agriculture

MDPI

Article

Phenolic Acid Composition and Antioxidant Activity of Whole and Defatted Seeds of Italian Hemp Cultivars: A Two-Year Case Study

Valeria Menga [1], Carmela Garofalo [1], Serafino Suriano [1], Romina Beleggia [1], Salvatore Antonio Colecchia [1], Domenico Perrone [2], Massimo Montanari [3], Nicola Pecchioni [1] and Clara Fares [1,*]

[1] Council for Agricultural Research and Economics, Research Centre for Cereal and Industrial Crops, S.S. 673, km 25.200, 71122 Foggia, Italy; valeria.menga@crea.gov.it (V.M.); garofalocarmela@tiscali.it (C.G.); serafino.suriano@crea.gov.it (S.S.); romina.beleggia@crea.gov.it (R.B.); colecchiasalvatore@tiscali.it (S.A.C.); nicola.pecchioni@crea.gov.it (N.P.)

[2] Council for Agricultural Research and Economics, Research Centre for Vegetable and Ornamental Crops, Via Cavalleggeri 25, 84098 Pontecagnano Faiano, Italy; domenico.perrone@crea.gov.it

[3] Council for Agricultural Research and Economics, Research Centre for Cereal and Industrial Crops, Bologna, Via di Corticella 133, 40128 Bologna, Italy; massimo.montanari@crea.gov.it

* Correspondence: clara.fares@crea.gov.it; Tel.: +39-0881-742-972; Fax: +39-0881-713-150

Abstract: The study investigated the nutritional profile and nutraceutical composition of the seeds (whole and defatted) of two monoecious hemp cultivars (Carmaleonte, Codimono) and one dioecious cultivar (CS) grown during the 2018 and 2019 crop seasons. The phenolic acid profiles, both free and bound, antioxidant activity (AA), protein content (PC), total phenolic content (TPC), β-carotene, lutein content, and condensed tannins (CT) were studied, and the effects of genotype (G), year (Y), and GxY interaction were also measured. The results indicated the stronger involvement of the year in the nutritional and antioxidant properties of the whole seeds than in those of the defatted seeds, as indicated by the analysis of the variance. The PC, TPC, AA, sum of phenolics free (SPF), and sum of phenolics bound (SPB) were significantly affected by year, while the lutein and some phenolic acids, free and bound (ferulic and *p*-coumaric acids and N-trans-caffeoyltyramine), showed significant effects of the genotype. In this respect, the Carmaleonte revealed the highest content of ferulic and *p*-coumaric acids, as well as CS of N-trans-caffeoyltyramine. A prevalence of Y effect over G was measured in the free and bound fraction of the phenolics of the whole seeds, in contrast to the defatted seeds, in which significant effects of GxY were also measured. Moreover, the Pearson's correlation coefficients indicated a strict involvement of precipitations in the variation of the phenolics accumulation, above all with bound *p*-hydroxybenzoic acid (r = 0.71 **), bound syringic acid (r = 0.69 *), bound N-trans-caffeoyltyramine (r = 0.64 *), and SPB (r = 0.60 *). As phenolics bound fractions have strong biological activities, (including antioxidant and anti-inflammatory activities) the high concentrations of N-trans-Caffeoyltyramine B in the CS defatted seeds suggest that it is valuable ingredient for functional foods.

Keywords: hemp seeds; defatted seed; phenolic acids profile; antioxidant activity; genotype; year

Citation: Menga, V.; Garofalo, C.; Suriano, S.; Beleggia, R.; Colecchia, S.A.; Perrone, D.; Montanari, M.; Pecchioni, N.; Fares, C. Phenolic Acid Composition and Antioxidant Activity of Whole and Defatted Seeds of Italian Hemp Cultivars: A Two-Year Case Study. *Agriculture* **2022**, *12*, 759. https://doi.org/10.3390/agriculture12060759

Academic Editor: Silvia Tavarini

Received: 10 May 2022
Accepted: 24 May 2022
Published: 26 May 2022

1. Introduction

Hemp (*Cannabis sativa* L.) is an annual herbaceous crop, known since ancient times as an important source of food, feed, fiber, and natural compounds with medical potential [1]. Hemp is a polymorphic species differentiated into intraspecific taxonomic groups by chemotype and crop type (for drug or fiber purposes) [2]. Naturally, hemp is a dioecious crop that is cultivated for several purposes in several agro-industrial fields (papermaking, cosmetics, biofuel, agriculture, and food). Monoecious cultivars have been developed as a result of previous breeding efforts [3]. The drug type, also known as marijuana, is mainly

bred for medical and recreational purposes [2]. The drug type and industrial hemp differ in the psychotropic molecule contents, i.e., cannabinoids (Δ9-tetrahydrocannabinol, THC) in their leaves and inflorescences. In the second type, the THC limit is determined by law; it cannot exceed 0.2% [4] in Europe and North America.

Hemp seeds have been extensively studied, and their high content of polyunsaturated fatty acids and high-quality proteins are well documented. They contain about 30% oil, 25% protein, fiber, vitamins, minerals, and polyphenols. Callaway [1] found that hemp-seed oil is a source of two essential fatty acids, α-linoleic acid (ALA) and LA (linoleic acid) (ω-3 and ω-6 fatty acids, respectively), and their biological metabolites (stearidonic and arachidonic acids, respectively). The lower omega-6/omega-3 ratio in hemp-seed oil (2–3/1) is more desirable for reducing the risk of many chronic diseases [1] and is considered favorable for satisfying dietary intake and maintaining a low risk of inflammatory diseases [5].

Among the minor components of liposoluble antioxidants, Chen et al. [6] found that hempseed oil is a rich source of tocopherols. The prevalence of γ-tocopherol over α-tocopherol was established recently [7], and both the compounds (vitamers of Vitamin E) exerted strong antioxidant activity and anti-inflammatory activities.

Furthermore, seeds are excellent sources of proteins with high nutritional value; edestin accounts for about 60–80% of the total storage protein content, while albumin accounts for the remaining portion. In addition, edestin contains all the essential amino acids and is easily digestible, as it lacks trypsin-inhibiting factors [8]. Among the essential amino acids, high levels of arginine, about twice those of soybean and egg proteins, were reported by Callaway [1]. Kim and Lee [9] carried out the isolation and characterization of edestin from a hemp cultivar in Korea and found it had high free radical scavenging activity (measured by DPPH assay).

