period (OSI-time) in samples of refined olive oil with α-tocopherol, especially at 0.01% and 0.1%, agrees with previous literature data, according to which analysis of the induction period carried out at 110 ° C does not have a positive correlation with the total tocopherol content (Baldioli et al., 1996; Sabolová et al., 2017).

5.6 Conclusions

For the two model systems of edible oils considered, which differed in terms of fatty acid composition and initial oxidative state, several interesting aspects were found. In particular, an increase in the peroxide value, perhaps linked with oxygenation because of the preparation/mixing of the oily solutions, was observed; furthermore, a certain additional pro-oxidant effect of CBD, confirmed by measuring the oxidation parameters, appeared evident. This pro-oxidant activity was also confirmed by the reduction of the OSI-time analysis, passing from 23.58 ± 0.32 (ROO) to 17.28 ± 0.18 h (ROO with 0.5% CBD) and from 4.93 ± 0.04 (SO) to 3.98 ± 0.04 h (SO with 0.5% CBD). In terms of free

radicals, the ESR assay did not show a relevant decrease in their concentration because of the addiction of CBD, with corresponding values of 76.94 ± 9.04 μM (for ROO) and 72.25 ± 4.13 μM (ROO with 0.5% CBD), and extremely similar for the model system made by the pure sunflower oil (SO, 17.91 ± 0.95 μM) and with the addition of 0.5% CBD (16.84 ± 0.25 μM). On the contrary, the DPPH• assay showed a higher scavenging capacity for CBD than for α-tocopherol, likely because of the presence of two hydroxyl groups in the CBD molecule. The addition of 0.1% and 0.5% α-tocopherol, even in the presence of an increase in the number of peroxides, exerted higher antioxidant activity in the more unsaturated system (SO), as shown by the forced oxidation test. In fact, OSI-time increased from 4.15 ± 0.07 to 6.28 ± 0.11 h for sunflower oil with 0.5% α-tocopherol, while the OSI-time of the refined olive oil substantially did not change (form 28.35 ± 0.28 to 28.23 ± 0.81 h). α-Tocopherol, at a concentration of 0.01%, did not have any protective effect, in either of the model systems. It is interesting to notice that the measure of the resistance at the forced oxidation (OSI-time analysis) can be interpreted in a certain coherence with that of the free radicals (by ESR assay), while the data of DPPH• assay showed a different trend. In other words, the higher scavenging activity of CBD, measured by DPPH•, does not seem to be related with a greater oxidative stability of the model system when added to it. This can be due to the different reaction kinetics of the species considered with respect to the DPPH• and of the peroxide radical forms ROO• present in the matrix. As highlighted in literature, the use of this (DPPH•), or similar scavenging test, to compare the antioxidant activities of different molecules is questionable. To provide an exhaustive framework, it will necessary to thoroughly investigate the conditions and concentration at which CBD shows an antioxidant or a pro-oxidant effect, knowing that the use of a single test could be misleading. Regarding the two model oily solutions considered herein, more or less unsaturated, it is essential to note that the CBD did not show a protective antioxidant action when added up to 0.5%. This evidence is useful to establish the shelf-life of oily solutions, very common and diffuse in the market, containing CBD and to also correctly formulate oily foods, medicines, or supplements containing CBD. Concerning shelf-life, it will be necessary to study the information given by the ESR-forced oxidation assay, such as coupling constants, type of radicals, and interaction among PBN, lipid radicals, and added molecules, to see if this test could be effectively and reliably use as predictive.

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Chapter 6

Sensory and instrumental characterization of cold-pressed hemp seed oils

6.1 Instrumental characterization of 13 commercial hemp seed oils

6.1.1 Details of the publication based on Paragraph 6.1

Title: Instrumental characterization of 13 commercial hemp seed oils

Authors: Matilde Tura[a], Mara Mandrioli[a], Enrico Valli[a], Tullia Gallina Toschi[a]

a Department of Agricultural and Food Sciences, Alma Mater Studiorum-University of Bologna, Viale Fanin 40, 40127 Bologna, Italy

Corresponding author: Enrico Valli -enrico.valli4@unibo.it

Submitted to: *Food Research International*

Abstract

Hemp seed oil, particularly if cold-pressed, can be a valuable source from a nutritional and sustainability point of view. Hemp seed show an optimal ratio of ω6:ω3 fatty acids, equal to around 3:1, and several minor components, such as terpenes, cannabinoids and tocopherols. Moreover, they are considered a co-product of hemp cultivation for textile sectors or for extracting cannabinoids. The present study is aimed to characterize 13 hemp seed oils (HSOs), also providing useful data about the bioactive compounds occurring in this product. Samples have been collected from the market, and the prices were from 14.20 to 55.96 €/L; 12 HSOs out of 13 were claimed as cold-pressed in the label. Firstly, the hydrolytic and oxidative states were investigated, according to the standards provided by the Codex Alimentarius: HSOs showed a wide range of free acidity values (from 0.89±0.02 to 4.58±0.03 mg KOH/g of oil) and peroxide values (ranging from 3.97±0.25 to 23.89±0.74 mEqO2/kg of oil). The analyzed HSOs highlighted a ratio of ω6:ω3 ranging from 2.60 to 3.67. Samples presented γ-tocopherol from 593.88 to 967.47 mg/kg and a wide range in the content of chlorophylls and carotenes from 0.78 mg/kg of oil to 75.73 mg/kg of oil and from 2.53 to 33.93 mg/kg of oil, respectively. Thus, probably indicating different extraction conditions (production plants, extraction temperatures and time). Cannabinoids were also present, in particular as CBDA (from 4.25 to 91.64 mg/kg), Δ9-THC (up to 5.29 mg/kg) and THCA (up to 5.00 mg/kg). Moreover, the analysis of the volatile profile highlighted the presence of some specific cannabis terpenes, such as α-pinene and β-pinene, possible freshness volatile markers.

Keywords: hemp seed oil, oxidative state, cannabinoids, vegetable oil, instrumental characterization, fatty acids, quality

6.1.2 Introduction

Industrial hemp (Cannabis sativa L.) is an annual herbaceous plant that produces small fruits botanically called “akenes” (Citti et al., 2018; Moczkowska et al., 2020), and it is economically a valuable source of fiber, foods and medicine (Moczkowska et al., 2020; Özdemir et al., 2021). Recently, hemp has regained much attention, mainly because of its nutritional and pharmaceutical values (Moczkowska et al., 2020); Özdemir et al., 2021). The cultivation of hemp was prohibited after the 1930s around the world, due to the presence of the psychoactive cannabinoid, Δ9-tetrahydrocannabinol (Δ9-THC) (Özdemir et al., 2021). At now, in Europe, only some varieties with a low content of Δ9-THC (<0.2%) are allowed to be cultivated as they are certified as non-drug variety, and they are usually called “industrial hemp” (REGULATION (EU) No 1307/2013; Montserrat-De La Paz et al., 2014; LEGGE 2 Dicembre 2016, n. 242; Moczkowska et al., 2020). Commonly, industrial hemp cultivars are characterized by a high content of cannabidiolic acid (CBDA), the acidic precursor of cannabidiol (CBD), for which it has been recognized a wide range of biological proprieties, such as anticonvulsive, anti-epileptic and antimicrobial (Izzo et al., 2020). Hemp seeds are a source of nutrients and minor compounds with biological properties; in particular, they contain 25-35% of lipids, 20-25% of proteins, 20-30% of carbohydrates, 10-15% of fiber and also minor components, such as vitamins (A, C and E) and minerals (Moczkowska et al., 2020). Moreover, among the different parts of the hemp plant, in Italy the seeds are the only ones authorized for food applications and as derivate products, such as hemp seed oil and flour (Ministero Della Salute, 2019; Spano et al., 2020). The cannabinoids are present in hemp seed only as cross-contamination due to the harvest and transformation processes (Spano et al., 2020). Literature regarding hemp seeds and their derivate products has recently increased (Spano et al., 2020). The high nutritional value of hemp seed oil is due to the presence of a high content of polyunsaturated fatty acids (PUFAs), in particular, linoleic acid (C18:2 ω-6) and α-linolenic acid (C18:3 ω-3) in a concentration of around 50% and 15-20%, respectively; thus, resulting in a perfectly balanced ratio of ω-6:ω-3, optimal for human nutrition (Griffin et al., 2006; Spano et al., 2020). Also, of no less importance is the presence of stearidonic acid (18:4 n-3) in a concentration range of around 0.5–2% (Mikulcová, Kašpárková, Humpolíček, & Buňková, 2017). The high content of PUFAs results in a high oxidative instability of hemp seed oil, which is among the most critical factors affecting the shelf-life of oils and fats. Oxidation can affect nutritional value, food colour and flavour, leading to off-flavours and unpleasant organoleptic characteristics (Izzo et al., 2020). Approximately 1.5-2% of the hemp seeds represent the unsaponifiable fraction, an essential source of minor components. In fact, since hemp seed oil is usually obtained by cold-pressing, a high amount of minor compounds is extracted with the oil (Montserrat-De La Paz et al., 2014; Liang et al., 2015). The presence of

antioxidant minor components, such as tocopherols, increases the nutritional value, the healthy proprieties of hemp seed oil and its shelf-life (Spano et al., 2020). For example, tocopherols act both as natural antioxidants against oxidative deterioration of the oil, but also as vitamin E in human body (Matthäus & Brühl, 2008). On the contrary, the oxidation process could be catalysed by the presence of a substantial amount of chlorophyll in hemp seed oil, also extracted by the cold pressing process (Liang et al., 2018). In fact, as a photosensitive pigment, chlorophyll is susceptible to photooxidation, acting as a pro-oxidant in oils and oily products. For this reason, a great content of chlorophyll could increase the susceptibility to oxidation, leading to rancidity (Liang et al., 2018) and quality deterioration of hempseed oil, which, therefore, necessitates storage in dark or opaque bottles (Aachary, Liang, Hydamaka, Eskin, & Thiyam-Holländer, 2016). Although hemp seed oils and hemp-based oily product are currently available on the market, the legislative framework for those products in the European Union is ambiguous, due to the absence of a regulation regarding the quality requirements for their marketing (Izzo et al., 2020). Only few studies have examined the characteristics of commercial hemp seed oils (Citti et al., 2018); Citti et al., 2019; Izzo et al., 2020; Jang et al., 2020; Kitamura et al., 2020; Spano et al., 2020), and little is known on the composition and quality of the hemp seed oils available on the market for consumers. On this basis, the present work aims at the chemical characterization of 13 commercial hemp seed oils, purchased them directly from the market. This is very relevant in order to evaluate their quality, including the oxidative state, at the time of purchase.

6.1.3 Materials and methods

6.1.3.1 Samples

A total of 13 samples of different commercial hemp seed oils were purchased from supermarkets, small speciality stores, and online shops. A more detailed description of the samples is reported in Table 6.1.3.1.1. After the collection and before the analyses, the samples were stored in the laboratory at room temperature in a place protected from direct light. The determination of free acidity, the peroxide value, the OSI (Oxidative Stability Index) test and the determination of conjugated diene and triene systems were carried out on each sample. Moreover, the composition of HSOs was also investigated by determining the cannabinoids composition, the chromatographic lipid profile, the fatty acid composition, the tocopherol content, the chlorophylls and carotenes composition and the volatile profile. Three independent analytical replicates were performed for each sample.

Table 6.1.3.1.1 Production, geographical and economic characteristics of the HSOs examined.

Sample	Seed cultivation*	Technological information*	Seed origin*	Sample origin*	Price per liter (€/L)
1	Organic	Cold-pressed and filtered, without the use of solvents	UE-Extra UE	Italy	14.20
2	Organic	Cold-pressed	Extra UE	Italy	28.52
3	Organic	Cold-pressed, not refined	Extra UE	Italy	26.40
4	Organic	Cold-pressed	UE	Italy	44.00
5	Organic	Cold-pressed	UE-Extra UE	Germany	46.00
6	Organic	Cold-pressed	China	Germany	55.96
7	*n.s.*	Cold-pressed, not refined	Poland	Poland	36.68
8**	*n.s.*	Cold-pressed, not refined	*n.s.*	Poland	30.94
9	Organic	Cold-pressed	UE	Italy	15.60
10	Organic	Cold-pressed and filtered, without the use of solvents	UE	Italy	19.96
11	Organic	Cold-pressed	UE	Netherlands	51.96
12	Organic	Cold-pressed	Extra-UE	Italy	26.00
13	Organic	*n.s.*	Italy	Italy	36.00

*Information reported in the label

**Sample 8 was the only one sold in a bottle of 500 mL, the other HSOs were purchased in bottles of 250 mL

n.s. not specified

6.1.3.2 Chemicals and reagents

All chemicals used were of analytical grade. Methanol CAS 67-56-1, chloroform CAS 67-66-3, acetonitrile CAS 75-05-8, water CAS 7732-18-5, orthophosphoric acid CAS 7664-38-2, potassium hydroxide CAS 1310-58-3, ethanol CAS 64-17-5, diethyl ether CAS 60-29-7, phenolphthalein CAS 77-09-8, acetic acid CAS 64-19-7, 2-propanol CAS 67-63-0, hexane CAS 110-54-3 and potassium iodide CAS 7681-11-0 were purchased from Sigma-Aldrich (St. Louis, MO, USA). While sodium thiosulfate 0.1 mol/L CAS 10102-17-7, starch CAS 9005-84-9 and isooctane CAS 540-84-1 were purchased from Carlo Erba Reagents (Milano, Italy).

6.1.3.3 Free acidity

The free acidity was determined by applying the method reported by the Codex Alimentarius (Codex Stan 19-1999). Results are expressed as mg of KOH needed to neutralize the free acids present in 1 gram of oil.

6.1.3.4 Peroxide value

The PV was determined according to the NGD C35-1976 method (NGD method C35), performing an iodometric titration. Results are expressed as mEq of active oxygen per kg of oil.

6.1.3.5 Spectrophotometric investigation in the ultraviolet region (K_{232} and K_{270})

The dienoic and trienoic conjugated fatty acid derivates were analysed by following the method described in ISO 3656:2011. The spectrophotometric analysis was performed using a Jasco (Tokyo, Japan) dual-beam spectrophotometer model V-550 UV-Vis. Quartz cuvette with an optical path of 10 mm was used. The spectrophotometric investigations were performed at 232 nm to determine diene conjugated systems and at 270 nm for the triene conjugated systems. A regular check was carried out for the accuracy and reproducibility of the absorbance and wavelength scales as well as for stray light.

6.1.3.6 Oxidative Stability Index (OSI)

The analysis was performed using an oxidative stability instrument (OMNION OSI-8 Decatur, IL, USA), by following the method described by the AOCS Official Method Cd 12b-92. 5 g of oil were weighed in a glass tube and heated to 110°C in the presence of a continuous airflow (150 mL/min). The samples are subjected to the accelerated oxidation test in standardized conditions; the induction period is measured in hours (Carrasco-Pancorbo et al., 2005).

6.1.3.7 Cannabinoids profile

The determination of cannabinoids was performed by using a HPLC Cannabis Analyzer for Potency Prominence-i LC-2030C (Shimazu, Kyoto, Japan) equipped with a reverse phase C18 column (Nex-Leaf CBX Potency 150 × 4.6 mm, 2.7 μm with a guard column Nex-Leaf CBX 5 × 4.6 mm, 2.7 μm), and an UV detector. The acquisition software was LabSolutions version 5.84 (Shimazu, Kyoto, Japan). 0.5 g of hemp seed oil were weighed into a 10 mL volumetric flask and were brought to volume with isopropanol. Samples were placed 10 minutes in an ultrasonic bath. Then, they were filtered with a 45 μm nylon filter. Samples were transferred into a HPLC vial, and the analysis was performed according to the method proposed by Mandrioli et al., 2019. Briefly, the detection was carried out at 220 nm, and gradient elution was used at flow rate of 1.6 mL/min with eluent mixture as follow: water + 0.085% phosphoric acid (A), acetonitrile + 0.085% phosphoric acid (B). Gradient elution: 70% of B up to 3 min, 85% of B to 7 min, 95% of B to 7.01 up to 8.00 min, and 70% of B up to 10 min. The quantification was performed by injecting a cannabinoids standard mix in the same conditions and by the construction of calibration curves for each cannabinoid. It was used the standard

mixture of phytocannabinoids 0.1% in acetonitrile: Cannabidiolic acid (0.01%) CAS 1244-58-2, cannabigerolic acid (0.01%) CAS 25555-57-1, cannabigerol (0.01%) CAS 25654-31-3, cannabidiol (0.01%) CAS 13956-29-1, tetrahydrocannabivarin (0.01%) CAS 31262-37-0, cannabinol (0.01%) CAS 521-35-7, tetrahydrocannabinolic acid (0.01%) CAS 23978-85-0, D-9-tetrahydrocannabinol (0.01%) CAS 1972-08-3, D-8-tetrahydrocannabinol (0.01%) CAS 5957-75-5, cannabichromene (0.01%) CAS Number 20675-51-8, purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Calibration curves used were: CBDA y=19141x-3707.2 (r2=0.9989), CBD y=12305x+235.25 (r2=0.9997), Δ9-THC y=15487x-1608.5 (r2=0.9997), THCA y=16462x+207.69 (r2=1).

6.1.3.8 Total lipid profile

This method allows the determination of the main lipid classes (free fatty acids (FFA), monoacylglycerols (MAG), free sterols (STE), diacylglycerols (DAG), esterified sterols (EST) and triacylglycerols (TAG). The different lipid classes were determined by following the method proposed by Gallina Toschi et al. (2014) and Tappi et al. (2020), using a GC-FID equipment. In particular, a fused silica capillary column (SE-52 MEGA, 10 m × 0.32 mm i.d. × 0.1 μm film thickness; Legnano, MI, Italy), coated with 95% dimethyl- and 5% diphenylpolysiloxane, was used. The temperature was programmed from 100 to 355°C at a rate of 5°C/min; the final temperature was kept for 20 minutes. The injector and FID temperatures were set at 355°C. The injection was performed in the split mode (1:25), and helium at a flow of 2,02 mL/min was used as the carrier gas. The different lipid classes were identified using different commercial standards. In particular, the standard mixture free fatty acids (GLC 406) were purchased from NuChek (Elysian, MN, USA). Triolein (≥99.0%, CAS 122-32-7), tripalmitin (≥99.0%, CAS 555-44-2), tristearin (≥99.0%, CAS 555-43-1), 1,3-diolein (≥99.0%, CAS 2465-32-9), 1,2(3)-dipalmitin (≥99.0%, CAS 26657-95-4), cholesteryl palmitate (≥97%, CAS 601-34-3), 1-oleoyl-rac-glycerol (≥99.0%, CAS 111-03-5), methyl tridecanoate (≥99.5%, CAS 1731-88-0), 5α-cholestan-3β-ol (dihydrocholesterol, ≥95%, CAS 80-97-7), cholesterol (≥99%, CAS 57-88- 5), stigmasterol (≥95%, CAS 83-48-7), β-sitosterol (≥95%, CAS 83-46-5) and betulinol (≥98%, CAS 473-98-3) were purchased from Sigma (St. Louis, MO, USA). Results were expressed as area percentages.

6.1.3.9 Fatty acid composition

The determination of the fatty acid composition was performed using a GC-FID instrument, particularly a GC8000 series (Fisons Instruments, Milan, Italy), interfaced with a computerized system for data acquisition (Chromcard Data System, ver. 2.3.1, Fisons Instruments). First, alkaline treatment was used by mixing 0.01 g of oil dissolved in 500 μL of *n*-hexane and 20 μL of 2 N

potassium hydroxide in methanol. The mixture was centrifuged, and the supernatant containing the fraction of interest was taken (BS EN ISO 12966-2:2017). A column RTX 2330 fused-silica column (30 m × 0.25 mm × 0.2 μm film thickness) (Restek, Bellefonte, USA) coated with 90% biscyanopropyl and 10% cyanopropylphenylpolyasiloxane was used. Oven temperature was programmed from 60°C to 240°C at a 4°C/min rate kept for 10 min. The injector and detector temperatures were both set at 240°C. Helium was used as carrier gas at a constant flow of 1.25 mL/min. The split ratio was 1:30. To identify peaks, retention times were compared with those of authentic reference compounds (reference standards GLC463 from Nu-Chek Prep. Inc., Elysian, MN, USA) injected under the same analytical conditions. Quantification was carried out with the internal standard method, using tridecanoic acid (CAS number 638-53-9, Sigma-Aldrich, St. Louis, Missouri, USA) as internal standard. Results are expressed as mg I.S./100 g of oil.

6.1.3.10 Tocopherols content

The determination was performed by using liquid chromatography coupled with a diode-array detector (HPLC-DAD). 0.5 g were solubilized in isopropanol, and 20 μL were injected into an RP-HPLC system equipped with a quaternary pump model HP 1260 and diode-array detector; the software for data processing was Chemstation for LC3D (Agilent Technologies, Palo Alto, CA, USA). The instrument was equipped with a column Cosmosil π NAP 150 mm × 4,6 mm Thermo Fisher, 5 μm (Nacalai-Tesque, Kyoto, Japan). The mobile phase and the elution gradient were the same reported by UNI/TS 11825:2021. The diode-array detector was set up at a wavelength of 292 nm. Quantification was carried out using calibration curves of α- and γ-tocopherols (CAS numbers 10191-41-0 and 54-28-4, respectively; Sigma-Aldrich, Missouri, USA), which were constructed with the external standard method, injecting solutions of known concentration in the range of 0.5 ppm to 50 ppm. The calibration curves were y=8.2165x-1.1032 (r^2=0.999) for α-tocopherol and y=10.226x-2.5615 (r^2=0.9997) for γ-tocopherol. δ-tocopherol was identified by using a standard while it was quantified by the use calibration curve of α-tocopherol.

6.1.3.11 Chlorophylls and carotenes content

The method used to determine chlorophylls and carotenes was the one adopted by Ward et al. (1994), that is the same reported in ISO 10519 and ISO 17932 with slight modifiications. Briefly, a sample quantity of 1 to 3 g of oil was dissolved in 10 mL of 3:1 v/v isooctane-ethanol mixture, the sample was filtered and the absorbance measured at 625, 665,705 and 446 nm in a cuvette of 1 cm quartz using a double-beam UV − visible spectrophotometer (JASCO model V-550, JASCO International, Tokyo, Japan). The reading was carried out against a blank consisting of triolein in solution with

isooctane-ethanol 3:1 v/v. The calculation of the chlorophyll and carotene content, expressed as β-carotene, was given by the following formulas:

Chlorophyll

$$Chl\ \left(\frac{mg}{kg}\right) = \frac{(k * Acorr * V)}{m * l}$$

Where:

Acorr (the corrected absorbance) is equal to A665-(A705+A625)/2;

A665 is the absorbance at 665 nm;

A705 is the absorbance at 705 nm;

A625 is the absorbance at 625 nm;

k is a constant which is equal to 13;

l is the path length, in centimetres, of the optical cell;

m is the mass expressed as grams of the test portion;

Carotene

$$Carotene\ \left(\frac{mg}{kg}\right) = \frac{383 * A}{l * \rho}$$

Where:

383 = 2 610 is the percentage solution extinction coefficient of β-carotene in isooctane at 446 nm;

A is the absorbance,

l is the path length, in centimetres, of the cell;

ρ represents the concentration expressed as grams per 100 mL used for absorption measurement.

6.1.3.12 Volatile compounds profile

This determination was performed by solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME/GC–MS). Volatile compounds were detected by quadrupolar mass-selective spectrometry (in the 30-350 amu mass range) coupled with GC by using a GCMS-QP2010 (Shimadzu Co.) equipped with an autosampler AOC-5000 Plus (Shimadzu Co.). The method reported by Bendini et al. (2015) was followed, with some modifications. Briefly, 2 g of oil were weighed into 20 mL vial. The sample has been conditioned for 20 minutes at 40°C and, after the conditioning, a divinylbenzene/carboxen/polydimethylsiloxane fiber (50/30μm, 2 cm long; Supelco Ltd, Bellefonte, PA, USA) was exposed to the sample headspace for 40 minutes and desorbed for 5 minutes at 260°C in the injector with a split ratio of 1:10. Analytes were separated on a ZB-WAX capillary column (30 m x 0.25 mm ID, 1.0 μm film thickness) supplied by Phenomenex (Castel Maggiore, Bologna, Italy). The oven temperature was held at 40°C for 10 min and increased to 200°C at 3°C min−1. Then, the

temperature increased to 250°C at 20°C min−1 and remained stable for 3 minutes. Helium was used as a carrier gas with a flow of 0.75 mL min−1. Peak identification was tentatively based on comparing mass spectrum data with spectra present in the National Institute of Standards and Technology library 2008 version (NIST®08), and only identifications that matched more than 90% were considered. Results are expressed in area x103/g of oil.

6.1.3.13 Statistical analyses

All the analytical determinations were performed on three replicates. Data are expressed as mean ± standard deviation (SD). One-way ANOVA (Tukey's HSD $p<0.05$) using XLSTAT software version 2018 (Addinsoft, New York, USA) was applied for the evaluation of statistically significant differences in the results among the 13 hemp seed oil samples. Principal component analysis (PCA) was performed on selected variables in order to graphically represent correlations. On the same variables also, Pearson Correlation matrix was carried out in order to highlight the statistically significant correlations.

6.1.4 Results and discussion

6.1.4.1 Quality parameters of HSOs

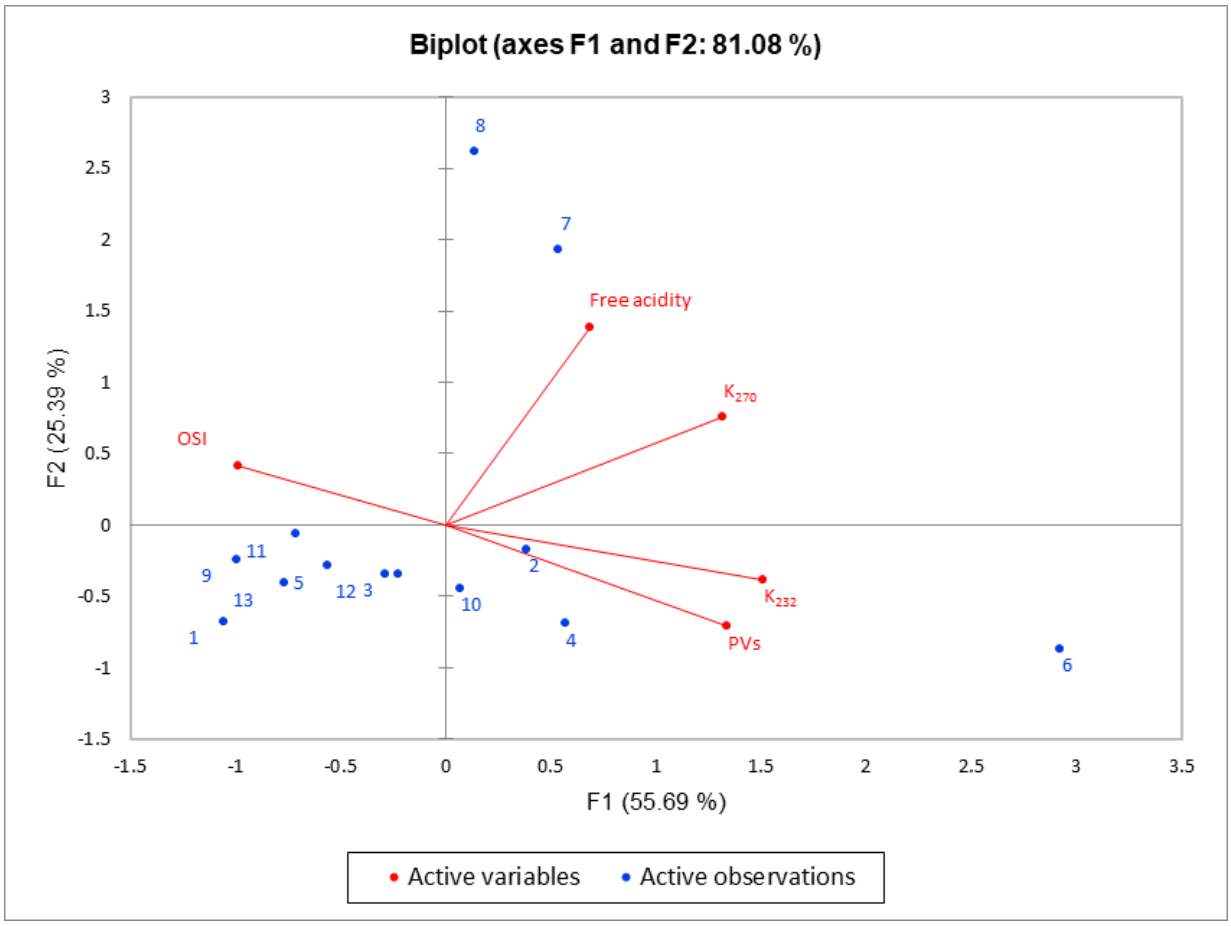
The hydrolytic and oxidative states of the 13 HSO samples were evaluated, and results are reported in Table 6.1.4.1.1.

Table 6.1.4.1.1. Results of free acidity, peroxide value, spectrophotometric investigation in the ultraviolet (K_{232} and K_{270}) and OSI time of the 13 hemp seed oil samples. Results are expressed as mean±standard deviation of three independent analytical replicates. Different letters in the same column indicates statistically significant differences (ANOVA Tukey's HSD test, $p\leq0.05$).

Sample	Free acidity (% of oleic acid)	Free acidity (mg KOH/g of oil)	PV ($mEqO_2$/kg of oil)	K_{232}	K_{270}	OSI time (hours)
1	0.70 ± 0.01^{f}	1.38 ± 0.02^{f}	5.91 ± 0.39^{d}	1.38 ± 0.15^{h}	0.16 ± 0.01^{g}	$4.82\pm0.21^{d,e}$
2	1.03 ± 0.03^{d}	2.04 ± 0.06^{d}	11.93 ± 0.51^{b}	3.24 ± 0.23^{b}	$0.38\pm0.02^{c,d}$	$5.83\pm0.37^{b,c}$
3	0.71 ± 0.00^{f}	1.40 ± 0.00^{f}	8.38 ± 0.31^{c}	$2.37\pm0.21^{d,e}$	$0.34\pm0.04^{d,e}$	$5.42\pm0.31^{c,d}$
4	$0.61\pm0.04^{g,h}$	$1.21\pm0.08^{g,h}$	7.98 ± 0.68^{c}	3.17 ± 0.20^{b}	$0.42\pm0.02^{b,c}$	$3.77\pm0.10^{f,g}$
5	0.56 ± 0.01^{h}	1.10 ± 0.02^{h}	4.40 ± 0.15^{f}	$1.84\pm0.05^{f,g}$	$0.34\pm0.01^{d,e}$	$4.77\pm0.06^{d,e}$
6	1.25 ± 0.01^{c}	2.49 ± 0.02^{c}	23.89 ± 0.74^{a}	5.02 ± 0.15^{a}	0.62 ± 0.03^{a}	3.37 ± 0.29^{g}
7	2.31 ± 0.02^{a}	4.58 ± 0.03^{a}	$4.65\pm0.15^{e,f}$	$2.39\pm0.11^{d,e}$	0.47 ± 0.00^{b}	$4.32\pm0.18^{e,f}$
8	2.15 ± 0.03^{b}	4.27 ± 0.06^{b}	3.97 ± 0.25^{f}	$2.02\pm0.07^{e,f}$	0.59 ± 0.01^{a}	$6.32\pm0.28^{a,b}$
9	0.45 ± 0.01^{i}	0.89 ± 0.02^{i}	6.15 ± 0.15^{d}	$1.87\pm0.10^{f,g}$	$0.30\pm0.01^{e,f}$	6.72 ± 0.12^{a}
10	0.94 ± 0.02^{e}	1.87 ± 0.03^{e}	11.87 ± 0.13^{b}	$2.54\pm0.19^{c,d}$	$0.32\pm0.00^{e,f}$	$5.27\pm0.23^{c,d}$
11	$0.66\pm0.01^{f,g}$	$1.31\pm0.02^{f,g}$	$5.65\pm0.38^{d,e}$	$1.87\pm0.10^{f,g}$	$0.33\pm0.01^{d,e}$	$5.83\pm0.32^{b,c}$

12	0.45±0.01^{i}	0.90±0.03^{i}	7.78±0.51^{c}	2.82±0.09^{b,c}	0.39±0.03^{c,d}	6.42±0.36^{a,b}
13	0.66±0.02^{f,g}	1.31±0.04^{f,g}	5.98±0.26^{d}	1.58±0.05^{g,h}	0.26±0.00^{f}	4.98±0.25^{d,e}

Free acidity represents an essential index both to evaluate the quality of edible oils and for their resistance to oxidation, also during storage. In particular, the free acidity of HSOs could be affected by the harvest type (mechanical or by hand) as well as by the post-harvesting processing, such as drying and storage conditions (Calzolari, Rocchetti, Lucini, & Amaducci, 2021). The Codex Alimentarius (Codex Stan 19-1981) indicates for cold-pressed vegetable oils a maximum of free acidity equal to 4 mg KOH/g of oil (equal to around 2% expressed as oleic acid). Only two samples showed value higher than this limit (samples 7 and 8). The determination of peroxide value is commonly used to assess the oxidative state of oils as it represents one of the measurement tests for the primary oxidation products (Barriuso, Astiasarán, & Ansorena, 2013). According to the Codex Alimentarius (Codex Stan 19-1981) the maximum peroxide value for vegetable oils not covered by individual standards is 15 mEqO2/kg of oil. It was found that only one sample (sample 6) showed a peroxide value higher than this limit (23.89±0.74 mEqO2/kg of oil), while the others were all below 11.93±0.51 mEqO2/kg of oil. Previous studies investigated the peroxide values of HSOs (Liang et al., 2018; Rapa et al., 2019; Izzo et al., 2020; Moczkowska et al., 2020), reporting results similar to our findings. K_{232} and K_{270} were evaluated to investigate the conjugated diene and triene systems, respectively (Spano et al., 2020). Those two indexes are related to the stabilization of the radical via double-bond rearrangement (Barriuso et al., 2013). Sample 6 showed the highest K_{232} (5.02±0.15) and, it presented the lowest OSI time (3.37±0.29 hours). The OSI time is used to evaluate the quality of oils, and it also gives an indication of their susceptibility to oxidation (Jebe et al., 1993; Cerretani et al., 2006). For this reason, it can be used to compare the resistance to oxidation of the thirteen different HSOs. A PCA was performed on several parameters (i.e. free acidity, peroxide value, spectrophotometric extinctions in the UV region (K_{232} and K_{270}) and OSI time) in order to graphically visualize the relation among those variables, related to the oxidative and hydrolytic state of HSOs. A Pearson correlation matrix was done in order to evidence the statistically significant correlations. Figure 6.1.4.1.1 (a) show that sample 6 was quite different from all the others considering the quality parameters, due to the highest peroxide value, K_{232} and K_{270} and the lowest OSI time (Table 6.1.4.1.1). While sample 13, which did not present any processing indications on the label, was similar to the majority of the samples. Moreover, a positive correlation between K_{232} and PVs as well as between K_{232} and K_{270} (Figure 6.1.4.1.1 b) was identified, as previously reported for canola and soybean oils (Barriuso et al., 2013). Also, it was evidenced that samples 7 and 8 were mainly characterized by high free acidity values (Figure 6.1.4.1.1 a).



(a)

Variables	Free acidity	PVs	K_{232}	K_{270}
PVs	-0.005			
K_{232}	0.149	**0.893**		
K_{270}	**0.648**	0.455	**0.698**	
OSI	-0.154	-0.456	-0.480	-0.246

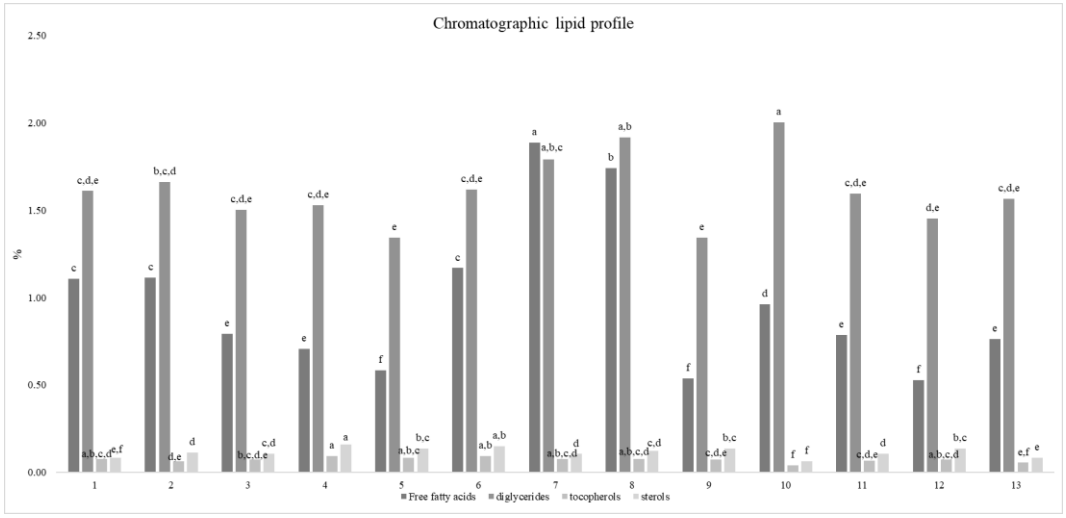
(b)

Figure 6.1.4.1.1. Principal Component Analysis (PCA) biplot performed with free acidity, peroxide values, OSI times and specific extinction in the UV region (K_{232} and K_{270}) (a) and Pearson correlation matrix (b) carried out on the same parameters (values in bold are statistically significant, $p<0.05$).

