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Effect of Malting on Antioxidant and Nutritional Properties of the Seeds of Two Industrial Hemp (*Cannabis sativa* L.) *cultivars*

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A Romina e Lara
che mi hanno sostenuto, supportato e sopportato
in questi faticosi, ma magnifici anni insieme.

It's not over 'till you're underground,
It's not over before it's too late.
– Letterbomb, Green Day

Short abstract

This PhD project deal with the evaluation of the impact of malting process on antioxidant, nutritional and antinutritional features of the seeds of two industrial hemp *cultivars* (*Futura 75* and *Secuieni jubileu*). Specifically, seeds were stepped for 5 h, germinated for 3 days and kilned at 50 or 70 °C for 6 h. Malted and unmalted seeds were analysed for total phenolic content, polyphenol profile, total antioxidant capacity, tocopherol composition, proximate composition, fatty acids profile, and antinutritional profile. Obtained data demonstrated that malting increased the protein content without affecting the fat amount, ash content, and the fatty acids profile of the seeds of both *cultivars*. Total phenolic content, tocopherol profile, and antioxidant capacity of the seeds were also improved. Differently to 50 °C, 70 °C kilning temperature resulted effective in significantly reducing the trypsin inhibitors' amount and increasing the content of N-*trans*-caffeoyltyramine and Cannabisin A. Therefore, based on our results, malting with 3-germination days and 70 °C as kilning temperature could be considered suitable transformation process' conditions for improving hemp seeds quality.

Keywords: *Cannabis sativa* L. seeds; industrial hemp; malting; antioxidant capacity; phenolic compound; tocopherols; antinutritional compounds.

Contents

Extended abstract

This PhD thesis dealt with the evaluation of malting technique applied to the seeds of industrial hemp (*Cannabis sativa* L.) to improve their nutritional and functional features, in order to develop a novel raw material usable in functional and healthy foods as well as to contribute to the revitalization of the *Cannabis sativa* L. crop.

C. sativa L., commonly hemp, is an herbaceous, anemophilous plant belonging to the *Cannabaceae* family. It is a multipurpose, sustainable, and low environmental impact crop which can be useful for several applications. Traditionally, *C. sativa* L. plants were cultivated primarily as a fibre crop for the production of textiles and ropes, especially in the western world. However, the progressive diffusion of synthetic fibres and the use of some narcotic strains for the production of intoxicant drugs led to forbid its cultivation. Only since the last two decades, there has been a reintroduction of the *C. sativa* L. cultivation exclusively for industrial purposes. For this aim, the cultivation of varieties popularly called "industrial hemp" or "fibre-type" hemp, which contains a level of Δ9-tetrahydrocannabinol (THC) – the only one psychoactive compound of the plant – less than 0.2% (for Europe) or 0.3% (for Canada and USA) has been authorised.

In the last decades, consequently to the recognition of the nutritional features and health benefits of *C. sativa* L., there has been a growing interest in the seeds of this plant which were once considered as a waste product of the fibre production, or, at most, used as animal feed. Therefore, the production of hemp seeds has been increased and they became a product with an important and growing potential market (Johnson, 2018; Crini et al., 2020; (Farinon et al., 2020; Leonard et al., 2020; Rupasinghe et al., 2020).

Despite the high variability in the hemp seed's composition due to the genotypes and environmental factors, hemp seed typically contains 25–35% lipids with a unique and perfectly balanced fatty acids (FAs) composition; 20–25% proteins easy to digest and containing all essential amino acids; 20–30% carbohydrates, whose main part is represented by insoluble dietary fibre; as well as vitamins, and minerals. In addition to its nutritional value, hemp seed is also rich in natural antioxidants and other bioactive components such as bioactive peptides, phenolic compounds, tocopherols, carotenoids, and phytosterols (Farinon et al., 2020). Hemp seed contains also some antinutritional compounds that could negatively influence its nutritional value, such as trypsin inhibitors (TIs) and phytic acid (Farinon et al., 2020).

For their nutritional and functional properties, hemp seed and its derivatives could represent potential ingredients for the development of new functional and/or healthy foods and some biotechnological transformations could be useful to improve their nutritional and functional values. Among these, it is possible to mention the malting process, an ancient biotechnological process traditionally performed on cereal grains, which consists of the following three main steps: (1) steeping to hydrate and reactivate the metabolism of the resting seeds, (2) germination during which the seed undergoes modifications including the activation of several enzymes involving in both catabolic and anabolic reactions, needed for the embryo's growth and (3) kilning to stop metabolic processes and ensure stability and storability of the dried product. Through by the entire process of malting, drastically changes of the seed's content and physic and biochemical compositions occur which are often associated with health benefits and therefore, they can be useful to improve the nutritional and functional quality of the malted seeds compared to unmalted, raw seeds. Indeed, different studies highlighted the benefits resulting

from germination and malting of cereal and pseudo-cereal grains and oilseeds such as linseed (Carvalho et al., 2016; Li et al., 2019).

Werz and colleagues found that germination induce the production of the anti-inflammatory prenylflavonoids Cannflavins A and B in the seeds of *Ermo* and *Finola* hemp varieties (Werz et al., 2014). In addition, Frassinetti and co-workers recently reported that germination of the seeds of *Futura 75* hemp *cultivar* (*cv*) for 3 and 5 days increased the total phenolic content and the antioxidant capacity of the hemp sprouts in comparison to the ungerminated seeds (Frassinetti et al., 2018). However, to the best of our knowledge, there is no study investigating the effect of the malting process on the nutritional, antinutritional and antioxidant properties of hemp seeds. Therefore, in this PhD thesis above all, the effect of germination on the antioxidant profile of the seeds of two certified industrial hemp varieties were evaluated in order to identify the best germination time for the malting procedure. Then, the hemp seeds germinated for the resulting best germination time, were underwent to two experimental malting procedures, which differ in the temperature used for the kilning step (*i.e.,* 50 °C or 70 °C). The industrial hemp varieties used in this study were *Futura 75* and *Secuieni jubileu.* The first one is a *cv* already described in the scientific literature, whose seeds are largely used in food preparation and have been already nutritionally and functionally characterized. The *Secueini jubileu* variety is a locally farmed hemp *cv* not described in the literature, which is grown in the Tuscia area with a good seeds' production.

The obtained data demonstrated that the malting influenced all assessed parameters in a similar way for both hemp varieties, inducing an increment in the protein content without affecting the fat content and ash amount, as well as the fatty acids profile of the seeds of both analysed *cultivars.* Total phenolic content (TPC), tocopherol profile and total antioxidant capacity of seeds were also improved after malting. In addition, malting with a kilning step performed at 70 °C resulted suitable to increase the content of N-*trans*-caffeoyltyramine and Cannabisin A – polyphenols defined as the major contributors to the antioxidant activity in hemp seeds (Irakli et al., 2019) – and to reduce the amount of the antinutritional TIs, although it did not affect the phytate content.

Overall, these results provide important information for both scientific literature and food industry. Indeed, to the best of our knowledge, it is the first study concerning nutritional and functional characterisation of *Secuieni jubileu* seeds as well as the effect of malting on hemp seeds, which as demonstrated here, could be very attractive for food industries, giving the opportunity to adopt a hemp seed's transformation product as ingredient to formulate nutritionally improved foods.

Introduction

1. *Cannabis sativa* L.: an overview

Cannabis sativa L. commonly hemp, is an herbaceous, anemophilous plant belonging to *Cannabaceae* family. It is considered one of the most ancient, cultivated plant and although currently there are no more traces of wild-type hemp, however, domesticated (*i.e.*, individuals of a species chose and selected by humans for characteristics making them useful to people) and ruderal (*i.e.*, forms growing outside of cultivation) hemp plants exist. The nowadays-domesticated form of *C. sativa* plants is widespread and cultivated throughout the world, from the Asian countries to Canada, United States (US), Europe and Africa. It is a multipurpose, sustainable, and low environmental impact crop, which can be useful for several application fields, from agricultural and phytoremediation to food and feed, cosmetic, building, and pharmaceutical industries. Indeed, from this highly versatile plant it is possible to obtain various products of industrial interest such as fibre and shives; bio-building and thermal insulated materials; seeds, flour and oil with important nutritional and functional features, as well as bioactive compounds of pharmacological interest (Andre et al., 2016; Salentijn et al., 2015) (**Figure 1**).

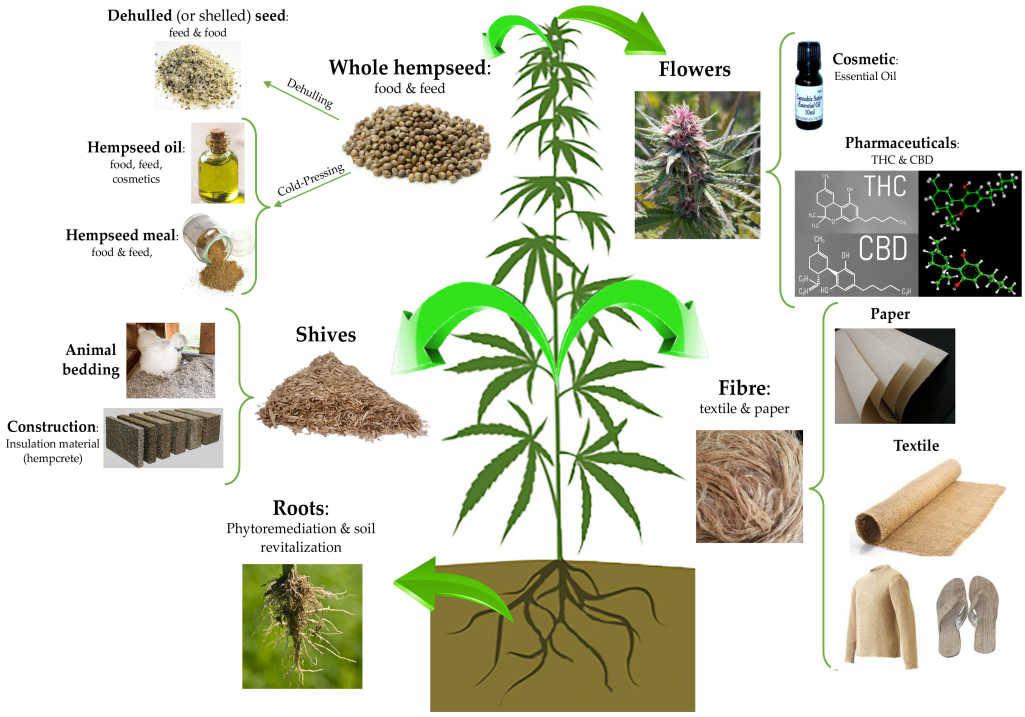

Figure 1. The manifold applications of hemp plant. Virtually, each part of this plant can be used in a specific industrial field: the seeds can be used in food, feed and cosmetical field as whole or dehulled, or it may be subjected to a cold press process to obtain an oil employed in food and cosmetic industries. From the stem it is possible to obtained both shives and fibre, useful for animal, building, paper and textile application. The hemp root system is highly developed in comparison to other herbaceous plant and this feature is suitable for the phytoremediation of soil from heavy metals. Hemp flowers can be used for ornamental purpose or to obtain products of cosmetic and pharmaceutical interest, such as essential oil composed by delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) pure extracts. From Farinon et al. 2020.

Essentially, *C. sativa* L. can be grown for three main purposes: industrial, narcotic/recreational, and medicinal (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2018) Traditionally, *C. sativa* L. plants were cultivated primarily as a fibre crop for the production of textiles and ropes, especially in the western world. Despite their high nutritional value, the seeds of this plant were initially considered as a by-product of the fibre production, and hence, they were mainly used as animal feed. From the first half of the 21th century the cultivation of this crop declined because of the progressive diffusion of synthetic fibres and the use of some narcotic strains of *C. sativa* L. plant for the production of intoxicant drug. Only since the last two decades there has been a reintroduction of the *C. sativa* cultivation exclusively for industrial purposes and in this context, Canada has been the first western country to restore this crop, followed by Europe and US. Nowadays, a growing interest for the seeds of *C. sativa* L. plant, commonly named hemp seeds, has been developed due to the increase knowledge about their high nutritional value and potential functionality. However, there is still lack of awareness and much confusion between "industrial hemp" and "drug hemp" especially among the public opinion, so that the research of the potential health benefits provided by hemp seed is still penalized by the negative reputation of drug hemp, which daunts interests and investments. Moreover, although in scientific literature there is a fairly good amount of information about the nutritional composition of hemp seeds, there is still lack of studies regarding the nutritional and health benefits related to the hemp seeds-based food products for human consumption. Therefore, before to deal with the nutritional and functional properties of hemp seeds – that is one of the main topic of this thesis – for clarity and to avoid misunderstanding, it is important to outline and well-define (i) the genetic and biochemical mechanisms underlining the difference between industrial hemp and drug hemp, and (ii) the legislative history and the evolution of legislation concerning the cultivation of *C. sativa* L. plants, and the utilization and transformation of *C. sativa* L. products in order to understand the correct, safety and legal use of *C. sativa* L. products.

1.1 Cannabinoids synthesis in C. sativa *L. plant*

The major discriminant factor related to the different intended use of *C. sativa* L. is the level of the two major and more known phytochemicals characteristic of this crop, namely the only one psychoactive and toxicant compound of the plant, delta-9- tetrahydrocannabinol (THC) and the non-psychoactive cannabidiol (CBD). Both of them belong to the cannabinoids' class which includes over of 100 secondary metabolites belonging to the family of terpenophenolic compounds, typical of all *C. sativa* L. plants. These compounds are synthesized, collected and stored in capitate-stalked glandular trichomes (**Figure 2**), that are specialized tiny secretory epidermal glands (Marks et al., 2009; Sirikantaramas et al., 2005), which are essentially present and abundant on the inflorescence of the female plant, whilst are present in lower number on leaves and stems, and absent on roots and seeds, therefore, these latter organs do not contain cannabinoids (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2018; Onofri et al., 2015; Staginnus et al., 2014)

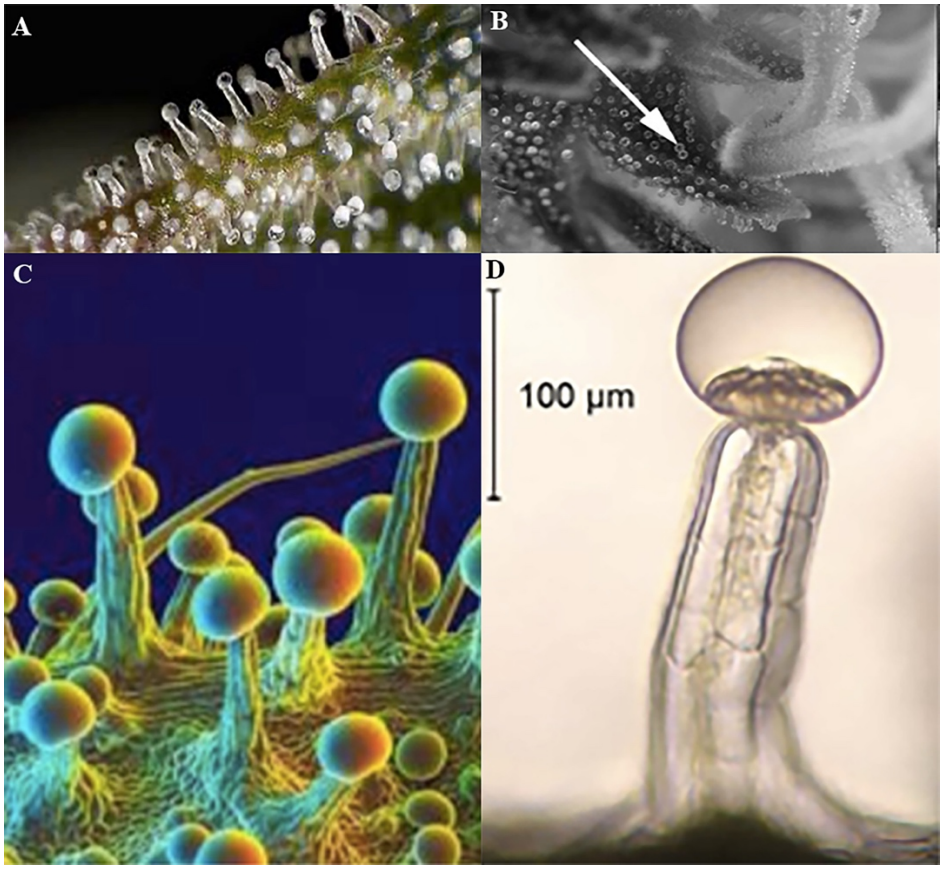

Figure 2. **(A)** Capitate-glandular stalked trichome on plant's surface **(B)** Capitate-glandular stalked trichomes (indicate by the arrow) on the bracts of pistillate florets (Marks et al., 2009) **(C)** Scanning electron microscope image of the capitate-glandular stalked trichomes (Hartsel et al., 2016) **(D)** Enlargement of a capitate-stalked trichome. From Andre et al., 2016.

A possible presence of cannabinoids in hemp seeds could occur during the harvesting process as result of physical contact with the resin secreted by the glandular trichomes located on the bracts that surround the seed (Citti et al., 2018; Escrivá et al., 2017). Hence, the presence of cannabinoids in hemp seed actually represents a contamination, and the level of this contamination depends on both the *cultivar* and the cleaning process of the seed. Reasonably, THC contamination in seeds from *C. sativa* L. varieties which produce low-THC level should be extremely low (Citti et al., 2018), anyway, the adoption of a method for the quantification of the possible cannabinoid's contamination and level in hemp seed products and food may be appropriate (Christinat et al., 2020; Citti et al., 2019; Meng et al., 2018).

C. sativa L. plants grown for an industrial purpose are cultivated to obtain fibre, seeds, and their derivatives. These plants are popularly called "industrial hemp" or "fibre-type" hemp and they low-THC level (*i.e.*, < 0.3 or 0.2% of dry weight of entire plant). Whereas *C. sativa* L. plants cultivated for narcotic/recreational purposes are characterized by high-THC level and those cultivated for medicinal purposes are characterized by contain high-THC and high-CBD levels.

Several works well-clarified the cannabinoids' biosynthetic pathway (Fellermeier et al., 2001; Fellermeier and Zenk, 1998; Gagne et al., 2012; Giroud, 2002; Taura et al., 2009). According to these studies, a common precursor of all the main cannabinoids exists and it is the cannabigerolic acid (CBGA) which can be synthesized from the

phenolic moiety of olivetolic acid and the terpenoid component of geranyl pyrophosphate (GPP). The first compound is originated in the cytosolic polyketide pathway by an aldol condensation of hexanoyl-CoA with three molecules of malonyl-CoA. This reaction is catalysed by polyketide synthase (PKS) enzyme in the presence of olivetolic acid cyclase (OAC) which has to function in concert with PKS to obtain the olivetolic acid. The GPP is synthesized by the plastidial methylerythritol phosphate (MEP) pathway and it is adopted for the synthesis of both cannabinoids and terpenes (Booth et al., 2017). One molecule of olivetolic acid is alkylated with one molecule of GPP by a geranylpyrophosphate:olivetolate geranyltransferase (GOT), to generate the CBGA precursor. CBGA is then converted into the acidic form of the three main cannabinoids, from which other related cannabinoid compounds will originate, namely tetrahydrocannabinol acid (THCA) that in the acidic form have no psychoactive activity, cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA). This conversion is catalysed by an oxidocyclase specific for each cannabinoid (THCA-synthase, CBDA-synthase, and CBCA-synthase, respectively) (**Figure 3**).

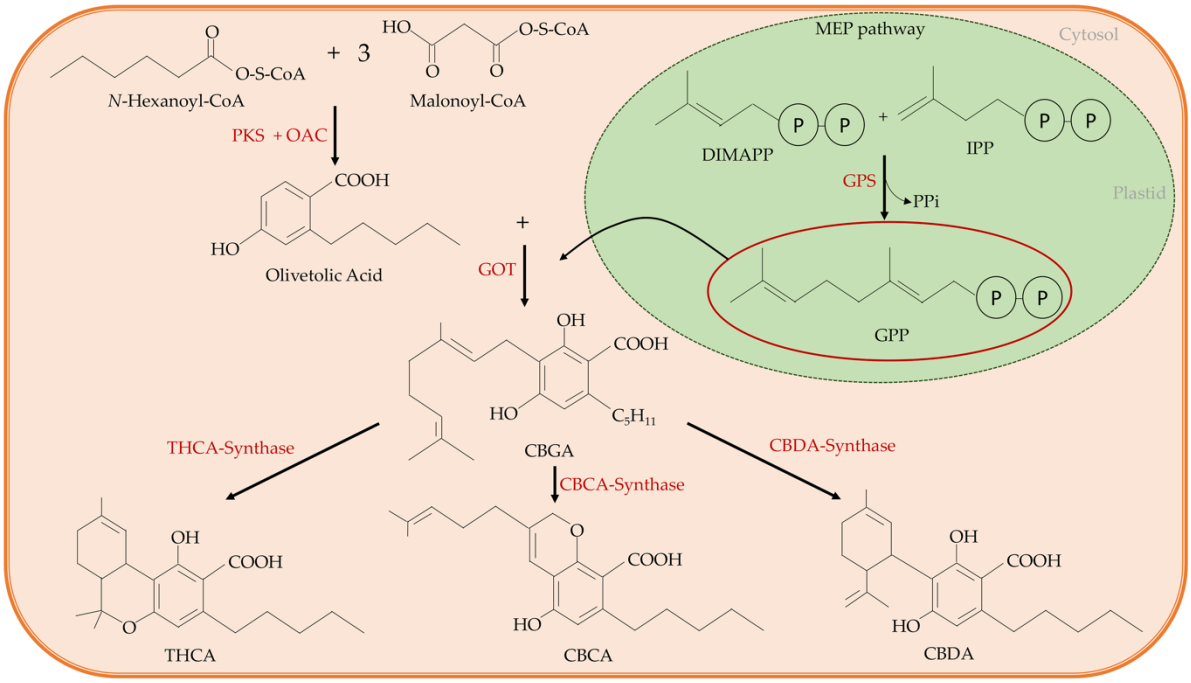

Figure 3. The molecular precursors, the products and the enzymes (in red colour) involved in the cannabinoid synthetic pathway in the cells of stalked glandular trichomes in *C. sativa* L. plant. See the 2.1 section for a detailed description. CBCA: cannabichromenic acid; CBDA: cannabidiolic acid; DIMAPP: dimethylallyl pyrophosphate; GOT: geranylpyrophosphate:olivetolate geranyltransferase; GPP: geranyl pyrophosphate; GPS: geranyl pyrophosphate synthase; GPS: geranyl pyrophosphate synthase; IPP: isopentenyl diphosphate; MEP: methylerythritol phosphate; OAC: olivetolic acid cyclase; PKS: polyketide synthase; PPi: Inorganic pyrophosphate; THCA: tetrahydrocannabinol acid. From Farinon et al. 2020.

1.2 Chemical phenotype and taxonomy of C. sativa *L.*

The elucidation of the cannabinoids' biosynthetic pathway has been essential to demonstrated that the concentration of each cannabinoids in the plant is genetically determined, so that various genotypes related to

different chemical phenotypes, diverging in types and concentration of cannabinoids (*i.e.*, cannabinoids profile), exist. These phenotypes are known as "chemotypes" or "biotypes" and three different principal chemotypes were commonly identified (Broséus et al., 2010; Pacifico et al., 2006; Staginnus et al., 2014) on the basis of the two main cannabinoids (*i.e.*, THC and CBD) content and ratio (**Figure 4**):

- Chemotype I characterized by a low CBD/THC ratio (0.00 – 0.05), due to high THC content (> 0.3% of dry weight of the reproductive part of the female plant at flowering). This chemical phenotype is also known as "drug type", "THC-predominant", or *C. sativa* L. subsp. *Indica* and the varieties belonging to this chemotype are those commonly grown for narcotic/recreational purposes.
- Chemotype II that has both the two main cannabinoids, CBD and THC, in a content ratio (CBD/THC) close to the unity (0.5 – 3.0), usually with a slight prevalence of CBD. This chemical phenotype is also named "intermediate type" or "THC-intermediate", and the *cvs* belonging to this chemotype are mainly grown for medicinal use.
- Chemotype III characterized by high CBD/THC ratio (15 – 25) due to high CBD amount and low THC content, not over than 0.3% of dry weight of the reproductive part of the female plant at flowering. This chemical phenotype is also known as "non-drug type", "fibre-type", "THC-predominant", or *C. sativa* L. subsp. *Sativa*, and the varieties belonging to this chemotype are cultivated for industrial purposes, namely, for fibre, seeds, and their derivatives.

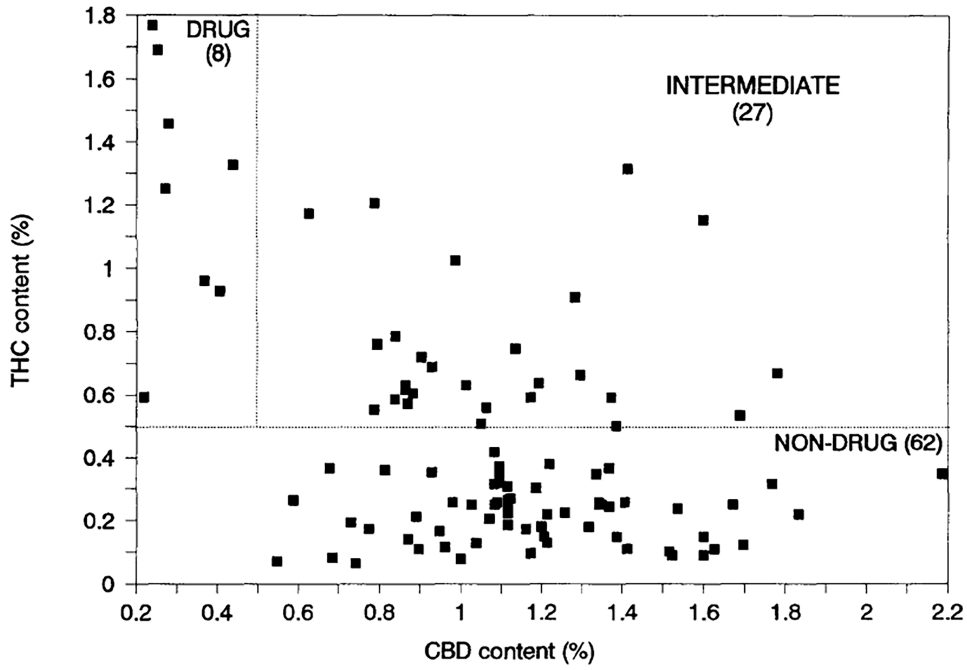

Figure 4. Scatter diagram of 97 *C. sativa* L. populations analysed by de Meijer and colleagues for their THC and CBD content. Thanks to this analysis the three chemotypes classification of *C. sativa* L. was been developed. This classification distinguishes three different chemotypes of *C. sativa* L. on the base of their THC and CBD content. CBCA: cannabichromenic acid; CBDA: cannabidiolic acid; THCA: tetrahydrocannabinol acid. From de Meijer et al., 1992.

De Meijer and colleagues (de Meijer et al., 2003) for the first time gave a clear genetic meaning to the tripartite distribution of the chemotypes within *C. sativa* L. population. Indeed, studying the inheritance of the chemotype traits of *C. sativa* L. plants, they identified the existence of a single locus named *B* locus, with two co-dominant alleles, named B_T and B_D, each of which codes for the THCA- and CBDA-synthase, respectively. Hence, according to this model, the THC-predominant phenotype (chemotype I) is related to the B_T/B_T genotype, the CBD-predominant phenotype (chemotype III) is determined by the B_D/B_D genotype, and the intermediate phenotype (chemotype II) is induced by the heterozygous state B_T/B_D. The same authors also showed that the value of the CBD/THC ratio in the heterozygous hybrids obtained from cross between parental homozygous pure-THC and pure-CBD, unexpectedly differed significantly and deviating from the expected 1.0 value. So, they speculated that some heritable factor could affect the balance between THCA- and CBDA-synthase in their competition to convert the CBGA precursor. In particular, the authors hypothesized that B_T and B_D alleles could be part of a wider allelic series coding for several isoenzymatic forms of THCA- and CBDA-synthase, respectively, with differential affinities for the CBGA substrate, resulting in significantly different CBD/THC ratios observed in the heterozygotes. Further studies pointed out the existence of several allelic variant of B_D (Cascini et al., 2019; Onofri et al., 2015; Pacifico et al., 2006) and B_T (Cascini et al., 2019; Kojoma et al., 2006; Onofri et al., 2015; Staginnus et al., 2014) genes coding for less or totally non-functional CBDA- and THCA-synthase, respectively, and which therefore, influence the cannabinoid profile of the plant and from a practical point of view, also represent an useful genetic marker to differentiate the drug type from the fibre type *C. sativa* L. plants. Interestingly, higher number of allelic variants of wild-type B_D locus was found in comparison to B_T locus. Hence, considering the high number of the cannabinoid synthase genes' allelic variants and the higher mutation rate of CBDA-synthase allele, Onofri and colleagues (Onofri et al., 2015) proposed a phylogenetic hypothesis according to which it is possible to consider all cannabinoids' allelic variants as a gene family and speculate that wild-type CBDA-synthase allele may be the ancestral form of this gene family, from which events of duplication would have led to a higher CBDA-synthase variation, resulting in the formation of the CBDA-synthase pseudogenes (*i.e.*, the CBDA-synthase allelic variants) and to the rise of new sequence coding for a new enzyme able to convert the CBDA substrate in a new product, the THCA. According to this evolutionary theory, it has also hypothesized that low-functionally THCA-synthase allele found in some fibre type (CBD-predominant) plants (Kojoma et al., 2006), could be an evolutionarily intermediate between CBDA-synthase ancestor and the fully functional THCA-synthase allele.

The high intrinsic genetic variability rate of *C. sativa* L. has been further accentuated by the long history of its domestication. Indeed, the different intended use of the *C. sativa* L. cultivation's products has led over the years, to an artificial phenotypic selection of specific features of the domesticated plants, useful for increasing the yield and/or the quality of the commercial interest's cultivation products (de Meijer, 2014). The direct consequence of this selection was the unaware artificial creation of the *C. sativa* L. varieties, each with specific genotypic and phenotypic features, which at first, induced the taxonomists and botanists to erroneously recognize two or three different species of *C. sativa* L., embracing a polytypic concept of the *Cannabis* genus (Hillig, 2005). To further complicate the taxonomic classification of the *Cannabis* genus, there has been also the fact that *C. sativa* L. is a crop which tend to exist in "crop-weed complexes", that is complexes of domesticated forms in cultivation and related ruderal (weedy) forms growing outside of cultivation, developing morphological characteristics also very different from those of the domestic progenitor, as a consequence of adaptation to the wild environment (Naraine et al., 2020). However, it must be considered that despite the high genetic variability of *C. sativa* L., the varieties

that genotypically and phenotypically differ, are interfertile. Therefore, taking into account the Darwinian definition of biological species – «a group of organisms that can reproduce with one another in nature and produce fertile offspring» – *C. sativa* L. varieties cannot be consider as different species of the *Cannabis* genus. For this reason, to date, the polytypic concept has been definitely given up and replaced by the monotypic one. According to this, a single species of *Cannabis* genus exists, namely *C. sativa* L., which includes several varieties or *cultivars* (*cvs*) that genotypical and phenotypical differ, but they all are interfertile and therefore, they belong to the same species (McPartland, 2018; Small and Cronquist, 1976).

From a practical point of view, the main discrimination factor among the *C. sativa* L. *cvs* is the THC content which, essentially for legal reasons, is a useful tool to discriminate between the drug type plants with high THC content, employed for medical or recreational purposes, and the fibre type plants with low THC content, commonly named industrial hemp.

1.3 Legislation of C. sativa *L. in Western World (USA, EU, Canada)*

Historically, industrial hemp or simply, hemp, that is *C. sativa* L. plants grown for fibre and/or seeds, was frequently cultivated over the world, mainly for the production of technical textiles, until the first half of the 21th century. In the US hemp was widely grown from the colonial period into the mid-1800s. In the early 1900s and prior to the late 1950s, hemp continued to be grown being considered as an agricultural commodity: US Department of Agriculture (USDA) supported its production and USDA researchers continued to publish information related to hemp production and also reported on hemp's potential for use in textiles and in paper manufacturing (Johnson, 2018). In Europe, at the end of 1950s, Italy was the second country in the world after Russia for the areas under hemp cultivation (over 100,000 hectares) and the world's best for the quality of the obtained products (Porto et al., 2015). However, following the discovery of the psychotropic activity of THC, and the increasing awareness of its deleterious effects on human health, many countries began to take measures in an effort to stem the use of *C. sativa* L. plants' flowers and leaves for their psychotropic effects. The first provision was taken in US and Canada. In US, between 1914 and 1933, 33 states passed laws restricting legal production to medicinal and industrial purposes only. In 1937 the Marihuana Tax Act defined hemp as a narcotic drug, without any distinguish between low THC plants (hemp) and high THC (drug hemp or simply, marijuana) ones: both were considered Schedule I controlled substances and it was required that farmers growing hemp hold a federal registration and special tax stamp. This effectively limited further production expansion, in fact, after 1943, production of hemp started to decline until the late 1950s when no production was recorded. Finally, in 1970 The Controlled Substances Act (CSA) was issued, and it placed the control of selected plants, drugs, and chemical substances under federal jurisdiction. Among the selected plants there was also *C. sativa* L. ones to which were given the statutory definition of marijuana and were put in the Drug Enforcement Administration (DEA) schedule of Controlled Substances (Johnson, 2018). In Canada the cultivation of hemp has been prohibited due to the presence of THC, in 1938 with the Canadian Opium and Narcotics Act (Vera and Hanks, 2004; Vonapartis et al., 2015). In 1961 the United Nation (UN) endorsed and adopted the Single Convention on Narcotic Drugs which established a universal system for limiting the cultivation, production, distribution, trade, possession and use of narcotic substances to medical and scientific purposes, with a special focus on plant-derived substances, among which cannabis. In the article 28, paragraph 2 of this convention, cannabis was defined as «the flowering or fruiting

tops of the *C. sativa* L. plant (excluding the seeds and leaves when not accompanied by the tops) from which the resin has not been extracted, by whatever name they may be designated». The same article described a system of control required if a country decides to permit the cultivation of *C. sativa* L. that is not for industrial or horticultural purposes (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2018; Sabet, 2011). Ten years later, in 1971, the UN endorsed also the Convention on Psychotropic substances which established an international control system for psychotropic substances, among which THC (United Nation, 1971). In line with these directives, in 1975 the Italian Republic issued the law n. 685/1975 introducing cannabis (intended as a drug product obtained from *C. sativa* L. plants) in the schedule of controlled substances.

The better knowledge of the biochemical and biomolecular features of the *C. sativa* L. species has made it possible to understand the genetics and biochemical mechanisms previously described, which are the basis of the cannabinoids' synthesis, and in particular of THC. Furthermore, thanks to the development of specific analysis techniques (*e.g.*, Gas Chromatography or Gas Chromatography–Mass Spectrometry), it is now possible to resolutely and accurately quantify the THC content of *C. sativa* L. plants, in order to distinguish between *cvs* with high and low THC content. For these reasons, nowadays the cultivation of industrial hemp has been reintroduced either in the US, Canada and Europe. Canada was one of the first country to restore the industrial hemp cultivation. Indeed, in 1994 it began to issue licenses for hemp as research crop and then, in 1998 the cultivation of hemp varieties containing less than 0.3% THC of the dry weight of leaves and flowering parts was legalized and it is currently permitted, provided that a license from the Office of Controlled Substances of Health Canada has been acquired. To date, Canada is the major hemp producing and exporting country, particularly of hemp-based foods, ingredients, and other related products (Johnson, 2018). In EU, the hemp cultivation reintroduction took place in 2013 with the EU regulation n. 1307/2013 that allowed the grown of *C. sativa* L. plants for industrial purposes only for those plants with low level of THC. According to this regulation, the granting of payments under the Common Agricultural Policy (CAP) is conditional upon the use of certified seeds of specific hemp varieties, that is *C. sativa* L. *cvs* with a THC content not exceeding 0.2% of the dry weight of leaves and flowering parts (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2018). Thus, EU has adopted more stringent parameters compared to Canada, to ensure the safety and protect the health of citizens. Different genotypes of industrial hemp with a THC content < 0.2% have been selected and registered, and currently, there are about 70 allowed industrial hemp varieties listed in the European Plant Variety Database as agricultural species (**Table 1**) (European Commission, 2020; Pavlovic et al., 2019). Some of these *cvs* are dioecious as *C. sativa* L. plant naturally occurs, others *cvs* are monoecious and are obtained by ancestral breeding (Galasso et al., 2016). Often, but not always, the monoecious varieties are adopted for seed production since they give a higher yield of the interest's product, whereas the dioecious *cvs* are mainly adopted for fibre production. Moreover, the industrial hemp varieties listed in the EU plant variety database are constantly updated based on the results of the annual THC-content's monitoring and the possible request for the introduction of new *cvs* with low THC amount.

Table 1. Industrial hemp *cultivars* registered and listed in the European Union Plant Variety Database and their origin. All these varieties are certified to contain less than 0.2% of THC of dry weight of leaves and flowering parts of the plant, according to the EU regulation 1307/2013.

No	Registered varieties	Origin	Sex type	No	Registered varieties	Origin	Sex type
1	Adzelvieši	Latvia	D*	33	Ivory	Nederland	M
2	Armanca	Romania	D	34	KC Bonusz	Hungary	M
3	Asso	Italy	D	35	KC Dora	Hungary	M
4	Austa SK	Latvia	M**	36	KC Virtus	Hungary	M
5	Balaton	Hungary	D	37	KC Zuzana	Hungary	M
6	Beniko	Switzerland Czech Republic Nederland Poland	M	38	KCA Borana	Hungary	M
7	Bialobrzeskie	Czech Republic Polan	M	39	Kompoliti	Hungary	D
8	Cannakomp	Hungary	D	40	Kompoliti Hibrid TC	Hungary	D
9	Carma	Italy	D	41	Lipko	Hungary	D
10	Carmaleonte	Italy	M	42	Lovrin 110	Romania	D
11	Chamaeleon	Nederland	D	43	Marcello	Nederland	M
12	Codimono	Italy	D	44	Markant	Nederland	M
13	Dacia Secuieni	Romania	M	45	Monoica	Switzerland Hungary	M
14	Delta-405	Spanish	M	46	Orion 33	France	M
15	Delta-llosa	Spanich	M	47	Rajan	Poland	M
16	Dioica 88	France	D	48	Ratza	Romania	M
17	Earlina 8 FC	France	M	49	Santhica 23	France	M
18	Eletta Campana	Italy	D	50	Santhica 27	France	M
19	Epsilon 68	France	M	51	Santhica 70	France	M
20	Fedora 17	Switzerland France	M	52	Secuieni Jubileu	Romania	M
21	Felina 32	France	M	53	Silvana	Romania	M
22	Fibrante	Italy	D	54	Succesiv	Romania	M
23	Fibrol	Hungary	M	55	Teodora	Romania	M
24	Fibror 79	France	M	56	Tiborszallasi	Hungary Italy	D
25	Finola	Finland	D	57	Tisza	Hungary	D
26	Futura 75	France	M	58	Tygra	Poland	M
27	Futura 83	France	M	59	Uniko B	Hungary	D
28	Fèrimon	Deutschland France	M	60	Uso-31	Nederland	M
29	Glecia	Italy	M	61	Villanova	Italy	D
30	Gliana	Italy	M	62	Wielkopolskie	Poland	M
31	Glyana	Poland	M	63	Wojko	Poland	D
32	Henola	Poland	M	64	Zenit	Romania	M

D*, Dioecious plant; M**, Monoecious plant.

Nowadays EU is the world's largest hemp producing market second only to Canada, with France, Netherlands, Lithuania and Romania as the major production centres (Johnson, 2018). According to the EU guidelines, also in Italy the cultivation of industrial hemp has been recently restored through the law n. 242/2016, and the subsequently circular of the Ministry of Agricultural, Food and Forestry Policies (MIPAAF) published on 14th of January 2017 that has delineated the conditions for hemp production, its commercialization and utilization for specific industrial purposes (Pavlovic et al., 2019).

Finally, in the US, the federal policy regarding hemp was significantly altered with the 2014 Farm Bill (Agricultural Act of 2014) that allowed the USDA and certain research institutions to grow hemp under an agricultural pilot program. Despite this act, the industrial hemp continued to be a niche's crop. The great novelty was four years later with the 2018 Farm Bill that has established a new federal hemp regulatory system under the USDA with the aim to facilitate the commercial cultivation, processing and marketing of hemp, and essentially treat hemp like any other agricultural commodity. Indeed, it removed hemp (*i.e.*, *C. sativa* L. varieties with a THC content < 0.3% of the dry weight of leaves and flowering parts) and their products – among which hemp seed – from the statutory definition of the drug marijuana and the DEA schedule of controlled substances, opening the hemp industry for business (Johnson, 2018). In **Figure 5** the main highlights about the *C. sativa* L. legislation in Canada, US, EU and Italy among the EU states, are illustrated.

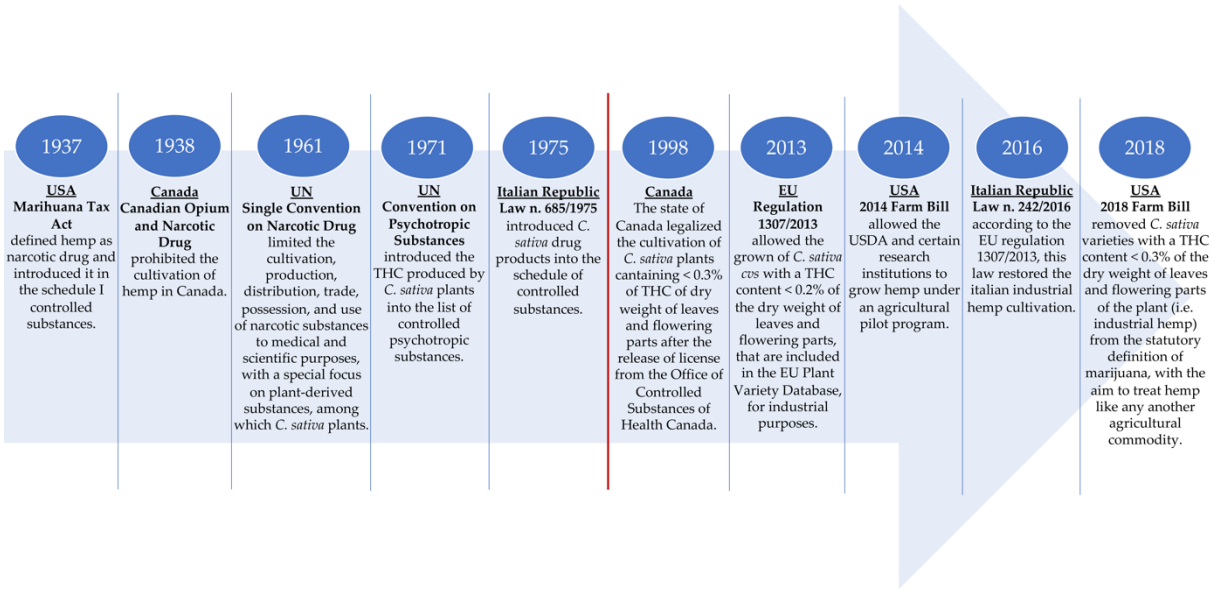

Figure 5. The main points of the legislation about *C. sativa* L. cultivation (in particular, industrial hemp) in US, Canada, and EU with a focus on Italian Republic among the EU states. The red line separates the legislation relating to the prohibition of *C. sativa* L. cultivation (left part of the red line) from that allowed the reintroduction of growing of this crop (right part of the red line). For more details, see the 2.3 section. US: United States; UN: United Nations; THC: delta-9- tetrahydrocannabinol; EU: European Union; USDA: United Stated Department of Agricultural. From Farinon et al. 2020.

2. Industrial hemp seed

As mentioned above, *C. sativa* L. crop has a prehistoric use as an important source of industrial interest's plant fiber. Nevertheless, in the last decades there has been a growing interest in the seed of the plant which is the fruit of hemp, commonly named seed, even if it is technically an achene namely an one-seeded dry fruits in which the pericarp is not as tightly joined to the seed, essentially similar to the cereal caryopsis (Naraine et al., 2020). Normally, the seeds of hemp grown for fibre production were considered as a waste product and at most, they were mainly used as animal feed (Kriese et al., 2004; Vonapartis et al., 2015). However, in recent times, with the growing recognition of their nutritional features and health benefits, the production of hemp seeds has been increased and these seeds have become a product with important and growing potential market (Karus and Vogt, 2004).

2.1 Hemp seed nutritional features

Hemp seed has commonly been claimed as one of the most nutritionally complete food sources due to its high nutritive traits. It can be consumed as such (whole, hulled seed) or dehulled (hemp seed kernel), as well as its processing products, including oil, flour, and protein powder. Despite the fact that some studies highlighted high variability in the hemp seed composition according to the genotypes and environmental factors (Galasso et al., 2016; House et al., 2010; Irakli et al., 2019; Lan et al., 2019; P. Mattila et al., 2018a; Mihoc et al., 2012; Porto et al., 2015; Vonapartis et al., 2015), it typically contains 25–35% lipids with unique and perfectly balanced fatty acids (FAs) composition, 20–25% proteins easy to digest and rich in essential amino acids, 20–30% carbohydrates, great part of which are constituted in dietary fibre, mainly insoluble, as well as vitamins, and minerals (**Table 2**).