Hemp seed is also a significant source of polyphenols, which act as strong antioxidant molecules [4,10]. Crescente et al. [2] pointed out the predominance of N-trans-caffeoyltyramine, N-trans-feruloyltyramine, and N-p-coumaroyltyramine, the first hydroxycinnamic acid amides, and isolated their derivatives.

The identification of compounds with predominant radical scavenging activity in defatted hemp seeds was intensively studied by Chen et al. [11]. The authors found that the antioxidants in the defatted seeds belonged to the phenolics and, among them, two compounds showed the highest activity, N-trans-caffeolyltyramine and Cannabisin B. More recently, Pojić et al. [12] characterized the hemp-seed meal obtained after oil extraction by measuring the distribution of the nutritional and antinutritional compounds in different meal fractions. Accordingly, they found that the cotyledon-containing fractions were richer in protein, lipids, and sugar than the hull-containing fractions, from which the highest contents of fiber and levels of antioxidant activity were recovered. Interestingly, they found that the cotyledon-containing fractions were the richest in *p*-hydroxybenzoic acid and catechin, and the hull-containing fractions had the highest contents of N-trans-caffeolyltyramine and Cannabisin B. All these nutritional characteristics have attracted the interest of the pharmaceutical and food industries to respond the increased demand for innovative natural compounds. Over recent years, it has become highly popular to enhance foods' nutritional properties by including functional additives [13].

Comparative studies on the seed compositions of hemp cultivars are available [3], and the effects of genotype and environment on seeds' composition were extensively studied [14], but few studies investigated the seeds' composition after oil extraction. Therefore, the present study was designed to examine the phytochemical composition, phenolic profiles (free and bound phenolic acids and some simple flavonoids), and antioxidant activity of the whole and defatted seeds of new hemp cultivars during two years of cultivation in the same location. An evaluation of the effect of the genotype and growing year on all the traits was also performed. Moreover, the pairwise correlations between the chemical compositions of the seed (whole and defatted) and precipitations (April–September) of the two years were obtained. The results of this study contributed to promoting the reintegration and valorization of hemp varieties into agricultural systems as sources of valu-

able ingredients for human diet, in view of the development of a circular and sustainable economy.

2. Material and Methods

2.1. Plant Materials and Agronomic Management

Three Italian hemp cultivars, two monoecious (Codimono and Carmaleonte) and one dioecious (CS), were grown at the experimental field of Rovigo (Italy) for two consecutive years (2018 and 2019). Details about the agro-botanical characteristics of the cultivars were described in the Supplementary Table S1. These cultivars were sown in triplicate on 31 May 2018 and on 19 April 2019 at Rovigo (latitude 45°08′90.92″ and longitude 11°76′74.19″) at field conditions. Briefly, the sowing density was 40 kg ha^{-1}, with an inter-row spacing of 25 cm on large plots (20.5 m^2) fertilized with 40 kg ha^{-1} of N; a selective herbicide treatment against monocotyledons was applied in pre-seeding, while a manual weed control was performed during the initial stages of crop development. The monoecious cultivars were harvested on 9 October 2018 and 16 September 2019, while the dioecious were harvested on 18 October 2018 and 27 September 2019.

2.2. Chemical Analysis

The frozen seeds (about 60 g) of the three hemp cultivars were ground with a household grinder. Half a portion of each sample was defatted by hexane (1/3, weight/volume) in a Soxhlet apparatus. The defatted hemp seed and the whole hemp seed were sealed in plastic bags and stored −20 °C until analysis and the moisture content was determined by drying at 130 °C, for 3 h. All the analyses were conducted in triplicate and expressed on dry matter.

2.3. Proximate Composition

Protein content (PC) was determined using the Dumas combustion nitrogen method, according to the AACC Approved Method 46–30.01 [15] and using a nitrogen/protein determinator (FP-528 Leco Corp., Saint Joseph, MI, USA). A factor of 5.7 was used to convert the nitrogen content into protein and expressed as grams per 100 g dry matter (g 100 g^{-1}).

2.4. Extraction of Phenolic Compounds

Phenolic compounds were extracted according to Beta et al. [16], with minor modifications. The samples (1 g) ground with a 1.0–millimeter sieve, were extracted using 8 mL methanol acidified with 1N HCl (80:20; vv^{-1}), for 30 min in an ultrasonic bath. The mixtures were centrifuged at 2000× for 15 min and were used for the determination of the total phenolic content (TPC), condensed tannins (CT), and antioxidant activity by ABTS assy.

2.5. Determination of Total Phenolic Content

Determination of total phenolic content was performed according to the procedure described by Fares and Menga [17]. Ferulic acid was used as the standard, and the data were expressed in mg ferulic acid equivalents g^{-1} (FA g^{-1}) on dry matter. A calibration curve was prepared by using increasing concentrations of ferulic acid (range from 50 to 250 mg L^{-1}, r = 0.997) (Sigma-Aldrich, Milano, Italy).

2.6. Determination of Condensed Tannins

The condensed tannins (CT) were determined according to the modified vanillin assay of Sun et al. [18] and expressed in mg catechin equivalents g^{-1} (CE g^{-1}) on dry matter.

2.7. Extraction and Determination of Carotenoids

Saponification was carried out for the extraction of carotenoids using methods previously described by Hussain et al. [19], with modifications. Briefly, whole-meal flour (1 g) was saponified with ethanol pyrogallol (2.5 mL, 60 g L^{-1}), sodium chloride (1 mL, 10 g L^{-1}), ethanol (1 mL, 95%), and potassium hydroxide (1 mL, 600 g L^{-1}). The tubes were placed in a

70 °C water bath for 30 min and mixed every 10 min during saponification. Afterwards, the tubes were cooled in an ice-water bath, and 7.5 mL sodium chloride (10 g L^{-1}) and 7.5 mL of n-hexane/ethyl acetate (9:1) were added. Next, the organic layer was separated by centrifuging at 1500 rpm for 5 min. Two additional extractions were carried out by adding n-hexane/ethyl acetate (9:1, 5 mL) in each extraction. The organic layer was evaporated to dryness and residue was dissolved in 2 mL of isopropyl alcohol (10%) in n-hexane. A sample volume of 50 µL was injected for chromatographic analysis.

