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Piano Nazionale di Ripresa e Resilienza
Missione 4 - Componente 2 - INVESTIMENTO 1.1 – "Fondo per il Programma Nazionale di Ricerca e Progetti di Rilevante Interesse Nazionale (PRIN)"

- NOrCa - Not Ordinary Cannabis - Exploring the chemical space around hemp (*Cannabis sativa* L.) waste and by-products from a circular economy perspective -

CUP code
F53D23011900001

Strategic emerging Topics
- SUSTAINABILITY AND PROTECTION OF NATURAL RESOURCES -

Mid-term report of the project

Milestone: 1 - Development of new green methods for the extraction of hemp waste and by-products

1 *- Extraction of the hemp by-products with sustainable strategies for further chemical characterization and bioactivity evaluation*	Period (two months): **1 – 7** (30 nov. 23 – 30 gen. 25)

Research Units Involved	Activities and Results
UNIFE UNIMORE	Milestone's first activities involved ultrasonic assisted extractions (UAE) and supercritical fluid extractions performed on hemp roots as the first green approaches to more sustainable extraction methodologies. Secondly, extractions were performed on exhausted hemp biomass residues after industrial processing. *Ultrasound assisted extractions (UAE)* Ultrasound-assisted extractions (UAE) were performed on hemp roots, one with EtOH and the other with n-hexane as solvent, as reported in Kornpointner et al. (2021 - doi:10.1016/j.indcrop.2021.113422), who compared traditional extractions with these organic solvents to an extraction with SFE. A sample (1 g) of powdered roots was extracted with 16 mL of EtOH under sonication for 90 min. The extract was centrifuged and filtered with a Buchner funnel. The filter cake was further extracted two more times with 16 mL of EtOH. The three supernatants were collected and dried with a Rotavapor. The same procedure was used for the extracts obtained with n-hexane. *Supercritical fluid extractions (SFE-CO_2)* SFE was performed on roots according to the operating conditions reported by Kornpointner et al. (2021 - doi:10.1016/j.indcrop.2021.113422), with some modifications. The extraction vessel was filled 2/3 with C. sativa roots (5 g) and 1/3 with diatomaceous earth as a dispersing agent. The extraction was conducted for 1 h under static conditions and for 1 h under dynamic conditions, with a pressure of 20 MPa and the oven containing the extraction cell set to a temperature of 60 °C. The carbon dioxide flow rate was set to 3 L/min. GC-MS analyses at the conditions described above for UAE extracts were then also performed on the SFE root extracts. *SFE on C. sativa aerial waste*

A DoE, extrapolated thanks to the software MODDE, was followed in order to optimize the $scCO_2$ extraction. A total of 21 SFEs was performed, at the following conditions:

- Sample: 6.5 g pulverized hemp aerial parts + 1.5 g dispersing agent
- Temperature: 40°C min – 70°C max
- Pressure: 150 bar min – 350 bar max
- CO_2 flow: 2 L/min min – 4 L/min max
- Time: 1 h min – 2 h max
- Co-solvent (EtOH): 0% min – 5% max

Results and discussion

Extractions performed on C. sativa roots

The yields calculated after three consecutive UAE extractions with both solvents were quite low: 1.40% with EtOH and 1.13% with n-hexane.

A preliminary analysis of the GC-MS spectra, performed by comparing the fragmentation patterns with an internal mass spectrometric library and the NIST database, allowed to identify 15 peaks with an area greater than 1%, corresponding to 95.83% of the total area. Identification and quantification analyses are ongoing.

SFE DoE (Superctitival Fluid Extractions – Design of Experiment)

These activities based on the DoE outcomes, planned to optimize SFE results, will be carried out in the following months.

Extractions of aerial parts by-products:

Extractions on *C. sativa* aerial waste (by-products), provided by Whole Lotta Hemp SRL, were performed via SFE, following a DoE previously described, and UAE as a reference for SFE results. Extraction conditions were as follows. For SFE:

- Sample: 6.5 g pulverized hemp aerial parts + 1.5 g dispersing agent
- Temperature: 40°C min – 70°C max
- Pressure: 150 bar min – 350 bar max
- CO_2 flow: 2 L/min min – 4 L/min max
- Time: 1 h min – 2 h max
- Co-solvent (EtOH): 0% min – 5% max

For UAE:

- Solid/liquid ratio: 1/16
- Solvent: EtOH 100%
- Time: 90 min in ultrasound bath

SFE extracts were evaluated in terms of quantitative yield (following) and antimicrobial activity (MS 4.4).