Table 2. Hemp seed nutritional characteristics (g/100 g)

Moisture	Fat	Proteins	CHO	Total DF	Insoluble DF	Soluble DF	Ash	Ref
1.1–7.2	26.9–30.6	23.8–28.0	n.a.	n.a.	n.a.	n.a.	5.1–5.8	(Vonapartis et al., 2015)[1]
4.1–4.3	32.8–35.9	24.3–28.1	32.5–37.5	n.a.	n.a.	n.a.	4.9–6.1	(Lan et al., 2019)[2]
6.7 ± 0.5	34.6 ± 1.2	25.6 ± 0.6	34.4 ± 1.5	33.8 ± 1.9	30.9 ± 1.5	2.9 ± 0.4	5.4 ± 0.3	(P. Mattila et al., 2018a)[3]
4.0–9.2	25.4–33.0	21.3–27.5	n.a.	n.a.	n.a.	n.a.	3.7–5.9	(House et al., 2010)[4]
6.5	35.5	24.8	27.6	27.6	22.2	5.4	5.6	(Callaway, 2004)[5]
8.4 ± 0.02	33.3 ± 0.1	22.5 ± 0.2	n.a.	n.a.	n.a.	n.a.	5.9 ± 0.03	(Oseyko et al., 2019)[6]
7.3 ± 0.1	24.5 ± 2.0	24.8 ± 1.1	38.1 ± 2.5	n.a.	n.a.	n.a.	5.3 ± 0.6	(Siano et al., 2018)[7]

[1]Analysed *cultivars*: Alyssa; Anka; CanMa; CFX1; CFX2; CRS1; Delores; Finola; Jutta; Yvonne
[2]Analysed *cultivars*: Canda; CFX1; CFX2; CRS1; Delores; Grandi; Joey; Katani; Picolo; X59
[3]Analysed *cultivars*: not specified by the authors
[4]Analysed *cultivars*: Crag; Finola; USO14; USO31
[5]Analysed *cultivars*: Finola
[6]Analysed *cultivars*: Hlesiia
[7]Analysed *cultivars*: Fedora
Where more than one *cultivar* has been analyzed, the maximum and minimum value of the obtained data range is shown. Where only one *cultivar* has been analyzed, the mean ± standard deviation is reported. n.a.: not available; CHO: carbohydrates; DF: Dietary Fiber.

In addition to its nutritional value, hemp seed is also rich in natural antioxidants and other bioactive component such as bioactive peptides, phenolic compounds, tocopherols, carotenoids and phytosterols, the content of which appear to be mostly affected by the environmental and agronomic factors and to a lesser extent, by genetic variability (Irakli et al., 2019). Moreover, hemp seed contains also some antinutritional compounds that could negatively influence its nutritional value. In the following subsections, these nutritional and antinutritional hemp seed's components are discussed in detail.

2.1.1. Hemp seed fat

2.1.1.1. Fatty acid composition

Fat represents the most important component of hemp seeds, particularly from an industrial point of view. Indeed, since hemp seeds are oilseeds, the oil is the main food product of industrial interest that it is possible to obtained from them. For this reason, hemp seed's fat is commonly called oil. Several studies (Callaway, 2004; Galasso et al., 2016; House et al., 2010; Lan et al., 2019; P. Mattila et al., 2018a; Oseyko et al., 2019; Siano et al., 2018; Vonapartis et al., 2015) demonstrated that the oil content of hemp seeds belonging to different *cvs*, ranges from 25 to 35% of the whole seed (**Table 2**). Galasso and colleagues (Galasso et al., 2016) and Irakli and co-workers (Irakli et al., 2019), analyzing the seeds belonging to different industrial hemp *cvs*, highlighted that this variation is mainly due to the genotype. In addition, Irakli and colleagues (Irakli et al., 2019) also observed that

even environmental condition such as geography, climatic conditions, and local agronomic factors, have an effect on the total oil content, though slighter than genotype. This evidence is in accordance with the findings of Kriese and colleagues (Kriese et al., 2004) and Mihoc and co-workers (Mihoc et al., 2012). In this latter study, the authors, analyzing the oil content of five Romanian industrial hemp *cvs* grown in two consecutive years in the same site, found that environmental conditions such as high monthly average temperature of 25–26 °C and a low rainfall level (223 mm during the flourishing of plants), resulted in an incomplete maturation of hemp seeds and in a decrease in oil content.

As regard the chemical composition of the hemp seed's fat, it is needed to consider that most publications on this argument are referred to the oil extracted from hemp seed through specific industrial methods, rather than to the whole seed, because of the high industrial relevance attributable to hemp seed oil. However, in this review we have been focused primarily on the whole hemp seed, so, except in the case of lack of literature data about whole hemp seed, we have considered only the publications about this matrix, excluding those specifically concern the oil. The literature data about the FA composition of the fat component of whole hemp seed are reported in **Table 3**.

Table 3. Hemp seed fatty acid profile (% of oil).

PA	SA	OA	LA	GLA	ALA	SDA	Σ SFA	Σ MUFA	Σ PUFA	n-6/n-3	Ref.
6.7–7.0	2.1–2.8	9.4–13.0	55.6–56.6	2.6–4.5	14.7–17.3	n.a.	9.6–10.3	n.a.	n.a.	n.a.	(Vonapartis et al., 2015)[1]
6.0–7.3	2.3–3.5	9.2–15.7	55.0–58.2	0.6–4.5	12.6–19.6	0.2–1.5	9.4–11.7	9.7–16.1	72.2–80.7	2.8–4.5	(Galasso et al., 2016)[2]
7.1–9.1	2.1–2.8	10.3–17.9	51.6–54.2	1.9–5.0	10.5–15.3	n.a.	n.a.	n.a.	n.a.	3.9–5.5	(Irakli et al., 2019)[3]
6.1–7.8	2.3–4.0	12.2–18.8	53.9–59.0	3.5–6.2	12.3–18.9	n.a.	n.a.	n.a.	72.0–78.6	3.2–5.0	(Lan et al., 2019)[4]
5.0	2.0	9.0	56.0	4.0	22.0	2	n.a.	n.a.	84.0	2.5	(Callaway, 2004)[5]
7.0±0.3	2.8±0.4	12.7±1.3	56.2±3.5	2.9±0.4	15.0±1.1	n.a.	10.9±0.5	13.1±1.3	75.1±3.7	4.0	(Siano et al., 2018)[6]
5.6±0.5	3.9±0.4	16.2±6.2	54.7±4.1	n.a.	16.2±4.0	0.5±0.01	10.9±0.7	17.5±6.3	72±4.3	3.2	(Vecka et al., 2019)[7]

[1]Analysed *cultivars*: Alyssa; Anka; CanMa; CFX1; CFX2; CRS1; Delores; Finola; Jutta; Yvonne

[2]Analysed *cultivars*: Carmagnola Selezionata (CS); Carmaleonte; Codimono; Fedora; Felina 32; Ferimon; Fibranova; Finola; Futura 75; Kc Dora

[3]Analysed *cultivars*: Bialobrzeskie; Felina 32; Tygra; Futura 75; Santhica 27; Fedora 17; Finola

[4]Analysed *cultivars*: Canda; CFX1; CFX2; CRS1; Delores; Grandi; Joey; Katani; Picolo; X59

[5]Analysed *cultivar*: Finola

[6]Analysed *cultivar*: Fedora

[7]Analysed *cultivars*: not specified by the authors

Where more than one *cultivar* has been analyzed, the maximum and minimum value of the obtained data range is shown. Where only one *cultivar* has been analyzed, the mean ± standard deviation is reported. PA: Palmitic Acid (16:0); SA: Stearic Acid (18:0); OA: Oleic Acid (18:2, n-9); LA: Linoleic Acid (18:2, n-6); GLA: γ–Linolenic Acid (18:3, n-6); ALA: α–Linolenic Acid (18:3, n-3); SDA: Stearidonic Acid (18:4, n-3); ∑–SFA: Total Saturated Fatty Acid; ∑–MUFA: Total Monounsaturated Fatty Acid; ∑–PUFA: Total Polyunsaturated Fatty Acid; n.a.: not available.

Overall, the literature data showed that hemp seed oil is characterized by high polyunsaturated fatty acids (PUFAs) content and low saturated fatty acids (SFAs) amount. More precisely, based on genotype and environmental factors, hemp seed oil contained up to 90% unsaturated fatty acids (Vonapartis et al., 2015), of which from 70 to over 80% is composed by PUFAs. The major monounsaturated fatty acid (MUFA) was Oleic Acid (18:1, n-9, OA) which it has been shown to reach the highest value (18.78%) in the Canadian *cv* Joey (Lan et al., 2019), whereas the lowest OA content (8.42%) was found in the Finola *cv* grown in Italy (Galasso et al., 2016). Generally, the amount of OA in hemp seed oil is showed to be higher than those found in chia seed (7%) (Marineli et al., 2014) and comparable to those present in linseed (15%) (Teh and Birch, 2013). Among PUFAs, Linoleic Acid (18:2, n-6, LA) was the most representative FA in hemp seed oil of all analyzed genotypes, accounting for more than half of total FA. The second prominent PUFAs was α-linolenic acid (18:3, n-3, ALA). Hence, hemp seed oil represents an especially rich source of these two fatty acids which are known as Essential Fatty Acids (EFAs), since they cannot be synthesized by mammals and therefore, must be acquired by the diet because they are necessary to maintain healthy human life. Indeed, LA and ALA are the precursors of the n-6 and n-3 PUFAs biologically active in animals, including humans, namely the long-chain PUFAs Arachidonic Acid

(20:4, n-6, AA) which derived by the conversion of LA, and Docosahexaenoic Acid (22:6, n-3, DHA) and Eicosapentaenoic Acid (20:5, n-3, EPA) obtaining from the ALA precursor (**Figure 6**). These biologically active forms of EFAs are necessary for many physiological processes, including maintenance of cell membrane structure, and cardiovascular health, the regulation of metabolic and inflammatory process through the synthesis of prostaglandins and leukotrienes, skin integrity, as well as the proper regulation of the brain's development and function. In this context, it is important to note that the conversion of both LA and ALA into their biologically active PUFAs derivatives occurs at a low conversion rate. Several studies (Callaway, 2004; Galasso et al., 2016; Irakli et al., 2019; Vonapartis et al., 2015) highlighted that the Finola *cv* was the hemp variety with the highest ALA amount compared to other *cvs*. The highest ALA content in the Finola *cv* was found by Callaway (22%) (Callaway, 2004) and the lowest by Irakli and colleagues (15.3%) (Irakli et al., 2019).

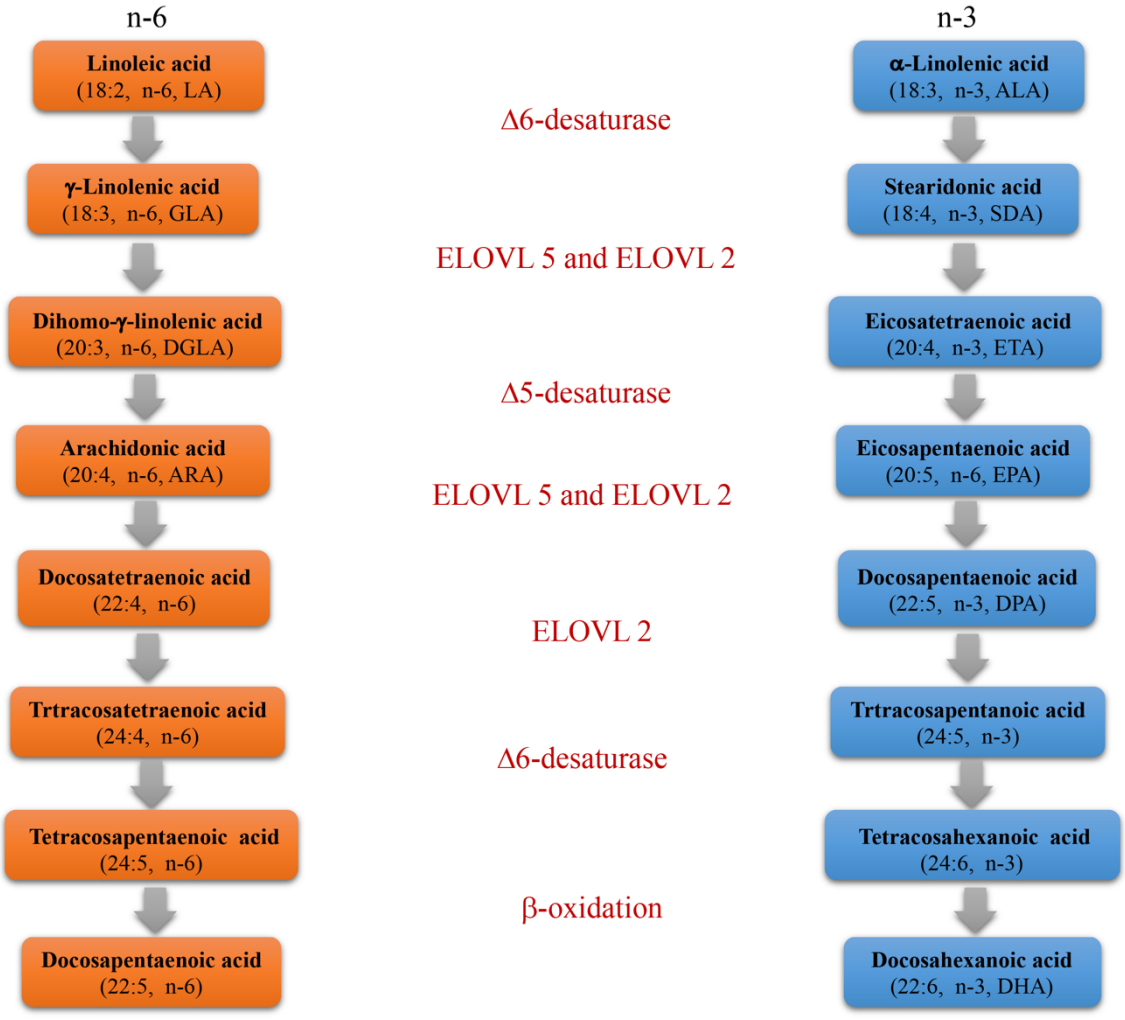

Figure 6. Schematic presentation of the PUFAs pathway. The n-6 PUFAs pathway is reported on the left. The n-3 PUFAs pathway is shown on the right. The enzymes involved in each reaction step is indicated in the middle of the image, in red color.

In addition to the absolute concentration of PUFAs in the diet, the n-6/n-3 ratio represents an important index to ensure the maintenance of an optimal state of health, and to prevent the onset of chronic-degenerative diseases characterized by a chronic inflammation status, like cardiovascular and neurodegenerative diseases, as well as cancer (Simopoulos, 2008). Despite the existence of different lines of thought about the healthiest n-6/n-3 ratio, according to the European Food and Safety Authority (EFSA) (EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2009), the ideal n-6/n-3 ratio has been established as 3:1 to 5:1 that is also the ratio found in the traditional Japanese and Mediterranean diets, where the incidence of coronary disease has been historically low (Callaway, 2004). The levels of LA and ALA found in the fat component of hemp seeds make them a food matrix with the ideal, low n-6/n-3 ratio ranging precisely from 3:1 to 5:1, which is useful for reducing the n-6/n-3 ratio in the diet, especially of industrialized countries, where there is an unhealthy average n-6/n-3 ratio of about 10:1, due to the too high n-6 and too low n-3 amount in the people's diet (Siano et al., 2018). In addition to LA and ALA, among n-6 and n-3 FAs, hemp seed oil also contains their respective biologic metabolites γ-Linolenic Acid (18:3, n-6, GLA) and Stearidonic Acid (18:4, n-3, SDA) that allow to bypass the first critical enzymatic step of δ-6-desaturase (**Figure 6**), facilitating the conversion into the biologically active form of long-chain PUFAs. Even in this case, Finola *cv* appeared as the hemp variety with the highest content of both GLA and SDA (Callaway, 2004; Galasso et al., 2016; Irakli et al., 2019; Vonapartis et al., 2015). Furthermore, GLA performs also an anti-inflammatory activity since it is rapidly converted in dihomo-γ-linolenic acid (DGLA; 20:3, n-6) in human body. DGLA is located in cell membrane where it can act as a precursor of anti-inflammatory metabolites and compete with AA for the synthesis of metabolites involving in the inflammatory response (Sergeant et al., 2016). In this context, it is important to consider that hemp seed oil is not the only one source of GLA, neither the plant source with the highest amount of GLA, in fact, one of the most representative source of this FA is the borage oil (19–23% of GLA) which however lacksn-3 PUFAs differently of hemp seed oil (Guil-Guerrero et al., 2018). As regard the SFAs, studies reported that the total amount of them is not over than 12% (Table 3), with a consequent recommended high (>10) PUFA/SFA ratio, considered beneficial to reduce the risk of atherosclerosis and coronary heart disease (Porto et al., 2015). The principal SFA was the Palmitic Acid (16:0, PA) with an amount ranging from 2 to 9%, followed by Stearic Acid (18:0, SA).

Concerning the variability in the hemp seed oil's FAs content, in literature there is some conflicting data. Particularly, is a common evidence that the FAs composition differs among *cvs*. Irakli and co-workers (Irakli et al., 2019) demonstrated that similarly to the oil content, the FAs profile of hemp seeds is drastically affected by genotype. The same authors found that the content of FAs of seven industrial hemp *cvs* approved for cultivation in Greece and grown in the same site for three consecutive years, was most affected by genotype for ALA primarily, followed by OA and PA (genotype contribution on the variance, 99.6%, 91.2%, 86.2%, respectively), whereas LA was the FA least affected (genotype contribution on the variance, 42%). Similar data was found by Vonapartis and colleagues (Vonapartis et al., 2015) for ten Canadian industrial hemp *cvs*, which did not obtained significant differences among the analyzed *cvs* for LA, but they found noticeable differences in the ALA concentration. Contrarily, Galasso et al. (Galasso et al., 2016) detected high and significant variability both for LA and ALA content of twenty hemp seed accessions and *cvs*.

2.1.1.2. UNSAPONIFIABLE MATTER

Generally, any oil matrix is composed by saponifiable and unsaponifiable matter. The former consists of all the oil substances containing an ester portion that can be removed by treatment with irreversible alkaline hydrolysis, including mono-, di-, and tri-acylglicerols and phospholipids. The latter, according to the ISO 18609:2000 definition, consists in all the substances dissolved in the product which cannot be saponified by the caustic alkalis, but are soluble in the ordinary fat solvent (*e.g.*, hexane). The unsaponifiable matter includes sterols, aliphatic and terpenic alchols, as well as fat-soluble vitamins. Literature data about the composition of the unsaponifiable matter of the fat component of whole hemp seed are very scarce, in fact, almost of them concern the oil of hemp seed. One of the first reports in which the unsaponifiable matter of the oil of hemp seed has been investigated in detail was the work of Montserrat-De La Paz and colleagues (Montserrat-de la Paz et al., 2014). They demonstrated that the unsaponifiable matter of hemp seed oil represents from 1.8 to 1.92% of the total oil and the most relevant compounds of this oil fraction are tocopherols and phytosterols. Tocopherols are naturally occurring, fat-soluble compounds with high antioxidant activity. They include the different isomers α-, β-, γ-, and δ-tocopherol and represent the dominant antioxidants in hemp seed oil, protecting it from oxidation thanks to their ability to scavenge free radicals. As said, most publications in which the tocopherols content has been assessed, concern the oil of hemp seed instead of whole hemp seed, and in fact, the obtained results are expressed referring to the oil (*e.g.*, mg/100 g of oil) rather than to the seed (*e.g.*, mg/100 g of seeds). Anyway, they provide important information about the content of these functional compounds and considering the lack of data referred to whole hemp seed, these publications have been considered in this specific case. The literature data obtained from the studies that evaluated the tocopherols profile of hemp seed and hemp seed oil are reported in Table 4 where the results expressed per 100 g of seeds have been separated from those expressed per 100 g of oil.

Table 4. Hemp seed oil unsaponifiable matter: tocopherols profile

Type of analyzed sample	Total tocopherols	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol	Ref.
mg/100 g seed						
Whole seed	n.a.	n.a.	n.a.	212.1–294.9	62.0–115.7	(Vonapartis et al., 2015)[1]
Oil	14.33–34.04	0.70–3.05	0.06–0.30	15.42–29.40	0.51–2.49	(Kriese et al., 2004)[2]
Whole seed	n.a.	n.a.	n.a.	4.6–11.3	0.6–1.3	(Irakli et al., 2019)[3]
Whole seed	90	5	n.a.	85	n.a.	(Callaway, 2004)[4]
Whole seed	n.a.	0.35 ± 0.09	n.a.	0.55 ± 0.13	n.a.	(Siano et al., 2018)[5]
mg/100 g oil						
Whole seed	61–135	n.a.	n.a.	n.a.	n.a.	(Galasso et al., 2016)[6]
Oil	56.28–58.22	23.4–24.62	31.6–32.20*		1.28–1.40	(Oseyko et al., 2019)[7]
Oil	n.a.	2.78 ± 0.01	n.a.	56.41 ± 0.02	n.a.	(Teh and Birch, 2013)[8]
Oil	80.28 ± 4.50	3.22 ± 0.65	0.81 ± 0.16	73.38 ± 2.86	2.87 ± 0.83	(Montserrat-de la Paz et al., 2014)[8]
Oil	n.a.	2.56 ± 0.06	0.60 ± 0.005	59.79 ± 1.21	3.97 ± 0.15	(Uluata and Özdemir, 2012)[8]
Oil	79.7	3.4	0.6	73.3	2.5	(Oomah et al., 2002)[9]
Oil	97.13	4.32 ± 0.65	n.a.	89.26 ± 1.92	3.55 ± 0.25	(Rezvankhah et al., 2018)[8]
Oil	92.97	4.71 ± 0.54	n.a.	84.11 ± 1.74	4.15 ± 0.44	(Rezvankhah et al., 2019)[8]

*β–tocopherol + γ–tocopherol.

[1]Analysed *cultivars*: Alyssa; Anka; CanMa; CFX1; CFX2; CRS1; Delores; Finola; Jutta; Yvonne

[2]Analysed *cultivars*: Beniko; Bialobrezeskie; Dneprovskaya odnodomnaya 14; Epsilon 68; Fasamo; Fedora 19; Fedrina 74; Felina 34; Ferimon; Fibrimon 56; Futura; FxT; GB14; GB15; GB16; GB17; GB18; GB20; GB21; GB22; GB23; GB24;GB26; GB27; GB28 ('Forose'); GB29; GB30 ('Bernburger'); GB31; GB32; GB33; GB36; Glera; Gluchivski-33; Gluchivski-46; Helvetica; Juso 14; Juso 31; Kompolti; Lipko; Lovrin 110; P51 (T. belt × Sz. belt); P52 (K. unisex × Kompoliti); P53 (F. belt × T. belt); P54 (K. unisex × UnikoB); P57 (Fibr. × (F × T); P59 (Kinai unisex); P60 (Fibr. × K. unisex); Ramo; Uniko-B (F1); Uso 14; Zolotonoshskaya-11

[3]Analysed *cultivars*: Bialobrzeskie; Felina 32; Tygra; Futura 75; Santhica 27; Fedora 17; Finola

[4]Analysed *cultivar*: Finola

[5]Analysed *cultivar*: Fedora

[6]Analysed *cultivars*: Carmagnola Selezionata (CS); Carmaleonte; Codimono; Fedora; Felina 32; Ferimon; Fibranova; Finola; Futura 75; Kc Dora

[7]Analysed *cultivar*: Hlesiia

[8]Analysed *cultivars*: not specified by the authors

[9]Analysed *cultivar*: Fasamo

Where more than one cultivar has been analyzed, the maximum and minimum value of the obtained data range is shown. Where only one cultivar has been analyzed, the mean ± standard deviation is reported. n.a.: not available.

Obviously, when the values are reported per 100 g of oil, the tocopherols amount results to be higher in comparison to the values referred to 100 g of seeds, as consequence of the concentration of these compounds in the extracted oil. In any case, all works reported γ-tocopherol as the most abundant isomer, followed by α-, δ- and β-tocopherols. Among these isomers, g-tocopherol has been suggested to be the most active antioxidants in lipids (Porto et al., 2015), and therefore, it contributes together with other antioxidant compounds like polyphenols, to provide high oxidative stability to hemp seed and especially, to its oil. Whereas, the α-isoform is considered as the only one tocopherol bioactive form, namely the form with vitamin E activity in the human body (EFSA NDA Panel, 2015). Regarding the daily intake of vitamin E as α-tocopherol, EFSA has been set an average intake (AI)

of 13 mg/day for males and 11 mg/day for females (EFSA NDA Panel, 2015). According to literature data (**Table 3**), the α-tocopherol content in whole hemp seed can reach up to 5 mg/100 g of seeds (Callaway, 2004). A serving portion of hemp seed correspond to 30 g (Lan et al., 2019), hence, the daily intake of a serving portion of whole hemp seed can contribute to satisfy up to 14% for women and 12% for men of the vitamin E DRV. Whereas, the g-tocopherol content found in the oil of hemp seed has been shown to be higher than those found in both linseed and canola seed oils (Teh and Birch, 2013).

The total tocopherol amount in hemp seed oil has been shown to be higher than that found in sunflower, sesame and amaranth oil (Oseyko et al., 2019). It can reach even value higher than 90 mg/100 g of oil based on the type of extraction process used to obtain oil from the seed. Indeed, on an industrial scale, various oil extraction techniques exist, each of which can differ for the performance in term of economic costs, extraction yield, and composition and physicochemical quality of the extracted oil (Devi and Khanam, 2019). One of these techniques is the cold-pressing method. It is a traditional and frequently used extraction process, which allows to extract oil without applying high temperatures and no require any organic solvent hazardous to human health and environment. However, it has low extraction efficiency and lead to obtain an oil with high chlorophyll level, probably due to intensive mechanical destruction of hemp seed cells during the pressing, and the consequent release of the pigments from chloroplasts. High amount of chlorophyll negatively influences the oil quality and shelf-life, since can lead to oil photooxidation due to the chlorophyll's role as a sensitizer (Aladić, 2015). To obtain a qualitatively better oil, it is possible to used alternative extraction process such as supercritical fluid extraction (SFE), ultrasound assisted extraction (UAE) or microwave assisted extraction (MAE). SFE is based on the use of a gaseous medium above or near its critical temperature and pressure to recover extracts from solid matrices. Carbon dioxide (CO_2) is the most commonly supercritical fluid used in the food industry for extraction from plant materials. Aladìc and colleagues (Aladić, 2015) demonstrated that this method of extraction can extract more efficiently the tocopherols than the traditional cold-pressing, leading to two- or three-fold higher γ-tocopherol content in the extracted oil. Another alternative extraction process that has been shown to improve both the yield and nutritional quality of the hemp seed oil was the enzyme-assisted cold-pressing (EACPM) method. Indeed, Latif and co-workers (Latif and Anwar, 2009) found that using this extraction technique, the level of total tocopherols in the hemp seed oil increase from 4.8 to 14.1% (based on the enzyme's type) in comparison to that obtained by cold-pressing method. Finally, UAE and MAE resulted to be even more efficient in increasing the extraction, not only of tocopherols (+17% and +11%, respectively), but also of other oil-soluble antioxidant compounds present in the hemp seeds, such as polyphenols, since the oil obtained through these techniques showed to have higher oxidation stability and antioxidant capacity then that extracted by SFE. This has been attributed either to the capacity of both UAE and MAE processes to extract oil at lower extraction temperature and a shorter process time (Rezvankhah et al., 2019, 2018). These methods can extract oil and oil-soluble components from seed using the effect of ultrasounds and microwaves respectively, on the seeds. The ultrasounds, propagating through the cells, generate the phenomenon of cavitation which provides a greater solvent penetration into the sample matrix increasing the contact between the sample and the solvent, and thereby, enhancing the efficiency (yield) and reducing the time of extraction. Whereas the microwaves can easily penetrate into the sample pores causing the solvent trapped in the pores to heat evenly and rapidly, in this way, the kinetics of extraction increases, leading to shorter extraction times, higher extraction rates, lower costs, and also less solvent use.

The extraction technique can have an influence on the tocopherol amount of the oil but not on that of the whole hemp seed. Factors that mainly affect the tocopherols amount in the seeds – and indirectly, also in the derived oil – are both genotype and above all, environmental factors. About this, Irakli and colleagues (Irakli et al., 2019) observed that the differences found in the tocopherols amount of seven industrial hemp *cvs* grown in Greece in three consecutive years, were mainly due to the effect of growing year (71% of the total variance) than the genotype (24% of the total variance). This, adding to the use of different extraction technique used for the tocopherol analysis from hemp seed, could explain the much lower tocopherol amount found by the authors in comparison to the very high tocopherols concentration obtained by Vonapartis and co-workers (Vonapartis et al., 2015).

Phytosterols are fat-soluble compounds found only in plants; they cannot be synthesized in human and are characterized to have a structure similar to that of cholesterol. Thanks to this chemical feature, when phytosterols are ingested, in the intestine they are able to reduce the cholesterol solubility excluding it from lipid micelles and to compete with free cholesterol uptake, thereby preventing the cholesterol intestinal absorption. To date, only three reports are investigated the phytosterols content and profile of whole hemp seed (Siano et al., 2018; Vecka et al., 2019) or hemp seed oil (Montserrat-de la Paz et al., 2014) (**Table 5**).

Table 5. Hemp seed oil unsaponifiable matter: phytosterol profile.

Type of analyzed sample	Total phytosterols	β–sitosterol	Stigmasterol	Campesterol	Ref.
			mg/100 g seed		
Whole seed	n.a.	53.61 ± 3.15.	2.47 ± 0.25	11.54 ± 1.03	(Siano et al., 2018)[1]
Whole seed	124 ± 12	79.7 ± 0.1	3.4 ± 0.9	7.3 ± 0.6	(Vecka et al., 2019)[2]
			mg/100 g oil		
Oil	279.37 ± 12.43	190.5 ± 5.93	10.02 ± 0.75	50.57 ± 3.20	(Montserrat-de la Paz et al., 2014)[2]

Data are expressed as mean ± standard deviation. n.a.: not available

[1]Analysed *cultivar*: Fedora

[2]Analysed *cultivars*: not specified by the authors

As noted for tocopherols content, the total phytosterols content results more concentrated when expressed for hemp seed oil rather than for whole seeds. Anyway, the most abundant phytosterol has been shown to be β–sitosterol with a value of 190.5 mg/100 g for hemp seed oil (Montserrat-de la Paz et al., 2014) and 79.7 (Vecka et al., 2019) and 53.61 mg/100 g (Siano et al., 2018) for the whole hemp seed belonging to two different *cvs.* β–sitosterol in the seed plays an important role in the fluidity of plant cell and membrane and has a role in cellular differentiation. In human it can be effective to reduce hypercholesterolemia, can have favourable effects against colon cancer, besides, it possesses antiviral, antifungal, and anti-inflammatory properties (Vecka et al., 2019). According to Vecka and colleagues (Vecka et al., 2019), the amount of β–sitosterol in whole hemp seed is higher than those found in linseed (79.7 *vs* 55.4 mg/100 g seeds) and is similar to the richest source of β–sitosterol analysed in the same study, namely pistachio (85.9 mg/100g seeds). Other two main phytosterols found in hemp

seed are campesterol and stigmasterol. The latter was described as a compound able to inhibit several pro-inflammatory factors and potentially effective in the prevention of osteoarthritis (Vecka et al., 2019).

Finally, other still little explored components of the unsaponifiable matter of hemp seed oil are carotenoids. Analysis of total carotenoids has been performed only by few papers (Aladić, 2015; Aladić et al., 2015; Oseyko et al., 2019) on the hemp seed oil obtained by CO_2 SFE, and cold-pressing process, whereas data on the carotenoids profile on whole hemp seed are reported only by Irakli and colleagues (Irakli et al., 2019). According to these papers, the total carotenoids of hemp oil has been found ranging from 7.8 to 8.2 mg/100 g for oil obtained by two different cold-pressing methods applied on Hlesiia *cv* (Oseyko et al., 2019), and amount to 3.15 or 12.54 mg/100 g for oil obtained by cold-pressing and CO_2 SFE, respectively (Aladić, 2015). In another work (Aladić et al., 2015), the amount of total carotenoids in oil obtained by CO_2 SFE was 3.42 mg/100 g. These differences may be due to the adoption of different CO_2 SFE parameters as well as, genotype and environmental factors as reported in the publication of Irakli and colleagues (Irakli et al., 2019). As said, this is the only one reports analysing the carotenoids profile of whole hemp seeds belonging to seven different *cvs*, showing that all analysed varieties contained the three main carotenoids lutein, zeaxanthin, and β-carotene with lutein as the most abundant carotenoid ranging from 1.4 to 3.4 mg/100 g seeds, followed by β-carotene (0.2–0.8 mg/100 g seeds) and zeaxanthin (0.2–0.5 mg/100 g seeds). In this case, the total carotenoids content was found ranging from 1.4 to 4.3 mg/100 g seeds with values significantly affected by both genotype and growing year.

2.1.2. Hemp seed proteins

The protein content of whole hemp seed can vary from 20 to 25% (Table 2) according to variety and environmental factors. This amount can further increase in some hemp seed-processed products such as dehulled seed and hemp seed meal or cake (also called hemp seed flour), that is, the remaining fraction of hemp seed obtained after expelling its oil fraction (House et al., 2010; P. Mattila et al., 2018a; Oseyko et al., 2019; Siano et al., 2018). Mattila and colleagues (P. Mattila et al., 2018a) demonstrated that in hemp seed the proteins are mostly located in the inner layer of the seed, in fact they found only a low quantity of total proteins in the hull. Therefore, the increase in the protein content of processed products can be explained as a consequence of protein concentration after removing some component of the whole seed that totally or almost lacks in protein, such as the hull, where most of the fibre is located and the removal of which leads to 1.5 times increase in both protein and oil amount. More proteins are also present when both hull and oil (the major component of whole hemp seed) are removed. Indeed, their removal leads to obtaining the hemp seed-processed product with the highest protein (up to over 50%) and the lowest fat content (even less than 10%, based on the type of extraction methods used) (House et al., 2010).

Research about the hemp seed proteins originated from the early 20th century and highlighted that the two main proteins of hemp seed are the storage protein albumin, a globular protein, and Edestin, a legumin. This latter is the most abundant component, constituting about 82% of total hemp protein content. It is a hexamer of about 300 KDa composed by six identical subunits, each of which is in turn made up of an acidic (AS) and a basic (BS) subunit with molecular weights of about 33.0 and 20.0 KDa, respectively, that are linked by one disulphide bond (Tang et al., 2006; Wang et al., 2008). In addition to Edestin, the other components that have been found were

albumin components (13% of total protein) and β–conglycinin (a vicilin-like protein) (up to 5% of total protein), the presence of which has been recently confirmed also by Pavlovic and co-workers (Pavlovic et al., 2019) and in a recent genome-wide study (Ponzoni et al., 2018) in which two albumin and one vicilin-like encoding genes in addition to three Edestin isoforms where identify, each encoded by a different gene, differing for the amount of sulphurated amino acids that are particularly abundant in the isoform-type 3. Nevertheless, in the report of Pavlovic and co-workers (Pavlovic et al., 2019), the authors found that Edestin amounted to about 65% of total hemp seed proteins. This result is in line with what was found from the HPLC and MS/MS analysis performed by Mamone and colleagues (Mamone et al., 2019), but it is less than what was obtained in the previous studies by Tang and colleagues (Tang et al., 2006) and by Wang and co-workers (Wang et al., 2008). Moreover, these latter authors also found that the solubility of hemp seed proteins at acidic pH was lower than that of soy proteins, and this may be due to the formation of covalent disulphide bonds among individual molecules of Edestin, resulting in insoluble protein aggregates. Another physio-chemical property investigated was the thermal one. It was found that the denaturation temperature of hemp seed proteins was 92 °C. This evidence is in agreement with what was observed by Raikos and colleagues about the effect of heat treatment (80 °C or over) on the structural characteristics of hemp seed proteins and consequently, on their digestibility (Raikos et al., 2015). Indeed, high temperatures can lead the proteins to unfold and to expose their hydrophobic groups, favouring protein-protein interactions instead of the protein-water ones, and, hence, the formation of insoluble protein aggregates to which digestive enzymes cannot access. In this context, Wang and co-workers, investigating on the *in vitro* digestibility of hemp seed protein isolates, had previously shown that untreated hemp seed proteins were much more digestible compared to soy proteins (Wang et al., 2008). The high digestibility of hemp seed proteins has been confirmed also by Mamone and colleagues, who observed that only a handful of peptides survived to an *in vitro* digestion process (Mamone et al., 2019). On the contrary, Lin and colleagues observed that heat pre-treatment on hemp seed proteins at 100 °C for 15 or 30 minutes, improved their digestibility (Lin et al., 2020). The authors explained this evidence as a consequence of the improvement in the digestive enzymes' bioaccessibility on target proteins through increased exposure of susceptible peptide bonds after protein unfolding.

Regarding the nutritional value of hemp seed proteins, it is important to consider that the nutritional quality of a protein is defined by its amino acid composition and by its digestibility and bioavailability. The protein's amino acid composition along with the individual's amino acid requirement, are important to establish the amino acid score, which is the relative contribution that the amino acids contained in the protein meet the individual's amino acid requirement, whereas, the protein digestibility is closely related to the bioavailability of its amino acids, since it measures the degree to which the protein is digested and their components – the amino acids – are absorbed by the gastrointestinal tract and, hence, introduced into the human body. Several authors investigated the hemp seed proteins' amino acid composition (**Table 6**) (Callaway, 2004; House et al., 2010; P. Mattila et al., 2018a; Tang et al., 2006; Wang et al., 2008). Some of them analysed the protein extracted from whole hemp seed while others examined commercial hemp seed protein isolate, so the obtained results are expressed on either a whole seed basis (g of amino acids per 100 g of seeds), or total protein basis (g of amino acids per 100 g of protein).

Table 6. Hemp seed protein amino acid content (g/100 g seed).

Ala	Arg	Asp	Cys	Glu	Gly	His*	Ile*	Leu*	Lys*	Met*	Phe*	Pro	Ser	Thr*	Trp*	Tyr	Val*	Ref.
1.28	3.10	2.78	0.41	4.57	1.14	0.71	0.98	1.72	1.03	0.58	1.17	1.15	1.27	0.88	0.20	0.86	1.28	(Callaway, 2004)[1]
1.23	2.76	2.13	0.31	4.58	1.24	0.64	0.62	1.53	1.28	0.50	1.02	1.14	1.27	0.85	n.a.	0.73	0.68	(Oseyko et al., 2019)[2]
0.94	2.69	2.33	0.36	3.83	1.02	0.58	0.86	1.47	0.84	0.56	1.01	0.89	1.12	0.79	n.a.	0.78	1.13	(P. Mattila et al., 2018a)[3]
0.96	2.28	2.39	0.41	3.74	1.06	0.55	0.80	1.49	0.86	0.56	1.03	0.90	1.19	1.01	0.23	0.68	1.14	(House et al., 2010)[4]

*Essential amino acids. Ala: alanine; Arg: arginine; Asp: asparagine; Cys: cysteine; Glu: glutamate/glutamine; His: histidine; Iso: isoleucine; Leu: leucine; Lys: lysine; Met: methionine; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; Val: valine; n.a.: not available.

[1]Analysed *cultivar*: Finola

[2]Analysed *cultivar*: Hlesiia

[3]Analysed *cultivars*: not specified by the authors

[4]Analysed *cultivars*: Crag; Finola; USO14; USO31

Amino acid composition obtained for hemp seed proteins by various authors, are in good agreement and highlighted (1) that hemp seed proteins contain all essential amino acids (EAAs) required by humans, and (2) that the most abundant amino acid is glutamic acid (3.74–4.58% of whole seed) followed by arginine (2.28–3.10% of whole seed). As previously anticipated, several authors (Callaway, 2004; Tang et al., 2006; Wang et al., 2008) compared the amino acid composition of hemp seed proteins to that of soy proteins and Casein. The soy proteins have been chosen since they are one of the most important and commonly used plant sources of high-quality proteins (Tang et al., 2006), whereas Casein is a highly digestible animal protein, considered a complete protein source, and therefore, it is commonly used as a reference protein to assess the nutritional quality of other proteins (House et al., 2010). Moreover, soy proteins and Casein are considered good sources of amino acids for infants. From this comparison, it emerged that hemp seed proteins have a good amount of sulphur-containing amino acids, higher than both Casein and soy proteins. Furthermore, in comparison to soy proteins, the amino acid content of hemp seed proteins was higher or similar, except for aspartic acid, glutamic acid, and lysine that resulted in being higher in soy proteins, whereas, if compared to Casein, the amino acid content of hemp seed proteins was higher or similar, except for tyrosine, leucine, methionine, and lysine that were more abundant in Casein. Among EAAs, only three (isoleucine, lysine, and phenylalanine) resulted in being lower in hemp seed proteins in comparison to casein, while the others appeared to be higher or similar and the proportion of EAAs to the total amino acids for hemp seed proteins resulted in being higher than soy proteins and similar to Casein. However, to understand if a protein can satisfy the human nutritional requirement, it is necessary to estimate the amino acid score for each amino acid element. The amino acid score for hemp seed proteins has been calculated in different papers (House et al., 2010; Tang et al., 2006; Wang et al., 2008) based on the Food and Agriculture Organization/World Health Organization (FAO/WHO) amino acid suggested requirements for children 2–5 years old. The results obtained from all reports agree with the evidence that lysine was one of the limiting amino acids since its score was lower than 1 (0.75 in (Tang et al., 2006); 0.72 in (Wang et al., 2008); and 0.62 in (House et al., 2010)). Instead, conflicting

results were found for other limiting amino acids of hemp seed proteins. In both the papers of Tang and co-workers and Wang and colleagues, others limiting amino acids were shown to be the sulphur-containing amino acid (methionine + cysteine) with an amino acid score of 0.65 (Tang et al., 2006) and 0.62 (Wang et al., 2008). Hence, comparing the sulphurated amino acid score with the lysine amino acid score, the former resulted in the first limiting amino acid followed by lysine. Contrarily, House and co-workers found that lysine was the first limiting amino acid followed by tryptophan (amino acid score, 0.87) and leucine (amino acid score, 0.94) and the limitation in the lysine content of hemp seed proteins, ranks hemp seed in the same range of the main cereal grains (House et al., 2010). These differences in the results may be related to the fact that the analyses were performed on different type of samples (*i.e.*, commercial hemp seed protein isolates and proteins extracted from whole hemp seed). Tang and colleagues have also calculated the amino acid score based on the FAO/WHO amino acid suggested requirements for children 10–12 years old and for adults, showing that, in these cases, there are no limiting amino acids (Tang et al., 2006).

House and colleagues investigated the *in vivo* digestibility of hemp seed proteins using a rat bioassay for protein digestibility, with the aim to evaluate the actual amino acid bioavailability. The authors observed that the *in vivo* protein digestibility of hemp seed proteins collocates hemp seed in the same range as the major pulse protein sources, such as lentils, and above cereal grain products. Furthermore, they also found that the presence of hull in the seed negatively affects the digestibility of the hemp seed proteins. In fact, removal of the hull fraction from the hemp seed led to an average increase in the protein digestibility from 85.2 to 94.9% (House et al., 2010). The authors supposed that the high fibre content of the hull may exert an influence on the digestibility of the proteins, but it is also possible that this decrease is, almost in part, due to the presence of some antinutritional factors such as phytate and trypsin inhibitors (Galasso et al., 2016; P. H. Mattila et al., 2018; Russo and Reggiani, 2013a), with a different distribution in the various parts of the whole hemp seed (see Subsection 3.1.6).

Overall, whole hemp seed can be considered a rich-protein source containing a protein amount higher or similar than other protein-rich products, such as quinoa (13.0%) (P. Mattila et al., 2018a), chia seeds (18.2–19.7%) (da Silva et al., 2017), buckwheat seeds (27.8%) and linseeds (20.9%) (P. Mattila et al., 2018a). Nutritionally, the protein fraction of hemp seed is highly digestible, has a good profile of EAAs required for infants similar to those of Casein except for lysine that is the first limiting amino acid in hemp seed proteins, even if this limitation is related only for the amino acid requirement for infants up to 5 years old who require a larger proportion of lysin. Furthermore, in addition to EAA profile, it is important to consider also the benefits provided by the amount of other non-EEAs such as arginine. In fact, arginine is a dietary precursor for the formation of nitric oxide (NO), a potent mediator of vascular tone, and therefore it is very important for the health of the cardiovascular system. Additionally, arginine and NO specifically, have been linked to optimal immune function and to muscle repair (House et al., 2010), so from this point of view, the proteins of hemp seed represent an important source of digestible arginine. Hence, whole hemp seed or their derived products such as hemp seed meal or hemp seed protein isolate can be considered a good viable, vegetable-based protein source for human diet.