The chromatographic separation of the compounds was achieved in normal phase with a Kromasil Phenomenex Si column (250 mm × 4.6 mm i.d., 5-micrometer particle size) (Torrance, CA, USA). The mobile phase was n-hexane/isopropyl alcohol (5%) at a flow rate of 1.5 mL min^{-1}. Spectrophotometric detection was achieved by means of a diode array detector set in the range of 350–500 nm. Peaks were detected at 450 nm. Carotenoids were identified through their characteristic spectra and comparison of their retention times with standard solutions. Carotenoids were expressed in µg g^{-1} on dry matter.

2.8. Antioxidant Activity Measured by ABTS Assay

The antioxidant activity (AA) was determined according to Fares and Menga [17]. The AA of extracts were expressed in µmol Trolox equivalent (TE) g^{-1} on dry matter.

2.9. Extraction and Determination of Free and Bound Phenolic Acids and Simple Flavonoids

The free and bound phenolic acids and simple flavonoids were extracted as described previously [17]. The quantification was based on their peak area of the following standards: protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, syringic acid, *p*-coumaric acid, N-trans-caffeoyltyramine (phenolic acids), naringenin, and epicatechin (simple flavonoids). The data from the free and bound phenolics (i.e., alkaline plus acid hydrolysates) were summed to give the sum of phenolics (SP) and expressed in µg g^{-1} on dry matter.

2.10. Statistical Analysis

Two-way analysis of variance (ANOVA) was carried out with respect to each parameter detected in the whole and defatted seed samples of three hemp cultivars collected in two growing seasons. Means discriminations were performed applying Tukey's multiple test or Student's *t*-test and statistically significant differences were determined at $p \leq 0.05$ probability level. Relationships between the precipitation (sum of rainfall from April to September) of each year and all the phytochemical compounds were examined using Pearson's correlation coefficients. All the statistical analyses were performed using the statistical package STATISTICA (StatSoft, Inc., version 7.1, Tulsa, OK, USA).

3. Results

3.1. Phytochemical Composition and Antioxidant Activity of Whole and Defatted Seeds: Effects of Genotype and Year

Table 1 shows the data of monthly rainfall and temperatures at the experimental field of Rovigo during the two growing seasons. The two years differed both in the amount of rain and in the average maximum temperature; the second year (2019) was wetter (427.6 mm) and cooler (25.3 °C) than the first year (2018) (mean rainfall 309.4 mm; 27.8 °C maximum temperature). The months of April and May of the second year were the wettest ever, with the lowest mean minimum and highest maximum mean temperatures.

Table 1. Monthly total rainfall and temperature (minimum and maximum) at the experimental field of Rovigo during the two growing seasons, 2018 and 2019.

	Rainfall (mm)		Min. Temperature (°C)		Max. Temperature (°C)	
	2018	**2019**	**2018**	**2019**	**2018**	**2019**
April	18.0	86.4	10.3	11.7	22.5	12.1
May	48.2	151.2	14.8	11.3	25.6	20.0
June	19.6	5.6	17.2	18.8	28.9	31.7
July	80.2	66.0	19.3	19.7	31.5	31.7
August	55.0	46.6	19.7	19.9	31.5	30.7
September	88.4	71.8	16.0	15.1	27.0	25.7
Mean			16.2	16.1	27.8	25.3
Total	309.4	427.6				

The three italian hemp cultivars evaluated in this study, two monoecious (Codimono and Carmaleonte) and one dioecious (CS), contained Δ9-tetrahydrocannabinol (THC) <0.2% in their leaves and inflorescence (data not shown), according to laws on their use for food purposes. An overview of the phytochemical compositions of the seeds is given in Table 2.

Table 2. Mean data with statistical significance for the experimental factors (genotype and year) on protein and phytochemical content of whole and defatted seeds of the three Italian hemp cultivars.

		PC	β car	Lutein	TPC	CT	AA
	Experimental Factors	**(%)**	**(μg g^{-1})**	**(μg g^{-1})**	**(mgFAg^{-1})**	**(mgCEg^{-1})**	**(μmolTEg^{-1})**
Whole seed	Carmaleonte	19.3 c	0.4	17.0 b	4.5	9.9	4.6
	Codimono	20.3 b	0.5	23.3 ab	3.9	8.8	6.0
	CS	21.9 a	0.5	33.9 a	4.4	8.8	5.3
	P	*0.0001*	*0.47 ns*	*0.023*	*0.19 ns*	*0.51 ns*	*0.13 ns*
	2018	18.0 B	0.6 A	22.9	4.8 A	9.9	6.4 A
	2019	23.0 A	0.4 AB	26.6	3.8 B	8.4	4.2 B
	P	*0.0001*	*0.009*	*0.34 ns*	*0.004*	*0.13 ns*	*0.003*
Defatted seed	Carmaleonte	26.7 b	0.2	17.4 c	5.0 a	13.8 a	8.4 b
	Codimono	25.9 b	0.4	32.5 b	3.8 b	8.6 c	11.3 a
	CS	29.4 a	0.3	40.4 a	4.4 ab	11.4 b	11.4 a
	P	*0.029*	*0.3 ns*	*0.000*	*0.02*	*0.001*	*0.000*
	2018	21.9 B	0.3	27.3 B	4.2	12.0	10.7
	2019	32.7 A	0.3	33.0 A	4.6	10.6	10.0
	P	*0.0001*	*0.58 ns*	*0.010*	*0.19 ns*	*0.06 ns*	*0.11 ns*

PC = protein content; β car = beta carotene content; TPC = total phenolic content; CT = condensed tannins; AA = antioxidant activity; FA = ferulic acid equivalent; CE = catechin equivalent; TE = Trolox equivalent. Different lower-case letters in the same column represent significant differences among genotypes (Tukey's test $p < 0.05$), different capital letters in the same column represent significant differences between years (Student's test $p < 0.05$); ns = not significant. All measurements were made in triplicate and expressed on dry-matter basis.