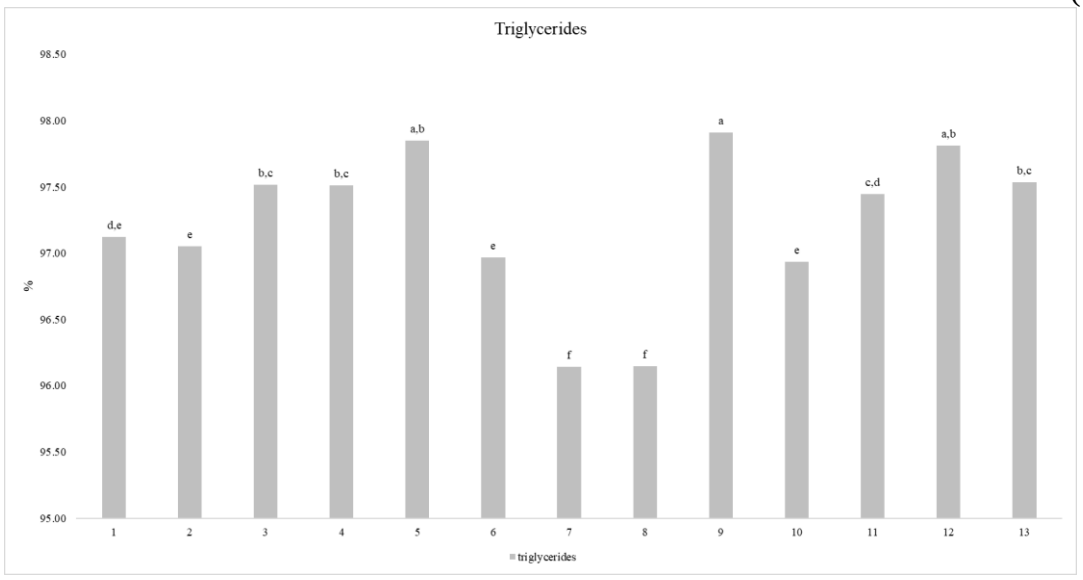
6.1.3.2 Lipidic composition

The main fatty acids detected in HSOs were linoleic acid (C18:2 n-6) which ranged from 38.48 mg/100 g to 52.16 mg/100 g, α-linolenic acid (C18:3 n-3) from 11.02 mg/100 g to 17.40 mg/100 g, oleic acid (C18:1 n-9) from 6.89 mg/100 g to 20.47 mg/100 g. In addition, a low amount of γ-linolenic acid (C18:3 n-6) and stearidonic acid (C18:4 n-3) was also found, from 0.98 mg/100 g to 4.43 mg/100 g and from 0.20 mg/100 g to 1.50 mg/100 g, respectively. Previous studies investigated the fatty acids composition of HSOs (Oomah et al., 2002; Petrović et al., 2015; Pratap Singh et al., 2020; Bartkiene

et al., 2021), and the herein presented results are in accordance with them. Furthermore, the lipidic profile by GC-FID was investigated (Figure 6.1.4.2.1), thus to compare the lipidic composition of the HSOs particularly in terms of free fatty acids, which could impact the oxidative stability of vegetable oils (González-Hedström, Granado, & Inarejos-García, 2020). The most represented class is triglycerides (above 96.14%). Sample 4 showed the higher content of tocopherols determined by HPLC-DAD and in the total lipid profile. The free fatty acids (FFAs) contribute to the free acidity value; moreover, a high presence of free fatty acids could determine a higher susceptibility to oxidation (González-Hedström et al., 2020). Samples 7 and 8 were characterized by the higher presence of free fatty acids (Figure 6.1.4.2.1) as well as the higher free acidity (Table 6.1.4.1.1).



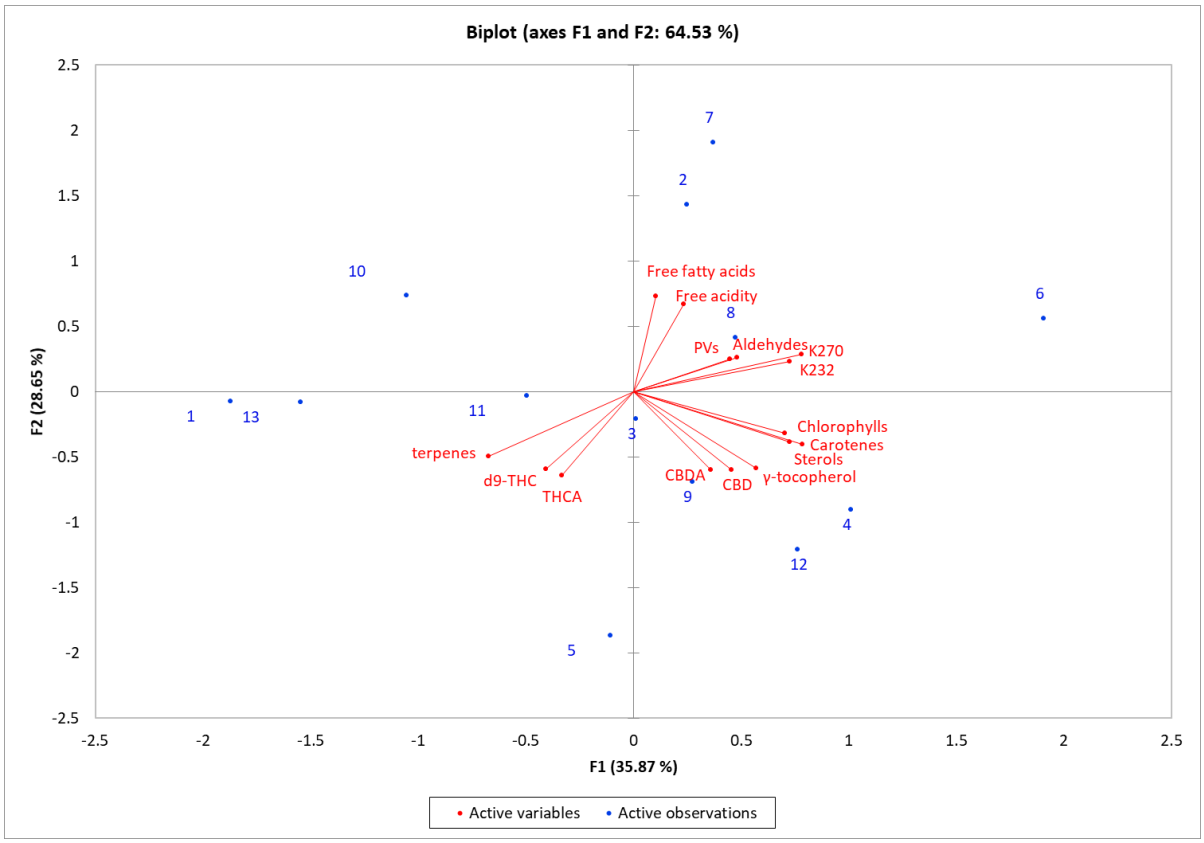
(a)



(b)

Figure 6.1.4.2.1. Total lipid profile of the 13 HSOs obtained by GC-FID analysis. Figure (a) reports the most representative lipid class (TAGs), while figure (b) shows the others classes founded (FFAs, diglycerides, tocopherols and sterols)

It was found that the Pearson's correlation value (Figure 6.1.4.2.2) between free fatty acids and free acidity was 0.968. Those two parameters are strongly related. Free acidity is defined as the amount of free fatty acids no longer linked to their parent triglyceride molecules (TAGs). For olive oil, this parameter is particularly affected by the action of enzymes, which determine the separation of fatty acids from TAGs (Ciafardini, Zullo, & Iride, 2006; Grossi et al., 2019). The same also happens during the storage of hemp seeds: the higher the relative humidity during the storage of hemp seeds, the more free fatty acids will increase. This is due to the greater activity of endogenous enzymes and moulds, and the process could be stimulated also by heat (e.g. if the seeds are stored at 30° or 35°C instead of 20-25°C) (Jian et al., 2019).



(a)

Variables	Free fatty acids	Sterols	Free acidity	PVs	K_{232}	K_{270}	γ-tocopherol	CBDA	CBD	d9-THC	THCA	Terpenes	Aldehydes	Chlorophylls
Sterols	-0.214													
Free acidity	**0.968**	-0.093												
PVs	0.010	0.212	-0.005											
K_{232}	0.104	0.504	0.149	**0.893**										
K_{270}	0.517	0.542	**0.648**	0.455	**0.698**									
γ-tocopherol	-0.282	**0.834**	-0.155	0.047	0.283	0.348								
CBDA	**-0.612**	**0.565**	-0.499	-0.133	0.063	-0.058	0.535							
CBD	-0.436	**0.640**	-0.293	-0.056	0.139	0.164	**0.642**	**0.903**						
d9-THC	-0.377	-0.165	-0.381	-0.221	-0.425	-0.317	0.152	-0.076	0.039					
THCA	-0.349	0.007	-0.310	-0.476	-0.479	-0.297	0.318	0.105	0.217	**0.787**				
Terpenes	-0.398	-0.358	-0.528	-0.353	**-0.584**	**-0.721**	-0.051	-0.111	-0.143	**0.785**	**0.663**			
Aldehydes	0.081	0.324	0.146	0.103	0.408	0.312	0.055	0.346	0.179	**-0.738**	-0.410	**-0.672**		
Chlorophylls	-0.007	**0.794**	0.120	0.251	0.455	**0.698**	**0.712**	0.214	0.391	0.161	0.066	-0.231	0.018	
Carotenes	-0.106	**0.843**	0.032	0.177	0.420	**0.646**	**0.746**	0.359	0.469	0.114	0.060	-0.258	0.132	**0.979**

(b)

Figure 6.1.4.2.2. Principal component analysis (PCA) biplot performed by the volatile aldehydes, and terpenes, γ-tocopherol, peroxide values (PVs), cannabinoids, sterols content, chlorophylls and carotenes, diene and triene conjugated systems (K_{232} and K_{270}).

Moreover, a negative correlation was found for triglycerides and free fatty acids (-0.981) (Figure 6.1.4.2.3); this could be due to the separation of fatty acids from the relative TAGs (Paradiso, Gomes, Nasti, Caponio, & Summo, 2010).

Variables	Free fatty acids	diglycerides	triglycerides	tocopherols	Sterols	Free acidity	C 14	C 16:0	C 16:1	C 17:0	C 18:0	C 18:1	C 18:2	C 18:3 γ	C 18:3	C 18:4	C 20:2	C 20:3 γ	C 24:0	C 22:5
diglycerides	**0.731**																			
triglycerides	**-0.981**	**-0.845**																		
tocopherols	0.059	-0.435	0.040																	
Sterols	-0.214	-0.535	0.273	**0.812**																
Free acidity	**0.968**	**0.695**	**-0.951**	0.081	-0.093															
C 14	-0.346	-0.532	0.404	0.271	**0.556**	-0.190														
C 16:0	-0.264	-0.209	0.285	-0.355	-0.176	-0.356	0.156													
C 16:1	0.015	-0.005	-0.005	-0.211	0.016	-0.039	-0.015	**0.824**												
C 17:0	-0.166	-0.024	0.143	-0.075	-0.190	-0.317	-0.327	0.490	0.303											
C 18:0	-0.075	0.167	0.053	**-0.671**	**-0.764**	-0.191	-0.048	0.468	0.071	0.176										
C 18:1	0.068	0.153	-0.074	-0.514	-0.339	0.000	0.045	**0.863**	**0.857**	0.227	0.516									
C 18:2	-0.400	-0.267	0.402	-0.031	-0.268	-0.472	-0.032	-0.224	**-0.663**	0.099	0.421	-0.470								
C 18:3 γ	-0.135	-0.438	0.188	**0.752**	**0,886**	-0.011	0.407	-0.306	-0.021	-0.319	**-0.813**	-0.413	-0.243							
C 18:3	0.070	0.059	-0.084	0.406	0.083	0.002	-0.430	**-0.599**	**-0.609**	0.193	-0.264	**-0.769**	0.511	0.157						
C 18:4	-0.065	-0.313	0.096	**0.762**	**0.845**	0.037	0.173	-0.435	-0.090	-0.236	**-0.902**	-0.535	-0.236	**0.955**	0.362					
C 20:2	-0.155	-0.309	0.176	**0.677**	**0.612**	-0.093	0.204	-0.519	-0.515	0.007	**-0.582**	**-0.794**	0.316	**0.680**	**0.715**	**0.735**				
C 20:3 γ	-0.084	-0.438	0.154	**0.695**	**0.854**	0.049	**0.726**	-0.097	0.041	-0.297	**-0.555**	-0.195	-0.255	**0.875**	-0.063	**0.731**	**0.584**			
C 24:0	-0.114	-0.441	0.176	**0.728**	**0.859**	0.027	**0.698**	-0.232	-0.114	-0.296	**-0.597**	-0.349	-0.147	**0.892**	0.058	**0.770**	**0.690**	**0.984**		
C 22:5	**0.737**	0.463	**-0.725**	0.350	0.265	**0.813**	0.127	-0.380	-0.176	-0.157	-0.418	-0.235	-0.381	0.252	0.125	0.275	0.323	0.383	0.395	

Figure 6.1.4.2.3. Pearson correlation matrix among lipid classes, fatty acids and free acidity ($p<0.05$)

Finally, also the ω6:ω3 fatty acids ratio was evaluated. HSOs generally present a unique ω6:ω3 ratio with an optimum of around 2.5:1-3:1, which is balanced from a nutritional point of view (Cerino et al., 2021). In the 13 HSOs analysed, the ratio was from 2.60 to 3.67 (Table 6.1.4.2.1), which is in line with what was previously reported in the literature (Anwar et al., 2006; Kiralan et al., 2010; Da Porto et al., 2012; Kazlauskienė et al., 2021) and confirms the optimal value from a nutritional point of view. Several factors could affect this ratio, such as climatic, cultivar, farming, light conditions and the origin of hemp seeds (Izzo et al., 2020). PUFAs could impact several biological activities, and the ratio of ω6:ω3 equal to 3:1 has been claimed to present health benefits in the human body, such as reducing cholesterol and high blood pressure and anti-inflammatory effect (Rezvankhah et al., 2019; Izzo et al., 2020).

Table 6.1.4.2.1. Fatty acid composition of the 13 HSOs. Results are expressed as mean±standard deviation of three independent replicates. Different letters in the same row indicate statistically significant differences (one-way ANOVA, Tukey's HSD $p<0.05$).

Fatty acids	**Sample**												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	(mg/100 g of oil)												
Miristic acid (C14:0)	0.03 ±0.00^{a}	0.03 ±0.00^{a}	0.03 ±0.00^{a}	0.03 ±0.00^{a}	0.04 ±0.00^{a}	0.03 ±0.00^{a}	0.03 ±0.00^{a}	0.04 ±0.00^{a}	0.04 ±0.00^{a}	0.03 ±0.00^{a}	0.03 ±0.00^{a}	0.04 ±0.00^{a}	0.03 ±0.00^{a}
Palmitic acid (C16:0)	5.93 ±0.22^{b}	7.12 ±0.05^{a}	5.65 ±0.40^{c,d,e}	4.99 ±0.04^{g}	5.60 ±0.06^{d,e,f}	5.37 ±0.08^{f}	4.95 ±0.12^{g}	5.41 ±0.07^{e,f}	5.78 ±0.12^{b,c,d}	5.37 ±0.09^{f}	5.97 ±0.08^{b}	5.98 ±0.04^{b}	5.86 ±0.07^{b,c}
Palmitoleic acid (C16:1 n-7)	0.10 ±0.00^{d,e,f}	0.37 ±0.01^{a}	0.11 ±0.01^{c,d,e}	0.10 ±0.01^{d,e,f}	0.11 ±0.00^{c,d,e,f}	0.11 ±0.00^{d,e,f}	0.10 ±0.00^{f}	0.10 ±0.00^{d,e,f}	0.12 ±0.00^{b,c}	0.10 ±0.00^{e,f}	0.12 ±0.00^{c,d}	0.13 ±0.01^{b}	0.10 ±0.00^{d,e,f}
Margaric acid (C17:0)	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.03 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00
Stearic acid (C18:0)	2.55 ±0.17	2.20 ±0.02	2.13 ±0.12	1.73 ±0.02	2.09 ±0.03	1.69 ±0.03	2.01 ±0.04	2.22 ±0.03	2.16 ±0.06	2.35 ±0.06	2.30 ±0.02	2.26 ±0.03	2.34 ±0.04
Oleic acid (C18:1 n-9)	11.61 ±0.61	20.47 ±0.29	11.51 ±0.82	6.87 ±0.12	10.16 ±0.10	6.89 ±0.12	9.53 ±0.20	12.33 ±0.13	11.22 ±0.30	11.66 ±0.26	11.64 ±0.14	11.74 ±0.10	10.51 ±0.18
Linoleic acid (C18:2 n-6)	52.16 ±2.36	38.48 ±0.47	45.30 ±3.06	45.19 ±0.64	47.04 ±0.69	45.27 ±0.81	44.74 ±0.96	42.58 ±0.56	46.82 ±1.16	47.30 ±1.22	48.70 ±0.63	48.97 ±0.16	48.18 ±0.66
γ-linolenic acid (C18:3 n-6)	1.09 ±0.06	2.31 ±0.06	1.72 ±0.05	4.27 ±0.13	4.34 ±0.07	4.43 ±0.08	2.84 ±0.07	2.04 ±0.01	3.07 ±0.08	1.10 ±0.03	1.04 ±0.01	3.30 ±0.02	0.98 ±0.08
α-linolenic acid (C18:3 n-3)	16.69 ±0.81	11.02 ±0.14	12.33 ±0.78	17.40 ±0.26	14.13 ±0.21	16.39 ±0.29	14.84 ±0.35	13.69 ±0.17	12.82 ±0.34	14.31 ±0.35	14.57 ±0.22	13.35 ±0.06	15.58 ±0.24
Stearidonic acid (C18:4 n-3)	0.20 ±0.03	0.51 ±0.01	0.34 ±0.02	1.50 ±0.04	1.05 ±0.02	1.36 ±0.02	0.82 ±0.02	0.44 ±0.01	0.68 ±0.02	0.26 ±0.01	0.22 ±0.00	0.73 ±0.00	0.23 ±0.00
Eicosadienoic acid (C20:2)	0.04 ±0.00	0.03 ±0.00	0.03 ±0.00	0.05 ±0.00	0.05 ±0.00	0.06 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00

Behenic acid (C22:0)	0.18 ±0.02	0.22 ±0.00	0.19 ±0.02	0.24 ±0.00	0.29 ±0.00	0.27 ±0.01	0.21 ±0.01	0.25 ±0.01	0.25 ±0.01	0.16 ±0.00	0.19 ±0.00	0.26 ±0.00	0.18 ±0.00
Lignoceric acid (C24:0)	0.07 ±0.01	0.08 ±0.00	0.07 ±0.01	0.10 ±0.00	0.13 ±0.00	0.12 ±0.00	0.09 ±0.00	0.10 ±0.01	0.10 ±0.00	0.06 ±0.00	0.08 ±0.00	0.11 ±0.00	0.07 ±0.00
SFA	8.81	9.69	8.11	7.13	8.19	7.52	7.31	8.06	8.37	8.01	8.62	8.69	8.52
MUFA	11.71	20.84	11.62	6.97	10.27	7.00	9.63	12.44	11.35	11.77	11.76	11.87	10.62
PUFA	70.38	52.59	59.92	68.66	66.92	67.83	63.54	59.11	63.71	63.18	64.78	66.67	65.20
Ratio ω6:ω3	3.13	3.49	3.67	2.60	3.33	2.76	3.01	3.11	3.65	3.31	3.34	3.67	3.09

SFA saturated fatty acids
MUFA monounsaturated fatty acids
PUFA polyunsaturated fatty acids

6.1.4.3 Volatile compounds profile

Terpenes are secreted in the glandular trichomes, and they present an entourage effect (synergic action) with cannabinoids (Pavlovic et al., 2018). The volatile profiles of the HSOs were quite different. In particular, sample 1 was characterized by a high content of terpenes, which was 66% expressed as relative peak area (RPA) of the total area counts, while sample 2 showed a high presence of aldehydes (Table 6.1.4.3.1). The primary terpenes detected were α-pinene and β-pinene, and also β-myrcene and β-ocimene were found in all the samples, in line with previous results (Pavlovic et al., 2018). Terpenes are considered contaminants in HSO, because their presence is due to the contact of hemp seed with leaves or bracts (Leizer et al., 2000). It was found a positive correlation (Figure 6.1.4.2.2) among THCA, δ9-THC and terpenes. Previous studies that investigated terpenes and cannabinoids in hemp plant and their relation, also found several correlations between THCA and several terpenes (e.g. ocimene) (Aizpurua-Olaizola et al., 2016; Namdar et al., 2019). Moreover, several aldehydes were detected in the samples, such as hexanal, pentanal, 2-butenal, trans-2-pentenal, heptanal, trans-2-hexenal, trans-2-heptenal, trans-2-octenal (in their respective trans or cis forms) and 2,4-heptadienal (Belitz, H. D., Grosch, W., 1999). Aldehydes are generally related to an advanced oxidative state. In fact, mainly starting from PUFAs, hydroperoxides are formed, which are subsequently decomposed to secondary oxidation compounds, among which we mainly find aldehydes. Several volatile aldehydes, such as hexanal, 2-heptenal and 2-octenal, are formed starting from linoleic acid (Poyato, Ansorena, Navarro-Blasco, & Astiasarán, 2014), the main fatty acid present in HSOs.

Table 6.1.4.3.1. Volatiles in the 13 HSOs. Results are expressed in area counts $\times 10^3$ for each compound. Data are the mean of three analytical replicates.

Compound	Sample												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Propanal	1047	2783	1085	2919	1128	1344	1743	785	870	2376	972	992	1425
Octane	-	-	356	-	-	-	-	-	-	-	-	-	-
2,4-dimethyl-Hexane	-	2997	-	863	266	651	1262	618	436	674	350	399	-
Acetone	198	682	1413	1278	630	333	1873	938	1460	272	1147	1557	233
Acetic acid, methyl ester	-	941	-	-	-	-	207	-	-	-	-	-	-
alpha-octene	-	1432	-	751	-	416	1264	-	-	-	-	-	222
trans-2-octene	-	6027	-	3369	437	2184	6091	294	-	1110	-	-	-
cis-2-octene	-	3357	-	2382	609	1378	2719	396	-	906	-	-	471
Butanal	473	-	193	-	-	-	-	349	-	-	-	-	-
Ethyl Acetate	-	815	-	-	-	-	-	-	-	-	-	-	-
Methanol	-	162	194	353	-	-	-	-	-	-	382	-	-
2-Butanone	332	-	292	638	-	4448	2777	467	-	334	505	268	-
2-methyl-Butanal	-	-	1014	417	-	-	-	621	1162	-	827	1559	-
3-methyl- Butanal	-	-	770	432	-	-	-	754	865	-	660	1256	-
2-propanol	-	-	160	-	-	-	-	417	-	-	-	211	146
3,5-Octadiene, isomer	-	1476	-	1195	-	-	2336	-	-	440	-	-	-
Ethanol	-	2621	399	582	913	86830	-	-	-	-	-	-	-
3,5-Octadiene, isomer	-	512	-	418	-	-	-	-	-	-	-	-	-
2,2,3,5-Tetramethylheptane	-	-	370	-	1589	-	-	-	-	-	-	-	-
2-Ethylfuran	-	-	-	-	-	-	500	443	-	-	-	-	-
Heptane	-	-	-	-	-	-	-	-	2271	10651	-	-	18657
2-Pentanone	-	3999	-	-	-	-	1017	1217	-	-	395	-	-
Pentanal	410	2408	1132	4452	1164	3033	2309	1807	1000	853	1720	1065	333
2,2,3-Trimethylnonane	-	-	173	-	991	-	-	-	-	-	-	-	-
1-Penten-3-one	-	-	-	430	-	-	149	-	-	-	-	-	-
2-butenol	-	-	-	-	651	-	269	-	339	-	413	389	-
α-pinene	12143	5052	10628	12427	17477	9277	1814	261	6261	9002	14416	10524	1194
1-Propanol	168	734	541	901	932	727	484	7673	657	-	680	756	191
2-Butenal	-	5429	179	4371	490	931	3106	-	-	1082	-	-	-
Toluene	-	-	167	-	375	-	-	441	-	-	433	160	-
Camphene	172	-	-	-	348	-	-	617	-	-	-	-	193
Hexanal	2168	34032	9649	42373	10499	33288	17673	206	10767	10797	14208	13733	3869
2-methyl-1-Propanol	123	-	-	-	-	-	-	14821	647	-	-	-	179

β-Pinene	5661	1434	3851	4717	4287	2984	586	217	1462	4187	6171	2077	5554
2-Pentanol	-	-	-	-	-	-	283	-	-	-	-	-	-
trans-3-penten-2-one	-	-	-	233	-	-	-	-	-	-	-	-	-
trans-2-pentenal	456	782	380	1044	265	1903	768	399	301	-	-	263	392
2-Butylfuran	-	-	-	-	-	-	220	-	-	-	-	-	-
1-butanol	-	-	220	283	219	494	208	454	-	-	323	-	-
1-Heptene oxide	-	-	-	830	-	668	-	-	-	-	-	108	-
1-Penten-3-ol	-	1345	920	1872	774	-	704	-	-	-	-	-	-
o-Xylene	-	-	38	-	-	101	-	-	-	181	-	-	-
p-Xylene	202	-	-	-	-	-	-	-	-	324	-	-	-
3-Carene	816	-	-	-	-	3796	-	2096	1958	3873	2024	-	822
β-myrcene	6484	4659	3349	24948	11520	9880	1270	4614	620	161	7706	2441	7024
2-Heptanone	135	655	355	587	227	1080	1640	2378	-	342	544	175	-
Heptanal	-	594	199	589	210	527	320	380	-	-	247	-	239
6-Methyl-1-heptanol	-	378	-	339	-	-	-	-	-	-	926	-	-
2-methyl-1-Butanol	271	-	819	-	945	891	269	1123	-	-	-	199	330
D-Limonene	3325	409	1660	3135	2116	3270	544	3201	-	2954	-	540	-
Eucalyptol	-	-	-	-	-	-	-	-	-	-	-	405	-
β-Phellandrene	581	-	387	1107	482	664	-	-	-	-	498	-	368
trans-hex-2-enal	-	4280	242	918	213	1269	165	267	-	478	-	281	111
2-Pentylfuran	-	-	-	-	-	-	711	2859	-	234	557	408	465
trans-beta-Ocimene	281	-	125	311	315	-	-	-	-	163	258	-	276
1-Pentanol	756	2939	2083	3899	3261	7009	1804	3810	2282	446	3187	2720	831
cis-beta-Ocimene	1230	1351	686	3914	2346	1961	2105	1469	484	963	1517	709	1416
o-Cymene	261	-	138	608	316	820	332	571	-	145	492	123	311
Acetoino	-	-	-	-	-	167	-	-	-	-	-	-	-
2-Octanone	-	424	-	-	-	367	982	770	-	198	-	-	-
(+)-4-Carene	1039	413	344	4349	1385	1774	348	663	-	307	1224	175	1216
cis-2-Penten-1-ol	-	263	149	-	-	374	-	-	-	178	-	-	188
5-Methyl-2-hexanol	172	-	-	-	-	-	327	497	-	-	342	-	-
4-Hexen-1-ol, acetate	-	409	-	-	-	-	-	-	-	-	-	-	-
trans-2-Heptenal	-	4746	522	3391	845	1037	2577	519	479	942	483	328	186
6-Octen-2-one, (Z)	-	-	-	-	-	-	189	-	-	-	-	-	-
1-Hexanol	4813	14999	10171	15074	15813	40643	10264	18255	13483	995	16813	15385	5490
cis-3-Hexen-1-ol	-	1744	-	-	-	-	-	-	-	-	-	-	-
trans-2-Hexen-1-Ol	-	1664	156	280	-	-	-	-	-	-	-	254	-
(3E)-3-Octen-2-one	-	341	123	258		458	-	-	-	-	132	102	-
trans-2-Octenal	-	420	161	470	209	503	257	-	-	123	155	173	-
Acetic acid	2423	7017	4230	5932	3068	8469	10533	9952	8032	3376	4916	2567	1873
1-heptanol	-	-	74	-	-	639	-	726	-	-	-	252	173
2,4-Heptadienal (isomer)	239	1422	-	725	-	1620	1080	-	-	-	175	-	325

2,4-Heptadienal (isomer)	-	313	149	361	-	429	-	-	-	557	-	-	-
trans-2-Hepten-1-ol	-	177	-	118	-	-	-	-	-	-	-	-	-
Formic acid	-	3098	-	2738	-	-	488	-	-	-	-	-	-
3,5-Octadien-2-one	399	554	248	-	-	1368	830	299	-	1141	269	183	297
Propanoic acid	432	1180	713	1614	509	2355	1142	1709	636	993	791	626	422
beta-Linalool	-	-	-	-	-	-	-	370	-	-	110	-	-
trans-2-Octen-1-ol	-	186	106	-	-	-	-	347	-	-	-	-	-
Carbitol	365	306	313	-	-	-	186	323	237	207	929	247	220
Butanoic acid	1334	1539	1253	-	648	3824	3285	1321	470	1321	251	519	1363
Caryophyllene	734	-	-	-	478	-	-	-	338	-	-	514	783
Pentanoic acid	-	400	163	527	-	609	517	888	-	-	303	129	-
Hexanoic acid	273	4196	962	4323	1376	6457	2864	3714	964	707	1520	1115	702
RPA terpenes	66	10	33	33	45	14	7	14	19	35	38	27	33
RPA aldehydes	10	41	24	37	17	18	31	6	26	27	22	30	12

6.1.4.4 Minor compounds

In HSO are usually found several minor compounds, such as tocopherols, chlorophylls, carotenes and cannabinoids. Tocopherols are known to preserve the lipid matrix from oxidation, preventing the oxidation of PUFAs-rich oils (Izzo et al., 2020), such as HSOs. The main tocopherol found in the 13 HSOs was γ-tocopherol (Figure 6.1.3.4.1), which ranged between 593.88 to 967.47 mg/kg.

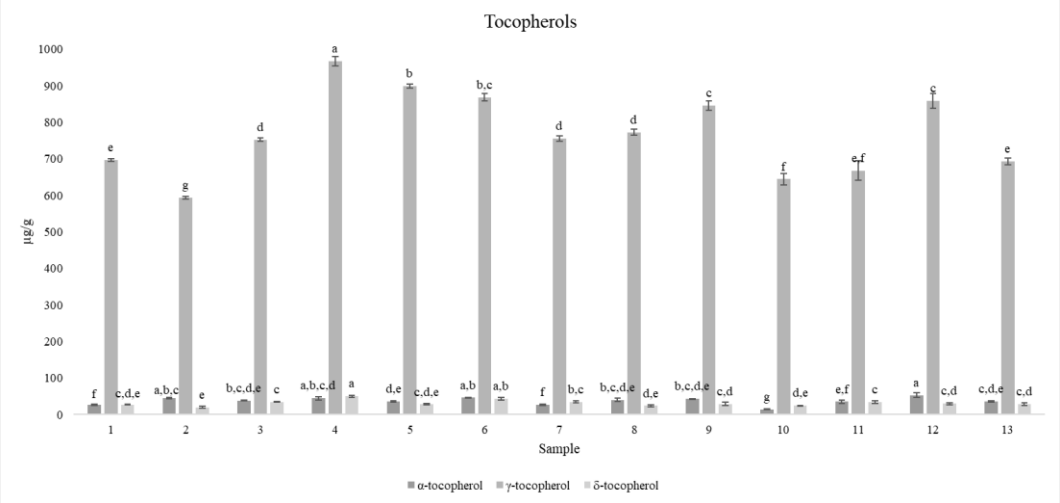


Figure 6.1.4.4.1. Tocopherols content expressed as µg/g. Results are reported as mean±standard deviation of three analytical replicates. Different letters indicate statistically significance differences (one-way ANOVA, Tukey's HSD $p<0.05$) among α-, γ- and δ-tocopherols.

Also, α-tocopherol and δ-tocopherol were found, ranged between 14.55 and 53.00 mg/kg and between 19.61 and 50.31 mg/kg, respectively. These results are in line with Özdemir et al. (2021), who reported content of α-,γ- and δ-tocopherol equal to 52.92, 707.47, 38.47 mg/kg, respectively. In another study conducted by Rezvankhah et al., 2018 the authors found similar results. The total chlorophylls and carotenes content in the 13 commercial HSOs were quantified, and results are reported in Figure 6.1.3.4.2. The total chlorophylls content detected in the HSOs showed a wide range from 0.78 mg/kg of oil to 75.73 mg/kg of oil. Our results followed what was reported by (Liang et al., 2015; Aachary et al., 2016), confirming the high differences in chlorophylls content among different HSOs (Izzo et al., 2020). The presence of chlorophylls could affect the oxidative stability of HSOs, due to their pro-oxidant and photosensitizer effects (Liang et al., 2018). Even if the only correlation found among chlorophyll content and oxidative parameters was with K_{270} index, it is interesting to highlight that sample 6, which showed thi higest chlorophyll content (Figure 6.1.4.4.2), also presented the lower OSI time (Table 6.1.4.1.1). For this reason, among the minor compounds naturally extracted by cold-pressing, chlorophylls are generally undesirable because they could reduce the shelf-life of HSOs (Izzo et al., 2020). Chlorophylls content could be decreased by refining and bleaching processes; on the other hand, the presence of chlorophylls affects the colour of the oil,

determining shades from light to dark green (Liang et al., 2018; Izzo et al., 2020). At the same time, it is well known that carotenoids present antioxidant activity (El-Abassy et al., 2010; Blasi & Cossignani, 2020). The total carotenoids content was found to be ranged between 2.53 mg/kg of oil and 33.93 mg/kg of oil. Lower results were recently reported by (Izzo et al., 2020), while other previous studies conducted by (Oomah et al., 2002; Pratap Singh et al., 2020) reported similar carotenoids total content. These differences could be mainly due to the variety, maturity of the seeds, climatic conditions and processing, such as bleaching (Rincón, Ramírez, & Orjuela, 2021). Samples 10 showed the lowest value of chlorophylls content and one of the lowest value of carotenes content; on its label, it was reported: "cold-pressed and filtered, without the use of solvents" (Table 6.1.3.1.1). This could indicate the application of a bleaching process without the use of chemicals, such as bleaching earths. The same can be hypothesized for sample 1; in fact, also this sample showed two of the lowest value of chlorophylls and carotenes contents, and on its label, the same technological indication was present (Table 6.1.3.1.1). On the other hand, samples 6 and 8 showed two of the highest chlorophylls and carotenes contents (Figure 6.1.4.4.2). Those 2 samples presented in the label the indication "cold-pressed"; only on sample 8 was also specified "unrefined". This is in line with previous literature, which indicate a content of chlorophyll up to 98.6 mg/Kg and of carotenoids 31.4 mg/kg in cold-pressed oils (Liang et al., 2015). A significant relation was found between sterols, chlorophylls and carotenoids (Figure 6.1.4.2.2).

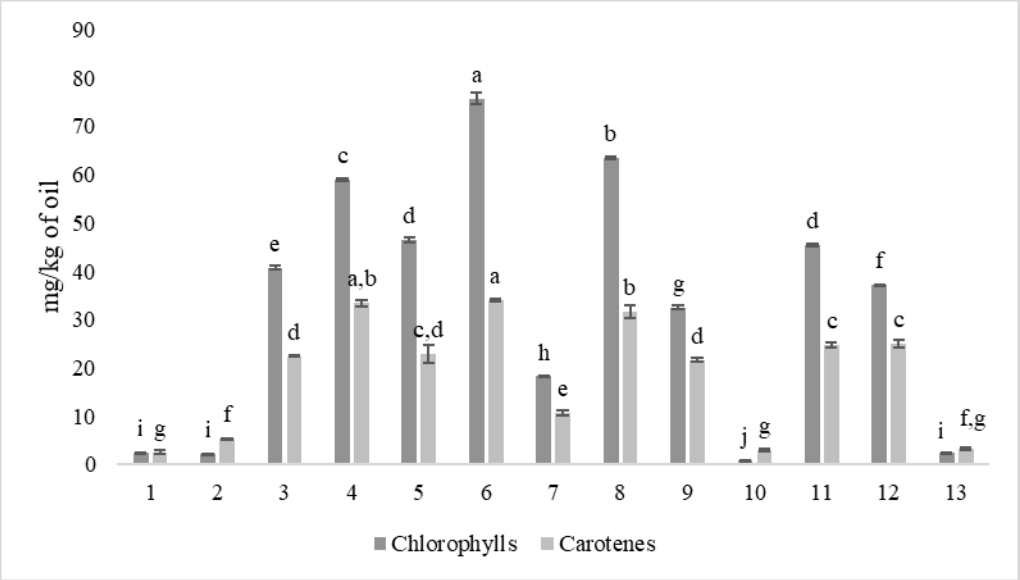
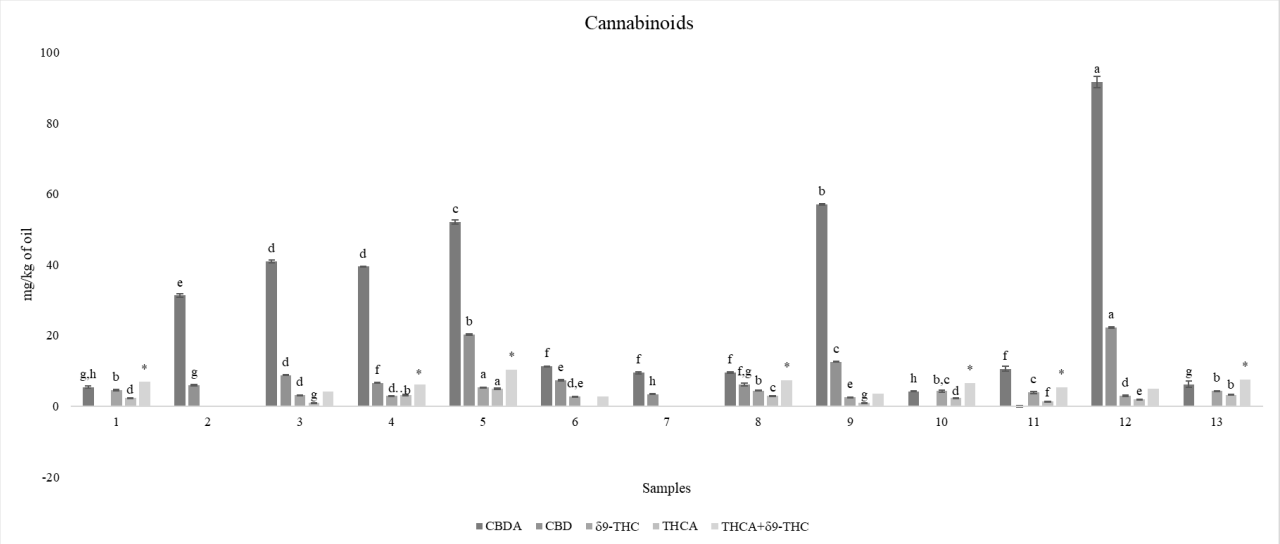


Figure 6.1.3.4.2. Chlorophylls and carotenes content in the 13 HSOs. Results are expressed as mean±standard deviation of three analytical replicates. Different letters indicate statistically significance differences (one-way ANOVA, Tukey's HSD $p<0.05$) among chlorophylls and carotenes total content in samples.