2.1.3. Hemp seed carbohydrate and dietary fibre

Dietary fibre is defined as the part of plant material in the diet, which is resistant to enzymatic digestion and that includes cellulose, non-cellulosic polysaccharides such as hemicellulose, pectin, gums, mucilage and a non-carbohydrate component, namely lignin. Nutritionally, the dietary fibre is considered an integral part of the carbohydrate fraction of a food matrix. As previously said, the total carbohydrate content of hemp seed can range between 20 and 30% (**Table 2**). Actually, only a few literature reports analysed the total carbohydrate and fibre of hemp seed. Among these, Callaway (Callaway, 2004) found that the total carbohydrate content of the whole hemp seed belonging to the Finola *cv* amounted to 27.6 g/100 g of seeds, whereas Mattila and colleagues (P. Mattila et al., 2018a) by analysing the nutritional value of some commercial protein-rich seeds, among which is hemp seed, found that the total carbohydrate content of whole hemp seed was similar to those found in the whole linseed (34.4 ± 1.5 g/100 g for hemp seeds and 29.2 ± 2.5 g/100 g for linseeds, respectively). However, the most interesting result obtained in both studies was that a great part of the total carbohydrate of the analysed hemp seed was constituted by dietary fibre, the most of which was the insoluble fraction (**Table 2**). In particular, Callaway found a Total Dietary Fibre content (TDF) of 27.6 g/100 g of seeds (Callaway, 2004), demonstrating that the entire carbohydrate fraction consisted in dietary fibre, whereas in the study of Mattila and co-workers, the TDF of hemp seed amounted to 33.8 ± 1.9 g/100 g of seeds, representing the 98% of the total carbohydrate (P. Mattila et al., 2018a). Since the remaining carbohydrate after excluding the amount of TDF is essentially starch, it is possible to assert that hemp seed represents a low-starch content food matrix and a good source of dietary fibre, above all, the insoluble fraction. The high content of Insoluble Dietary Fibre (IDF) was found also by Multari and co-workers for hemp seed flour, which resulted in an IDF content of 25.49 g/100 g, whereas the Soluble Dietary Fibre (SDF) content was 0.16 g/100 g, thus highlighting that hemp seed is one of the richest sources of IDF among several high-protein crops such as green pea, buckwheat, and fava bean (IDS, 8.69 ± 0.07 g/100 g, 6.98 ± 0.01 g/100 g, and 9.39 ± 0.30 g/100 g, respectively) (Multari et al., 2016). These results were rather consistent with those obtained by Callaway (IDF, 22.2 g/100 g; SDF, 5.4 g/100 g) (Callaway, 2004) and by Mattila and colleagues (IDF, 30.9 ± 1.5 g/100g; SDF, 2.9 ± 0.4) (P. Mattila et al., 2018a) for the amount of SDF and IDF in whole hemp seed.

An estimate of the IDF could be obtained also through the analysis of Neutral Detergent Fibre (NDF), which measures the amount of the cell wall components, namely, hemicellulose, cellulose and lignin. Vonapartis and colleagues (Vonapartis et al., 2015) reported that the average concentration of NDF in hemp seed of ten industrial hemp *cvs* grown in Canada, was 35.7 g/100 g and this result was in good agreement to that obtained by House and colleagues (NDF, 32.1 g/100 g) (House et al., 2010). To note, it has been demonstrated that much of the fibre fraction of whole hemp seed is located in the hull, as the fibre content obtained for dehulled hemp seed was significantly lower than that found for the whole hemp seed (House et al., 2010; P. Mattila et al., 2018a).

As previously said, the high fibre content of whole hemp seed can negatively affect the protein digestibility; however, it is also important to take into account that the consumption of dietary fibre provides several health benefits in the human body. Indeed, dietary fibre, including the insoluble fraction, is considered a functional product acting as a probiotic, among others. In particular, it has been shown that it can improve insulin sensitivity; can reduce appetite and food intake, thus decreasing the risk of obesity and diabetes; and can lower the blood total cholesterol and low-density lipoprotein (LDL); moreover, because of the dietary fibre resists to digestion in the

small intestine, it reaches the large intestine, where it is fermented by the gut microbiota, to produce short chain fatty acids with anti-carcinogenic and anti-inflammatory properties (Lattimer and Haub, 2010).

Hence, considering the health benefits derived by the ingestion of dietary fibre together with the considerable amount of fibre in hemp seed, it would merit consideration as an ingredient to enrich the fibre content of foods, thereby improving their nutritional value. In this context, it should be taken into account that the use of whole hemp seed (as it or defatted) would be more appropriate since almost all the fibre is located in the hemp seed hull.

2.1.4. Hemp seed minerals

The total mineral content of a food matrix is indicated by the amount of ash that is the inorganic matter of the sample. Minerals are considered as micronutrients because their dietary requirement is relatively low (1–2500 mg/day depending on the mineral's type); nevertheless, they are needed to maintain optimal health, playing physiological and structural essential roles. Based on their dietary requirements, minerals can be classified as macro-elements (*i.e.*, minerals needed in amount of > 50 mg/day), including phosphorous (P), potassium (K), magnesium (Mg), calcium (Ca); and sodium (Na), and as micro-elements or in-trace elements (*i.e.*, minerals needed in amounts of < 50 mg/day) like iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn).

Although different authors analysed the total ash amount in several varieties of hemp seeds (**Table 2**), highlighting that they are a good source of total minerals also in comparison with other oilseeds like chia seeds (da Silva et al., 2017) and linseeds (Mueller et al., 2010) (4.9–6/100 g for hemp seeds, 4.56–5.07 g/100 g for chia seeds and 3.5 g/100 g for linseeds), only a few authors investigated their mineral profile (Callaway, 2004; Lan et al., 2019; P. Mattila et al., 2018a; Mihoc et al., 2012; Oseyko et al., 2019; Siano et al., 2018) showing that they can be considered a good source of useful macro- and micro-elements.

In general, the mineral profile of seeds can widely vary based on environmental condition, mineral soil composition, the use or not of fertilizers, the type of fertilizer if used, as well as the plant variety. However, except to Siano and colleagues (Siano et al., 2018), other authors who have investigated the mineral profile of hemp seeds belonging to different varieties and grown in various countries, obtained consistent and comparable values (Callaway, 2004; Lan et al., 2019, p. 201; Mihoc et al., 2012; Oseyko et al., 2019). The major macro-elements found in hemp seeds were P, K, Mg, Ca, and Na, whereas among the in-trace elements, Fe, Mn, Zn, and Cu have been reported (**Table 7**).

Table 7. Hemp seed mineral composition value (mg/100 g) of different industrial hemp *cultivars*.

P*	K*	Mg*	Ca*	Na*	Fe*	Mn*	Zn**	Cu**	Cd**	Ref.
1160	859	483	145	12	14	7	7	2	n.a.	(Callaway, 2004)[1]
890	n.a.	240	90	n.a.	7	6	6	n.a.	n.a.	(Oseyko et al., 2019)[2]
n.a.	252	268	94	6.8	10	4	5	0.5	n.a.	(Siano et al., 2018)[3]
n.a.	463–2821	237–694	144–955	n.a.	113–240	6–11	4–9	n.a.	0.1–0.4	(Mihoc et al., 2012)[4]
1014–910	727–866	430–482	94–121	22–27	11–13	12–15	10–11	0.8–0.9	n.a.	(Lan et al., 2019)[5]
1170	921	496	127	n.a.	4	11	7	1.9	0.0015	(P. Mattila et al., 2018a)[6]

*Macro-element. **In-trace element. Where more than one *cultivar* has been analysed, the maximum and minimum values of the obtained data range are shown. Where only one *cultivar* has been analysed, the mean ± standard deviation is reported. P: phosphorous; K: potassium; Mg: magnesium; Ca: calcium; Na: sodium; Fe: iron; Mn: manganese; Zn: zinc; Cu: copper; Cd: cadmium. n.a.: not available.

[1]Analysed *cultivar*: Finola

[2]Analysed *cultivar*: Hlesiia

[3]Analysed *cultivar*: Fedora

[4]Analysed *cultivars*: Zenit; Diana; Denise; Armanca; Silvana

[5]Analysed *cultivars*: Canda; CFX1; CFX2; CRS1; Delores; Grandi; Joey; Katani; Picolo; X59

[6]Analysed *cultivars*: not specified by the authors

Considering either EFSA's Dietary Reference Value (DRV) (EFSA NDA Panel, 2017) and the Food and Nutrition Board of the Institute of Medicine's Dietary Reference Intake (DRI) (Lan et al., 2019), and the mineral content of hemp seeds reported for different hemp *cvs*, it is possible to claim that hemp seeds represent an excellent natural source of P, K, Mg, Ca, Fe, Zn, Cu, and Mn. Particularly, the amount of P, the most abundant mineral found in hemp seeds, resulted in being higher also than that found in niger seeds (*Guizotia abyssinica* (L.f.) Cass.) and linseeds (*Linum usitatissimum* L.), which are oilseeds like hemp seeds and are considered optimal phosphorous' sources (P average content, 784.64 mg/100 g and 461.35 mg/100 g, respectively) (Deme et al., 2017). The level of K in hemp seeds is in general higher compared to that found in linseeds (568.91 mg/100 g) (Deme et al., 2017), and is equivalent to that observed in hazelnut (863 mg/100 g), thought to be an optimal source of this macro-element (Köksal et al., 2006). Interestingly, the high K amount along with a relatively low Na content, leads to a high K/Na ratio, which is believed to be related to cardioprotective effects as it promotes a high K intake considered to be inversely related to blood platelet aggregation and stroke incidence. Mg also contributes to hearth function and health, so that a Mg deficiency can lead to cardiac dysfunction. The amount of this mineral in hemp seeds resulted in being similar to walnut (Mg range: 381–443 mg/100 g) which is one of the most important sources of Mg (Tapia et al., 2013). Among the in-trace elements, Fe is of particular importance considering its essential role for human health and its widespread dietary deficiency. Interestingly, the hemp seed's Fe content resulted to be exceptionally high in the study of Mihoc and colleagues (Mihoc et al., 2012) in comparison to others works (**Table 7**), but this may be due to the very high Fe level in the experimental growth soil (1900–2043 mg/100 g). Lan and co-workers highlighted that the hemp seed's Fe content is much higher than

cereal grains, and for this, hemp seeds could be used to enrich cereal food products, ameliorating iron deficiency (Lan et al., 2019). In the same study, the authors have also calculated the percent contribution of minerals per serving of hemp seeds (30 g of seeds) to Reference Daily Intake (RDI) for adult males from age 19 to 30, finding that the highest % RDI supplied by hemp seeds of the analysed industrial hemp *cvs*, was for Fe and Mn (average % RDI, 46.68 and 169.14, respectively), concluding that all analysed varieties represent an excellent sources especially of Fe, Mn, Cu, Zn, P, and Mg. Additionally, an increase in the content of some minerals in hemp seed kernel after dehulling has been shown. Particularly, in the Hungarian Hlesiia *cv*, hull removal led to an increase in phosphorous (1.5 times), iron (1.25 times), and zinc (2 times) (Oseyko et al., 2019). Whereas Mattila and co-workers observed that whole hemp seed held 30–65% fewer macro-elements and Zn among in-trace elements in comparison to hemp hulls, whilst Cu and Mg were more evenly distributed in both the seed's kernel and hull (P. Mattila et al., 2018a).

It is known that hemp plant with its wide root system is among the plants appropriate for phytodepuration, namely, decontamination of soil from the high and toxic level of heavy metals such as cadmium (Cd). For the non-smoking population, the major source of exposure to Cd is food and among the most important Cd food sources can be mentioned integral cereals, potatoes, seafood, fungi, and oilseeds. A dietary exposure to Cd in humans leads to a relatively low, but efficient absorption of the metal in the kidney and liver, where it has a very long biological half-life ranging from 10 to 30 years. Cd is primarily toxic to the kidney, especially to the proximal tubular cells, where it accumulates over time and may cause renal dysfunction. Cd can also cause bone demineralization, either through direct bone damage or indirectly, as a result of renal dysfunction. Moreover, the International Agency for Research on Cancer has classified Cd as a human carcinogen (group 1) after that data on human exposure to Cd in the general population have been statistically associated with increased risk of some cancer types such as lung, endometrium, bladder, and breast cancers (European Food Safety Authority (EFSA), 2009). Taking into account the high capacity of hemp to absorb minerals from soil, including heavy metals, Mihoc and colleagues (Mihoc et al., 2012) evaluated the hemp plant capacity to translocate the absorbed Cd into the seeds in a soil with high Cd content (> 0.3 mg/100 g) in the absence or presence of one of two different fertilizers (K and P fertilizers). They analysed the seeds belonging to five industrial hemp *cvs* approved for growth in Romania and they showed that hemp is able to absorb and translocate to the seeds from 130 to 400 µg/100 g of Cd, with the highest values obtained for plants treated with both K and P fertilizers. Considering the highly cadmium toxicity for human health, the European Food Safety Authority's Panel on contaminants in the food chain has set the tolerable weekly intake (TWI) for cadmium at 2.5 µg/Kg of body weight (European Food Safety Authority (EFSA), 2009), whereas the FAO/WHO recommended 100 µg/Kg in cereals and legumes and up to 300 µg/Kg in medicine as the cadmium upper limit for safe human consumption (Mihoc et al., 2012). Hence, the value of Cd found in hemp seeds by Mihoc and colleagues (Mihoc et al., 2012) far exceeds the upper limit set by the FAO/WHO, even if according to the EFSA TWI value, for an adult man with an average body weight of 70 Kg, the TWI for Cd is 175 µg, and 30 g (a serving portion) of hemp seeds belonging to plants grown in a soil with high Cd content, has a Cd level below the TWI (39–120 µg of Cd). In addition, the medium Cd content in soil is generally of 0.05–0.08 mg/100 g, namely from 6 to 37.5 times lower than that used in the work by Mihoc and colleagues (Mihoc et al., 2012). Indeed, in another work investigating the heavy metal content in twenty-one hemp seed samples collected from north western Turkey, the Cd content in the samples was found to be within the range of 0.5–

2.3 µg/100 g, much lower than the upper limit recommended from the FAO/WHO (Korkmaz et al., 2010). Similarly, Mattila and colleagues observed that the Cd level in whole hemp seed was 1.5 µg/100 g (P. Mattila et al., 2018a). In their study, Mihoc and co-workers also investigated whether and how the mineral composition of soil, the presence or absence of different fertilizers and the seeding distance could influence the minerals profile of the analysed hemp seeds (Mihoc et al., 2012). They observed that both fertilizers at different levels of administration, decreased the absorption of Zn and Mn due to both the soil's pH increase which limited the availability of minerals from soil, and the mineral-phosphate complexes forming for the P excess owing to the use of fertilizer. Furthermore, the authors showed that the content of Ca, Mg, and K in the seeds was mainly affected by varieties. The uptake of Ca by the plants was disturbed also by fertilizer, whereas K uptake was influenced also by further factors, such as seeding distance and environmental factors such as soil moisture and temperature.

2.1.5. Hemp seed antinutritional compounds

The antinutritional factors are biological compounds present in human or animal foods that reduce nutrients' bioavailability or food intake, or the metabolism of which may lead to the release of toxic products, thereby contributing to impaired gastrointestinal and metabolic performance. Generally, compounds such as saponins, phytic acid, alkaloids, certain oligosaccharides, protease inhibitors, cyanogenic glycosides, glucosinolates, and tannins are traditionally incorporated into this group. Referring to hemp seed, only a few reports (Galasso et al., 2016; P. H. Mattila et al., 2018; Russo and Reggiani, 2013a, 2015a) investigated about the antinutritional compounds in this seed, and the antinutritional factors that have been considered were phytic acid, trypsin inhibitors, condensed tannins, cyanogenic glycosides, and saponins (**Table 8**).

Table 8. Hemp seed antinutritional compounds.

Phytic Acid*	Condensed Tannins**	Trypsin Inhibitors§	Cyanogenic Glycosides†	Sapoins‡	Ref.
4.5–7.6 *	n.a.	10.8–27.8*	n.a.	n.a.	(Galasso et al., 2016)[1]
6.2–7.7 *	214.0–456.0*	10.8–27.7*	50.0–170.0 *	47.0–70.0 *	(Russo and Reggiani, 2015a)[2]
5.3 ± 0.4 *	105.0 ± 4.0 **	n.a.	n.a.	n.a.	(P. Mattila et al., 2018b)[3]
6.1–7.4*	135.0–214.0*	18.2–28.4*	60.0–240.0*	5.9–8.1 *	(Russo and Reggiani, 2013a)[4]

*g/100 g. **mg/100 g. §U/mg. [4]ppm. †On defatted matter. ‡On whole seed. Where more than one *cultivar* has been analysed, the maximum and minimum values of the obtained data range are shown. Where only one *cultivar* has been analysed, the mean ± standard deviation is reported. n.a.: not available.

[1]Analysed *cultivars*: Carmagnola Selezionata (CS); Carmaleonte; Codimono; Fedora; Felina 32; Ferimon; Fibranova; Finola; Futura 75; Kc Dora

[2]Analysed *cultivars*: Carmagnola; Carmagnola Selezionata (CS); Fibranova; Futura 75; Felina 32; Ferimon

[3]Analysed *cultivars*: not specified by the authors

[4]Analysed *cultivars*: Carmagnola; Carmagnola Selezionata (CS); Fibranova; Fedora 17; Felina 32; Ferimon

Phytic acid is a natural plant compound acting as the major storage form of P as well as the primary P reserve for the plant. Indeed, it constitutes 60 to 70% of the total P in cereal grains, oilseeds, and legumes and generally comprises 1–5% weight in cereals, oilseeds, legumes, and nuts (Gupta et al., 2015). From a chemical point of view, PA is a myo-inositol hexakisphosphate (IP6) composed by an inositol ring esterified with six phosphate groups that are not internally connected (**Figure 7**); hence it has up to 12 replaceable protons allowing it to bind with multivalent cations (*i.e.*, Zn^{2+}, $Fe^{2+/3+}$, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+}), positively charged proteins, and in some cases, starch through hydrogen bonds (Bohn et al., 2008), forming insoluble complexes known as phytates.

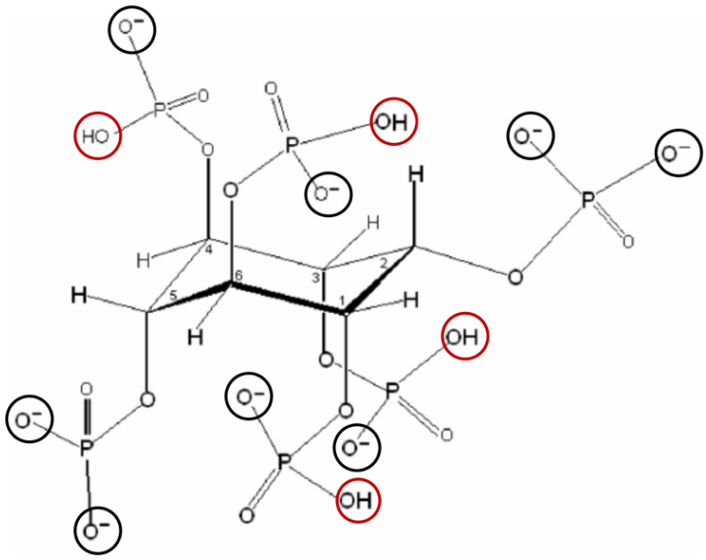

Figure 7: myo-inositol-1,2,3,4,5,6-hexakis phosphate (phytic acid, PA) at pH 6–7. Under physiological conditions phytic acid is highly charged: eight of twelve hydroxyl groups (black circles) carry negative charges that are counterbalanced most likely by multivalent cations and positively charged proteins. The red circles show other potential binding sites, negatively charged at lower pH values, for a maximum of 12 potential binding sites (Schlemmer et al., 2009).

Both phytate-mineral and phytate-protein complexes are not readily absorbed by monogastric animals including humans because of their inability to metabolize phytic acid due to the lack of a sufficient level of phytate-degrading enzymes in their digestive tract. Thus, these complexes negatively affect the bioavailability of dietary and endogenous minerals and proteins as well as the digestive enzymes' activity; therefore, for these reasons, phytic acid is considered an antinutrient. However, phytic acid may also have beneficial effects in the prevention and treatment of several pathological conditions related to oxidation and cancer (P. Mattila et al., 2018b), maybe due to its ability to chelate Fe ions, that lead phytic acid to inhibit iron-driven hydroxyl radical (–OH•) formation and, hence, to strongly suppress lipid peroxidation and the oxidation of other biomolecules. Hence, for optimum physiological benefit of phytic acid, the phytate content in the upper part of the gut should be low to avoid negative effects on mineral and protein absorption and bioavailability, but at the same time, it should be high enough to use its antioxidant and anticancer activity. However, to date, accurate and clear

information on the phytate dosage required to elicit beneficial effects in human is still limited. In accordance with Russo and Reggiani (Russo and Reggiani, 2015a), to increase the safety for feed and food formulation, it is preferable to reduce the phytic acid content to at least 0.45 g/100 g of defatted sample. According to literature data, the results obtained by different authors about the phytic acid content in hemp seed (**Table 8**) are in good agreement and show that the level of this antinutritional compound ranges between 4 and 8% of the dry weight of the defatted matter. Galasso and colleagues (Galasso et al., 2016) found a significant variability in the phytate content among different hemp *cvs*, and Russo (Russo and Reggiani, 2013a) showed that French monoecious varieties had lower content than Italian dioecious ones, but this evidence is exactly the opposite of what Galasso and co-workers found analysing twenty hemp seed *cvs* and accessions of different origin, among which, the French varieties have been to be those with the highest phytic acid content (Galasso et al., 2016). These differences in the results could be explained by the significant influence of genotype, growing year and the interaction between these two factors on the antinutritional compounds' amount, phytic acid included (Russo and Reggiani, 2015a). Compared to other seeds, the content of phytic acid in hemp seed seems to be higher. For example, Russo and Reggiani, and Russo and co-workers highlighted that the phytic acid content of hemp seed was higher than those found in soy bean (2%) (Russo and Reggiani, 2013a, 2015a), and Mattila and colleagues, comparing different type of oilseed, legumes, and pseudo-cereal grains, observed that oilseeds in general, including hemp seed, linseed, and rapeseed, form a group with a higher phytic acid content in comparison to other legumes (*e.g.*, fava bean and lupin) and pseudo-cereal grains (*e.g.*, quinoa and buckwheat), and among the analysed oilseeds, whole hemp seed was that with the highest phytic acid level (P. Mattila et al., 2018b).

Condensed tannins (CTs) are phenolic compounds (flavonoid oligomers, specifically), ubiquitous in the plant kingdom, designated as proanthocyanidins. They are considered as antinutritional compounds because of their ability to form insoluble complexes with proteins and minerals, negatively affecting nitrogenous compounds uptake and absorption of minerals, and thus reducing the nutritional value of foods (Russo and Reggiani, 2015a). Moreover, based on their concentration, CTs can affect the palatability of the meal. Nevertheless, being phenolic compounds, in the monomeric form, they also have some beneficial effects on health acting not only as antioxidants, but also as anticarcinogenic, cardioprotective, anti-microbial and anti-diabetic compounds (P. Mattila et al., 2018b). According to Russo, the attention threshold for CTs is 1% of dry weight of the product (Russo and Reggiani, 2013a). Although the amount of CTs found in hemp seed meal (**Table 8**) has been shown to be higher than other plant sources, for example soy (10 mg/100 g), the hemp seed meal's CTs content is however below than the attention threshold, so it neither is toxic, nor affects the hemp seed meal palatability (Russo and Reggiani, 2013a). Russo and Reggiani [73] found significant variability for the CTs content in monoecious and dioecious *cvs*, with a double level in the monoecious varieties than dioecious ones (Russo and Reggiani, 2015a). Mattila and co-workers evaluating the amount of CTs in whole hemp seed and in the hull of hemp seed, observed that the hull had slightly higher CTs content than whole hemp seed, even if the difference was rather small (105 ± 4 mg/100 g and 144 ± 5 mg/100 g, respectively). The same authors also found that CTs in hemp seed were essentially epi-catechin polymers (*i.e.*, procyanidins) (P. Mattila et al., 2018b).

Trypsin inhibitors (TIs) are a type of endogenous antinutritional factor commonly present in various crops, seeds, and vegetables, among which are pulses and legume seeds, nuts, oilseeds, and cereal grains. They can have a negative impact on the nutritional value of food and feed products as they affect protein utilization and digestion in human and animals. Their role in the plant is to protect the plant against microbial proteinases and to regulate

the activity of endogenous proteinases. From a nutritional point of view, the ingestion of TIs reduces the digestibility of food proteins and the bioavailability of amino acids because they can pass unaltered through the stomach as they are stable against pepsin and at low pH (Weder and Kahleyss, 2003), and in the small intestine, they are able to inhibit the biological activity of the digestive enzymes trypsin and chymotrypsin—both important for the digestion of protein in living organisms—by competitive binding and in this way, they lead to the formation of an irreversible trypsin/chymotrypsin enzyme-TI complex. TIs have been largely studied mainly in legume seeds, particularly in soybean, as it is one of the richest sources of these antinutrients. Guillamòn and colleagues (Guillamón et al., 2008), investigating the TIs content in different types and varieties of legume seeds, identified three groups of legume seeds according to the TIs content:

- the highest content group including different soy (*Glycine max*) *cvs* with a TIs content ranging from 43 to 84 U/mg of the sample;
- the medium content group corresponding to common bean (*Phaseolus vulgaris*) varieties containing from 17 to 51 U/mg of the sample;
- the lowest content group including lentil (*Lens culinaris*) and fava bean (*Phaseolus vulgaris*) *cvs* containing from 3 to 10 U/mg of the sample.

Although most of the works regarding TIs are performed on soy and legume seeds in general, some literature data about the amount of these antinutritional compounds in oilseeds such as Sacha inchi (*Plukenetia volubilis* L.) (Bueno-Borges et al., 2018) and linseed (Russo and Reggiani, 2013b) are also reported. The TIs amount in hemp seed meal reported by different authors (**Table 8**) was found to range between 10.8 and 28.4 U/mg, and the differences in the TIs content is probably due to both genotype and growth conditions (Galasso et al., 2016; P. Mattila et al., 2018b; Russo and Reggiani, 2013a). These values are similar to those found for the TIs content obtained for linseed meal (13.8–24.7 U/mg) by Russo and Reggiani (Russo and Reggiani, 2013b) and, considering the grouping made by Guillamòn, they would place both linseed and hemp seed in the medium content group of TIs. Interestingly, Pojić and co-workers highlighted that the inner fraction of hemp seed, containing predominantly cotyledon particles, is the part of the seed with the highest TIs activity compared to the fraction containing the hull particles (Pojić et al., 2014). By contrast, Mattila and colleagues found that the TIs activity between whole hemp seed and hemp seed hull was similar, indicating that TIs are equally located through the hemp seed's kernel and hull (P. Mattila et al., 2018b).

Cyanogenic glycosides are nitrogen-containing secondary metabolites that serve to the plant as a protective device against predators such as the herbivores. The level of cyanogenic glycosides produced by the plant is dependent upon the age and variety of the plant, as well as the environmental factors (Francisco and Pinotti, 2000). They are considered toxicants for humans because, structurally, they are glycosides of 2-hydroxynitriles that can be hydrolysed by the enzyme β-glucosidase into cyanohydrin, which is unstable and thus dissociates, leading to the toxic hydrocyanic acid. Although this is a violent poison, oral intake of cyanogenic glycosides (*e.g.,* via food) is not necessarily toxic, particularly in the short-term intake. Indeed, hydrolysis of the cyanogenic glycosides in the digestive tract or by the liver leads to a slow release of hydrocyanic acid that is readily detoxified by the body. According to Russo, at levels below 100 ppm, the cyanogenic glycosides are not harmful to health (Russo and Reggiani, 2013a). The amount of these antinutrients in hemp seed has been investigated only in two reports (Russo and Reggiani, 2013a, 2015a), showing that, in the hemp seed meal, their level significantly varies according to genotypes (Russo and

Reggiani, 2013a) and monoecious or dioecious *cvs*, with monoecious varieties containing lower cyanogenic glycosides than dioecious ones (Russo and Reggiani, 2015a). Moreover, these studies highlighted that the hemp seed meal of some hemp *cvs* has a cyanogenic glycosides content above the safety limit, but since this value refers to hemp seed meal, it is resulted to be more concentrated compared to the whole seed.

Finally, saponins comprise a large family of structurally related compounds containing a steroid or triterpenoid aglycone (sapogenin) linked to one or more water-soluble oligosaccharide moieties. At larger quantities, these compounds are gastric irritants, and they can exert toxic properties causing haemolysis of red blood cells. However, at low doses, they may have a plasma cholesterol lowering effect in humans, as well as strong cytotoxic effects against cancer cell lines, and are important in reducing the risk of many chronic diseases (P. Mattila et al., 2018b). The FAO/WHO established a safety limit of 12% of saponins for quinoa seeds which are among the main saponins' plant sources (Joint FAO/WHO Food Standards Programme, 2018). Russo and Reggiani found that the saponins amount in hemp seed meal of different *cvs* was well below than 12%, and lower than other plant sources such as linseed and soy (Russo and Reggiani, 2013a, 2015a), despite the results they obtained from two separate studies being very different, with the level of saponins showed in one work (Russo and Reggiani, 2015a) almost ten-fold higher than that obtained in the other work (Russo and Reggiani, 2013a).

Concluding, among the various antinutritional compounds analysed in hemp seed, TIs and, above all, phytic acid were the most abundant, based on a comparison with other edible seeds. Overall, it has been highlighted that all the analysed antinutritional compounds are positively correlated, and therefore, it is possible that the biosynthetic pathways of these compounds in hemp are expressed simultaneously during the seed's development (Russo and Reggiani, 2015a).

2.2. Hemp seed Functional Features

2.2.1. Phenolic compounds

2.2.1.1. COMPOSITION STUDIES

Phenolic compounds are plant-derived secondary metabolites that are commonly produced by the plants as defence agents against biotic and abiotic stress, such as the attack of predatory species and UV light, respectively. These compounds, thanks to their chemical structures, have intrinsic antioxidant activity; therefore, they may protect cell constituents against oxidative damage and, hence, limit the risk of various degenerative diseases associated with oxidative stress. Indeed, in the human body they can exert a multitude of physiological activities, among which are cardioprotective and anti-inflammatory effects. Often, literature values about total antioxidant capacity and total phenolic content of a matrix are difficult to absolutely compare, due to the variety of analytical and extraction methods and the wide use of different reference compounds in the quantitation. Hence, in this review, we quantitatively compared the literature data obtained about these parameters by different authors, only if possible.

The first investigations about the antioxidant power and the total phenolic content (TPC) of hemp seed were performed on hemp seed oil. As previously said, the most abundant antioxidants in this product are tocopherols, especially the γ-isoform which confers to this oil a high oxidative stability despite the high level of unsaturated fatty acids that are highly susceptible to oxidation. However, together with tocopherols, also phenolic compounds contribute to the high oxidative stability of hemp seed oil (Smeriglio et al., 2016; Teh and Birch, 2013; Uluata and

Özdemir, 2012; Yu et al., 2005). Indeed, it has been shown that hemp seed cold-pressed oil represents a good dietary source of antioxidants which could have potential application in health promotion and disease prevention from oxidative damage, and therefore, it could be used to improve the quality and stability of food products. Smeriglio and colleagues (Smeriglio et al., 2016) evaluating in detail the polyphenolic profile and the antioxidant properties of cold-pressed hemp seed oil of the Finola *cv*, found that this product contained significant amounts of polyphenols, especially flavonoids such as flavanones, flavonols, flavanols, and isoflavones, the most representative and abundant of which were naringenin, kaepferol-3-*O*-glucoside, epicatechin, and daidzein, respectively. The presence of flavonoids in hemp seed cold-press oil was demonstrated also by Faugno and co-workers (Faugno et al., 2019) who investigated the polyphenol profile of cold-pressed oil of Uso 31 *cv*. The authors used the LS-ESI-MS/MS technique to assess the polyphenol qualitative and quantitative compositions of the oil and to investigate the influence of some agronomic practices on the quantitative variability of polyphenolic compounds. They found that, among phenols, the most representative compounds were dihydroxybenzoic acid, caffeoyl-tartaric acid isomers, *p*-coumaric acid, *N*-caffeoyltyramine, and *N*-feruoyltyramine, whereas, among the flavonoid subfamily, they found the glycosides of quercetin, kaempferol and isorhamnetin. Moreover, the authors observed that the highest production of polyphenols has been obtained without pre-seeding fertilization and with 60-plant/m^2 crop density, whereas when the plant density was halved, the abundance of phenolic compounds was compromised. In general, as said, the quantitative variability of polyphenols is affected by the influence of multiple biotic and abiotic factors, which take place in the biosynthetic pathway of these compounds, as reported also by Irakli and colleagues (Irakli et al., 2019), who highlighted that the amount of phenolic compounds in the whole hemp seed is affected by genotype, growing year, and the interaction between these factors. However, the growing year and so, the environmental and agronomical conditions, were the factor that most affected the variability of the TPC (contribution to total variance, 59%). In **Table 9**, the literature data about the TPC extracted by different methods but analysed by the same technique (Folin–Ciocolteau) and expressed using the same reference compound (gallic acid) are reported, and they show the high variability of the parameters.

Table 9. Total phenolic content (TPC) of hemp seed cold-pressed oil or whole hemp seed.

Type of sample	TPC*	Ref.
Whole hemp seed	13.68 – 51.60	(Vonapartis et al., 2015)[1]
Whole hemp seed	3.82 – 7.80	(Irakli et al., 2019)[2]
Whole hemp seed	0.77 ± 0.04	(Siano et al., 2018)[3]
Cold-pressed oil	1.88 ± 0.003	(Teh and Birch, 2013)[4]
Whole hemp seed	0.96 ± 0.35	(P. Mattila et al., 2018b)[5]
Cold-pressed oil	2.68 ± 0.09	(Smeriglio et al., 2016)[6]
Cold-pressed oil	0.44 ± 0.001	(Yu et al., 2005)[7]
Whole hemp seed	0.92 ± 0.04	(Moccia et al., 2019)[8]
Whole hemp seed	2.33 ± 0.07	(Frassinetti et al., 2018)[9]

*mg GAE/g. Where more than one *cultivar* has been analysed, the maximum and minimum values of the obtained data range are shown. Where only one *cultivar* has been analysed, the mean ± standard deviation is reported. GAE.: gallic acid equivalent.

[1]Analysed *cultivars*: Alyssa; Anka; CanMa; CFX1; CFX2; CRS1; Delores; Finola; Jutta; Yvonne

[2]Analysed *cultivars*: Bialobrzeskie; Felina 32; Tygra; Futura 75; Santhica 27; Fedora 17; Finola

[3]Analysed *cultivar*: Fedora

[4]Analysed *cultivars*: not specified by the authors

[5]Analysed *cultivar*: not specified by the authors

[6]Analysed *cultivar*: Finola

[7]Analysed *cultivar*: not specified by the authors

[8]Analysed *cultivar*: Fedora

[9]Analysed *cultivar*: Futura 75

Although tocopherol was reported as the main antioxidant for hemp seed oil, it would not be the same for the whole hemp seed. Indeed, Irakli and co-workers (Irakli et al., 2019) observed a significant positive correlation between the TPC and antioxidant activity of hemp seeds belonging to seven industrial hemp *cvs* approved for cultivation in Greece and only weak correlation between γ-tocopherol and antioxidant activity, concluding that the antioxidant capacity of hemp seed is due, mainly, to phenolic compounds rather than to the tocopherols. The high positive relation between the TPC and the radical scavenging activity of hydroalcoholic hemp seed extract was also reported by Chen and colleagues (Chen et al., 2012). These results can be explained by the fact that polyphenols are polar, hydro-soluble compounds and, thus, that only a few of them are isolated during oil extraction whereas most remain associated to the hemp cake (Moccia et al., 2019). Conversely, the tocopherols, that are fat-soluble, are extracted more efficiently, and hence, are more abundant in oil. This is in accordance with the results of Siano and co-workers (Siano et al., 2018) and Moccia and colleagues (Moccia et al., 2019) who have analysed and compared the TPC and the radical scavenging activity of whole hemp seed, hemp seed flour, and hemp seed oil of Fedora *cv*, showing that, although the values obtained for whole hemp seed and hemp seed flour were similar for both evaluated parameters, those for hemp seed oil were significantly lower. Regarding the TPC of whole hemp seed, Galasso and colleagues (Galasso et al., 2016) highlighted that the value obtained for hemp seed were slightly higher than those found for linseed (5.88–10.63 mg Caffeic Acid Equivalent (CAE)/g of dry weight of defatted hemp seed vs. 4.64–9.40 mg CAE/g of dry weight of defatted linseed), which is considered a

matrix rich in total phenols among the oilseeds (Russo and Reggiani, 2015b). In addition, the TPC and the total antioxidant capacity of whole hemp seed could increase consequently the germination of seed, leading to a derived product –the hemp seed sprouts – enriched with beneficial bioactive compounds (Frassinetti et al., 2018; Werz et al., 2014).

Various authors found that in whole hemp seed, polyphenols are mainly located in the hull rather than in the kernel (Chen et al., 2012; P. Mattila et al., 2018b; Pojić et al., 2014) and that the main phenolic compounds that have been detected in *C. sativa* L. seeds are lignans, phenols derived from the shikimic acid biosynthetic pathway. Particularly, they originate from cinnamic acid derivatives which are biochemically related to the metabolism of phenylalanine. Lignans detected in hemp seed are also called phenylpropionamides due to their chemical components and belong to two main groups known as phenolic amides and lignanamides (Irakli et al., 2019). The first ones are made by an ammine moiety (tyramine, derived from the tyrosine oxidative decarboxylation) linked to a phenolic moiety (a hydroxycinnamic acid, such as coumaric, caffeic or ferulic acid). The second ones are derived by a random polymerization of phenolic amides. Some of these phenols are found exclusively in the *C. sativa* L. plant. More than 20 kinds of phenylpropionamides, most of which are lignanamides, have been isolated from hemp seed. Chen and co-workers (Chen et al., 2012) isolated and identified two hemp seed lignans with strong *in vitro* antiradical activity and protective effect against *in vitro* oxidation of human LDL in an ethanol extract of hemp seed hull. These compounds were the phenolic amide *N-trans*-caffeolyltyramine and the lignanammide cannabisin B, which were identified as the most abundant phenolic compounds in the hull fraction of hemp seed also by Pojic´ and colleagues (Pojić et al., 2014) who in addition, showed that the most abundant polyphenols in the cotyledon inner fraction of hemp seed were catechin and *p*-hydroxybenzoic acid. The other phenylpropionamides identified as the most abundant in hemp seed of different varieties were the lignanamides cannabisin A (Irakli et al., 2019), cannabisin F, and grossamide (P. Mattila et al., 2018b). Also, Yan and co-workers (Yan et al., 2015) isolated and identified a total of 14 phenolic compounds characteristic of the hemp seed, twelve of which were lignanamides. Further phenylpropionamides recently isolated from hemp seed were cannabisin I (Bourjot et al., 2017), cannabisin Q, and coumaroylaminobutanol glucopyranoside (CLG) (Zhou et al., 2018b), corresponding to the glycoside of 4-((E)-p-coumaroylamino)butan-1-ol previously described by Yan and colleagues (Yan et al., 2015).

2.2.1.2. BIOLOGICAL EFFECTS

Concerning the functional effect, most of the phenolic compounds isolated from hemp seed have been shown to have high radical scavenging activity compared to quercetin, and, in addition, the phenolic amide *N-trans*-feryoryltyramine and especially, the lignanamides 3,3 -demethyl-grossamide and 3,3′-demethylheliotropamide were also able to *in vitro* inhibit the acetylcholinesterase (AChE) enzyme at a concentration of 100 μg/mL, thus exhibiting properties similar to the medicines used for the treatment of mild to moderate Alzheimer's disease (AD) such as galanthamine (Yan et al., 2015). Bourjot and co-workers (Bourjot et al., 2017) found that, among the phenolic amides they have extracted from hemp seed flour, *N-trans*-caffeoyltyramine had the highest antioxidant and arginase inhibitor activities. In this regard, the inhibition of arginase could raise NO bioavailability that ameliorates endothelial functionality, and it can also reduce oxidative stress which plays a central role in the onset and progression of endothelial dysfunction involved in various diseases, including cardiovascular ones. However, the most important biological effects attributable to hemp seed phenylpropionamides are the anti-inflammatory

and neuroprotective activities. In this context, Zhu and co-workers (Zhu et al., 2018) had recently isolated and characterized the chemical and functional features of two unique hemp seed bioactive compounds named sativamides A and B. According to the authors, from a chemical point of view, they are non-lignanamide compounds characterized by a benzo-angular triquinane core (*i.e.*, a core consisting of fused five-membered hydrocarbon rings and arranged in an angular position), derived from *N-trans*-caffeoyltyramine. Interestingly, it has been shown that pre-treating of the SH-SY5Y human neuroblastoma cell line with 50 μM of sativamides A or B, reduced the cell death caused by endoplasmic reticulum stress that has been shown to play an important role in neurodegenerative diseases, such as Parkinson's disease and AD. The same cell line was also used by Maiolo and colleagues (Maiolo et al., 2018) to create a cellular model of Parkinson's disease, on which the neuroprotective effect of various natural compounds, among which is *N-trans*-caffeoyltyramine extracted from hemp seed, was tested. The obtained results highlighted that 150 μM of the hemp seed's phenolic amide was able to prevent the cell death induced by up to 150 μM of H_2O_2 treatment and due to oxidative stress and mitochondrial dysfunction caused by this toxic compound. Oxidative stress and mitochondrial dysfunction are thought to contribute to cell death in Parkinson's disease; therefore, the resulting effect of 150 μM *N-trans*-caffeoyltyramine can be considered as potentially neuroprotective.

The neuroprotective effect of different hemp seed phytochemicals has been shown to be related particularly to the anti-inflammatory and antioxidant action of some of these compounds on microglia cells which are immune cells of the central nervous system that are involved in regulating the immune responses in the brain, thus playing an important role in brain infection and inflammation. The persistent and/or over-activation of these cells is often related to neuronal damage and onset of neurodegenerative diseases, such as AD and Parkinson's disease, and multiple sclerosis, that in fact, are characterized by chronic inflammation and oxidative stress. About this, Zhou and colleagues (Zhou et al., 2018b) investigated the anti-neuroinflammatory and neuroprotective action of each of the twenty phenolic compounds that they have isolated from hemp seed, on the release of the inflammatory cytokine TNF-α from Lipopolysaccharide (LPS)-stimulated BV2 microglia cells, treated with 15 μM of each interest compounds. The authors highlighted that, among the analysed phenylpropionamides, those with a higher inhibitory activity on the Tumor Necrosis Factor α (TNF-α release were lignanamides and compared to resveratrol—the positive control—the much stronger inhibitory action was found for the CLG. To understand the molecular mechanism underlying the neuroprotective action of the most bioactive hemp seed phenylpropionamides, various studies were performed by different authors. More specifically, to date, the investigated compounds were CLG (Wang et al., 2019b), grossamide (Luo et al., 2017), and cannabisin F (Wang et al., 2019a). In all cases, BV2 microglia cells were pre-treated with 5, 10, or 15 μM of each tested compound 1 h before the addition of 100 ng/mL of LPS for 24 h. After 24 h of LPS exposure, it has been shown that all three lignanamides were able to decrease the release of the inflammatory cytokines IL-1β, IL-6, and TNF-α in a dependent-manner by the inactivation at different level of the NF-κB inflammatory pathway. Moreover, CLG and cannabisin F were also found to have the ability to promote the expression of nuclear factor erythroid 2-related factor 2 (Nfr-2), a protein important in modulating redox homeostasis, in regulating the cellular antioxidant response against reactive oxygen species (ROS) and in attenuating oxidative stress. Normally, this protein is located in the cytoplasm, but in response to oxidative stimuli, Nrf2 transferred to the nucleus and activated downstream genes involved in the antioxidant defence. Although LPS stimulation leads to an increased production of ROS by microglia-stimulated cells, it has been demonstrated that the pre-treatment with CLG or cannabisin F

upregulated the Nfr-2 pathway, thus exerting a beneficial antioxidant effect (Wang et al., 2019b, 2019a). In addition, it has also shown that cannabisin F was able to enhance its inhibitory action on the NF-κB transcription factor even by inhibiting the downregulation of the silent information regulator transcript-1 (SIRT1) induced by LPS. SIRT1 is a histone deacetylase that inhibits NF-κB transcriptional activity through deacetylation of its p65 subunit, thus leading to the reduction of inflammatory cytokines. LPS stimulation induces the downregulation of SIRT1, increasing the inflammatory status, but this can be counteracted by cannabisin F (Wang et al., 2019a). The anti-neuroinflammatory and neuroprotective effect of hemp seed phenols was observed also *in vivo* on an LPS-induced neuroinflammatory model mice (Zhou et al., 2018a). In particular, the beneficial effects were obtained after three weeks of treatment with 1 g/Kg/day of hemp seed extract (concentration of the extract, 233.52 ± 2.50 μg/mg) and consisted of a reduced expression of the inflammatory cytokines IL-1β, IL-6, and TNF-α in the brains and the prevention of LPS-induced damage to the hippocampal nerve cells as well as an improvement in the animal's learning, memory, and their cognitive function. The phenylpropionamides isolated from hemp seed and the literature studies about their biological action are listed in **Table 10**.

Table 10. Hemp seed phenylpropionamides and their biological effect.