The PC varied significantly according to the genotype as well as the year of cultivation in the whole and defatted seeds. In the whole seeds, the lowest value was measured in Carmaleonte (19.3%), and the highest in CS (21.9%). The highest PC was found in 2019, both in the whole (23.0%) and the defatted seeds (32.7%), compared to 2018 (18.0% in the whole seeds and 21.9% in the defatted seeds). The PC increased by 37%, 27%, and 34% in defatted seeds of Carmaleone, Codimono, and CS, respectively, keeping the variety ranking unchanged.

β-carotene and lutein were the unique carotenoids detected in the samples, with a high predominance of lutein; conversely, β-carotene was present in scarce and insignificant amounts. The highest lutein content was found in the CS (33.9 μg g^{-1} and 40.4 μg g^{-1} in whole and defatted seed, respectively), while Carmaleonte had the lowest content (17.0 μg g^{-1} and 17.4 μg g^{-1} in the whole and defatted seeds, respectively). In all the

samples, an increase in lutein content was observed in the defatted seeds; by contrast, β-carotene showed a decrease (50%, 20%, and 40% for Carmaleonte, Codimono, and CS, respectively).

The total polyphenol content (TPC) of the whole seeds did not show any significant differences among the genotypes. The mean content in the whole and defatted seeds was 4.3 μg g^{-1} FA and 4.4 μg g^{-1} FA, respectively. In this regard, Carmaleonte and CS showed comparable contents, both of which were higher than that of Codimono. Significant differences were observed solely in the whole seeds due to the year effect (with a content that was 20.8% higher in 2018).

The condensed tannins (CT) showed significant differences only between the varieties of defatted seeds, with the highest (13.8 mg CEg^{-1}) and the lowest (8.6 mg CEg^{-1}) content in Carmaleonte and Codimono, respectively.

The antioxidant activity (AA) showed significant differences between the varieties only in the defatted seeds. Codimono and CS possessed the highest values (11.3 μmol TEg^{-1} and 11.4 μmol TE g^{-1}, respectively). Between the two years, only the AA of the whole seeds from 2018 differed significantly, with a value of 6.4 μmol TEg^{-1}, which was in line with what was observed for the TPC.

The relative weight of the single and combined influence of the genotype (G), year (Y), and G × Y interaction is shown in Table 3. Generally, a prevalent effect of genotype was observed in the defatted seeds when compared to the whole seeds. In the whole seeds, the effect of genotypic variance was solely significant for the lutein and total carotenoids (TC) (about 64%; $p < 0.001$) and for PC (15%; $p < 0.01$). The year was the most important variance component for the PC, β-carotene, TPC, and AA, accounting for a range of 43–82%.

Table 3. Relative weight of the single and combined influences of genotype (G), year (Y), and G × Y interaction.

	Whole Seed				Defatted Seed			
	G (%)	**Y (%)**	**GXY (%)**	**E (%)**	**G (%)**	**Y (%)**	**GXY (%)**	**E (%)**
PC	15.0 **	81.8 ***	2.1 *	1.2	6.5 **	85.0 ***	7.4 **	1.1
β carotene	6.7	56.0 **	13.9	23.4	24.6	3.0	20.1	52.3
Lutein	64.0 *	4.6	6.0	25.4	81.1 ***	7.3 **	8.3 *	3.3
TC	63.5 *	5.1	6.0	25.4	81.6 ***	7.1 *	7.8 *	3.5
TPC	11.0	48.3 **	25.7 *	15.0	63.9 *	8.8	2.3	25.0
CT	8.6	17.3	39.6	34.5	68.9 **	7.5	14.5	9.1
AA	11.6	43.3 **	32.8 *	12.3	73.3 ***	4.0	15.3 *	7.3
Vanillic acid F	43.0	4.5	17.2	35.3	43.2	9.9	9.2	37.8
Epicatechin F	17.0	0.1	9.8	73.1	32.4 *	41.1 **	16.0	10.5
N-trans-caffeoyltyramine F	25.7 *	18.7 **	47.6 **	8.0	48.2 *	27.4 *	9.4	15.0
Naringenin F	13.7	19.6 *	47.7 *	19.0	61.1 *	18.1 *	3.1	17.8
SPF	19.9	12.9 *	55.3 **	11.9	41.7 **	35.2 ***	17.5 *	5.6
Protocatechuic acid B	43.1	4.5	17.2	35.3	43.2	9.9	9.2	37.8
p-Hydroxybenzoic acid B	3.1	50.7 *	9.8	36.4	30.0 *	39.4 **	20.7 *	9.9
Vanillic acid B	6.7	39.7 *	29.2	24.3	16.0 **	35.7 ***	45. 3 ***	3.0
Caffeic acid B	19.6	1.4	1.0	78.1	20.8	55.1 **	7.5	16.5
Ferulic acid B	65.1 *	3.6	7.9	23.4	14.9	0.1	21.4	63.6
Syringic acid B	20.9	48.0 **	15.4	15.7	39.0 *	24.1 *	20.6	16.3
p-Coumaric acid B	48.2 **	27.4 **	17.1 *	7.3	29.0 **	32.4 **	37.9 **	0.7
Naringenin B	43.6	10.9	16.4	29.0	37.9	6.3	25.7	30.1
N-trans-caffeoyltyramine B	42.0 ***	40.9 ***	12.8 *	4.3	43.6 ***	19.7 ***	34.5 ***	2.2
SPB	34.6 **	36.4 **	20.8 *	8.2	36.6 ***	13.2 **	47.3 ***	3.0

PC = protein content; TC = total carotenoids; TPC = total phenolic content; C T = condensed tannins; AA = antioxidant activity; SPF = sum phenolics free; SPB = sum phenolics bound; F = free fraction; B = bound fraction; E = Error. Asterisks: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

The effect of the G × Y interaction was solely significant for the PC, TPC, and AA ($p < 0.05$) but contributed less than Y, and slightly over 25% of the total variance.