Finally, the cannabinoids profile was also determined to assess if the content of tetrahydrocannabinol (as sum of Δ9-THC and tetrahydrocannabinolic acid THCA) was lower than the limit established by the Italian legislation for HSO equal to 5 ppm (Ministero della Salute, 2019). Cannabinoids are considered "contaminants" in HSO, since their presence is due to contamination from contact among hemp seeds and inflorescence, bracts of leaves (Citti, Pacchetti, Vandelli, Forni, & Cannazza, 2018). Form a legislative point of view, there is no specific regulations regarding the analytical parameters for assessing the quality and authenticity of hemp seed oils, despite the growing interest in food products obtainable from hemp. In fact, only a few countries (such as Canada, Swiss, United Kingdom, Germany and Italy) have established a limit of THC in hemp seed oil, but there are no uniform indications (Kladar et al., 2021). Seven out of 13 HSOs showed a content of Δ9-THC+THCA above the Italian limit (Figure 6.1.3.4.3), between 5.35 mg/kg (sample 11) and 10.30 mg/kg (sample 5). The main cannabinoid found in all the samples was cannabidiolic acid (CBDA), which is reported to be one of the principal cannabinoids in hemp cultivated for industrial purposes (such as for food) (Citti et al., 2018). Figure 6.1.3.4.3 shows that samples 1 and 10, as for chlorophylls and carotenes, had the lowest content in CBDA (5.45 mg/kg and 4.25 mg/kg respectively). Sample 12 showed the highest content of CBDA (91.64 mg/kg). The herein presented results highlighted that HSOs can be extremely different in terms of cannabinoids content, in particular regarding the CBDA rate. Results are in accordance with Citti et al. (2018), in fact they found a CBDA range between 2.26 mg/kg and 821.1 mg/kg. This could be due to differences in processing (e.g. cleaning of the seeds) and hemp varieties (Citti et al., 2019). Sample 9 and 12 were mainly characterized by a high content of CBDA (Figure 6.1.4.2.2 (a)) while sample 5 stood out for the highest content of THCA and δ9-THC (Figure 6.1.4.2.2 (a) and Figure 6.1.4.4.3)



*Sum of Δ9-THC and THCA above the limit established by the Italian legislation (Ministero della Salute, 2019).

Figure 6.1.4.4.3. Cannabinoid profile of the 13 HSOs. Results are expressed as mean±standard deviation of three analytical replicates. Different letters for each cannabinoid indicate statistically significant differences (one-way ANOVA, Tukey's HSD $p<0.05$).

6.1.5 Conclusions

The quality parameters and composition of 13 HSOs collected from the market were evaluated. Two out of the 13 HSOs showed a free acidity value higher than the limit provided by the Codex Alimentarius. Another one sample presented a peroxide value higher than the limit set by the Codex. The chlorophylls content was extremely variable, and this could also have an impact on the oxidative stability of the HSOs. Even if only one positive correlation was highlighted between chlorophyll content and oxidation parameters (i.e. with K270), it would be interesting to investigate a possible relationship between resistance to forced oxidation and chlorophylls content by analyzing a greater number of samples. Also in terms of carotenoids, tocopherols and cannabinoids samples showed a high variability, probably related not only to different conditions of extraction but also to the hemp genotype and maturity of the seeds. Considering the differences in terms of quality and composition of commercial HSOs, it appears relevant to establish further parameters, besides the peroxides and free acidity, related to the quality, purity and authenticity for this product that can give guarantees to the consumer and to provide strategies to producers in order to market high quality HSOs.

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6.2 Sensory attributes and consumers insights of cold-pressed hemp seed oils

This paper will be submitted soon.

Abstract

The sensory attributes of 15 commercial cold-pressed hemp seed oils (HSOs) (15 samples) were assessed. To this aim, first the profile sheet was defined and a specific training of the panellists was carried out thus reaching a satisfactory performance level of the panel. Moreover, the consumers' attitudes with regards to hemp seed oils were also investigated. To do this, a focus group with the involvement of 8 subjects was performed. Several descriptors were highlighted both by the panel and interviewees, such as "rancid", "grass" (or herbaceous), sunflower/pumpkin seeds. The panel identified 46 descriptors, among them 13 were selected for the sensory sheet and categorized as pleasant and unpleasant. On the other hand, the interviewees reported that the technology (extraction process/refining) the colour of hemp seed oil represent drivers of choice in the purchase.

Keywords: hemp seed oil, descriptive analysis, focus group, panel training, consumers, attributes, colour

6.2.1 Introduction

In the last years, a growing interest was observed for cold-pressed oils, followed by an increase in the presence of these products on the market (Chew, 2020). Currently, the most common are olive oil, sunflower oil, palm oil, rapeseed oil and soybean oil, and less usual oils, hemp seed oil, flaxseed oil, and pumpkin seed oil, among others (Kotecka-Majchrzak, Sumara, Fornal, & Montowska, 2020). The global cold-pressed oil market is expected to increase from $ 24.62 (2018) to $ 36.40 (2026), that represents an annual growth of 5.3% (Kotecka-Majchrzak et al., 2020). The Codex Alimentarius clearly defines cold-pressed oils as "*obtained, without altering the oil, by mechanical procedures only, e.g. expelling or pressing, without the application of heat. They may have been purified by washing with water, settling, filtering and centrifuging only*" (Codex Stan 210-1999, 2019). One of the most remarkable differences between cold-pressed and the respective refined oils is related to the organoleptic characteristics, which are lost in the refining process (Gaca, Kludská, Hradecký, Hajšlová, & Jeleń, 2021). Cold-pressed hemp seed oil (HSO) is a rich source of polyunsaturated fatty acids (PUFAs) (Liang et al., 2018), which are generally present in a ω6:ω3 around 2.5:1-3:1, considered optimal from a nutritional point of view and recommended for healthy diets (Siudem, Wawer, & Paradowska, 2019). Cold-pressed HSO also presents several minor bioactive compounds, such as tocopherols, which are powerful antioxidants and contribute to prevent anti-cardiovascular diseases (Teh & Birch, 2013). In addition, more than 150 terpenes, mainly monoterpenes and sesquiterpenes, were identified in different cannabis plants varieties (Micalizzi et al., 2021). The volatile profile is responsible for the aroma of the product, even if not all the volatiles contribute in the same way or are characterized by a specific odour. The oil flavour is based on the composition of volatiles as well as on their odour thresholds, meaning that a lower concentration does not necessarily correspond to a lower aromatic incidence of that compound and vice versa. For these reasons, an oil may show a high concentration for some volatiles without influences on the flavour and a very low concentration of others with high sensory impact (Genovese et al., 2019; Gaca et al., 2021). Thus, the sensory impact depends on the "odour activity value", which is the ratio between the concentration of a volatile and its odour threshold (Genovese et al., 2019). The volatiles could be originated from the plant, or they are formed during production processes or storage of oils, and some of them could present a negative impact on the sensory quality (e.g. aldehydes, ketones, esters and furan derivatives) (Genovese et al., 2019; Gaca et al., 2021) determining the presence of off-flavour, such as rancid (Piochi, Cabrino, & Torri, 2021). The sensory decay of oils also affects the consumers' acceptance of these products (Piochi et al., 2021). Even if several authors reported sensory attributes related to hemp seed oils, to the authors' knowledge, no studies have been published focused on the development of a sensory profile of HSO. Several authors mentioned that high-quality hemp seed oils

are characterized by a dark to light green colour, nutty flavour and, sometimes, by a slightly bitter aftertaste (Matthäus & Brühl, 2008; Citti, Pacchetti, Vandelli, Forni, & Cannazza, 2018; Cerino et al., 2021; Soroush et al., 2021). While Sova, Lutsenko, Korchmaryova, & Andrusevych (2018) reported that the sensory quality indicators for hemp seed oils are the transparency, light green colour, without off-flavours and off-taste. Krist (2020) reported that cold-pressed hemp seed oils present a greenish-yellow colour, herbaceous, aromatic, green and nutty odour and herbaceous and nutty flavour. Callaway & Pate (2009) highlighted that a good-quality HSO has a light green colour to an olive-like colour and a nutty smell, while an old oil is usually from cleat green to yellow with a fishy and painty smell. Trained sensory panels are helpful for assessing the quality of foods (Tomic et al., 2010). In particular, Quantitative Descriptive Analysis (QDA®) is a useful sensory descriptive method to identify and quantify the sensory characteristics of a product; thus, it also defines sensory standards for quality control (Stone & Sidel, 1998). Several critical issues related to the QDA® have been previously reported in the literature. In particular, they are related to the training of the judges and the stability of the judgements (Cartier et al., 2006). For this reason, it is essential to detect the lack of precision (repeatability) and/or disagreement (reproducibility) as well as the ability of the tasters to discriminate different samples (Næs, 1990; Tomic et al., 2010). To this aim, a helpful open-source program, called PanelCheck, allows a fast and efficient analysis of the sensory data (Tomic et al., 2010). On the other hand, it is also crucial to assess the consumers' opinions and acceptance of HSO that was not early investigated to authors' knowledge. A useful way to investigate the consumers' point of view on a preliminary basis is focus group (FG). The FG is a qualitative techinique that allows to collect data on the topic; in fact, participants discuss in a non-threatening and friendly environment (Roascio-Albistur, Gámbaro, & Ivankovich, 2019). This technique presents several advantages (e.g. rapid and efficient test, people are more willing to participate when they are in a group than alone), and disadvantages (e.g. homogeneity of the results, polarization attitude, compliance and group think) (Roascio-Albistur et al., 2019). The aims of the present work were to develop a sensory sheet and train a panel of assessors for tasting HSO, by following ISO 13299:2010. In addition, the sensory characteristics of 15 HSOs were evaluated by the panel. Finally, a preliminarly investigation about the consumers opinions and attitude about edible hemp seed oil were investigated by FG.

6.2.2 Materials and methods

The samples used for the sensory evaluation were 16 different commercial cold-pressed hemp seed oils (HSOs), purchased at supermarkets, small sector shops or online, all packed in 250 mL amber glass bottles, except S8 that was sold in a 500 mL amber glass bottle (Table 6.2.2.1). 14 of the 16 HSOs were organic, while for 2 samples the farming system was not specified on the label. After the purchase and before the analyses, they were stored closed at room temperature in a place protected from direct light to avoid deterioration of the product and, once opened, in the refrigerator until the best before date reported on the label.

Table 6.2.2.1. Main information present on the label of the sampled commercial cold-pressed hemp seed oils

Samples code	Label information	Packaging
CP-BIO1	Cold-pressed hemp seed oil filtered, without the use of solvents and organic	Dark glass bottle (250 mL)
CP-BIO2	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO3	Cold-pressed, not refined and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO4	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO5	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO6	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO7	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO8	Cold-pressed and filtered, without the use of solvents and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO9	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO10	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)

CP-BIO11	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO12	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO13	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP-BIO14	Cold-pressed and organic hemp seed oil	Dark glass bottle (250 mL)
CP1	Cold-pressed and not refined hemp seed oil	Dark glass bottle (250 mL)
CP2	Cold-pressed and not refined hemp seed oil	Dark glass bottle (500 mL)

For the FG only one sample of HSO was tasted by the interviewees, which was supplied by Enecta S.r.l. It was chosen to taste only one sample because 6 of the respondents lived in Bologna while 3 were in little place located in Abruzzo. In this way, the moderator directly gave the sample to interviewees located in Bologna, while the other 3 already have the same sample at home. The discussion about the color of HSOs was performed by sharing 22 photos of different HSOs photographed from different perspectives.

6.2.2.1 Sensory analysis

The whole procedure for selection, training and monitoring the assessors, and choosing specific and suitable descriptors, measure scales and evaluation of results was made according to ISO 13299:2010 (ISO 13299, 2016). All the samples were evaluated at least in duplicate by a panel composed of 9 assessors (5 male and 4 females, mean age 37 years). The panellists and the panel leader were recruited from the staff and Ph.D. students of the Department of Agricultural and Food Science (University of Bologna) and they had previous experience in sensory descriptive analysis. During such former involvement in sensory analysis, they performed screening tests, such as ranking on the perception of the basic tastes and selected olfactory attributes (e.g., rancid), to determine the adequacy of their sensory skills. The panel was led by a panel leader, who organized the sessions, prepared samples and the sensory room for tasting. In addition, the panel leader also monitored the performance of the panel, prepared the reference materials for the attributes to be evaluated as well as the sensory sheets. The panel worked in a sensory laboratory and each assessor carried out the tasting in a single sensory booth. During the training phase, each assessor received three HSO samples during each session and they were first asked to find the perceivable product descriptors by identifying any

appearance, aroma, taste, and flavour attributes to describe the oil samples. For the definition of a list of attributes, four testing sessions were carried out. Then, a free discussion among panelists was done to decide which descriptors were redundant or not understood by everyone (so they should be removed from the list of attributes) or if there were terms that should be added. Thus, the final list of attributes was defined and the panel detailed each one (Table 6.2.2.1.1). The panel leader identified possible standards for the proper rating of selected attributes. Subsequently, sixteen sessions of training on rating intensities were carried out. During these sessions, the reference materials were presented to each assessor to develop a proper recognition of attributes in the samples and the ability to rate their intensities on an unstructured scale. The assessors were asked to taste three different hemp seed oils, to identify the attributes, and to rate their intensitites on a 100 mm unstructured scale with two anchor points, namely 0 (not perceivable) on the left and 100 (perceivable at saturation level) on the right. According to the Regulation COI/T.20/Doc. N° 15/Rev10 (2018) for sensory analysis of olive oil, the panel leader entered the assessment data and checked if the robust coefficient of variation were lower than or equal to 20.0%. It was chosen to evaluate a maximum of 3 samples per session because some of the products under examination showed very intense sensory characteristics: increasing the number of samples, there could be the risk of sensory fatigue. Disposable white plastic glasses were used to taste the samples, pouring out around 15 g of oil. Tasters were advised to follow several rules befor the evaluation, such as not smoke or drink coffee at least 30 minutes before the time set for the test and the other indications given by the International Olive Council (IOC) for the assessment of virgin olive oil (COI/T.20/Doc. No 15/Rev. 10 2018, paragraph 9.4). Samples were tested at room temperature. First, the assessors were asked to analyse the samples visually. Subsequently, the tasting procedure was the same reported by the IOC for the assessment of virgin olive oil (COI/T.20/Doc. No 15/Rev. 10 2018, paragraph 10.1), with the only exception that disposable glasses were used, for this reason, before the olfactory and gustatory phase, the panellists were asked to warm the sample by holding the glass in their hands, cover and roll it. Between samples, panellists had to rinse their mouths with sparkling or natural water and unsalted crackers. PanelCheck open-access software was used to monitor the performances of the panel; if the panel leader identified anomalous results, the taste was repeated.

Table 6.2.2.1.1. Sensory attributes and reference standard materials, acronyms and anchor points used in the sensory description of cold-pressed hemp seed oil samples.

	Descriptors	Definitions	References	Anchor points
Appearance	Yellow colour	Intensity of yellow colour	A selected cold-pressed hemp seed oil 	Weak (20%)
			A selected cold-pressed hemp seed oil 	Average (40%)
			A selected cold-pressed hemp seed oil 	Strong (80%)
	Green colour	Intensity of green colour	A selected cold-pressed hemp seed oil 	Weak (20%)
			A selected cold-pressed hemp seed oil 	Average (60%)
			A selected cold-pressed hemp seed oil 	Strong (80%)
Taste	Sweetness	Gustatory sensations caused by sucrose or other sugars and felt mainly on the tongue	Sucrose 100 g/kg in water	Strong (100%)
	Nutty	Flavour reminiscent hazelnut in a cup	Fresh hazelnuts in a cup	Strong (100%)

Odour (orthonasal and retronasal sensations)	Toasted nutty	Flavour reminiscent toasted hazelnut in a cup	Toasted hazelnuts (cooked in the oven at 150°C for 25 minutes)	Strong (100%)
	Sunflower/pumpkin seeds	Flavour reminiscent sunflower and/or pumpkin seeds	Mixture of 50% of pumpkin seeds and 50% of sunflower seeds in a cup	Strong (100%)
	Hay	Flavour reminiscent hay	Dried hay in a cup	Strong (100%)
	Rancid	Characteristic flavour of strongly oxidised oils or fats	COI standard for rancid of olive oil	Strong (90%)
	Paint	Flavour reminiscent paint, siccative oils, linoleum	A selected cold-pressed hemp seed oil subjected to a forced oxidation (Rancimat/Oxidative Stability Instrument, 24 hours at 110°C) in a disposable glass	Strong (100%)
	Burnt	Flavour reminiscent burnt dried fruits	Mixture of burnt sunflower seeds, hazelnuts and pumpkin seeds (cooked in the oven at 200°C for 1 hour and 30 minutes) in a cup	Strong (100%)
	Fish	Flavour reminiscent fish oil	Fish oil in a disposable glass	Strong (100%)
Tactile attributes	Pungency	Sensation similar to burning felt in the throat or diffusely in the oral cavity	Capsaicin 0.8 mg/kg in water	Strong (100%)

6.2.2.2 Focus group

Given the health emergency dictated by the COVID-19 pandemic, the sessions were held online through Microsoft Teams and were recorded through the same platform. Befor starting each session the privacy statement was read by the moderator and it was asked to each subjet if he/she accept it.

Subjects who consume/buy or, at least, know hemp seed oil were selected and interviewed. None of the focus group participants had previous experience as a panel taster. The discussion on the product in question was carried out also by tasting a sample of hemp seed oil. The focus group on hemp seed oil was carried out to investigate, through the use of an online discussion, consumption habits, satisfaction and expectations regarding this product. The participants in the focus group were 9 people, aged between 22 and 69 years. The participants, as previously reported, all knew the product. The group consisted of 3 men and 6 women, coming from different Italian regions, particularly Emilia-Romagna, Tuscany, Abruzzo and Puglia. The duration of the three interview sessions was around 90 minutes, and they were held on three different days. The discussion was mediated by a moderator, who had the task of guiding the argument without influencing the subjects, but at the same time facilitating and encouraging good discussions and probing declarations of the participants (Duerlund, Andersen, Grønbeck, & Byrne, 2019). During the first session, the discussion was focused on some general aspects, namely the purchasing and consumption habits of the product. The second session focused on evaluating the colour of hemp seed oil. In fact, for this purpose, a presentation with images of different oils was prepared by the moderator and submitted to the group to start the discussion. Finally, for the third session, focused on the recognition of taste and aroma descriptors of the product under examination, the subjects tested the same sample of hemp seed oil. The moderator explained to the participants the correct tasting procedure: the same followed by the panel (COI/T.20/Doc. No 15/Rev. 10 2018, paragraph 10.1). Then, asked the participants to warm the glass containing the sample with their hands, and to taste the sample following the previous instructions. The group then discussed the found sensory attributes, indicating which they considered pleasant and which unpleasant.

6.2.3 Results and discussion

6.2.3.1 Sensory lexicon

The panel has developed a sensory vocabulary during 4 tasting sessions and a final discussion. A total of 46 descriptors were identified and depicted in a three-tiered wheel (Figure 6.2.3.1.1).

Figure 6.2.3.1.1. Cold-pressed hemp seed oils sensory wheel.

The outer tier attributes are specific for cold-pressed hemp seed oils; the secondary descriptors that group-specific attributes are the second tier. Finally, the inner tier contains the 4 primary sensory modalities: appearance, taste, aroma, and tactile/trigeminal sensations. Accordingly to De Pelsmaeker, De Clercq, Gellynck, & Schouteten (2019), descriptors were selected, to develop the sensory sheet, based on the frequency quotation (≥5%). This percentage was calculated by dividing the number of quotations for an attribute by the total possible number of quotations (Cartier et al., 2006). Sorting procedure as an alternative to quantitative descriptive analysis to obtain a product sensory map. In Table 6.2.3.1.1 the frequency quotations for each descriptor reported during the 4 initial sessions are shown.

Table 6.2.3.1.1. Complete list of preliminary descriptive terms generated during the 4 sessions carried out for the definition of a common vocabulary

Attributes		Frequency (%)
Appearance	Yellow	9.2%
	Green	7.7%
	Brown	0.8%
	Olive green	0.4%
Taste	Sweet	7.1%
	Bitter	5.0%
	Salty	0.1%
Odour (orthonasal and retronasal sensations)	Rancid	11.7%
	Toasted nutty	5.7%
	Hay	5.3%
	Sunflower seeds	5.2%
	Burnt	5.1%
	Fish	5.0%
	Nutty	5.0%
	Paint	5.0%
	Seeds	4.9%
	Grass	1.7%
	Mouldy	1.3%
	Toasted	1.1%
	Pumpkin seeds	1.0%
	Boiled vegetables	0.9%
	Thistle	0.8%
	Cucumber	0.6%
	Herbs	0.6%
	Wood	0.6%
	Coffee	0.5%
	Vegetable	0.5%
	Cannabis	0.4%
	Chamomile	0.4%
	Lavender	0.3%
	Liquorice	0.3%
	Cabbage	0.2%
	Dried fruit	0.2%
	Peanuts	0.2%
	Pistachio	0.2%

	Sesame	0.2%
	Winey	0.2%
	Mint	0.1%
	Resin	0.1%
	Roots	0.1%
	Tobacco	0.1%
	Walnut	0.1%
	Walnut kernel	0.1%
	Wet soil	0.1%
Other sensations (tactile and trigeminal)	Pungent	5.5%
	Astringent	0.4%

Consequently, the panel generated 2 appearances, 2 taste, 8 aromas, and 1 tactile descriptive terms. Several descriptors reported by the panel were in line with what previous published in literature (Matthäus & Brühl, 2008; Callaway & Pate, 2009; Citti et al., 2018; Sova et al., 2018; Krist, 2020; Cerino et al., 2021; Soroush et al., 2021). The most frequent (>5%) attributes highlighted by the panel were: yellow and green colours, as regards the appearance; sunflower/pumpkin seeds, nutty, toasted nutty, hay, sweet, bitter, pungent in terms of olfactory and gustatory evaluation. The olfactory defects found significantly by the tasters in some samples were: rancid, paint, burnt and fish. The fish notes may be due to the presence of some sulfur-containing compounds, such as dimethyl trisulfide, or trimethylamine or to fungal infection of seeds that are not correctly dried (Callaway & Pate, 2009; Zhang et al., 2021). The paint flavour could be related to the release of free fatty acids from triglycerides and their subsequent oxidation (Callaway & Pate, 2009), which can lead to the formation of volatile compounds, secondary oxidation products, such as aldehydes and aliphatic ketones (Poyato, Ansorena, Navarro-Blasco, & Astiasarán, 2014). It is well known that the oxidation of linoleic acid leads to the formation of hexanal, 2-heptenal, 2-octenal, (E,Z)-2,4-decadienal and (E,E)-2,4-decadienal, while starting from linolenic acid (E,Z)-2,4-heptadienal and (E,E)-2,4-heptadienal are usually produced (Poyato et al., 2014). Several of those compounds were previously related to off-flavours, such as (E,E)-2,4-decadienal with deep-fried notes, (E)-2-heptenal with oxidized aroma and 2,4-heptadienal with rancid (Bendini, Cerretani, Salvador, Fregapane, & Lercker, 2009). Some of these attributes agree with what was previously found in the literature. Callaway & Pate (2009) reported a characteristic smell of paint in strongly oxidized hemp seed oils as well as a hint of fish, also attributable to an advanced oxidative process. According to the literature, freshly produced cold-pressed hemp seed oil is characterized by notes of citrus, mint and pepper (Callaway & Pate, 2009). Citrus and mint notes can be given by limonene while pepper was related to β-caryophyllene (Cital,

Kramer, Hughston, & Gaynor, 2021). The panellists identified only mint among these descriptors. It is important to underline that the sensory characteristics can vary both according to the variety of the seeds and according to the environmental conditions of their growth, and how the seeds are dried and stored (Callaway & Pate, 2009). The flavour of hHSO is linked to the presence of terpenes in the aromatic profile (Zhou et al., 2017). For this reason, a crucial point of the production process is the drying of hemp seeds: it is essential to dry them slowly at low temperature (<25°C) to preserve the terpenic fraction and to prevent the formation of off-flavour related to the oxidation, such as reminiscent of jute sacks or jute rope, but also paint or fish (Callaway & Pate, 2009).

Firstly, a specific sensory profile sheet for cold-pressed HSOwas developed (Figure 6.2.3.1.2) after defining a common vocabulary (Table 6.2.2.1.1). The panel used this sensory sheet during the training for recognition of attributes and to score their intensities. The panel identified several pleasant attributes (reported in Figure 6.2.3.1.2 as “positive attributes”) and unpleasant descriptors (reported in Figure 6.2.3.1.2 as “negative attributes”). In particular, the training was carried out only on the attributes reported in Table 6.2.2.1.1. As regards the indication “other” on the sensory profile sheet (Figure 6.2.3.1.2), related to both pleasant and unpleasant descriptors, it is referred to several attributes identified only by a few assessors during the first 4 sessions, namely the sessions related to the definition of a vocabulary. The panel decided to report also those attributes on the sensory profile sheet, as additionals in order to describe the sample more thoroughly. On the other hand, since not all tasters were able to identify them in the same way, it was decided to include them as additional descriptors without perform a specific training.

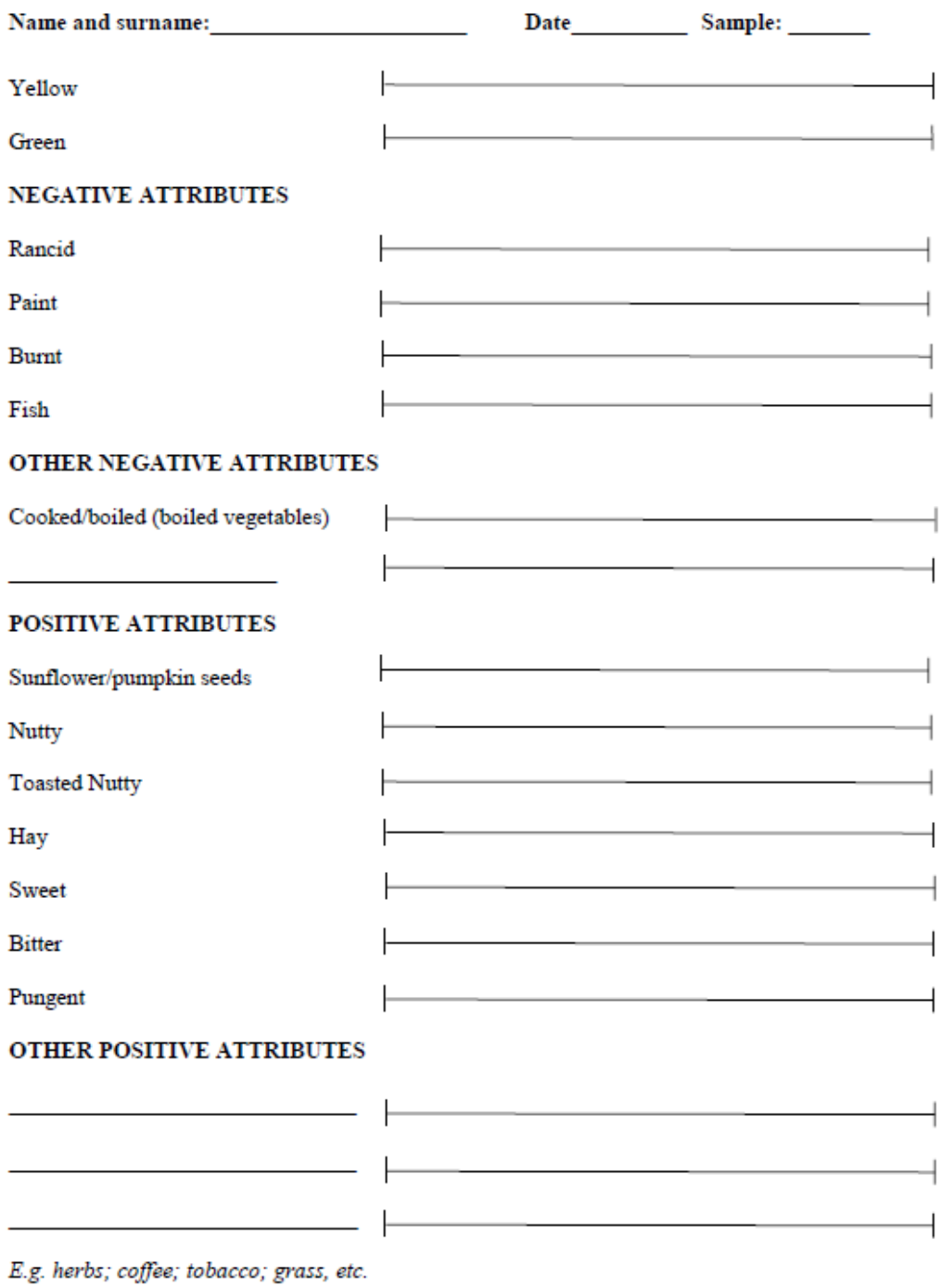


Figure 6.2.3.1.2. Sensory profile sheet specifically developed for cold-pressed hemp seed oils. The intensity of each attribute is evaluated with the use of a 100 mm unstructured scale with two anchor points 0 (not perceivable) and 100 (perceivable at the level of saturation).

6.2.3.2. Evaluation of the panel performances

The monitoring of the training of the panel was done by using the PanelCheck software. The software provides an intuitive and easy-to-use graphical user interface that handles all statistical computations in the background and visualises results in different types of plots (Tomic et al., 2010). During the training, 16 sessions were necessary to reach the panellists' appropriate reproducibility and repeatability levels. In particular, during the training sessions, the panel had difficulties related to the evaluation of colour. For this reason, it was decided to present three different references for each colour (yellow and green) and to assess again the samples until the misalignment has disappeared. The elaboration of the sensory data with PanelCheck software gave important information regarding the panel's discriminatory ability, alignment, and reproducibility. To evaluate these parameters and follow the workflow proposed by Tomic et al. (2010), a three-way ANOVA was performed to assess the importance of each descriptor in detecting significant sensory differences among samples. Only the significant attributes ($p \leq 0.05$) were considered for further analyses. In particular, as reported in Table 6.2.3.2.1, only the attribute “bitter” and “pungent” showed a p values$\geq$0.05, indicating that those are not important descriptors to detect sensory differences among HSO samples. Moreover, since the replicates were served systematically, which means one replicate per session, the replicate effect was evaluated (Table 6.2.3.2.1), thus indicating whether there is a significant systematic variation in the data based on the session (Tomic et al., 2010). No replicate effects were highlighted (three-way ANOVA, $p \leq 0.05$), as well as no assessor*replicate interactions were detected. On the other hand, several product*replicate significant interactions have been found, in particular for the descriptors “sunflower/pumpkin seeds”, “nutty”, “hay”, “rancid”, “paint” and “burnt” (Table 6.2.3.2.1). This can be related to a certain instability of the sample during the time, which should influence the panel results. In fact, although the samples have been stored in the refrigerator and protected from light between one session and another, this interaction may indicate that there has been a decay of the matrix, for example, in relation to the progress of the oxidative phenomenon. Hemp seed oils are prone to oxidation due to their composition (especially in terms of fatty acids) (Izzo et al., 2020).

Table 6.2.3.2.1. Results of the mixed model 3-way ANOVA for product effect, assessor effect and replicate effect.

Attribute	Yellow	Green	Sweet	Bitter	Pungent	Sunflower/ pumpkin seeds	Nutty	Toasted nutty	Hay	Rancid	Paint	Burnt	Fish
Product effect													
F-values	129.65	440.24	35.84	1.02	2.03	104.92	36.02	414.88	51.15	25.87	57.07	23.51	344.54
p-values	0.000***	0.000***	0.000***	0.057	0.057	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***	0.000***
Assessor effect													
F-values	1.89	1.81	1.07	-	-	0.75	1.92	0.14	1.09	0.93	1.40	0.79	1.42
p-values	0.076	0.091	0.395	-	-	0.650	0.379	0.996	0.379	0.495	0.215	0.613	0.213
Replicate effect													
F-values	1.28	0.25	0.48	-	-	0.06	0.65	0.00	1.55	0.06	0.00	0.39	0.06
p-values	0.264	0.618	0.490	-	-	0.810	0.423	1	0.220	0.811	0.955	0.538	0.812
Assessor*Replicate interaction													
F-values	0.71	0.43	1.81	-	-	0.86	0.80	1.39	0.87	1.22	0.72	1.10	1.81
p-values	0.682	0.899	0.091	-	-	0.555	0.605	0.218	0.543	0.305	0.674	0.377	0.092
Product*Replicate interaction													
F-values	1.23	1.59	0.61	-	-	6.98	3.28	1.67	2.46	2.85	3.36	2.10	1.51
p-values	0.299	0.146	0.756	-	-	0.000***	0.003***	0.123	0.022*	0.009**	0.003**	0.049*	0.171

* p≤0.05

**p≤0.01

***p≤0.00

Subsequently, the Tucker-1 plots were evaluated (Figure 6.2.3.2.1).

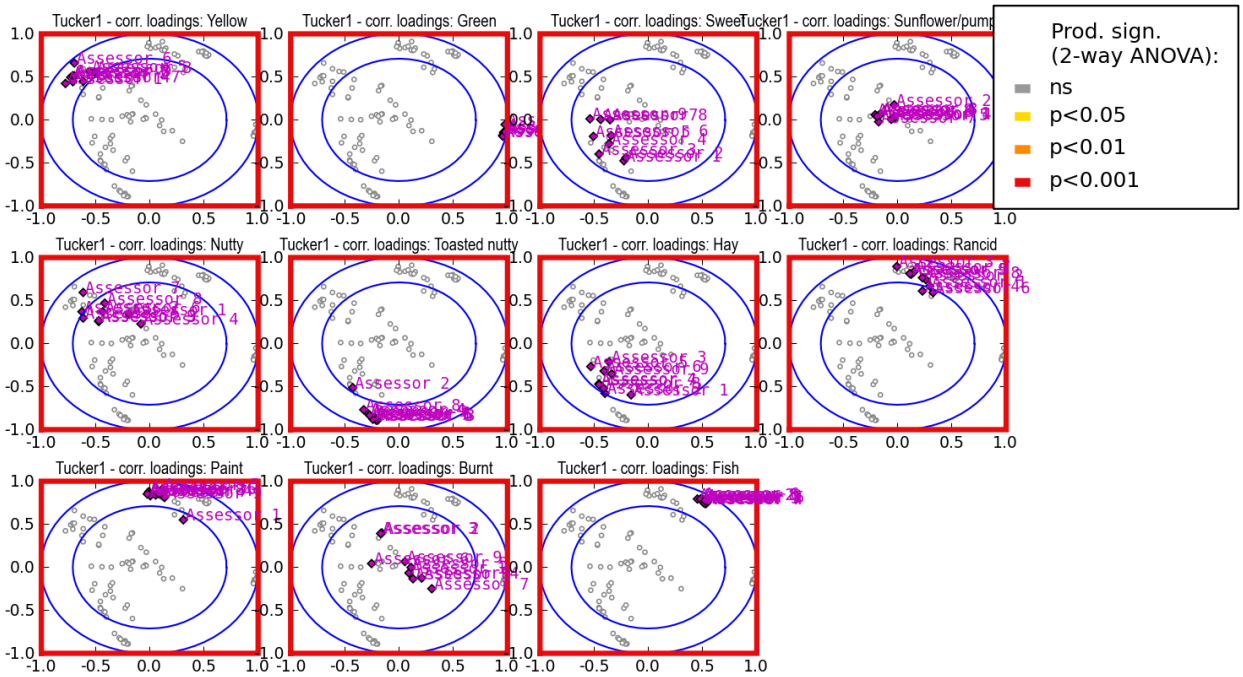
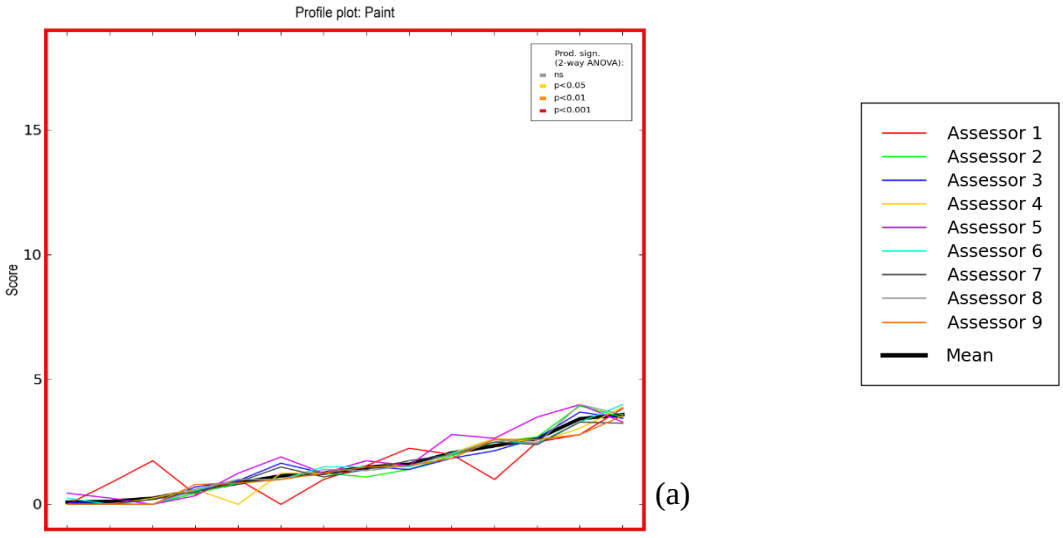


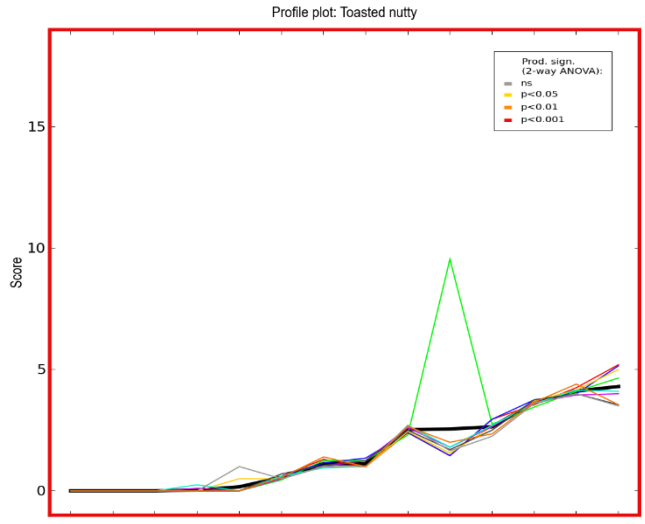
Figure 6.2.3.2.1. Evaluation of panel performance by PanelCheck software. Consensus among the assessors of hemp seed oil samples

The Tucker-1 plot is a two-dimensional graphic that provides a visual representation of the level of agreement among the assessors and an estimate of the information that each provides to describe the products and differentiate them. The graphic shows two ellipses; the inside one represents 50% of the explained variance between the samples, while the outer ellipse represents 100% of the explained variance. The positioning of the panellists in the chart is crucial, and, ideally, these should be located in the outermost area (which indicates 100% of the variance), meaning that the attribute is correctly used to describe the sample. However, the location between one judge and the other also conveys very important information regarding consent within the panel. Therefore, the more the points are grouped, the greater the agreement within the panel will be approximately the use of a certain attribute (Tomic et al., 2010). The panel reached great performances and consensus (Figure 6.2.3.2.1) for attributes “yellow”, “green”, “rancid”, and “fish” dots are located in the outer ellipse and very close to each other. As regards “paint” and “toasted nutty” assessor 1 and assessor 2, respectively, are relatively distant from the others, but most of the panellists showed good performances. On the other hand, the agreement among the judges is also good for the attributes “sweet”, “sunflower/pumpkin seeds”, and “hay” but the assessors are positioned in the internal ellipse. Lastly, for attributes “nutty” and “burnt” the agreement between most of the assessors is good but they are positioned in the inside ellipse and some dots (e.g. assessors 2 and 3 for burnt descriptor) are positioned away from others.