A preliminary series of GC-MS on SFE extracts is being performed, following the previously applied method (Kornpointner et al. 2021).

Results

Aerial by-product parts confirmed to be much richer than roots and shives as reported in the table below.

Sample	Technique	Yield %
Shives (UPO)	UAE	2,64
Shives (WLH)	UAE	1,93
Roots (UPO)	UAE	1,28 ± 0,15
Roots (UPO)	SFE	0,39
Aerial parts (WLH)	UAE	7,96 ± 0,17

Table 1: Yield comparison of previous extractions on different C. sativa samples.

As activity to optimize the extraction processes, a design of experiment plan has been performed as reported in the table below.

The DoE is summarized in the following table:

Exp Name	Run Order	Incl/Excl	Temp	Time	Flow	Co-solvent	Pressure	Extraction Yield (%)
N1	19	Incl	40	1	2	0	350	3,88
N2	7	Incl	70	1	2	0	150	1,62
N3	3	Incl	40	2	2	0	150	5,39
N4	5	Incl	70	2	2	0	350	6,11
N5	18	Incl	40	1	4	0	150	6,31
N6	11	Incl	70	1	4	0	350	6,77
N7	13	Incl	40	2	4	0	350	4,13
N8	6	Incl	70	2	4	0	150	3,09
N9	20	Incl	40	1	2	5	150	9,22
N10	10	Incl	70	1	2	5	350	6,63
N11	8	Incl	40	2	2	5	350	7,34
N12	17	Incl	70	2	2	5	150	10,48
N13	4	Incl	40	1	4	5	350	6,58
N14	2	Incl	70	1	4	5	150	8,96
N15	15	Incl	40	2	4	5	150	7,94
N16	14	Incl	70	2	4	5	350	4,70
N17	9	Incl	40	1	2	0	350	5,83
N18	1	Incl	70	1	2	0	150	2,50
N19	21	Incl	55	1,5	3	2,5	250	6,74
N20	16	Incl	55	1,5	3	2,5	250	6,78
N21	12	Incl	55	1,5	3	2,5	250	6,58

Table 2: Design of experiment followed for SFEs on C. sativa aerial parts, extrapolated thanks to the software MODDE, and yields.

SFEs gave variable yields between 1,62-10,48%. Highest yields were obtained with experiment N12, followed by N9 and N14. All of these used a 5% EtOH as a co-solvent and a pressure of 150 bar. N9 was extracted at 40°C for 1 hour, with a flow of 2 L/min. N12 was extracted at 70°C for 2 hours, with a flow of 2 L/min. N14 was extracted at 70°C for 1 hour, with a flow of 4 L/min.
Extracts will also be evaluated in terms of triterpenoids concentration, mainly phytosterols and cannabinoid.

A series of GC-MS analysis is currently in progress and results still must be elaborated. So far, there seems to be an abundance of CBD in aerial parts extracts.

Consistent with the above-mentioned extraction activities, further extractions of the residual bioactive compounds (cannabinoids, polyphenols and terpenes) have been performed from hemp biomasses obtained after industrial extraction of cannabidiol (CBD) with ethanol (EtOH) (Materia Medica Processing, Siena), from now on called Biom_EtOH, and supercritical fluid extraction (Exenia group srl, Torino), from now on called Biom_SFE. The same procedure was applied to hemp plant waste, including roots (RO15B, RO15C, RO15D, RO15E, RO16A), hurds (canapulo) (HU17A, HU18A, HU18D, HU19B) and aerial parts.