In the defatted seeds, the effect of genotypic variance was significant for the PC, lutein, TC, TPC, CT, and AA, with a lower contribution of 6.5% for the PC and over 60% for the others. The Y contribution to the total variance of the PC accounted for 85%, while a lower contribution was found from the lutein (7.3%). The effects of the G × Y interaction, although significant, represented less than 15% of the total variance for the PC, lutein, and AA.

3.2. Phenolic Composition in Whole and Defatted Seeds: Effects of Genotype and Year

Table 4 reports the phenolic composition based on the identification and quantification of the following standards: protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, syringic acid, *p*-coumaric acid, N-trans-caffeoyltyramine (phenolic acids), naringenin, and epicatechin (simple flavonoids). The sum of the free phenolics (183.8 μg g^{-1} in the whole seeds and 201.7 μg g^{-1} in the defatted seeds) was lower than the sum of the bound phenolics (455 μg g^{-1} in whole seeds and 719.7 μg g^{-1} in the defatted seeds). In the phenolic-free fraction of the whole seeds, only N-trans-caffeoyltyramine was, on average, the most significantly represented, ranging from 67.7 μg g^{-1} (Carmaleonte) to 108.8 μg g^{-1} (CS). CS differed from Carmaleonte and Codimono, which had an average content of about 37.8% and 34%, respectively. In addition, the difference between the two years was also significant, and in 2019, the content was greater than 30%. Epicatechin was the second most representative phenol, but no significant difference was found among the varieties or between the years. No significant differences for naringenin were observed between the varieties, whereas the effect of the year was highlighted, and 2019 presented an average content that was 28% higher than that of 2018.

In the defatted seeds, considering the free fraction of the phenolics, except for vanillic acid, significant differences were observed both among varieties and between years. Among the varieties, CS had the highest values of epicatechin (95.5 μg g^{-1}) and N-trans-caffeoyltyramine (91.4 μg g^{-1}), while Codimono had the highest content of naringenin (12.2 μg g^{-1}). Considering the years, in 2019, significantly higher values were found.

Regarding the bound fraction, the N-trans-caffeoyltyramine and *p*-coumaric acid were by far the most representative in the whole and defatted seeds. In the whole seeds, significant differences between varieties were found: CS showed the highest content of N-trans-caffeoyltyramine, with a concentration almost double (427 μg g^{-1}) that of the other two cultivars, while Carmaleonte showed the highest content of *p*-coumaric acid (141 μg g^{-1}). For the years, significant differences were observed for the *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, and N-trans-caffeoyltyramine, with a prevalence of 2019 compared to 2018. In the defatted seeds, significant differences between the varieties were observed, and CS showed the highest content of N-trans-caffeoyltyramine (723 μg g^{-1}), *p*-hydroxybenzoic acid (23.9 μg g^{-1}), and vanillic acid (10.7 μg g^{-1}). Moreover (data not reported in Table 4), the sum of all the phenolics of the bound fraction (SPB) was more abundant in the defatted seeds, with respect to the whole seeds and, on average, CS showed the highest sum of phenols in the whole and defatted seeds (771 μg g^{-1} and 935 μg g^{-1}, respectively).

Table 4. Phenolic acids (free and bound) and some simple flavonoid contents determined in whole and defatted seeds of the three Italian hemp cultivars during 2018 and 2019 cropping years.

		Phenolics												
		(Free Fraction μg g^{-1} dm)						**(Bound Fraction, μg g^{-1} dm)**						
	Experimental factors	Vanillic acid	Epicatechin acid	Naringenin	N-trans-caffeoyltyramine	Protocatechuic acid	*p*-Hydroxybenzoic acid	Vanillic acid	Caffeic acid	Ferulic acid	Syringic acid	*p*-Coumaric acid	Naringenin	N-trans-caffeoyltyramine
Whole Seed	Carmaleonte	34.9	65.1	8.0	67.7 b	8.7	16.3	6.2	2.2	2.9 a	3.0	140.9 a	1.1	226.2 b
	Codimono	38.3	47.2	8.2	71.4 b	9.6	16.2	5.7	2.1	0.7 b	2.8	116.2 b	0.8	242.8 b
	CS	28.8	62.1	10.7	108.9 a	7.2	15.5	6.1	2.0	2.4 ab	2.3	96.6 b	1.7	426.7 a
	P	*0.09 ns*	*0.53 ns*	*0.2 ns*	*0.01*	*0.09 ns*	*0.78 ns*	*0.5 ns*	*0.51 ns*	*0.02*	*0.07 ns*	*0.002*	*0.06 ns*	*0.000*
	2018	35.3	58.6	7.5 B	66.8 B	8.8	14.7 B	6.7 A	2.1	2.2	2.3 B	131.5 A	1.0	208.8 B
	2019	32.7	57.7	10.5 A	98.6 A	8.2	17.3 A	5.2 B	2.1	1.8	3.2 A	104 4 B	1.4	388.3 A
	P	*0.42 ns*	*0.94 ns*	*0.05*	*0.01*	*0.42 ns*	*0.03*	*0.02*	*0.75 ns*	*0.37 ns*	*0.005*	*0.003*	*0.18 ns*	*0.000*
Defatted Seed	Carmaleonte	28.6	73.8 b	8.7 b	63.6 b	7.1	19.0 b	11.3 a	2.5	4.6	3.1 ab	207.2 a	1.6	358.6 b
	Codimono	33.3	82.1 ab	12.2 a	80.6 ab	8.3	20.5 ab	8.3 b	3.0	4.2	3.8 a	181.5 ab	1.8	377.2 b
	CS	26.8	95.5 a	8.5 b	91.4 a	6.7	23.9 a	10.7 a	3.0	4.1	2.4 b	156.1 b	4.7	723.4 a
	P	*0.101*	*0.014*	*0.011*	*0.013*	*0.10ns*	*0.015*	*0.04*	*0.08 ns*	*0.53 ns*	*0.025*	*0.007*	*0.09 ns*	*0.0001*
	2018	30.9	73.8 B	8.9 B	69.9 B	7.7	18.8 B	12.1 A	2.5 B	4.5	3.5 A	203.6 A	2.1	373.7 B
	2019	28.2	93.9 A	10.7 A	87.2 A	7.1	23.5 A	8.2 B	3.2 A	4.5	2.7 B	159.5 B	3.3	599.1 A
	P	*0.25 ns*	*0.02*	*0.048*	*0.016*	*0.25 ns*	*0.002*	*0.0001*	*0.004*	*0.93 ns*	*0.024*	*0.001*	*0.30 ns*	*0.0003*

Different lower-case letters in the same column represent significant differences between genotypes (Tukey's test, $p < 0.05$). Different capital letters in the same column represent significant differences between years (Student's test $p < 0.05$); ns = not significant. All measurements were made in triplicate and expressed on dry-matter basis.