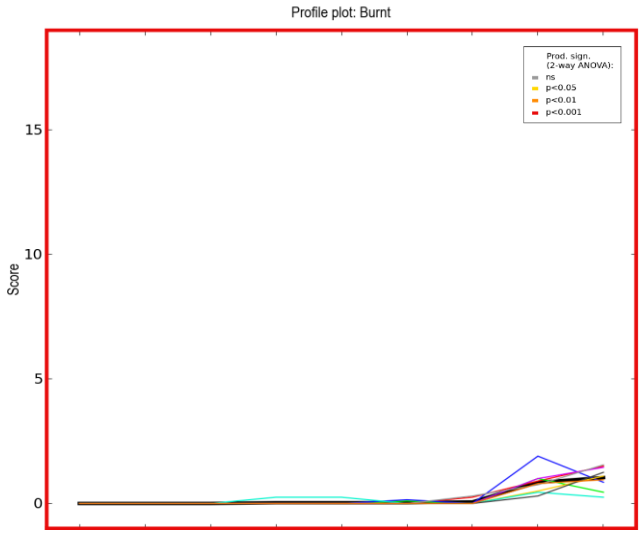
A deeper assessment was done for the attributes “paint”, “toasted nutty” and “burnt” to investigate the behaviour of the panelists positioned away from the clusters in the Tucker-1 plots. In particular, Figure 6.2.3.2.2 evidences that assessor 1 stands out from the others because of higher or lower scoring on intensities of several samples (Figure 6.2.3.2.2 a). Assessor 2 is clearly away from the others because of higher scoring on intensities of some tested samples (Figure 6.2.3.2.2 b). Assessors 2 and 3 (Figure 6.2.3.2.2 c) stand out from the others because of lower and higher scoring on intensities of some samples, respectively. Also, the p*MSE plots (Figure 6.2.3.2.3) confirm that assessors 2 and 3 showed different performances for the two abovementioned attributes. However, a grat level of agreement and training of the panel has been reached, even if it will be necessary to perform more training sessions for panelists 2 and 3, in order to specifically train them on the attributes that they scored differently from the panel.



(a)



(b)



(c)

Figure 6.2.3.2.2. Profile plots of attributes "paint" (a), "toasted nutty" (b) and "burnt" (c). Assessor 1 stands out from the others because of higher or lower scoring on intensities of several samples (a). Assessor 2 clearly stands out from the others because of higher scoring on intensities of some tested samples (b). Assessors 2 and 3 stand out from the others because of higher (assessor 3) or lower (assessor 2) scoring on intensities of some samples.

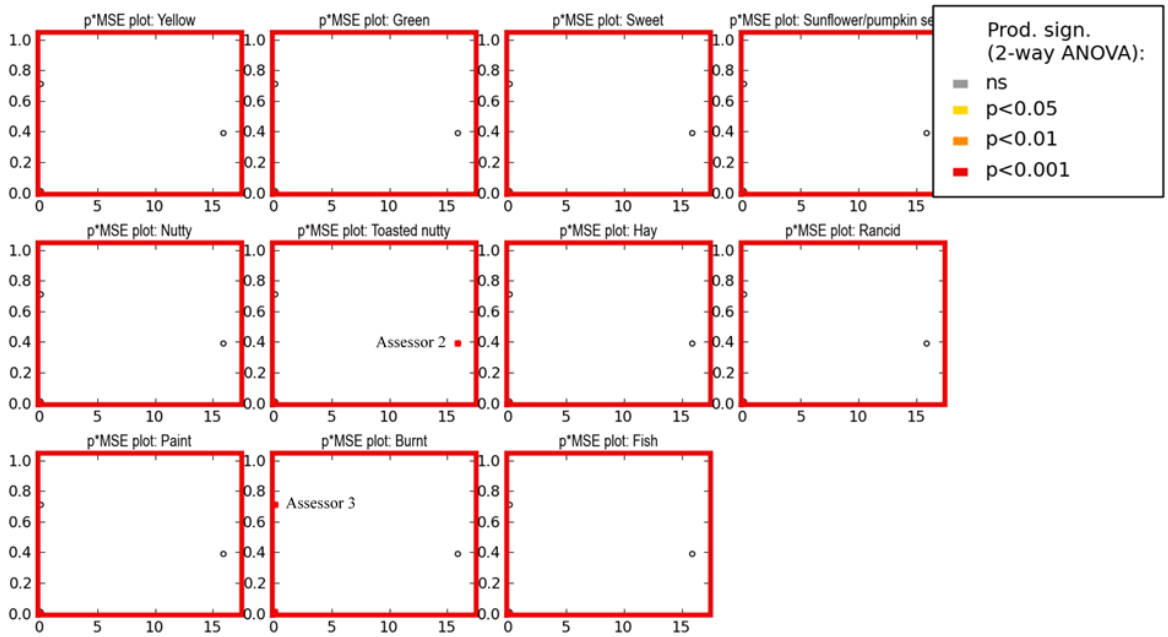


Figure 6.2.3.2.3. p*MSE plot for all the attributes. Assessors 2 and 3 performed differently from the others for attributes "toasted nutty" and "burnt", respectively.

6.2.3.3. Focus group

First session: During the first session of the focus group, the discussion was directed to the purchasing and consumption habits of vegetable oils. It was evident that the most used oil by the whole group of subjects involved was extra virgin olive oil, both as a condiment and for cooking. In particular, all the participants declared that they purchase extra virgin olive oil directly or indirectly from small and medium-sized companies, preferring it to large retailers. This first part of a general nature was followed by a discussion focused on the product in question, namely HSO. Firstly, it was asked where they usually buy this product, and different answers were given. Some interviewees buy or would purchase HSO from companies that produce and market only hemp-derived products, others in supermarkets. One interviewee stated that she bought it from a cooperative, considering it more reliable in producing and marketing "crude" oil, cold-pressed and unrefined. Subsequently, however, she purchased hemp seed oil in pharmacies or shops dedicated to selling organic products and foods for special diets. Next, the participants discussed some characteristics of the hemp seed oils they use most. All the participants agreed that their consumption is directed towards oils characterized by a dark colour, especially green, rather than yellow or clear-colourless and characterized by intense olfactory and gustatory attributes. A yellowish colour, according to them, recalls a “tasteless” product. On the other hand, as regards the mode of consumption, conflicting opinions emerged. For half of the participants in the focus group, the use of HSO is closely related to the benefits they think it brings in relation to the content of ω6 and ω3 fatty acids (Jacobson, Glickstein, Rowe, & Soni, 2012). Hemp seed oil is, in fact, very rich in unsaturated fatty acids, such as linolenic acid and α-linolenic acid, which can constitute up to 80% of total fatty acids (Matthäus & Brühl, 2008). The ratio between ω-3 and ω-6 fatty acids is about 1:3 in hemp seed oil, an optimal value from a nutritional point of view (Cerino et al., 2021). As part of an ideal diet, fat consumption should not exceed 15-20% of total caloric intake, and about 1/3 of these fats should be essential. This goal can be easily achieved by consuming 3-5 tablespoons of hemp oil (Tringaniello, Cossignani, & Blasi, 2021). In particular, as previously mentioned, it has been shown that ω-3 PUFAs positively influence the prevention of cardiovascular diseases (Pizzini et al., 2017). Therefore, it is substantially used as a food supplement or "nutraceutical" products. For the other half of the participants, the oil is appreciated and can be offered as a substitute for extra virgin olive oil in raw condiments and fish and meat marinades. Finally, one participant use it as a cosmetic product in addition to its "nutraceutical" and food use. The use of hemp seed oil as a "nutraceutical" food or as an alternative condiment is in accordance with what is reported by ISMEA (2018). The consumers interviewed (ISMEA, 2018) also attribute to oils more recently introduced to the market, such as hemp seed oil, nutritional properties even

better than those of extra virgin olive oil. Subsequently, the discussion shifted to the variability of the price and the sensory characteristics of hemp seed oil: the interviewees wondered if the range of oil prices on the market, from about 20 €/L up to 100 €/L (Spano et al., 2020), actually corresponds to such a different level of quality. However, a fully shared answer was not given to this question, but most of the participants hypothesized that there is often no such correspondance. It should be noted that some participants declared that they always use the same hemp oil as they believe to know the related supply chain. In this regard, it is also important to underline that there is still no specific regulation regarding the parameters for assessing the quality and authenticity of cold-pressed hemp seed oils, except the THC limit set only by a few countries (such as Canada, Swiss, United Kingdom, Germany and Italy) (Kladar, Čonić, Božin, & Torović, 2021). In fact, although this product is of growing commercial interest, to date there is only a specific limit on the acceptable level of Δ9-THC in hemp seeds, hemp seed oil or processed foods (Jang et al., 2020), equal to 5 mg/kg (Ministero della Salute, 2019). About the quality characteristics, the Codex Alimentarius (Codex Alimentarius, 2019) indicates for cold-pressed vegetable oils, not coverd by individual standards, the free acidity limit, equal to 4 mg KOH/g of oil, and that of the peroxide value, equal to 15 milliequivalents of active oxygen per kg of oil. Hemp seed oils must comply with safety requirements and comply with the general labelling rules for food. As with many other edible oils, information on the the botanical variety can be omitted on the label, as it is not mandatory or allowed to report them (Spano et al., 2020). As for the composition in fatty acids, it is not possible to report it on the label, but only as the total content of saturated and unsaturated fatty acids, thus giving the consumer this kind of nutritional indication. It emerged that 4 participants in the focus group appreciated the sensory characteristics of some of these oils. These subjects have had the opportunity to taste and compare more samples before the focus group. The others who use it for "nutraceutical" purposes do not appreciate its sensory characteristics. A fascinating aspect concerns the methods of consumption. Those who appreciate these oils are generally also willing to use them as a condiment, while those who do not like hemp oil from a sensory point of view are not inclined to think that it can be used as a condiment. The reason given by the latter is that it is characterized by a "very intense flavour" which could, therefore “cover that of the food being seasoned".

Second session: The central theme of the second session was the colour of hemp seed oil. Concerning the colour of the hemp plant from which the product is extracted, all participants expected the oil to be green, in particular olive green and bright. Most of the participants involved in the focus group believed that the yellow hemp seed oil may have undergone a refining process or had been obtained from a different extraction process than the green one; others have not raised the problem, not having

the preconceived idea that the different colour is a sign of lower quality. Then, the moderator showed pictures of different hemp seed oils to the participants (Figure 6.2.3.3.1).

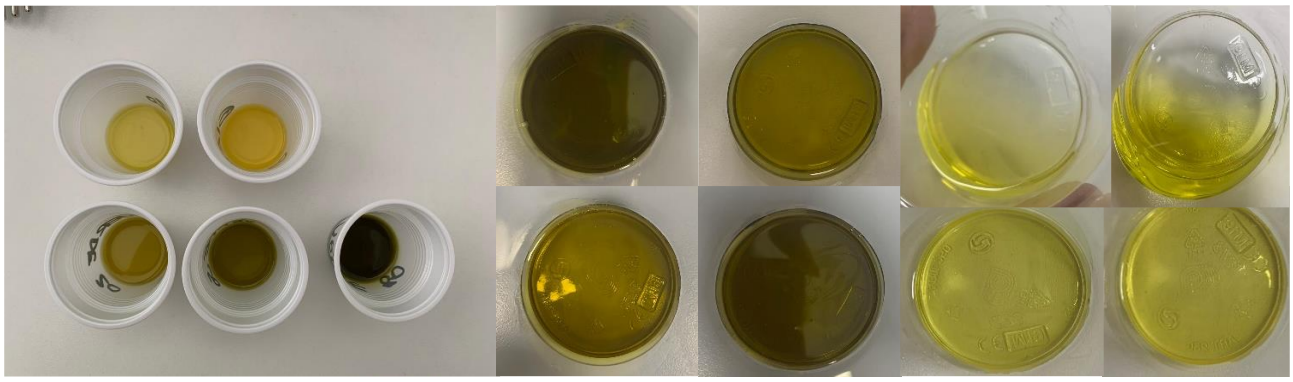


Figure 6.2.3.3.1. Colour of different hemp seed oils found on the market.

Looking at the series of samples of different shades of green, all consumers preferred a dark green colour of the product, not only with respects to yellow HSOs but also compared to the ligh green ones. If visible, HSO of an intense yellow or straw yellow colour would not be purchased by more than half of the participants, while only two subjects would purchase it only if accompanied by proven quality guarantees (e.g. certainty of the production process). The discussion then moved on to the hemp seed oil label; all the participants would have liked to have wording on the label about the colour of the product or a distinction of the products based on colour. This was very curious given that no such indication is provided as compulsory indication in the label for any vegetable oil and that neither for extra virgin olive oil the yellow or green colour represent an element of higher or lower quality within the above mentioned extra virgin commercial category. Regarding the label, one of the participants moved the discussion on the other indications that he/she would appreciate on the label. All the interviewees agreed that the words "cold-pressed" and "unfiltered" were the main ones. The interviewed subjects agreed that the colour they expect from an unfiltered cold-pressed oil is bright green, while for a filtered cold-pressed oil, the colour may be less intense, e.g. lighter green or yellowish. Furthermore, it emerged that the oil with the wording "cold-pressed" on the label would be bought by everyone, while the presence of the term "filtered" is experienced by 7 of 9 subjects as an index of poor/lower quality, capable of reducing the native characteristics of the product, as the filtration is wrongly connected to a process that is not only mechanical but also chemical based. At this point, the moderator asked if the participants, while storing the hemp seed oil they consume, had ever experienced any changes in the product. All the participants then pointed out that they usually keep hemp seed oil in the refrigerator, and then some of them stated that during this storage, in some cases, they had noticed differences compared to when they first opened the bottle. In particular, 4 of

9 participants have reported a change in colour from green to brown, the formation of a deposit on the bottom of the bottle and the appearance of an unpleasant odour. Others (6 of 9), however, showed only a slight browning without noticing any deposit on the bottom. The formation of deposits is associated by many participants with a natural course of the product obtained and is not perceived as a decline in quality. On the other hand, those who consumed only samples of oils purchased from large retailers found neither sediments nor deposits; this suggests that a filtration step was carried out for these oils during the production process. From this session, therefore, it emerged how colour could influence the choice of some consumers. Furthermore, a "rawer" HSO is considered of high quality by the 4 participants, even if it changes colour over time or if it separates sediment during storage. The freshly produced hemp oil is bright green due to the presence of chlorophyll which , can degrade during storage, thus causing colour changes, with a tendency to the appearance of yellow-brown tones, as reported by Matthäus & Brühl (2008) and Leonard, Zhang, Ying, & Fang (2020). The conservation of the oil carried out in the refrigerator, in many cases, is not sufficient to prevent alterations on the product, as evidenced by many of the participants. In fact, they found unpleasant and pungent odours and a hint of rancid after a few months after opening the bottle, even if stored in the refrigerator. Previous studies reported that a sensory note similar to paint, reminiscent of linoleum (Callaway & Pate, 2009) attributable to aged oil has a negative sensory impact. This attribute was highlighted also by the trained panel. Previously, it was reported that it is suggested to store HSO in open bottles for a period no longer than two months. For this reason, it would be practical to use small bottles for packaging, as they contain quantities of oil that can be easily consumed in a short time (Matthäus & Brühl, 2008).

Third session: The third session was focused on the sensory evaluation of HSO. All participants had the same sample of hemp seed oil available, as it was previously provided by the moderator to each of them by in-person delivery or courier. Initially, each participant was asked to observe, smell and taste a small amount of oil before starting the discussion. In terms of visual analysis, all the participants defined the colour of the hemp seed oil under examination as dark and bright green. 6 of 9 of the interviewees have reported olfactory notes similar to cooked vegetables, such as chicory and wild herbs. Many of the participants found rancid, medium-low intensity, and other olfactory notes attributable to pumpkin seeds and hay, as well as astringent and sweet taste. 4 of 9 of the participants perceived the toasted, and specific notes reminiscent of peanut and hazelnut. In addition, one of the subjects stated that he perceived the characteristic smell of the hemp plant. 7 of th 9 interviewees perceived bitterness and pungent, some of them compared this perception to that felt after tasting some extra virgin olive oils, albeit less pleasant, while 2 subjects did not perceived the bitterness. There were also different opinions regarding persistence after gustatory evaluation. Some of the

participants reported a pleasant sensation despite the persistence of the flavour, but most of them found this persistence as unpleasant. At this point, each participant was asked by the moderator to classify each perceived attribute as negative or positive, depending on the fact that it was considered a pleasant or unpleasant note, respectively. Sweetness, olfactory notes attributable to hazelnut, peanut and pumpkin seeds were among the positive attributes cited by all the participants. Among the negative attributes the rancid was identified by all; in addition, the herbaceous is described as annoying for some subjects. The bitterness was perceived as negative by most of the subjects who perceived it. In fact, 5 of the 7 participants described this taste as "negative and persistent", while for 2 subjects the bitter taste was pleasant. In any case, the consumers who use HSO as a nutraceutical food affirmed that bitterness and pungent are not attributes that affect the overall appreciation of the HSO, even if perceived as unpleasant. This is mainly due to the purpose for which these subjects use hemp seed oil: They still appreciate the product for the benefits to which they associate it more than for a sensory aspect. It is known that the attributes of bitter and spicy are difficult for the consumer to appreciate, unless there is familiarity (Rébufa, Pinatel, Artaud, & Girard, 2021). On the other hand, they are positive attributes in virgin olive oils because they are linked to the presence of polyphenols, for which it is also possible to indicate a health claim. In particular, bitterness is mainly related to the presence of oleuropein and p-HPEA-EA in virgin olive oils (Cliceri, Aprea, Menghi, Endrizzi, & Gasperi, 2021). It would be interesting to understand the etiology of bitterness als in HSOs.

6.2.3.4. 3.3 Comparison between attributes emerged from the trained panel and focus group

During the focus group sessions, some attributes emerged that had also been found by the panel trained for the tasting of HSOs. In particular, the panel found differences in the colour of the hemp seed oils examined, defining two different colour intensity scales, one for yellow and one for green. Also the subjects participating in the focus group, particularly during the second session, after observing some photos shared by the moderator (Figure 6.2.3.3.1) of various hemp seed oil samples, indicated the same colour attributes. Regarding the gustatory and olfactory evaluation, the positive attributes found by consumers and indicated in the sensory sheet by the trained panel were sweet, hazelnut, toasted, pumpkin seeds, grass, and pungent. In addition, bitterness was also a positive attribute for 2 of the interviewees, as indicated by the panel. As for the negative attributes identified by the focus group participants, only rancid and cooked/boiled vegetables were also reported by the panellists. It is essential to underline that many of the tasters trained for the sensory evaluation of hemp oils are also part of tasting panels for other food products, such as virgin olive oils. For this reason, many of them know how to use a specific sensory vocabulary in a better way than the subjects

involved in the focus group who did not have previous experience in sensory analysis as taster. In fact, panellists were able to identify a higher number of sensory attributes in the hemp seed oils than the interviewees. The attribute “green colour”, highlighted both by the trained panel and the subjects participating in the focus group, was also previously reported in the literature. As discussed above, unrefined and cold extracted hemp seed oil are generally dark green related to the presence of chlorophyll (Teh & Birch, 2013).

6.2.4. Conclusions

At now and to the authors’ knowledge, the investigation presented herein is the first dealing with the sensory evaluation of cold-pressed hemp seed oils by following ISO 13299:2010. A specific sensory wheel for such oils was developed, and a panel was trained to assess these products. Several significant (three-way ANOVA, $p<0.05$) attributes were found by the tasters in the context of panel training, such as “yellow”, “green”, “sweet”, “sunflower/pumpkin seeds”, “nutty”, “toasted nutty”, “hay”, “rancid”, “paint”, “burnt” and “fish”. A good consensus among the panellists was reached, and also a specific tasting sheet was set up. Regarding the interviewees in the focus group, interesting results were related to the information they would like on the label and the drivers of buying. In particular, the involved subjects appreciated if the technology (extraction process/refining) is reported in the label because most of them think that a high quality hemp seed oil has to be cold-pressed. Moreover, they would like an indication about the colour of the oil, as they think that the cold-pressed one has to be green. This is a quite curious result because even if the sensory assessment is a legal requirement for establishing the commercial category of other oils, i.e. for virgin olive oils the colour does not represent low or high quality for this product. The consumer perceptions about hemp seed oils need to be deeply investigated in the future, also in relation to the renewed attention on such oils. At the same time, the assessment of the sensory quality by descriptive approaches also represents crucial aspects for the valorization of high-quality hemp seed oils.

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6.3 Sensory evaluation of cold-pressed hemp seed oil conduced remotely during Covid-19 pandemic

6.3.1 Details of the publication based on paragraph 6.3

Title: Remote testing: sensory test during Covid-19 pandemic and beyond

Authors: Caterina Dinnella[a,*], Lapo Pierguidi[a], Sara Spinelli[a], Monica Borgogno[b], Tullia Gallina Toschi[c], Stefano Predieri[d], Giliana Lavezzi[e], Francesca Trapani[f], Matilde Tura[c], Massimiliano Magli[d], Alessandra Bendini[c] and Erminio Monteleone[a]

[a] Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence, Italy
[b] Mérieux NutriSciences Italy
[c] Department of Agricultural and Food Science, Alma Mater Studiorum-University of Bologna, Italy
[d] Institute of BioEconomy, National Research Council, Italy
[e] Kerry Ingredients & Flavours | EMEA Region, Italy
[f] Enrico Giotti S.p.A. a subsidiary of McCormick & Company, Inc.

Corresponding author: Caterina Dinnella – caterina.dinnella@unifi.it

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Abstract

Restrictions adopted by many countries in 2020 due to Covid-19 pandemic had severe consequences on the management of sensory and consumer testing that strengthened the tendency to move data collection out of the laboratory. Remote sensory testing, organized at the assessor's home or workplace and carried out under the live online supervision of the panel leader, represents a trade-off between adequate control and the convenience of conducting testing out of the lab. The Italian Sensory Science Society developed the "Remote sensory testing" research project aimed at testing the effectiveness and validity of the sensory tests conducted remotely through a comparison with evaluations in a classical laboratory setting. Guidelines were developed to assist panel leaders in setting up and controlling the evaluation sessions in remote testing conditions. Different methods were considered: triangle and tetrad tests, Descriptive Analysis and Temporal Dominance of Sensations tests, all of which involved trained panels, and Check-All-That-Apply and hedonic tests with consumers. Remote sensory testing provided similar results to the lab testing in all the cases, with the exception of the tetrad test run at work. Findings suggest that remote sensory testing, if conducted in strict compliance with specifically developed sensory protocols, is a promising alternative to laboratory tests that can be applied with both trained assessors and consumers even beyond the global pandemic

Keywords: sensory analysis, discrimination tests, descriptive analysis, TDS, CATA, liking, home test

6.3.2 Introduction

Covid-19 pandemic has impacted our lives tremendously from several points of view. This includes food behaviours and preferences (Marty, de Lauzon-Guillain, Labesse, & Nicklaus, 2021; Li, Kallas, Rahmani, & Gil, 2021), but also how sensory and consumer testing are conducted due to the restrictions to limit the spread of the virus. In fact, due to government health and safety directives, having assessors gathering in a facility may not be possible, or may be perceived as unsafe by the assessors. This was very clear in the period of strict lockdowns adopted by many countries in 2020 but has consequences that also impact the management of sensory testing even when less restrictive governmental measures are adopted. The push to move out of the laboratory and to conduct more and more home use testing in sensory studies has been observed far before the pandemic (Nogueira-Terrones, Tinet, Curt, Trystram, & Hossenlopp, 2006; Martin, Visalli, Lange, Schlich, & Issanchou, 2014; Galmarini, Symoneaux, Visalli, Zamora, & Schlich, 2016); and predicted as a growing trend in the future (Meiselman, 2013). Furthermore, data collection out of the lab is being made easier by internet technology, which permits data collection anywhere, anytime and in real time. Recently the possibility of conducting sensory tests in the assessor's own vehicle has been proposed as a valid alternative to lab sensory booth setting allowing for participants to feel safe from the risk of COVID-19 while performing sensory evaluations (Seo, Buffin, Singh, Beekman, & Jarma Arroyo, 2021). Live video calls were used for conducting observational studies on children's food preference and intake and were proposed as a valid instrument for food behavioural studies at participant's home even beyond pandemic (Venkatesh & DeJesus, 2021). Internet technology, together with the constraints of the global pandemic, allowed the setup of a solution that represents a trade-off between adequate control and the convenience of conducting testing at home: remote sensory testing. Remote sensory testing is sensory testing organised out of the lab, for example at assessor's home or workplace. However, remote sensory testing radically differs from home use tests as it includes a constant control of the conditions in which the test is performed. The panel leader is in fact connected in videoconference with the assessors for all the duration of the test, having always the possibility to monitor the evaluations and to interact with the assessors, similarly to what happens in a lab environment. These specificities make remote sensory testing a very useful tool, particularly for sensory tests that require highly controlled conditions, such as with trained assessors. While remote sensory testing has been more and more applied starting from the spring/summer 2020, in particular by sensory test providers, food and personal/home care industries, and some attempts of operative

guidelines were made (White Paper, Compusense, 2020), at present there is no published scientific literature that supports the validity of this methodology. The Italian Sensory Science Society (SISS) has responded to this urgent need, that was very large not only for research centers and universities but particularly for industries, by developing the "Remote sensory testing" research project aimed at testing the effectiveness and validity of the sensory tests conducted remotely through a comparison with evaluations in a classical laboratory setting. Five different discriminant or descriptive methods were considered: triangle and tetrad tests (Lawless & Heymann, 2010); Descriptive Analysis (Lawless & Heymann, 2010); Temporal Dominance of Sensations (Pineau,, Cordelle, & Schlich, 2003); Check-All-That-Apply (Adams, Williams, Lancaster, & Foley, 2007; Meyners & Castura, 2014). All the tests involved trained panels apart from Check-All-That-Apply that involved consumers with no specific preliminary sensory training. An hedonic test with consumers was also considered. The aim of this paper is to present the results of this study and to illustrate the guidelines developed for remote sensory testing.

6.3.3. General Methods

6.3.3.1 Overview of the experimental plan

The research project was run in 2020 and involved, on a voluntary basis, six sensory laboratories of public and private organizations belonging to SISS across Italy. A working group, open to SISS members from the laboratories who joined the project, selected the sensory methods to be included in the project to cover quality control activities, descriptive methods performed by a trained panel, and a consumer study. Internal (assessors recruited from personnel of the organization running the test) and external panels (assessors recruited out of the organization running the test) took part in the study. Internal panels were selected as an example of procedures generally adopted by food and food ingredient companies for quality control purposes. To be consistent with the usual conditions of internal panel activities, at the working place and during working hours, the assessor's workstation was selected as remote testing location (RT-W). Assessors' home was selected as the remote testing location (RT-H) for external panels. The working group revised the procedure applied for data collection in lab conditions and defined the procedure for remote testing conditions. Each laboratory team performed data analysis relevant to the sensory test conducted under its own responsibility. The experimental plan and the testing dates are summarized in Table 6.3.3.1.

Table 6.3.3.1. Summary of sensory evaluations performed in lab (LAB) and remote testing (RT) conditions: type of test, samples (product and sample number), location and dates (period) for lab and remote testing conditions (home -H, at work -W), number of assessors and experimental design.

Test	Sample	LAB			RT			Experimental design
		Location	Assessors (n)	Period	Location	Assessors (n)	Period	
Study 1 Discrimination tests								
Tetrad test	Lime Flavour (2 samples)	Kerry (Mozzo, Bergamo Italy)	36	Jul 2019	W	36	Oct 2020	within-subjects
Triangle test	Orange Flavour (2 samples)	Giotti McCormick (Scandicci, Firenze, Italy)	36	Feb 2020	W	36	Jun 2020	between-subjects
Study 2 Descriptive Analysis								
Re-training	Coffee descriptor standards	Mérieux-Nutriscience (Prato, Italy)	23	Jan 2020	H	23	Apr 2020	within-subjects and between-subjects
	Mocha Coffee (3 samples)	Mérieux-Nutriscience (Prato, Italy)	15	Jan 2020	H	24	May 2020	within-subjects and between-subjects
Evaluations	Hemp seed oils (4 samples)	Dept. of Agricultural and Food Sciences, University of Bologna (Bologna, Italy	9	May-Jun 2020	H	9	Jun-Jul 2020	within-subjects
Study 3 Temporal Dominance of Sensations								
	Chewing gums (3 samples)	CNR-Istitute for Bioeconomy (Bologna, Italy)	9	3rd week of June	H	9	4th week of June	within-subjects
Study 4 Check All That Apply								
	Gluten free breads(4 samples)	SensoryLab, Dept. of Agriculture, Food, Environment and Forestry, University of Florence(Firenze, Italy)	60	Nov 2018	H	60	Apr 2020	between-subject

Sensory tests in lab settings during 2020 were performed according to Italian government regulations to control for virus spread, that include: controlled access to the lab after testing for the absence of COVID-19 symptoms; compliance with the minimum interpersonal distance of 1.8 m; wearing masks apart while testing the sample; environment and individual workstation sanitization after every use.

6.3.3.2 Procedure for remote testing

Evaluations in remote condition (RT) were performed by video call from the assessor's home (H) or at work (W), i.e. at the company of the participating research partners of SISS, where sensory testing is usually performed by the trained panellists, but in their own office instead that in the sensory lab) under the guidance of the panel leader. An "evaluation box" with all the equipment needed was delivered to the assessor's home for RT-H evaluations, while a tray with the samples under evaluation, water and crackers for rinsing procedure was brought to the assessor desk in his/her own office by sensory lab personnel for RT-W. Video calls were operated using multimedia platforms (Microsoft Teams). Data were collected with a paper evaluation sheet.

6.3.3.3 General guidelines for panel leaders

Panel leaders were provided with general guidelines for setting up and controlling the evaluation sessions. A list of preliminary documents to lead the remote testing was defined, which included the list of the participants in the session with their contact (email and mobile number, to contact the assessors in private during evaluations in case of need) and the MasterCard to assist assessors in the correct sample evaluation order. Guidelines specified that panel leaders were responsible for supervising the preparation of the "evaluation box" (RT-H) or the "tray" (RT-W) to be delivered to assessors. They were recommended to include in the box all the equipment needed for carrying out the evaluation in order to standardize as much as possible the evaluation conditions. Thus, it was suggested to include in the box three-digit coded vessels for sample evaluation, small white paper towels to cover the working surface, napkins, cutlery if necessary, and unsalted crackers for the mouth rinsing procedure. Moreover, panel leaders were asked to select the most appropriate packaging to assure sample stability and avoid leakage during transportations. It was recommended to minimize the time between sample preparation and evaluation and to eventually perform preliminary tests with sensory lab personnel to identify the maximum time allowed between preparation and evaluation to avoid sample perceptual changes. Panel leaders were also requested to make available to assessors the instructions for the correct sample storage and handling before evaluation. Panel leaders were recommended to schedule remote testing sessions and "evaluation box" delivery controlling for time

between sample shipping and evaluation. They were invited to run the sessions with a maximum of six participants per time to allow the easy monitoring of the assessor's behaviour during evaluation and facilitate eventual corrective action toward a single assessor. They were also recommended to define the evaluation's duration time considering the time needed for checking for the workstation setting up and managing possible delays due to assessor connection difficulties. Guidelines also included detailed instructions on how to plan and carry out the video call on popular multimedia platforms. Finally, panel leaders were recommended to identify (or act themselves as) a contact person for assessor's assistance requests before the evaluation session. Health status of personnel in charge for sample preparation both for RT-W and RT-H was daily controlled for the absence of COVID-19 symptoms according to the measures adopted from the Italian government to limit the spread of the virus (Gazzetta Ufficiale della Repubblica Italiana, n. 61, anno 161°). Sample handling accomplished the procedures reported in "Interim provisions on food hygiene during the SARS-CoV-2 epidemic" from ISS COVID-19 Working group on Veterinary public health and food safety (ii, 17 p. Rapporto ISS COVID-19 n. 17/2020).

6.3.3.4 General instructions to assessors

Assessors received by e-mail the general instructions to participate in the sensory evaluations. They were requested to have a stable internet connection and an appropriate device allowing for both audio and video connection (tablet or pc) to participate in the experiment. They were recommended to have their mobile phone available during the evaluation. Assessors were informed that they would have received instructions for video-call connection and for setting up their workstation at home in the days immediately preceding the test. They were also informed that an "evaluation box" would have been delivered at home with all the equipment needed for setting up the workstation and with instructions for sample storage (RT-H) or, alternatively, that a tray would have been delivered to them by sensory lab personnel (RT-W). A text message advised the assessors that the evaluation box had been shipped, and they were asked to let the sensory laboratory contact person know when they had received it. They were instructed to set-up the workstation in a quiet room, possibly with a window in order to assure ventilation, where they could be alone during the whole test; they were also instructed to choose a working surface wide enough (i.e. 90x60 cm) to comfortably position the connection device and all the equipment needed for the evaluations. Furthermore, they were asked to avoid cooking and using household cleaners one hour before the test and to follow the general behaviour rules preceding sensory evaluations (e.g., do not smoke, eat or drink, apart from water, prior to the test). Assessors were asked to sign the informed consent attached to the message and send it back to the sensory lab. A contact was provided for further clarifications, if needed.

6.3.3.5 General procedure for remote testing

The panel leader opened the video call, checked that the name of the participants in the video call was included in the assessor list and individually tested audio and video connection ensuring that the assessor's faces were visible in the frame. Then, assessors declared that they signed the informed consent and confirmed they had no allergies or intolerances to the sample ingredients. The panel leader invited assessors to position all the equipment delivered in the "evaluation box" on the working surface (e.g., a table or a desk), to access the software for data acquisition and to position the samples according to the evaluation order (the first sample on the left). The panel leader individually checked with assessors the sample positioning according to the MasterCard. Then, evaluation aim and modality were recalled, assessors were told that they were not allowed to talk while the evaluation was ongoing and were instructed to use their mobile to contact the panel leader for assistance (with microphones of the video call muted to avoid interferences with the other panelists' evaluations). Assessors were invited to open the data acquisition software page in full screen mode (so that they could not see each other anymore while their faces were all visible to the panel leader), mute their microphone and start the evaluation. Once assessors completed the evaluation, they were requested to share their screen with the panel leader to show the final page of the session and were allowed to quit the video call. The panel leader monitored the assessor's behaviour during the evaluation and in case of incorrect actions privately contacted the assessor and drew his/her attention on the strict compliance to the evaluation procedure. Equipment, environmental conditions and panel leader activities for sensory testing at assessor's home are summarized in Table 6.3.3.5.1.

Table 6.3.3.5.1. Summary of equipment, environmental conditions and panel leader activities to perform sensory evaluations at assessor's home (RT-H).

Equipment and environment	
"Evaluation box" content	• Three-digit coded vessels · • white paper towel· • napkins and cutlery· • unsalted crackers
Evaluation station	• quiet room with possibility of air exchange· • wide working surface·

	• avoid cooking/household cleaner use 1 h before evaluation start
Panel leader "To do list"	
Days preceding evaluation	• evaluation box delivery· • instruction to assessors for sample handling • instruction to assessors for video call connection • collection of informed consent forms from assessors
Before evaluation start	• participant list and document check· • audio and video connection test· • workstation equipment and organization inspection • sample positioning and sample order assistance • reminder to assessors about evaluation aim and modality • reminder to assessors that talking is not allowed
During evaluation	• data acquisition page in full screen mode • assessor's microphone muted • monitor assessor behaviour
End of evaluation	• final page on assessor screen check • closing connection

6.3.4 Study 2 – Descriptive Analysis (DA)

6.3.4.1 Materials and Methods

Participants

Nine trained assessors (44.4% women, mean age 37.0±13.15) with previous experience in descriptive sensory testing, mainly on olive oil, participated in hemp seed oil (HSO) evaluations.

Samples

Four hemp seed oils (HSO1, HSO2, HSO3, HSO4) were selected for evaluation to cover the sensory variability of HSO on the Italian market. The evaluation box for HSO tasting in RT-H condition included pre-weighted samples (20 ml) in three digit coded sealed containers, disposable glasses identified by the same three-digit codes, the same standards for colour and flavour attributes used for training, the evaluation sheet, napkin and crackers for rinsing procedure and instructions for sample storage (6-8°C, room temperature 40 min before evaluation).

Evaluations

Trained assessors for HSO evaluation participated in 20 training sessions held at the sensory lab (in 2019) consisting of the generation of a list of attributes describing HSO sensory profile (four sessions) and panel calibration (sixteen sessions). For term generation, assessors were asked to taste sixteen samples representative of the sensory variability of HSO on the market. Samples used for training were purchased both in large scale distribution and retail stores considering production process as the main selection criterion. Only oils obtained by mechanical or physical processes (i.e. labelled as "cold-pressed" or "obtained only by mechanical/physical processes") were selected. The panel consensus was reached on a list of nine attributes: yellow, green, rancid, paint, roasted, fishy, sunflower/pumpkin seeds, toasted hazelnuts, and hay. An evaluation sheet was then set up following the official olive oil evaluation sheet layout (COI/T.20/Doc. No 15/Rev. 2 September 2007). To facilitate the calibration of the panel on descriptors, participants were familiarized with standard solutions prepared to induce a moderate intensity (corresponding to the central point of a 100 mm unstructured scale) of the seven flavour descriptors. Reference HSO samples were provided as a standard for yellow (two references corresponding to 20 and 60 on the scale) and green (two references corresponding to 50 and 80 on the scale).

Procedure

The trained panel participated in eight sessions for HSO evaluations; four sessions were held in the lab and four sessions were held in RT-H condition. Two samples (in replicates, i.e. assessed twice) were evaluated in each session. Colour and flavour standard solutions were made available to assessors before evaluation. They were instructed to observe colour and smell flavour standards in order to help identification and ratings of the relevant sensations in HSO samples. In lab evaluation conditions, samples (20 ml) were presented in disposable glass coded with random three-digit codes. In RT-H conditions, assessors were instructed to fill the provided disposable glass with the corresponding sample (20 ml). The presentation order of the samples was randomized among assessors using a balanced Latin square design. Assessors were asked to take a sip and rate descriptors' intensity on the paper evaluation sheet. An unstructured 100 mm scale was used for intensity ratings (0=extremely weak; 100=extremely strong). After each sample, subjects were asked

to take a short break and rinse their mouths with water and crackers. HSO data was collected on a paper evaluation sheet. In RT-H conditions assessors were asked to take a picture of the sheet using their mobile and send it to the Sensory Lab contact person. Evaluations in lab conditions were performed in individual booths under white light.