Extraction of compounds (cannabinoids and polyphenols)

A portion of 0.25 g of sample was added with 10 mL of EtOH as the extraction solvent and the resulting mixture was extracted at room temperature for 15 min, under magnetic stirring [Pellati et al., 2018]. After the filtration of the solution by a paper filter, the residue was extracted twice more with the same procedure with 10 and 5 mL of extraction solvent, respectively. The filtrates of the three extractions were then combined and brought to 25 mL with the solvent in a volumetric flask. Before the injection in the HPLC system, the extracts were filtered by using a 0.45 µm PTFE filter into a HPLC vial and a 3 µL aliquot was injected for analysis. Regarding GC-MS analysis, a 1.5 µL aliquot was injected in the GC system.

References

Kornpointner C, Sainz Martinez A, Marinovic S, Haselmair-Gosch C, Jamnik P, Schröder K, Löfke C, Halbwirth H. 2021. Chemical composition and antioxidant potential of Cannabis sativa L. roots. Industrial Crops and Products. 165:113422. https://doi.org/10.1016/j.indcrop.2021.113422

Pellati F, Brighenti V, Sperlea J, Marchetti L, Bertelli D, Benvenuti S. New Methods for the Comprehensive Analysis of Bioactive Compounds in *Cannabis sativa* L. (hemp). Molecules 2018;23:2639. https://doi.org/10.3390/molecules23102639.

Gas chromatographic analyses

Qualitative analysis of the chemical constituents of the extracts was performed by GC-MS, with a method optimized for the separation of triterpenoids, as reported by Kornpointner et al. (2021 - doi:10.1016/j.indcrop.2021.113422). A Varian VF-5 column (30 m x 0.25 mm x 0.25 µm) was used. The flow rate was set to 1.3 mL/min, with helium as carrier gas. The temperature program was as follows: 1 min at 100 °C as initial conditions, a ramp of 5 °C/min up to 325 °C and then 15 min at 350 °C. The GC was coupled with an EI(+) mass detector (E = 70 eV) and the total ion current was measured between 35 and 750 m/z, after a solvent signal delay of 6.5 min. The setpoint temperatures of the injector, trap and transfer line were 250, 150 and 290 °C. Both extracts were analyzed by this method and gave similar results, but the n-hexane extract had

some impurities; therefore, the EtOH extract was mainly considered. The ethanol extract was solubilized in 1 mL of EtOH, filtered with a 0.22 µm PTFE filter and then concentrated to 200 µL before injection.

Saponification of C. sativa seed oil

Characterization analyses were performed on hemp oil obtained from pressed seeds. The extraction material and oil were provided free of charge by companies in the hemp production chain.

Hemp Seed Oil (HSO) was centrifuged at 18,000 rpm for 5 minutes, to separate the oil from the seed residue. The unsaponifiable fraction of HSO was isolated following conventional procedures, according to Montserrat-de la Paz et al. (2014 - doi:10.1021/jf404278q). A sample of 2.5 g of HSO and 20 mL of 2N KCl in EtOH 80% vol were placed in a flask placed on a hot plate at 80°C for 1 hour. After cooling, 50 mL of H2O was added and the solution was then transferred to a separating funnel and extracted 3 times with 40 mL of Et2O each. The ether extracts were collected in another separating funnel and washed 3 times with 60 mL of H2O each, until the last aqueous fraction was neutral. The washed ether fraction was then treated with anhydrous Na2SO4, filtered and dried with a rotavapor. The unsaponifiable fraction was injected into GC-MS according to the method previously described [Kornpointner et al., 2021 - doi:10.1016/j.indcrop.2021.113422].

Results and discussion

Gas chromatographic analyses

The most abundant compounds in root extracts are β-sitosterol (peak 11) and friedelin (peak 15), followed by campesterol, stigmasterol and stigmast-4-en-3-one. Peak 14 was identified as α-friedelanol, while the presence of its β-epimer, epifriedelanol, is often mentioned in the literature; this will be the subject of further analysis. A low amount of squalene was identified (peak 8), relevant for its applications as an adjuvant in vaccine formulations. β-amyrin, mentioned in the literature, could correspond to peak 12, which is still uncharacterized. A couple of phthalic structures were identified (peaks 5 and 7), but they are probably contaminations due to contact with plastic material, such as syringes or tips. The yield after SFE on C. sativa roots was 0.39%. GC-MS analysis of SFE root extract (without EtOH) is currently the subject of further investigation.