The relative weights of the single and combined influence of G, Y, and G × Y interaction are shown in Table 3. Considering only the most representative phenolics, in the free fraction of whole seed, the G effect was significant only for the N-trans-Caffeoyltyramine (26%). The Y effect was also significant, and it contributed to the total variance of 18.7%, 19.6%, and 12.9% for the N-trans-caffeoyltyramine, naringenin, and sum of phenolics free (SPF), respectively. The effect of the G × Y interaction was prevalent, and it contributed to the total variance of 47.6%, 47.7%, and 55.3% for the N-trans-caffeoyltyramine, naringenin, and sum of phenolics free (SPF), respectively. In the bound fraction of the whole seeds, the effects of G were significant and prevalent for the ferulic acid, *p*-coumaric acid, and N-trans-caffeoyltyramine, while the contribution of Y to the total variance was the same as that of G for the N-trans-caffeoyltyramine and the sum of phenolics bound (SPB), accounting for about 41% and 36%, respectively. The effect of Y was prevalent and significant for the vanillic, syringic, *p*-hydroxybenzoic, N-trans-caffeoyltyramine, and *p*-coumaric acid (range 27–51% of total variance). The effects of the G × Y interaction, when significant, were low and negligible.

In the defatted seeds (free and bound), the significant effects of G and Y were observed for most of the representative phenolics. In the free fraction, the G effect was significant for almost all the compounds detected, with the exception of the vanillic acid, while in the bound fraction, the G effect was significant for six compounds with a range of 16–43.6% and, for SPB, with a range of 36.6% ($p < 0.001$). The Y effect was the highest and most significant for the free epicatechin, accounting for 41.1% ($p < 0.01$). For the bound fraction, the most representative phenolics ranged from 19.7%, for the N-trans-caffeoyltyramine, to 55.1%, for the caffeic acid. The effect of the G × Y interaction was strongest in the defatted seeds, ranging from 20.7%, for the *p*-hydroxybenzoic acid, to 34.5%, for the N-trans-caffeoyltyramine.

3.3. Correlations

The correlations of the precipitations (sum from April to September for each year) of the two cropping years and the phytochemicals of the whole and defatted seeds are summarized in Table 5, with the Pearson's correlation coefficients (r value). In the whole seeds, the precipitations were positively correlated with the PC (r = 0.9 ***), bound *p*-hydroxybenzoic acid (r = 0.71 **), bound syringic (r = 0.69 *), bound N-trans-caffeoyltyramine (r = 0.64 *), and SPB (r = 0.60 *); negative and significant correlations were found with the β carotene (r = −0.75 **), TPC (r = −0.70 **), AA (r = −0.66 *), and bound vanillic acid (r = −0.63 *). In the defatted seeds, significant positive correlations were found with the SPF, *p*-hydroxybenzoic acid bound, free epicatechin, bound caffeic acid, and PC (range of r from 0.6 * to 0.92 ***), while a negative and significant correlation was observed with the bound vanillic acid (r = −0.6 *).

Table 5. Pairwise correlations between chemical compositions of seeds (whole and defatted) and precipitations (April–September) of the two years.

	Whole Seeds	**Defatted Seeds**
PC	0.90 ***	0.92 ***
β-carotene	−0.75 **	-
p-Hydroxybenzoic acid B	0.71 **	0.63 *
TPC	−0.70 **	-
Syringic acid B	0.69 *	-
AA	−0.66 *	-
N-trans-caffeoyltyramine B	0.64 *	-
Vanillic acid B	−0.63 *	−0.60 *
SPB	0.60 *	-
Epicatechin F	-	0.64 *

Table 5. *Cont.*

	Whole Seeds	Defatted Seeds
Caffeic acid B	-	0.74 **
SPF	-	0.60 *

Results are expressed as Pearson correlation coefficients (r value); *** $p < 0.001$; ** $p < 0.01$; * $p < 0.5$; PC = protein content; TPC total phenolic content; AA antioxidant activity; SPB = sum phenolics bound; SPF = sum phenolics free.

4. Discussion

Hemp is a versatile industrial crop due to its richness in proteins, oil, and polyphenols, which act as strong antioxidant molecules [4,10], and there is a deep interest in exploring the potential application of these compounds in cosmetics and foods. In this regard, hemp seeds contain several antioxidant compounds (phenolic acids, lignans, and flavonoids) and proteins that represent valuable ingredients for several food enrichments [12]. Here, the mean protein contents of the whole and defatted seeds showed a wide variability among the cultivars, but the ranking was the same for the whole and defatted seeds. Moreover, the PC mean contents measured in the whole seeds were similar to those found by Irakli et al. [14] in a three-year study on seven hemp cultivars. The increased PC in defatted seeds was also observed by Callaway [1] in the Finola cultivar and by Pojić et al. [12], who found a progressive increase in PC in the cotyledon-containing fractions of defatted hemp. Thus, the seeds of the three cultivars analyzed in this study represented an excellent source of proteins, whose content increased in defatted meal.