Data analysis

Intensity data collected in lab and RT-H conditions were independently submitted to a 3-way ANOVA Mixed model (fixed factors: samples and replicates; random factor: assessors) with interactions. A further two-way ANOVA model (samples and conditions) was computed to test the effect of condition on HSO significant attributes. Post-hoc Fisher (LSD) multiple comparison tests were carried out to determine significant differences between samples in each condition. The significance level was fixed at 95% ($p \leq 0.05$). ANOVA models were performed using XLSTAT, (version 2021.3.1, Addinsoft, NY, USA).

6.3.5 Results

Descriptive Analysis

Analysis on data from HSO evaluations separated by condition showed a significant sample effect on the same six (yellow, green, fishy, pumpkin seeds, toasted hazelnut and hay) out of eight attributes ($p \leq 0.002$). A significant assessor*sample interaction was found for fishy in lab conditions ($F=2.20$ $p=0.029$). However, considering the low F values for this interaction compared to the F values for the product effect ($F=41.27$), it could be assumed that the interaction effect is negligible (Næs et al., 2010). F values for fishy, toasted hazelnuts and hay were very similar in both conditions; F values for yellow was higher in lab than in RT-H condition while the opposite was observed for green and sunflower seeds (Fig. 6.3.5.1).

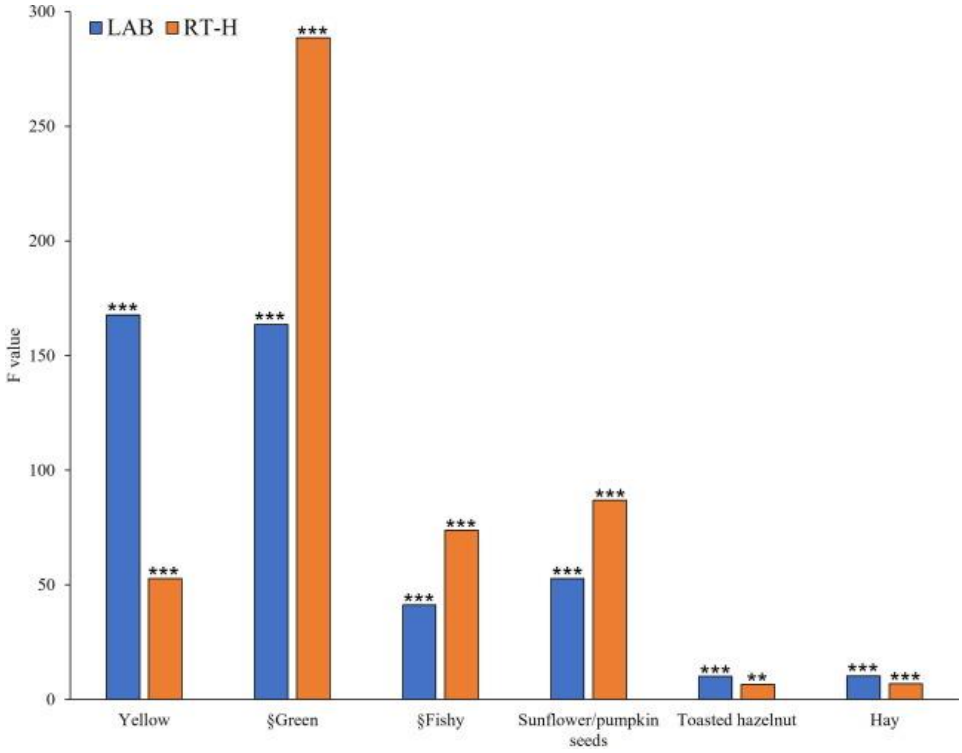

Figure 6.3.5.1. Three-way ANOVA on hemp seed oil (HSO) intensity data: F-values of sample effect in lab (LAB) and remote sessions at home (RT-H). Significance: ***=$p<0.001$, **=$p<0.01$, *=$p<0.05$. § indicates that F value was $x10^{-1}$

The effect of condition on HSO sample evaluation for discriminating attributes was further investigated. The comparison between data collected in the lab and in RT-H conditions showed a significant effect of samples for all attributes ($p\leq0.001$). Furthermore, a significant effect of evaluation conditions ($F=4.4$; $p=0.038$) and interaction sample*condition ($F=5.7$; $p=0.001$) for yellow was reported: no significant difference in yellow intensity evaluated in lab and remote conditions were found for HSO2 and HSO4 while HSO1 and HSO3 were rated higher in remote than in lab conditions (Fig. 6.3.5.2).

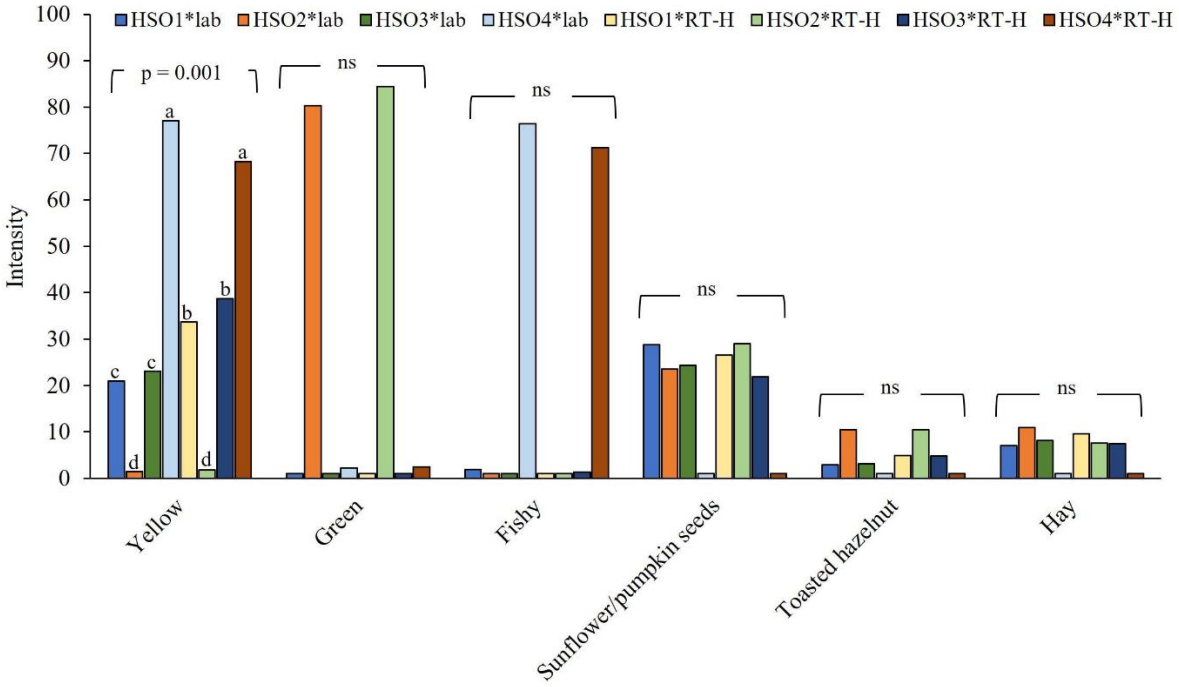

Figure 6.3.5.2. Two-way ANOVA model on hemp seed oils (HSO1, HSO2, HSO3 and HSO4) intensity data: comparisons of mean sample scores between lab (LAB) and remote sessions at home (RT-H). P values for product*condition interaction effects are reported. Different letters indicate a significant difference at $\alpha = 0.05$ as determined by Fisher's least significant difference (LSD).

6.3.6 Discussion

Hemp seed oil was selected as a "simpler case" in which samples are delivered as ready to be evaluated. Results from hemp seed oil descriptive analysis indicated that the sensory profile of samples obtained in the two conditions are very similar, as well as the assessor performance. These findings clearly indicate that descriptive analysis in remote conditions represents an alternative to the lab evaluation when samples are provided ready to be used. However, a significant effect of the evaluation conditions on colour evaluation was found. Light conditions at home cannot be easily standardized. When colour represents a target attribute, further effort should be made by experimenters to provide assessors with appropriate light devices to overcome this source of variability in respect to the lab conditions. However, results from descriptive analysis indicate a substantial match between sensory descriptions obtained in classical laboratory settings and in remote conditions at the assessor's home. This confirms previous evidence of the reliability of descriptive data from food sensory evaluations carried at the assessor home (Martin et al., 2014). Previous research using an Internet panel found some disagreement between the results of the Internet and lab reference panels and a poorer performance of the former panel (Nogueira-Terrones et al., 2006). In their study however the assessors were left free to choose the time for the evaluations and the contacts

with the panel leader were only possible by phone and email (so not live). The results of our study show instead that live remote testing ensures satisfactory performance of the home panel. It should also be noted that Nogueira-Terrones et al. (2006) recruited new assessors and the whole process of training was done online, while in our case a re-training of the panel was performed. This suggests that training is a key factor when performing a remote descriptive analysis.

6.3.7 Conclusions

Remote sensory testing was found to be appropriate for studies with trained panellists but can also be useful with studies with consumers, when there is a need for a control of the testing conditions and a conventional home use test (in which the product is evaluated in natural conditions) is not optimal. Furthermore, consumer testing in remote conditions can help in overcoming logistic limitations when data from different regions or different countries need to be collected (i.e. cross cultural comparison). Sample characteristics limit the possibility of remote testing to products that can be handled and shipped without any hazard for participant safety and that in general show relatively high stability (physico-chemical, microbiological and sensory). Finally, it is worth noting that remote testing is time saving for participants in sensory evaluation (no time needed for travelling to sensory lab facilities) and more flexible than lab testing, and thus may facilitate participant recruitment, availability, and motivation.

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Chapter 7

Evolution of the hemp seed oils composition during storage

7.1 Instrumental evaluation of the changes in the hemp seed oil composition during 3 months of storage

7.1.1 Details of the publication based on Paragraph 7.1

Title: Changes in the composition of a cold-pressed hemp seed oil during three months of storage

Authors: Matilde Tura[a], Mara Mandrioli[a], Enrico Valli[a,*], Rosamaria Cristina Rubino[b], Dylan Parentela[c], Tullia Gallina Toschi[a]

[a] Department of Agricultural and Food Sciences, Alma Mater Studiorum - Università di Bologna, Viale G. Fanin 40, Bologna (BO), 40127, Italy
[b] Enecta Srl, Viale E. Panzacchi 17, Bologna (BO), 40136, Italy
[c] -GreenValley Sa, Via Casale, snc, Castel di Ieri (AQ), 67020, Italy

Corresponding author: Enrico Valli -enrico.valli4@unibo.it

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Abstract

Several analytical parameters were evaluated to investigate changes in the composition of a cold-pressed hemp seed oil over a period of 3 months of storage. The environmental conditions applied for the conservation were 12 hours of light and 12 of darkness at room temperature, to mime a grocery or supermarket shelf, and the oil was stored in amber glass bottles. The peroxide value was quite low on freshly produced oil (2.66 ± 0.29 mEqO2/kg of oil) and decreased after 3 months (1.35 ± 0.08 mEqO2/kg of oil), as confirmed by free radical concentration, while no other statistically significant (one-way ANOVA, Tukey's HSD test, $p<0.05$) differences were observed (e.g. conjugated diene and triene systems, Oxidative Stability Index and free acidity). The fatty acid, cannabinoids and tocopherols composition did not statistically change; expect for δ-tocopherol, for which a decrease was observed. The overall results did not show a strong effect of photooxidation on the oil, despite its high degree of unsaturation.

Keywords: Hemp seed oil, oxidation, storage condition, shelf-life, cold-pressing, cannabinoids, tocopherols, polyunsaturated fatty acids

7.1.2. Introduction

Hemp (*Cannabis sativa* L.) is a versatile plant that can be grown to obtain different products, for example fiber or seeds, and can also be used for food and nutraceutical purposes (Rapa et al., 2019). Hemp seeds have been quite recently reconsidered because they represent a valuable industrial crop and for their nutritional value, which is why they are appreciated in many European countries, but also in Asia and Canada (Mikulec et al., 2019). Hemp seeds are composed of protein (22-25%), lipids (30-35%), consisting mainly in polyunsaturated fatty acids (PUFAs), and carbohydrates (35-37%) (Mikulec et al., 2019; Rapa et al., 2019). Hemp seeds are usually transformed into flour or oil; in order to obtain an oil with higher nutritional and sensory qualities, hemp seeds are commonly cold-pressed. Cold pressing is a technology that does not involve the use of heat treatment or solvent (Codex Stan 210-1999; Lutterodt et al., 2010). Cold pressing aids in preserving minor compounds that, in contrast, could be reduced during a refining process; in fact, a challenge that concerns virgin oils is to maintain low the free acidity value, because the action of lipases can increase it. For this reason, they can be subjected to a refining process. The maximum free acidity value admissible for a cold pressed vegetable oil is 4.0 mg KOH/g of oil (Codex Stan 19-1999): the seeds must be in perfect condition to obtain an oil that respects this limit; for example, high relative humidity and temperature increase enzymatic activity, and thus the free fatty acid content in the oil (Jian et al., 2019). When the free acidity is higher than this limit, mild refining can be applied to cold-pressed seed oils. In this case, according to Codex Alimentarius (Codex Stan 210–1999) they can not be defined as "cold-pressed" oils. On the other hand, thanks to this soft refining process, the oils are deacidified but, at the same time, they still present similar visual and olfactory sensory attributes to unrefined oils (Bendini et al., 2011).

The main PUFAs present in hemp seed oil are linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3) (Da Porto et al., 2012), approximately 50-70% and 15-25% respectively (Esmaeilzadeh Kenari & Dehghan, 2020). LA and ALA are considered as essential because humans cannot produce them on their own: they must have been taken with the diet, and the ratio in which they are present in hemp seed oil, namely 3:1, is optimal from a nutritional point of view (Da Porto et al., 2012; Teh & Birch, 2013). Furthermore, hemp seed oil contains a significant amount of stearidonic acid (range from 0.5 to 2%) (Mikulcová et al., 2017), which has a positive impact on human health. It has become clear that dietary stearidonic acid increases eicosapentaenoic acid more efficiently than ALA. For this reason, it is also of interest for individuals who suffer from a deficit in Δ-6-desaturase function, for whom an additional supplementation of stearidonic acid can improve the formation of eicosanoids (Matthäus & Brühl, 2008). The presence of γ-linolenic acid (GLA, 18:3 n-3) also makes hemp seed oil suitable as ingredient in cosmetic products (Da Porto et al., 2012). One of the main factors

influencing the quality of edible oils is the oxidative phenomenon, which can occur during storage (Liang et al., 2018), and is one of the causes of flavor deterioration of oils and oily products (Uluata & Özdemir, 2012). During storage, fatty acids can undergo oxidative degradation, determining the progressive formation and accumulation of hydroxyperoxides (odorless molecules) and then secondary products, such as hexanal or 2-butenal (Poyato et al., 2014), which are associated with off-flavors. Moreover, oxidation products can also have a harmful impact on human health (Frankel, 1983). Cold-pressed hemp seed oil also contains bioactive compounds (i.e. tocopherols, polyphenols, phytosterols and carotenoids) that have health benefits (Teh & Birch, 2013), which can reduce oxidation (Liang et al., 2015). However, high quantities of PUFAs make the oil prone to oxidation, thus the quality must be monitored, also for safety concerns (The & Birch, 2013). Quality control must take place throughout the entire supply chain, since unsaturated fatty acids easily react with free radicals and oxygen giving rise to oxidation products (Sapino et al., 2005). Accordingly, the determination of peroxide value and conjugated dienoic fatty acid derivates are often used to monitor the oxidative state of oils (Teh & Birch, 2013).

Although the production and marketing of hemp seed oil has recently increased, not much is known about how hemp seed oil behaves during storage and under certain photooxidation conditions. Also, considering that in the hemp seed oils on the market the *best before date* is not defined in a homogeneous way, the assessment of the changes in the composition of hemp seed oil during storage could be useful for companies that market this product. For these reasons, this investigation evaluated the changes in the oxidative state of cold-pressed hemp seed oil by mimicking market conditions during 3 months of storage on a supermarket shelf. The evolution of several analytical parameters was monitored, and in particular peroxide value (PV), free acidity, oxidative stability index (OSI), spectrophotometric investigation in the ultraviolet (K_{232} and K_{268}), and the concentration of free radicals by forced oxidation test with electronic spin resonance (ESR) spectroscopy. In addition, the fatty acid composition of hemp seed oil was analyzed by GC-FID, the tocopherols content and the cannabinoids profile were investigated by HPLC-DAD and HPLC-UV, respectively. These aspects can be useful for companies to provide information about storage.

7.1.3. Materials and Methods

7.1.3.1 Samples

Hemp seed oil was obtained by cold pressing of hemp seeds (Futura 75 variety), dried and not skinned, using a screw press with 20 kg/hour capacity (Plus model, engine power of 3.0 kW, rpm 15-70, voltage 400 V 3-ph, dimensions 480 x 480 x 620 mm, and weight 145 kg) supplied by Tecnoimpianti Srl (Ospedaletto Euganeo, Padua, Italy). The oil was filtered with cotton gauze; then,

given the amount of sediment still present, it was centrifuged in a 50 mL Falcon type tube for 20 minutes at 4000 rpm. A centrifuge model PK 120-ALC supplied by the Opto-Lab company (Modena, Italy) was used. The hemp seed oil was divided into 10 amber glass bottles of 100 mL each (named from T0 to T9); these, equipped with screw caps, were filled to the brim to reduce head space and to limit oxidative phenomenon due to the presence of oxygen. Samples were analyzed every 10 days during three months of storage, as reported by Matthäus & Brühl, 2008. The samples were kept inside a controlled environment (at room temperature), protected from external ambient light. In order to simulate shelf conditions in a supermarket, and also according to literature (Kanavouras & Coutelieris, 2006; Pristouri et al., 2010; Garitta et al., 2018), a photoperiod of 12 hours of light and 12 hours of dark was applied (Prescha et al., 2014), at room temperature, by using LED lighting with an intensity of 270 lux, according to the minimum lighting value of 200 lux reported by UNI EN 12464-1:2011. An electrically powered timer allowed for control of the photoperiod. Bottles were rotated every 10 days. For all analyses, three analytical replicates were performed. Free acidity, peroxide value, spectrophotometric investigation in the ultraviolet, oxidative stability index and the determination of free radicals by ESR were performed every 10 days; while, the determination of fatty acid composition, the determination of cannabinoids and tocopherols content were evaluated only on samples T0 and T9, in order to assess possible differences between the beginning (named time 0, T0) and the end of the storage period (named time 9, T9). At each time the sample was directly analyzed, without storage it in the refrigerator.

7.1.3.2 Free acidity

The method described by the Codex Alimentarius (Codex Stan 19-1999) was applied for determination of the free acidity. The acidity index is defined as mg of KOH needed to neutralize the free acids present in 1 gram of oil.

7.1.3.3 Peroxide value

The PV determination was carried out according to the NGD C35-1976 method (NGD method C35), performing an iodometric titration. In fact, by following an oxide reduction reaction in the presence of starch as an indicator, it is possible to measure the quantity of peroxide present in the sample. Results are expressed as mEq of active oxygen per kg of oil.

7.1.3.4 Spectrophotometric investigation in the ultraviolet

The analysis of dienoic and trienoic conjugated fatty acid derivates was performed by following the method described in the ISO 3656:2011. The spectrophotometric analysis was performed using a

Jasco dual beam spectrophotometer model V-550 UV-Vis (Jasco, Tokyo, Japan). A quartz cuvette with an optical path of 10 mm was used. The determination is based on spectrophotometric analysis of 0.1 g of oil diluted in 10 mL of iso-octane. The spectrophotometric investigations were performed at 232 nm and 234 nm for the determination of diene conjugated systems at 262, 264, 266, 268, 270, 272, and 274 nm for the triene conjugated systems.

7.1.3.5 Oxidative stability index

The analysis was performed using an oxidative stability instrument (OMNION OSI-8 Decatur, IL, USA), by following the method described by the AOCS Official Method Cd 12b-92. Five g of oil was weighed in a glass tube and heated to 110°C in the presence of continuous air flow (150 mL/min). Through this continuous measurement, the instrument extrapolates the data relative to the induction period from the initial phase of oxidation in which it assumes an exponential trend, known as OSI-time (Jebe et al., 1993). With this test, the samples are subjected to the accelerated oxidation test, in standardized conditions; the induction period is measured in hours.

7.1.3.6 Electron Spin Resonance spectroscopy

The determination of free radicals was performed by following an internal method. Briefly, one mL of oil was added with 40 µL of 2.5 M *N*-tert-butyl-α-phenylnitrone (PBN, ≥98% VWR-International, Milan, Italy) in ethanol, vortexed for one minute, and readings were taken with microESR STANDARD V 2.0 (Bruker BioSpin GmbH, Rheinstetten, Germany) heating the sample to 110°C for 108 minutes, and recording the spectra obtained every 7 minutes. The standard for the calibration curve used was constructed by TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy, free radical) solutions in mineral oil (Sigma-Aldrich, Missouri, USA) at concentrations of 1.0, 2.5, 5.0, 10.0, 20.0, 35.0, and 50.0 µM. Results were expressed as µM.

7.1.3.7 Fatty acid composition

The determination of the fatty acid composition was performed using a GC Shimadzu (Tokyo, Japan) 2010 Plus instrument equipped with a flame ionization detector 2010 Plus (Shimadzu, Tokyo, Japan). First, alkaline treatment was used by mixing 0.01 g of oil dissolved in 500 µL of *n*-hexane and 20 µL of 2 N potassium hydroxide in methanol. The mixture was centrifuged and the supernatant containing the fraction of interest was taken. The applied GC conditions were the same reported by Nsir et al., 2017, by using helium as carrier gas and a fused silica BPX-70 capillary column (10 m length, 0.1 mm i.d., 0.2 µm film thickness), purchased from SGE (Ringwood Victoria, Australia); with a total time of analysis of 5 minutes. To identify peaks, retention times were compared with those of

authentic reference compounds (reference standards GLC463 from Nu-Chek Prep. Inc., Elysian, MN, USA) injected under the same analytical conditions. Only for stearidonic acid (C18:4 n-3) was a comparison with the literature performed (Mottram et al., 1997). Results were reported as % of total fatty acids (area %).

7.1.3.8 Determination of cannabinoids

The determination of cannabinoids was performed by using a HPLC Cannabis Analyzer for Potency Prominence-i LC-2030C equipped with a reverse phase C18 column, Nex-Leaf CBX Potency 150 × 4.6 mm, 2.7 µm with a guard column Nex-Leaf CBX 5 × 4.6 mm, 2.7 µm (Shimazu, Kyoto, Japan), UV detector and acquisition software LabSolutions version 5.84 (Shimazu, Kyoto, Japan).

0.5 g of hemp seed oil were weighed into a 10 mL volumetric flask and were brought to volume with isopropanol. Samples were placed 10 minutes in an ultrasonic bath and were then were filtered with a 45 µm nylon filter. Samples were transferred into a HPLC vial and the analysis was performed according to the method proposed by Mandrioli et al. (2019). The quantification was performed using a calibration curve for each cannabinoid, with the external standard method, by the injection of the standards (Phytocannabinoid Mixture 10 (CRM), Cayman Chemical, Michigan USA) in the range of 0.5 µg/mL to 20 µg/mL for CBDA and in the range of 0.05 µg/mL to 10 µg/mL for the other cannabinoids. Results were reported as mg/kg of oil.

7.1.3.9 Determination of tocopherols

This determination was performed by following the UNI/TS 11825:2021, by using liquid-chromatography coupled with a diode-array detector (HPLC-DAD). In brief, 0.5 g of oil were solubilized in isopropanol and 20 µL was injected into the HPLC system equipped with a quaternary pump model HP 1260; the software for data processing was Chemstation for LC3D (Agilent Technologies, Palo Alto, CA, USA). The instrument was also equipped with a column Cosmosil π NAP 150 mm × 4.6 mm Thermo Fisher, 5 µm (Nacalai-Tesque, Kyoto, Japan). The mobile phase was solvent A, methanol–water with 0.2% of H_3PO_4 (90:10 v/v), and solvent B, acetonitrile (100%), eluted in gradient with a flow rate of 1.0 mL/min. The diode-array detector was set up at 292 nm. Quantification was carried out using a calibration curves of alfa and gamma tocopherols (CAS numbers 10191-41-0 and 54-28-4, respectively; Sigma-Aldrich, Missouri, USA) which were constructed with the external standard method, injecting solutions of known concentration in the range of 0.5 ppm to 50 ppm. The identification of δ-tocopherol was performed by the injection of a standard (CAS number 119-13-1; Sigma-Aldrich, Missouri, USA) while the quantification was done by using the curve constructed with the standard solutions of α-tocopherol. Results were expressed as mg/kg of oil.

7.1.3.10 Statistical analysis

One-way ANOVA (Tukey's HSD test, $p<0.05$) was applied for the evaluation of statistical significance differences during the storage period using XLSTAT software version 2018 (Addinsoft, New York, USA).

7.1.4. Results and Discussion

7.1.4.1 Hydrolytic rancidity

The free acidity remained lower than the maximum limit reported by the Codex Alimentarius which, for vegetable oils obtained by cold pressing, is 4.0 mg KOH/g of oil (Codex Stan 19-1999), i.e. 2% of oleic acid (Rovellini et al., 2013). In particular, from a value of 1.78 ± 0.18 mg KOH/g for the oil freshly produced (T0), it reached a value of 2.00 ± 0.07 mg KOH/g of oil (1.01 ± 0.04 expressed as % of oleic acid) at the end of the storage (T9). Thus, no significant variations were observed during the storage test (Table 7.1.4.1.1), in line with the previous observations of Prescha et al. (2014), who showed that the free acidity of a hemp seed oil did not undergo significant changes over time, remaining stable up to 6 months. Our results are in accordance with Spano, et al. (2020), which reported that most of the hemp seed oils analyzed had a free acidity value lower than 2.22 mg KOH/g.

Table 7.1.4.1.1. Results of free acidity, peroxide value, spectrophotometric investigations in the ultraviolet region (K_{232} and K_{268}) and oxidative stability index (OSI time) at the different storage time. Data are expressed as mean±standard deviation of three replicates.
Different letters in the column indicate significant differences (Anova, Tukey's HSD test, $p<0.05$).

Time (days)	Free acidity (mg KOH/g of oil)	Peroxide values (mEq O_2/kg of oil)	K_{232}	K_{268}	OSI time (hours)
0	1.78±0.18^{a}	2.66±0.29^{a}	2.58±0.40^{b,c}	0.45±0.05^{b,c}	3.28±0.12^{a,b}
10	1.88±0.02^{a}	1.48±0.17^{b,c}	3.82±0.62^{a}	0.73±0.07^{a}	3.00±0.23^{b}
20	1.90±0.07^{a}	1.62±0.18^{c}	1.97±0.12^{c,d}	0.43±0.01^{b,c}	3.17±0.13^{b}
30	1.98±0.03^{a}	1.49±0.05^{b,c}	2.50±0.28^{b,c}	0.47±0.03^{b,c}	2.95±0.13^{b}
40	1.98±0.03^{a}	1.79±0.13^{b}	1.63±0.05^{d}	0.40±0.02^{c}	3.12±0.14^{b}
50	1.95±0.08^{a}	1.10±0.12^{c}	2.03±0.14^{b,c,d}	0.51±0.03^{b}	3.18±0.20^{a,b}
60	1.84±0.12^{a}	1.46±0.17^{b,c}	1.62±0.15^{d}	0.42±0.03^{b,c}	3.18±0.16^{a,b}
70	1.88±0.07^{a}	1.11±0.09^{c}	2.76±0.11^{b}	0.47±0.02^{b,c}	3.63±0.21^{a}
80	2.00±0.04^{a}	1.60±0.12^{b}	2.45±0.09^{b,c}	0.49±0.00^{b,c}	3.03±0.03^{b}
90	2.00±0.07^{a}	1.35±0.08^{b,c}	2.51±0.12^{b,c}	0.51±0.02^{b}	3.13±0.15^{b}

The starting value of free acidity of a mechanically extracted and non-refined hemp seed oil, which has to be lower than 4.0 mg KOH/g of oil, is one of the most stringent parameters and is difficult to accomplish; once the oil is bottled this value remains constant. The free acidity depends on the quality of the seeds and the storage conditions. Even if official guidelines for the correct storage of hemp seeds do not exist to the authors' knowledge, it is important to monitor the environmental conditions (such as temperature, relative humidity and storage time) and other parameters related to the seeds (e.g. moisture content and dockage percentage) (Jian et al., 2019) in order to obtain a good hydrolytic quality of the freshly produced oil that is lower than the legal limit.

7.1.4.2 Oxidation of hemp seed oil

The PV, starting from 2.66 ± 0.29 mEq O_2/kg of oil at T0, showed significant fluctuations, but did not increase during 3 months of storage. The fluctuations in peroxides are due to their instability, which are able to quickly react with other compounds to generate radical forms, and in particular

hydroperoxy and alkoxy (Frankel, 1983) that in turn can evolute or degrade into more stable secondary oxidation products (Xu et al., 2014). The highest value at T0 of peroxides can be interpreted as a trigger effect generated by the accidental high oxygenation of the oil occurring during the production and bottling phases. In fact, as reported by Parenti et al. (2007), there is a strong influence of the initial quantities of dissolved oxygen on the oxidative state of the oil. In contrast, the non-increase of the number of peroxides can be explained by the decrease of the dissolved oxygen concentration generated both by: *i)* its consumption in forming secondary oxidation products (Frankel, 1983) and *ii)* its volatilization in the headspace without any replacement if the bottle is correctly closed (Parenti et al., 2007). For these reasons, the PV were, during the 3 months of storage, lower than the maximum limit established for cold-pressed vegetable oils by the Codex Alimentarius (Codex Stan 19-1999), i.e. 15 $mEqO_2$ /kg of oil. In particular, the results were always lower than 2.66 ± 0.29 $mEqO_2$/kg of oil and the highest value was detected in the freshly produced oil (T0), which is accidently "oxygenated" during the post-production phases. This shows how contact of the oil with oxygen in the bottling phase is critical and should be reduced and controlled to guarantee a longer shelf-life of the oil (Del Nobile et al., 2003; Parenti et al., 2007).

The determination of conjugated dienes and trienes was carried out to evaluate the presence both of primary and secondary oxidation compounds. However, since no specific limits are established for those oxidation compounds for hemp seed oil by either the Codex Alimentarius or European and Italian regulations, the results obtained were compared with data in the literature. As reported in Table 7.1.4.2.1, hemp seed oil is characterized by a high percentage of linoleic and α-linolenic fatty acids (Dimić et al., 2009; Baldini et al., 2018), which are unsaturated fatty acids that are particularly prone to oxidation (Madhujith & Sivakanthan, 2018).

Table 7.1.4.2.1. Fatty acid composition of the sample at the beginning (day 0) and at the end (day 90) of the storage period. Data are expressed as mean±standard deviation (area %) of three replicates.

Fatty acid	**T0** **(area %)**	**T9** **(area %)**
Palmitic acid (C16:0)	7.68±0.03	8.36±0.18
Trans-palmitoleic acid (*trans* C16:1 n-7)	0.02±0.00	0.02±0.00
Palmitoleic acid (C16:1 n-7)	0.12±0.01	0.13±0.01
Margaric acid (C17:0)	0.05±0.00	0.05±0.01
Stearic acid (C18:0)	2.73±0.04	2.96±0.07
Oleic acid (C18:1 n-9)	13.41±0.03	14.47±0.33
Vaccenic acid (C18:1 n-7)	0.78±0.02	0.89±0.06
Linoleic acid (C18:2 n-6)	55.84±0.04	52.18±0.04
γ-linolenic acid (C18:3 n-6)	1.10±0.03	1.16±0.05
α-linolenic acid (C18:3 n-3)	16.36±0.04	17.67±0.43
Stearidonic acid (C18:4 n-3)	0.77±0.02	0.84±0.04
Arachidic acid (C20:0)	0.32±0.03	0.32±0.02
Gadoleic acid (C20:1)	0.37±0.03	0.37±0.00
Behenic acid (C22:0)	0.30±0.02	0.38±0.06
Lignoceric acid (C24:0)	0.15±0.00	0.17±0.01
SFA	11.23	12.24
MUFA	14.70	15.88
PUFA	74.07	71.85
ω6/ω3	3.32	2.88

The analysis of the conjugated dienic fatty acid derivatives was firstly carried out at two wavelengths (232 and 234 nm); 232 nm was chosen due to the greater absorbance seen at this wavelength

compared to 234 nm, in accordance with the literature (Rovellini et al., 2013). The results showed that the value at T1 (10 days of storage) was significantly different from the others, as reported in Table 7.1.4.1.1. During storage, no significant differences were detected between the initial (T0) and final (T9) values of conjugated dienes and trienes (one-way ANOVA, Tukey's HSD test, $p<0.05$). The highest value, observed at T1 (10 days of storage), could be linked with the evolution of peroxides registered at T0, which formed during the bottling phase.

Also, the determination of conjugated trienoic fatty acid derivatives was carried out at different wavelengths (262, 264, 266, 268, 270, 272, and 274 nm). As for the diene systems one wavelength was selected to perform the analyses; in particular, 268 nm was chosen for measurement as it showed the highest absorbance value. At K_{268}, T1 showed an absorption that was higher than the others, while the difference between the initial (T0) and final value (T9) was not statistically significant (Table 7.1.4.1.1). Liang et al. (2018) also reported that hydroperoxides and dienes accumulated more during the initial phases of hemp seed oil oxidation; the subsequent small variations could thus indicate limited changes in the oxidative state of α-linolenic acid (Oomah et al., 2002), coherent with what emerged from determination of fatty acids, which did not show a decrease in α-linolenic acid (Table 7.1.4.2.1) and substantially in accordance with what previously reported in the literature (Prescha et al., 2014).

Thus, it was possible to confirm, also through the evolution of secondary compounds, that 3 months of light/dark storage, such as in a grocery shop or a supermarket, did not leads to significant oxidation. The lower values of K_{232} and K_{268} at T2 (20 days of storage) could be due to a partial degradation of the dienes and trienes formed between T0 and T1 (10 days of storage) due to the formation of volatile compounds, such as hexanal or 2-butenal (Frankel, 1983) and to a substantial steady state of oxidation from T2 (20 days of storage) to T9 (3 months). In fact, during the storage period, free radicals attack the double bond of unsaturated fatty acids, resulting in the formation of a conjugated bond even if some of the double bonds were destroyed during autoxidation (Nyam et al., 2013). Results of K_{232} and K_{268} are in accordance with data reported by Oomah et al. (2002), which found a K_{232} equal to 2.61 and a K_{270} value equal to 0.54.

The results of the forced oxidation, measuring the oxidative stability index (OSI) time, showed a short induction period for the hemp seed oil, as predictable from the fatty acid composition, but this remained nearly constant during the storage period considered. In fact, although it appeared slightly higher (3.63 ± 0.21 hours) for T7 (70 days of storage), the value was not significantly different from T0 (Table 7.1.4.1.1). OSI times were higher with respect to those reported by Uluata & Özdemir (2012), which was 1.32 hours at the same temperature (110°C) with a higher air flow (20 L/h), but were comparable to those reported by Irfan et al. (2019), which was 3.12 ± 0.02 hours, conducted at

a higher temperature (120°C) and air flow (20 L/h). The oxidative stability of cold-pressed oils as well as their shelf-life, are critical factors for their potential food applications. Lipid oxidation depends on several factors, such as the fatty acids composition, the presence of antioxidants and the storage conditions (Parker et al., 2003). It is amply recognized that the oxidative chain reaction mediated by free radicals is the main mechanism of lipid peroxidation. In fact, free radicals can be generated in the first phase of the oxidative chain, called the initiation phase, and lead to the acceleration of lipid oxidation in the next phase, called propagation. ESR is a technique that determines unpaired electron species, such as that of free radicals, and for this reason it is widely used in investigations on lipid peroxidation by quantifying radicalization and accumulation in experimental systems (Lutterodt et al., 2010). Regarding the results from ESR spectroscopy analysis, performed by microESR at a lower ESR signal intensity, which corresponds to a lower radical concentration (Lutterodt et al., 2010), Velasco et al. (2004) found low progression of the oxidative phenomenon in the lipid matrix. However, it should be highlighted that the instrument only measures the radical forms and the lower concentration of free radicals found for sample T9 (3 months) with respect to other samples (Figure 7.1.4.2.1) could be linked to the degradation of peroxides into neutral secondary compounds (not new radical forms).

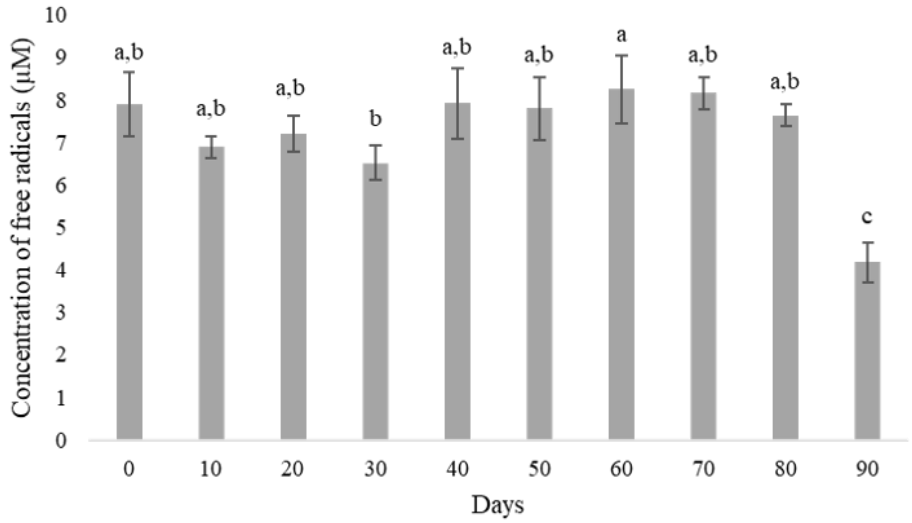

Figure 7.1.4.2.1. Determination of free radicals by electron spin resonance spectroscopy expressed as mean±standard deviation of three replicates after 108 minutes of forced oxidation assay at 110°C. Different letters indicate significant differences (Anova, Tukey's HSD test, $p<0.05$).

On the other hand, the oscillation of the concentration of free radicals during the storage test could be due to the instability of the radicals themselves, whose concentrations, if the exponential

propagation phase does not begin, remain within an interval far from the exponential propagation phase. This possibility and the experimental evidence would therefore be in agreement with Raitio et al. 2011, according to which the results of the ESR, in terms of concentration of free radicals, agree with the number of peroxides. What was observed can be defined as an "oxidative wave", which however does not give rise to subsequent degeneration of the oil. In fact, the "oxidative wave", recorded using official parameters (peroxides, K_{232} and secondary products by K_{268}) and the microESR trace, showed a crest in the first 10 days (from T0 to T1) with no further increase in the following 3 months of storage.