Saponification and analyses of HSO (Hemp seed Oil)

The yield of the unsaponifiable fraction of HSO was 2.32%. A preliminary analysis of the GC-MS spectra, performed by comparing the fragmentation patterns with an in-house mass spectrometric library and the NIST database, allowed to identify 10 peaks with an area greater than 1%, corresponding to 95.05% of the total area (24 peaks greater than 0.1%). The most abundant compound is β-sitosterol (peak 20, 64.04%), followed by campesterol (peak 18). Several other sterols are detected, not all identified yet, all exiting after 40 minutes. Another relevant compound is phytol (peak 10), followed

	by squalene (peak 15) and γ-tocopherol (peak 17). The detection of BHT (peak 2) is worth investigating, as it was unexpected in this oil sample (perhaps due to contamination during the sample preparation phase by the supplier company). *SFE DoE (Superctitival Fluid Extractions – Design of Experiment)* Design of Experiment (DoE) analyses to optimize SFE results have been started and are currently ongoing. In general, root results were consistent with literature data [Almeida Neto et al., 2023 - doi:10.1002/cbdv.202201039; Kornpointner et al., 2021 - doi:10.1016/j.indcrop.2021.113422; Jin et al., 2020 - doi:10.1038/s41598-020-60172-6; Elhendawy et al., 2018 - doi:10.1159/000495582; Slatkin et al., 1971 - doi:10.1002/jps.2600601232], showing a prevalence of triterpenoids. Further characterization work is ongoing and an SFE with scCO2 doped with 10 vol% EtOH will be performed as soon as the instrument is operational (currently awaiting repair), to more fully compare the results of SFE and UAE with organic solvents. The results on the unsaponifiable fraction of HSO are also consistent with literature data [Montserrat-de la Paz et al., 2014 - doi:10.1021/jf404278q], showing a prevalence of sterols and tocopherol. Further purification on the unsaponifiable fraction will be performed to further highlight these results. *Chemical characterization of extracts obtained from industrial hemp biomasses* Another related activity carried out in parallel by UR UNIMORE focused on the extraction and characterization of residual bioactive compounds (cannabinoids, polyphenols and terpenes) from hemp biomasses obtained after industrial extraction of cannabidiol (CBD) with ethanol (EtOH) (Materia Medica Processing, Siena), hereafter referred to as Biom_EtOH, and extraction with supercritical fluid (Exenia group srl, Turin), hereafter referred to as Biom_SFE. The extracts were treated as in [Pellati et al., Molecules 2018;23:2639 doi.org/10.3390/molecules23102639] and subsequently analyzed. In particular, a portion of 0.25 g of industrial waste biomass was spiked with 10 mL of EtOH as extraction solvent and the resulting mixture was extracted at room temperature for 15 min, under magnetic stirring. Before injection into the HPLC system, the extracts were filtered using a 0.45 µm PTFE filter into an HPLC vial and a 3 µL aliquot was injected for analysis. For GC-MS analysis, a 1.5 µL aliquot was injected into the GC system. After filtration of the solution, the residue was extracted two more times with the same procedure with 10 and 5 mL of extraction solvent, respectively. The filtrates of the three extractions were then combined and made up to 25 mL with the solvent in a flask. Before injection into HPLC, the extracts were filtered using a 0.45 µm PTFE filter into an HPLC vial and a 3 µL aliquot was injected for analysis. For GC-MS analysis, a 1.5 µL aliquot was injected into the GC system. Detailed results are reported in the section dedicated to the activities of the individual RUs.
***2 -** Application of the Design of Experiments (DoE) to optimise the extraction conditions*	Period (two months): **2 – 8** (31 gen. 24 – 30 mar. 25)
Research Units Involved	**Activities and Results**
UNIPV	Extractions of Cannabis roots and shives with supercritical CO2 (also mixed with DMSO) during these early stages of the project do not reveal the presence of a significant amount of cannabinoids. CBD cannabis by-products were

	further subjected to CO_2 supercritical extraction, but no advantages over less expensive techniques (i.e., extraction with ethanol) were found.