Compound	Biological Effect	Ref.
N-trans-caffeolyltyramine	Strong *in vitro* scavenging DPPH radical and ORAC antioxidant activity. LDL protection against *in vitro* oxidation. *In vitro* AChE and arginase inhibitory activity. *In vivo* anti-neuroinflammatory action. *In vitro* prevention of H2O2-induced cell death.	(Bourjot et al., 2017; Chen et al., 2012; Maiolo et al., 2018; Yan et al., 2015; Zhou et al., 2018a)
N-trans-feryoryltyramine	*In vitro* scavenging DPPH radical activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
N-trans-caffeolyoctopamine	*In vivo* anti-neuroinflammatory action.	(Zhou et al., 2018a)
N-trans-cumaroyltyramine	*In vivo* anti-neuroinflammatory action.	(Zhou et al., 2018a)[4]
Cannabisin A	*In vitro* scavenging DPPH radical activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
Cannabisin B	Strong *in vitro* scavenging DPPH radical and ORAC antioxidant activity. LDL protection against *in vitro* oxidation. *In vitro* arginase inhibitory activity.	(Bourjot et al., 2017; Chen et al., 2012; Zhou et al., 2018a)
Cannabisin C	*In vitro* scavenging DPPH radical activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
Cannabisin D	*In vitro* scavenging DPPH radical activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
Cannabisin E	*In vivo* anti-neuroinflammatory action.	(Zhou et al., 2018a)
Cannabisin F	*In vitro* scavenging DPPH radical and ORAC antioxidant activity. *In vivo* anti-neuroinflammatory action. *In vitro* inhibition of the NFκB inflammatory pathway, activation of Nfr-2 antioxidant pathway and upregulation of SIRT1.	(Bourjot et al., 2017; Wang et al., 2019a; Zhou et al., 2018a)
Cannabisin M	*In vitro* scavenging DPPH radical activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
Cannabisin N	*In vivo* anti-neuroinflammatory action.	(Zhou et al., 2018a)
Grossamide	*In vitro* inhibition of the NFκB inflammatory pathway. *In vivo* anti-neuroinflammatory action.	(Luo et al., 2017; Zhou et al., 2018a)
3,3-demethyl-grossamide	*In vitro* scavenging DPPH radical activity. *In vitro* AChE inhibitory activity. *In vivo* anti-neuroinflammatory action.	(Yan et al., 2015; Zhou et al., 2018a)
3,3-demethylheliotropamide	*In vitro* AChE and arginase inhibitory activity.	(Yan et al., 2015)
Sativamides A	*In vitro* reduction of ER stress-induced cell death.	(Zhu et al., 2018)
Sativamides B	*In vitro* reduction of ER stress-induced cell death.	(Zhu et al., 2018)
4-((E)-p-coumaroylamino)butan-1-ol	*In vitro* inhibition of TNF-α release.	(Zhou et al., 2018b)
Cumaroylaminobutanol glucopyranoside	*In vitro* inhibition of TNF-α release. *In vitro* inhibition of the NFκB inflammatory pathway and activation of Nfr-2 antioxidant pathway.	(Wang et al., 2019b; Zhou et al., 2018b)

2.2.2. Bioactive peptides

Together with phenolic compounds, other kinds of hemp seed's functional compounds are the bioactive peptides. The evidence of the existence of these peptides has been demonstrated by the findings about the bioactive features observed for the products obtained by the hydrolysis of hemp seed proteins. Indeed, it has been shown that, although hemp seed proteins have limited bioactive properties, their hydrolysis provides hydrolysates with higher bioactivity (Logarušić et al., 2019; Rodriguez-Martin et al., 2019; Teh et al., 2016), including antioxidant (A. Girgih et al., 2014; Girgih et al., 2011; Lin et al., 2020; Logarušić et al., 2019; Rodriguez-Martin et al., 2019; Tang et al., 2009; Teh et al., 2016; Wang et al., 2009), antihypertensive (Malomo et al., 2015; Teh et al., 2016), antiproliferative (Lin et al., 2020; Logarušić et al., 2019), hypocholesterolemic (Aiello et al., 2017; Zanoni et al., 2017), anti-inflammatory, and neuroprotective (Malomo and Aluko, 2016; Rodriguez-Martin et al., 2019) effects. This evidence suggests that bioactive peptides are encrypted in the protein native structure and are released during the hydrolysis process. Depending on the hydrolysis conditions (*i.e.*, type of proteases used, period, and time of hydrolysis), hydrolysates with different kinds and efficiency degrees of activity can be obtained. This is due to the fact that hydrolysis conditions can influence the type of obtained peptides, namely the size and the amino acid profile – and hence, the structure – which in turn, influence the activity and the function of peptides. In this context, literature data agree that important factors which affect the bioactivities of the peptides are their molecular weight and amino acid composition. In general, low-molecular weight peptides are more bioactive than the high-molecular weight ones, since they more easily escape enzymatic degradation in the gastrointestinal tract, and therefore, they can be absorbed and introduced as such into the blood stream than larger peptides. Moreover, small peptides are likely more easily able to interact with specific target sites, such as an enzyme's active site, in comparison to larger peptides. Instead, the peptide's amino acid composition is fundamental to define its structure and, therefore, to influence the strength and the efficiency of the interaction with the peptide's target site; in fact, the amino acids composition is the basis of the peptide's structure-function relationship.

About hemp seed protein hydrolysates, different reports (Malomo et al., 2015; Malomo and Aluko, 2016; Tang et al., 2009; Teh et al., 2016; Wang et al., 2008) confirmed that the type and level of proteases used as well as the time of hydrolysis affect the degree of hydrolysis, the amino acids profile, and the molecular weight of the obtained peptides due to the fact that different proteases have different specificities, and therefore, they will produce different types of peptides with different activities. Moreover, these publications demonstrated that, overall, independently to the hydrolysis' type, hemp seed proteins are highly susceptible to proteolysis and most of the liberated bioactive peptides have a molecular weight less than 10 KDa and a high hydrophobicity rate. More specifically, Tang and colleagues (Tang et al., 2009) and Wang and co-workers (Wang et al., 2009) observed that hemp seed hydrolysates with a higher degree of hydrolysis, a higher amount of hydrophobic amino acids, and a higher surface hydrophobicity possessed higher *in vitro* antioxidant activity (particularly, the α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging and Fe^{2+} chelating abilities). Their results were consistent with those obtained by Logarušić and colleagues (Logarušić et al., 2019), who also found that the obtained hydrolysates showed cytotoxic and antiproliferative activity on HeLa cancer cells and a stimulatory effect on the proliferation of normal HaCaT cells. However, the effective concentration at which the hydrolysates exerted these functional activities dependeds on the hydrolysis condition, thus highlighting that different hydrolysis methods generate peptides with different structure and, hence, different activity. Similar results were obtained also by Rodriguez-Martin and colleagues (Rodriguez-Martin et al., 2019), who in addition to the *in vitro* antioxidant activity

demonstrated that specific hemp seed hydrolysates' fractions possessed neuroprotective action, being able to down-regulate the expression levels of TNF-α and IL-6 inflammatory cytokines and to increase the mRNA levels of the anti-inflammatory IL-10 gene at the concentration of 100 μg/mL in LPS-stimulated BV2 microglia cells. Furthermore, Malomo and colleagues investigated both *in vitro* and *in vivo* hypotensive (Malomo et al., 2015) and *in vitro* neuroprotective (Malomo and Aluko, 2016) activity of hemp seed protein hydrolysates obtained by different hydrolysis methods, highlighting that the hydrolysates' activity was determined mostly by the type and sequence of peptides' amino acids rather than by the degree of hydrolysis that is related to the level of the proteases used and the time of the hydrolysis process. Particularly, the authors observed that the hypotensive effect of hemp seed protein hydrolysates was due to their angiotensin-converting enzyme (ACE) and renin-inhibitory activity, which seemed to be related to high proline and phenylalanine content, respectively, though also other additional peptides' structural factors were likely to be involved in the overall functional effect. In the other work (Malomo and Aluko, 2016), the authors showed that hemp seed hydrolysates exert an *in vitro* neuroprotective action through inhibition of the activity of the acetylcholinesterase enzyme (AChE) and observed that the most active hydrolysates were those with the highest negatively charged amino acids amount. Also, arginine was the second most abundant amino acid found in the hydrolysates with higher AChE inhibitory action, and according to the authors, this was probably due to the fact that it is responsible for binding the peripheral anionic site (PAS) of AChE that is believed to be an important enzyme binding site for inhibitors, thus highlighting the importance of the structure-function relationship. Nevertheless, none of these reports that have investigated the bioactive features of hemp seed protein hydrolysates elucidated the amino acids profile and structural features of peptides responsible for the investigated functional effects, nor the specific relationship between their function and structure.

In this regard, Aiello and co-workers (Aiello et al., 2017) found that hemp seed peptides with a hypocholesterolaemic effect contained 8−10 amino acid residues and were characterized by a high hydrophobicity rate, a hydrophobic N-terminus, and a negatively charged C-terminus. These were identified as features essential to interact with 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCoAR), the enzyme that mediates the rate-limiting step of the intracellular cholesterol production. These peptides seemed to be generated especially through peptic hydrolysis of hemp seed proteins because it has been observed that, in comparison to hydrolysates obtained using other peptidases, the peptic hydrolysate exerted a significantly higher hypocholesterolaemic activity on HepG2 cells already at a concentration of 0.25 mg/mL (Zanoni et al., 2017). This hypocholesterolaemic effect has been attributed to a statin-like mechanism, since like statins, the peptic hemp seeds hydrolysate was able to decrease the cholesterol level (1) by acting as direct inhibitor of HMGCoAR and (2) by increasing the level of sterol-responsive element binding protein 2 (SREBP2), a transcription factor playing a crucial role in the regulation of genes involved in the cholesterol homeostasis, such as the low-density lipoprotein receptor (LDLR) coding gene. Therefore, the increase in the SREBP2 level leads to an increase in the LDLR expression on the HepG2 surface and to a consequent increase in the circulating cholesterol uptake.

As said, another bioactive property attributed to hemp seed protein hydrolysate was the anti-hypertensive activity (Malomo et al., 2015). Publications investigating the structural features of the hydrolysate's component peptides found that most bioactive peptides able to inhibit renin and/or ACE contained three or four amino acids and some particular structural characteristics related to its sequence. They had a good hydrophobicity/hydrophilicity balance; contained hydrophobic amino acids at the C-terminal, aromatic, or branched-chain amino acid residues near the C-terminal; and hydrophobic residues at the N-terminal. Moreover,

when a branched-chain, hydrophobic amino acid residue and/or an ionizable functional group were present at the N-terminal or near it, the ACE-inhibitory activity may be enhanced (Aiello et al., 2017; A. T. Girgih et al., 2014c, 2014b; Orio et al., 2017). Hemp seed peptides with these structural features that has been shown to possess *in vitro* ACE-inhibitory activity were the Glycine-Valine-Leucine-Tyrosine (GVLY), Leucine-Glycine-Valine (LGV) (Orio et al., 2017), Tryptophan-Valine-Tyrosine-Tyrosine (WVYY), and Proline-Serine-Leucine-Proline-Alanine (PSLPA) peptides, whereas the peptides Tryptophan-Tyrosine- Threonine (WYT), Serine-Valine-Tyrosine-Threonine (SVYT), and Isoleucine-Proline-Alanine-Glycine- Valine (IPAGV) showed dual inhibition of ACE and renin enzymes (Girgih et al., 2014b), with the tripeptide WYT exhibiting the strongest renin inhibition related to the presence in its sequence of the main structural features characteristics of renin-inhibitor peptides, namely one hydrophobic side chain (tryptophan) at the N-terminal, bulky chains (tyrosine) at the C-terminal, one hydrogen bond donor (tryptophan), and two hydrogen bond acceptors (threonine and tyrosine) (Girgih et al., 2014a). Girgih and colleagues found that the hemp seed peptides inhibited ACE through a mixed-type model of enzyme inhibition, implying that peptides bound to both active and non-active sites, thus blocking the substrate binding or producing changes in the enzyme's conformation, which reduced the substrate affinity with the active site (Girgih et al., 2014b). These interactions between peptide and ACE were mainly stabilized by electrostatic and hydrophobic interactions and hydrogen bond formation. Whereas the hemp seed peptides inhibited renin enzyme by an uncompetitive model of enzyme inhibition, implying that peptides bound to the renin–substrate complex, causing reduction in the reaction velocity as well as the catalytic efficiency. Here too, the main involved interactions were the electrostatic and hydrophobic ones, as well as multiple hydrogen bonds. Of note, these bioactive peptides originated by simulated gastrointestinal digestion of hemp seed proteins. In addition, the *in vivo* hypotensive ability of the WVYY, PSLPA, WYT, SVYT, and IPAGV peptides were assessed after oral administration of 0.5 mg/mL of each peptide to spontaneously hypertensive rats. The results demonstrated that all analysed peptides were able to significantly reduce the systolic blood pressure (SBP) of animals, but PSLPA, SVYT, and IPAGV exerted a long-lasting SBP-lowering effect, indicating a more efficient absorption coupled with strong binding to target enzymes and resistance to structural inactivation by gastrointestinal tract or blood circulatory system proteases (Girgih et al., 2014b).

Hydrophobicity represents a particularly important feature for all bioactive peptides in general and for the antioxidant ones in particular. Indeed, along with the short length of the peptides, hydrophobicity is essential to facilitate the crossing of the intestinal barrier and to enhance the peptides permeability through the cell membrane via hydrophobic association with the lipid bilayer, and in antioxidant peptides, it promotes the effective interaction with free radicals and their inhibition, thus achieving potent antioxidant effects (Aiello et al., 2017). It has been shown that the hydrophobic amino acids such as valine, alanine, and leucine, represent 50% of the total amino acids in antioxidant hemp seed peptides and that they are distributed on both sides of the peptide chain (Lu et al., 2010). The hydrophobic, aromatic amino acids such as phenylalanine and tryptophan are also abundant since they can enhance the metal chelating ability due to the several binding sites on their aromatic rings (Girgih et al., 2013). Another amino acid residue recurring in the sequence of antioxidant peptides, is histidine, especially located at the N- or C-terminal where it can enhance the radical scavenging activity due to its imidazole ring that is effective in metal-ion chelation (Girgih et al., 2013; Lu et al., 2010).

Finally, Ren and co-workers identified two hemp seed peptides with α-glucosidase inhibitory activity and therefore, with antidiabetic property (Ren et al., 2016). These peptides were the dipeptide Leucine-Arginine (LR)

and the pentapeptide Proline-Leucine-Methionine-Leucine-Proline (PLMLP). Even in this case, the hydrophobic amino acids gave a great contribution to the performance in the inhibition of α-glucosidase activity, and the P and L residues seemed to be particularly important; in fact, they were been considered as vital amino acids playing α-glucosidase inhibitory action separately or synergistically.

In conclusion, it is important to highlight that, although the *in vitro* and, in part, *in vivo* (animal) studies have demonstrated the functional effects of hemp seed protein hydrolysates and of single isolated peptides, human studies investigating the peptides' stability into the gastrointestinal tract and their bioavailability, namely the capacity to reach the target site in the active and functional form, are still lacking and they should be investigated.

2.3. Hemp seed Dietary Supplementation

Considering the antioxidant and anti-inflammatory properties related to the hemp seed's components, different efforts were performed to investigate whether hemp seed dietary supplementation could have the ability to counteract chronic-degenerative diseases characterized by chronic inflammatory and oxidative stress conditions, such as cardiovascular and neurodegenerative diseases. Until now, most of the studies have been conducted on animal models and only a few literature reports have been found on humans, underlining the requirement to evaluate the effects of the supplementation with hemp seeds and their derivatives on human health and to understand their potentiality as ingredient for functional foods. With this in mind, in the following subsections, literature publications about the effects of hemp seed and hemp seed products-based supplementation in animals' and humans' studies have been reported.

2.3.1. Animal studies

According to the WHO, cardiovascular diseases (CVDs) are the first cause of worldwide death (World Health Organization, 2017). Atherosclerosis, hypercholesterolaemia, and hypertension represent among the major risk factors of CVDs and both hypercholesterolemia and hypertension, along with chronic inflammation and oxidative stress, are considered as the factors that promote the process of atherosclerosis (Mendis et al., 2011); hence, their prevention would reduce the risk of atherosclerosis and CVDs onset. Diet represents an important non-pharmacological tool that can reduce cardiovascular risk and, thus, improve health outcomes of people at risk, and in this context, the antioxidant and anti-inflammatory properties of whole hemp seed could represent an effective and efficient dietary intervention to promote the treatment and prevention of the CVDs. Therefore, some authors investigated the potential anti-hypercholesterolaemic, anti-hypertensive, and anti-atherosclerotic ability of hemp seeds or hemp seed-derived products such as hemp seed oil, hemp seed flour, or hemp seed protein hydrolysate, after dietary administration, mainly on rats', mice's, or rabbits' CVDs models.

Regarding this, Kaushal and colleagues (Kaushal et al., 2020) evaluated whether 1 month of supplementation of 10% whole hemp seeds in Wistar rats fed high-fat, pro-hypercholesterolemic diet had the ability to ameliorate hypercholesterolaemia-associated cardiovascular effects. The obtained results highlighted that when 10% whole hemp seeds were added to a normal, balanced diet, they did not cause any changes in the serum lipid parameters, the aorta's wall histology, and the redox and inflammatory status of the animals in comparison to the control (*i.e.*, rats fed a normal, balanced diet without hemp seeds supplementation), however, the supplementation of a high fat diet with 10% hemp seeds led to a significantly ameliorated effect of serum parameters (*i.e.*, decreased total cholesterol, LDL, and triglycerides levels), redox and inflammatory status, as well as the prevention in the

development of the aortic changes related to atherosclerosis. As said, inflammation and oxidative stress are involved in the atherosclerosis changes. It is known that high-fat diet is responsible for the activation of the inflammation inducible genes such as cyclooxygenase-2 (COX-2), which lead to increase the expression of pro-inflammatory prostaglandins Prostaglandin E (PGE). The COX-2 inflammatory pathway is closely related to the redox status because it can be activated by oxidative stress and vice versa (*i.e.*, the COX-2 inflammatory pathway can generate oxidative stress). Based on the obtained results, the authors asserted that 10% hemp seeds supplementation was able to exert the anti-hypercholesterolaemic effects through redox-sensitive modulation of the inflammatory pathway of PGs biosynthesis. In particular, it would seem that the antioxidant properties of hemp seed, which include the ability to scavenge free radicals, to chelate metal ions, and to inhibit the LDL oxidation, may improve the redox status in the high-fat diet induced hypercholesterolaemic rats, and thus, it can induce a redox shift of the COX-2 pathway toward the production of anti-inflammatory PGs of D- and J-series.

The beneficial effect of the short-term (20 days) hemp seed feeding in Wistar rats on the prevention of hypercholesterolaemia and coronary artery disease was observed also by Karimi and co-workers (Karimi and Hayatghaibi, 2006). Indeed, they found that the hemp seed dietary treatment did not significantly affect the mean fasting triglyceride, total cholesterol and albumin levels, but it was able to improve the blood lipid and protein profiles, since it significantly decreased the mean fasting serum LDL level, and significantly increased the mean fasting serum HDL and total protein levels. These actions were attributed to the high and low contents of PUFAs and SFAs, respectively, in hemp seeds, as well as to their relatively high amount of phytosterols, especially β-sitosterol. Actually, Seo and colleagues (Seo et al., 2012) showed that also hydrophilic components of hemp seeds could exert an anti-atherosclerotic effect. In their study, the authors treated via intragastric inoculation Apo-E Knock Out (KO) mice atherosclerotic animal models, with a hemp seed water extract for 14 weeks. The lack of Apo-E in these genetically modified mice led to increase in total plasma cholesterol and triglycerides and decrease of serum HDL-cholesterol level and, as consequence, generated atherosclerotic lesions similar to those found in humans. The treatment with hemp seed water extract reduced the atherosclerotic plaque formation, as well as the serum level of total cholesterol and LDL-cholesterol and increased the serum HDL-cholesterol level. Moreover, a reduction in the serum level of the inflammatory cytokine TNF-α demonstrating a decrease in the inflammatory status related to atherosclerosis, was also found. As previously said, all these findings have been generally attributed to the hemp seed lipidic fraction, that is missing in the hemp seed water extract used in this study, and therefore, the authors concluded that some hydrophilic compounds of hemp seed, presumably phenolics, possessed the observed anti-atherosclerotic action, but the specific compounds and the molecular mechanisms by which they exerted this protective action still need to be elucidated.

The ability of the dietary hemp seeds supplementation to reduce the dietary cholesterol uptake in the presence of an enriched-cholesterol diet, was observed also by Lee and co-workers (Lee et al., 2011) in *Drosophila melanogaster* and was attributed to the ability of some hemp seed components, PUFAs LA and ALA *in primis*, to down-regulate the expression of the Niemann-Pick C1-Like 1 (NPC1L1) gene, coding for a key intestinal cholesterol absorption protein. By contrast, Prociuk and colleagues (Prociuk et al., 2008) found that 8 weeks of 10% hemp seeds supplementation of a cholesterol-enriched diet in albino New Zealand rabbits did not decrease the cholesterol plasma level but led to an approximately 12-fold increase in the plasma GLA level, resulting in a normalization of the platelet aggregation. Indeed, it was shown that the enriched-cholesterol diet without hemp seeds supplementation increased the cholesterol plasma level and the platelet aggregation, raising the risk of acute

coronary syndrome. The addition of hemp seeds to the diet was however able to inhibit the platelet aggregation despite the presence of high circulating cholesterol, and this effect was exactly attributed to the high GLA plasma level. All these results that showed an overall beneficial effect of hemp seeds dietary supplementation on atherosclerosis are still in contrast to those found by Gavel and co-authors (Gavel et al., 2011). They investigated the effect of the addition of 10% hemp seeds to the cholesterol-enriched diet of albino New Zealand rabbits on atherogenesis and aortic contractile function, and they found that this dietary supplementation would not be able to protect the aortic vessel from the atherosclerotic plaque formation. In this case, the hemp seeds supplementation provided only mildly beneficial effects against contractile disfunction associated with atherosclerotic vessel.

In female subjects, the risk of coronary heart disease and other CVDs related to the rise of the concentration of circulating total cholesterol and LDL-cholesterol, can increase as a consequence of menopause, which is associated also to other relative complications related to the oestrogen deficiency, like depression, anxiety, and possibly impaired cognition. In this context, Saberivand and colleagues (Saberivand et al., 2010) demonstrated that whole hemp seeds dietary supplementation (until 10%) in a balanced diet of the ovariectomized rat model of menopause, may improve the menopausal complications including the cardiovascular ones. In particular, the authors have shown that 3 weeks of dietary supplementations with 1%, 2%, or 10% of hempseeds were able to conserve the triglycerides and total cholesterol plasma level close to the control group (non-ovariectomized rats), preventing the dyslipidaemia and the weight gain that usually occurs after ovariectomy (or menopause) as a consequence of the decline of the oestrogen levels. Indeed, it was found that the decline of plasma oestrogen levels was prevented by the hemp seeds dietary treatment, probably thanks to the presence of phytoestrogens, such as β-sitosterol, in hemp seeds. In addition, the authors observed that the hemp seeds dietary supplementation was also capable of exerting an antidepressant activity, as well as of preventing the accrual of blood calcium and thus preventing the bone loss and promoting the bone homeostasis.

As previously underlined, along with hypercholesterolemia, hypertension represent another main CVDs risk factor. It is defined as a systolic blood pressure (SBP) higher than 140 mmHg or a diastolic blood pressure (DBP) higher than 90 mmHg (Girgih et al., 2014a). The *in vitro* studies which demonstrated the existence of hemp seed peptides with anti-hypertensive properties (see Section 2.2.2) led researchers to evaluate the *in vivo* hypotensive ability of hemp seed products on the spontaneously hypertensive rats (SHRs). This animal model is characterized by high plasma ACE and renin enzyme activities as well as a decrease in the antioxidant defence and higher oxidative stress in comparison to the normotensive rats. A diet supplemented with 1% of hemp seed proteins hydrolysate was found to be effective both to prevent the hypertension development in SHRs and to act as treatment to reduce the SBP in SHRs with established hypertension through the reduction of the plasma ACE activity (Girgih et al., 2014a). Moreover, the same supplemented diet led also to a reduction of the oxidative stress in SHRs normalizing the plasma total antioxidant capacity, which reached a value similar to normotensive rats, and enhancing the cellular antioxidant defences, namely the catalase and superoxide dismutase enzymes' activity (Girgih et al., 2014). The anti-hypertensive and antioxidant effects were not so marked in the SHRs fed a diet supplemented with 1% hemp seed protein isolate instead of the hemp seed protein hydrolysate. This evidence was explained with the fact that the already hydrolysed peptides are more bioactive or more bioavailable than those obtained by the gastrointestinal digestion of hemp seed proteins (Girgih et al., 2014; Girgih et al., 2014a), suggesting the advantage of the use of hemp seed protein hydrolysate rather than whole hemp seed or hemp seed proteins isolate as an ingredient to formulate functional foods or nutraceuticals for the prevention and treatment

of hypertension. Interestingly, the hypotensive effect of these hemp seed products was not observed in normotensive rats, suggesting an action directly related to the higher ACE plasma activity than the physiological level (Girgih et al., 2014a).

Other animal studies were performed to explore the potential beneficial effect of the dietary intake of hemp seeds and their products on neurodegenerative diseases, generally associating with aging, oxidative stress, and neuroinflammation, such as AD, cognitive deficit, memory impairments and multiple sclerosis (MS). Most of these studies were carried out after that epidemiological evidence highlighted that some Chinese populations, which habitually consume hemp seeds as an integral part of their daily diet, are characterized by significant numbers of long-lived centenarians' individuals with good cognition and few health problems (Chen et al., 2017; Li et al., 2018). About this, Lee and co-workers (Lee et al., 2011) found that a *Drosophila melanogaster* model of AD reared in a hemp seed meal media, significantly suppressed the cytotoxicity of amyloid β42 (Aβ42) peptides accumulation, ameliorating the eye degenerative phenotype, and this neuroprotective effect of hemp seed meal was attributed to the PUFAs LA and ALA. Further evidence has demonstrated that the daily inclusion and long-term consumption of hemp seeds into the diet may promote the longevity and the lifespan extension; may improve cognitive function; and may prevent age-related diseases, including obesity, diabetes, and cancer in old mice, as a consequence of the adjustment of some nutritional imbalances typical of the western diet (*e.g.*, the n-6/n-3 PUFAs ratio), and the capability to regulate the expression of genes involved in the regulation of 5′AMP-activated protein kinase (AMPK) signalling that can reduce the oxidative stress and can inhibit the inflammatory response induced by the NF-κB pathway (Li et al., 2018). It has been also shown that daily oral administration of hemp seed oil on an MS mice model can have positive effects on the treatment of this chronic-inflammatory and neurodegenerative disease characterized by wide demyelination, since it can exert immunomodulatory action, in particular the anti-inflammatory one, and can improve the re-myelination of neuronal myelin sheath in the brain, with a protective effect on the central nervous system (CNS) (Rezapour-Firouzi et al., 2018). This protective effect of hemp seed oil in CNS tissues seems to be a consequence of (i) the increase in the intake of n-3 PUFAs and the n-6 anti-inflammatory GLA and of (ii) the reduction of the n-6/n-3 PUFAs ratio and SFAs intake, which are all factors able to regulate the mTOR signalling pathway in order to shift the immune response from pro- to anti-inflammatory one, thus reducing the chronic inflammation, which is an important factor for MS onset and development (Rezapour-Firouzi et al., 2019, 2018). However, in many of the reports that demonstrated the neuroprotective effect of hemp seeds or derivatives dietary supplementation, the hemp seeds' supplement was not the only one administrated. In fact, hemp seeds were often administrated in combination with other products such as bitter vegetable (*Sonchus oleraceus*) (Li et al., 2018) or evening primrose oil (Rezapour-Firouzi et al., 2019, 2018), and therefore, based only on these data, it is not possible to understand whether the beneficial effects can be obtained also with the solely ingestion of the hemp seeds supplement or whether they are closely related to a co-operative action of the both administrated supplements.

Hence, although different evidence of an effective beneficial action of hemp seeds or hemp seed derivatives on CVDs and neurodegenerative disease animal models is present in scientific literature (**Figure 8**), further studies are needed to clarify the exact molecular mechanisms involved, as well as the type and the doses of the more efficient hemp seed product which are necessary to exert the *in vivo* beneficial function, all essential factors to develop a potentially functional food.

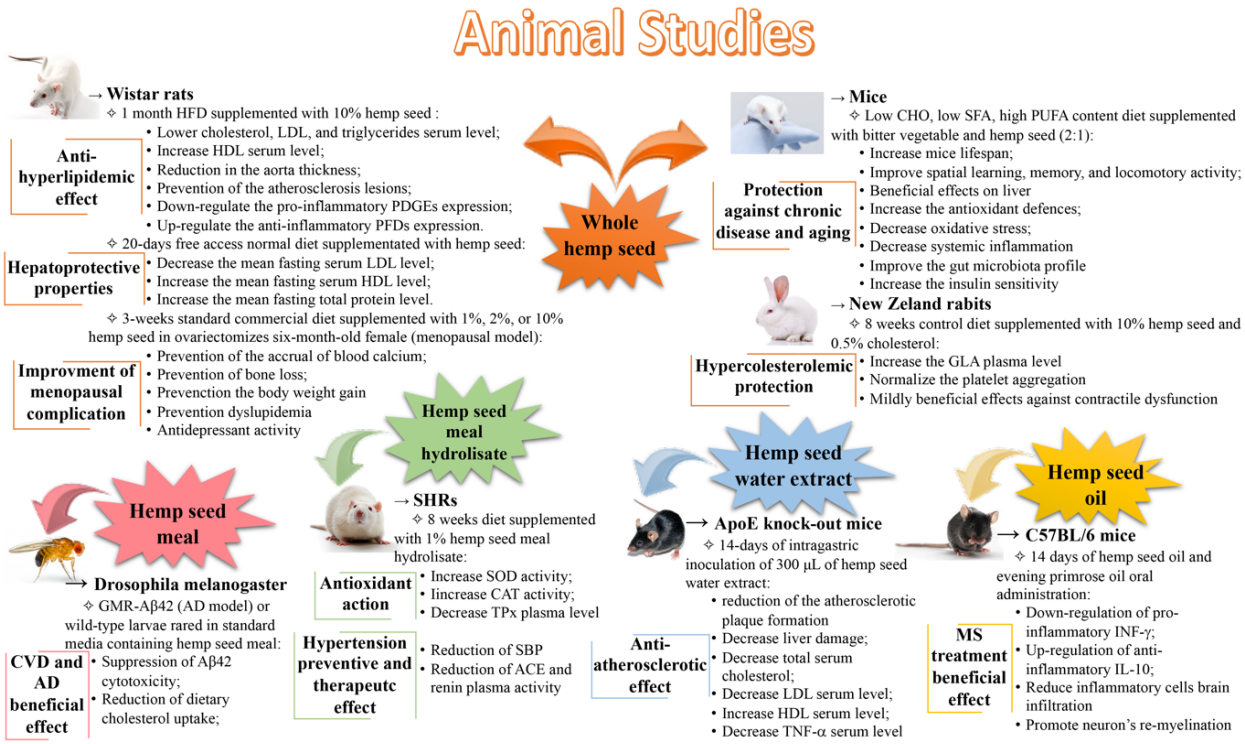

Figure 8: Literature studies on the effect of hempseed or hempseed products' dietary supplementation on animal models. See the main text for more details. ACE: angiotensin I-converting enzyme; AD: Alzheimer Disease; CAT: Catalase; CHO: Carbohydrate; CVD: Cardiovascular Disease; GLA: γ- linolenic acid; GOT: glutamic oxaloacetic transaminase; HDL: High Density Lipoprotein; IFN-γ: Interferon γ; LDL: Low Density Lipoprotein; MS: Multiple Sclerosis; PGD: Prostaglandin D; PGE: Prostaglandin E; PUFA: Polyunsaturated Fatty Acid; SBP: Systolic Blood Pressure; SFA: Saturated Fatty Acid; SHRs: Spontaneously Hypertensive Rats; SOD: Superoxide dismutase; TG: Triglyceride; IL: Interleukin; TNF-α: Tumor Necrosis Factor α; TPx: Total Peroxide.

2.3.2. Human studies

Although the studies about hemp seeds and hemp seed products dietary supplementation in animals are relatively abundant in scientific literature, only few papers have faced the issue of the hemp seeds and derivatives dietary supplementation in human nutrition and its effects on human health, and all of the few investigations performed to date, were carried out exclusively with hemp seed oil as a supplement.

One of the first studies which investigated the beneficial effect of hemp seed oil dietary supplementation was that of Callaway and co-workers (Callaway et al., 2005) in which 8-weeks oral consumption of 30 mL/day of hemp seed oil in patients with diagnosis of atopic dermatitis was adopted to evaluate the potential ability of this oil to ameliorate the patients' symptomatology and medicinal usage. The results of this research highlighted that, contrarily to the olive oil treatment used as comparison, the hemp seed oil dietary assumption can effectively improve the skin quality and the pathology's symptoms leading to decrease of skin dryness, itchiness, and dermal medication usage by patients. The authors attributed these findings to the increase in the GLA, SDA, and LA plasma levels as a result of the hemp seed oil supplementation. Indeed, both GLA and SDA have anti-inflammatory action which can be beneficial for atopic dermatitis. Particularly, GLA has been shown able to decrease the production of the pro-inflammatory leukotriene B4 (LTB4) by polymorphonuclear neutrophils (PMN), through increasing the plasma level of its biological metabolite, namely the anti-inflammatory DGLA which can directly suppress the LTB4 PMN's production in the skin. Furthermore, SDA can increase the level of EPA in both erythrocytes and plasma phospholipids contributing to the anti-inflammatory effect, whereas the increase of the LA plasma level can raise the ceramide amount in the skin's stratum corneum, reducing the loss of moisture and improving the skin's dryness, since LA acts as precursor of ceramide 1, an important component of the skin's lipid lamellar sheet. Finally, the total plasma PUFAs amount of the patients significantly increased after the oral daily assumption of hemp seed oil, and this can have facilitated the formation of new skin cells by dermal stem cells, contributing to the obtained amelioration of atopic dermatitis.

Other works employed the hemp seed oil as a dietary treatment/supplementation to explore its potential cardiovascular benefits on humans. In this regard, Schwab and colleagues (Schwab et al., 2006) observed that 4-weeks daily supplementation of 30 mL of hemp seed oil was able to reduce the total TGs plasma level and to induce significant changes in the composition of serum cholesteryl-esters (CEs) and TGs of healthy volunteers. Particularly, the content of LA, ALA, and GLA increased whilst the OA concentration significantly decreased after the hemp seed oil treatment. In contrast, the proportion of AA and DHA in both TGs and CEs did not change after hemp seed oil intake as well as the plasma glucose, insulin, and haemostatic factors, including fibrinogen, coagulation factor VIIa (FVIIa), and plasminogen activator inhibitor-1 (PAI-1). Interestingly, the hemp seed oil treatment led to reduction of the TC/HDL-cholesterol ratio which is considered an important index to predict the risk of coronary heart disease. Contrarily, Kaul and colleagues (Kaul et al., 2008) found that the daily assumption of 2 capsules, each containing 1 g of hemp seed oil, in healthy volunteers for 12 weeks neither significantly affected any blood lipid parameters (*i.e.*, the TGs, TC, LDL, and HDL plasma levels), nor induced any changes in the LA and ALA plasma concentrations as well as in the inflammatory and platelet aggregation markers, namely TNF-α and C-Reactive Protein (CRP), and collagen and thrombin-induced platelet aggregation, respectively. The authors hypothesized that these findings could be due to a limited and low gastrointestinal absorption of hemp seed oil's LA and ALA, or to the fact that a larger dosage and a longer experimental period may be necessary to obtain significant changes in the evaluated parameters. It is also important to note that these studies have been performed

on healthy subjects, and therefore, they do not exclude potential beneficial actions in a specific pathological status. Furthermore, this evidence also introduces the importance and the necessity of identifying the appropriate dosage and vehicle/matrix that will have benefits in healthy people too as a preventive tool. In the context of the usage of hemp seeds or their products for a specific pathological target groups, a recent report of Del Bo' and colleagues (Del Bo' et al., 2019) explored the effects of hemp seed oil supplementation in the modulation of hyperlipidaemia and CVDs risk in children and adolescents (6–16 years old) with primary hyperlipidaemia. In more detailed, the test group was daily supplemented with one hemp seed oil soft capsule containing 3 g of oil, which provided 700 mg of ALA, 1400 mg of LA, 30 mg of SDA, and 100 mg of GLA, with 2.3 g total PUFAs and a PUFAs/SFAs ratio of 7.7:1. As a consequence of this treatment, it has been shown that, although the hemp seed oil supplementation after 8 weeks did not significantly change the plasma lipid profile of the volunteers, it was able to improve the red blood cell (RBC) FAs composition, significantly increasing the total n-3 PUFAs, including the LC-n-3 PUFAs EPA, DPA, and DHA and decreasing the n-6/n-3 PUFAs ratio, as well as the total amount of SFAs and MUFAs. Considering that an altered FAs composition in RBCs has been shown to be positively related with coronary heart disease, atherosclerosis-related diseases, arterial hypertension, and dyslipidaemia (Novgorodtseva et al., 2011), and that SFAs have been reported to have hypercholesterolemic activity in adults, the observed effect of hemp seed oil supplementation could have a contribution in counteracting the negative consequences of hyperlipidaemia on the cardiovascular system. Interestingly, in this work was also noted that the dietary intake of higher ALA amounts led to an increase in the RBCs' LC-n-3 PUFAs derived by the ALA conversion (EPA, DPA, and DHA) in comparison to the control group, and this evidence could indicate that hemp seed oil supplementation may stimulate the endogenous conversion of ALA into its biologically active derivatives. According to the authors and in agreement with Kaul and colleagues (Kaul et al., 2008), the lack of differences between the control and the test group on the serum lipid markers, may be dependent on the food matrix/vehicle used and/or the length of the intervention. This evidence has again highlighted the importance to understand the molecular mechanisms through which hemp seed components can exert their beneficial effect on human health, the level of each functional component needed in order to obtain an effective and clinically detectable action, and the more suitable matrix able to maximize the gastrointestinal bioavailability and structurally preservation of these components in the human body.

Finally, another three literature works about the hemp seed oil dietary supplementation in humans have been performed on patients affected by MS (Rezapour-Firouzi et al., 2014, 2013b, 2013a). As described in the previous subsection (Section 2.3.1), the same authors have used an MS mouse model in order to understand the molecular mechanisms underlying the beneficial effects of the co-supplementation of hemp seed oil and evening primrose oil on this neurodegenerative, immune-mediated pathology (Rezapour-Firouzi et al., 2019, 2018). In previous works performed on humans, these authors observed that the co-supplementation of hemp seed oil and evening primrose oil in a 9:1 ratio, used alone, or in association to a "hot-natured" diet, was able to ameliorate the inflammatory status of MS patients, thus exerting a beneficial effect on this pathological condition. In particular, the authors explained that MS is a Th1-mediated autoimmune disease, which therefore, is characterized by an unbalance Th1/Th2 immune responses and high levels of pro-inflammatory IL-6, IFN-γ and IL-17 cytokines. This latter cytokine is produced by the Th17 cells which, hence, are involved in the development of the pathology, together with Th1 cells (Rezapour-Firouzi et al., 2013b, 2013a). A "hot-natured" diet is characterized by the consumption of foods with low cholesterol, hydrogenated, *trans*, and saturated FAs; a plenty of fruits and

vegetables, nuts and seeds without additives, olive and grapes oils as the main oils of the diet, fish and seafood, and unrefined carbohydrates and dairy products with honey; contrarily, it excludes the consumption of sugar and refined carbohydrates, alcohol, and smoke. It has been shown that the consumption of the "hot-natured" diet is related to a deviation of the immune system toward the Th2 responses. In addition, both hemp seed oil and evening primrose oil possess anti-inflammatory features, related to the high PUFAs content and the perfectly balanced n-6/n-3 PUFAs ratio of the former and to the about 9% of GLA content of the latter. Based on all this evidence, Rezapour-Firouzi and co-workers in their studies decided to adopt the "hot-natured" diet co-supplemented with 18–21 g of hemp seed oil and evening primrose oil in a 9:1 ratio, as a potential useful dietary intervention for the treatment and attenuation of MS (Rezapour-Firouzi et al., 2013b, 2013a). The authors found that both types of dietary intervention (*i.e.*, the "hot-natured" diet co-supplemented with hemp seed oil and evening primrose oil, and the co-supplementation of hemp seed oil and evening primrose oil alone) for 6 months have exerted an effective anti-inflammatory action, leading to a significant increase in the IL-4 cytokine level on MS patients and to an improvement of the degree of the Th2/Th1 ratio, the expanded disability status scale (EDSS), and the relapse rate, which are all indexes of the disease severity, and thus performed a beneficial action in the patients, without exhibiting any side effects (Rezapour-Firouzi et al., 2014, 2013b, 2013a). However, only the "hot-natured" diet associated with the hemp seed and evening primrose oils co-supplementation resulted in having also the ability to significantly decreased the level of the pro-inflammatory IFN-γ and IL-17 cytokines. Indeed, contrarily, the hemp seed oil and evening primrose oil co-supplementation alone showed a decreased trend in the level of these cytokines, which though, was not statistically significant, at least after 6 months of the treatment (Rezapour-Firouzi et al., 2013b). In a later report, the same authors (Rezapour-Firouzi et al., 2014) also demonstrated that both "hot-natured" diet co-supplemented with hemp seed and evening primrose oils and the co-supplementation of hemp seed and evening primrose oils alone were able to improve the liver function in the MS patients, which generally showed a serious liver injury as a consequence of the drug treatment, but the former dietary intervention was more efficient than the latter.

In conclusion, in addition to the fact that there is still a lack of studies about the effects of whole hemp seed dietary intake on either healthy human subjects or patients with a specific pathological status, to date, the few works present in the scientific literature (**Figure 9**) are referred exclusively to hemp seed oil supplementation and they are rather inconclusive and not very comparable due to the employment of different types of experimental designs, as well as different dosages, experimental periods, and administration methods of the supplement. Therefore, it appears necessary to deepen and expand the research and the knowledge in this area.

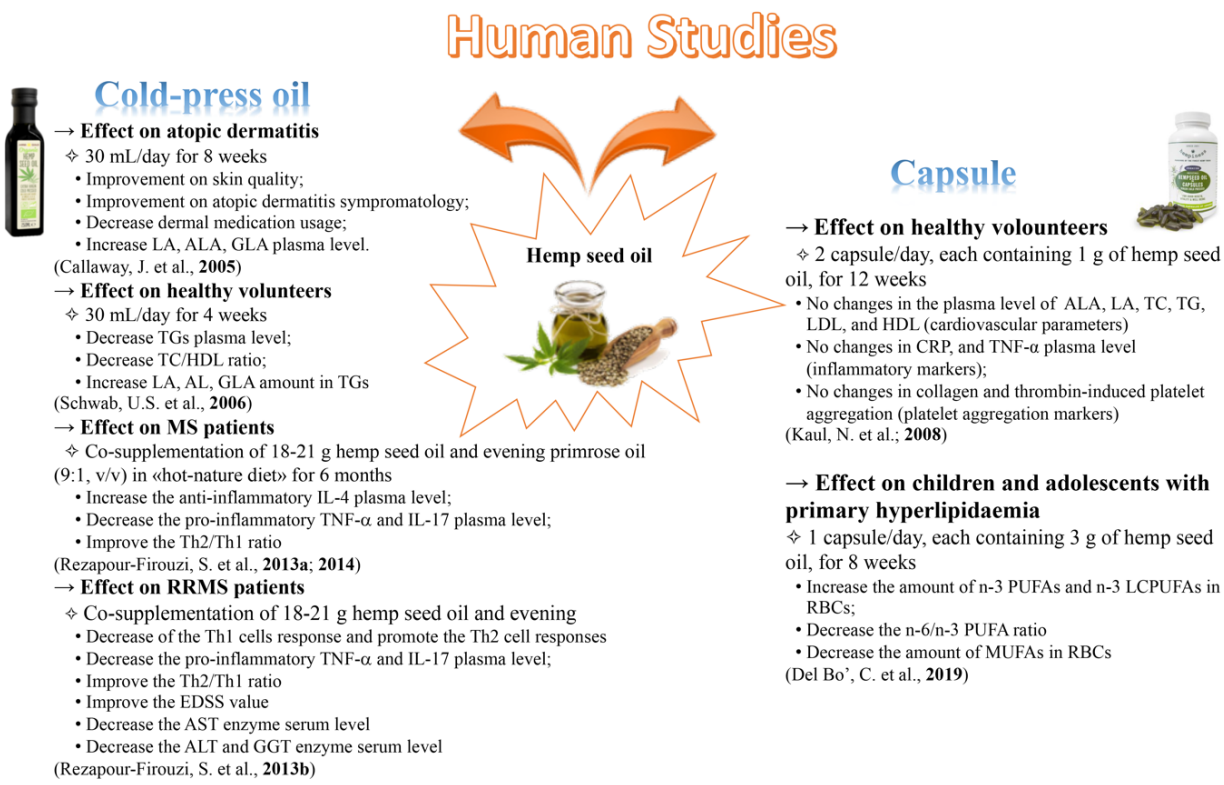

Figure 9: Literature studies on the effect of hempseed or hempseed products' dietary supplementation on humans. ALA: α-linolenic Acid; ALT: (SGPT) alanine-aminotransferase; AST: aspartate- aminotransferase; EDSS: Extended Disability Status Score; GGT: gamma-glutamyl transferase; GLA: γ-Linolenic Acid; HDL: High Density Lipoprotein; LA: Linoleic Acid; LCPUFAs: Long Chain Polyunsaturated Fatty Acids; LDL: Low Density Lipoprotein-Cholesterol; MS: Multiple Sclerosis; MUFAs: Monounsaturated Fatty Acids; PUFAs: Polyunsaturated Fatty Acids; RBCs: Red Blood Cells; RRMS: Relapsing-Remitting Multiple Sclerosis; SFAs: Saturated Fatty Acids; TC: Total Cholesterol; TGs: Triglycerides.