7.1.4.3 Changes in the composition of hemp seed oil

The determination of fatty acids, carried out exclusively on samples T0 (beginning of storage) and T9 (end of storage), showed a slightly decrease in linoleic acid (Table 7.1.4.2.1), equal to -6.05%, suggesting that it could be the main oxidation substrate during the storage period considered.

This phenomenon, even if very low, could be due to the nature of the fatty acid C18:2 n-6 which, being polyunsaturated and present in high percentages, can be more subject to oxidation than other fatty acids (Frankel, 1983; Yun & Surh, 2012), also considering that the predicted half-life of ω-3 and ω-6 fatty acids is around 3-5 days at 25°C (Singh et al., 2020). The data, expressed as an internal percentage, are in accordance with what reported in the literature with reference to the fatty acid composition of hemp seed oil (Petrović et al., 2015), and in particular with oils obtained with the Futura 75 variety (Dimić et al., 2009; Baldini et al., 2018). A change in the total content of PUFAs was highlighted (Table 7.1.4.2.1) during the storage, which also led to a reduction in the ω6:ω3 ratio. The main cannabinoid found in hemp seed oil was cannabidiolic acid (CBDA), which likely arises from contact of the seed with the resin produced by the epidermal glands located on inflorescences, leaves, and bracts (Citti et al., 2018); in fact, variety Futura 75, according to the literature, shows a high content of CBDA (Brighenti et al., 2017). More in general, the content of CBDA was consistent with previous data reported in the literature and higher than that of cannabidiol (CBD) (Citti et al., 2018), while data reported by Izzo et al. (2020) showed a slightly high content of CBDA with respect to our results. Moreover, cannabigerolic acid (CBGA) was also found and its concentration was in accordance with what reported by Citti et al., 2019. No statistically significant differences were found regarding the cannabinoid content during 3 months of storage (Table 7.1.4.3.1).

Table 7.1.4.3.1. Cannabinoid content in samples T0 and T9 determined by RP-HPLC-UV. The concentration is expressed as mg/kg and the results are reported as mean±standard deviation of three replicates.

Different letters in rows indicate significant differences (Anova, Tukey's HSD test, $p<0.05$).

Cannabinoid	Concentration	
	T0 (mg/kg)	T9 (mg/kg)
CBDA	184.20±1.22[a]	184.74±1.20[a]
CBGA	24.54±0.30[a]	24.61±0.28[a]
CBD	23.60±0.69[a]	23.67±0.71[a]
δ^9-THC	3.66±0.14[a]	3.67±0.15[a]
δ^8-THC	n.q.	n.q.
THCA	6.56±0.14[a]	6.58±0.15[a]
CBDA/CBD	7.81	7.80

n.q. not quantified (<LOQ) according to the method validation parameters reported by Mandrioli et al. (2019)

According to Italian legislation (Decree of 4th of November 2019), the limit of tetrahydrocannabinol (THC), as sum of δ^9-tetrahydrocannabinol and tetrahydrocannabinolic Acid (δ^9-THC+THCA), in hemp seed oil is 5 ppm. The results showed that the amount of δ^9-THC+THCA was approximatively twice this limit (Table 7.1.4.3.1). Even if a confirmation of the result by gas or liquid chromatography, coupled with a mass spectrometric detector, which are the official methods reported by the European legislation (Commission Recommendation EU n° 2016/2115) would be necessary, this represents a point of attention for the producers of cold-pressed and non-refined hemp seed oil. It is well known that THC+THCA can be effectively removed during the refining process, but for cold-pressed/virgin hemp seed oils the only way to not exceed the limit is to perform careful seed cleaning (e.g. removal of bracts, pieces of inflorescences and impurities such as dust) before extracting the oil (Matthäus & Brühl, 2008). Citti et al. (2018) suggested that the ratio between CBDA and CBD can be a marker of storage. Our results showed that this ratio was not affected by the storage conditions applied; in fact, it was the same at the beginning and the end of the test (Table 7.1.4.3.1). According to Citti et al. (2018) at 25°C the decarboxylation was predicted to occur in less than 2 months, but our results suggested that at this temperature it is slower. However, it would be necessary to carry out the conservation test on more samples to confirm or not this result and to verify the effective usefulness of this ratio as marker for the storage. In fact, the cannabinoids are considered as contaminant of hemp seed oils and, if seeds have been perfectly cleaned from bracts or parts of the plant (Petrović et al., 2015; Citti et al., 2018), they may be absent or present in very low concentrations. Regarding the tocopherol composition, γ-tocopherol was quantitatively more present compared to α-tocopherol and

δ-tocopherol, both at the beginning (T0) and end of 3 months of storage (T9). At T0, the content of γ-tocopherol was 794.66 ± 5.19 μg/g and α-tocopherol was 38.73 ± 1.45 μg/g, while δ-tocopherol was 29.22 ± 1.03 μg/g which are substantially in accordance with the literature (Özdemir et al., 2020). The content of γ-tocopherol and α-tocopherol did not decrease during 3 months of storage, while δ-tocopherol decreased slightly (approximatively -13%) (Table 7.1.4.3.2).

Table 7.1.4.3.2. Tocopherol content in sampled T0 and T9 determined by HPLC-DAD. The concentration is expressed as mg/kg, and the results are reported as mean±standard deviation of three replicates.
Different letters in rows indicate significant differences (Anova, Tukey's HSD test, $p<0.05$).

Tocopherol	Concentration	
	T0 (mg/kg)	T9 (mg/kg)
δ -Tocopherol	29.22±1.03[a]	25.40±1.85[b]
γ -Tocopherol	794.66±5.19[a]	791.87±3.24[a]
α -Tocopherol	38.73±1.45[a]	35.97±3.26[a]

7.1.5. Conclusions

The post-production phases linked with high oxygen exposure, such as bottling or filtration, leave a clear mark on the oxidative behavior of hemp seed oil. The presence of antioxidant components (e.g. tocopherols), likely undergoes a quenching effect. In fact, the oil, in amber glass and hermetically closed bottles, with a limited head space, stored on the shelves for 3 months in a dark-light cycle (room temperature), simulating supermarket conditions did not oxidize significantly. The previously mentioned "oxidative wave", recorded by measuring peroxides, and then secondary products (K_{268}) and the microESR trace, showed a crest after 10 days from bottling, but with no further increase in the following 3 months of storage. Unlike other oils (e.g. olive oil), and disrespectful of the high degree of unsaturation, photo-oxidation, that can partially act also when amber glass bottles are used, did not seem to significantly affect the quality of cold-pressed hemp seed oil in the first 3 months of storage. These evidences, focused on bottling and the short subsequent storage of the oil, can be useful for producers albeit, for greater certainty of the results the presented test procedure should be repeated for other production batches with different initial parameters. In fact, as mentioned above, the quality of cold-pressed oils largely varies depending on the quality of seeds, their composition (e.g. antioxidants, bioactive compounds), their moisture content, the degree of maturity and the extraction conditions applied by the producers.

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7.2 Instrumental evaluation of the changes in 9 hemp seed oils composition during accelerated oxidation test

7.2.1 Details of the publication based on Paragraph 7.2

Title: Evaluation of hemp seed oils stability under accelerated storage test

Authors: Matilde Tura[a], Diana Ansorena[b,*], María Icíar Astiasarán[b], Mara Mandrioli[a] and Tullia Gallina Toschi[b]

[a] Department of Agricultural and Food Sciences, Alma Mater Studiorum - Università di Bologna, Viale G. Fanin 40, Bologna (BO), 40127, Italy
[b] Department of Nutrition, Food Science and Physiology, School of Pharmacy, Universidad de Navarra, Irunlarrea 1, 31008 Pamplona, Spain

Corresponding author: Diana Ansorena-dansorena@unav.es

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Abstract

Accelerated thermal oxidation (60°C, 18 days) has been applied to nine types of cold-pressed hemp seed oils to monitor the evolution of the samples during oxidative deterioration. In particular, several volatiles identified at the beginning of the accelerated storage (at time 0), such as the predominant α-pinene and β-pinene, gradually decreased during the accelerated storage period. On the other hand, aldehydes (hexanal, (E)-2-hexenal, heptanal, (E,E)-2,4-hexadienal, (E)-2-heptenal, (E,E)-2,4-heptadienal, (E,Z)-2,4-heptadienal, 2-octenal, nonanal, nonenal, 2,4-nonadienal, (E,E)-2,4-decadienal and 2,4-decadienal), ketones (1-octen-3-one, 3-octen-2-one, (E,E)-3,5-octadien-2-one and 3,5-octadien-2-one), acids (propionic acid, pentanoic acid, hexanoic acid and heptanoic acid) and 2-pentyl-furan increased during the accelerated storage, as principal markers of oxidation. Moreover, fatty acid composition, total phenols, tocopherols and cannabinoids profile were also evaluated, as well as the evolution of peroxide values and TBARs.

Keywords: hemp seed oil, accelerated storage, volatile compounds, SPME, fatty acids

7.2.2 Introduction

Cannabis sativa L. is an annual plant that produces small seeds, botanically named "achenes". Cold pressing of these seeds allows obtaining hemp seed oil, which is mainly used as food (Citti et al., 2018). Hemp seed oil (HSO) is a source of polyunsaturated fatty acids (PUFAs), in particular, linoleic acid (18:2 n-6, generally present at 55%) and α-linolenic acid (18:3 n-3, generally present 20%). Moreover, also γ-linolenic acid (18:3 n-6; approximately 1–4%) and stearidonic acid (18:4 n-3; ranges from 0.5–2%) were identified in this oil (Mikulcová, Kašpárková, Humpolíček, & Buňková, 2017). The cold pressing process also extracts minor compounds naturally present in hemp, i.e. antioxidants such as phenols and tocopherols (Dimić, Romanić, & Vujasinović, 2009; Teh & Birch, 2013; Faugno et al., 2019). The evaluation of the total phenolic and tocopherols contents could be useful to evaluate the differences among samples and during storage in terms of antioxidants (Okogeri & Tasioula-Margari, 2002). On the other hand, it also determines the presence of high chlorophyll content, which is a photosensitive pigment that could affect the quality of hemp seed oil during storage (Aachary, Liang, Hydamaka, Eskin, & Thiyam-Holländer, 2016; Liang et al., 2018). In fact, the presence of chlorophyll as well as the great content of PUFAs, which are highly prone to oxidation, could lead to oxidative degradation during storage of hemp seed oil, also at room temperature due to the low activation energy required (Frankel, 1984; Liang et al., 2018).

In addition, several cannabinoids had been found in hemp seed oil even if hemp seeds did not contain cannabinoids. In fact, their presence is due to the contact of hemp seed with the resin located on flowers, leaves or bracts, so they are considered as "impurities" or "contaminants" of hemp seed oil (Citti et al., 2018). Although these compounds are present only in small quantities, they have medical interest due to their bioactive activities, such as anti-convulsive and anti-epileptic effects (Maroon & Bost, 2018). Cannabinoid acids such as cannabidiolic acid (CBDA) convert to corresponding neutral forms through a decarboxylation reaction that is catalyzed by heat. Hence, the changes of CBDA/CBD ratio in HSO can be considered as a useful indicator for monitoring HSO storage life (Pratap Singh et al., 2020).

The lipid oxidation process involves a complex series of chemical reactions, which lead to the formation of primary (commonly measured as peroxide value and UV absorbance at 232 nm) and secondary (commonly measured with several indices, such as conjugated diene and triene, p-anisidine value and thiobarbituric acid value) oxidation products (Barriuso, Astiasarán, & Ansorena, 2013; Xu, Yu, Li, Chen, & Wang, 2018; Liang et al., 2018). Furthermore, the oxidation process could also be sensory detected; in fact, it determines the formation of off-flavours in the oil, such as rancid (Gaca, Kludská, Hradecký, Hajšlová, & Jeleń, 2021). The aroma of the oil is formed by the presence of volatile compounds, which have different odor thresholds, meaning that a high or low concentration

of volatile compounds does not directly impact the oil's sensory quality. The oil flavour could be affected by volatile compounds from the plant and volatiles deriving from chemical changes during storage, such as oxidation. Several classes of volatile compounds could impact the quality deterioration, e.g. aldehydes, ketones, esters and furan derivatives, and specific compounds are usually identified as markers of lipid oxidation, e.g. hexanal and nonanal (Gaca et al., 2021). It is widely known that the oxidation borne by linoleic acid conducts to the production of hexanal, 2-heptenal, 2-octenal, (E,Z)-2,4-decadienal and (E,E)-2,4-decadienal, while the oxidation of linolenic acid can give rise to produce a more significant proportion of (E,Z)-2,4-heptadienal and (E,E)-2,4-heptadienal (Poyato et al., 2014). Several volatile compounds that could be formed during lipid oxidation, such as 4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal, come from the oxidation of ω-3 and ω-6 polyunsaturated groups, also present a harmful impact on human health (Goicoechea & Guillén, 2014). One of the primary analytical techniques used for the volatiles analysis is solid-phase microextraction (SPME) thus because it is rapid, solvent-free and sensitive (Vichi, Pizzale, Conte, Buxaderas, & López-Tamames, 2003; Xu et al., 2018; Gaca et al., 2021). Moreover, oxidation determines the quality degradation of the oil during storage, also from the nutritional point of view (Dedebas, Ekici, & Sagdic, 2021).

This study aimed to evaluate the changes in the characteristics of hemp seed oil during accelerated thermal oxidation storage, focusing on the modifications in the volatile profile. Moreover, another goal was to determine the main volatile markers of lipid oxidation and freshness in hemp seed oil. The progress of the oxidation was monitored by performing several analyses related to the oxidation and the composition of hemp seed oils. In fact, to the authors' knowledge, although the interest in hemp seed oil is increasing, there are still few studies in the literature investigating changes during storage, focusing on the volatile profile.

7.2.3 Materials and methods

7.2.3.1 Samples and accelerated storage

Nine types of different cold-pressed hemp seed oils (food grade) from the Italian market were employed. Oils were distributed in glass vials (2 g in each vial) and oxidized at 60°C (Gaca et al., 2021) in the oven for a total period of 18 days. In these conditions (Schaal oven conditions), one day at 60ºC is equivalent to one month at room temperature. Sampling and analyses were performed every 3 days. The samples were named with a number (from 1 to 9, for the types of oils), and the oxidation time was indicated by "T" followed by the number of days of accelerated oxidation, from T0 (i.e.

time 0 or 0 days of accelerated oxidation test) to T18 (i.e. time 18 or 18 days of accelerated oxidation test).

7.2.3.2 Peroxide value

Peroxide value (PV) was determined at 510 nm by following the methods reported by Shantha & Decker (1994). A calibration curve with cumene hydroperoxide was used for quantification ($y=7.9473x+0.0363$; $r^2 = 0.9983$). Results were expressed as $mEqO_2$/kg of oil.

7.2.3.3Determination of TBARs

TBARs value was determined at 532 nm according to Gutiérrez-Luna, Ansorena, & Astiasarán (2020). A calibration curve with 1-1-3-3-tetrahydroxypropane was used for the quantification ($y=30850x+0.1866$; $r^2=0.9904$). Results were expressed in mg malonaldehyde MDA/kg sample. Moreover, data were also recorded at 390 nm, thus in order to evaluate a possible interference of aldehydes, as reported by Poyato et al. (2014).

7.2.3.4 Determination of total phenols

Total phenolic content was determined at 765 nm. 1 g of oil was added with 20 mL of n-hexane and 20 mL of methanol:water (80:20). After the separation of the two phases, the lower phase was recovered and dried with a rotavapor at 40°C. Then, the dried phase was recovered with 5 mL of distilled water. 100 µL of the samples were added to 7.9 mL of distilled water, with 500 µL of Folin-Ciocalteau reactive. After 2 minutes of waiting, 1.5 mL of Na_2CO_3 saturated solution was added and then the samples were stored for 2 hours in the dark. After 2 hours, the absorbance of 300 µL was read at 765 nm (FLUOStar Omega spectrofluorometric analyzer, BMG Labtechnologies, Offenburg, Germany). A calibration curve with gallic acid was used for the quantification ($y=0.9423x+0.0077$; $r^2=0.9998$). Results were expressed as mg of gallic acid/kg of oil.

7.2.3.5 Fatty acid composition

The determination of fatty acid composition was conducted according to the AOAC official method (2002). A Perkin-Elmer Autosystem XL gas chromatography equipped with a capillary column SPTM-2560 (100 m x 0.25 mm x 0.2 µm) and flame ionization detector was used. The GC-FID conditions were set as reported by Gutiérrez-Luna et al. (2020). The identification of fatty acid methyl ester (FAME) was made by comparing the retention times of the peaks in each sample with those of

standard pure compounds. Individual methylated standards from Sigma (St. Louis, MO, USA) were used.

7.2.3.6 Volatile profile

Volatile compounds were analyzed by headspace solid-phase microextraction (HS-SPME) combined with gas chromatography-mass spectrometry (GC–MS) by following the method reported by Gayoso et al. (2017), with some modifications. The SPME fiber coating used was divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) (50/30 lm film thickness, Supelco). 2 g of oil was weighed into a 25 mL headspace vial. The sample was equilibrated at 40°C for 15 min and the adsorption time, with the fiber exposed to the headspace of the sample, was 60 min at the same temperature. The desorption time for the fiber in the injection port of the gas chromatograph was 30 min. The GC–MS instrumentation used was GC 6890 N coupled to a mass selective 5973 detector (Agilent Technologies, Santa Clara, US). Volatiles were separated using a capillary column HP-5MS, 5% phenyl methyl siloxane (30 m long x 0.25 mm inner diameter x 0.25 µm film thickness, Agilent Technologies, Santa Clara, US). Chromatographic conditions were as follows: the oven temperature was held for 5 min at 42°C, then increased to 120°C at 3°C min-1 and to 250°C at 10°C min-1 (5 min hold); injector temperature, 270°C; detector temperature 280°C; ion source temperature, 230°C; quadrupole mass analyzer temperature, 150°C. Helium was used as carrier gas at 1 mL min-1. The mass spectrometer was operated by electronic impact at 70 eV, and ions were scanned over the m/z range of 33–350 at a rate of 4.43 scan/s. Kovats Index (KI) was calculated for each detected peak using the following formula:

$$KI = [100 * \left(tR(i) - \frac{tR(z)}{tR(z+1)} - tR(z) \right)] + 100z$$

Where:

z is the number of carbon atoms in alkane z;

tR(i) is the retention time of compound i;

tR(z) is the retention time of alkane z;

tR(z+1) is the retention time of alkane z + 1.

The identification of each peak was made taking into account the KI reported in the literature (Kondoyan & Berdague, 1996) and comparing their mass spectra with the one of a commercial library (Wiley 275.L, Mass Spectral Database). In the case of overlapping peaks, the quantification of the corresponding compound was done by a specific ion and considering the relative proportion in which this ion is present in each compound. Results are expressed in area counts $x10^3$/g of oil. Samples were analyzed in triplicate.

7.2.3.7 Tocopherols

The determination of tocopherols was performed by applying the method reported by Tura, Mandrioli, & Toschi (2019). Identification of tocopherols was performed by injecting standard of γ-tocopherol (CAS number 54-28-4; Sigma-Aldrich, Missouri, USA) and α-tocopherol (CAS number 119-13-1; Sigma-Aldrich, Missouri, USA). Quantification was carried out using a calibration curve constructed with the external standard method, injecting solutions of known concentration in the range of 0.5–100 mg/mL for both α-tocopherol ($y = 38.811x+41.366$; $r^2=0.998$) and γ-tocopherol ($y=123.04x+109.1$; $r^2 = 0.9985$).

7.2.3.8 Cannabinoids

A total of 500 mg of oil was weighed in a 10 mL flask, solubilized, brought to volume with isopropanol, vortexed for 1 min, and placed in an ultrasonic bath (Branson 2150) for 10 min. Next, the solution was filtered through a 0.45 µm nylon filter. The determination of cannabinoids was performed following the method proposed by Mandrioli, Tura, Scotti, & Toschi (2019). Quantification was carried out using a calibration curve constructed with an external standard, injecting solutions (Phytocannabinoid Mixture 10 (CRM), Cayman Chemical, Michigan USA) of known concentration in the range 0.05–5 µg/mL (CBDA $y=15844x-2893.9$, $r^2=0.9997$; CBGA $y=16409x-2591.7$, $r^2=0.9996$; CBG $y=14113x-2002.3$, $r^2=0.9994$; CBD $y= 12877x-281.01$, $r^2=0.995$).

7.2.3.9 Data Analysis

Samples were analyzed in triplicate and the results are shown as mean±standard deviation. Only for the fatty acids profile, samples were prepared in duplicate and each replicate was injected twice. Data were statistically analyzed by using the software XLSTAT Addinsoft (2018.XLSTAT statistical and data analysis solutions. Paris, France. https://www.XLSTAT.com) version 2018.1.1.

7.2.4 Results

7.2.4.1 Peroxide value

All the samples, at time 0, presented PVs lower than the maximum value reported in the Codex Alimentarius for cold-pressed vegetable oils, equal to 15 $mEqO_2$/kg of oil. Samples 1 and 3, after 15 days of accelerated oxidation, showed PVs upper than the limit (Codex Stan 210-1999) (Figure 7.2.4.1.1).

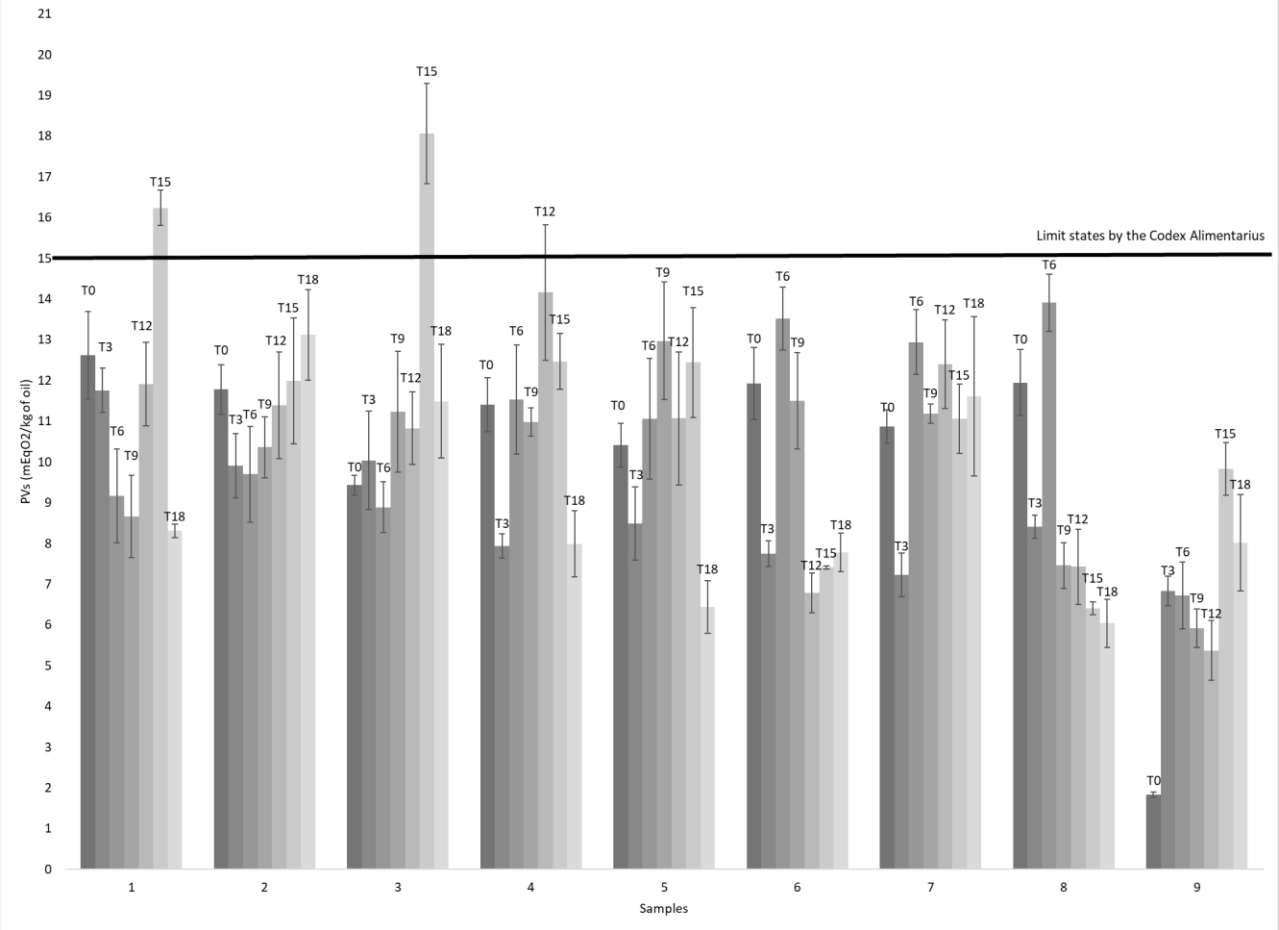


Figure 7.2.4.1.1. Results of the peroxide value expressed as $mEqO_2$/kg of oil during the accelerated oxidation test (from T0 to T18, during 18 days of heating at 60°C). Data are reported as mean±standard deviation of 3 independent replicates.

7.2.4.2 TBARs

The level of lipid oxidation in the hemp seed oils was also evaluated by measuring the thiobarbituric acid-reactive substances (TBARs), before the accelerated oxidation test (Time 0-T0) and after 18 days of oxidation in the oven (Time 18-T18). TBARs generally reflect the level of the secondary

products from lipid peroxidation, with a positive association with lipid peroxidation (Yu, Zhou, & Parry, 2005). At the end of the accelerated oxidation period (after 18 days), the TBARs values decreased in all the 9 types of hemp seed oil (Table 7.2.4.2.1).

Table 7.2.4.2.1. Results of the TBARs expressed as mg MDA/kg of oil at the beginning (T0) and at the end (T18) of the accelerated oxidation test. Data are reported as mean±standard deviation of 3 independent replicates. Different letters in row indicates statistically significant differences (one-way ANOVA, $p<0.05$, Tukey HSD).

Sample	T0 mg MDA/kg of oil	T18 mg MDA/kg of oil
1	11.28±0.69^{a}	9.62±0.74^{b}
2	11.04±1.58^{a}	9.06±0.47^{b}
3	13.27±1.78^{a}	8.50±0.66^{b}
4	11.56±1.70^{a}	9.65±1.42^{b}
5	11.54±0.28^{a}	8.12±1.19^{b}
6	11.37±1.57^{a}	10.25±1.14^{a}
7	12.25±1.15^{a}	7.17±0.73^{b}
8	12.01±0.97^{a}	9.98±1.32^{b}
9	17.15±0.83^{a}	6.87±0.99^{b}

7.2.4.3 Total phenolic compound

As reported in Table 7.2.4.3.1, the total phenolic content decreased during the accelerated oxidation. In fact, at the end of the accelerated storage period (i.e. 18 days at 60°C), all the samples showed a lower phenolic content in comparison to time zero (i.e. before the heating in the oven), although samples 4 and 8 did not show statistically significant differences (one-way ANOVA, $p<0.05$, Tukey HSD).

Table 7.2.4.3.1. Results of the determination of total phenols expressed as mg gallic acid/kg of oil. Data are reported as mean±standard deviation of 3 independent replicates. Different letters in rows indicate statistically significant differences (one-way ANOVA, $p<0.05$, Tukey HSD).

Sample	T0 mg gallic acid/kg of oil	T18 mg gallic acid/kg of oil
1	50.37±2.03[a]	22.14±1.78[b]
2	106.50±8.80[a]	16.95±0.82[b]
3	45.58±2.71[a]	11.28±1.15[b]
4	15.39±0.79[a]	12.76±1.16[a]
5	16.75±0.41[a]	10.89±0.28[b]
6	30.76±6.60[a]	7.90±0.21[b]
7	25.70±1.07[a]	7.10±0.49[b]
8	12.08±1.96[a]	11.80±2.25[a]
9	186.78±4.57[a]	60.10±0.63[b]

7.2.4.4 Fatty acids

The main fatty acids were: linoleic acid (ranged from 46.24% to 51.25% at time 0, and from 46.25% to 53.13% at time 18), followed by α-linolenic acid (from 10.61% to 17.03% at time 0, and from 9.75% to 16.82% at time 18), oleic acid (from 7.05% to 13.10% at time 0, and from 8.09% to 13.07% at time 18). Those results are in line with what is reported in the literature (Parker, Adams, Zhou, Harris, & Yu, 2003; Da Porto, Decorti, & Natolino, 2015; Alonso-Esteban et al., 2020). Moreover, as described in previous studies (Callaway, Tennilä, & Pate, 1996; Callaway, 2004; Mikulcová et al., 2017), also the presence of γ-linolenic acid and stearidonic acid was detected in all the samples (Table 7.2.4.4.1).

Table 7.2.4.4.1. Results of the determination of fatty acids expressed as g/100 g of oil. Data are reported as mean±standard deviation of 4 injections of 2 independent replicates. Different letter in row indicates statistically significant differences between time 0 and time 18 (one-way ANOVA, $p<0.05$, Tukey HSD).

	Sample 1		**Sample 2**		**Sample 3**		**Sample 4**		**Sample 5**		**Sample 6**		**Sample 7**		**Sample 8**		**Sample 9**	
Fatty acid	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**	**T0**	**T18**
									g/100 g of oil									
Myristic acid	0.03 ±0.00	0.03 ±0.01	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.02 ±0.00	0.03 ±0.00	0.03 ±0.01	0.04 ±0.00	0.04 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00
Palmitic acid	5.78^{b} ±0.34	6.62^{a} ±0.08	6.16 ±0.13	6.45 ±0.07	5.89 ±0.07	6.24 ±0.24	6.24 ±0.04	5.96 ±0.27	6.30 ±0.08	6.42 ±0.02	5.43^{b} ±0.12	6.21^{a} ±0.07	6.46^{b} ±0.18	7.05^{a} ±0.14	5.45^{b} ±0.11	6.17^{a} ±0.17	5.47^{b} ±0.03	6.40^{a} ±0.08
trans-palmitoleic acid	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.04 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00	0.03 ±0.00
Palmitoleic acid	0.10 ±0.01	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.01	0.11 ±0.00	0.10 ±0.00	0.11 ±0.00	0.11 ±0.00	0.10 ±0.00	0.11 ±0.00	0.10 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.11 ±0.00	0.12 ±0.01
Stearic acid	2.25 ±0.14	2.61 ±0.00	2.50 ±0.06	2.65 ±0.04	2.56 ±0.03	2.76 ±0.10	2.58 ±0.02	2.45 ±0.14	2.54 ±0.03	2.62 ±0.01	1.92 ±0.04	2.22 ±0.03	2.55 ±0.07	2.81 ±0.06	1.99 ±0.04	2.29 ±0.08	1.98 ±0.02	2.33 ±0.02
Oleic acid	11.46^{b} ±0.67	12.32^{a} ±0.10	10.97^{b} ±0.25	11.54^{a} ±0.07	10.84^{b} ±0.14	11.50^{a} ±0.53	13.10^{a} ±0.03	12.29^{b} ±0.57	12.88 ±0.15	13.07 ±0.12	7.05^{b} ±0.24	8.09^{a} ±0.05	11.13^{b} ±0.25	12.07^{a} ±0.20	7.67^{b} ±0.17	8.57^{a} ±0.15	8.44^{b} ±0.03	9.80^{a} ±0.07
Vaccenic acid	0.77 ±0.05	0.88 ±0.01	0.70 ±0.02	0.73 ±0.01	0.67 ±0.01	0.71 ±0.03	0.82 ±0.00	0.78 ±0.04	0.84 ±0.01	0.86 ±0.01	0.75 ±0.02	0.85 ±0.01	0.65 ±0.01	0.69 ±0.01	0.75 ±0.02	0.83 ±0.01	0.77 ±0.00	0.88 ±0.01
trans-linoleic acid	0.06^{a} ±0.00	0.01^{b} ±0.00	0.09^{a} ±0.00	0.05^{b} ±0.00	0.07 ±0.00	0.07 ±0.00	0.03^{a} ±0.00	0.01^{b} ±0.00	0.04 ±0.00	0.04 ±0.01	0.06^{a} ±0.01	0.02^{b} ±0.00	0.05^{a} ±0.00	0.02^{b} ±0.00	0.04^{a} ±0.00	0.02^{b} ±0.00	0.03 ±0.00	n.d.
cis-trans linoleic acid	0.31 ±0.01	0.32 ±0.00	0.24 ±0.00	0.25 ±0.00	0.24 ±0.00	0.24 ±0.00	0.29 ±0.01	0.27 ±0.00	0.30 ±0.01	0.28 ±0.01	0.29 ±0.00	0.30 ±0.00	0.23 ±0.00	0.24 ±0.00	0.28 ±0.00	0.30 ±0.01	0.29 ±0.01	0.30 ±0.00
trans-cis linoleic acid	n.d.	n.d.	0.05 ±0.00	0.05 ±0.00	0.04 ±0.00	0.05 ±0.00	0.05 ±0.00	0.04 ±0.00	0.06 ±0.01	0.04 ±0.00	n.d.	n.d.	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00
Linoleic acid	48.63^{b} ±3.27	50.95^{a} ±0.85	50.67^{b} ±1.06	52.05^{a} ±1.64	50.67^{b} ±0.34	52.01^{a} ±2.54	48.10^{a} ±0.42	46.25^{b} ±0.32	49.87^{a} ±0.59	47.57^{b} ±1.87	49.95^{b} ±2.37	52.32^{a} ±0.07	51.25^{b} ±1.04	53.13^{a} ±0.64	49.40^{b} ±1.20	52.32^{a} ±0.69	46.24^{b} ±0.39	51.47^{a} ±0.04
Arachidonic acid	0.72 ±0.05	0.84 ±0.01	0.57 ±0.01	0.61 ±0.01	0.58 ±0.01	0.62 ±0.03	0.72 ±0.01	0.69 ±0.04	0.71 ±0.01	0.74 ±0.01	0.75 ±0.02	0.87 ±0.01	0.59 ±0.02	0.64 ±0.02	0.76 ±0.02	0.87 ±0.03	0.74 ±0.01	0.86 ±0.01

γ-linolenic acid	2.38 ±0.21	2.60 ±0.14	0.39 ±0.01	0.37 ±0.01	0.37 ±0.02	0.38 ±0.03	1.59 ±0.06	1.37 ±0.04	1.58 ±0.02	1.35 ±0.16	3.39 ±0.34	3.83 ±0.03	0.37 ±0.01	0.37 ±0.00	3.68 ±0.12	3.53 ±0.03	3.59 ±0.02	3.85 ±0.08
Eicosenoic acid (n-9)	0.24 ±0.02	0.29 ±0.00	0.22 ±0.01	0.24 ±0.00	0.25 ±0.00	0.27 ±0.02	0.27 ±0.00	0.26 ±0.02	0.28 ±0.00	0.28 ±0.00	0.28 ±0.01	0.33 ±0.00	0.25 ±0.01	0.27 ±0.01	0.28 ±0.01	0.32 ±0.01	0.27 ±0.00	0.32 ±0.00
α-linolenic acid	11.38 ±0.89	11.51 ±0.27	14.96 ±0.25	14.47 ±0.12	15.27 ±0.17	15.28 ±0.89	10.91 ±0.30	9.75 ±0.71	11.94 ±0.14	10.61 ±0.86	15.17 ±1.23	16.73 ±0.10	15.05 ±0.31	15.02 ±0.01	17.03 ±0.48	16.82 ±0.09	15.22^{b} ±0.18	16.36^{a} ±0.12
Stearidonic acid	0.49 ±0.05	0.52 ±0.03	0.12 ±0.00	0.14 ±0.02	0.13 ±0.01	0.18 ±0.02	0.32 ±0.02	0.26 ±0.02	0.34 ±0.01	0.26 ±0.04	1.04 ±0.13	1.14 ±0.02	0.16 ±0.03	0.14 ±0.01	1.21 ±0.06	1.04 ±0.03	1.07 ±0.01	1.12 ±0.04
Eicosadienoic acid (n-6)	0.12 ±0.01	0.10 ±0.02	0.09 ±0.01	0.06 ±0.02	0.10 ±0.02	0.07 ±0.01	0.11 ±0.01	0.08 ±0.01	0.08 ±0.00	0.08 ±0.01	0.16 ±0.02	0.10 ±0.02	0.12 ±0.02	0.06 ±0.01	0.20 ±0.00	0.19 ±0.02	0.12 ±0.00	0.13 ±0.00
Behenic	0.18^{b} ±0.02	0.24^{a} ±0.01	0.10 ±0.01	0.13 ±0.01	0.19^{a} ±0.03	0.12^{b} ±0.02	0.23 ±0.01	0.23 ±0.02	0.23 ±0.01	0.26 ±0.00	0.21^{b} ±0.00	0.28^{a} ±0.00	0.11^{b} ±0.01	0.14^{a} ±0.01	0.22^{b} ±0.00	0.26^{a} ±0.00	0.21^{b} ±0.01	0.28^{a} ±0.01
Brassidic	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.05 ±0.00	0.05 ±0.00	0.04 ±0.00	0.04 ±0.00	0.05 ±0.00	0.05 ±0.00	0.05 ±0.00	0.05 ±0.00
Nervonic	0.04^{b} ±0.00	0.09^{a} ±0.01	0.03^{b} ±0.00	0.08^{b} ±0.00	0.03^{b} ±0.00	0.07^{a} ±0.00	0.04^{b} ±0.00	0.08^{a} ±0.01	0.03^{b} ±0.00	0.10^{a} ±0.00	0.04^{b} ±0.00	0.07^{a} ±0.00	0.03^{b} ±0.00	0.07^{a} ±0.00	0.07 ±0.00	0.07 ±0.00	0.04^{b} ±0.00	0.07^{a} ±0.00
Lignoceric	0.14^{b} ±0.01	0.21^{a} ±0.01	0.10 ±0.00	0.10 ±0.00	0.09^{b} ±0.01	0.14^{a} ±0.01	0.16^{b} ±0.01	0.20^{a} ±0.01	0.15^{b} ±0.00	0.23^{a} ±0.03	0.16^{b} ±0.01	0.23^{a} ±0.00	0.09 ±0.00	0.09 ±0.00	0.13^{b} ±0.01	0.20^{a} ±0.00	0.14^{a} ±0.01	0.10^{b} ±0.00
SFA	10.52	11.45	10.59	10.88	10.45	10.71	11.41	11.51	11.08	11.83	9.96	10.21	10.88	11.43	9.43	10.20	9.95	10.44
MUFA	14.82	15.15	13.62	14.05	13.46	13.87	16.64	16.59	15.96	16.93	9.83	10.05	13.58	14.16	9.87	10.52	11.30	11.81
PUFA	73.40	72.14	75.03	74.29	75.36	74.50	70.85	70.84	71.87	70.17	78.27	77.80	74.82	73.70	78.70	77.48	76.81	75.88
n-3	13.36	12.74	17.08	16.17	17.42	16.90	12.84	12.14	13.64	12.59	18.07	17.92	16.96	16.24	19.15	17.97	18.04	17.38
n-6	59.90	59.29	57.85	58.06	57.82	57.52	57.87	58.60	58.14	57.47	60.01	59.76	57.73	57.40	59.32	59.29	58.62	58.36
*n*6/*n*3	4.48	4.65	3.39	3.59	3.32	3.40	4.51	4.84	4.26	4.57	3.32	3.33	3.40	3.53	3.10	3.30	3.25	3.36
pufa/sfa	6.99	6.30	7.09	6.84	7.21	6.95	6.21	6.16	6.49	5.94	7.87	7.62	6.88	6.45	8.35	7.60	7.72	7.27
pufa+mufa/sfa	8.40	7.62	8.37	8.13	8.50	8.25	7.67	7.61	7.93	7.37	8.86	8.60	8.13	7.69	9.39	8.63	8.85	8.40
trans	0.52	0.45	0.51	0.46	0.48	0.59	0.55	0.50	0.54	0.50	0.52	0.49	0.44	0.40	0.50	0.48	0.52	0.46

nd not detected, SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids

7.2.4.5 Volatile profile

The volatile compounds detected during the accelerated storage in the nine types of oils are reported in Table 7.2.4.5.1. Several compounds related to oxidation, such as 2,4-nonadienal, (*E*,*E*)-2,4-decadienal and 2,4-decadienal were observed in the samples after 3, 6, 9, 12, 15 and 18 of accelerated storage while they were not always detected at the beginning (time 0). In Table 7.2.4.5.2 the correlation matrix (Pearson) among accelerated storage time and volatiles and the relative *p*-values are reported.