Milestone: 2 - Exploring hemp biodiversity to isolate novel high-value bioactive compounds

1 - *Fractionation strategies of lipophilic compounds* | Period (two months): **2 – 6** (31 gen. 24 – 30 nov. 24)

Research Units Involved	Activities and Results
UNIUPO	The strategies for the isolation of lipophilic fraction is based on a physical separation of the unsaponifiable fraction from the saponifiable (saturated triglycerides and waxes) with a cold methanolic filtration. The unsaturated triglycerides are collected with a vacuum filtration on RP as a stationary phase. The considered lipophilic waste material were roots, the hemp shives and the by-product from hemp oil. *Extractions and Isolation of roots from Cannabis sativa L.* Roots from C. sativa (site of collection Cuccaro – Monferrato) 331 g, cleaned from residual interference and powdered, were extracted with acetone affording an acetonic extract that was subsequently defatted and de-waxed by vacuum filtration with Celite and cold MeOH obtaining FE1013A, 837 mg of residual fraction after evaporation of solvent with a rotary evaporator and FE1013B, 385 mg as a residue on the paper filter. The residual vegetable material was extracted a second time in the same vertical percolator with MeOH following the same protocol of vacuum filtration to obtain a defatted fraction FE1013C, 1.4 g and FE1013D, 33 mg as a residue on the paper filter and identified by 1H NMR as unsaturated triglycerides. *Further fractions purification* Fraction FE1013A, consisting of 837 mg was further purified by flash chromatography with Isolera One on RP18 silica gel (60 g, solvent A: MeOH 0.03% formic acid, solvent B: H2O 0.03% formic acid gradient from 80:20 to 95:5) to afford the following fractions:

Fractions	UV λ 254 nm	Quantity mg	Comments
FE1015A	-	83.8	Unsaturated fatty acid

FE1015B	-	44.5	Fatty acid+aromatic compound
FE1015C	+/-	37.9	Interesting mixture
FE1015D	+/-	60.4	Fatty acids+interesting compound
FE1015E	+/-	81.8	β-sitosterol+ another compound similar to FE1016A

Fraction FE1013C, consisting of 1.41 g was further purified by flash chromatography with Isolera One on RP18 silica gel (60 g, solvent A: MeOH 0.03% formic acid, solvent B: H2O 0.03% formic acid gradient from 80:20 to 95:5) to afford the following fractions:

Fractions	**UV λ 254 nm**	**Quantity mg**	**Comments**
FE1016A	+/-	39.2	β-sitosterol + compound UV visible
FE1016B	-	15.1	β-sitosterol

Extractions and defatting of "hemp shives"

Hemp shives (the internal woody part of the plant stem that remains when the bark fiber is removed) from C. sativa (provided by Assocanapa - Carmagnola) 500 g, powdered, was extracted with acetone affording an acetonic extract. This was subsequently defatted and dewaxed by vacuum-filtration with cold MeOH and Celite to obtain FE1014A, 4.66 g of residual fraction after evaporation of solvent with a rotary evaporator and FE1014B, 478 mg as a residue on the paper filter and identified by 1H NMR as β-sitosterol.

The residual vegetable material was extracted a second time in the same vertical percolator with MeOH following the same protocol to obtain a defatted fraction FE1014C, 8.98 g without any solid residue on paper filter.

Further fractions purification

A portion of fraction FE1014A, consisting of 1.69 g was further purified by flash chromatography with Isolera One on RP C-18 (60 g, solvent A: MeOH 0.03% formic acid, solvent B: H2O 0.03% formic acid gradient from 80:20 to 95:5) to afford the following fractions:

Fractions	UV λ 254 nm	Quantity mg	Comments
FE1018A	+/-	24	Pre-cannabinoids
FE1018B	+/-	43	Pre-cannabinoids
FE1018C	+/-	296	Mainly composed by unsaturated fatty acids
FE1018D	+/-	213	Phenolics and unsaturated fatty acids

The fractions were sent to UNIMORE RU for further analysis.

Study of the oil by-products

140 g of oil by-product from seeds of Cannabis sativa L. were centrifuged and the liquid supernatant has been divided by decantation from the solid residue (S2 - 33.97 g) after washing with petroleum ether to remove all the oily residue obtaining an oily fraction of 102.5 g (O1). The solid residue has been extracted with acetone to obtain an acetonic extract (A1- 2.45 g).