β-carotene and lutein were the unique carotenoids detected in the samples and, in contrast to other research [14], we did not find traces of zeaxanthin, while the amounts of β-carotene and lutein in the whole seeds were comparable. The observed increases in the PC and lutein contents of the defatted seeds were probably the direct consequence of the nutrient concentration; by contrast, for the β-carotene, a significant decrease was observed in the defatted seeds (50%, 20%, and 40% for Carmaleonte, Codimono, and CS, respectively). We ascertained that the two main carotenoids detected in the italian hemp-seed cultivars showed opposite behavior, as the β-carotene content was strongly influenced by the year, in contrast to the lutein, which had a genotypic variance of over 60%. These results are consistent with the study by Irakli et al. [14], and the robustness of these results is confirmed by the fact that although the meteorological trends of the two studies were quite different, as indicated by the three drier and warmer years compared to our two-year trial (the mean rainfall was 234 mm, 168 mm, and 248 mm, and the mean temperature maximum was 34 °C, 35 °C, and 33 °C, for the years 2016, 2017, and 2018, respectively), the conclusions reached were the same. Moreover, the results confirmed the key role of the environment on the accumulation of antioxidants (TPC and AA), as previously ascertained by Menga et al. [20] in a large set of cereal species.

The total polyphenol content (TPC) of the whole seeds showed low differences between the genotypes, which become more evident and significant in the defatted seeds, even though the mean contents observed here were lower than those reported by Galasso et al. [3]. These observations could be ascribed to the different panels of the genotypes investigated. Moreover, it should be considered that the TPC depicts a very small portion of the polyphenolic profile [21], and the assessment of TPC by the Folin–Ciocalteu method always showed values lower than the total content of phenolic acids, as evidenced by the analysis of the phenolics reported in Table 4. Condensed tannins have a positive role in human health and are involved in suppressing oxidative stress, which is important for the pathogenesis of cancer and influences the apoptosis of cells [22]. In this study, a greater content of CT was observed in comparison to the findings of Russo et al. [23]. These differences could be attributable to the different genotypes used in the two studies, as confirmed by the strong effect of G measured in the defatted seeds, indicated in Table 3 (68.9% $p < 0.01$).

The presence of phenolic compounds in food has gained much attention in recent years [4,10]. The main reason for this relates to their antioxidant properties and health benefits, such as their promotion of cardiovascular health and protection against cancers and neurodegeneration illness [2]. Several studies have pointed out that N-trans-caffeoyltyramine and cannabisin A, B and C are the main polyphenols in hemp seeds [11,12,14], which are also responsible for the protective effect against the in vitro oxidation of human low-density lipoprotein. The phenolic profile detected in this study included protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid, syringic acid, *p*-coumaric acid, N-trans-caffeoyltyramine (phenolic acids), naringenin, and epicatechin (simple flavonoids). Comparable results and similar phenolics profiles were found by Pojic et al. [12] in the whole-seed and defatted-seed fractions. Here, we observed that the greatest accumulation of N-trans-caffeoyltyramine in the free and bound fractions was recorded in the second year, which was wetter (427.6 mm) and cooler (25.3 °C) than the first year (mean rainfall 309.4 mm; 27.8 °C maximum temperature). The CS cultivar had the highest content of N-trans-caffeoyltyramine in the free and bound form, with similar concentrations found in the cultivar Felina in the study by Irakli et al. [14]. In general, almost all the phenolics studied here were higher in the second year compared to the first year, and, according to the significant correlation with rainfall, it seems that the wetter weather conditions promoted the accumulation of phenolics. There are few studies with which to compare these results, but in this study, we found a slight prevalence of the Y effect over G, which was more visible in whole seeds, which is in agreement with the research by Irakli et al. [14]. Glynn et al. [24], studying the response of willow (Salix spp. L.) to drought and irrigation, found that the total phenolics were greater in well-irrigated plants. Farming practices, and biotic and abiotic stresses are also directly involved in modulating the accumulation of these phytochemicals [25]. Here, we found a significant correlation between the rainfall and the whole-seed composition, indicating the probable involvement of precipitation in the accumulation of phenolics in hemp seeds.

5. Conclusions

The results indicated the strong involvement of the growing year in the nutritional and antioxidant properties of the whole seeds compared with the defatted seeds, as indicated for the PC, TPC, AA, sum of phenolic free (SPF), and sum of phenolic bound (SPB). Nevertheless, the lutein and some phenolic acids, both free and bound (ferulic *p*-coumaric acids and N-trans-Caffeoyltyramine), showed a significant effect of genotype. In this respect, Carmaleonte displayed the highest content of ferulic acid and CS of N-trans-caffeoyltyramine.

The strong effect of the growing year was probably caused by the different amounts of precipitation over the two years, as indicated by the positive and significant correlation with the most abundant phenolics detected in the whole seeds. The high concentrations of N-trans-Caffeoyltyramine recovered in the CS defatted seeds suggest that it is a valuable ingredient for functional foods as phenolics' bound fractions have strong biological activities, including antioxidant and anti-inflammatory properties.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture12060759/s1, Table S1. Agronomic characteristics of the cultivars and detailed content of CBD (Cannabidiol) and THC (Δ9-tetrahydrocannabinol).