Table 7.2.4.5.1. Volatile detected along the heat treatment for the nine oil samples. Results are expressed in area counts $\times 10^3$/g for each compound; the ion used for the quantification of several compounds is indicated, as well as the Kovats index for each volatile.

Volatile compound	Ion[1]	KI[2]	Sample 1							Sample 2						
			Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Alkenes																
1-octene		792	1277	1293	1145	1354	1412	1589	1642	251	775	1042	1398	1318	1371	251
2-(*E*)-octene		808	4616	6134	3141	4627	5746	5935	5197	722	2308	3109	3578	3714	4454	522
2-(Z)-octene		815	2004	1892	2096	2537	3047	3659	3619	1007	826	1590	2071	2766	2698	164
Aldehydes																
Hexanal		804	62642	69294	63908	62382	65111	70458	68678	46097	42367	52322	48300	56688	40303	47374
(*E*)-2-hexenal		850	1195	1619	2902	6815	6589	9063	9338	876	4832	7891	8087	8470	6904	8421
Heptanal		901	4246	4734	4823	4099	4402	4364	4506	2449	2015	2673	3312	3644	2727	3202
(*E*,*E*)-2,4-hexadienal	81	909	-	544	1485	4906	4488	6143	5584	-	1880	4177	4951	4416	4773	4842
(*E*)-2-heptenal		954	4503	17346	41234	67046	61070	63238	61349	990	52483	73398	69376	73565	65314	61248
(*E*,*E*)-2,4-heptadienal	81	995	1259	5544	20169	45755	51556	42852	39634	1818	33207	52792	57483	54103	67017	57168
(*E*,*Z*)-2,4-heptadienal		1009	-	5529	10155	65459	65110	71301	81457	280	23462	59649	90353	118685	106074	113161
2-octenal		1056	3130	4309	8989	28801	29959	30908	32614	1277	6129	15727	24999	21993	16634	23357
Nonanal		1103	3023	2538	2483	5658	5499	6451	7322	669	1368	3352	3712	5568	2902	3607
Nonenal		1158	571	553	757	6511	8159	18210	23701	94	461	2090	3724	3476	3280	8396
2,4-nonadienal		1192	-	-	-	1133	927	1313	1714	-	-	851	1438	1204	1116	1976
(*E*,*E*)-2,4-decadienal		1291	-	-	470	5483	6373	7440	8048	-	502	2985	4414	4700	4244	6836
2,4-decadienal		1317	-	-	280	10472	11805	15846	18040	-	1073	6390	10912	11425	9224	17547
Ketones																
2-heptanone		890	10028	10856	9246	6034	6032	6230	5972	3326	3070	3817	3627	3759	2865	3379
1-octen-3-one		977	1223	1947	2509	3993	3653	3511	2996	-	915	1747	1812	2151	2045	1624
3-octen-2-one		1038	4481	5164	7430	12091	12407	13276	13174	-	4065	7840	9136	9058	9011	10588
(*E*,*E*)-3,5-octadien-2-one		1070	1858	6740	15865	24816	28097	21593	20348	663	14107	21482	21855	29210	29186	23179
3,5-octadien-2-one		1091	1138	1747	6373	21508	22368	23413	26688	171	6977	16722	21333	20227	22089	27858
Alcohols																

3-methyl-1-butanol		727	154	120	-	-	-	-	-	413	-	-	-	-	-	-
1-pentanol		762	16661	15297	12239	6503	6337	5317	4308	6527	5768	5754	5258	5604	4665	4430
1-hexanol		873	127496	106758	73273	30852	27493	21410	17800	37524	17140	9863	8067	7156	8564	5584
1-octen-3-ol		979	1608	4010	13182	21842	18746	20333	20263	709	17065	22550	21252	21931	16945	14509
Acids																
Propionic acid	74	726	-	-	13199	15285	14945	13531	13164	158	10719	16437	16517	24198	17579	16839
Butanoic acid	60	801	11687	16246	15639	10532	9512	8953	8666	13247	14244	11665	10075	12459	10914	8776
Pentanoic acid	60	904	6145	6327	7149	7497	7545	7370	7345	1029	2050	4143	3957	4648	3121	4181
Hexanoic acid	60	992	32923	45230	51900	59979	59261	58943	58810	4441	10707	18202	26236	20660	19617	29525
Heptanoic acid	60	1082	2259	7385	9186	10161	9096	9633	9526	213	3644	4408	4205	4986	4341	4154
Furans derivatives																
2-pentyl-furan	81	991	21427	12458	16002	47213	38143	56228	58561	-	9813	26200	27308	33666	16635	27634
Terpenes																
α-pinene		929	8604	7685	5571	2817	2650	2344	2283	36608	16750	9966	8512	7865	9372	7005
β-pinene		971	3074	2695	2596	1238	1458	1235	1251	19832	10076	6400	5776	5175	6395	4899
δ-3-carene	93	1006	949	908	778	441	415	387	358	1932	1326	962	742	669	858	632
para-cymene	93	1021	2566	2616	2456	1654	1497	1527	1523	1427	1597	1724	1913	2134	1942	5439
Limonene		1025	2971	2445	2102	1715	1633	1537	1566	17951	13712	12257	10593	10345	11033	10060
(*E*)-β - ocimene		1047	2232	1757	2200	1902	1768	1788	1797	2794	2598	2515	2421	2168	2540	2127
Terpinolene		1085	5239	3585	2495	1672	965	1467	1296	4182	4473	3855	2696	3025	3016	3093
(*E*)-caryophyllene		1420	1264	1175	1140	619	482	456	475	967	1005	858	515	695	548	375
Bergamotene (isomer)		1439	172	160	173	196	162	191	204	70	241	63	312	90	95	95

Volatile compound	Ion[1]	KI[2]	Sample 3							Sample 4						
			Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Alkenes																
1-octene		792	1813	1184	1296	1108	1243	1453	1585	2701	577	845	1111	1258	1260	1930
2-(E)-octene		808	4280	3929	3079	3331	4092	4385	3853	4684	2679	3437	4267	4742	3851	2197
2-(Z)-octene		815	3867	2183	2427	2181	2323	3011	3088	3209	1583	2277	2631	3080	2843	4276
Aldehydes																
Hexanal		804	18512	43587	39625	36235	36835	37284	42003	33222	50275	60870	70953	72552	77201	113722
(E)-2-hexenal		850	915	4321	6535	4721	5083	6138	7460	1383	5640	6589	8147	9014	9747	14231
Heptanal		901	1256	2220	1970	1764	1776	1747	2951	1249	2372	3043	3760	3596	4233	5728
(E,E)-2,4-hexadienal	81	909	62	1603	2634	2365	2454	3498	4009	236	5174	6415	6260	6185	5485	9277
(E)-2-heptenal		954	907	61914	70486	58127	55930	54667	57562	3971	81176	84493	75316	77349	71335	101564
(E,E)-2,4-heptadienal	81	995	1885	40069	60777	66743	62208	69072	63409	1190	51001	52411	44562	42380	33372	47128
(E,Z)-2,4-heptadienal		1009	85	31430	71900	64371	57550	4817	108082	3704	49285	64707	76372	75684	82854	127569
2-octenal		1056	-	7252	12287	12174	10414	14083	3208	-	20072	28284	29722	28473	28097	41185
Nonanal		1103	434	1277	2108	1734	1654	2341	3281	787	3218	5343	6624	6317	7206	11341
Nonenal		1158	149	565	1602	1378	1357	3736	7821	-	1685	5980	18355	23332	36120	62660
2,4-nonadienal		1192	-	-	-	316	404	906	1577	-	1245	2253	3023	2906	3325	4539
(E,E)-2,4-decadienal		1291	-	659	2834	2792	2670	416	6349	-	3436	5651	7636	7270	8080	13724
2,4-decadienal		1317	-	1242	6417	5709	5061	894	15717	-	5690	11602	18035	17138	21547	37008
Ketones																
2-heptanone		890	1963	3001	2689	2647	2679	2824	3290	1904	2593	2680	3376	2989	2352	5247
1-octen-3-one		977	-	854	1279	926	990	1047	1205	434	2565	3720	3185	2898	2216	2972
3-octen-2-one		1038	-	4949	7876	7697	7381	10250	12408	-	5994	7798	9360	4954	5028	7532
(E,E)-3,5-octadien-2-one		1070	823	20630	27603	36844	35491	34646	30849	1593	20383	20905	18098	16381	13645	20819
3,5-octadien-2-one		1091	-	9904	19406	23132	22125	33003	40272	-	13432	19390	19731	19008	21139	34514

Alcohols																
3-methyl-1-butanol		727	197	-	-	-	-	-	-	947	365	254	-	-	-	-
1-pentanol		762	2162	6368	5511	4733	4680	3707	3839	3733	5191	4558	3557	3070	2647	3535
1-hexanol		873	15495	6748	4687	4665	5089	3491	2753	12688	3793	2714	2039	1875	1350	1530
1-octen-3-ol		979	357	19802	23069	18887	18386	15552	16182	2012	22477	24110	22675	22694	21443	28875
Acids																
Propionic acid	74	726	-	15669	20472	20102	21722	22780	21547	-	11397	12491	12037	11596	10131	14897
Butanoic acid	60	801	9493	10600	8820	9056	9849	7804	6975	4203	3293	3156	12486	3085	2881	4356
Pentanoic acid	60	904	305	1229	1755	1740	1708	2081	3781	857	2372	3277	3630	3064	12428	5201
Hexanoic acid	60	992	866	7759	13187	13009	12098	17814	30795	3445	17302	24210	26922	23490	24189	38840
Heptanoic acid	60	1082	-	2173	2460	2740	2606	2489	2726	-	2263	2390,115	10269	2235	2499	4706
Furans derivatives																
2-pentyl-furan	81	991	-	9828	18992	13407	11983	16727	27386	-	20347	29070	43481	44434	60081	85247
Terpenes																
α-pinene		929	39742	16676	10855	11313	12656	9158	6687	70091	33273	28899	25953	24912	22770	24780
β-pinene		971	21577	10496	7881	7749	8763	6424	4889	29314	14308	7881	7749	10458	9731	12598
δ-3-carene	93	1006	1613	1078	829	870	899	686	4804	2333	829	870	1624	1502	2005	4804
para-cymene	93	1021	1531	1826	1869	1805	1646	2004	5211	7161	6552	6321	10209	5503	4862	12953
Limonene		1025	19949	15338	13172	13512	14209	11845	12086	33134	21513	18413	16817	15904	14599	20296
(E)-β -ocimene		1047	5140	4478	4313	4664	4800	3809	3413	20345	12977	11450	10313	9768	8633	13830
Terpinolene		1085	6917	6248	5511	6032	6350	4667	4280	32112	20087	15183	13610	11707	12353	18091
(E)-caryophyllene		1420	3035	3124	2510	2490	2507	1760	1424	40220	25171	17609	13715	11218	11043	17289
Bergamotene (isomer)		1439	255	617	591	332	385	303	302	4543	4707	4559	4556	4060	4224	6710

Volatile compound	Ion[1]	KI[2]	Sample 5							Sample 6						
			Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Alkenes																
1-octene		792	1828	651	752	941	1120	977	74	1803	6551	2475	3058	3551	3760	3874
2-(E)-octene		808	936	2143	2475	2587	3452	3029	5311	4874	2645	5946	8575	9119	10416	10730
2-(Z)-octene		815	-	-	1191	1320	2117	1790	3329	-	2083	5137	7105	8182	9056	8403
Aldehydes																
Hexanal		804	30184	50535	65699	76635	86329	86925	135559	65268	43505	58679	66388	61232	62012	60217
(E)-2-hexenal		850	731	3625	5409	6849	8513	9274	13882	1437	3040	7256	8333	9443	10422	10836
Heptanal		901	1415	2251	3122	3495	3885	3997	6164	2394	1985	3427	4630	4459	4168	4313
(E,E)-2,4-hexadienal	81	909	153	4695	7762	6902	6008	5045	7871	132	2043	6728	7320	8290	8005	7255
(E)-2-heptenal		954	-	50166	76728	62160	67507	52341	86424	5379	38130	77400	82767	76619	83701	82125
(E,E)-2,4-heptadienal	81	995	1117	30293	45472	40711	35613	32034	40163	1582	26727	82702	90501	89239	88743	81878
(E,Z)-2,4-heptadienal		1009	1361	24196	49169	56690	60072	60659	98646	1582	1344	20696	91259	131287	146401	152645
2-octenal		1056	3969	9451	20447	22110	21708	19919	34539	3070	7865	25155	39953	32497	34397	36152
Nonanal		1103	681	2793	5618	6850	7745	7418	12612	1510	1531	3355	5329	5452	5691	6174
Nonenal		1158	-	-	3981	17202	32996	42259	76043	342	546	2597	6015	8091	9978	13503
2,4-nonadienal		1192	-	653	1956	350	2463	2335	4134	-	-	-	-	-	-	-
(E,E)-2,4-decadienal		1291	-	-	3526	252	5209	5303	9018	-	674	6494	10145	11443	12164	13664
2,4-decadienal		1317	-	691	6411	412	11617	12747	23246	-	580	9452	18965	22307	24000	28665
Ketones																
2-heptanone		890	2774	3272	3642	3795	3920	3685	5305	4133	3713	4775	5539	5174	5264	5268
1-octen-3-one		977		2336	4155	3109	3632	2111	3126	670	1744	5130	6345	5831	5438	670
3-octen-2-one		1038	8830	14920	19857	19729	19248	17501	25385	3560	5073	11159	13128	13666	13395	14816
(E,E)-3,5-octadien-2-one		1070	2874	32801	38290	34437	28264	26673	35660	-	13663	33987	30987	32474	29680	28451
3,5-octadien-2-one		1091	-	17070	26442	30243	29318	30739	51107	-	5144	5792	7397	6391	7756	8163
Alcohols																

3-methyl-1-butanol		727	651	-	-	-	-	-	-	309	-	-	-	-	-	-
1-pentanol		762	3644	4367	4178	3624	3044	2455	3165	7892	6329	8002	7680	7468	6475	6057
1-hexanol		873	9284	3635	2039	1572	1254	949	1112	69264	39831	32579	24861	24459	22543	19254
1-octen-3-ol		979	2572	12478	22154	19264	20222	16113	23387	2290	13513	29462	30129	28770	29305	28613
Acids																
Propionic acid	74	726	-	12626	14203,6	13957	9988	12532	17467	-	5310	16659	18356	19523	19563	18378
Butanoic acid	60	801	3949	3444	3404	3295	3313	3031	4589	3713	7184	9811	9575	9506	9271	8409
Pentanoic acid	60	904	1079	2656	3682	3897	4040	3638	5929	1429	2372	4582	7113	6214	6351	6341
Hexanoic acid	60	992	4592	17432	26420	28720	28559	27045	42971	8901	15882	33774	46393	43028	43955	46407
Heptanoic acid	60	1082	190	2713	3437	3493	3369	3437	5595	127	2294	4573	5005	4699	4838	5053
Furans derivatives																
2-pentyl-furan	81	991	-	-	28035	40424	49725	54352	88260	8112	10601	24205	33614	33640	33493	35289
Terpenes																
α-pinene		929	81883	50323	38111	34399	32598	31246	32083	25534	16780	16202	12999	12753	12760	11578
β-pinene		971	34338	21088	33977	14210	13524	12578	17188	11673	8979	8566	7352	6981	6720	5898
δ-3-carene	93	1006	5332	3454	2383	2183	2052	1886	2508	4293	3386	3441	2739	2756	2714	2490
para-cymene	93	1021	8861	8344	12155	11202	11150	10108	15196	2403	2471	2737	7913	8437	8023	7591
Limonene		1025	37132	28761	23595	21941	18755	17969	24241	12974	11255	13393	13903	13036	12983	15517
(E)-β -ocimene		1047	-	15950	13635	12468	11328	10283	14804	8112	6624	8362	7126	7058	6987	6633
Terpinolene		1085	27185	21154	13141	12752	11246	11995	16788	11500	10156	12281	10230	11108	10329	9709
(E)-caryophyllene		1420	53095	39526	25080	20313	16643	15627	24710	1304	1212	1598	1387	1261	1103	1065
Bergamotene (isomer)		1439	6050	6402	6602	6500	6016	5804	9584	-	-	-	-	-	-	-

Volatile compound	Ion[1]	KI[2]	Sample 7							Sample 8						
			Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Alkenes																
1-octene		792	378	761	1521	1997	2287	2372	2557	1946	1499	2640	3293	3133	3985	4388
2-(E)-octene		808	3839	2821	4207	4022	4796	5086	5613	3810	2447	5999	7828	8410	11830	12774
2-(Z)-octene		815	2859	1322	3100	3325	4097	4243	4325	2235	2024	5001	6808	7640	9393	9944
Aldehydes																
Hexanal		804	24168	36898	46876	48812	51229	50944	52120	60005	51230	54850	54741	57499	58360	59456
(E)-2-hexenal		850	432	3872	9704	10950	11847	11462	11856	1837	3997	6698	7637	8128	10119	10957
Heptanal		901	1255	1776	2402	3537	3783	3576	3927	2227	2280	3112	2974	3516	3870	4177
(E,E)-2,4-hexadienal	81	909	-	1148	4068	5170	5355	4683	5428	-	3562	8007	8490	8305	9347	8200
(E)-2-heptenal		954	2140	23761	63186	75377	78766	75165	77298	5134	27579	58354	69304	78179	79883	79724
(E,E)-2,4-heptadienal	81	995	9979	40753	87515	85008	80969	80211	72908	946	36200	81852	90816	89396	88156	83870
(E,Z)-2,4-heptadienal		1009	1875	33626	112457	151247	159055	165147	165689	-	23797	90460	119781	135477	161530	169121
2-octenal		1056	1079	7334	16878	23822	25219	23744	23372	2614	8049	25704	28423	35442	34154	35708
Nonanal		1103	-	1275	2957	4180	4345	4266	3974	971	1517	3405	4560	5160	6157	6410
Nonenal		1158	-	244	2171	5635	7905	8471	10446	-	-	2107	4150	6520	10393	13011
2,4-nonadienal		1192	-	-	-	-	2040	2284	2178	-	-	1297	2095	2186	2860	2892
(E,E)-2,4-decadienal		1291	-	646	5147	8937	9485	9968	9939	-	-	5788	9226	10514	12393	12213
2,4-decadienal		1317	-	1132	11904	24118	27321	27806	29263	-	-	8760	11197	19088	25023	26269
Ketones																
2-heptanone		890	-	-	-	-	-	-	-	4521	4124	4245	3996	4735	4748	5080
1-octen-3-one		977	-	751	2689	2110	2259	2223	1994	630	2841	6662	7158	5882	5621	6199
3-octen-2-one		1038	-	5439	12869,5	15604	16346	17361	17096	3038	5422	10420	11890	12584	14655	14841
(E,E)-3,5-octadien-2-one		1070	1757	21059	37683	33590	31161	33649	29854	-	21198	33109	35257	34797	32023	29639
3,5-octadien-2-one		1091	-	1972	3901	4487	5340	5823	4952	-	-	5299	27034	31531	36711	36599
Alcohols																

3-methyl-1-butanol		727	355	992	1942	1314	2415	4197	2240	878	653	2740	2354	2185	2545	2561
1-pentanol		762	2559	4737	5931	5592	5691	5192	5053	12016	8386	7701	6626	6379	6035	5372
1-hexanol		873	7356	3546	3720	3035	3002	2927	2868	80186	42281	21665	18282	16293	15178	14127
1-octen-3-ol		979	-	16919	32162	30591	29827	27681	26454	2142	17502	30694	29852	28183	29956	30309
Acids																
Propionic acid	74	726	-	13735	25075	26391	25235	27130	25266	-	7596	16197	18794	20044	20632	20114
Butanoic acid	60	801	6339	9416	11484	10373	10263	10343	10155	5426	8859	8158	8037	7736	8088	7879
Pentanoic acid	60	904	190	1684	3678	5031	5342	5130	4980	1194	2180	4157	4391	5219	5339	5660
Hexanoic acid	60	992	808	7875	19197	28634	30327	30732	30282	4806	13661	27517	32232	37820	38863	40626
Heptanoic acid	60	1082	-	1739	3623	3344	3395	3295	2968	-	2297	3082	3623	3682	3643	3705
Furans derivatives																
2-pentyl-furan	81	991	-	-	24547	37736	40499	37063	39376	-	-	19575	23073	27203	28120	32105
Terpenes																
α-pinene		929	21406	8292	8398	7109	6738	6808	6507	50728	27569	15353	13131	12761	11375	11467
β-pinene		971	10351	5330	5966	5057	5041	5095	4523	17312	11005	6722	5522	5636	4921	4789
δ-3-carene	93	1006	1509	1001	1097	919	862	889	836	11113	8034	4967	4414	4121	3867	3766
para-cymene	93	1021	938	941	1465	1608	1621	1640	1499	3698	4220	3994	3700	9919	10572	9325
Limonene		1025	8737	7763	9698	8924	8775	8993	8040	18220	16025	12504	11572	11829	10889	10328
(E)-β -ocimene		1047	-	-	-	-	-	-	-	13406	12259	9717	9981	9806	7904	7717
Terpinolene		1085	4346	3727	4865	4463	4461	4685	4766	-	-	-	-	-	-	-
(E)-caryophyllene		1420	1023	980	1352	1132	1032	1009	862	1689	1679	1248	1206	1084	919	820
Bergamotene (isomer)		1439	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Volatile compound	Ion[1]	KI[2]	Sample 9						
			Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
Alkenes									
1-octene		792	21722	8095	4945	4387	4750	4845	4653
2-(E)-octene		808	83333	31320	14022	17435	18800	20483	19748
2-(Z)-octene		815	70800	25639	15690	13620	13949	15204	14898
Aldehydes									
Hexanal		804	46589	52164	65886	86575	87469	102370	111396
(E)-2-hexenal		850	3893	4147	3758	1214	7265	10031	12534
Heptanal		901	2974	2231	2421	2708	2871	2918	3722
(E,E)-2,4-hexadienal	81	909	-	1658	5366	8520	8717	10242	11128
(E)-2-heptenal		954	73582	85798	73737	65516	60403	63992	65532
(E,E)-2,4-heptadienal	81	995	41761	99891	137971	127719	130425	121482	85322
(E,Z)-2,4-heptadienal		1009	9402	48862	113429	166438	168800	193527	216637
2-octenal		1056	2304	27628	94503	133690	145154	153966	140736
Nonanal		1103	116	2726	3703	5837	5882	6341	6846
Nonenal		1158	-	1644	4005	5678	15996	22773	35679
2,4-nonadienal		1192	-	-	-	-	-	-	-
(E,E)-2,4-decadienal		1291	-	4612	10720	16151	17418	16500	15784
2,4-decadienal		1317	-	3578	8669	25742	26684	27589	33994
Ketones									
2-heptanone		890	658	2129	-	-	-	-	-
1-octen-3-one		977	1498	1331	2692	2671	2027	1725	1135
3-octen-2-one		1038	-	-	-	-	-	-	-
(E,E)-3,5-octadien-2-one		1070	-	6107	10854	13154	13577	13258	9353
3,5-octadien-2-one		1091	-	1860	5527	8697	8909	10079	12428
Alcohols									

3-methyl-1-butanol		727	-	-	-	-	-	-	-
1-pentanol		762	553	-	-	-	-	-	-
1-hexanol		873	1304	1468	1462	1214	1211	1294	1333
1-octen-3-ol		979	746	17601	22170	23444	20931	22510	24124
Acids			-						
Propionic acid	74	726		3561	7260	8935	8932	10049	8685
Butanoic acid	60	801	3497	4558	4129	4009	3745	4129	3811
Pentanoic acid	60	904	408	1333	1967	2354	2624	2711	2553
Hexanoic acid	60	992	1039	5871	13686	87220	23750	24728	24085
Heptanoic acid	60	1082	-	1968	2798	3261	3464	2939	2845
Furans derivatives									
2-pentyl-furan	81	991	1777	7193	13858	20997	19745	25239	38068
Terpenes									
α-pinene		929	675	-	-	-	-	-	-
β-pinene		971	-	-	-	-	-	-	-
δ-3-carene	93	1006	-	-	-	-	-	-	-
para-cymene	93	1021	-	-	-	-	-	-	-
Limonene		1025	-	-	-	-	-	-	-
(E)-β -ocimene		1047	-	-	-	-	-	-	-
Terpinolene		1085	-	-	-	-	-	-	-
(E)-caryophyllene		1420	-	-	-	-	-	-	-
Bergamotene (isomer)		1439	-	-	-	-	-	-	-

Table 7.2.4.5.2. Correlation matrix (Pearson) and relative *p*-values (Pearson) among volatiles detected during the accelerated storage of nine cold-pressed hemp seed oils analyzed at 7 different times (0, 3, 6, 9, 12, 15, 18 days).

Volatile compounds	Time	*p*-values (Pearson)
1-octene	-0.094	0.464
2-(*E*)-octene	-0.066	0.608
2-(*Z*)-octene	-0.054	0.675
Hexanal	**0.482**	**< 0.0001**
(*E*)-2-hexenal	**0.843**	**< 0.0001**
Heptanal	**0.600**	**< 0.0001**
(*E*,*E*)-2,4-hexadienal	**0.708**	**< 0.0001**
(*E*)-2-heptenal	**0.640**	**< 0.0001**
(*E*,*E*)-2,4-heptadienal	**0.488**	**< 0.0001**
(*E*,*Z*)-2,4-heptadienal	**0.752**	**< 0.0001**
2-octenal	**0.398**	**0.001**
Nonanal	**0.734**	**< 0.0001**
Nonenal	**0.615**	**< 0.0001**
2,4-nonadienal	**0.582**	**< 0.0001**
(*E*,*E*)-2,4-decadienal	**0.719**	**< 0.0001**
2,4-decadienal	**0.809**	**< 0.0001**
2-heptanone	0.004	0.973
1-octen-3-one	**0.326**	**0.009**
3-octen-2-one	**0.516**	**< 0.0001**
(*E*,*E*)-3,5-octadien-2-one	**0.559**	**< 0.0001**
3,5-octadien-2-one	**0.667**	**< 0.0001**
3-methyl-1-butanol	0.099	0.439
1-pentanol	**-0.278**	**0.027**
1-hexanol	**-0.253**	**0.045**
1-octen-3-ol	**0.619**	**< 0.0001**
Propionic acid	**0.648**	**< 0.0001**
Butanoic acid	-0.059	0.648
Pentanoic acid	**0.547**	**< 0.0001**
Hexanoic acid	**0.546**	**< 0.0001**
Heptanoic acid	**0.447**	**0.000**
2-pentyl-furan	**0.741**	**< 0.0001**
α-pinene	**-0.417**	**0.001**
β-pinene	**-0.381**	**0.002**
δ-3-carene	-0.167	0.191
para-cymene	**0.254**	**0.045**
Limonene	-0.209	0.101
(*E*)-β -ocimene	-0.063	0.626
Terpinolene	-0.173	0.175
(*E*)-caryophyllene	-0.192	0.131
Bergamotene (isomer)	0.043	0.740

7.2.4.6 Tocopherols

The tocopherols detected in all the samples were α-tocopherol and γ-tocopherol. Results are shown in Table 7.2.4.6.1 and are in accordance with literature (Oomah, Busson, Godfrey, & Drover, 2002; Matthäus & Brühl, 2008; Liang, Appukuttan Aachary, & Hollader, 2015) except for sample 9. In fact, sample 9 showed a higher content of α-tocopherol with respect to the other samples. According to literature the main tocopherol naturally present in hemp seed oils is γ-tocopherol, while the main one detected in sample 9 was α-tocopherol.

Table 7.2.4.6.1 Tocopherols content in the nine hemp seed oil samples during the accelerated oxidation test. Results are reported as mean±standard deviation of three replicates and expressed as µg/g.

Samples	Time (days)													
	0		3		6		9		12		15		18	
	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol	α-tocopherol	γ-tocopherol
1	32.09 ±0.78	641.91 ±29.25	-	350.13 ±5.74	-	83.08 ±2.39	-	76.23 ±1.41	-	69.93 ±1.53	-	69.59 ±1.68	-	70.38 ±1.80
2	28.28 ±0.85	568.68 ±39.34	-	65.86 ±0.76	-	56.25 ±0.30	-	60.95 ±1.39	-	54.83 ±1.09	-	58.34 ±2.40	-	60.03 ±1.88
3	45.98 ±4.15	569.99 ±16.46	-	65.62 ±0.63	-	65.48 ±0.41	-	64.76 ±0.30	-	64.27 ±1.21	-	65.74 ±2.08	-	65.65 ±2.07
4	21.02 ±0.28	376.28 ±10.72	-	40.40 ±0.84	-	44.64 ±1.68	-	46.15 ±0.73	-	47.36 ±0.73	-	49.03 ±3.46	-	50.28 ±1.14
5	41.53 ±3.09	650.52 ±14.68	-	103.65 ±2.90	-	65.23 ±3.58	-	73.80 ±1.72	-	68.63 ±0.83	-	72.57 ±3.44	-	67.71 ±1.30
6	65.92 ±4.86	906.63 ±65.75	-	301.72 ±3.88	-	212.29 ±8.22	-	200.12 ±3.34	-	209.57 ±5.87	-	195.09 ±6.47	-	195.57 ±8.28
7	59.30 ±1.46	622.24 ±12.12	-	79.69 ±10.22	-	66.57 ±1.61	-	73.37 ±1.31	-	70.54 ±0.96	-	85.92 ±5.93	-	86.53 ±1.16
8	49.81 ±0.63	893.81 ±7.78	-	384.12 ±4.77	-	300.00 ±5.10	-	233.05 ±11.29	-	206.03 ±8.11	-	207.02 ±7.70	-	237.66 ±8.34
9	1095.62 ±45.21	816.31 ±53.49	-	327.68 ±3.27	-	83.29 ±2.46	-	59.30 ±1.68	-	55.50 ±1.75	-	51.84 ±1.73	-	53.52 ±0.59

7.2.4.7 Cannabinoids

The main cannabinoid present in all the samples was cannabidiolic acid (CBDA). Several samples also showed the presence of cannabidiol (CBD) and cannabigerolic acid (CBG); while only in sample 1 it was found cannabigerol (CBG). As reported in Figure 7.2.4.7.1, the content of CBDA decreased from time 0 to time 18, in all the samples. On the contrary, the concentration of CBD increased in several samples, as for samples 4 and 5, for example. Those two samples also showed the highest content of cannabinoids with a concentration of CBDA equal to 166.46±1.01 µg/g in sample 4T0, 184.62±12.85 µg/g in sample 5T0. Sample 5 also showed a great increase in terms of CBD from time 0 to time 18 (passing from 70.43±5.31 µg/g at time 0 to 157.95±3.70 µg/g at time 18).

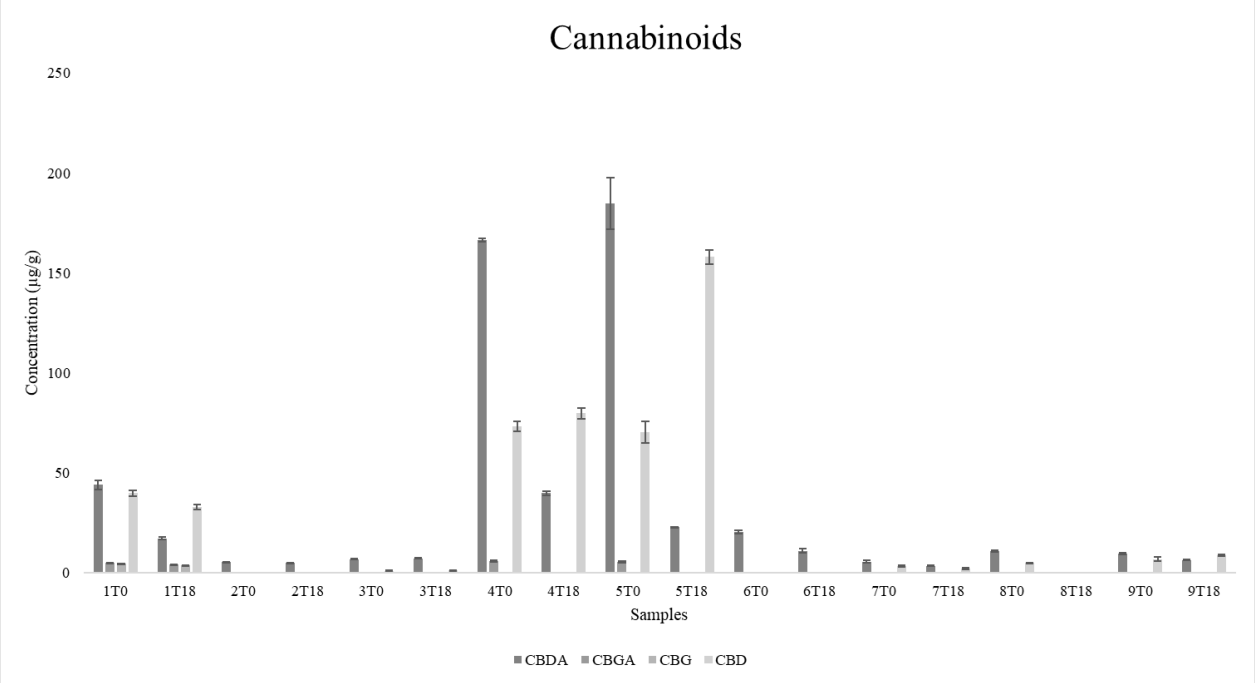


Figure 7.2.4.7.1. Cannabinoid content (µg/g) in the nine hemp seed oils at time 0 (T0) and time 18 (T18) of the accelerated oxidation test.

7.2.5 Discussion

7.2.5.1 Oxidative state and composition of the hemp seed oils at the initial stage of the accelerated oxidation test

Before starting the accelerated oxidation test, the initial oxidative status and composition of the samples were evaluated (Table 7.2.5.1.1). Results obtained for PVs at time zero are in accordance with several authors in literature for hemp seed oil (Matthäus & Brühl, 2008; Latif & Anwar, 2009; Liang et al., 2018; Spano et al., 2020).

Table 7.2.5.1.1. Results of the PVs, TBARs, Total Phenolic content, classes of volatiles, tocopherols and CBDA/CBD ratio before submitting samples to the accelerated test. Different letter in columns indicates statistically significant differences among samples (one-way ANOVA, $p<0.05$, Tukey HSD).

Sample	PVs ($mEqO_2$/kg of oil)	TBARs (mg MDA/kg of oil)	Aldheydes (area counts $\times10^3$)	Ketones (area counts $\times10^3$)	Acids (area counts $\times10^3$)	Furans (area counts $\times10^3$)	Terpenes (area counts $\times10^3$)	Total phenols (mg gallic acid/kg of oil)
1	12.62 ± 1.07^{a}	11.28 ± 0.69^{a}	79310^{b}	18728^{a}	53014^{a}	21427^{a}	27076^{g}	50.37 ± 2.03^{c}
2	$11.78\pm0.60^{a,b}$	11.04 ± 1.58^{a}	54553^{c}	4161^{d}	19997^{b}	-	85764^{e}	106.50 ± 8.8^{b}
3	9.43 ± 0.25^{c}	13.27 ± 1.78^{a}	22471^{f}	$2786^{e,f}$	$10665^{c,d}$	-	99763^{d}	45.58 ± 2.71^{c}
4	$11.41\pm0.66^{a,b}$	11.56 ± 1.70^{a}	44554^{d}	$3931^{d,e}$	$8506^{d,e}$	-	241727^{b}	15.39 ± 0.79^{e}
5	$10.42\pm0.54^{b,c}$	11.54 ± 0.28^{a}	$38251^{d,e}$	14477^{b}	9810^{d}	-	270385^{a}	16.75 ± 0.41^{e}
6	$11.93\pm0.88^{a,b}$	11.37 ± 1.57^{a}	81114^{b}	8363^{c}	14170^{c}	8111^{b}	77792^{e}	30.76 ± 6.60^{d}
7	$10.87\pm0.41^{a,b,c}$	12.25 ± 1.15^{a}	$30949^{e,f}$	1757^{f}	$7336^{d,e}$	-	48310^{f}	25.70 ± 1.07^{d}
8	$11.95\pm0.81^{a,b}$	12.01 ± 0.97^{a}	73734^{b}	8189^{c}	$11426^{c,d}$	-	116165^{c}	12.08 ± 1.96^{e}
9	1.83 ± 0.06^{d}	17.15 ± 0.83^{a}	141284^{a}	2156^{f}	4944^{e}	1777^{c}	675^{h}	186.78 ± 4.5^{a}

Sample	C18:1 (g/100 g of oil)	C18:2 n-6 (g/100 g of oil)	C18:3 n-3 (g/100 g of oil)	C18:3 n-6 (g/100 g of oil)	γ-tocopherol (µg/g)	α-tocopherol (µg/g)	CBDA/CBD
1	11.50^{b}	$48.62^{a,b,c}$	11.40^{c}	2.43^{b}	641.91 ± 29.25^{b}	$32.09\pm0.78^{b,c}$	1.10^{d}
2	10.90^{c}	$50.37^{a,b}$	14.89^{b}	0.39^{d}	568.68 ± 39.34^{b}	$28.28\pm0.85^{b,c}$	-
3	10.80^{c}	$50.58^{a,b}$	15.31^{b}	0.37^{d}	569.99 ± 16.46^{b}	$45.98\pm4.15^{b,c}$	6.58^{a}
4	13.08^{a}	$48.20^{a,b,c}$	10.99^{c}	1.61^{c}	376.28 ± 10.72^{c}	21.02 ± 0.28^{c}	$2.27^{b,c}$
5	12.92^{a}	$50.04^{a,b}$	11.98^{c}	1.58^{c}	650.52 ± 14.68^{b}	$41.53\pm3.09^{b,c}$	$2.62^{b,c}$
6	7.12^{f}	$47.63^{b,c}$	15.53^{b}	3.51^{a}	906.63 ± 65.75^{a}	65.92 ± 4.86^{b}	-
7	11.05^{b}	50.96^{a}	14.96^{b}	0.37^{d}	622.24 ± 12.12^{b}	$59.30\pm1.46^{b,c}$	$1.72^{c,d}$
8	7.72^{e}	$49.76^{a,b}$	17.17^{a}	3.71^{a}	893.81 ± 7.78^{a}	$49.81\pm0.63^{b,c}$	$2.17^{b,c}$
9	8.45^{d}	46.35^{c}	15.27^{b}	3.59^{a}	816.31 ± 53.49^{a}	1095.62 ± 45.21^{a}	1.44^{d}

Most of the samples showed similar values (9.43-12.62), with only sample type 9 showing very low values (1.83). Sample 9 also showed the highest TBARs values and the highest presence of aldehydes, which, representing secondary oxidation products (Poyato et al., 2014) could highlight a bad oxidative state of the oil, that could be also in relation to the low PV value. In fact, it is well known that peroxides are not stable compounds, and generally, their concentration in the oils increases until it reaches a maximum value and, then, decreases as they degrade to secondary oxidation products (Bajoub, Bendini, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2018). The highest content of ketones, acids and furans was detected in sample 1. Since also those compounds are generally related to the oxidation (Morales, Luna, & Aparicio, 2005; Guillen & Goicoechea, 2008), their presence could indicate a worse oxidation state of this oil compared to the others. In addition, the lowest content of terpenes was detected in the head space of sample 9. According to literature, most of the volatile compounds linked to oxidation have a low odor threshold, and the main contribution to rancid defects comes from aldehydes (Morales et al., 2005). However, terpenes can positively contribute to the aroma of hemp seed oil, bringing aromas such as hop, pine, lime and spicy (Sommano, Chittasupho, Ruksiriwanich, & Jantrawut, 2020). For these reasons, sample 9 could be mainly characterized by off-flavor related to oxidation.