O1 was subsequently defatted and dewaxed by vacuum-filtration with cold MeOH and Celite to obtain O2, 23.45 g of residual fraction after evaporation of solvent with a rotary evaporator and O3, 65.46 g as a residue on the paper filter and identified by 1H NMR as triglycerides of unsaturated fatty acids.

A portion of fraction O2 consisting in 5 g was further vacuum filtered with RP C-18 (30 g) with MeOH and acetone to afford the following fractions:

Fractions	UV λ 254 nm	Quantity mg	Comments
O1-a	-	2.49	Free unsaturated fatty acids

O1-b	-	1.17	Free unsaturated fatty acids with trace of other compounds

A portion of fraction A1 consisting in 1 g was further vacuum filtered with RP C-18 (30 g) with MeOH and acetone to afford the following fractions:

Fractions	UV ʎ 254 nm	Quantity mg	Comments
A2-a	-	377	Free unsaturated fatty acids
A2-b	-	5.7	Free unsaturated fatty acids with trace of other compounds

Fractions were sent to UNIMORE for further analysis.

2 - *Isolation of secondary metabolites from optimized extracts*	Period (two months): **3 – 8** (1 apr. 24 – 31 mar. 25)
Research Units Involved	**Activities and Results**
UNIUPO	A slight delay in obtaining results is due to the delay in obtaining leaves by-products by hemp companies (Whole Lotta Hemp, Parma, Italy and Canvasalus, Rovigo and Dimensione Canapa, Monferrato due to the weather and harvesting condition). Overcoming the availability challenge by synthetic methods to obtain rare cannabinoids: 1. General procedures to obtain CBDA esters with monoterpenoid alcohol: A solution of CBDA (100 mg, 0.279 mmol) in dry dichloromethane (5 mL) was treated with a catalytic quantity of p-toluenesulfonic acid (p-TSA) and N,N'-dicyclohexylcarbodiimide (DCC, 115.13 mg, 2 equiv/mmol) after the addition of the isoprenyl alcohol (1.2 eq/mmol). The reaction was followed by TLC on silica using a mobile phase composed of PE–EtOAc (80:20, v/v) by monitoring the disappearance of CBDA. After stirring for 1 h at room temperature, the solvent was evaporated under reduced pressure, and the residue dissolved in toluene and placed at 8 °C for 3 h to remove by filtration the dicyclohexylurea (DCU). After solvent evaporation at the rotary

	evaporator, the final residue was purified with HPLC on silica (PE–EtOAc gradient from 95:5 to 90:10 v/v) to afford the isopropyl cannabidiolates. This protocol led to the semisynthesis of: borneoil- , fenchil-, geranil-, neril-cannabidiolate. 2. General procedure to obtain minor derivatives of CBG-like cannabinoids: To a solution of each different synthons (1.5 molar equival) in DCE (5 mL/mmol isoprenyl alcohols) isoprenyl alcohols (1 molar equival) and acidic alumina (2g/mmol isoprenyl alcohol), previously dried at 200 °C for 6 h, were added (Fig. 4). The reactions were stirred at 80 °C followed by TLC (silica, petroleum ether-EtOAc 90:10) and finally filtered in a sintered funnel on a bed of Celite® and evaporated at reduced pressure. Each residue is purified by LPC on silica (5 g, petroleum ether-EtOAc gradient from 100:0 to 90:10) and then on HPLC (silica, petroleum ether-EOAc isocratic elution 90:10) to afford the cannabinoids: CBNR, sesqui-CBG, CBGB, CBNRB, CBNRV, CBNRP, CBGP.
3 - *Optimization of the isolation procedures and chemical characterization of the obtained compounds through chromatographic and spectroscopic techniques*	Period (two months): **4 – 11** (1 giu. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIMORE UNIPV	Different chromatographic methods were optimized for the chemical characterization of the main classes of bioactive compounds in hemp, including cannabinoids, polyphenols and terpenes. The detailed conditions for the HPLC and GC analysis are described below. *UHPLC-HRMS analysis of cannabinoids* Qualitative analysis of hemp extracts was performed by means of ultra high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). The analyses were performed on a Thermo Scientific (Massachusetts, United States) UHPLC Ultimate 3000 equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electro-spray ionization (HESI) source. An Ascentis Express C18 column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was used. A ternary (A/B/C) multistep gradient (solvent A: 0.1% HCOOH in H2O, solvent B: 0.1% HCOOH in ACN and solvent C: MeOH) was used. On the basis of a previous work [Berman et al., 2018 - https://doi.org/10.1038/s41598-018-32651-4], the multistep gradient program was established as follows: 0-2 min from 50 to 67% B which was kept for 4 min, 6-10 min from 67 to 90% B which was kept for 4 min, 14-15 min from 90% to 50% B, which was kept for 5 min for re-equilibration of the system prior to the next injection. Solvent C was kept constant at 5% throughout the run. A flow rate of 0.3 mL/min was used. The column temperature was 30 °C. The injection volume was 3 µL. *HPLC-UV analysis of cannabinoids*