Author Contributions: Conceptualization, C.F.; methodology, C.F., V.M., S.S. and C.G.; formal analysis, C.F. and S.S.; investigation, V.M., S.S., C.G. and M.M.; writing—original draft preparation, C.F.; writing—review and editing, C.F., R.B., V.M., C.G. and S.A.C.; visualization, D.P. and R.B.; supervision, N.P. and D.P.; project administration, C.F.; funding acquisition, C.F. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported with the special grant, MAIDET (Metodi Analitici Innovativi per la DEterminazione del Thc in matrici vegetali), supported by Italian Ministry of Agricultural, Food and Forestry Policies (MIPAAF).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Callaway, J.C. Hemp seed as a nutritional resource: An overview. *Euphytica* **2004**, *140*, 65–72. [CrossRef]
2. Crescente, G.; Piccolella, S.; Esposito, A.; Scognamiglio, M.; Fiorentino, A.; Pacifico, S. Chemical composition and nutraceutical properties of hempseed: An ancient food with actual functional value. *Phytochem. Rev.* **2018**, *17*, 733–749. [CrossRef]
3. Galasso, I.; Russo, R.; Mapelli, S.; Ponzoni, E.; Brambilla, I.M.; Battelli, G.; Reggiani, R. Variability in seed traits in a collection of *Cannabis sativa* L. genotypes. *Front. Plant Sci.* **2016**, *7*, 688–697. [CrossRef] [PubMed]
4. Frassinetti, S.; Moccia, E.; Caltavuturo, L.; Gabriele, M.; Longo, V.; Bellani, L.; Giorgi, G.; Giorgetti, L. Nutraceutical potential of hemp (*Cannabis sativa* L.) seeds and sprouts. *Food Chem.* **2018**, *262*, 56–66. [CrossRef]
5. Walker, C.G.; Jebb, S.A.; Calder, P.C. Stearidonic acid as a supplemental source of omega-3 polyunsaturated fatty acids to enhance status for improved human health. *Nutrition* **2013**, *29*, 363–369. [CrossRef]
6. Chen, T.; He, J.; Zhang, J.; Zhang, H.; Qian, P.; Hao, J.; Li, L. Analytical Characterization of Hempseed (Seed of *Cannabis sativa* L.) Oil from Eight Regions in China. *J. Diet. Supll.* **2010**, *7*, 117–129. [CrossRef]
7. Monserrat de la Paz, S.; Marin-Anguilar, F.; Garcia-gimenez, M.D.; Fernandez-Arche, M.A. Hemp (*Cannabis sativa* L.) seed oil: Analytical and phytochemical characterization of the unsaponifiable fraction. *J. Agric. Food Chem.* **2014**, *62*, 1105–1110. [CrossRef]
8. Tang, C.H.; Ten, Z.; Wang, X.S.; Yang, X.Q. Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. *J. Agric. Food Chem.* **2006**, *54*, 8945–8950. [CrossRef]
9. Kim, J.-J.; Lee, M.-Y. Isolation and characterization of edestin from Cheungsam hempseed. *J. Appl. Biol. Chem.* **2011**, *54*, 84–88. [CrossRef]
10. Martinez, J.R.; Monserrat-de la Paz, S.; De la Puerta, R.; Garcia-Gimenez, M.D.; Fernandez-Arche, M.A. Characterization of bioactive compounds in defatted hempseed (*Cannabis sativa* L.) by UHPLC-HRMS/MS and antiflammatory activity in primary human monocytes. *Food Func.* **2020**, *11*, 4057–4066. [CrossRef]
11. Chen, T.; He, J.; Zhang, J.; Li, X.; Zhang, H.; Hao, J.; Li, L. The isolation and identification of two compounds with predominant radical scavenging activity in hempseed (seed of *Cannabis sativa* L.). *Food Chem.* **2012**, *134*, 1030–1037. [CrossRef]
12. Pojić, M.; Mišan, A.; Sakač, M.; Hadnađev, T.D.; Šarić, B.; Milovanović, I.; Hadnađev, M. Characterization of Byproducts Originating from Hemp Oil Processing. *J. Agric. Food Chem.* **2014**, *62*, 12436–12442. [CrossRef]
13. Lourenço, S.C.; Moldão-Martins, M.; Alves, V.D. Antioxidants of Natural Plant Origins: From Sources to Food Industry Applications. *Molecules* **2019**, *24*, 4132. [CrossRef]
14. Irakli, M.; Tsaliki, E.; Kalivas, A.; Kleisiaris, F.; Sarrou, E.; Cook, C. Effect of genotype and growing year on the nutritional, phytochemical and antioxidant properties of industrial hemp. *Antioxidants* **2019**, *8*, 491. [CrossRef]
15. American Association of Cereal Chemists (AACC). *Approved Method of AACC*, 11th ed.; Methods 46-30.01 (protein); American Association of Cereal Chemists (AACC): St Paul, MN, USA, 2012.
16. Beta, T.; Nam, S.; Dexter, J.E.; Sapirstein, H.D. Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal. Chem.* **2005**, *82*, 390–393. [CrossRef]
17. Fares, C.; Menga, V. Effects of toasting on the carbohydrate profile and antioxidant properties of chickpea (*Cicer arietinum* L.) flour added to durum wheat pasta. *Food Chem.* **2012**, *131*, 1140–1148. [CrossRef]
18. Sun, B.S.; Ricardo Da Silva, J.M.; Spranger, I. Critical factors of vanillin assay for catechins and proanthocyanidins. *J. Agric. Food Chem.* **1998**, *46*, 4267–4274. [CrossRef]
19. Hussain, A.; Larsson, H.; Kuktaite, R.; Olsson, M.E.; Johansson, E. Carotenoid Content in Organically Produced Wheat: Relevance for Human Nutritional Health on Consumption. *Int. J. Environ.* **2015**, *12*, 14068–14083. [CrossRef]
20. Menga, V.; Fares, C.; Troccoli, A.; Cattivelli Land Baiano, A. Effects of Genotype, Location and Baking on the Phenolic Content and Some Antioxidant Properties of Cereal Species. *Int. J. Food Sci.* **2010**, *45*, 7–16. [CrossRef]
21. Fares, C.; Platani, C.; Baiano, A.; Menga, V. Effect of processing and cooking on phenolic acid profile and antioxidant capacity of durum wheat pasta enriched with debranning fractions of wheat. *Food Chem.* **2010**, *119*, 1023–1029. [CrossRef]
22. Koleckar, V.; Kubikova, K.; Rehakova, Z.; Kuca, K.; Jun, D.; Jahodar, L.; Opletal, L. Condensed and hydrolysable tannins as antioxidants influencing the health. *Mini Rev. Med. Chem.* **2008**, *8*, 436–447. [CrossRef]
23. Russo, R.; Reggiani, R. Variability in antinutritional compounds in hempseed meal of Italian and French varieties. *Plant* **2013**, *1*, 25–29. [CrossRef]
24. Glynn, C.; Ronnberg-Wastljung, A.C.; Julkunen-Tiitto, R.; Weih, M. Willow genotype, but not drought treatment, affects foliar phenolic concentrations and leaf-beetle resistance. *Entomol. Exp. Appl.* **2004**, *113*, 1–14. [CrossRef]
25. Cohen, S.D.; Kennedy, J.A. Plant Metabolism and the Environment: Implications for managing phenolics. *Crit. Rev. Food Sci. Nutr.* **2010**, *50*, 620–643. [CrossRef]