2.4. Hemp Seed in the Food Industry

In the field of food industry and technology, due to their unique nutritional quality and composition, hemp seeds have been exploited as ingredient or means to ameliorate the nutritional and healthy grade of food, in order to obtain healthy and potentially functional foods. In this context, the first efforts in the employment of these seeds by the food industry concerned their use as a means to increase the quality of animal derived food products, namely the use of hemp seed or hemp seed's derivatives (*i.e*., hemp seed oil or flour/cake/meal) as livestock feed supplement to ameliorate the quality composition of animal-based food products (*e.g*., meat, eggs, and milk). Only later, hemp seeds and their derivatives have also been used as ingredient to directly enrich daily consumed foods (*e.g*., bakery products).

2.4.1 Hemp seed as feed

As means to increase the quality of animal-derived food products, hemp seeds and hemp seed derivatives, especially hemp seed oil, were used as supplements of the laying hens or broiler chickens' normal diets to obtain eggs or meat enriched with n-3 PUFAs, including the n-3 long chain PUFAs (LCPUFAs) biologically active in humans, like EPA and DHA, due to the ability of the laying hens or broiler chickens to endogenously convert dietary ALA into these LCPUFAs and to deposit them into egg yolk or muscle tissues. In this context, several experiments were performed to assess either the safety of hemp seeds or hemp seed derivatives as feed ingredients in poultry diets as well as their efficacy as means to nutritionally ameliorate the FAs profile of egg yolk and meat, as an alternative to other n-3 PUFAs sources such as flaxseed, flaxseed oils, or marine oils, and microalgae products. Overall, these studies demonstrated that the performance of laying hens and broiling chickens, including feed intake, egg weight, egg production, feed conversion ratio, body weight, or weight gain, was not affected (Gakhar et al., 2012; Jing et al., 2017; Mierliță, 2019; Neijat et al., 2014) or was ameliorated (Bazdidi et al., 2016) by the dietary supplementation with hemp seeds (up to 30%) or hemp seed oil (up to 12%), and therefore, these products can be included in the bird's diet without compromising their safety and efficacy. Interestingly, the hens' performance was more positive if hemp seeds were subjected to a heat treatment (specifically 120 °C for 60 min) performed before to supplement the hens' diet (Konca et al., 2019). The safety of the inclusion of these supplementary products to poultry diet was further confirmed by the absence of the markers of hepatic and muscular damage after the hemp seed product supplementation (Bazdidi et al., 2016; Neijat et al., 2014). In addition, literature reports agree that the inclusion of hemp seed products to the poultry diet effectively improved the nutritional value of the eggs and meat increasing their total n-3 PUFAs amount and PUFA/SFA ratio and resulting in a better-balanced n-6/n-3 PUFAs ratio in comparison to the poultry food products obtained by animals fed a normal diet. More specifically, it has been demonstrated that the total n-3 PUFAs, the individual n-3 PUFA ALA, as well as the n-3 LCPUFAs biologically active in humans, namely EPA, DPA (Docosapentaenoic Acid), and DHA, were increased in both poultry eggs (Gakhar et al., 2012; Goldberg et al., 2012; Jing et al., 2017; Mierliță, 2019; Neijat et al., 2016a, 2016b; Raza et al., 2016; Shahid et al., 2015) and meat (thigh and breast) (Jing et al., 2017) as the inclusion of the hemp products rose and the n-6/n-3 PUFAs ratio, normally high in common eggs, was reduced [152,157,158,161]. Neijat and colleagues found that, as consequence of the dietary hempseed and hempseed oil supplementation to the laying hens' control diet (wheat-barley-soybean meal-based), the ALA content increased in a dose-dependent manner and this increase was higher in the TGs than in the phospholipids (PLs) that, therefore, seem to be less responsive in ALA enrichment in comparison to TGs (Neijat et al., 2016b).

The authors found also an increase in the total amount of n-3 LCPUFAs synthesized from ALA (*i.e.*, EPA, DPA, and DHA) in response to the increase in the ALA dietary intakes, reflecting the endogenous synthesis from ALA in hens' liver and the subsequent deposition in egg yolk. However, contrarily to ALA, this growth did not occur in a dose-dependent fashion for LCPUFAs, such as DHA, which in fact, was accumulated mainly in PLs, until a saturation level (Neijat et al., 2016a, 2016b). Regarding this, it is important to consider that the conversion of ALA to DHA occurs through the sequential actions of the enzymes Δ-6 desaturase encoded by the fatty acid desaturase 2 (FADS2) gene, elongase encoded by the elongase 5 (ELOVL5) gene, and Δ-5 desaturase encoded by the fatty acid desaturase 1 (FADS1) gene (**Figure 6**). Gakhar and co-authors evaluated the effect of the consumption of hemp seeds or hemp seed oil by hens on the expression of these three main biosynthetic genes involved in the PUFAs metabolism and found that the FADS1 and FADS2 expressions were reduced by the increasing inclusion of hemp seed products in laying hens' diets, thus providing a possible explanation to the DHA saturation in the egg yolk of hens supplemented with hempseed products (Gakhar et al., 2012). Interestingly, Jing and colleagues showed that the increase in the n-3 PUFAs ALA, EPA, DPA, and DHA occurred both in the egg yolk and in the meat of laying hens' and broiler chickens fed a hemp seed oil supplemented diet, but this increase took place to a different extent based on the food product (Jing et al., 2017). Indeed, in comparison to the respective control, the enrichment efficiency for total n-3 PUFAs, ALA, and EPA appeared to be higher in egg yolk than in chicken meat. Furthermore, the DPA amount was higher than DHA in meat, contrarily to egg yolk, where the DHA level was higher than DPA even without supplementation, and after hemp seed oil supplementation, the difference between DPA and DHA content in egg yolk or meat became more evident. This finding was attributed to a differential expression and activity of FADS2 and ELOVL5 enzymes that are involved in the conversion of DPA to DHA rather than an effect of the supplementation. In particular, based on the results, it was hypothesized that these enzymes may be low in the muscle tissues of chicken and thus, inhibiting the metabolism of DPA to DHA. Interestingly, Mierliță and colleagues (Mierliță, 2019) found that the egg yolk produced by hens fed a diet supplemented with whole hemp seeds contained a higher ALA and EPA amount and a more remarkable decrease in n-6/n-3 PUFAs ratio in comparison with the egg yolk produced by hens fed a diet supplemented with hemp seed cake. Hence, the authors concluded that whole hemp seeds supplementation was more effective in ameliorating the nutritional quality of egg yolk than the hemp seed cake one.

With regard to the effect of hemp seed products supplementation on the n-6 PUFAs content of the poultry eggs and meat, Shahid and co-workers found an increase in the total n-6 PUFAs amount of egg yolk (Shahid et al., 2015), whereas, Neijat and co-authors observed that, although the level of n-6 LA was higher especially in the TGs, in comparison to the control, the n-6 AA amount decreased as the hemp dietary inclusion increased, reflecting the metabolic competition of n-3 ALA and n-6 LA for the Δ-6 desaturase enzyme, responsible to their conversion in the corresponding LCPUFAs, with the greater affinity of n-3 ALA than n-6 LA, and a consequent favour of n-3 PUFAs metabolism (Neijat et al., 2016b). Similar data was found also by Mierliță and colleagues (Mierliță, 2019). Furthermore, it has been also observed that among n-6 PUFAs, the hemp seed products supplementation led to an enrichment of eggs and meat with the GLA (Jing et al., 2017; Park et al., 2014), conferring to these food products a further unique features related to the biological anti-inflammatory and anti-proliferative properties of GLA that have important implications for human health. Similar GLA-enrichment was also shown in the muscle of ducks fed a 15-20% hempseed cake-supplemented diet (Juodka et al., 2018).

Regarding MUFAs and SFAs, literature publications agree that the MUFAs content, mainly OA, decreased with the increase of the level of the hemp seed products to the diet, probably due to the inhibition effect of a PUFAs-rich diet on the activity of Δ-9 desaturase, which is essential for the conversion of PA to OA (Jing et al., 2017; Konca et al., 2019; Mierliță, 2019; Neijat et al., 2016b). Whereas, about the effect on SFAs there are some contrasting results. Some authors found a reduction in the SFAs (Konca et al., 2019; Raza et al., 2016), as well as in the cholesterol amount (Park et al., 2014) in egg yolk after hemp seed supplementation. Other researchers (Mierliță, 2019; Neijat et al., 2016b) showed that the overall levels of SFAs and cholesterol of egg yolk were not influenced by a dietary treatment with hemp seeds, hemp seed oil, or hemp seed cake.

Similar findings about the positive effect of the hemp seed or derivative dietary supplementation on the nutritional quality of egg yolk and meat were also shown for quails (Yalcin et al., 2018). In this case, 6-week supplementation with up to 20% whole hemp seeds, allowed to obtain n-3 PUFAs enriched quail eggs with a reduction of MUFAs – OA in particular – and SFAs – PA in particular – as an additional advantage. In the same study, the quality of the meat of the 5-week whole hemp seed supplemented quails was also analysed, and a decrease in thawing and cooking loss were observed with the increase of the dietary hemp seed amount, demonstrating that hemp seeds supplementation can ameliorate also the meat quality other than the eggs FA profile. In addition to the safety for animals and the efficacy in obtaining more nutritionally beneficial food products, the inclusion of hemp seed products into the poultry diet was tested also for the potential negative impacts on the sensory characteristics of the resultant products. Indeed, it was found that dietary supplement as flaxseed or fish oils can compromise the sensory quality of the poultry meat and eggs due to the presence of fish smell/taste. Instead, Goldberg and colleagues demonstrated that the inclusion of up to 20% hemp seeds or up to 10% hemp seed oil in the poultry's diet did not have any adverse effects on the eggs' sensory quality (Goldberg et al., 2012). Similar results were obtained by Konca and co-workers [155] for eggs produced by hens supplemented with 15% raw or heat-treated whole hemp seeds for 12 weeks (Konca et al., 2019).

Hemp seeds supplementation in animals' feed has been shown to be a useful tool also to improve the yield, the quality, and the nutritional value of some milks, such as the goat and sheep ones, and in particular, to positively modify the milk's FAs content. This by increasing the total concentration of LCPUFAs, n-3 PUFAs, and conjugated linoleic acids (CLAs), and concomitantly by decreasing the concentration of SFAs and the n-6/n-3 PUFAs ratio, with a putative beneficial effect on human health (Iannaccone et al., 2019; Mierliță, 2016; Pojić et al., 2015). Mierliță (Mierliță, 2018) found also an increase in the tocopherol amount and an improvement of total antioxidant capacity of milk obtained from sheep fed a diet supplemented with hemp seed (180 g/day) or hemp seed cake (480 g/day), in comparison to that obtained by sheep fed a control diet (hay diet completed with high-energy concentrate and sunflower meal), thus demonstrating that despite the increase of the PUFAs content in milk, the safety of this product was not negatively affected by lipid oxidation. The same author also showed that, between whole hemp seeds and hemp seed cake supplementation, the latter was more effective in ameliorating the quality and the nutritional value of the milk, as it was resulted in a significantly lower atherogenic index and a higher hypocholesterolemic/hypercholesterolemic index. Cozma and colleagues (Cozma et al., 2016) found a reduction in the lactose content of the milk produced by goat fed a diet supplemented with hemp seed oil (93 g/day). Contrarily, Iannaccone and co-workers (Iannaccone et al., 2019) showed an up-regulation in the genes involved in lactose biosynthesis, based on the results of RNA sequencing analysis of the whole-blood

transcriptome of lactating sheep fed either a control diet or a 5% hemp seed supplemented diet, and this result was in accordance with the increase in the lactose amount found in the resulting milk.

2.4.2 Hemp seed as ingredient for enriched/fortified foods

As means to directly enrich or fortify daily foods, hemp seeds and their derivatives have been assessed as ingredient added to daily consumed foods, including bakery products such as bread, cookies, crackers, and energy bars, as well as meat and meat products.

Regarding the enrichment of bread with hemp seeds or derivatives, some works have evaluated the impact on the nutritional, structural, and textural characteristics of both dough and bread, consequentially to the addition of hemp seeds or their derivatives. From a technological/rheological point of view, the main issue of the usage of non-conventional ingredient in the bakery industry in order to fortify the bakery products, is their adverse effect on the dough rheological and baking properties, mainly as a result of the dilution of wheat gluten proteins. Consequently, this can negatively affect the end product quality (volume, texture, crumb and crust properties, and colour) that may ultimately affect lowering the consumers' acceptability. Studies about the enrichment of the bread dough with hemp seed flour agree that the addition of hemp seed flour in the wheat flour significantly reduced the dough's water absorption, stability, strength, and starch gelatinization, in a proportional manner to the level of hemp flour added, as a consequence of the dilution of gluten amount in the dough (Pojić et al., 2015; Švec and Hrušková, 2015a, 2015b). Pojić and colleagues (Pojić et al., 2015), and Švec and co-authors (Švec and Hrušková, 2015b) found that the decrease in the water absorption of the bread dough had a negative impact also on the time required for dough development, but nevertheless, the dough's stability and strength were not significantly affected by the addition of up to 10% of hemp seed flour. Pojić and others (Pojić et al., 2015) also noted that the increase in the hemp seed flour content up to 20%, negatively affected both the dough's stability and strength.

The changes in the dough's rheological properties, resulting in the addition of hemp seed flour, led to a negative impact on the bread quality. In this regard, Pojić and colleagues (Pojić et al., 2015) showed that the bread volume was significantly impaired as the level of hemp seed flour increased (**Figure 10**). This result was found also by Hrušková and co-workers and by Švec and colleagues for bread enriched with 10%, 15%, or 20% of hemp seed flour (Hrušková et al., 2013; Švec and Hrušková, 2015b), and was confirmed also by Mikulec and co-authors for bread containing 15%, 30%, or 50% of hemp seed flour (Mikulec et al., 2019) (**Figure 10**), as well as by Wang and colleagues for bread enriched with 5%, 10%, or 15% extruded hemp-rice flour (EHR), made by the extrusion of the whole hemp seeds (30%) together with the rice flour (70%) (Wang et al., 2011). According to the authors, the decrease in the bread's volume could be due to the partial replacement of wheat flour with a non-glutinous one, that may dilute the gluten protein of wheat flour and prevent its extension, or to the increased content of fibre, which reduces the ability to retain fermentation gases.

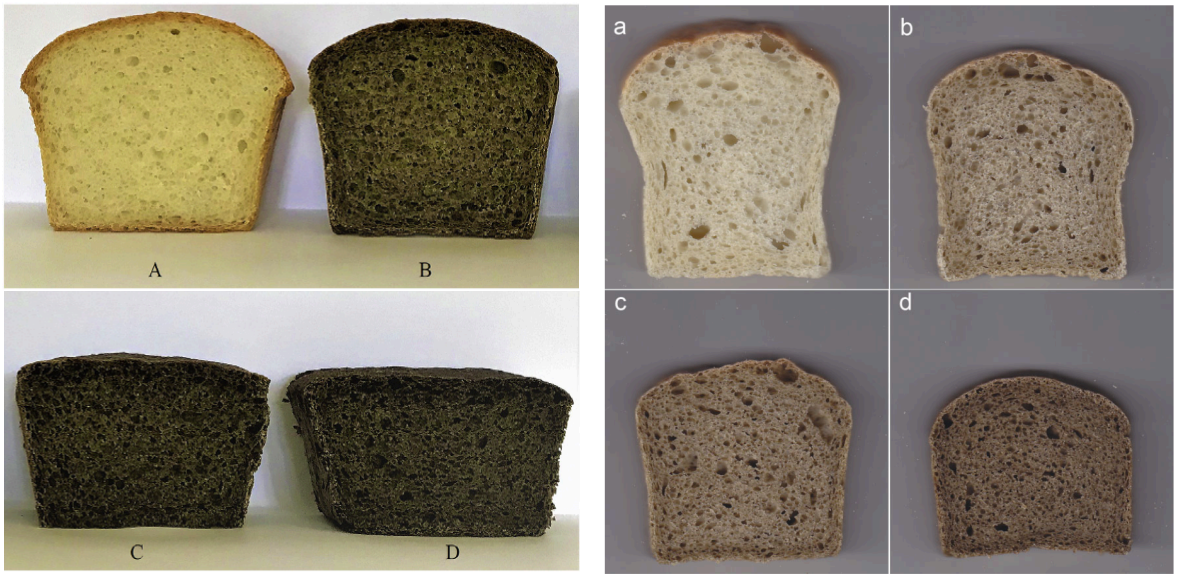

Figure 10: Transversal cut of bread enriched with hemp seed flour obtained by Mikulec et al., 2019 (on the left) and Pojić et al., 2015 (on the right). The higher the dough's hemp seeds flour content, the lower the bread volume, and the darker the bread colour. Left: A: wheat bread; B: bread with 15 % addition of hemp seed flour; C: bread with 30 % addition of hemp seed flour; D: bread with 50 % addition of hemp seed flour. From Mikulec et al., 2019. Right: a: wheat bread; b: bread with 5% addition of hemp seed flour; c: bread with 10% addition of hemp seed flour; d: bread with 20% addition of hemp seed flour. From Pojić et al., 2015.

However, Wang and colleagues showed that the addition of 15% of EHR led to a significantly increase of the bread volume in comparison to 10% EHR-containing bread (Wang et al., 2011). The researchers attributed this evidence to the higher fat content present in the 15% of EHR. This hypothesis was later confirmed by another work of the same authors in which the properties of the dough and the bread made from extruded and non-extruded hemp-rice flour were compared (Wang et al., 2013). The obtained results showed that the presence of whole hemp seeds in the bread dough did not impair the specific bread volume in comparison with the control. A similar finding was noted also by Švec and co-workers who found that the volume of the bread enriched with whole hempseeds powder (*i.e.*, ground whole hempseeds) was higher than those obtained for the breads enriched with other defatted hemp seed products (*i.e.*, hempseed flour or hempseed protein concentrates) (Švec and Hrušková, 2015b). This fact has been attributed to the interactions between lipids and proteins and to the influence of the fat in terms of enhancing dough expansion during proofing (Pojić et al., 2015).

The addition of hemp seed flour to the wheat flour also changed the physical and rheological properties of the bread's crust and crumb. The colour of both of them became darker after the hemp seed flour addition (Mikulec et al., 2019; Pojić et al., 2015; Švec and Hrušková, 2015b; Wang et al., 2011). Švec and colleagues asserted that the 5% enrichment has conferred a pleasant coffee brown colour, which intensified to dark brown by increasing the amount of hemp seed flour (Švec and Hrušková, 2015b), whereas, Wang and co-workers observed a significantly decreased in the lightness of both crust and crumb only for the breads enriched with an EHR amount of at least 10%, since the 5%-EHR bread did not show any significant colour's differences compared to the control

bread (Wang et al., 2011). Similar changes in the crust and crumb bread's colour were observed also after the addition of whole hemp seeds powder (Wang et al., 2013). Interestingly, in this paper, the authors noted that the hemp seed extrusion process and the extrusion temperature can further emphasize the lack of the lightness. Regarding the textural properties of bread crumb, Pojić and co-workers found that the presence of hemp seed flour as an ingredient of the bread's mixture significantly decreased the crumb cohesiveness and increased the crumb chewiness and hardness (Pojić et al., 2015). Analogously, Mikulec and co-authors found an increase in the hardness of the crumb after the addition of hemp seed flour (Mikulec et al., 2019). Contrarily, Wang and colleagues showed that the 15% EHR-bread displayed significantly lower hardness compared to the samples enriched with a lower EHR amount (*i.e.*, 5% and 10%) (Wang et al., 2011), nevertheless, in their later publications, the same authors observed no significantly differences on the crumb hardness in the whole hemp seeds powder-containing breads, and this might be attributed to the influence of hemp oil, which probably enhanced crumb texture and crust tenderness (Wang et al., 2013). Interestingly, Pojić and colleagues found that the presence of hemp seed flour up to 10% in bread formulation did not significantly affect the elasticity and the resilience of the crumb, unlike the addition of 20% of hemp seed flour (Pojić et al., 2015).

Concerning the nutritional quality of the hemp seed flour or whole hemp seed powder-enriched bread, it has been demonstrated that the addition of hemp seed product significantly ameliorated the bread nutritional quality, since it proportionally increased the total protein (Hrušková et al., 2013; Mikulec et al., 2019; Pojić et al., 2015; Švec and Hrušková, 2015b, 2015a) and fat (Pojić et al., 2015) contents, as well as the total, soluble, and insoluble dietary fibre (Švec and Hrušková, 2015b). About this, it is interesting to note that the increase in the dietary fibre was found to be higher if the enrichment was made with whole hemp seed powder rather than hemp seed flour (Švec and Hrušková, 2015b). Furthermore, Pojić and co-workers found that the hemp seed flour enrichment also led to an increase in the content of the macro-elements, especially of Mg and Ca and of the micro-elements such as Mn, Cu, and above all, Fe and Zn. At the same time, the Na amount, the complex carbohydrates, and total metabolizable energy of the bread significantly decreased. This nutritional improvement was more marked for the 20% hemp seed flour-enriched bread (Pojić et al., 2015). Also, an increase in the total phenolic content was observed by Mikulec and others as a consequence of adding the hemp seed flour, and the most abundant polyphenols included epicatechin, ferulic acid, and protocatechuic acid (Mikulec et al., 2019). However, according to Švec and co-workers, the addition of 20% of hemp product (*i.e.*, hemp seed flour or whole hemp seeds powder) has conferred a less acceptable, partially bitter taste to the bread; by contrast, the addition of 10% hemp seed product did not have any negative impact on the sensorial parameters (Švec and Hrušková, 2015b). Mikulec and co-workers found that also the 15% addition level of hemp seed flour had an overall acceptability (Mikulec et al., 2019). Therefore, also considering that the negative impact of the presence of the hemp seed flour on the bread quality seems to be attenuated with 10% enrichment, the literature generally agrees that the 10% or 15% substitution level with hemp seed flour should accomplish requirements in terms of providing the balance between bread quality, sensorial, and nutritional properties.

Since hemp seed is naturally gluten-free, it could be potentially employed as an ingredient to improve the poor nutritional value of the gluten-free products. To date, only a few papers in the literature investigated this issue by evaluating the impact of replacing to a certain extent, the main ingredient of the gluten-free recipe with hemp seed derivatives, mainly hemp seed flour or proteins. For example, Korus and colleagues assessed the effects of replacing 10% or 20% of gluten-free starch corn with hemp seed proteins or flour in the dough of gluten-free bread

(J. Korus et al., 2017), and the consequences of the substitution with 20%, 40%, or 60% of hemp seed flour in the preparation of gluten-free cookies (A. Korus et al., 2017) on the rheological, qualitative, sensorial, and nutritional properties of the relative food products. Nutritionally, both studies highlighted that the addition of hemp seed product led to significantly improvement of the nutritional value of the gluten-free food, since it rose, at high extent, the content of total proteins and total dietary fibre, including soluble and insoluble, with higher increase especially in the insoluble fraction as well as fat and minerals (A. Korus et al., 2017; J. Korus et al., 2017). Moreover, for the biscuits, it has shown that the carbohydrate content and the energy value of the products decreased proportionally to the level of substitution. Furthermore, the authors also found an enrichment in the antioxidant profile of the hemp seed flour-substituted cookies that had significantly higher values of total phenolic (up to 2-fold higher) and flavonoid (up to 2-fold higher) compounds compared to the control, with a resulting rise in the total antioxidant capacity (up to 3-fold higher) (A. Korus et al., 2017). Hence, from a nutritional point of view, either hemp seed flour or proteins may be considered valuable ingredients for the formulation of improved gluten-free products. Regarding the rheological properties of the bread's and cookies' dough, the authors showed that for the bread mixture, the substitution of corn starch with hemp seed flour resulted in a weakening of dough structure, which became more susceptible to deformation; contrarily, the substitution with hemp seed proteins reinforced the dough structure. These differences have been attributed to the differences in the interaction and the binding between the hemp seed components and water in the dough. Indeed, the authors explained that proteins absorbed more water, and hence, the addition of hemp seed proteins could prevent the fulfil hydration of all dough component, thus resulting in dough strengthening. By contrast, hemp seed flour contains other components besides proteins, and these significantly modified water binding, thus differently influencing the rheological properties of final dough (J. Korus et al., 2017). For the cookies' dough, the authors observed a significant increase in the overall hydration properties of the mixture, demonstrated by the higher values of water absorption, water retention, and oil absorption capacity in comparison to the control. These findings were associated to the increase of both the protein (4-fold higher) and total fibre content (20-fold higher) in the dough, which significantly affected the hydration properties of the biscuits' blend (A. Korus et al., 2017). In addition, contrary to what was observed for the enrichment with hemp seed flour of gluten-containing bread, the addition of hemp seed flour led to a significant increase in the quality of the gluten-free bread, as well as an improvement in the bread sensory acceptability, nutritional quality, and storage. Particularly, the authors found an increase in the bread volume, resulting in the addition of both hemp seed products. This increase was more evident with raising amounts of hemp seed products in the dough, and it was more pronounced in the case of hemp seed proteins addition. This is probably due to the absence of gluten proteins, and so, hemp seed proteins can act as surface-active and foaming agents, thus allowing to retain and stabilize the gas bubbles in the dough and bread. Interestingly, the bread volume was shown to be negatively related to the crumb hardness that, therefore, it was the lowest in the control bread and the highest in the 20% hemp seed protein-enriched bread. The limited hardness in the enriched bread was maintained also during the storage, thus limiting the bread's aging. Contrarily, in the biscuits, the volume decreased after the hemp seed flour substitution, whereas the cookies hardness increased, reaching the highest value in the 60% hemp seed flour enriched cookies (A. Korus et al., 2017). In both bread and biscuits, the colour of crumb and cookies became darker after the hemp seed product substitution as the substitution level increased; this was found to positively affect the product sensory acceptability (A. Korus et al., 2017; J. Korus et al., 2017). Moreover, for the gluten-free bread, either the enrichment with hemp seed flour or hemp seed protein conferred an aroma and taste considered

better than the control, nevertheless the taste acceptability was significantly reduced by 20% hemp seed flour (J. Korus et al., 2017). As regard the biscuits, the sensory panel highlighted a significant reduction in the sensorial acceptability of the hemp seed flour cookies than the control one, especially at a high level (60%) of substitution (A. Korus et al., 2017).

Similar results about the water absorption properties, colour, and nutritional and antioxidant changes after the addition of hemp seed flour in the mixture, were obtained by Radočaj and colleagues for gluten-free crackers in which brown rice flour was partially substituted with 10%, 20%, 30%, or 40% of hemp seed flour (Radočaj et al., 2014). In this work, the authors underlined the possible employment of hemp seed flour as a functional ingredient usable to mix with other functional raw materials such as green tea leaves, as it was done in this study, to enhance the nutritional and healthy values of daily food and to develop potentially functional foods, accessible also to the coeliac people. Indeed, with this report, the researchers demonstrated that the addition of hemp seed flour is the direct responsible to some nutritional improvement that was even better than those observed for other fortified or enriched products. For example, it was found that the addition of hemp seed flour permitted to obtained crackers with a content of proteins, fibre, and minerals higher than that found for crackers enriched with pulse flour. Moreover, the addition of hemp seed flour led to enforcement of the crackers with essential FAs that lack in other analogous products. This remark was in line with the findings by Norajit and colleagues, who investigated the effects of the addition of hemp seed flour, or whole hemp seeds powder, at three different levels (20%, 30%, or 40%), on the nutritional quality and the antioxidant activity of rice flour bars (Norajit et al., 2011). Interestingly, comparing the nutritional and functional differences (*i.e.*, antioxidant activity) between the rice bars enriched with hemp seed flour and that enriched with whole hemp seeds powder, the authors showed that the rice bar with whole hemp seeds powder had significantly higher total phenolic and flavonoid contents, as well as better antioxidant properties, than the rice bar blended with hemp seed flour.

A few studies investigated the incorporation of whole hemp seeds or derivatives products (*i.e.*, de-hulled hemp seeds, hemp seed flour, and hemp seed proteins) in meat products in order to ameliorate their nutritional value. In this context, Zając and Świątek [179] used the whole hemp seeds as added ingredients of liver pâtés with the aim to improve its FA profile (Zając and Świątek, 2018). Actually, although the total fat content of the tested pâtés did not significantly change, its FA profile was significantly improved since MUFAs and SFAs decreased compared to the control whilst the PUFAs increased. Moreover, the total n-3 PUFAs was almost 6-fold higher than the control product, and even though its level was too low to label the product as a source of n-3 PUFAs accordingly to the EU regulation 1924/2006, nevertheless, its significantly higher amount conferred to the final product a better nutritional value in comparison with the traditional pâtés. The authors also assessed the texture and sensorial properties of the experimental product and demonstrated that the addition of whole hemp seeds significantly raised the hardness, chewiness, and the adhesiveness of the pâtés without significantly changed its cohesiveness. Despite these texture variations, the inclusion of whole hemp seed did not modify the sensory parameters compared to the control.

Zając and colleagues [180] also investigated the effects of the incorporation of 5% of either whole hemp seeds, de-hulled hemp seeds, hemp seed flour, or hemp seed proteins in pork loaves, on nutritional quality, sensorial acceptability, and physical features of the obtained product (Zając et al., 2019). From a nutritional point of view, the obtained data showed that, among all hemp seed additives, only the whole hemp seeds have led to a significant increase in protein, ash, and total fibre content, without significantly changing the total fat amount, whereas, hemp

seed flour and proteins were the additives that have led to the highest enrichment in the amounts of K, Mn, and Fe, and of Ca, Mg, and Zn, respectively, even though a significant increase in the macro- and micro-elements in comparison to the control, was observed also for the others hemp seed additives, in spite of in a minor extent. Furthermore, predictably, only the inclusion of whole hemp seeds and de-hulled hemp seeds led to a significant improvement in the FAs profile, through decreasing the total MUFAs and SFAs, increasing the total PUFAs, both n-3 PUFAs (4-times and 3-times higher in whole and de-hulled hemp seeds enriched sample, respectively) and n-6 PUFAs (2-times higher) and decreasing the n-6/n-3 PUFAs ratio. The improvement of the FA profile of the pork loaves enriched with whole or de-hulled hemp seeds, led these samples to have also the lowest thrombogenicity and atherogenicity index. Hence, overall, these two enriched products appeared to be healthier and nutritionally better in comparison to the other enriched pork loaves. Nevertheless, a Thiobarbituric Acid Reactive Sunstances (TBARS) analysis showed that the de-hulling process made the pork loaves enriched with de-hulled hemp seeds more prone to oxidation during the storage in comparison to the other samples, probably due to the removal of the hull, that as previously said, it has shown to be the hemp seed fraction where most of polyphenols are located (Chen et al., 2012; P. Mattila et al., 2018b; Pojić et al., 2014). However, among all evaluated samples, the de-hulled hemp seeds enriched one was the sample which has received the higher consumers' acceptance, even though, interestingly, the authors found that the consumers' willingness to buy the meat enriched with hemp seed products increased after providing the information about the healthy ingredient and therefore, highlighted the importance to furnish the right awareness to consumers about the hemp seed's health benefits.

Finally, hemp seed proteins have been also used as an additive for yogurt by Dabija and colleagues with the aim to obtain a new, fortified, daily product with high nutritional value (Dabija et al., 2018). In this study, the authors found that among different tested vegetable source proteins (*i.e.*, hemp seed proteins, pea proteins, gluten wheat, soy proteins, and pumpkins flour proteins), hemp seed proteins have been shown to be those with the lowest syneresis value, which is an index of the expulsion grade of serum, considered as a negative acceptable parameter. This could be related to the high fibre content of hemp seed proteins that improved the viscosity and reduced the serum separation levels, as it was confirmed by the rheological analysis. Moreover, the addition of hemp seed proteins also induced a reduction in the pH value and an increase in the acidity of the fortified yogurt. Both features can be related to an improvement of the growth of the bacteria present in the yogurt. In addition to these positive changes, no significant differences were found about the acceptance of the fortified sample with hemp seed proteins in comparison to the control sample.

Hence, in view of the above, it is clear that there are efforts by researchers to understand the structural, nutritional, and functional roles of the hemp seeds and derivative utilization in the development of new heathier and nutritionally improved foods, also for specific intended groups of people such as the coeliac ones. However, further investigations are needed for a better comprehension of the most appropriate hemp seed product types, matrices, and dosages in order to develop a food product with a greater improvement of nutritional and functional properties, without negatively affecting the physical and sensorial properties of the food product. In-depth investigations are also necessary to identify the most functional hemp seed's compounds, their molecular mechanisms, and their targets in order to comprehend the most adequate concentration of the functional compounds and the vehicle/matrix most suitable to preserve their structure and functionality into the gastrointestinal tract to maximize their bioavailability in the human body and to allow the compounds to reach the

target sites. Only in this way, the regular ingestion of the appropriate quantity of food product could actually exert a preventive and beneficial clinically detectable effect, as required in order to claim a food as a functional food.

2.4.3 Hemp seed current market trend

After years of prohibition, since 1990s the recognition of the industrial hemp as an agricultural commodity has renewed the interest in this crop and its products in several developed Countries such as Canada, USA, as well as many European Countries as UK, France, the Netherlands, Germany, Spain and Italy. The growing interest in the industrial hemp cultivation is related also to the environmental benefits associated to this crop. Indeed, hemp is considered a low input crop, valuable crop for the bio-based economy, since it has low pesticide and herbicide requirements and adaptability to a wide range of agronomic conditions, including marginal agricultural lands. In accordance with the European Industrial Hemp Association (EIHA), hemp is one of the very few crops in Europe that is cultivated on non-organic farms, without the use of any agrochemicals. Strong, fast growing hemp crops are able to suppress weeds without chemical support and the crop does not suffer from any pests or diseases that would warrant a spray (Karus, 2017). The low environmental impact of this cultivation is in agreement with European regulations about organic agriculture (Regulation 2018/848) and the 17 Sustainable Development Goals (SDG) established by the United Nations for the global agenda 2030 (United Nations, 2015) (Giupponi et al., 2020). Despite this, hemp still remains a niche crop, cultivated for a global acreage of 330,000 acres in 2017, represented less than 0.5% of total crop production acreage (Johnson, 2018). However, the multiple possibilities of use and the low environmental impact of hemp makes it a promising crop with a growing market potential, as well as an excellent boost for an economic and environmental recovery (Giupponi et al., 2020).

The global market for hemp consists of more than 25,000 products in nine submarkets, including agriculture, textiles, recycling, automotive, furniture, food and beverages, paper, construction materials, and personal care (Johnson, 2018). Interestingly, globally, there has been a trend in shifting the interest in growing hemp from the technical fibre to the seed production – especially in the Western Countries – and in the use of hemp seed as ingredient in food products, beverages or as nutritional supplements, with Canada and France as the world leader in hemp seed production (Crini et al., 2020; Johnson, 2018). Particularly, in Canada almost all hemp is grown for seed only (Karus, 2017) and since 1998s, there has been an increasing trend of acreage in hemp cultivation, reaching a record of nearly 140,000 acres (≈ 56,700 ha) in 2017 (**Figure 11**). Furthermore, the annual retail sales of all Canadian-derived hemp seed products have been estimated between $20 million and $40 million, and the number of businesses active in the sector has grown over the past few years (Johnson, 2018).

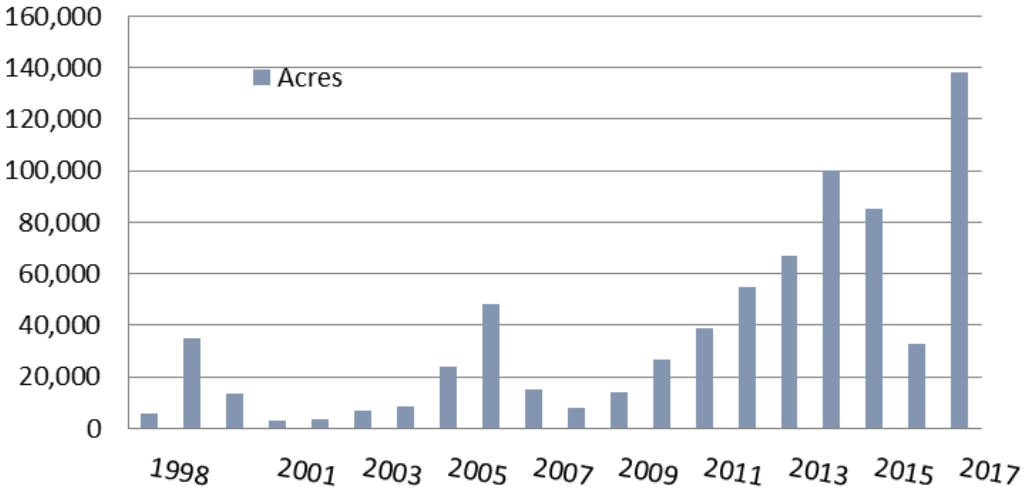

Figure 11: Canadian hemp acreage, 1998–2017. Source: Agriculture and Agri-Food Canada data, "Industrial Hemp Statistics," and "Industrial Hemp Production in Canada". From Johnson, 2018

In contrast to Canada, in Europe hemp seeds have been mainly a by-product of hemp crops traditionally grown in central or southern Europe for technical fibre production and only small areas were used exclusively for hemp seed production. However, in the last decade more and more producers in Europe started to cultivate hemp for seed production only, so that from 2010 to 2013 the production of seeds increased by 92% (from 6,000 to 11,500 tonnes) driven by the increasing demand from the food market (Karus, 2017) (**Figure 12**).

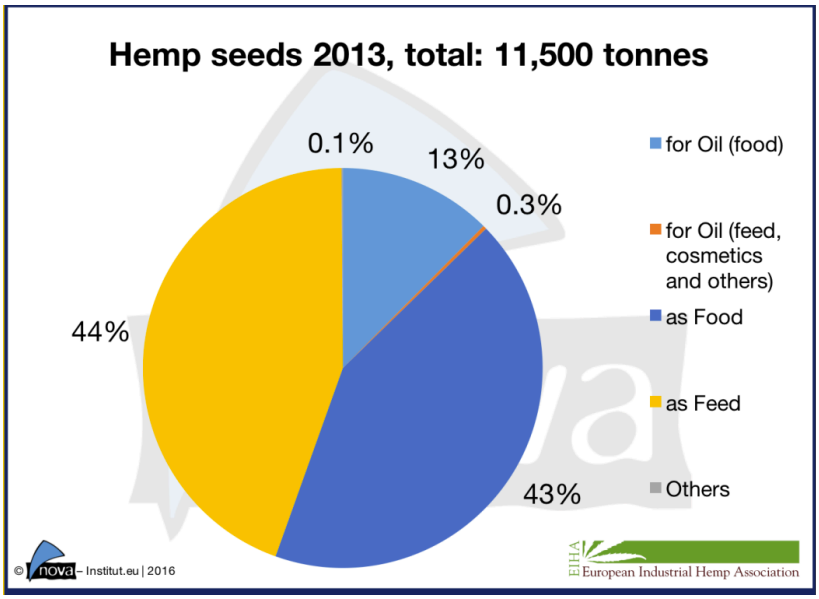

Figure 12: Applications for European Hemp Seeds harvested in 2013 (11,500 metric tonnes) (nova/EIHA 2016). Most of hemp seeds are used as human food (almost 60%) as whole or de-hulled seeds (43%), followed by hemp seeds for oil production (13.3%) used for both human foods and cosmetics. The whole seed, the cheapest and less processed product, is used also for animal feed (44%). From Karus, 2017.

The interest in investigating the potential use of industrial hemp in food and nutraceuticals has been growing in the scientific research too, as reported by Rupasinghe and co-authors (2020) based on the number of abstracts found in the CAB international database in the last 20 years (**Figure 13**).

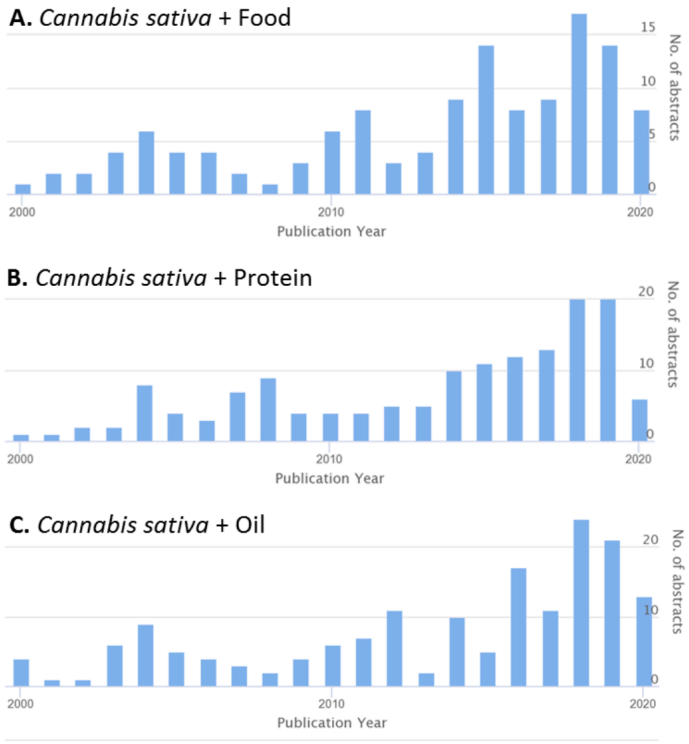

Figure 13: Number of abstracts in the CAB international database in the last 20 years. Keyword research: (**A**) *Cannabis sativa* + Food, (**B**) *Cannabis sativa* + Protein, (**C**) *Cannabis sativa* + Oil. From Rupasinghe et al., 2020.

The increasing interest and renewal of hemp seeds in foods could be related to several factors, which can be broken down as follow:

- The increasing in the investigation and knowledge concerning the nutritional and bioactive properties of hemp seeds, including antioxidative, antihypertensive, antimicrobial, anticholesterolemic, and also antitumoral activities, which have attracted an ever-growing attention not only from scientists, but also from the food industry and consumers (Crini et al., 2020). Nevertheless, more studies need to be done to better understand the effective and specific functionality and mechanisms of action of hemp seeds and their bioactive components in human body, especially when the seeds are an integral part of a food matrix.
- The increasing consumers' interest and trend in changing their dietary habits and buying selected foods with specific health-promoting properties, although not fully acceptable in terms of sensory attributes, in order to improving and maintaining their health status (Crini et al., 2020; Zając et al., 2019).
- The increasing market demand for healthier, organic, green, vegan, gluten free, and no-impact on the environment products (Crini et al., 2020; Economic Intelligence Unit (EIU), 2019). These are all claims which can be ascribed to hemp seed-based foods.

In response to interest from domestic hemp producers, the Economic Intelligence Unit (EIU)[1] has investigated the global markets for hemp products. This market analysis highlighted that between 2012 and 2018 there has been a consistent global increase in the number of new hemp retail products, with foods and cosmetic/beauty-care products as the key growth fields. In the food field, the snack market is found to be an ideal target for hemp producers, as it has showed to be a rapidly growing market within the broader category of the healthy foods, along with the skincare products in the cosmetics field. There has also been growing diversity of products launched across all categories in both food and cosmetic/health care fields (**Figure 14**) (Economic Intelligence Unit (EIU), 2019).

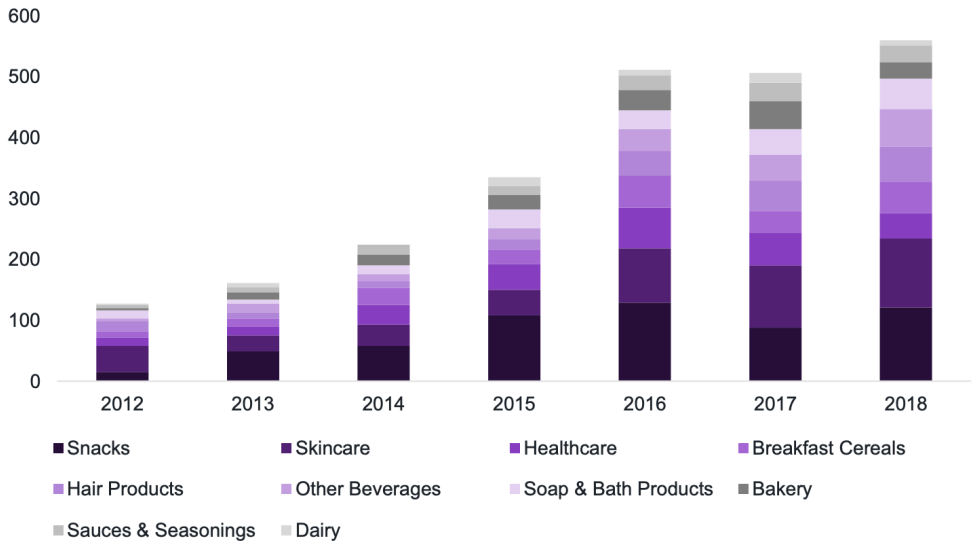

Figure 14: Globally hemp products launched by year (2012–2018). From Economic Intelligence Unit (EIU), 2019.

The strong market growth recorded for hemp seed-based snack reflects the alignment with the aforementioned consumer trends, such as a desire for quick, organic, healthy products, free from animal ingredients or chemical additives. The EIU market analysis also reported that from 2018 to 2023, this product category is forecast to grow strongly, at a Compound Annual Growth Rate (CAGR)[2] of 4.5% in the USA which represent a key market for hemp products, being the Country where the 26% (*i.e.* 1/4 of all hemp products globally launched and captured in the EIU's dataset) of new hemp products was launched between 2012 and 2018, the 90% of which were constituted by the hemp seed-based snack bars (Economic Intelligence Unit (EIU), 2019). Together with the USA, other industrialised Western countries leading the hemp and hemp seeds market, include Canada, France, Germany, and the United Kingdom (UK). Analogously to the USA, also here the hemp seed-based snacks

[1] Economic Intelligence Unit (EIU): The world's leading resource for economic and business research, forecasting and analysis.