The HPLC-UV analyses of the extracts were performed on an Agilent Technologies (Waldbronn, Germany) modular model 1260 Infinity II system, consisting of a quaternary pump, a manual injector and a UV variable wavelength detector. Chromatograms were recorded by using an Agilent OpenLab ChemStation (Rev. C.01.10). Regarding the chromatographic conditions, an Ascentis Express® C18 column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA) was chosen [Durante et al., 2022 - https://doi.org/10.1016/j.jpba.2022.115037]. The mobile phase used in this method was composed of 2 mM ammonium formate in H2O + 0.1% HCOOH (A) and 0.1% HCOOH in ACN (B). This was eluted at the following gradient: 0-20 min from 70% to 90% B, which was held for 5 min, with a subsequent post-running time of 10 min. The flow rate was set at 0.2 mL/min. The injection volume was 3 µL. Chromatograms were recorded at the wavelength of 210 and 220 nm for the detection of neutral cannabinoids and cannabinoic acids, respectively.

UHPLC-HRMS analysis of polyphenols

The qualitative analysis of polyphenols was performed by UHPLC-HRMS. The analyses were carried out on a Thermo Scientific (Massachusetts, United States) UHPLC Ultimate 3000 equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted column compartment and a Q-Exactive Orbitrap mass spectrometer with a heated electro-spray ionization (HESI) source. Separation of the analytes was achieved on an Ascentis Express C18 column (150 mm × 3.0 mm I.D., 2.7 µm, Supelco, Bellefonte, PA, USA), with a mobile phase composed of 0.1% HCOOH in both H2O (A) and ACN (B) [Caroli et al., 2023 - https://doi.org/10.1016/j.jpba.2023.115723]. The gradient elution was modified as follows: 0–20 min from 2% to 25% B, 20–30 min from 25% to 40% B, 30–40 min from 40% to 80% B which was kept for 5 minutes, 45–55 min from 80% to 90% B which was kept for 5 min. The post-running time was 10 min. The flowrate was 0.3 mL/min. The column temperature was set at 30 °C. The sample injection volume was 4 µL. MS acquisition was carried out with a heated electro-spray ionization source operated in both the positive and in the negative ion mode. As to the MS detector, the source parameters were set as follows: sheath gas (N2) 40, auxiliary gas (N2) 30, auxiliary gas temperature 290 °C, electrospray voltage 3.5 kV (+) and 3.2 kV (−). The analyses were acquired at a resolving power of 70.000 full width at half maximum (FWHM). The other mass analyzer parameters were set as follows: scan range m/z 100-1000, automatic grain control (AGC) target 1×106 ions in the Orbitrap analyzer, ion injection time 243 ms and isolation window for the filtration of the precursor ions m/z 3.0. The fragmentation of precursor ions was performed at 20, 30 and 50 as normalized collision energies (NCE).