The main fatty acids (oleic, linoleic, α- linolenic and γ-linolenic acids) were also evaluated in order to highlight differences among samples. In particular, the principal fatty acid was C18:2 n-6 (linoleic acid), which showed the lowest value in sample 9.

Phenolics greatly affect the stability and nutritional characteristics of oil samples and might prevent their deterioration through quenching of radical reactions responsible for lipid oxidation (Abuzaytoun & Shahidi, 2006). The total phenolic content was in accordance with what was found in the literature (Yu et al., 2005; Siger, Nogala-kalucka, & Lampart-Szczapa, 2008; Izzo et al., 2020). The highest phenolic content was detected in sample 9, which was significantly different from all the other oils (Table 7.2.5.1.1).

A high content of γ-tocopherol characterizes hemp seed oil, around 80-90% of the total amount of tocopherols (80-150 mg/100 g) in comparison with many edible oils (e.g. olive oil), which is a naturally present antioxidant in this oil (Liang et al., 2015). The content of γ-tocopherol was in accordance to literature for all the samples (Özdemir et al., 2021), while the content of α-tocopherol detected in sample 9 was greatly higher to previous studies (Izzo et al., 2020; Leonard, Zhang, Ying, & Fang, 2020; Özdemir et al., 2021).

Finally, the ratio between cannabidiolic acid (CBDA) and cannabidiol (CBD) was also considered since it could be a helpful index for hemp seed oil storage. This is related to the decarboxylation reaction which determines the conversion of CBDA (i.e. the acid form naturally produced by the

hemp plant) to CBD (i.e. the neutral form) (Pratap Singh et al., 2020). Only samples 2 and 6 showed the presence of neither CBDA nor CBD, and in sample 3, the higher ratio of CBDA/CBD was detected. This can be interpreted differently: the sample may have been extracted at lower temperatures or a fresher oil than the others. However, the presence of cannabinoids is strictly related to cross-contamination with flowers/leaves or a bad selection of the bracts (Citti et al., 2018). For this reason, even if the ratio CBDA/CBD could be a helpful index, it cannot be considered alone: some hemp seed oils do not show the presence of cannabinoids (Figure 7.2.4.7.1 and Figure 7.2.5.1.1). Several parameters evaluated and discussed in previous lines highlighted that the initial state and composition of sample 9 were very different from the other oils tested.

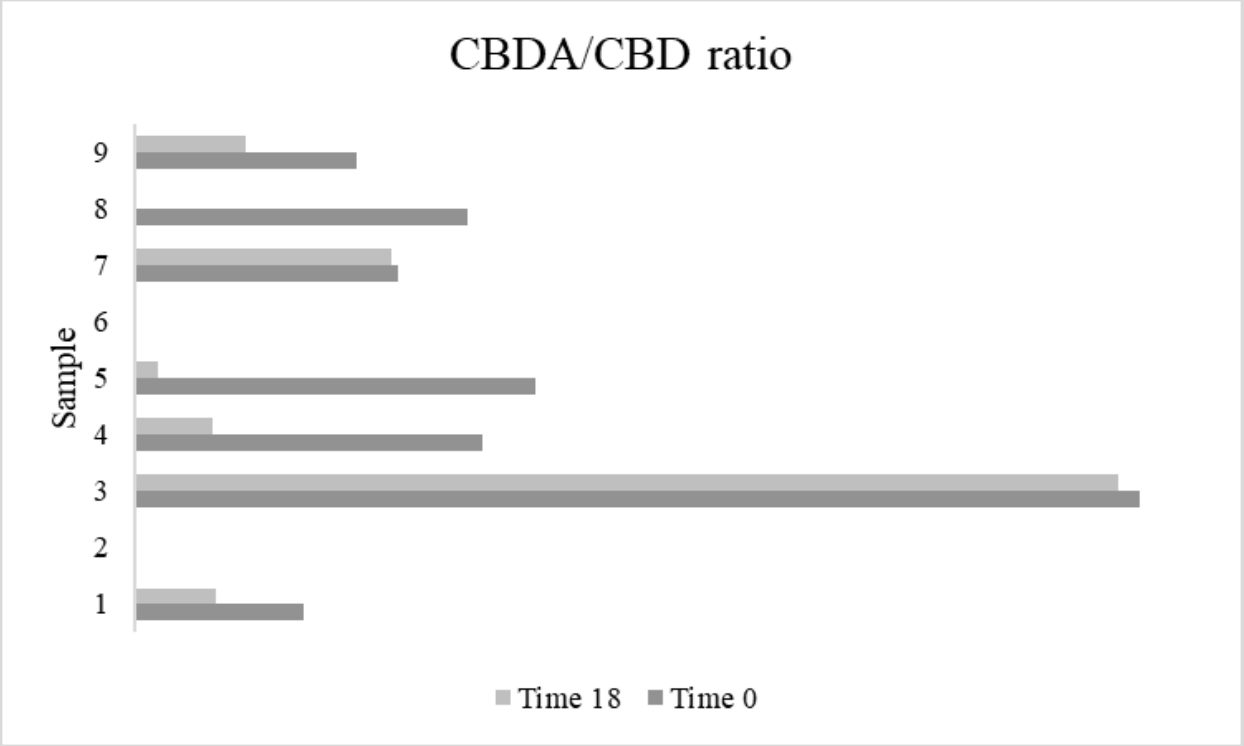
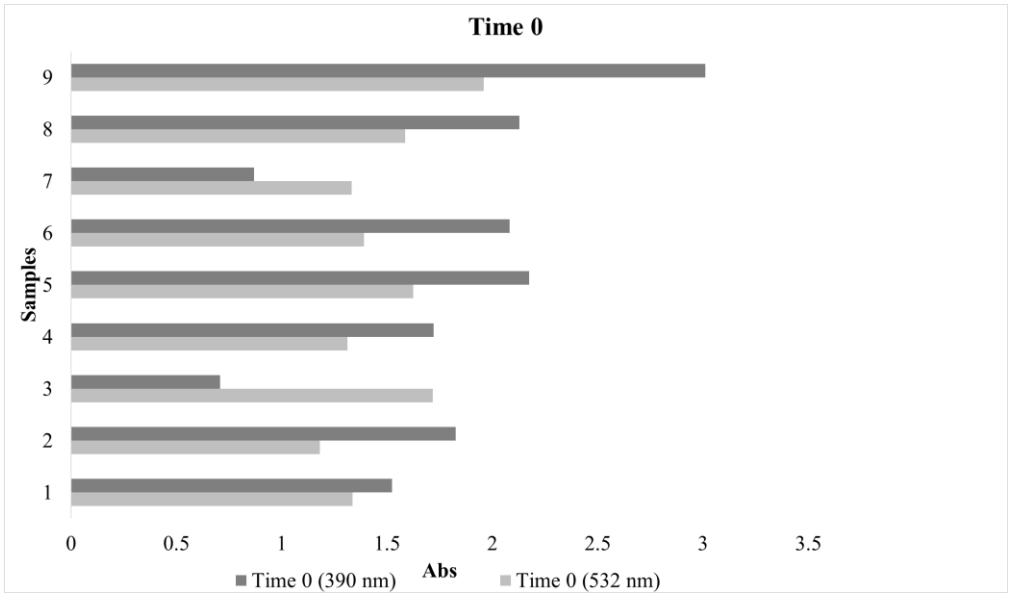


Figure 7.2.5.1.1. Ratio between cannbidiolic acid (CBDA) and cannabidiol (CBD) at the beginning (Time 0-T0) and at the end (Time 9-T9) of the accelerated oxidation test.

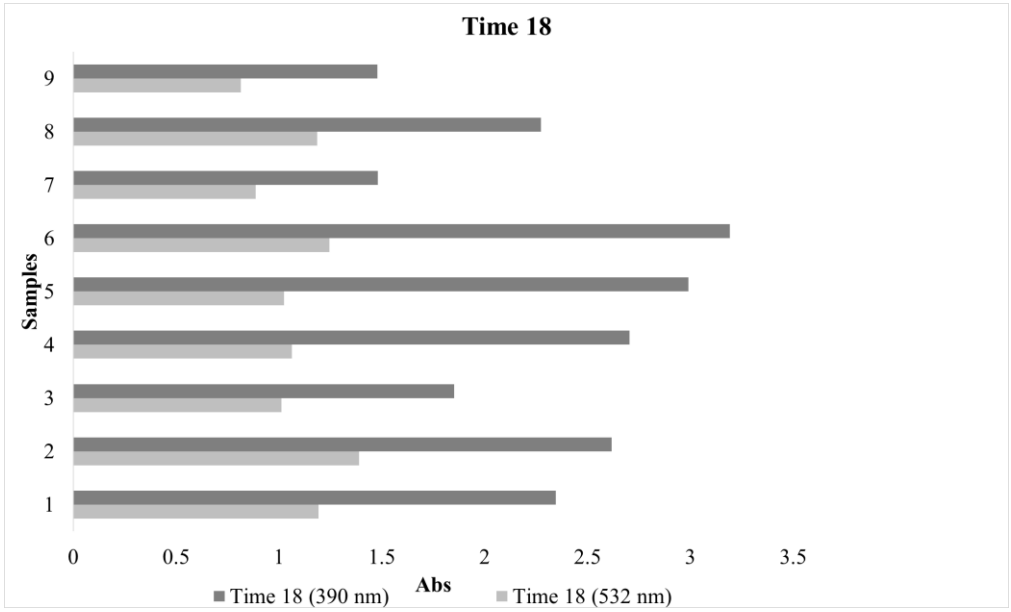
7.2.5.2 Evolution of the oxidative state and composition of the hemp seed oils during the accelerated heating test

As reported in Figure 7.2.4.1.1, the PVs increased during the accelerated oxidation test and, then, they decreased at the end. Only samples 2, 7 and 9 showed an increase in the PVs until the end accelerated storage. Regarding TBARs, data showed a decrease of this value during the accelerated oxidation test in all samples, except in sample 6, where the decrease was not statistically significant (Table 7.2.4.2.1). Poyato et al. (2014) reported that the presence of high content of aldehydes during storage gives rise to the formation of yellow chromophores determining a significant absorbance at

390 nm instead of at 532 nm, which is the clasical wavelength used for measuring TBARs. This could explain the decrease of TBARs registered; in fact, the absorbance at 390 nm was higher than at 532 nm, also in our case (Figure 7.2.5.2.1).



(a)



(b)

Figure 7.2.5.2.1. Absorbance measured at 390 and 532 nm for samples at time 0 (a) and at time 18 (b).

Total phenolic content decreased during the test in 7 out of the 9 types of samples (Table 7.2.4.3.1), with sample 9 maintaining the highest phenolic content at the end of the treatment. These decreases could be related to the oxidation process revealed by the analysis of volatile compounds and the destruction of the phenols acting as antioxidants. Regarding these volatiles, aldehydes significantly increased during the heating treatment. Among them, two saturated species were detected: heptanal and nonanal (Table 7.2.4.5.1), showing an increment during heating, especially in the case of nonanal.

Their presence could be related to the decomposition of hydroperoxides formed by the autoxidation of oleic acid (Vichi et al., 2003). (E)-2-hexenal was present also at the beginning of the storage in all the samples and increased during the accelerated oxidation test, thus could be related to two different phenomena: the linoleate autoxidation decomposition and the enzymatic oxidation of linolenic acid (Morales, Rios, & Aparicio, 1997; Vichi et al., 2003; Muik, Lendl, Molina-Díaz, & Ayora-Cañada, 2005). Hemp seed oils are rich in ω-3 and ω-6 fatty acids (Supplementary material Table 2) (Petrović, Debeljak, Kezić, & Džidara, 2015; Izzo et al., 2020) and the secondary products of lipid oxidation, such as volatiles, greatly depend on the fatty acid substrate (Nogueira, Scolaro, Milne, & Castro, 2019). According to Nogueira et al. (2019) several volatile compounds related to oxidation of ω-3 rich oils are (E,E)-2,4-heptadienal; (E,E)-3,5-octadien-2-one and (E,E)-2,4-decadienal, which are potentially toxic volatiles, while of ω-6 rich oils are (E,E)-2,4-heptadienal and nonanal; in fact, those compounds were detected in the nine types of oxidized oils (Table 7.2.4.5.1). The most abundant terpenes were α-pinene and β-pinene, this result is in line with Zhou et al. (2017). Moreover, as reported in Table 7.2.4.5.1, sample type 9 showed a very different terpenes profile compared to the other samples. In fact, α-pinene was the only terpene detected in this type of oil, while for the others also β-pinene, δ-3-carene, ρ-cymene, limonene, (E)-β -ocimene, terpinolene and (E)-caryophyllene were identified.

Regarding tocopherols, the content of α-tocopherol during oils storage usually decrease, in particular, if the overall oxidative status of the oil is not good (e.g. high PVs), resulting in a decrease of the nutritional value of the oil (Psomiadou, Tsimidou, & Boskou, 2000). In all the samples, α-tocopherol were detected only at time 0 (before starting the accelerated oxidation test), while γ-tocopherol was always identified and it showed a significant decrease (Table 7.2.4.6.1). The main decrease was among 3-9 days depending on the samples and then the amounts were stable until the eighteenth day. This reduction of tocopherols in the matrix could be related to the antioxidant activity of those compounds, slowing down the oxidation process in the oil (Liang et al., 2015).

Results showed that the most abundant cannabinoid was CBDA (Figure 7.2.4.7.1), which is in line with previous literature (Citti et al., 2018; Pratap Singh et al., 2020). This cannabinoid, found in all the samples, showed a reduction during the accelerated storage test. According to Pratap Singh et al. (2020), this decrease occurred due to the decarboxylation reaction of CBDA. Citti et al. (2018) hypothesised that at a temperature under 100°C the decarboxylation of CBDA leads only to the formation of CBD, increasing this cannabinoid leading to a nearly constant sum of CBDA+CBD; while at a temperature above 100°C it also determines the formation of unknown products or the evaporation of the neutral cannabinoid. For some samples, our results showed different trends in comparison to Citti et al. (2018): in particular, a decrease of CBDA with no increase in terms of CBD

was registered in samples 1, 4, 6 and 8, even if the temperature of the accelerated oxidation was 60°C; samples 2, 3 and 7 showed a constant sum of CBDA+CBD without substantial changes in their content; while in samples 5 and 9 it was highlighted a decrease of CBDA with a consequent increase of CBD content. Moreover, a decrease in the CBDA/CBD ratio was detected in the majority of the samples, only for sample 3 no differences were found (Figure 3). Samples 2 and 6 did not show the presence of CBD, for this reason the CBDA/CBD ratio was not reported.

7.2.5.3 Evaluation of the oxidation and freshness volatiles markers

In order to select volatile compounds as possible markers of oxidation and freshness during the accelerated storage period, the amount of each compound was monitored during the test and correlated with the storage time.

Table 7.2.4.5.2 shows the Pearson value, p-values and significance of the correlation between the area counts x103 of each compound and the storage time (reported in days). All the aldehydes detected showed a positive and significant correlation with the accelerated oxidation time, confirming to be markers of oxidation of edible oils (Morales et al., 1997; Vichi et al., 2003; Muik et al., 2005; Poyato et al., 2014; Gaca et al., 2021). Moreover, several ketones (1-octen-3-one, 3-octen-2-one, (E,E)-3,5-octadien-2-one and 3,5-octadien-2-one), acids (propionic acid, pentanoic acid, hexanoic acid and heptanoic acid) and furans (2-pentyl-furan) showed a positive correlation with storage time. Also, those compounds were reported to be related to the oxidative phenomena in edible oils (Pavlovic et al., 2018; Liu, Li, Cheng, & Liu, 2020). Among them, the principal oxidation volatile markers were (E)-2-hexenal, (E,E)-2,4-hexadienal, (E,Z)-2,4-heptadienal, nonanal, (E,E)-2,4-decadienal, 2,4-decadienal and 2-pentyl-furan, showing the highest Pearson values. On the other hand, 2 terpenes (α-pinene and β-pinene) were inversely related to the accelerated oxidation time, thus indicating them as possible markers of the freshness of the cold-pressed hemp seed oils. 1-pentanol and 1-hexanol were also inversely correlated with storage time (days), suggesting that the accelerated storage conditions could also determine further oxidation of the alcohols, according to Vichi et al. (2003). Figure 7.2.5.3.1 reports the volatile compounds detected at each time of the storage period for the nine types of oils.

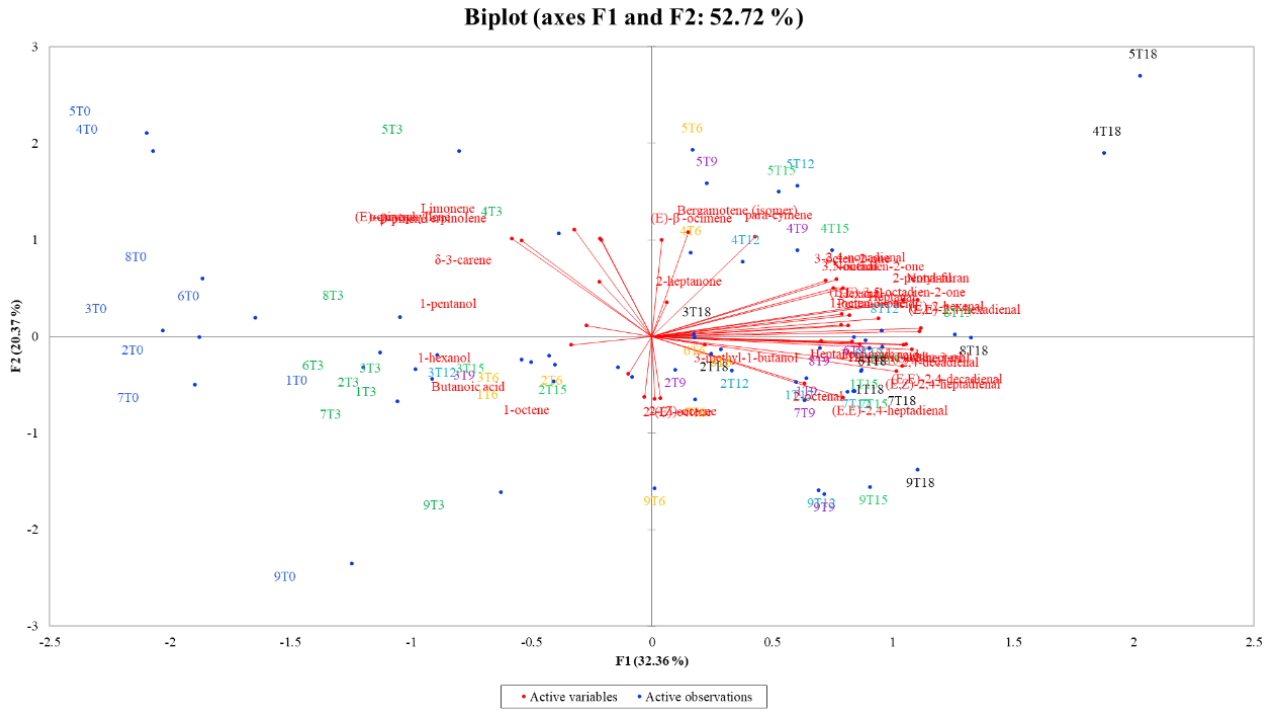


Figure 7.2.5.3.1. Bi-plot of the Principal Component Analysis (PCA) for volatiles identified in all the samples at each time of analysis (0, 3, 6, 9, 12, 15, 18 days of accelerated storage).

At time 0 (before starting the accelerated oxidation), samples are separated from all the other storage times, suggesting that they presented a very different volatile profile than the oxidized samples. Moreover, it is possible to notice also that the samples at time 3 (3 days of accelerated oxidation test) are also grouped in the same PCA quadrants as T0, and separated from the others, showing an intermediate oxidation status. In fact, they were mainly characterized by 1-octene, 1-pentanol, 1-hexanol, butanoic acid, α-pinene, β-pinene, limonene, terpinolene and (*E*)-caryophyllene. On the contrary, samples from time 6 (6 days of accelerated oxidation test) to time 18 (18 days of accelerated oxidation test) were not separated among them and they clustered with aldehydes, several ketones and acids and 2-pentyl-furan (Figure 7.2.5.3.1).

In Figure 7.2.5.3.2, samples at time 0 and time 18 were represented and correlated with marker volatiles, PVs, TBARs, total phenols, α-tocopherol and γ-tocopherol. The PCA showed that samples at time 0 were well separated from those at time 18 and they were characterized by a high content of tocopherols and phenols, which are the main antioxidant naturally present in hemp seed oil (Liang et al., 2015), as well as by α-pinene and β-pinene, which have been identified as volatile markers of freshness. The higher content of PVs, which characterized samples at time 0, could be explained by the instability of peroxides. In fact, it is well known that those compounds reduced their concentration during oxidation because of the degradation into secondary oxidation products.

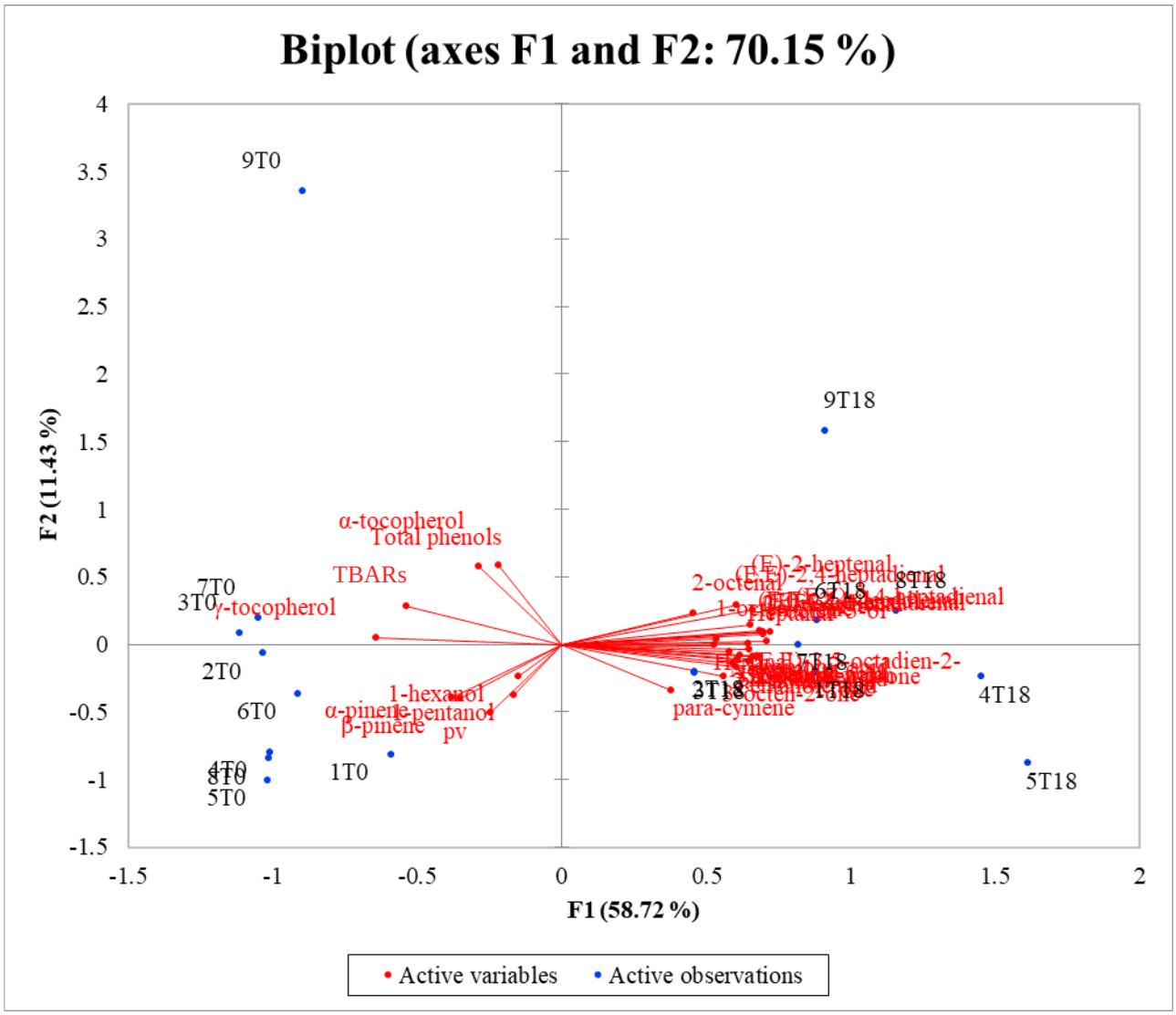


Figure 7.2.5.3.2. Representation of the principal component analysis (PCA) for volatile aldehydes, acids, ketones, alcohols and furan (selected as marker of oxidation), terpenes (as marker of freshness), tocopherols, peroxide values (PVs), TBARs and total phenols of the nine hemp seed oils at 0 and 18 days of the accelerated oxidation (60°C). (T0, 0 days of accelerated storage; T18, 18 days of accelerated storage).

7.2.6 Conclusions

During the accelerated storage test of 9 types of commercial hemp seed oils (60°C for 18 days), selected as representative of different qualities available on the market, similar and different rates of the oxidative process were highlighted. In particular, one hemp seed oil, even before the start of the accelerated oxidation test, showed a high amount of secondary oxidation products (TBARs), as it was probably already oxidized. For all samples, the rise of specific volatile compounds was found to be highly related with the progress of the oxidative process. It consisted in (E)-2-hexenal, (E,E)-2,4-

hexadienal, (E,Z)-2,4-heptadienal, nonanal, (E,E)-2,4-decadienal, 2,4-decadienal and 2-pentyl-furan. The increase of these compounds can be considered inversely proportional to the freshness of the hemp oil, as is the decrease of specific naturally occurring terpenes (in particular α-pinene and β-pinene,). Tocopherols and total phenols, acting as antioxidants, also registered a remarkable decrease during the accelerated storage test. By the correlation between peroxide value, TBARs, volatiles, phenols and cannabinoids, it was highlighted that the sole determinations of primary oxidation products and TBARs were not enough to evaluate the oxidative changes occuring during the test of accelerated storage. It was found that samples on the eighteenth day (end of the test) were mainly characterised by oxidation volatiles markers not detectable by the TBARs determination and low content of antioxidant compounds (i.e. average reduction of 86% of antioxidant compounds).

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Chapter 8 Conclusions and outlook

Chapter 8. Conclusions and outlook

The present thesis dealt with the assessment of food, phytotherapeutic, and pharmaceutical hemp products from instrumental and sensory points of view. To fulfil this objective, different research activities were carried out, some of which related to the determination of cannabinoids, to the antioxidant activity of cannabidiol (CBD), and characterization of hemp seed oils.

In particular:

- A HPLC-UV method for rapid and cost-efficient determination of the 10 main cannabinoids in *Cannabis sativa* L. inflorescences was developed. The extraction of cannabinoids was performed by using methanol-chloroform (9:1 v/v) as the extraction solvent. Following this, the solution has to be dried and recovered in 500 µL acetonitrile. The separation was performed by RP-HPLC-UV with water + 0.085% phosphoric acid (A) and acetonitrile + 0.085% phosphoric acid (B) as eluent mixtures, while detection was carried out at 220 nm. Quantification was done by an external standard method through the construction of calibration curves using pure standard chromatographic reference compounds. The main cannabinoids dosed (g/100 g) in actual samples were cannabidiolic acid (CBDA), CBD, and Δ9-THC (Sample L11 CBDA 0.88±0.04, CBD 0.48 ± 0.02, Δ9-THC 0.06±0.00; Sample L5 CBDA 0.93±0.06, CBD 0.45±0.03, Δ9-THC 0.06±0.00). Several validation parameters were assessed. In particular, the method demonstrated a very high repeatability, reproducibility, and recovery.
- The antioxidant activity of CBD, added to model systems of refined olive and sunflower oils at different concentrations, was evaluated. In particular, the peroxide value, oxidative stability index (OSI time), electron spin resonance forced oxidation, and DPPH• assays were performed. The free acidity was also examined. Using the same analytical scheme, CBD was compared with α-tocopherol and showed a higher scavenging capacity, measured by DPPH• assay, but did not confer a better oxidative stability to the oil. α-tocopherol (from 0.01% to 0.1%) did not show any significant antioxidant or pro-oxidant effects either in sunflower oil or in refined olive oil, while at 0.5% it produced e.g. an increase of more than 30% of the OSI time of sunflower oil (from 4.15±0.07 to 6.28±0.11 h), and a decrease of 86% (from 83.33±4.56 to 11.23±0.28) of free radicals (µM) in refined olive oil (ESR-forced oxidation assay). On contrast, the addition of 0.5% CBD caused a worsening of the oxidative stability of refined olive oil and sunflower oil, the OSI times of which were reduced by 27% (from 23.58 ± 0.32 to 17.28 ± 0.18 h) and 19% (from 4.93 ± 0.04 to 3.98 ± 0.04 h), respectively. Furthermore, 0.5% of CBD did not substantially reduce the concentration of free radicals

(μM) as for α-tocopherol, either in sunflower oil or in refined olive oil. This has to be taken into account in the formulation of phytotherapeutic CBD products, which are generally oily solutions with different concentrations of CBD. In some cases it would be advisable to add antioxidant compounds, such as tocopherols or phenolic compounds (e.g. polyphenol extracts from olive processing by-products or hydroxytyrosol) to guarantee a longer shelf-life. Obviously, any additions should be evaluated based on the CBD concentration, type of oil used as a matrix, and eventual regulatory restrictions.

- The evaluation of hemp seed oils from an instrumental and sensory point of view was also carried out. Firstly, 13 hemp seed oils (HSOs) from the market were characterized, thus providing data about their quality and the bioactive compounds naturally present in this product. HSOs differed in the free acidity value; two of the 13 HSOs showed a free acidity value higher than the limit established by the Codex Alimentarius, equal to 4 mg KOH/g of oil. Also, for one of the 13 HSOs a peroxide value higher than the limit reported by the Codex Alimentarius (20 $mEqO_2$/kg of oil) was registered. Samples also differed in the K_{232} and K_{270} indexes (from 1.38±0.15 to 5.02±0.15 and from 0.16±0.01 to 0.62±0.03, respectively) as well as in the OSI time (from 3.37±0.29 to 6.72±0.12 hours). The samples showed an excellent ω6:ω3 ratio ranging from 2.60 to 3.67, which is considered optimal from a nutritional point of view. They also presented an extremely variable content of chlorophylls and carotenes (from 0.78 to 75.73 mg/kg of oil and from 2.53 to 33.93 mg/kg of oil, respectively), as well as γ-tocopherol (from 593.88 to 967.47 mg/kg) and cannabinoids, in particular regarding the content of CBDA (from 4.25 to 91.64 mg/kg), Δ9-THC (from 0.00 to 5.29 mg/kg) and THCA (from 0.00 to 5.00 mg/kg). Moreover, the analysis of the volatile profile highlighted the presence of some specific cannabis terpenes, such as α-pinene and β-pinene. In addition, the sensory quality of cold-pressed HSOs (15 samples) was assessed by following the ISO 13299:2010. A sensory profile sheet was defined and specific training of the panellists was carried out in order to obtain a satisfactory performance level of the panel monitored through the use of PanelCheck open-source software. The panel identified 46 descriptors, of which 13 were selected for the sensory sheet and categorized as “positive” or “negative”, and all the attributes were graphically represented in a sensory wheel. The most frequent (>5%) attributes highlighted by the panel were: yellow and green color in terms of appearance; sunflower/pumpkin seeds, nutty, toasted nutty, hay, sweet, bitter, pungent, and astringent in terms of olfactory and gustatory evaluation. The olfactory defects significantly found by the tasters in some samples were rancid, paint, burnt, and fish. Moreover, consumers’ attitudes with regards to hemp seed oils were preliminarily investigated using a focus group with 8

subjects. The interviewees reported that the extraction process and the color of hemp seed oil represent drivers of choice, and they were convinced that, for this product, price is not directly related to the quality of the product. Finally, due to Covid-19 restrictions adopted by many countries in 2020, remote sensory testing, organized in a location chosen by the assessor (home or the office) and carried out under the live on-line supervision of the panel leader, was performed. The trained panel participated in 8 sessions for evaluation of HSOs; four sessions were held in the lab and four were held in remote conditions. Two samples (in two replicates) were evaluated in each session. Results from the descriptive analysis of HSOs indicated that the sensory profile of samples obtained in the two conditions were almost the same, as were assessor performances. These findings suggest that descriptive analysis in remote conditions represents an alternative to lab evaluation when samples are provided in a ready to use format. However, a significant effect of evaluation conditions on color evaluation was found. Light conditions at home cannot be easily standardized. When color represents a target attribute, further efforts should be made by experimenters to provide assessors with appropriate light devices to overcome this source of variability with respect to lab conditions.

- HSO changes during storage were assessed. To do this, two different experiments were performed. The first concerned investigation of changes in the composition of a cold-pressed HSO during a storage period of 3 months. In this case, the environmental conditions applied for the conservation were 12 hours of light and 12 of darkness at room temperature to mimic a supermarket shelf. The peroxide value was quite low on freshly produced oil (2.66 ± 0.29 mEqO2/kg of oil) and decreased after 3 months (1.35 ± 0.08 mEqO2/kg of oil), as confirmed by free radical concentrations, while no other significant (one-way ANOVA, Tukey's HSD test, $p<0.05$) differences were observed (e.g. conjugated diene and triene systems, OSI, and free acidity). The fatty acid, cannabinoid, and tocopherol composition did not significantly change, expect for δ-tocopherol for which a decrease was observed. The overall results did not show a strong effect of photooxidation on the oil, despite its high degree of unsaturation. The second investigation was an accelerated storage test, conducted at 60°C for 18 days on 9 HSOs. The main aim of this study was to assess the volatile oxidation and freshness markers. In particular, several volatiles identified at the beginning of the accelerated storage (at time 0), such as the predominant α-pinene and β-pinene, gradually decreased during the accelerated storage period. On the other hand, aldehydes (hexanal, (E)-2-hexenal, heptanal, (E,E)-2,4-hexadienal, (E)-2-heptenal, (E,E)-2,4-heptadienal, (E,Z)-2,4-heptadienal, 2-octenal, nonanal, nonenal, 2,4-nonadienal, (E,E)-2,4-decadienal and 2,4-decadienal), ketones (1-octen-3-one, 3-octen-2-one, (E,E)-3,5-octadien-2-one and 3,5-octadien-2-one), acids (propionic acid,

pentanoic acid, hexanoic acid and heptanoic acid), and 2-pentyl-furan increased during accelerated storage, as principal markers of oxidation. For evaluation of oxidative quality of HSOs, the absence of terpenes (in particular pinenes) and assessment of volatile compounds seems to be the most diagnostic method. In fact, the peroxides were subject to fluctuations, and in some cases decreased, and determination of TBARs was unsuitable for HSOs because it could be affected by the presence of interferents if the product is in an advanced oxidative state. It was found that samples on the 18th day were mainly characterized by oxidation volatiles markers that were not detectable by TBARs determination and a low content of antioxidant compounds (i.e. average reduction of 86% of antioxidant compounds).

This Ph.D. project highlighted that the rapid HPLC-UV method may be a good choice for determination of cannabinoids in *Cannabis sativa* L. inflorescences. In fact, it has the sensitivity and accuracy to discriminate samples with amounts of Δ-9-THC and Δ-8-THC (total THC content) that are below the limit of 0.2% from those that are subjected to legal restrictions in many EU countries, with a total THC content above 0.6%, which cannot be classified as hemp. Due to its simplicity and rapidity, it can be used to check raw material or crops during the harvesting period.

Moreover, this Ph.D. project showed that CBD, which is often added to different oils, can act as pro-oxidant of this matrix, even at low concentrations. Considering the many oily products added with CBD are on the market, it is necessary to define quality parameters, also in relation to the acceleration of oxidative degradation that can be caused by CBD. Finally, the need to define standards and parameters to evaluate the quality of HSOs is increasing. In fact, the research carried out within the context of this Ph.D. project has highlighted that differences are present among the HSOs already on the market. These differences are noticeable in terms of quality and composition of HSOs analyzed. Moreover, the genuineness and authenticity of this product should be assessed in the future. In fact, this is necessary not only to establish qualitative parameters, but also indicators that allow the confirmation, for example, of cold pressing or the variety of hemp used. Such indications could provide greater guarantees to consumers. In this regard, the work carried out during the Ph.D. project has also shown that consumers would like to purchase exclusively cold-pressed hemp seed oils. However, at the moment there is a lack of specific quality control markers and parameters adopted at European or international level.