[2] Compound Annual Growth Rate (CAGR): Is a "performance index" for an investment, defined as the "rate of return that would be required for an investment to grow from its beginning balance to its ending balance, assuming the profits were reinvested at the end of each year of the investment lifespan".

represent the most productive category, with large market demand, indicating strong profit opportunity for these products (**Figure 15**).

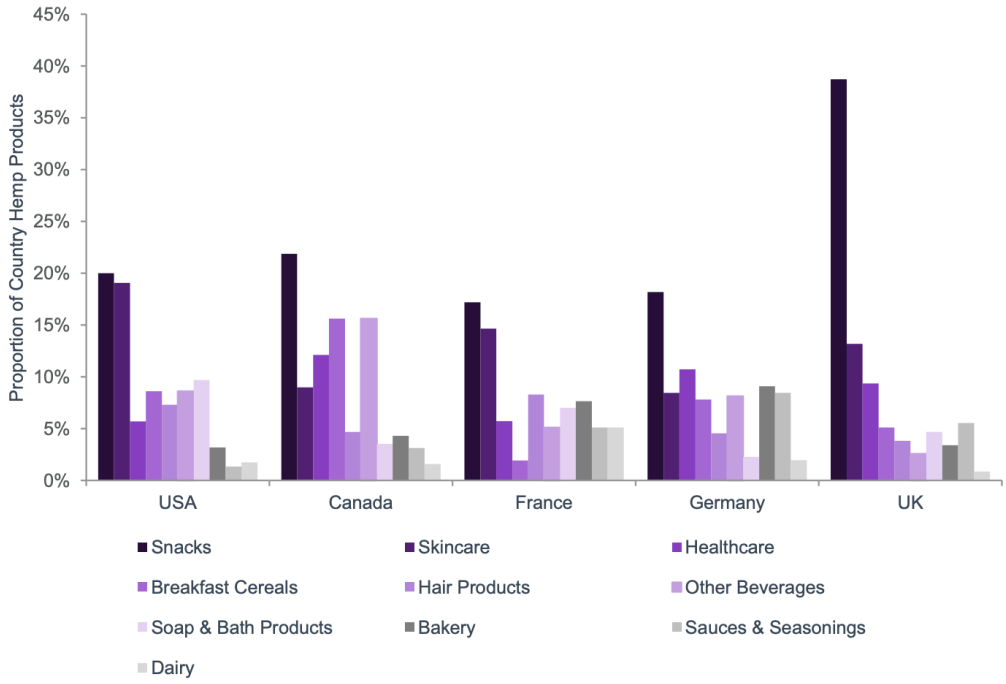

Figure 15: Top ten hemp seed-based product categories breakdown by the five largest industrial hemp producers Countries. From Economic Intelligence Unit (EIU), 2019.

Interestingly, the five largest retail markets are all countries that have legalised the hemp cultivation, suggesting that clear political and economic hemp legislation results in a high proportion of hemp farmers and stakeholder and is essential to promote the development of an internal hemp market (Crini et al., 2020; Economic Intelligence Unit (EIU), 2019; Giupponi et al., 2020; Karus, 2017). At the same time, the consumer awareness represents another important factor influencing the hemp industry development and growth, being the consumer himself leads the market demand (Karus, 2017). Therefore, it is very important to further investigate the knowledge and to provide right information concerning the industrial hemp and the industrial hemp seed, *e.g.*, by improving the agronomic practices and breeding programs to develop high seed-yielding *cultivars* keeping THC level as low as possible, in order to increase its competitiveness as compared to other oilseeds; by exploring the nutritional and functional features of the seeds of different hemp *cultivars*; as well as by identifying the best hemp seeds' transformation practises able to enhance and optimise their health benefits.

3. The malting process

Malting is an ancient biotechnological process traditionally performed on cereal grains, primarily barley, consisting in the limited germination of seeds under controlled conditions (Briggs, 1998). Its main objective is to modify the composition and physical structure of the grain and allow synthesis or activation and mobilisation of a series of endogenous enzymes, in order to hydrolyse biopolymer molecules such as starch and protein (Taylor and Emmambux, 2008), and produce a product – the malt – with more fermentative substances useful for production of beer, whisky and other beverages (MacLeod and Evans, 2016). Indeed, approximately 96% of the malt produced in the world is used as raw material to make beer. Approximately 3% of the world's malt is use in Scotland for whisky production. The rest is used as ingredient in the food and bakery industry as dry material which can be milled for producing flour that can be applied in the formulation of products as snacks or bread, to improve their palatability and nutritional value (Guido and Moreira, 2013; Taylor and Emmambux, 2008).

Malting procedure basically involves the three steps of (1) steeping, (2) germination, and (3) kilning, during which drastically changes of the seed's content and physic and biochemical compositions occur and can be useful to improve the overall nutritional and functional quality of the malted seeds (Hübner and Arendt, 2013).

The steeping, the first stage of the malting process, aims to achieve a rapid and even uptake of water and oxygen by the seed, in order to stimulate the embryo to become active and use the oxygen dissolved in the steeping water for respiratory purposes. This stage consists of alternating periods when the grain is immersed in water, named "under-water periods" and periods with the water drained from the grain, referred to as the "air rest periods". The immersions (*i.e.*, the under-water periods) allow the embryo to absorb water and the air-rest periods allow the water to be distributed into the non-respiring endosperm (MacLeod and Evans, 2016). Both periods are needed to achieve satisfactory malting results, in fact the seeds are normally in a state of quiescence which ensures their long-term survival during storage and implies that they are low in moisture and relatively inactive metabolically; water absorption and seed's hydration is essential for enzyme's activation, breakdown, translocation, and use of reserve storage material, and therefore, the under-water period is a basic requirement for the onset of germination (Shaban, 2013). At the same time, the embryo's respiration produces heat and carbon dioxide, as well as oxygen consumption, and in case of prolonged failure of the oxygen supply, the embryo's growth and enzyme production will stop, therefore, the air-rest period is important to keep alive the embryo, stimulate its growth, and avoid to negatively affect the germination rate (Guido and Moreira, 2013). Most seeds have a critical moisture content for germination to occur and once this is attained in the seed, sufficient water is present to initiate germination. The seed is committed to germinate and cannot turn back (Shaban, 2013). Usually, in the malting of cereal grains the steeping step overlap the germination step to a certain extent, because steeping is considered ended when most of seeds are "chitted", that is they have the tip of the rootlet breaking through the husk (Guido and Moreira, 2013). However, it is important to take into account that in germination *sensu stricto* is completed once the seed coat is protruded by the radicle, and the following growth is referred to as seedling establishment or post-germinative growth. Hence, physiologically, germination and post-germinative growth are actually, clearly separate events, even if as a whole, they are called as "germination process" by cereal scientists and brewers (Mäkinen and Arendt, 2015) and in this thesis the same terminology is used.

The germination process is characterized by the growth of the embryo, manifested by the increase in length of the rootlet (Guido and Moreira, 2013). Biochemically, the activation and mobilisation, as well as *de novo*

synthesis of a wide range of hydrolytic enzymes occur, resulting in extensive phytochemical changes on the seed's composition needed to support the growth of the young plant (Nelson et al., 2013). These changes induce variation on the seed's nutritional profile, including the nutrient bioavailability and digestibility, as well as the production of secondary metabolites with possible bioactivities. In particular, the high molecular weight reserves (*i.e.,* macromolecules) deposited in the storage tissues of the seed, are converted into soluble and more available forms (Mäkinen and Arendt, 2015). The hydrolytic enzymes activated during this step include starch-degrading enzymes (*e.g.*, α-amylases, α-glucosidases, β-amylases), cell-wall-degrading enzymes (*e.g.,* endo-β-glucanases, xylanases), lipases, proteases, and phytases.

The seed's modification induced by the enzymes' activity during germination has been investigated mainly on cereal grains representing the matrix on which the malting process has been extensively studied since many years. Cereals are starchy seeds; therefore, their main storage reserve is the starch located in the seed's endosperm. The endosperm is surrounded by the aleurone layers and is divided from the embryo by the scutellum (**Figure 16**). In cereal grains, the most significant changes induced by germination on the seed's composition occurs in starch. In the initial stages of germination, the scutellum releases gibberellines, hormones which promote germination by stimulating *de novo* synthesis and release of the hydrolytic enzymes mainly located in the scutellum and aleurone layer (Guido and Moreira, 2013). Thus, starch-degrading enzymes act on starch stored in the endosperm as amylose and amylopectin, leading to its partial hydrolysis into glucose, maltose, maltotriose and a wide range of dextrins (Lemmens et al., 2018b; Mäkinen and Arendt, 2015; Nelson et al., 2013).

As previously said, the hydrolytic enzymes releasing during germination also include proteases (*e.g.*, endo-, carboxy-, amino-, and dipeptidases), lipases, and phytases. The former activate functional proteins (*e.g.*, β-amylase, phytate) and degrade the grain's storage proteins such as globulins, prolamins and glutenin's, which are located in the protein bodies of embryo, endosperm and aleurone (Shewry and Halford, 2002), into low molecular weight peptides and free amino acids, which support the seed's metabolic requirements (Lemmens et al., 2018b; Nelson et al., 2013). Lipases degrade the grain's stored triglycerides that are found in the embryo, aleurone and scutellum, to yield free fatty acids and glycerol, providing substrates for β-oxidation, gluconeogenesis, and carbon skeletons for seedling development and photosynthesis (Nelson et al., 2013) (**Figure 16**). Finally, phytases are enzymes appointed to hydrolyse phytate located in the protein bodies found mainly in the aleurone cells (Hübner and Arendt, 2013; Lemmens et al., 2018b). The increasing in phytate activity levels during germination results in a dephytinization process important to make phosphate, mineral elements, and myoinositol available for plant growth and development (Lemmens et al., 2018b).

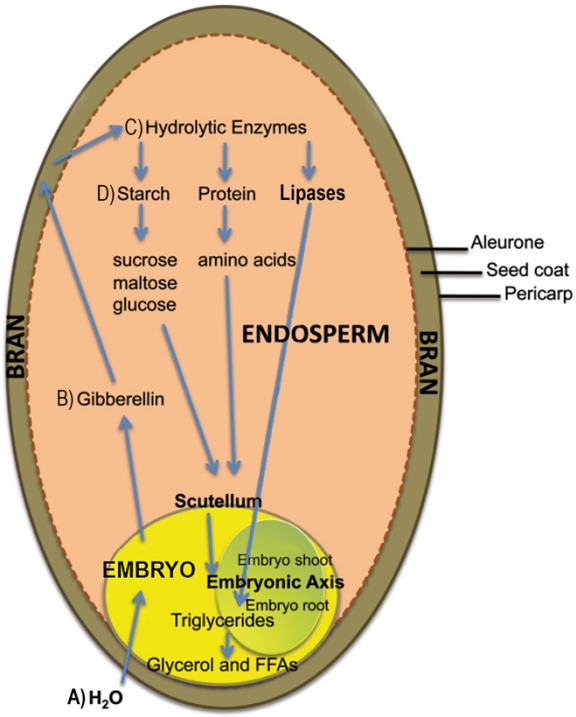

Figure 16: Diagrammatic representation of a longitudinal section of a cereal grain showing the sequence of major metabolic events occur during germination. Structurally, the cereal grain is mainly composed by the starchy endosperm which represent the most relevant component. The endosperm is surrounded by the aleurone layer and fibrous seed coat (*i.e.,* the bran). Within the starchy endosperm is located the embryo and the scutellum that is set between the endosperm and embryo. Storage nutrients for the embryo growth are starch (in the endosperm), proteins (in the protein bodies located in endosperm, embryo, and aleurone), and lipids (in the embryo, aleurone, and scutellum). (A) Water imbibition facilitates seed rehydration, in turn stimulating embryonic gibberellin releases from scutellum that promotes hydrolytic enzymes' gene expression. (C) Enzymes such as amylases, proteases, and lipases are secreted into the endosperm, whilst enzyme inhibitors decrease activity. (D) Useable nutrient substrates are catabolized from storage molecules (starch, protein, and triglycerides) for the embryo developing. From Nelson et al., 2013.

In addition to the changes in the seed's storage reserve (*i.e.*, starch, proteins, lipids), also alterations in the seed's structure take place during germination as a result of the breakdown cell walls. In fact, degradation of cell wall polysaccharides occur by the action of *de novo* synthesis of cell-wall-degrading enzymes, in order to facilitates the passage of hydrolytic enzymes located in the scutellum and aleurone (*e.g.*, amylases and peptidases) to their substrates (starch and protein, respectively) in the starchy endosperm, (Lemmens et al., 2018b). This degradation leads not only to thinning the seed's cell walls, but also to changes in the seed's dietary fibre content (as discussed in the next subsection), as the dietary fibre components are an integral part of the plant cell walls.

In cereal, germination is usually maintained until the rootlets reaches a length of between 1.5 and 2 times the original length of the grain (Carvalho et al., 2016a). At this stage, the material is called "green malt" (Briggs, 1998; Guido and Moreira, 2013; Özcan et al., 2018) and it is wet (approximatively, 40-50% moisture), easily damaged and not suitable for long storage. Hence, although some nutritional and antioxidant traits resulted better in green malt than in malt (*i.e.*, green malt after the kilning process) (Özcan et al., 2018) however, the heat treatment

performed in the subsequent step of malting, is essential to obtain a stable product, easier for storage and transport, and to develop and stabilize colour, aroma, and flavour (MacLeod and Evans, 2016). Indeed, the final stage of malting, namely kilning, consists of a heat treatment similar to a typical industrial drying process, which halts germination and grain growth, dries the grain to constant and low moisture for long lasting storage, arrests the activity of hydrolytic enzymes, and thus, the metabolic processes, and develops flavour and aroma (MacLeod and Evans, 2016). The composition of malt changes during kilning, so that, while the enzyme activity declines, its colour, aroma, flavour, and extractable polyphenol content too, increases (Guido and Moreira, 2013). In fact, kilning condition plays important role in influencing the final malt organoleptic but also antioxidant, character, so careful control of this stage allows a range of several malt types to be produced from the malting process (MacLeod and Evans, 2016).

Particularly, the heat treatment could be performed at constant temperature, or at temperature increasing slowly and in a stepwise fashion. Usually, three phases can be identified (Guido and Moreira, 2013; MacLeod and Evans, 2016):

1. The *free drying* or *withering* phase during which moisture is removed from green malt relatively quickly, reducing it from approximately to 12%, using kiln temperatures relatively low (*e.g.*, 50 - 60 °C).
2. The *intermediate* or *falling rate* phase during which malt is dried from 12% to 4% in a much slower process.
3. The *bound water* or *curing* phase occurring at the end of the drying process, when the kiln temperature may be raised to value even higher than 80 °C for 1 or 2 h.

Generally, when white, pale (lager) malt with enzymes' survival has to be produced, conventional kilning is performed at low/intermediate temperatures between 60 °C and 95 °C, or nevertheless lower than 100 °C (Carvalho et al., 2016b). While, when darker (specialty) malts have to be produced, higher temperatures are applied (110-250 °C) and the kilning step are commonly named "roasting" (Briggs, 1998; Carvalho et al., 2014; Guido and Moreira, 2013; MacLeod and Evans, 2016). In this case, all enzymatic activity is destroyed, but a very characteristic and strong flavours and colours malt is produced (MacLeod and Evans, 2016). Actually, flavours and colours development always occur in malting after the heat treatment, as result of the non-enzymatic browning process, also known as the Maillard reaction (MR). It represents one of the most complex non-enzymatic process, consisting of a reaction between carbonyl group of reducing sugar and an amino compound (*e.g.*, amino acid, or amino group of peptides or proteins) which initially originates the relatively stable Amadori Rearrangement Product (ARP), including aldosamines and ketosamines. The latter products may condense with another sugar to yield diketosamines. Subsequent breakdown of the diketosamines occurring in the "intermediate phase" of the process, initiates a flood of chemical reactions which continuously produce new intermediates highly reactive, such as reductones and other (di)carbonyl compounds. Some of these products may react and polymerise to form new Maillard Reaction Products (MRPs), including low-molecular-weight (LMW) (< 10 KDa) and high molecular weight (HMW) (> 300 KDa) substances also known as melanoidins (MLDs) (**Figure 17**) (Briggs, 1998; Carvalho et al., 2014; Hodge, 1953). The LMW MLDs include acids, alcohols, aldehydes, ketones, and esters and are the primarily responsible for the characteristic malt flavours and aromas; the HMW MLDs are generated by the polymerisation of LMW compounds occurring during the late stage of Maillard reaction, and contribute to the malt's colour (Briggs, 1998; MacLeod and Evans, 2016).

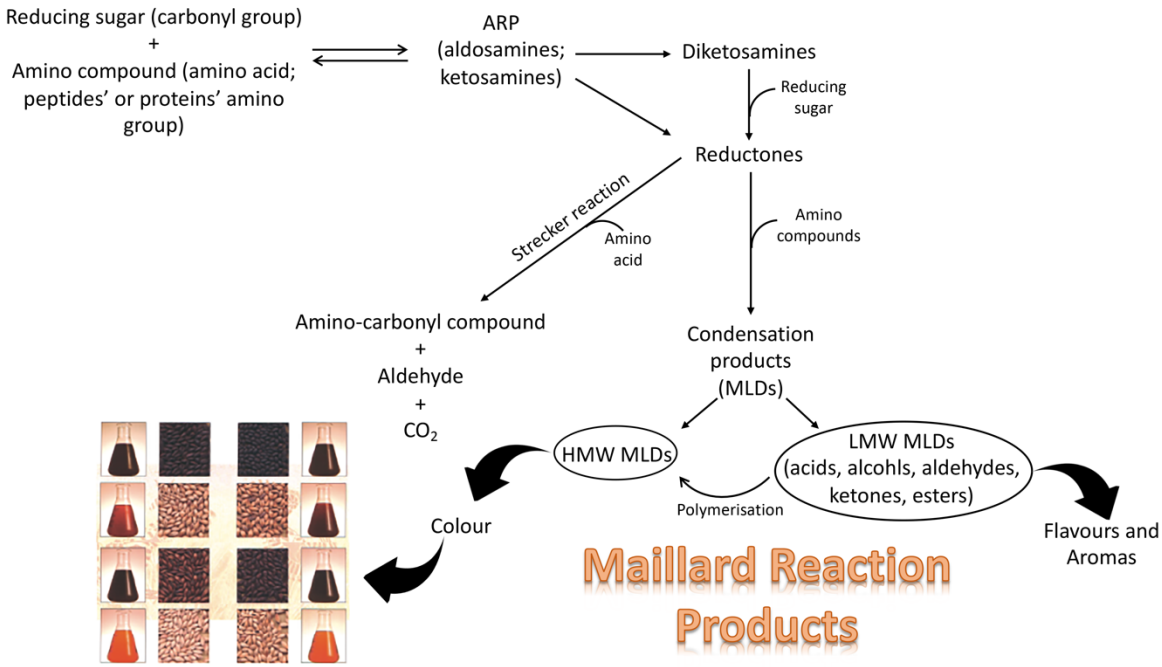

Figure 17: The main chemical steps involved in the production of Maillard Reaction Products (MRPs) occurring during the heat treatment of malting. Among these, the HMW MLDs are those contribute to the malt's colour, whereas the LMW MLDs contribute to develop malt's flavours and aromas. Based on the temperature's value adopted during the heat treatment, different types of MRPs will be produced, thus obtaining different types of malt, differing in the organoleptic features (*i.e.*, taste, colour, flavours, aromas). ARP: Amadori Reaction Products; HMW: High Molecular Weight; LMW: Low Molecular Weight; MLDs: Melanoidins.

Independently to the kilning temperature used, the heat treatment performed at the end of malting is important to improve the taste of the final product also thanks to the removal of sulphur compounds, such as dimethyl sulphide (DMS), that is responsible for an unpleasant "cooked vegetable" taste. Indeed, during kilning, DMS precursor, which is formed during grain germination, is converted to free DMS which is volatilized and lost (Guido and Moreira, 2013).

Once kilned, the green malt is converted in a friable, easily to mill and storable product, which can be used both as fermentable material to produce fermented beverages (*i.e.*, beer and whiskey), as well as ingredient to enrich the bakery products' doughs, thus improving their organoleptic, nutritional and antioxidant features.

3.1. Effect of malting on the seed's nutritional features

Numerous studies have shown that the nutritional and chemical profile is altered following malting of cereal grains especially as consequence of the profound changes occurring during the germination step to the seed's content. In cereal grains, one of the most notable nutrient alterations during malting is the conversion of starch to simple sugars through the amylolysis process. Nutritionally, amylolysis is considered to increase the starch digestibility as makes it more susceptible to enzymatic attack during digestion (Lemmens et al., 2018b; Mäkinen and Arendt, 2015). Indeed, some studies reported an increase in starch digestibility of cereals, pseudo-cereals and

legumes such as, rice, millet, pea, chickpea, and black gram, after germination (Archana et al., 2001; Bishnoi and Khetarpaul, 1993; Jood et al., 1988; Kaur et al., 2015). However, this could be related not only to the starch degradation, but also to the inhibition of α-amylase inhibitors and other antinutrients that negatively affect the starch digestion and bioavailability, like phytic acid, as well as to the starch gelatinization occurring after cooking, which make it even more readily available for digestion (Mäkinen and Arendt, 2015). Actually, some evidence stated that the *in vitro* starch digestibility decreases by 58% in bread made with germinated brown rice flour (Cornejo et al., 2015). This finding was attributed to the increase in the relative portion of crystalline starch than amorphous ones after germination. Indeed, the amorphous starch is more susceptible to the digestive enzymes in the gastrointestinal tract, differently, the crystalline starch is less accessible for pancreatic amylase due to its structure, and thus, it is hydrolysed to a lesser extent (Cornejo et al., 2015).

Conversion of starch into simple sugar could also potentially affect the glycaemic response, theoretically elevating postprandial levels of blood glucose. However, preliminary *in vivo* studies indicated blood glucose effects are improved following germinated-grains consumption compared with non-germinated counterpart, in healthy adults (Andersen et al., 2008), and overweight or obese males (Mofidi et al., 2012).

From a technologically point of view, the presence of reducing sugar obtained by starch hydrolysis and high amylolytic activity of malt represent an useful tool for the bakery industry to promote the dough's leavening, improving it's volume, and to exert an anti-staling effect extending the shelf-life and freshness of the final product (Mäkinen et al., 2013; Poutanen, 1997).

Together with starch, dietary fibre represents another carbohydrate fraction undergoes modification during malting, especially during the germination step. As previously said (see the paragraph: 2.1.3. "Hemp seed carbohydrate and dietary fibre"), dietary fibres are defined as the edible part of plants or analogous carbohydrates, which resists the hydrolysis by alimentary tract enzymes (Dietary Fiber Definition Committee of the American Association of Cereal Chemists, 2001). A wide range of health effects are ascribed to dietary fibre consumption, some of which related to their solubility, viscosity, particle size, and/or water-holding capacity (Lemmens et al., 2018b). Indeed, while not broken down by the human enzymes, the dietary fibre – mostly the soluble ones – are utilised by the bacteria in the colon, acting as substrates for microbial fermentation. Principal fermentation products include short-chain fatty acids (mainly acetate, propionate, and butyrate) which can, in turn, promote colonic health by providing energy for the colonocytes (Bränning and Nyman, 2011) and by decreasing gut permeability, as well as by ameliorating the postprandial glycaemic responses (Weickert et al., 2006) and lowering plasma lipid concentrations (Keenan et al., 2007). Furthermore, dietary fibre – essentially, the insoluble fraction – have a role in promoting the intestinal motility and digestive regularity, by decreasing the intestinal transit time, thus counteracting the constipation (McRorie and McKeown, 2017; Soliman, 2019). It also can prolong the post-meal satiety, promoting the weight loss especially in obese and overweight subjects (Soliman, 2019).

In addition, dietary fibre has also a technological effect, mainly caused by water binding and increased viscosity of solution (Hübner and Arendt, 2013) which can positively affect the dough's leavening.

The impact of germination on the composition (*i.e*., the type) and content of dietary fibre is widely variable and mainly depends on species and *cv*, as well as processing condition (*i.e.*, temperature and time), with increased, decreased, and no change reported for several grains. For instance, it was reported that in wheat, the total dietary fibre content decreased after 2 days of germination, but significantly increased after 7 days of germination compared with non-germinated grains (Koehler et al., 2007). Contrarily, 3 days of germination did not significantly

affect the total fibre content in barley grains (Teixeira et al., 2016), whereas 4 days of germination generally increased the fibre content – particularly the soluble fraction – in legumes (Mäkinen and Arendt, 2015).

Proteases represent other enzymes activated during the germination, which contribute to modify the seed's composition. Quantitatively, activation of proteases and the resulting protein hydrolysis, does not cause relevant changes in protein content even if some studies reported slight, but significant modification in the crude protein content of various cereals and pseudocereals, and often, the outcomes were contrasting. Indeed, some works have reported a significant decrease of 3% and 10% of the protein content in wheat (Seguchi et al., 2010), sorghum (Afify et al., 2012) and rice (Esa et al., 2011; Mohan et al., 2010; Veluppillai et al., 2009) after germination. This decrease was attributed to the leaching of water-soluble peptides in the steeping water. In contrast, other studies have reported an increase in the protein content of 5% and 10% in germinated barley (Donkor et al., 2012; Teixeira et al., 2016), oats, wheat, rye (Donkor et al., 2012), rice (Donkor et al., 2012; Ohtsubo et al., 2005; Pal et al., 2016; Watanabe et al., 2004), and millet (Hejazi and Orsat, 2016; Mbithi-Mwikya et al., 2000). In this case, the increase has been ascribed to *de novo* synthesis of proteins and enzymes (Aguilar et al., 2019), or by the loss of carbohydrates through respiration (Lemmens et al., 2018b). Besides the quantitative aspect, the most important modifications at the expense of the seed's proteins resulting by malting, is the improvement in the protein solubility and digestibility. This is explained not only by the hydrolysis of high molecular weight proteins into small peptides and free amino acids, easier to digest and absorbed, but also by the inactivation and reduction of the level of some antinutritional compounds, which limit proteins' digestibility through forming complexes with proteins themselves – thus making them unavailable to proteolytic, digestive enzymes – or with proteolytic enzymes inhibiting them, such as tannins and trypsin inhibitors (Lemmens et al., 2018b). Reduction in tannins level after germination has been observed in various grains, among which sorghum (Ogbonna et al., 2012) and finger millet (Hejazi and Orsat, 2016) and was attributed to the leaching of tannins into the steeping water. Inhibition of trypsin inhibitors activity and reduction in trypsin inhibitors content after germination and malting were related to the action of specific peptidases activated during germination (Mbithi-Mwikya et al., 2000; Murugkar, 2014), and to the heat treatment performed during kilning (Bueno-Borges et al., 2018). In addition, hydrolysis of storage proteins occurring during germination of cereal grains has been reported to have a benefit effect for people with gluten sensitivity, since it leads to degradation of gliadin peptides and decreases the gluten content (Nelson et al., 2013).

Increasing activity levels of endogenous phytases during the germination step contribute to ameliorate the proteins' digestibility and bioavailability and above all, make malting a promising strategy for improving mineral bioavailability in malted grains-containing products (Lemmens et al., 2018b). Indeed, attempts to remove phytate by the means of germination have been explored in a variety of grains, as well as in legumes. In general, the results greatly depend on the species in questions and the germination adopted conditions (essentially, germination time and temperatures) (Hübner and Arendt, 2013; Lemmens et al., 2018b; Mäkinen and Arendt, 2015). For instance, in finger millet (Hemalatha et al., 2007), oat (Hübner and Arendt, 2013) and chickpea (Mäkinen and Arendt, 2015) very little or no degradation of phytate was observed after germination, probably due to high phytate content and/or low phytate activity in the native seeds. By contrast, 37% and 68% phytate breakdown in barley and rye, respectively was reported as a result of germination for 3 days at 22 °C (Centeno et al., 2001), and degradation up to 87% of phytate in sorghum (Mahgoub and Elhag, 1998) and 30% in rice (Egli et al., 2002) after germination for 4 and 3 days, respectively was observed. It was reported that relatively long germination time (from 3 to 5 days) is required to improve the phytases activity level (Lemmens et al., 2018b). Several *in vitro* studies based on

the human gastrointestinal tract's simulated digestion demonstrated that a decrease in phytate content in cereals led to a significant increase in mineral bioavailability, especially for iron and zinc (Afify et al., 2011; Larsson et al., 1996; Lemmens et al., 2018a). During malting, the mineral content of seeds is influenced not only by the phytases activation and activity level occurring during germination, but also by the steeping step. In fact, several papers reported that during steeping, minerals can be lost due to leaching (Afify et al., 2011; Briggs, 1998; Hübner et al., 2010; Lintschinger et al., 1997; Udeh et al., 2018a) and the loss is relatively high (about 30%) when the cereal grains are steeped at 30 °C (Lemmens et al., 2018b). Hence, malting could be actually an useful strategy for reducing and potentially improving the mineral bioavailability, but to obtained optimal results, it is necessary identify the best condition for each individual species and variety.

Finally, concerning the fate of seed's lipid after malted, some contrasting evidence has been reported. Indeed, literature generally agree that the lipid content of cereal grains decrease after malting as result of the hydrolysis of triglycerides occurring during germination, due to the increase in lipase activity (Bravi et al., 2012; Lemmens et al., 2018b). Actually, a 8% to 15% decrease in the lipid content was reported for millet, barley and oat after germination, and the decrease was found to be more marked when higher germination temperatures (≥ 25° C) were adopted (Lemmens et al., 2018b). Analogously to other hydrolytic enzymes' activity, the lipolytic activity too is stopped in the final step of malting (*i.e.,* the kilning), therefore, it has been reported that in barley the total lipid content of malted seeds does not vary significantly when compared with barley's green malt (Bravi et al., 2012). By contrast, some findings showed an increase in the lipid content of barley, oat and rye after germination (Özcan et al., 2018), making the total lipid content of the green malt significantly higher compared to unprocessed grains. A decrease in the barley's green malt lipid content was obtained after kilned the green malt. Nevertheless, although lower compared to green malt, the lipid content of malted barley was found to be higher in comparison to unprocessed barley (Özcan et al., 2018). These differences with other literature reports were attributed to the fact that different malting conditions, including temperature, moisture, and germination time, as well as the seed's variety, could have different impact on the level of lipid degradation (Özcan et al., 2018).

3.2. Effect of malting on the seed's antioxidant profile

Various studies reported that processes as germination and kilning may have a positive impact not only on the nutritional properties of grains, but also on their secondary metabolism, leading to a general increase in their antioxidant properties. Effectively, one of the most important challenge of malting is to conserve or maximize the levels of endogenous antioxidant compounds (*e.g.*, polyphenols) which have a crucial role in delaying or preventing lipid oxidation in foods and beverages during processing and storage, as well as a health positive effect by protection against the pathologies linked to oxidative stress.

Literature evidence concerning cereals' and pseudo-cereals' studies have ascribed the increase in the antioxidant activity during malting, to three main factors (**Figure 18**):

1. *De novo* synthesis of phenolic compounds during germination (Cevallos-Casals and Cisneros-Zevallos, 2010; Krahl et al., 2008), resulting by the activation of phenilpropanoids pathway, and important to protect the growing embryo from environmental stress and oxidative processes derived by the embryo's high metabolic rate (Özcan et al., 2018).
2. Releasing of bound phenolic compounds as a result of the enhanced hydrolytic enzymes activity, as well as the dismantling of the cell wall occurring during germination (Dvořáková et al., 2008; Inns et al., 2007; Sharma and Gujral, 2010).
3. Formation of MRPs such as MLDs and reductones resulting to the heat treatment (*i.e.*, kilning or roasting) (Carvalho et al., 2016a; MacLeod and Evans, 2016).

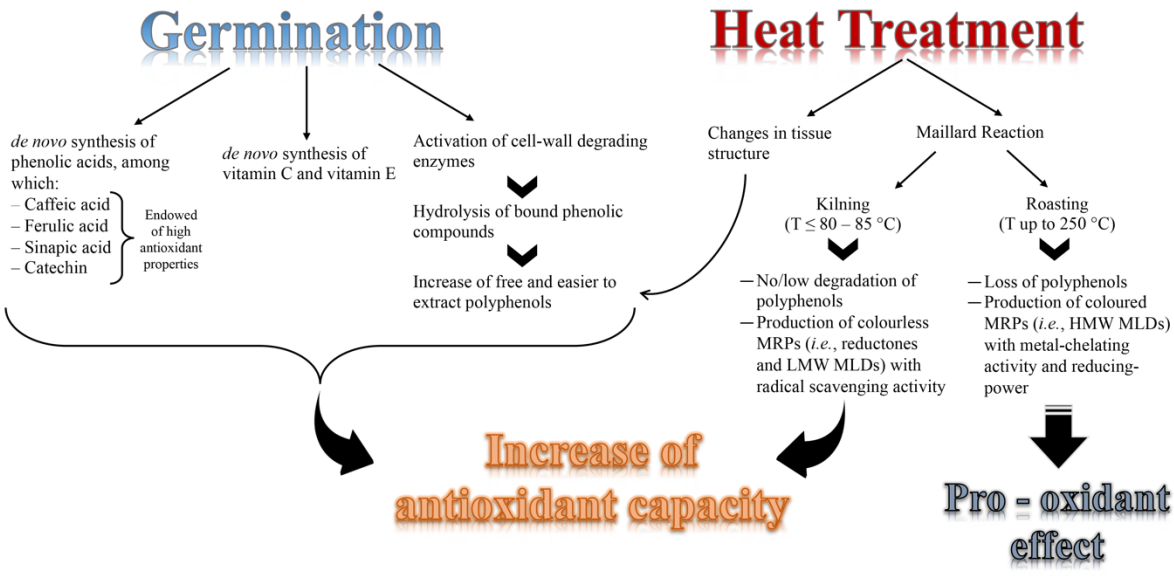

Figure 18: Main factors occurring during malting, which affect the antioxidant profile of malted seeds. For more details, see the main text. HMW: High Molecular Weight; LMW: Low Molecular Weight; MLDs: Melanoidins; MRPs: Maillard Reaction Products; T: Temperature.

Polyphenols are one of the most important endogenous antioxidant compounds found in seeds. Therefore, in general, the increase of TPC is related to a concomitant improvement in the antioxidant activity of seeds. Indeed, significant positive correlation between the TPC value and the results of *in vitro* antioxidant capacity assays, such as $ABTS^{\bullet+}$ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay), DPPH (2,2-diphenyl-1-picrylhydrazy radical scavenging activity), FRAP (Ferric Reducing Antioxidant Power), and CUPRAC (Copper ion Antioxidant Capacity) used to analyse the ability of sample to scavenge radical species ($ABTS^{\bullet+}$ and DPPH assays) and its reducing power (FRAP and CUPRAC assays), was reported by several papers.

Ondrejovič and colleagues found that the TPC of different types of cereals including wheat, barley, and oat, was 1.2 – 3.4-fold higher in their malts than in unprocessed counterpart. A concomitant increase of antioxidant activity was revealed (Ondrejovič et al., 2014). Analogously, phenolic compounds content were found significantly higher after malting of quinoa (Aguilar et al., 2019; Carciochi et al., 2016; Pilco-Quesada et al., 2020), barley (Carvalho et al., 2015; Özcan et al., 2018), and buckwheat grains (Molinari et al., 2018; Terpinc et al., 2016). Here too, a positive correlation between the increased TPC value and the antioxidant capacity was observed. The obtained higher TPC value found in malted samples was primarily attributed to the modifications occurring during germination process (Pająk et al., 2019, 2014; Sharma and Gujral, 2010). Effectively, a significant increase in both bound and free phenolic compounds was reported for wheat, barley, sorghum, rye, and rice after germination (Lemmens et al., 2018b). Indeed, during germination phenolic acids are biosynthesised and cell wall-degrading enzymes are activated and can hydrolyse phenolic compounds bound to cell walls resulting in an increased level of free phenolic compounds (Carvalho et al., 2015; Inns et al., 2007; Lemmens et al., 2018b; Samaras et al., 2005) which are considered antioxidants more effective (Ti et al., 2014) and easier to extract (Adebiyi et al., 2017) than their bound counterpart. An improved efficiency of phenolic compounds extraction also results after malting as consequence of the changes in tissue structure caused by kilning which have been shown to increase the ease of extracting compounds located in the over layers of the grain, such as phenolics (Do et al., 2015).

In addition to the increase in the TPC value, greater polyphenol diversity as well as differences in polyphenol types were observed after germination or malted (Nelson et al., 2016; Terpinc et al., 2016). For instance, Nelson and co-authors found an increase in the content of coumaric and ferulic acids, catechin and epicatechin after malting wheat, while the amount of luteolin and epigallocatechin decreased (Nelson et al., 2016). In buckwheat seeds, a great increase of rutin, orientin, vitexin, isovitexin and caffeic acid after 88 h of germination was reported, but a general negative impact of 80 °C kilning on all these phenolics were obtained, although their final amounts were still considerably higher than in raw buckwheat seeds (Terpinc et al., 2016). Özcan and colleagues also observed that 80 °C kilning negatively affected the TPC of barley green malt despite of the TPC value of the malt still was significantly higher than barley raw grains (Özcan et al., 2018). Contrarily, in other works was found that kilning of barley led to an increase of polyphenol levels and particularly, of ferulic acid (Carvalho et al., 2016a; Inns et al., 2007). Among polyphenols, ferulic acid together with catechin, caffeic acid and sinapic acid that was found raised after malting, were reported as the principal contributors to the antioxidant properties of malt (Carvalho et al., 2016a).

However, it has to take into account that the increasing trend in the TPC content after malting has not always been found. For instance, a decrease in TPC after malting of finger millet and sorghum *cvs* was observed and this outcome was ascribed to the loss of hydrolysed phenolic compounds during germination, or to leaching of

polyphenols into the soaking medium, or even, to degradation of polyphenols during the adopted kilning condition (Khoddami et al., 2017; Obadina et al., 2017; Udeh et al., 2018b). A negative impact of a high kilning temperature (190 °C) on the TPC value was also observed in malted quinoa extracts by Carciochi and co-workers (Carciochi et al., 2016).

Interestingly, some studies have reported a weak positive correlation between TPC value and antioxidant activity of malted cereals (Ondrejovič et al., 2014), as well as an unexpected increase in the antioxidant properties associated to a decrease of the TPC of malt (Carvalho et al., 2016a). Therefore, it was suggested that in addition to polyphenols, other compounds might contribute to the antioxidant capacity of malt, including vitamin C and E which was *de novo* synthesised during germination (Carvalho et al., 2016a). Effectively, it was reported that antioxidant capacity can improve during malting probably also due to the formation of new antioxidants, such as MRPs (Carvalho et al., 2016a; Terpinc et al., 2016). As previously described, MRPs include the HMW, and the LMW MLDs, as well as the reductons. These substances are also called "heat-induced antioxidants" (Samaras et al., 2005) whose antioxidant capacity is often ascribed to the metal-chelating properties, reducing powers, and radical-scavenging capacity (Carvalho et al., 2014). As previously described, the HMW MLDs are the main contributors to the dark malt's colour. Interestingly, Samaras and co-workers by comparing the antioxidant activity with changes in composition and colour of kilned and roasted barley malts, found that the antioxidant activity of malts increased with their colour and has been attributed to the formation of MRPs. Furthermore, the authors observed that the antioxidant activity of malts of low colour (*i.e.*, pale malts) was largely due to compounds that do not absorbed in the visible region, such as polyphenols – whose levels decreased with high temperature during heat treatment – with colourless MRPs like reductones, and LMW MLDs also play a role. Contrarily, in roasted, coloured malts, HMW MLDs instead of polyphenols, were found to be the main responsible for the majority of the antioxidant activity (Samaras et al., 2005). Particularly, the main antioxidant capacity of HMW MLDs has been reported to be ascribed to their metal-chelating properties (due to their anionic nature) as well as, their high reducing power. Indeed, by the analysis of MRP fractions isolated from roasted and kilned malts according to their molecular weight, it has been demonstrated that the HMW MLDs isolated from roasted malt (roasting temperature ≈ 230 °C) exhibited approximately 4-fold higher reducing power than the LMW MLDs from kilned malt (kilning temperature ≈ 80 – 85 °C) (Carvalho et al., 2014). In addition, both roasted and kilned malt have demonstrated to possess radical scavenging activity, but roasted malt exhibited lower radical scavenging activity than kilned one (Carvalho et al., 2016b) and it was also found that the scavenging activity decreased with the increase in the content of HMW MLDs (Carvalho et al., 2014). Hence, the higher the heat treatment temperatures, the lower the radical scavenging activity, and the higher the reducing power of malt. The high reducing power of roasted malt, attributed to their high HMW MLDs content, has been shown to give pro-oxidant effect to malt (Carvalho et al., 2016b). In fact, in the presence of iron ions in the oxidated form (*i.e.*, Fe^{3+}), the pronounced reducing properties of roasted malt leads to the reduction of Fe^{3+} to Fe^{2+}. Then, the reduced iron ions (*i.e.*, Fe^{2+}) can generate hydroxyl radicals ($^{\bullet}OH$) by Fenton reaction as follow:

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + {}^{\bullet}OH + H_2O$$

Hydroxyl radicals is known to be one of the most reactive oxygen species, which can damage biomolecules and contribute to oxidative stress.

To conclude although MRPs are usually considered as antioxidant compounds, the chemical properties of each MRPs types can give them an antioxidant or pro-oxidant features. Since, as explained, based on the heat treatment

temperatures used during the last step of malting, degradation of endogenous phenolic compounds and formation of HMW MLDs with high reducing power but pro-oxidant effect can occur, it seems very important to identify the kilning conditions that on one hand promote the increase in the phenolic compounds with high antioxidant activity (*e.g.*, catechin, ferulic acid, caffeic acid and sinapic acid) and reduce the heat degradation of thermolabile antioxidant compounds including vitamins, and on the other hands, lead to the development of new "heat-induced" antioxidant compounds such as LMW MLDs and reduttones. In doing so, a product not only nutritionally improved but also with high antioxidant properties could be obtained and the incorporation of which into food and beverages at levels that do not adversely affect the organoleptic and sensorial features (*e.g.*, flavour and colour) of the final product may delay or prevent lipid oxidation and extend product shelf life, as well as enrich its health benefit.

Materials and Methods

4. Plant materials

The seeds of two approved and certified industrial hemp *cvs Futura 75* (French monoecious) and *Secuieni jubileu* (Romanian monoecious) were used. *Futura75* seeds were kindly provided by Molino Crisafulli (Caltagirone, CT, Italy); *Secuieni jubileu* seeds were purchased by Agricola Matteucci (Civita Castellana, VT, Italy). *Futura 75* is a *cv* already described in the scientific literature, whose seeds are largely used in food preparation, whilst *Secuieni jubileu* is a locally farmed hemp *cv* not still described in the literature, which is grown in the Tuscia area with a good seeds' production. For each *cv*, seeds were sifted and those with a diameter higher than 3 mm were selected and stored at –20 °C until the analyses were carried out.

5. Malting procedure

5.1. Steeping

Selected seeds (100 g) were steeped with deionized, sterile water in a ratio of 1:15 (w/v) at room temperature in the dark. The steeping process were carried out for 5 h with an air-rest period of 5 min every 30 min.

At the end of the steeping step, an aliquot (20 g) of steeped seeds was collected and stored at –20°C. The remaining seeds were germinated.

5.2. Germination

The germination process was performed in the dark, at 24 °C and 85% relative humidity, in an incubator (F.LLI GALLI, *High performance* incubator mod. 2800, Milan, Italy). Briefly, steeped seeds (80 g) were placed in a single layer, between two layers of wet filter paper (Whatman No. 1) on perforated germination trays. The seeds were moistened with sterile, distilled water each day (40 mL). In order to evaluate the best germination time according to the seeds' antioxidant profile, germination was carried out for 18 h; 42 h; 66 h; 90 h (**Figure 19**) and after each germination time, an aliquot (about 20 g) of each sample was freeze-dried, milled and stored at -80 °C until the analysis.

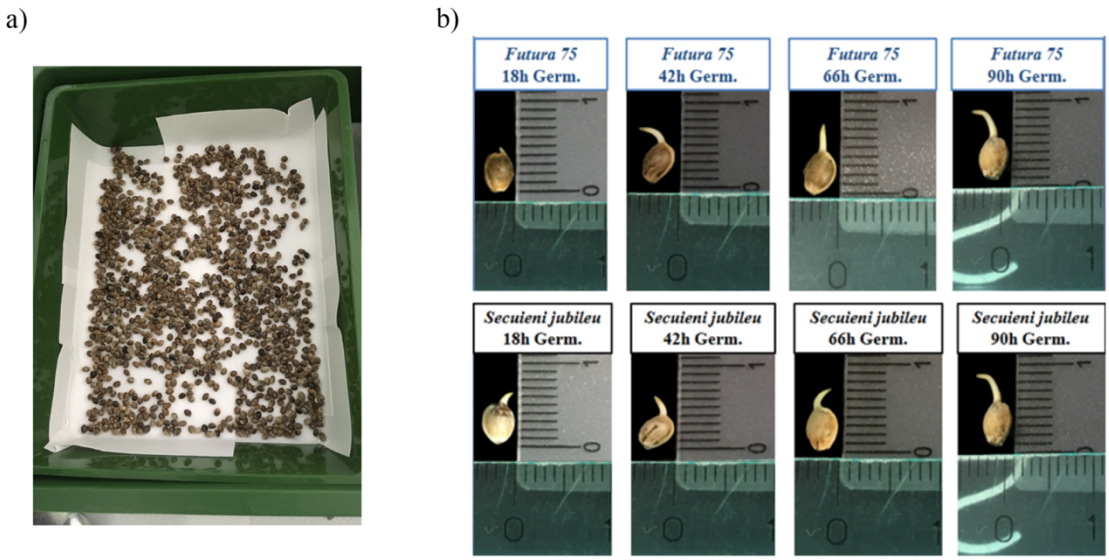

Figure 19: a) *During the germination step, a single layer of hemp seeds was placed on two layers of wet filter papers in a perforated germination tray. Above the seeds other two layers of wet filter papers were placed (not shown).* b) *Seeds of* Futura 75 *(at the top) and* Secuieni jubileu *(at the bottom)* cultivars *after 18 h; 42 h; 66 h; and 90 h of germination.*

Once the best germination time was individuated, seeds germinated for the best germination time were collected and divided in two fractions: the first one was stored at –20 °C for further analyses, the second one was subjected to the kilning step in order to complete the malting process.

5.3. Kilning

Seeds germinated for the best germination time were dried in a static oven (TCN 30, ARGO LAB, Modena, Italy) for 6h, at 50 °C or 70 °C as kilning temperature. Then, kilned seeds were immediately collected and stored at –20°C until analyses.

6. Steeping characteristics

During the steeping step, the increase in the moisture content of hemp seeds was estimated at different soaking intervals. Briefly, for each hour of steeping, about 3 g of seeds for each *cv* were collected and the moisture content of the samples was assessed in triplicate, in a static oven (TCN 30, ARGO LAB, Modena, Italy), at 105 °C until constant weight, according to Frassinetti et al. (2018).

7. Sample preparation

For all experiments, the seeds' samples were freeze-dried in a freeze-dryer (FreeZone 2.5, Labconco, Kansas City, MO, USA) and then ground by IKA® A11 basic Analytical mill. The ground seeds were immediately used or stored at –80 °C until analyses.

8. Evaluation of antioxidant features

8.1. Extracts' preparation

For the analyses of TPC and antioxidant activity, ground freeze-dried samples were extracted according to Frassinetti et al. (2018). Briefly, 1 g of sample was extracted with aqueous ethanol 80% (1:10, *w/v*), in the dark, for 4 h. Then, the samples were centrifuged at 6000 rpm, for 20 min, at 4°C. The supernatant was collected and used for the determination of TPC and total antioxidant capacity analysis. For the RP-HPLC analysis of phenolic compounds, samples were extracted according to (Siano et al., 2018) with some modification. Specifically, extraction was performed with 80% aqueous ethanol (1:10 *w/v*) for 30 min at RT using an ultrasonic bath (DU-100 Digital Ultrasonic cleaner, ArgoLab, Carpi, MO, Italy). The extract was then centrifuged at 3,500×g for 10 min and the supernatant was collected. Extraction was repeated for further 4 times. Pooled supernatants were dried in a rotary evaporator (Rotavapor® R-100, BÜCHI, Labortechnik, AG, Switzerland). Before injection, the dried phenolic extract was solubilized with methanol contained 0.5%formic acid and filtered through Whatman PTFE syringe filter 0.2 µm.

8.2. Determination of total phenolic compounds content

The TPC was determined using a modification of the Folin–Ciocalteu standard method (Singleton and Rossi, 1965) adapted for 96-wells plates and an automatic microplate reader (Infinite 2000, Tecan, Salzburg, Austria). Briefly, the assay was conducted by mixing to 30 µL of deionised water, 10 µL of ethanolic extract, 10 µL of Folin–Ciocalteau reagent, and 200 µL of 30% Na_2CO_3. After 30 min at room temperature, the absorbance of the mixture was measured at 725 nm. A standard curve with gallic acid (10-320 µg/mL) was prepared and the final results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (*dw*) of sample.

8.3 Determination of polyphenol profile composition by RP-HPLC analysis

Determination of polyphenol compounds was performed according to Smeriglio and co-authors (Smeriglio et al., 2016) with some modification. A Shimadzu Prominence-i LC-2030C 3D system equipped with autosampler, binary pump, column oven and a photodiode (PDA) detector was used. 10 µL of extracts or external standards were separately injected into a Kinetex Evo C_{18} column (150 × 4.6 mm, 5 µm; Phenomenex, Bologna, Italy) pre-heating at 30 °C. The separation was performed using a binary mobile phase consisted of methanol (A) and water/acetic acid, (97:3 *v/v*) (B), at 1.2 mL/min, combined in a gradient as follows: 0 min, 90% B; 0–6 min, 90–85% B; 6–10 min, 85% B; 10–12 min, 85–77% B; 12–13 min, 77–67% B; 13–18 min, 67–60% B; 18–45 min, 60–

0% B. Separations were monitored at λ 310 nm and 260 nm, and the peaks were integrated using the LabSolutions software (vers. 5.71 SP2, Shimadzu). N-*trans*-caffeoyltyramine and *p*-hydroxybenzoic acid were identified comparing their retention times and UV spectrums with the external standards. Quantification was based on external standard method and the results were expressed as µg/g of *dw* of sample. Whereas cannabisin A and cannabisin B were tentatively identified based on their UV spectrum due to the lack of the corresponding commercial standards and the results were expressed as µg of *trans*-cinnamic acid equivalents (CAE)/g of *dw* of sample. Calibration curves were performed in the range of 1–20 µg/mL (for N-*trans*-caffeoyltyramine, cannabisin A, cannabisin B and *trans*-cinnamic acid) or 0.025–1 µg/mL (for *p*-hydroxybenzoic acid).

8.4. Determination of tocopherol composition by RP-HPLC analysis

8.4.1. Tocopherol extraction

Tocopherols were extracted after micro-saponification according to (Górnaś et al., 2015) with minor modification. Briefly, 0.3 g of freeze-dried sample were placed into a 15 mL tube, followed by 0.05 g of ascorbic acid, 2.5 mL of ethanol, and 0.25 mL of aqueous potassium hydroxide 60% (v/v). The tube was immediately closed, vortexed vigorously for 10 sec and incubated in a water bath at 80 °C for 25 min. After the first 10 min of incubation, samples were vortexed for 10 sec. After 25 min of incubation, samples were immediately cooled in ice for 5 min, 2.5 mL of sodium chloride 1% was added to each tube and the samples were mixed for 5 sec on the vortex. Then, 2.5 mL of a *n*-hexane:ethyl acetate (9:1, *v/v*) solution was added and the samples were vortexed for 15 sec and centrifuged for 5 min, at 1000 × g, at 4 °C. The organic layer was transferred to a round bottom flask and the residue was re-extracted twice. The pooled organic fractions were evaporated in a vacuum rotary evaporator (Rotavapor® T-100, Büchi, Switzerland) at 40 °C and dissolved in 1 mL ethanol.

8.4.2. Tocopherol determination and quantification

The tocopherol isomers were separated through a reverse phase chromatographic analysis performed by using an HPLC Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a PDA detector. An amount of 20 µl of extract was loaded into a Dionex Acclaim 120 C_{18} column (150 mm × 4.6 mm, 5 µm; Thermo Fisher Scientific, Waltham, MA, USA) and eluted in isocratic mode with 0.8 ml/min MeOH (Urvaka et al., 2019). The run time of each sample was 30 min. Tocopherols were detected at 295 nm. Calibration curves (10-200 µg/mL) were plotted using γ-, δ-, and α-tocopherol standards (Sigma Aldrich, St. Louis, MO, USA). Quantification of individual tocopherols was performed based on the resulting curves. Individual tocopherol concentration was summed up in order to obtain the total tocopherol amount.

8.5. Determination of total antioxidant capacity

The total antioxidant capacity was assessed by both Cupric Reducing Antioxidant Capacity (CUPRAC) and $ABTS^{\bullet+}$ radical scavenging activity assays. For both assays, a standard curve with Trolox (4.7-300 µmol) was prepared and the antioxidant capacity of each sample was determined by comparison with the Trolox standards and expressed as µmol of Trolox equivalents (TE) per g of *dw* of sample.

8.5.1. CUPRAC assay

The CUPRAC assay was performed according to the method described by Rafi and co-workers (Rafi et al., 2018) adapted for 96-well plates and an automatic reader (Infinite 2000, Tecan, Salzburg, Austria). Briefly, 40 µL of appropriately diluted sample's ethanolic extract, 50 µL of 10 mM $CuCl_2$, 50 µL of 7.5 mM neocuproine and 60 µL of 1 M NH_4CH_3COO (pH 7) were added to a 96-well plate with total volume of 200 µL. Incubation of the mixture was carried out for 1 h, and after that, the absorbance was recorded at 450 nm using an automatic microplate reader (Infinite 2000, Tecan, Salzburg, Austria).

8.5.2. $ABTS^{\bullet+}$ radical scavenging activity assay

The $ABTS^{\bullet+}$ (2,2 -azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was evaluated by the OxiSelect™ Trolox Equivalent Antioxidant Capacity (TEAC) Assay Kit ($ABTS^{\bullet+}$) (Cell Biolabs INC.) following the manufacturer's instruction. Specifically, 25 µL of appropriately diluted sample's ethanolic extract and 150 µL of diluted $ABTS^{\bullet+}$ reagent (1:50 in 75% aqueous ethanol) were added to a 96-well plate. The mixture was incubated for 5 min in an orbital shaker and after that, the absorbance was immediately recorded at 405 nm in an automatic microplate reader (Infinite 2000, Tecan, Salzburg, Austria).

9. Evaluation of nutritional features

9.1. Proximate composition

Proximate composition of seeds was determined according to the standard procedures of the Association of Official Analytical Chemists International (AOAC, 2005). Moisture content was determined by heating 1 g of sample at 102°C in an oven (TCN 30, ARGO LAB, Modena, Italy) until constant weight. Crude protein content (conversion factor: N x 6.25) was estimated using the Kjeldahl method (SpeedDigester K-425 and Distillation Unit K-350, BÜCHI, Labortechnik, AG). Crude fat was extracted using a Soxhlet Extraction System B-811 (BÜCHI, Labortechnik, AG) with petroleum ether (40/60 boiling point) as solvent. Ash was determined in a muffle furnace at 550 °C for 4h. Total carbohydrates were determined by difference (*i.e.,* 100 – (g [protein + fat + ash] in 100 g of *dw* sample)). The energy value was calculated on fresh weight, using the Atwater factor (FAO, 2003), as follow:

$$\text{Energy value (Kcal)} = (\%\,Protein \times 4) + (\%\,Fat \times 9) + (\%\,Carbohydrate \times 4)$$

9.2. Fatty acids profile

Lipid extraction was performed according to Costantini et al. (2014). Briefly, 1 g of milled and lyophilized samples (raw and malted at 50 °C and 70 °C) were dissolved with 10 mL chloroform/methanol (2:1 v/v). Then, 500 µL of each extract was dried by nitrogen and trans-methylated by BF3 (Santa Cruz Biotechnology Inc., Dallas, Texas, 75220, USA), before being heated under reflux with methanol at 72°C for 30 min. The obtained fatty acid methyl esters (FAMEs) were extracted with n-hexane and evaporated by nitrogen flux to dryness. Transmethylated samples were dissolved in *n*-hexane, and their compositions were determined by GC/MS analysis. Specifically, GC/MS analyses were carried out on a gas chromatograph with a flame ionization detector (FID) directly coupled

to a mass spectrometer (MS) Perkin Elmer Clarus 500 model (Waltham, MA, USA). GC was equipped with a 60 m×0.25 mm, film thickness of 0.5 µm, Restek Stabilwax polar capillary column. Helium was used as the carrier gas at a flow rate of 1.0 mL/min with constant flow. The injector was set to a 280 °C and the oven temperature was programmed from 170° C, at rate of 3° C/min to 260° C for 10 min. 2 µL of each sample were injected into the column in splitless mode; the mass spectra were recorded at 70 eV (EI) and were scanned in the range of 40–500 m/z. The ion source and the connection parts temperature were 220 °C. The GC-TIC mass spectra were obtained by the TurboMass data analysis software. Electronic integration measurements of peak areas by the FID detector were used to generate quantitative data of all the compounds without the use of an internal standard or correction factors and expressed in percentages; the identification was performed by matching their mass spectra with those stored in the Wiley and NIST 02 mass spectra libraries database.

10. Evaluation of antinutritional factors

Analyses of antinutritional factors trypsin inhibitors (TIs) and phytic acid were performed on defatted material obtained by using *n*-hexane at a solvent to sample ratio of 1:10 (*w/v*). Briefly, samples were vigorously stirred for 30 min in an orbital shaker (VDRL mod. 711, Tecnochimica moderna s.r.l., Monterotondo, RM, Italy). Then, the *n*-hexane was decanted and removed. The procedure was repeated three times. Finally, samples were left to dry overnight under a fume hood. The obtained defatted, fine powder was collected and stored at -20 °C until analysis.

10.1. Trypsin inhibitors (TIs) activity analysis

Trypsin inhibitors (TIs) were extracted overnight from 0.1 g of defatted whole milled hemp seeds (sample to hexane ratio 1:10, w/v) with 25 mL 2 mM glycine buffer, pH 11.0, containing 2 mM NaCl, 10 mM urea and 25 mM EDTA. The extract was centrifuged for 15 min at 4 °C and 14 000 rpm. TIs activity (TIA) was measured as described by Galasso et al. (2016). 200 µL of trypsin solution (40 µg/mL) were added to 200 µL of extract and the mixture was incubated in a water bath at 37 °C for 3 min. Then 500 µL of N-benzoyl-DL-arginine p- nitroanilide hydrochloride (BAPNA) solution (0.4 mg/mL, pre-warmed to 37 °C) used as trypsin substrate, were added to start the reaction. The reaction was stopped after the addition of 100 µL 30% (v/v) acetic acid, after an incubation at 37 °C for 10 min, and the mixture was centrifuged for 5 min at 10 °C and 10 000 rpm. The trypsin's activity was measured reading the absorbance at 410 nm on Uvikon spectrophotometer (942, Kontron Instruments, Zurich, Switzerland). One unit of trypsin inhibitor (TIU) was defined as 0.01 decrease in absorbance at 410 nm compared with the control (without inhibitor) under the assay condition. Results were expressed on defatted material in order to make them comparable with other literature data.

10.2. Phytic acid content analysis

Phytic acid was extracted overnight from defatted (sample to hexane ratio 1:10, *w/v*) whole milled hemp seeds (raw and malted at 50 °C and 70 °C) with 0.66 M HCl and analysed with the Phytic acid assay kit (Megazyme®) according to the manufacturer's instructions. Hemp seed phytate content was express as g/100 g of *dw* of defatted material. Results were expressed on defatted material in order to make them comparable with other literature data.

11. Statistical analysis

Malting treatment were repeated three times and each sample was analysed in triplicate (n = 3) or duplicate (n = 2). The results are presented as means ± standard deviation. The obtained data were analysed by one-way analysis of variance (ANOVA), followed by Tukey as post hoc test, using R software (Version 1.0.153). In order to assess differences between the analysed varieties and due to the treatment (*i.e.,* malting) within each *cv* a one-way analysis of variance for each variable parameter (*i.e.,* variety or treatment) was performed. Differences were considered significant at the $p \leq 0.05$ level.

Results and Discussion

12. Steeping characteristics of hemp seeds

The moisture content of *Futura 75* and *Secuieni jubileu* seeds during the steeping step, is shown in **Figure 20**.

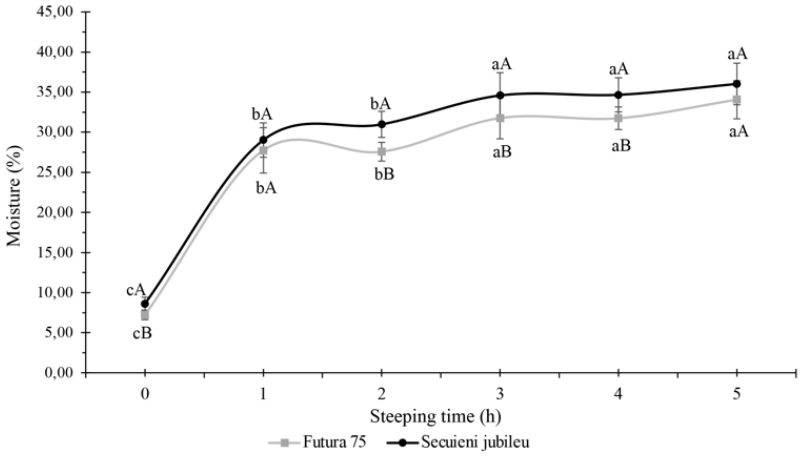

Figure 20: *Steeping characteristics of* Futura 75 *and* Secuieni jubileu *seeds. Data are means ± standard deviation of at least three replicates (n = 3). Different letters indicate significant differences ($p \leq 0.05$) according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among different steeping time and uppercase letters represent comparison between* cultivars *at the same steeping time.*

For the seeds of both analysed varieties the water uptake was very rapid during the early stage of steeping (*i.e.,* in the first steeping hour), increasing over than three-fold compared to raw seeds (from 7.2% to 27.7% for *Futura 75* seeds and from 8.6% to 29.0% for *Secuieni jubileu* seeds) (**Figure 20**). This rapid imbibition is related to the fact that dry seeds have low water potential due to their low moisture content and this causes rapid water influx when they come into contact with water (Weitbrecht et al., 2011). After 3 h of steeping, the seed's moisture content of both *cvs* increased significantly, reaching a four-fold higher value compared to raw seeds. Furthermore, after 3 h of steeping moisture reached a plateau, as shown by the absence of significant changes. Interestingly, moisture content of *Secuinei jubileu* seeds increased faster than *Futura 75* seeds, that reached a value statistically comparable to the *Secuinei jubileu* seeds only after 5 h of steeping (**Figure 20**). Shaban reported that seeds have a critical moisture content for germination that is different and specific for each species. Once this critical moisture content is attained in the seed, sufficient water amount is present to initiate germination, therefore, the seed is committed to that event and cannot turn back (Shaban, 2013). In literature there is no information concerning the critical moisture content for hemp seeds, however, we found that after 5 h of steeping some seeds of both *cvs* were "chitted" showing the testa rupture and radicle protrusion, so proving that the germination phase was started. Hence, according with our results, the hemp seeds' critical moisture content amounted around 34% and 36%.

13. Best germination time evaluation

In order to identify the best germination time for the malting process depending on the seed's antioxidant profile, *Futura 75* and *Secuieni jubileu* steeped seeds were germinated for 18 h, 42 h, 66 h, and 90 h. Then, the TPC and the ABTS radical scavenging activity of ethanolic extracts of seeds untreated, steeped and germinated for the four germination times were analysed by Folin–Ciocalteu and TEAC assays, respectively. The obtained results are depicted in **Figure 21**.

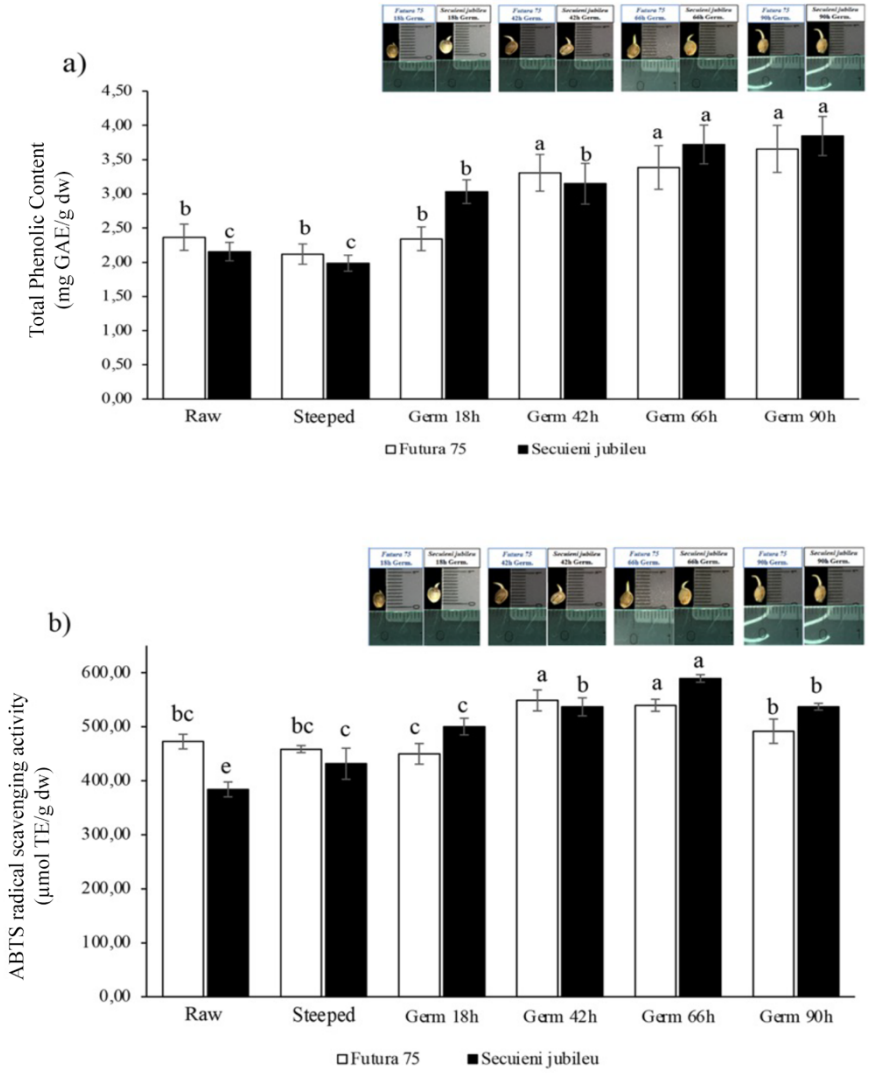

Figure 21: *Antioxidant profile of seeds Raw, Steeped, and germinated for 18 h (Germ 18h), 42 h (Germ 42h), 66 h (Germ 66h), and 90 h (Germ 90h) of* Futura 75 *and* Secuieni jubileu *hemp* cultivars. *a) Total phenolic content (TPC) (mg GAE/g dw) b) ABTS radical scavenging activity (µmol TE/g dw). Data are means ± standard deviation of at least three replicates (n = 3). Different letters within the same* cultivar *indicate significant differences ($p \leq 0.05$) among samples. $ABTS^{\bullet+}$: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). GAE: Gallic Acid Equivalents. TE: Trolox Equivalents. dw: dry weight.*

As shown, both TPC (**Figure 21a**) and ABTS radical scavenging activity (**Figure 21b**) increased during germination with a similar trend and reached the highest value after 90 h and 66 h of germination, respectively. Interestingly, the increase was more remarkable for *Secuieni jubileu* seeds (+ 78% for TPC; + 50% for TEAC) than *Futura 75* (+ 55% for TPC; + 13% for TEAC). These results are in accordance with the fact that the antioxidant activity is closely related to polyphenols, compounds with strong antioxidant power. In fact, it is found a positive correlation between TPC and TEAC ($R^2 = 0.56$) by the Pearson's analysis.

The increase of TPC during the germination was reported for several species (Cevallos-Casals and Cisneros-Zevallos, 2010) and it might be due to *de novo* biosynthesis of phenolic compounds by the activation of phenylpropanoids pathway, or the releasing of bound phenolic compounds by the action of enzymes activated during the germination. Several authors reported also a higher antioxidant capacity in sprouts in comparison to seeds for many plant species (Cevallos-Casals and Cisneros-Zevallos, 2010; Pająk et al., 2014), including *C. sativa* L. (Frassinetti et al., 2018) and this is probably due to the rise in the content of antioxidant compounds such as vitamins and polyphenols during germination, other than in their type and chemical nature.

Differently to the TPC value that reached the highest value after 90 h of germination (**Figure 21a**), the antioxidant capacity as resulted by the TEAC assay, gradually increased reaching the highest value after 66 h of germination followed by a slightly but significant, decrease when undergone to prolonged germination time (**Figure 21b**). We can assume that this is could be due to a degradation of antioxidant compounds different to polyphenols, such as tocopherols or bioactive peptides, occurred at prolonged germination time. Indeed, tocopherols are chain-breaking compounds with high antioxidant power, abundant in hemp seeds. Some literature data had shown an increase of the tocopherols content during early germination, followed by a decrease during long germination time (Gan et al., 2017). In addition, it was reported that the hydrolysis of hemp seeds' proteins produced small peptides with antioxidant activity and the degree of peptides' bioactivity depend on the hydrolysis time (Teh et al., 2016). Since during germination the activation of hydrolytic enzymes occurs, it is possible that a shorter germination time could produce bioactive and antioxidant peptides further hydrolysed to smaller molecules no more bioactive after long germination time. Overall, these results showed that the first step of the malting process (*i.e.,* the steeping step) did not cause any changes in the TPC and ABTS radical scavenging activity of the analysed seeds, whereas the germination step effectively improved the antioxidant profile of both *Futura 75* and *Secuieni jubileu* hemp seeds. Therefore, according to our results, 66 h of germination seems to be the best time for both *cvs*. For these reason, three days of germination has been adopted to perform the whole malting process.

15. Antioxidant features of raw and malted hemp seeds

15.1. Total phenolic compounds content of raw and malted hemp seeds

Phenolic compounds are considered among the most important contributors to the antioxidant capacity of seeds. They are secondary metabolites synthesized by plants in their normal development and especially as response to stress conditions. Several studies performed on different plant species, highlighted that processes such as germination and malting have a positive impact on the antioxidant values of a seed, as consequence to the rise in the content of antioxidant compounds such as polyphenols and tocopherols (Dabina-Bicka et al., 2011; Li et al., 2019; Pająk et al., 2019; Terpinc et al., 2016).Therefore, after identifying the best germination time according to

the antioxidant profile of *Futura 75* and *Secuieni jubileu* seeds (**Figure 21**), we evaluated the effect of each step of the malting process on the TPC, as well as the tocopherol and antioxidant capacity of the analysed hemp seeds, after malting at either 50 °C or 70 °C. The results for TPC of the raw, steeped, 3-days germinated and malted seeds of the two hemp varieties are reported in **Figure 22**.

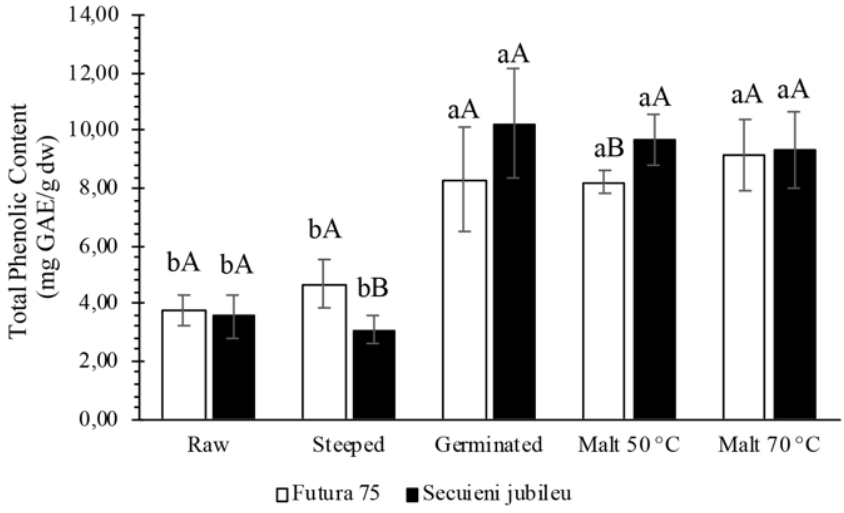

Figure 22: *Total phenolic content (TPC) of untreated (Raw), Steeped, Germinated, and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C) seeds of* Futura 75 *and* Secuieni jubileu *hemp* cultivars *expressed as milligrams of gallic acid equivalents per gram of dry weight of sample (mg GAE/g dw). Data are means ± standard deviation of at least three replicates (n = 3). Different letters within the same cultivar indicate significant differences ($p \leq 0.05$), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among raw, steeped, germinated, and malted seeds and uppercase letters represent comparison between* cultivars.

There were no statistically significant differences for the TPC value of the raw seeds of both analysed *cvs*, which amounted to 3.76 ± 0.55 and 3.56 ± 0.76 mg GAE/g *dw* for *Futura 75* and *Secuieni jubileu*, respectively. These values were similar, even if slightly higher, to that obtained by Frassinetti and co-authors (2018) for the seeds of *Futura 75 cv*, whilst Irakli and colleagues (2019) reported an almost two-fold higher value for the seeds of the same *cv*. However, in the paper of Irakli and co-authors the analysis was performed on defatted seeds instead of whole seeds, that is the seeds after removing the fat fraction. When the fat fraction is removed from a matrix, the remaining components, including phenolic compounds, are more concentrated in the resulting defatted matter in comparison to the whole matter. So, the TPC analysis performed on defatted matter normally gives higher value than the analysis performed on the same amount of whole matter. This could explain the higher TPC value obtained by Irakli and co-workers for the *Futura 75* seeds compared to our results referred to the whole seeds. When compared to other oilseeds such as flaxseeds and chia seeds, the TPC value we found for both *Futura 75* and *Secuieni jubileu* raw seeds resulted to be over three-fold higher (Beltrán-Orozco et al., 2020; Pająk et al., 2019).

Regarding the malting treatment, 5 h of steeping did not affect the TPC level of the seeds of both *cvs*. In this context, Khandelwal and co-authors (2010) reported a negative impact of steeping on the TPC of some pulses (*i.e.,* red gram, Bengal gram, green gram and lentil) as consequence of their leaching into the soaking water and/or the

formation of insoluble complexes with proteins or polymerization of lower molecular weight phenolic compounds. By contrast, Cevallos-Casals and Cisneros-Zevallos (2010) showed that for some edible seeds such as fava, sunflower, mung bean, fenugreek, broccoli, and alfalfa, the imbibition represents an active period of phenolic synthesis, thus induces an increase in the amount of TPC. Similarly to our results, 8 h of steeping of two buckwheat varieties' seeds had no statistically significant influence on the TPC level (Terpinc et al., 2016).

A significant ($p \leq 0.05$) increase in the TPC level was observed after three days of germination and it was maintained after the heat treatment performed at either 50 °C or 70 °C. Specifically, after three days of germination, the TPC value increased two-fold (+ 121%) in the *Futura 75* seeds and three-fold (+ 188%) in the *Secuieni jubileu* seeds in comparison to untreated (raw) seeds. In this latter *cv*, the TPC level reached its maximum value after three days of germination (10.26 ± 1.87 mg GAE/g *dw*); then it was not significantly reduced after the heat treatment, so that *Secuieni jubileu* malted seeds have an increase in the TPC level up to 173% comparing to unmalted counterpart. For the *Futura 75* variety, the TPC level reached its maximum value after malting at 70 °C increasing up to 144% in comparison to the unmalted seeds, although there were no significant differences between malting and germinated seeds. Frassinetti and colleagues (2018) demonstrated that three days of germination performed at 24 °C led to an increase of 116% in the *Futura 75* seeds, similarly to our results. It was suggested that the increase in phenolic compounds during malting could be attributed to *de novo* synthesis of phenolic compounds during germination induced by enzymatic systems activation (*e.g.* enzymes involved in the phenylpropanoids pathway), as well as to structural changes in plant cell wall caused by heat treatment, able to improve the release of glycosylated/esterified phenolic compounds (Aguilar et al., 2019; Carciochi et al., 2016). In fact, some investigations reported that the heat treatment of germinated seeds can further increase the TPC values in the final malt, but the value of processing temperature is an important factor to take into account, since high processing temperature can negatively affect the TPC value of sample (Carciochi et al., 2016; Terpinc et al., 2016). Based on our results, the two alternative adopted kilning temperatures treatments did not have negative impact neither induced further increase in the hemp seeds' TPC, resulting in unchanged TPC values statistically significant for both *cvs*.

15.2. Polyphenol compounds composition of raw and malted hemp seeds

Ethanol extracts of unmalted and malted seeds at 50 °C and 70 °C were subjected to RP-HPLC analysis and the content of four main phenolic compounds detected by chromatographic separation (**Figure 23**) and successfully identified and quantified are presented in **Table 11**.

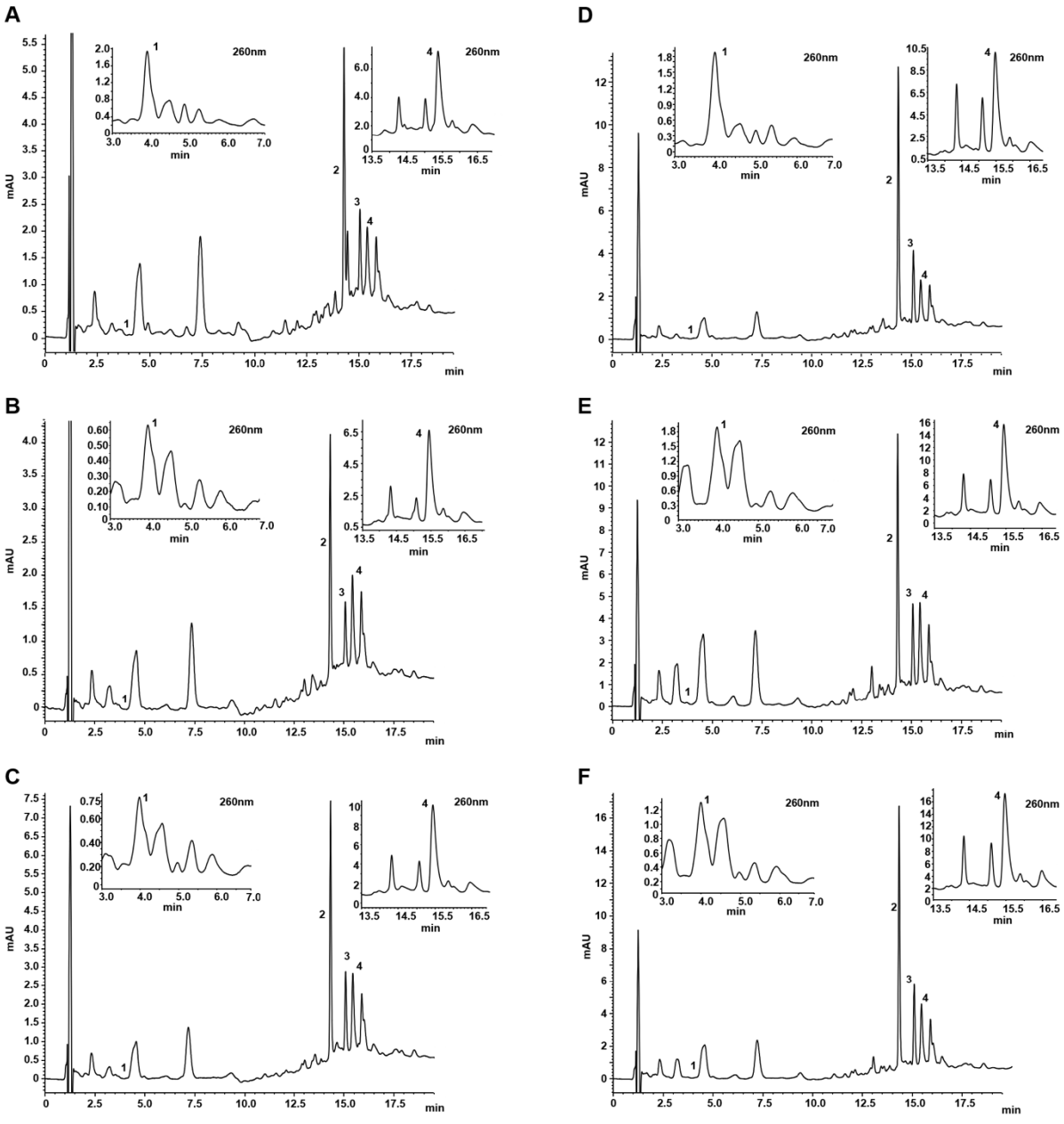

Figure 23: *Chromatograms of Futura 75 and Secuieni jubileu seeds' ethanol extracts. A) Secuieni jubileu unmalted (raw) seeds; B) Secuieni jubileu malted at 50 °C seeds; C) Secuieni jubileu malted at 70 °C seeds; D) Futura 75 unmalted (raw) seeds; E) Futura 75 malted at 50 °C seeds; F) Futura 75 malted at 70 °C seeds. The chromatograms area expansion of the integrated peaks at 260 nm was also reported. 1, p-hydroxybenzoic acid; 2, N-trans-caffeoyltyramine; 3, Cannabisin B; 4, Cannabisin A.*

Table 11: *Phenolic compounds (µg/g dw) identified in ethanol extract of Futura 75 and Secuieni jubileu hempseeds cultivars, untreated (Raw seed) and malted at either 50 °C (Malt 50 °C) or 70 °C (Malt 70 °C), by RP-HPLC analysis. Data are means ± standard deviation of two (n = 2) replicates. Different letters indicate significant differences ($p \leq 0.05$), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among treatments and uppercase letters represent comparison between cultivars.*

Hempseed *cultivar*	*N-trans*-caffeoyltyramine	Cannabisin A*	Cannabisin B*	*p*-hydroxybenzoic acid
Futura 75				
Raw Seed	69.28 ± 0.60^{bA}	88.34 ± 0.43^{bA}	18.95 ± 1.79^{bA}	7.46 ± 0.17^{aA}
Malt 50 °C	60.04 ± 2.44^{cA}	121.14 ± 9.91^{abA}	16.00 ± 0.64^{bA}	6.14 ± 0.20^{bA}
Malt 70 °C	91.13 ± 1.22^{aA}	140.93 ± 15.98^{aA}	25.24 ± 1.96^{aA}	5.50 ± 0.15^{bA}
Secuieni jubileu				
Raw Seed	35.45 ± 2.42^{bB}	57.58 ± 3.70^{bB}	9.64 ± 2.17^{aB}	6.81 ± 0.86^{aA}
Malt 50 °C	31.06 ± 0.67^{bB}	68.66 ± 0.43^{bB}	7.42 ± 0.06^{aB}	3.27 ± 0.01^{bB}
Malt 70 °C	42.47 ± 2.59^{aB}	90.59 ± 5.55^{aA}	11.17 ± 0.50^{aB}	2.95 ± 0.12^{bB}

*Expressed as µg *trans*-cinnamic acid equivalents (CAE)/g of dry weight (*dw*).

Major polyphenols found in hempseeds are lignans belonging to the two groups of phenolic amides and lignanamides (Farinon et al., 2020). As previously described, the first ones are derived from the phenylpropanoid pathway and are made by an ammine moiety (tyramine, derived from the tyrosine oxidative decarboxylation) linked to a phenolic moiety (a hydroxycinnamic acid, such as coumaric, caffeic or ferulic acid). While lignanamides are a result of oxidative, random coupling between phenolic amide monomers.

N-*trans*-caffeoyltyramine and *p*-hydroxybenzoic acid were identified by comparing the retention times and spectra obtained from the extracts' chromatogram with those of the corresponding external standards. Whereas cannabisin A (UV λ_{max} in EtOH 260 nm) and cannabisin B (UV λ_{max} in EtOH 310 nm) were identified on the basis of their spectral characteristics, according to literature data (Irakli et al., 2019; Moccia et al., 2019; Pojić et al., 2014; Siano et al., 2018). Hence, quantification of both N-*trans*-caffeoyltyramine and *p*-hydroxybenzoic acid was based on their external standards calibration curve, while Cannabisin A and Cannabisin B were expressed in *trans*-cinnamic acid equivalents (CAE) due to the lack of corresponding external standards.

Accordingly with other literatures data (Alasalvar et al., 2021), we found that the most abundant phenolic amide in both *Futura 75* and *Secuieni jubileu* raw seeds was N-*trans*-caffeoyltyramine accounting for 69.28 ± 0.60 and 33.45 ± 2.42 µg/g *dw*, respectively. Whereas the most abundant lignanamide in both the analyzed varieties was the Cannabisin A (88.34 ± 0.43 and 57.58 ± 3.70 µg/g *dw* for *Futura 75* and *Secuieni jubileu* raw seed, respectively), followed by Cannabisin B (18.95 ± 1.79 and 9.64 ± 2.17 µg/g *dw* for *Futura 75* and *Secuieni jubileu* raw seed, respectively). Although this profile was qualitatively comparable to that obtained in other studies (Alasalvar et al., 2021), we found values quantitatively slightly lower. The differences could be explained by different varieties and quantification methods, as well as the environment that represent an important factor largely influence the plants' polyphenol profile. In addition to N-*trans*-caffeoyltiramine, Cannabisin A and Cannabisin B, we also detected *p*-hydroxybenzoic acid in lower quantities.

Malting significantly rose the content of N-*trans*-caffeoyltyramine and Cannabisin A for both *cvs*, especially for the *Futura 75* one, and particularly, when the kilning temperature was 70 °C. These polyphenols were defined as the major contributors to the antioxidant activity in hempseeds (Irakli et al., 2019), hence, their increase may

play an important role to the total antioxidant activity of malted seeds. In cereal, changes in the polyphenols profile after malting was reported and attributed both to polyphenols' *de novo* biosynthesis and release of bound polyphenols, resulting in the increase of polyphenols' extractability and bioavailability (Carvalho et al., 2016a; Lemmens et al., 2018b). Actually, N-*trans*-caffeoyltyramine is found to be mainly located in the hempseeds hull, so their increase after malting at the higher kilning temperature (*i.e.*, 70 °C) may be effectively related to the thermal-induced changes in the structure of the molecules connected to bound phenolic compounds and consequently increase in its release. Interestingly, in both *cvs* the amount of *p*-hydroxybenzoic acid significantly ($p \leq 0.05$) decreased after malting. *p*-hydroxybenzoic acid is a phenolic acid and considering that phenolic acid are constituents of lignanammide, it could be speculated that *p*-hydroxybenzoic acid may be addressed and used in the lignanammide biosynthesis pathway. Chromatograms of malted and unmalted sample (**Figure 23**) show the presence of additional peaks we were unable to identified and that overall, probably contributing to the TPC and antioxidant activity values. Hence, further and more resolutive analysis could be appropriated

15.3. Tocopherol profile of raw and malted hemp seeds

Together with phenolic compounds, tocopherols represent another class of important antioxidants involved in the reproduction and physiology, and effective growth accelerator, which act synergistically with polyphenols to protect against lipid oxidation and thus, they can prevent the onset of chronic-degenerative diseases related to oxidative stress, when included in the diet. Since tocopherols are thermolabile compounds, we focused on analysing the effect of the two different kilning temperature of the malting procedure on the tocopherols profile of both *Futura 75* and *Secuieni jubileu* seeds, in order to investigate any possible negative impact of the heat treatment. The obtained results are depicted in **Figure 24**.

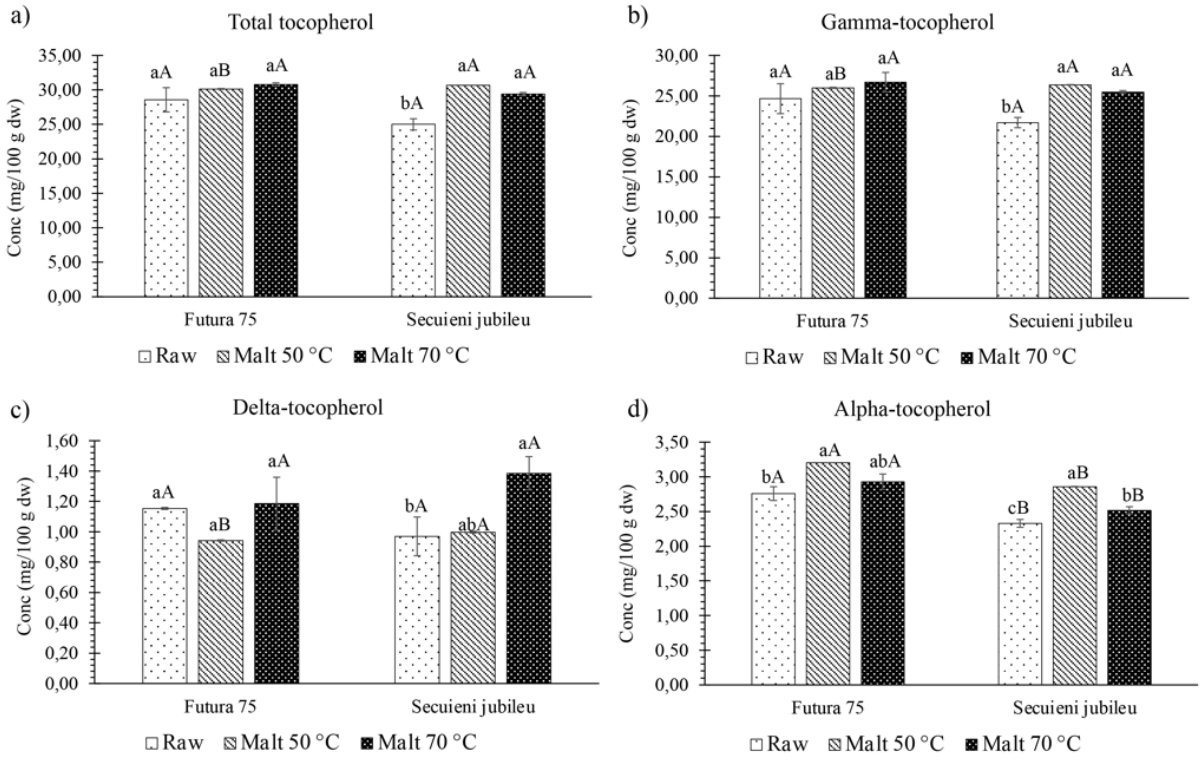

Figure 24: *Tocopherols' composition of untreated (Raw) and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C) seeds of* Futura 75 *and* Secuieni jubileu *hemp* cultivars *expressed as milligram of tocopherol per 100 grams of dry weight sample (mg/100 g dw). a) Total tocopherol; b) gamma-tocopherol; c) delta-tocopherol d) alpha-tocopherol. Data are mean ± standard deviation of two replicates (n = 2). Different letters indicate significant differences (p ≤ 0.05) within the same* cultivar *according to the one-way analysis of variance. Lowercase letters represent Tukey's test comparison among malted and raw seeds. Uppercase letters represent comparison between* cultivars.

The total tocopherol amount was 28.57 ± 1.76 and 25.00 ± 0.82 mg/100 g *dw* of *Futura 75* and *Secuieni jubileu* raw seeds, respectively, and there were no significant differences between *cvs* (**Figure 24a**). According to previous studies, among the tocopherols' isoform, hemp seed includes the α-, δ-, and γ-tocopherols, the latter of which is

the most abundant, representing almost the 90% of the total tocopherol, followed by α- and δ-tocopherol, present in lower concentration (Galasso et al., 2016; Vonapartis et al., 2015). Interestingly, we found slightly but significant differences only for the α-tocopherol which resulted higher in the *Futura 75* raw seeds than in the *Secuieni jubileu* ones (2.76 ± 0.10 vs 2.33 ± 0.06 mg/100 g *dw*, respectively). The values we found were comparable to that reported for the fat fraction extracted from hemp seeds (Blade et al., 2006; Oomah et al., 2002), and pointed out that hemp seeds of both analysed varieties are a source of tocopherol as good as other edible seeds traditionally considered an excellent source of these compounds, such as flaxseed (Trela et al., 2019). Furthermore, considering the contribution of each isomers to the vitamin E biological activity, as defined by the European Food Safety Authority (alpha-tocopherol, 1.0 mg; beta-tocopherol, 0.5 mg; gamma-tocopherol, 0.1 mg; delta-tocopherol, 0.03 mg) (EFSA NDA Panel, 2015), the medium vitamin E content was 4.88 and 4.13 mg/100 g *fw* in *Futura 75* and *Secuieni jubileu* raw seeds, respectively. The vitamin E average intake (AI) set by EFSA is 13 mg/day for males and 11 mg/day for females (EFSA NDA Panel, 2015), hence, a serving portion (*i.e.* 30 g) of hemp seeds would contribute to satisfying the vitamin E AI up to 11.3% for males and 13.3% for females in the case of *Futura 75 cv* and up to 9.5% for males and 11.3% for females in the case of *Secuieni jubileu* variety.

Malting at 50 °C and 70 °C did not significantly affect the total tocopherol content in the *Futura 75* seeds; conversely a significant increase in the total tocopherol of *Secuieni jubileu* seeds was observed after malting at 50 °C (+ 22%) and 70 °C (+ 18%) (**Figure 24a**). Similar results were found for the γ-tocopherols amount (**Figure 24b**). Concerning the δ-tocopherol content, it was found unchanged in the *Futura 75* malted seeds, instead a growing, but not significant, trend was obtained for *Secuieni jubileu* seeds malted at 50 °C. The δ-tocopherol amount was found to be significantly higher (+ 43%) in the *Secuieni jubileu* seeds malted at 70 °C in comparison to raw seeds (**Figure 24c**). As regard the α-tocopherol, its amount significantly increased after malting at 50 °C and 70 °C compared to unmalted seeds, for both *cvs*, and the increase was more remarkable for *Secuieni jubileu* variety than *Futura 75* (Malt 50 °C: + 16% for *Futura 75* and + 23% for *Secuieni jubileu*; Malt 70 °C: + 6% for *Futura 75* and + 8% for *Secuieni jubileu*). However, the value found for samples Malt 70 °C was lower than those for samples Malt 50 °C (**Figure 24d**), indicating that α-tocopherol is probably more susceptible to heating. The significant increase in the α-tocopherol amount in the malted samples is accompanied with an increment of the vitamin E biological activity which ranged from 5.26 to 5.82 and 5.63 mg/100 g *dw* in the *Futura 75* Malt 50 °C and Malt 70 °C, respectively and from 4.52 to 5.57 and 5.11 mg/100 g *dw* in the *Secuieni jubileu* Malt 50 °C and Malt 70 °C, respectively. This indicated that malting of hemp seeds using a kilning temperature of 50 °C could help to improve the vitamin E bioactivity in hemp seed, especially for the *Secuinei jubileu cv*.

To the best of our knowledge, this is the first study exploring the changes in the tocopherols profile of hemp seeds after malting, therefore, comparison with other literature data is rather difficult. Moreover, limited investigation and conflicting results about this topic are present also regarding other seeds commonly malted, such as barley. For example, Dabina-Bicka and colleagues (2011) reported a significant increase by 38% in the content of vitamin E during malting of barley, attributed to the beginning of biochemical reactions in the grains during the germination stage. By contrast, Do and co-workers (2015) reported that the vitamin E content of most barley genotypes was reduced after malting and this was attributed to the leaching of vitamin E at steeping step according to the sensitivity to water of the grains, as well as to the instability of this vitamin to water, oxygen and heat, which are all factors applied to the sample during malting. In addition, the paper of Frias and co-authors, (2005)

highlighted that up to 9 days of germination of lupin induced an increase in the content of α-tocopherol, a decrease in the γ-tocopherol amount and did not affect the level of δ-tocopherol, and were accompanied with an increment of the vitamin E activity. Similarly, an increase in the α-tocopherol content and a decrease in the γ-tocopherol amount was observed in flaxseed after 5 days of germination, whereas the total tocopherol content reached the highest value after 1 and 2 days of germination (Li et al., 2019). These results were been attributed to the synthesis of tocopherol as material needed for the growth of young plants, that was subsequently consumed in the process of scavenging phospholipid peroxyl radicals and/or as growth accelerator (Do et al., 2015; Li et al., 2019).

Overall, malting conditions, genotype, and species probably have an important role in affecting the total tocopherol content and tocopherols profile of a seed and further investigations concerning the changes in all vitamin E isomers, as well as total vitamin E content, across each malting step (*i.e.,* steeping and germination) would be interesting to explore in more detail in future studies, to better understand the best condition for malting of hemp.

15.3. Antioxidant activity of raw and malted hemp seeds

As previously mentioned, both phenolic compounds and tocopherols act as antioxidants, having a primary role in determining the total antioxidant capacity of the sample. We evaluated the antioxidant capacity of ethanol extracts through two assays commonly used for food matrix: the TEAC and the CUPRAC assays. The TEAC assay is based on the ability of antioxidants to scavenge the $ABTS^{\bullet+}$ radical to ABTS via Single Electron Transfer (SET) or Hydrogen Atom Transfer (HAT) mechanism by lipophilic and hydrophilic antioxidants of sample. While in the CUPRAC method the antioxidant activity of sample is measured by evaluating the power of antioxidant compounds in reducing Cu^{2+} into Cu^{+}. Analogously to TEAC assay, also the CUPRAC assay allows to determine lipophilic and hydrophilic antioxidants simultaneously, since charged chromophores used in this method (*i.e.,* Cu^{2+}-Neocuproine complexes) are water and organic soluble (Rafi et al., 2018). For this reason, TEAC and CUPRAC assays seem to be methods particularly suitable for evaluating the total antioxidant capacity of a sample such as hemp seeds, rich in both lipophilic and hydrophilic antioxidants, including tocopherols and phenolic compounds. Differences in the antioxidant capacity values obtained from these two methods where mainly due to the chemical differences involved in the antioxidant activities' measurements by each method. Results of TEAC and CUPRAC assays of raw, steeped, 3-days germinated, and malted seeds of *Futura 75* and *Secuieni jubileu cvs* are presented in **Figure 25**.

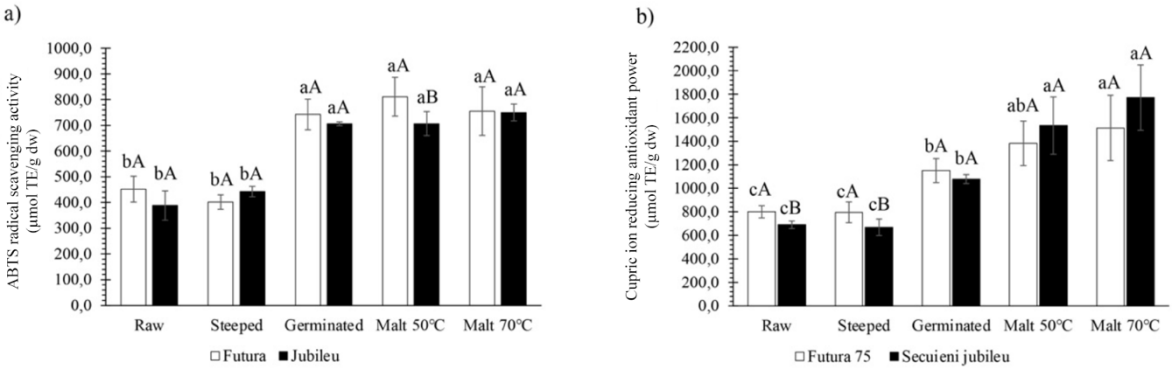

Figure 25: *Antioxidant activity of untreated (Raw), Steeped, Germinated, and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C) seeds of* Futura 75 *and* Secuieni jubileu *hemp* cultivars*. a) ABTS$^{\bullet+}$ radical scavenging activity (µmol TE/g DW); b) Cupric ion reducing antioxidant capacity (CUPRAC) (µmol TE/g dw). Data are means ± standard deviation of at least three replicates (n = 3). Different letters within the same* cultivar *indicate significant differences ($p \leq 0.05$), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among raw, steeped, germinated, and malted seeds and uppercase letters represent comparison between* cultivars*. ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). TE: Trolox Equivalent. dw: dry weight.*

Differences in the extraction methods and in the analytical procedure used for the analysis, as well as the scarcity of investigation concerning the antioxidant capacity of whole hemp seeds, make the comparison between our results and literature reports rather difficult. For example, Irakli and colleagues (2019) reported a mean value of 734.2 mg TE/100 g in defatted flour for the seeds of *Futura 75*, measured by ABTS assay, equivalent to 29.33 µmol TE/g, whereas Galasso and co-authors (2016) obtained a value of 168 µmol TE/g in *Futura 75* seeds' defatted flour, using a different analytical method. Compared to these results, we found higher values for both *Futura 75* and *Secuieni jubileu* raw seeds (TEAC: 451.93 ± 50.42 and 388.06 ± 56.99 µmol TE/g *dw*, respectively; CUPRAC: 800.07 ± 51.55 and 689.86 ± 31.88 µmol TE/g *dw*, respectively): this could be related to the fact that we analysed whole seeds, differently to the previous authors that performed the analyses on defatted seeds, thus removing all or a large amount of lipophilic antioxidants potentially present in the seed, including tocopherols. Frassinetti and colleagues (2018) assessed the antioxidant power of *Futura 75* whole seeds' ethanol extract (*i.e.,* without defatting) and obtained a value of 134 ± 3 µmol TE/g *dw* by ORAC assay. Although this analysis was performed on whole seeds instead of defatted material, the value was similar to that found by Galasso and co-workers (2016) and lower than obtained by us. The differences could be due to the use of different technique for evaluating the antioxidant capacity. Overall, our results showed that the ABTS scavenging activity, measured by TEAC assay, of the raw seeds of both analysed *cvs* are similar (**Figure 25a**), whereas slight but significant difference ($p \leq 0.05$) was found for the reducing power measured by CUPRAC assay, resulting higher in the *Futura 75* raw seeds than *Secuieni jubileu* ones (**Figure 25b**).

Since phenolic compounds and tocopherols are the two major class of antioxidants in hemp seeds, it is reasonable to hypothesize that an increase in their amount during malting may be related to a concomitant increase in the seeds' total antioxidant activity. Therefore, we evaluated the ABTS scavenging ability and the reducing power of *Futura 75* and *Secuieni jubileu* seeds subjected to steeping, 3-days of germination and kilning at 50 °C or 70 °C. Results obtained by the TEAC assay (**Figure 25a**) revealed that the ABTS scavenging ability of analysed

samples had a trend similar to that found for TPC (**Figure 22**): it was not affected by 5 h of steeping, and significantly increased by 64% and 82% in *Futura 75* and *Secuieni jubileu cvs*, after 3-days of germination. Kilning at 50 or 70 °C did not result in lower antioxidant scavenging activity for the seeds of both *cvs*. Similarly, the reducing power of seeds did not significantly change in both varieties after steeping, whereas the germination induced an increase by 44% and 56% of the *Futura 75* and *Secuieni jubileu* seeds' reducing power, respectively (**Figure 25b**). Interestingly, the heat treatment at 50 °C and especially at 70 °C led to a further increase in the CUPRAC value of the seeds of both *cvs*, resulting 2-folds (*Futura 75*) and 2.5-folds (*Secuieni jubileu*) higher than the untreated seeds after malting at 70 °C. Hence, the increase in the CUPRAC value after malting was more remarkable for the *Secuieni jubileu cv* than *Futura 75* ones, due to the fact that *Secuieni jubileu* raw seeds had a CUPRAC value significantly lower than *Futura 75* raw seed, but after malting, *Secuieni jubileu* malted seeds reached values statistically comparable to those of *Futura 75* malted seeds (**Figure 25b**). In general, an increase in the antioxidant activity of seeds was reported as consequence of the metabolic changing which occur during germination, including the increase activity of endogenous hydrolytic enzymes able to split reserve nutrients into simpler component with antioxidant activity, as well as *de novo* biosynthesis and accumulation of bioactive compounds such as tocopherols and phenolic compounds (Lemmens et al., 2018b). In fact, Frassinetti and co-authors (2018) reported an increase of the ORAC assay value by 78% in the seeds of *Futura 75* after three days of germination, and this result was in good agreement with our findings. The increase in the antioxidant activity in hemp seeds after germination could be related not only to the observed higher TPC value, but also to the production of antioxidant peptides by hydrolysis of hemp seed's proteins, occurring by the activation of hydrolytic enzymes during germination. Indeed, some reports demonstrated that the hydrolysis of hemp seed's proteins provides hydrolysates with higher bioactivity, including antioxidant effects (Farinon et al., 2020).

Some studies reported that according to the temperature, thermal treatment could negatively affect the antioxidant activity of seeds (Carciochi et al., 2016; Terpinc et al., 2016), but neither 50 °C nor 70 °C showed to be deleterious for the antioxidant activity of both *Futura 75* and *Secuieni jubileu* seeds. On the contrary, an increased reducing power after kilning was revealed by CUPRAC assay, and it could reflect a higher presence of donor electron compounds in the malted seeds, generated following the heat treatment. It is known that in cereal grains thermal treatment performed on germinated seeds led to the production of Maillard reaction products (MRP), resulting by complex reactions between carbonyl-containing compounds (*e.g.,* reducing sugars) and amino-containing compounds (*e.g.,* amino acids or peptides), which are generated by the hydrolysis of reserve substances such as starch and proteins in the germinated seeds. MRP have shown to possess antioxidant activity, acting as reducing compounds (Manzocco et al., 2000). Temperature and time represent important factors in regulating the rate of Maillard reaction and the properties of MRP. An increase in temperature leads to an increase in the rate of the Maillard reaction and hence, in the amount of MRP, with a concomitant increase in the reducing power of sample, as reported by Carciochi and colleagues (2016) for quinoa seeds malted at different kilning temperature. This evidence is in accordance with the increase in the reducing power found in hemp seeds after the thermal treatment, however the kilning temperature used in this study was lower than 100 °C and to date, there is no evidence about the production of MRP in hemp seeds. So, further investigation about metabolomic profile's changing induced by malting could be appropriate to better understand this result.

16. Nutritional features of raw and malted hemp seeds

16.1. Proximate composition of raw and malted hemp seeds

In order to evaluate whether malting may affect the nutritional properties of hemp seeds, the proximate composition of raw (*i.e.,* unmalted) and malted seeds of the two analysed hemp varieties were assessed and the obtained results are presented in **Table 12**.

Table 12: *Proximate composition (g/100 g dw) and energy value (Kcal/100 g of edible portion), of* Futura 75 *and* Secuieni jubileu *hemp seeds* cultivars, *untreated (Raw seed) and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C). Data are means ± standard deviation of at least two (n = 2) replicates. Different letters indicate significant differences (p ≤ 0.05), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among treatments and uppercase letters represent comparison between* cultivars.

Hemp seeds *cultivar*	Moisture	Protein[1]	Fat	Carbohydrate[2]	Ash	Energy value
Futura 75						
Raw Seed	7.20 ± 0.58^{aB}	23.88 ± 0.05^{bA}	32.73 ± 1.27^{aA}	39.02 ± 1.29^{aA}	4.38 ± 0.04^{aB}	506.50 ± 5.49^{bA}
Malt 50 °C	3.15 ± 0.07^{bA}	25.65 ± 0.72^{abA}	35.34 ± 1.16^{aA}	34.87 ± 1.85^{aA}	4.15 ± 0.04^{bB}	542.57 ± 6.02^{aA}
Malt 70 °C	1.15 ± 0.09^{cA}	26.06 ± 0.50^{aA}	34.75 ± 0.06^{aA}	34.92 ± 0.60^{aA}	4.29 ± 0.04^{abB}	550.11 ± 0.66^{aA}
Secuieni jubileu						
Raw Seed	8.61 ± 0.81^{aA}	21.50 ± 0.32^{bB}	33.20 ± 1.17^{aA}	39.20 ± 0.83^{aA}	6.11 ± 0.02^{aA}	490.47 ± 3.32^{bA}
Malt 50 °C	2.85 ± 0.47^{bA}	22.62 ± 0.13^{aB}	34.44 ± 0.45^{aA}	36.91 ± 0.28^{abA}	6.04 ± 0.03^{aA}	530.60 ± 0.15^{aA}
Malt 70 °C	1.24 ± 0.11^{cA}	22.64 ± 0.04^{aB}	35.24 ± 0.88^{aA}	35.75 ± 1.07^{bA}	6.38 ± 0.15^{aA}	544.28 ± 5.96^{aA}

[1]Conversion factor: 6.25. [2]As difference (*i.e.,* 100 – (g [protein + fat + ash] in 100 g of *dw* sample)).

The moisture content of the raw seeds was significantly higher for the *Secuieni jubileu cv* than *Futura 75* ones, but for both *cvs* it significantly ($p \leq 0.05$) decreased after the malting process as consequence of the kilning. The higher the kilning temperature, the more marked the moisture decrease. In malting, the kilning step aims to remove water in order to inactivate the enzymes and stop the germination, thus prevent further growth of the embryo and modification of the seeds' content. Moreover, it reduces the moisture content of the germinated seeds until 5% or below, a condition important to ensure stability during storage (Carvalho et al., 2016a). As expected, the lowest moisture temperature was reached at the kilning temperature of 70 °C (1.15 ± 0.58 g/100 g *dw* and 1.24 ± 0.11 g/100 g *dw* for *Futura 75* and *Secuieni jubileu*, respectively), nevertheless, also the kilning performed at 50 °C produced an equally stable product, with a moisture content higher than that observed for samples kilned at 70 °C, but however lower than 5% (3.15 ± 0.07 g/100 g *dw* and 2.85 ± 0.47 g/100 g *dw* for *Futura 75* and *Secuieni jubileu*, respectively).

Fat is the most representative nutritional component of the *C. sativa* L. seed as it is an oilseed. In this study, the crude fat of *Futura 75* and *Secuieni jubileu* seeds accounted for over 30% of the dry matter, without differences between the two varieties. The values obtained are in good agreement with previously published data (Farinon et al., 2020) and highlight that both the varieties can be considered an excellent source of oil. Several papers reported

a decrease in the fat amount after germination or malting of different type of seeds, including cereal grains (Adebiyi et al., 2017; Aguilar et al., 2019; Chaturvedi N, 2015; Obadina et al., 2017; Singh et al., 2018) and flaxseed (Li et al., 2019), as consequence of the increased lipolytic activities and the utilization of fat in order to obtain the energy required for the biochemical and physiological modifications occurring during germination. However, we obtained a slight, but not significant, increase in the fat amount for both *cvs* after malting. Similar result was reported by Khang and colleagues (2016) for oat seeds after 5 days of germination. Özcan and co-workers (2018) also obtained a significant increase in the oil content of barley grains after germination. These differences could be due to the malting condition which may differently influence the level of lipid degradation.

Concerning the protein content, slight but significant difference between the two varieties was found, being the seeds of *Futura 75 cv* those with the highest amount. Anyway, the values obtained for both *cvs* ranging between 20 and 25% *dw* according to other papers (House et al., 2010; Lan et al., 2019). Differences in the hemp seeds' protein content can be related to genetic and environmental factors (Irakli et al., 2019). When compared to the unmalted seeds, the protein content of hemp seeds significantly ($p \leq 0.05$) increased by 7.5% and 9.2% for the seeds of *Futura 75* and by 5.2% and 5.3% for the seeds of *Secuieni jubileu* after malting at 50 °C and 70 °C, respectively. Works concerning the protein content of seeds after malting process are conflicting. Some studies reported significant decreased in the protein amount of malted seeds in comparison to unmalted ones, due to the fact that during germination, proteins are catabolized into soluble amino acids which can be lost by lixiviation (Aguilar et al., 2019; Lemmens et al., 2018b; Singh et al., 2018). Other studies showed an increase in the crude protein content of malted seeds (Adebiyi et al., 2017; Chaturvedi N, 2015; Hejazi and Orsat, 2016; Obadina et al., 2017; Pilco-Quesada et al., 2020) attributed to the synthesis of enzymes and proteins needed for the embryo growth (Aguilar et al., 2019), or by loss of carbohydrates through respiration (Lemmens et al., 2018b). Interestingly, Mäkinen and Arendt (2015) reported that during germination of cereals, pseudocereals, and legumes an increase in the albumins content occurred as consequence of *de novo* synthesis. Therefore, it could be speculated that the increase in the protein content after malting may be due to albumin synthesis during the germination step, also considering that albumin is one of the most abundant proteins in hemp seed (Callaway, 2004). Despite the increase in the protein content after malting, the amount of protein in the *Secuieni jubileu* seeds malted at 50 °C and 70 °C remained lower when compared to *Futura 75* malted seeds.

Analogously to protein, the energy value of malted seeds showed little but significant increased for both *cvs*, which could be actually related to the enrichment in the protein content after malting. Nevertheless, no differences between the *cvs* neither in the kilning temperature used for malting were observed. Similar result was obtained by Adebiyi and co-authors after malting pearl millet grains (Adebiyi et al., 2017).

The carbohydrates content was similar in the raw seeds of *Futura 75* and *Secuieni jubileu cvs* and it represented up to 39% of dry matter. This result is consistent to other literature data which demonstrated that the great part of carbohydrates was constituted by dietary fibre, the most of which was the insoluble fraction (Farinon et al., 2020). After malting, the carbohydrates showed a decreasing trend, but the change was significant only for the seeds of *Secuieni jubileu* malted at 70 °C. Similarly, Obadina and colleagues (2017) found a decreasing trend in the carbohydrates content of the pearl millet malted flour and this was attributed to the enzymatic hydrolysis of starch granules into free simple sugars used as energy source for the growing embryo. Nevertheless, as previously said, the great part of total carbohydrates in hemp seed is constituted by dietary fibre instead of starch (Farinon et al., 2020), therefore, the decrease in the carbohydrates amount of *Secuieni jubileu* seeds malted at 70 °C is unlikely

due to starch hydrolysis occurring during germination. By contrast, other studies reported an increase in the carbohydrates content of different types of seeds after malting, explained by the increase in the crude fibre for building the dry matter during the growth and development of the new plant (Adebiyi et al., 2017; Laxmi et al., 2015). Interestingly, Koehler and co-authors observed that the total dietary fibre of wheat grains increased after 7 days of germination compared with non-germinated counterpart, but after 2 days of germination the value were trending downwards (Koehler et al., 2007). As previously explained (see the section 3 "The malting process") during the germination step the activation of wall-cell degrading enzymes takes place and led to the breakdown cell walls and degradation of cell wall's polysaccharides including cellulose, hemicellulose, and lignin (Lemmens et al., 2018b) which all are constituents of dietary fibre. Hence, it is possible to speculate that early germination tends to induce a decrease in the total fibre content as result of the hydrolytic enzymes activity on the seed's wall cell; when germination is further prolonged, the catabolic to anabolic shift occurs to promote the growth of the new seedling. In this case, an increase in the total dietary fibre could be explained by the formation of new primary cell walls (Lee et al., 2007). Anyway, it would be interesting to evaluate the changes in the carbohydrates profile (*i.e.,* soluble and insoluble fibre, and starch content) of raw and malted hemp seeds in future investigation, to better understand the impact of malting on this nutritional component.

Regarding the ash amount, Table 11 shows significant ($p \leq 0.05$) difference between *Secuieni jubileu* and *Futura 75* seeds and *Secuieni jubileu* was the *cv* with the highest content. Ash represents the sample's mineral amount which can widely vary based on environmental condition, mineral soil composition, the use or not of fertilizers, the type of fertilizer if used, as well as the hemp variety (Farinon et al., 2020). Literature data reported a decrease in the ash contents after malting due to lixiviation during steeping and/or germination steps (Aguilar et al., 2019), assimilation of available minerals into cell constituents, or their utilization as coenzymes during germination (Obadina et al., 2017). Nevertheless, the malting condition applied in this study, did not induce any significant ($p \leq 0.05$) change in the ash content of *Secuieni jubileu* malted seeds, for both types of kilning temperatures. By contrast we obtained a significant decrease for *Futura 75* seeds after malting.

16.2. Fatty acids composition of raw and malted hemp seeds

The fatty acids composition and the percentages of SFA, MUFA, and PUFA of *Futura 75* and *Secuieni jubileu* raw and malted at 50 °C and 70 °C are given in **Table 13**.

Table 13*: Fatty acids composition (% of oil) of* Futura 75 *and* Secuieni jubileu*, untreated (Raw seed) and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C). Data are means ± standard deviation of two (n = 2) replicates. Different letters indicate significant differences (p ≤ 0.05), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison among treatments and uppercase letters represent comparison between cultivars.*

	Futura 75			***Secuieni jubileu***		
	Raw Seed	Malt 50 °C	Malt 70 °C	Raw Seed	Malt 50 °C	Malt 70 °C
PA (16:0)	8.33 ± 0.16^{aA}	7.91 ± 0.08^{bB}	8.55 ± 0.01^{aA}	8.08 ± 0.06^{bA}	8.51 ± 0.00^{aA}	8.10 ± 0.01^{bB}
PAL (16:1, n-7)	0.13 ± 0.04^{bA}	0.11 ± 0.01^{bB}	0.23 ± 0.01^{aA}	0.22 ± 0.04^{aA}	0.33 ± 0.01^{aA}	0.27 ± 0.01^{aA}
SA (18:0)	2.86 ± 0.05^{aA}	2.76 ± 0.08^{aA}	2.84 ± 0.02^{aA}	2.19 ± 0.08^{aB}	2.28 ± 0.00^{aB}	2.14 ± 0.01^{aB}
OA (18:1, n-9)	17.72 ± 0.04^{aA}	15.28 ± 0.04^{bA}	14.76 ± 0.05^{cB}	14.33 ± 0.04^{cB}	15.00 ± 0.08^{bA}	15.25 ± 0.01^{aA}
LA (18:2, n-6)	57.92 ± 0.24^{bB}	61.41 ± 0.12^{bA}	61.17 ± 0.02^{aA}	61.15 ± 0.81^{aA}	60.62 ± 0.02^{aA}	61.06 ± 0.01^{aA}
GLA (18:3, n-6)	1.23 ± 0.01^{aA}	1.04 ± 0.02^{bA}	1.12 ± 0.03^{bA}	1.28 ± 0.38^{aA}	1.03 ± 0.01^{aA}	1.03 ± 0.01^{aA}
ALA (18:3, n-3)	11.38 ± 0.04^{aA}	11.11 ± 0.03^{bB}	11.10 ± 0.01^{bB}	12.40 ± 0.37^{aA}	11.82 ± 0.04^{aA}	11.76 ± 0.01^{aA}
ARA (20:0)	0.44 ± 0.00^{bA}	0.52 ± 0.01^{aA}	0.25 ± 0.01^{cB}	0.37 ± 0.04^{aA}	0.42 ± 0.01^{aB}	0.41 ± 0.02^{aA}
∑ SFA	11.6	11.2	11.6	10.6	11.2	10.6
∑ MUFA	17.9	15.4	15.0	14.6	15.3	15.5
∑ PUFA	70.5	73.6	73.4	74.8	73.5	73.9
PUFA/SFA	6.1	6.6	6.3	7.0	6.6	6.9
n-6/n-3	5.2	5.6	5.6	5.0	5.2	5.3

PA: Palmitic Acid; PAL: Palmitoleic Acid; SA: Stearic Acid; OA: Oleic Acid; LA: Linoleic Acid; GLA: γ-Linolenic Acid; ALA: α-Linolenic Acid; ARA: Arachidic Acid; SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid; PUFA: Polyunsaturated Fatty Acid.

For both analyzed *cvs*, the oil fraction was characterized by high unsaturated fatty acids content, with the PUFA fraction amounted to 70.5% and 74.8% of total fatty acids, and MUFA content represented 17.9% and 14.6% of total fatty acids found in *Futura 75* and *Secuinei jubileu* raw seed's oil, respectively. SFA was found to be the lowest fatty acids of the raw hemp seeds' oil for both *cvs*, amounted to 11.6% and 10.6% of total fatty acids of *Futura 75* and *Secuieni jubileu* varieties, respectively. The principal SFA found in the raw seeds of both *cvs* was the PA, followed by SA, while the major MUFA was OA. Regarding the essential fatty acids, the raw seeds of *Futura 75* and *Secuieni jubileu cvs* contained on average 57.92% and 61.15% of LA, respectively and 11.38% and 12.40% of ALA, respectively. Among the minor unsaturated fatty acids, GLA was found in the raw seeds in an average percentage of 1.23% and 1.28% of *Futura 75* and *Secuieni jubileu* total fatty acids, respectively. These results are in agreement with previous reports (Galasso et al., 2016; Irakli et al., 2019; Vonapartis et al., 2015) and demonstrated that the oil fraction of the analyzed hemp seed's *cvs*, including the underexplored *Secuieni jubileu* variety, has good nutritional value, related to the high content of PUFA, the presence of anti-inflammatory GLA, and low amount of SFA, resulting in relatively high PUFA/SFA ratio. In addition, the n-6/n-3 ratio was found to be equal to 5:1 for the raw seeds of both varieties. This is in accordance with what reported by Irakli and co-

authors (2015) and confers to both *Futura 75* and *Secuieni jubileu* raw seeds good, nutritionally balanced and healthy amount of n-6 and n-3 fatty acids. Interestingly, we found that the SA and OA amount were significantly lower, while the LA content was significantly higher in *Secuinei jubileu* raw seeds than *Futura 75* ones. It was demonstrated that the fatty acid composition of hemp seed was highly affected by genotype (Galasso et al., 2016; Irakli et al., 2019) and between the two most abundant fatty acids in hemp seeds, ALA was found to be more affected by genotype than LA (Irakli et al., 2019; Vonapartis et al., 2015). Contrarily, for the raw seeds we obtained that LA but not ALA was significantly different between the two *cvs*.

Fatty acids' profile of hemp seed represents one of the most important nutritional features of this oilseed. Therefore, although the total lipid content of both *Futura 75* and *Secuieni jubileu* hemp seed was not significantly affected by malting, we assessed the fatty acids' composition of the seeds of both *cvs* after malting, to evaluate whether and how malting could potentially affect it. No remarkable changes were observed for both *cvs*. Specifically, some slight, but significant ($p \leq 0.05$) differences were noticed in the *Futura 75* seeds, where a decreased in OA, GLA, and ALA content was observed after malting, independently to the kilning temperature, while a slight increase was observed for ALA, but only with a kilning temperature of 70 °C. Whereas, in *Secuieni jubileu* seeds, the most relevant modification was found for OA which slightly increased after malting, differently to *Futura 75* seeds. Variation in the fatty acid changes after malting among different varieties was reported in barley too and was attributed to different biosynthesis of lipid during the germination step (Bravi et al., 2012).

16.3. Antinutritional compounds of raw and malted hemp seeds

The nutritional profile of a food matrix can be negatively affected by some antinutritional compounds that reduced the bioavailability of some nutrients such as proteins and minerals, including phytate and trypsin inhibitors (TIs). In this study we assessed the content of these antinutritional compounds in the raw seeds of *Secuieni jubileu* and *Futura 75* and evaluated the effect of the malting process with two different kilning temperatures on these parameters. The obtained results are graphically reported in **Figure 26**.

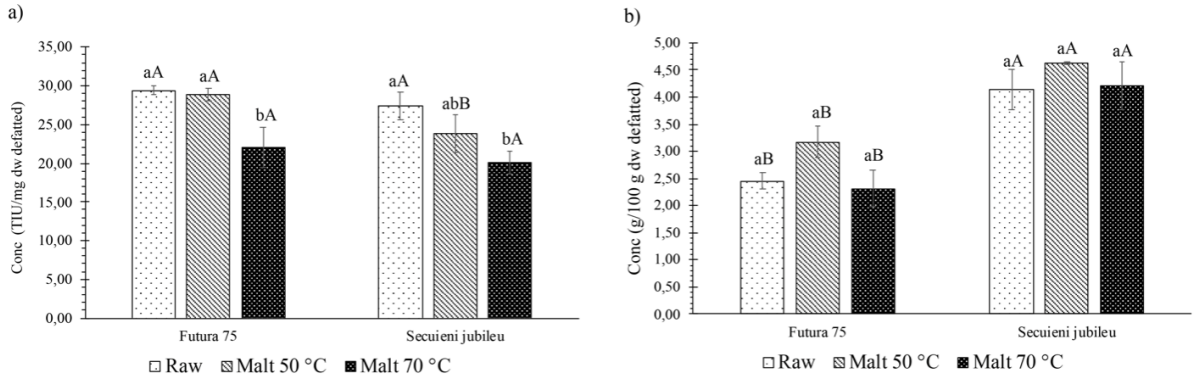

Figure 26: *Antinutritional Compounds* of Futura 75 *and* Secuinei jubileu *defatted hemp seeds untreated (Raw) and malted at 50 °C (Malt 50 °C) and 70 °C (Malt 70 °C). a) Trypsin Inhibitors Activity (TIU/mg dw). b) Phytic acid (g/100 g dw). Data are means ± standard deviation of three replicates (n = 3). Different letters indicate significant differences ($p \leq 0.05$), according to one-way analysis of variance. Lowercase letters represent Tukey's test comparison between raw and malted seeds and uppercase letters represent comparison between* cultivars*. dw: dry weight. TIU: Trypsin inhibitors unit; dw: dry weight.*

The TIs amount was almost the same for the raw seeds of both *Futura 75* and *Secuieni jubileu cv* and amounted to 29.42 ± 0.51 and 27.38 ± 1.72 TIU/mg *dw* of defatted sample, respectively (**Figure 26a**). These results are in good agreement with those obtained for the seed of *Futura 75 cv* by Russo and Reggiani (2015a) and by Galasso and colleagues (2016), and according to the classification of Guillamón and co-workers (2008), hemp seeds would be placed in the group including edible seeds with medium content of TIs, ranging from 17 to 51 TIU/mg. Methods as thermal treatment, soaking and germination have shown to be efficient to inactivate the TIs in soybean (Vagadia et al., 2017). Since all these represent steps of malting, it is reasonable to assume that they may be useful as well also for the inactivation of hemp seed's TIs. Therefore, we evaluated the TIs activity in hemp seeds of both *Futura 75* and *Secuieni jubileu* before and after malting. As depicted in **Figure 26a**, malting induced significant decrease in the content of TIs in the seeds of both varieties in comparison to the unmalted counterpart. Particularly, the kilning step performed at 70 °C appeared to be the more efficient than 50 °C, especially for the *Futura 75* seeds. Indeed, Malt 70 °C samples showed a decrease of the TIs amount by 25.0% and 26.6% for the *Futura 75* and *Secuieni jubileu cvs*, respectively. Bueno-Borges (2018) previously demonstrated that a thermal treatment performed at 80 °C on the "Sacha inchi" oilseed reduced the TIs activity by 26.3% compared to control (*i.e.*, untreated seeds), thus obtaining a comparable result. Since the Malt 50 °C samples did not show significant

differences from the unmalted raw seeds for both analysed *cvs*, it is possible to assert that differently to soybean, the hemp seed's TIs are more susceptible to thermal treatment than germination and soaked processes.

The phytic acids content was significantly higher in the *Secuieni jubileu* raw seed than *Futura 75* one, amounting to 4.14 ± 0.37 g/100 g *dw* of defatted sample and 2.45 ± 0.16 g/100 g *dw* of defatted sample, respectively (**Figure 26b**). Gupta and co-authors (2015) reported that the phytic acid content of oilseeds varies from 1.0 to 5.4 g/100 g *dw*, whilst Mattila and co-workers (2018) observed that the phytic acid content of commercial whole hemp seeds (variety not specified) amounted to 5.3 ± 0.4 g/100 g of defatted sample, accordingly to our results. Galasso and co-authors obtained a phytate amount of 7.5 g/100 g defatted sample for the seeds of *Futura 75 cv* (Galasso et al., 2016). Although this result is slightly higher than those we have found for the same variety, the orders of magnitude were quite similar, also considering that environmental factors influence the phytate content of the seed. Steeping, germination and kilning are all processes which may be useful to reduce the phytase content in some seeds since they can increase the activity of endogenous phytase, inducing extensive phytate hydrolysis (Hejazi and Orsat, 2016; Lemmens et al., 2018b). However, we did not obtain significant differences in the phytate content after malting the seeds of both analysed *cvs* (**Figure 26b**). This could be due to the fact that the effect of germination/malting on the phytate content of seeds depends on the species as well as the parameters used for each step of the malting process (*i.e.*, time and temperature) (Mäkinen and Arendt, 2015). Analogously, Udeh and colleagues (2018) did not observe any decrease in the phytate content of the finger millet grains after malting. Our results could be ascribed to a low phytase amount in hemp seed or to the fact that the adopted malting conditions may not be suitable enough to induce an adequately high phytase activity. It was reported that phytatses have a temperature *optimum* between 37° C and 55° C in cereal (Lemmens et al., 2018b). Thus, it is possible that modifying steeping and/or germination temperature to those optimal for hemp seed phytase action can enhance phytate breakdown. Nevertheless, more investigation concerning the hemp seed's phytase activity would be required before making any changes to the process; in addition, account should be taken that increasing steeping temperatures could boost the loss of minerals by leaching, and it could negative affect the seed's germinability (Lemmens et al., 2018b). Despite its antinutritional activity, phytic acid has also health beneficial effects related to its ability to chelate iron ions (Kumar et al., 2010; Nissar et al., 2017). Hence, for optimum physiological benefits, the phytate content in the upper part of the gut has to be low to avoid negative effect on minerals and proteins absorption and bioavailability, but at the same time, it has to be high enough to use its beneficial actions. Unfortunately, to date, accurate and clear information on the phytate dosage required to elicit beneficial effects in human is still missing. Russo and Reggiani (2015) proposed the amount of 0.45 g/100 g of defatted sample as phytate upper limited safety value. Based on this, the phytate level in both raw and malted seeds of *Futura 75* and *Secuieni jubileu cvs* would be too high and therefore, further investigation about efficient methods to reduce the phytate content of hemp seeds would be appropriate.

Conclusion and feature perspectives

This PhD work has experienced for the first time the effect of the malting process on the nutritional, antinutritional and antioxidant profile of the seeds of the revitalised *C. sativa* L. crop. This is one of the oldest domesticated plants known to humans. Evidence of the human use of this crop are dated to prehistoric times and according to Sorrentino (Sorrentino, 2021), hemp traces was reported in Italy in Etruscan findings and during the Roman Empire where hemp fibre was used for the construction of sails, nets and ropes. Then, during the Middle Ages and the following centuries hemp crop had a broader diffusion especially in the Po Valley and in great part of Central Italy up to Campania and Basilicata. For thousands of years, hemp was primarily cultivated as a worldwide fibre crop: hemp fibres were used for clothing and shoes, cordages, carpets and tarps, maritime ropes, sails and nets, and paper production. Additionally, hemp hurds were employed for bedding of animals and as straw for agricultural purposes. The leaves were also used for mulch, composting and animal bedding, while the seeds especially in the western world, were considered as a waste product of fiber production, and at most, used as animal feed. Only in China and in some other countries of the Middle East, they were consumed also as food. Subsequently, in the middle of the nineteenth century, the competition from other natural fibres such as cotton and jute, and the diffusion of synthetic fibers, led to a progressive decrease of hemp cultivation, until the total ban that was introduced in most western countries, due to the use of some narcotic strains of *C. sativa* L. plant, for the production of intoxicant drugs. These strains are also known as “marijuana” or “drug-type hemp” and are characterized by the presence of high level (≥ 10–30%) of THC the only one compounds of the plant with psychoactive properties. Contrarily, the *C. sativa* L. strains commonly called “industrial hemp” refer to the non-psychoactive varieties of the plant, containing less than 0.2–0.3% THC, that are value considered safety for human health. Hence, from 1990s a legal regulation of the industrial hemp growing has been introduced and a consequently renewal in the cultivation of this crop from an agricultural, industrial and scientific point of view throughout the world, especially in the western world (Crini et al., 2020; Farinon et al., 2020). The renaissance of the industrial hemp cultivation has been further encouraged by the fact that hemp is a valuable crop for the bio-based economy, since it is considered as a low-cost, ecological, sustainable and multi-use plant, each part of which can virtually be useable. Indeed, it was reported that the global market for industrial hemp has more than 25,000 products in nine main submarkets, including textiles, agriculture, automotive, food and beverages, paper, furniture, construction, recycling, and personal care (Crini et al., 2020). However, several years of prohibition unfortunately introduced a lot of confusion and social, political and moral controversies concerning the grown of industrial hemp, which is often incorrectly associated with hemp for narcotics purposes. Therefore, provide the right information appears particularly advantageous to spread the correct knowledge concerning this topic and avoid misconception, as well as to help the authorities to make the most appropriate and awareness decisions in the developing legislative framework concerning the industrial hemp cultivation, transformation and market.

One of the most emerging industrial hemp products is the hemp seed. As previously said, the seeds of *C. sativa* L. plant were previously considered as a waste product of the fibre production, especially in the western Countries, but relatively recent researchers concerning the chemical composition and nutritional value of hemp seeds highlighted their high nutritional and health beneficial power, related to the optimal nutritional composition

including unique and perfectly balanced FA profile, a n-6/n-3 ratio optimal for human nutrition, the presence of high biological value proteins easy to digest and rich in essential amino acids, as well as a high amount of macro and micro-elements. In addition, hemp seeds also contain various healthy bioactive components such as tocopherols and phytosterols, polyphenols and bioactive peptides with antioxidant, anti-inflammatory, neuroprotective, anti-hypertensive, anti-proliferative, and hypocholesterolaemic activities, assessed mainly by *in vitro* studies (Farinon et al., 2020).

Due to their nutritional features and health-promoting bioactive components, hemp seeds could be considered as a valuable source of dietary supplements and/or ingredient for functional foods' preparation. However, to date, knowledge about the technological transformation of hemp seeds is rather limited and the scientific research concerning this field is only just beginning. In this respect, most of the studies have focused on the hemp seed oil production and have investigated the various extraction techniques able to optimize the yield and the quality of the hempseed oil; some works have evaluated the nutritional and rheological properties of the hemp seed cake remaining after the hemp seed oil production and its potential utilization as additive/ingredient in the bakery industries (Farinon et al., 2020; Leonard et al., 2020; Rupasinghe et al., 2020). Nevertheless, only very few studies were performed about the whole hemp seeds utilization in the food industries (Babiker et al., 2021; Frassinetti et al., 2018; Leonard et al., 2021; Werz et al., 2014).

This PhD work has fitted in this context, being it was been aimed to investigate the impact of the ancient biotechnological process of malting – traditionally performed on the cereal grains – on the seeds of the two industrial hemp varieties of *Futura 75* and *Secuieni jubileu*. In this study, we chose the two relatively low kilning temperature of 50 °C and 70 °C in order to avoid thermal degradation of the hemp seed's lipid fraction according to Gao and co-worker (Gao and Birch, 2016). The obtained data demonstrated that the malting influences all assessed parameters in a similar way for both hemp varieties, increasing their radical scavenging activity and reducing power, improving their tocopherol profile and TPC value. Concerning the polyphenols, malting with the higher tested kilning temperature (*i.e.*, 70 °C) significantly increased the amount of N-*trans*-caffeoyltyramine and Cannabisin A, two polyphenols typical of hemp seeds, considered as the major contributors to the hemp seeds' antioxidant activity. Nutritionally, we observed that malting increased the seeds' protein content without affecting the fat content, ash amount, and FAs profile. The kilning temperature of 70 °C resulted useful also to reduce the amount of the antinutritional compounds TIs, although it did not significantly affect the phytic acid content.

Overall, the results of this PhD work provide important information for both scientific literature and food industry. Indeed, to the best of our knowledge, this is the first study concerning the nutritional and functional characterisation of *Secuieni jubileu* seeds of which there is no information in the scientific literature. In addition, for the first time, the effect of malting on the hemp seeds were evaluated, proving that this process could be very attractive for the food industries, giving the opportunity to adopt a hemp seed's transformation product as ingredient to formulate nutritionally improved foods. To conclude, additional investigation about this transformation process, including metabolomic analysis, could be suitable to better understand the benefits of malting on the nutritional and functional features of hemp seeds and how this process may be further optimized.

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