HPLC-UV analysis of polyphenols

The quantitative analysis of polyphenols was performed by HPLC-UV [Caroli et al., 2023 - https://doi.org/10.1016/j.jpba.2023.115723]. Analyses were performed on a Shimadzu (Kyoto, Japan) Prominence UFLC XR System equipped with a vacuum degasser, a binary pump, a thermostatted autosampler, a thermostatted

column compartment and a Shimadzu SPD-10A VP HPLC System UV-VIS Detector. The HPLC column and the applied chromatographic conditions were the same as those reported for the UHPLC-HRMS system. UV/Vis spectra were set in the range 190–600 nm. Chromatograms were acquired at 210 and 342 nm.

GC-MS analysis of terpenes

The qualitative analysis of terpenes was carried out on a 7890 B GC System (Agilent Technologies, Germany), coupled with a 5977 B mass spectrometer (Agilent Technologies, Germany). Compounds were separated on a capillary column Agilent 19091S-433 (30 m x 0.25 mm I.D., 0.25 µm film thickness, Agilent Technologies). The oven temperature was initially set at 45 °C, then increased to 170 °C at a rate of 3 °C/min, this was then increased to 200 °C at a rate of 6 °C/min and finally, it was increased to the final temperature of 280 °C, at a rate of 13 °C/min, that was kept for 10 min. The injection volume was 1.5 µL with a 1:10 split ratio. Helium was used as the carrier gas, at a flow rate of 1.2 mL/min. The injector and the transfer line temperature were set at 250 °C. Electron ionization (EI) at 70 eV was used to perform MS detection, operating in the full-scan acquisition mode in the m/z range 50-600. This method was the result of the optimization of one previously used in our laboratory [Anceschi et al., 2022 - https://doi.org/10.1002/ptr.7357].

Standard solutions for HPLC-UV quantitative analysis of cannabinoids

Calibration curves of cannabidivarin (CBDV), cannabidibutol (CBDB), cannabidiolic acid (CBDA), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabidiol (CBD), cannabichromevarin (CBCV), cannabinol (CBN), Δ9-tetrahydrocannabinol (Δ9-THC), Δ8-tetrahydrocannabinol (Δ8-THC), cannabichromene (CBC) and Δ9-tetrahydrocannabinolic acid (Δ9-THCA), were prepared by diluting stock standard solutions (1 mg/mL) with the EtOH at the desired concentrations. An aliquot of 3 µL of each reference solution was used for the HPLC analysis and the injections were performed in duplicate for each concentration level. All the external standard calibration curves were constructed at six calibration levels by plotting the peak areas of the analytes vs. their concentration, as follows: 2.5–50 µg/mL for CBDV, CBDB, Δ9-THC and Δ8-THC; 2.5–100 µg/mL for CBDA, CBGA, CBG, CBD, CBN, and Δ9-THCA; 1.4–53.6 µg/mL for CBCV; 1.7–66.5 µg/mL for CBC. The correlation coefficient r2 was higher than 0.998-0.999.

Standard solutions for HPLC-UV quantitative analysis of polyphenols

Calibration curves of cannflavin A (CFL-A) and cannflavin B (CFL-B) were prepared by diluting stock standard solution (1 mg/mL) with EtOH at the desired concentrations. An aliquot of 4 µL of each reference solution was used for the HPLC analysis and the injections were performed in duplicate for each concentration level. All the external standard calibration curves were constructed at six calibration levels by plotting the peak areas of the analytes vs. their concentration at the concentration range of 2.5-100 µg/mL.

	Going in-depth of the results achieved and documented in the previous reporting, taking inspiration from two recently published works 1,2, CBD has been treated with a common Lewis acid (boron trifluoride diethyl etherate, BF3OEt2) at room temperature in different solvents to isolate secondary cannabinoids to be used as standards for the GC-MS analysis of cannabinoids-containing products. Alongside the reported employed solvents (namely acetonitrile and toluene) two other different solvents have been employed (MTBE and α,α,α–trifluorotoluene); CBD concentrations have been set at either 60 mg/mL or 10 mg/mL, and different BF3OEt2 equivalents have been employed (0.5, 1.2 or 5.1 equivalents). All reactions conditions have been investigated with reaction times from 30 minutes up to 48 hours at ambient temperature. The results obtained from the performed explorative reactions are investigated by a chemometric approach (PCA) using the previously listed conditions as variables, to evidence the differences between the products that forms in the different reaction media. Consequently, the data obtained from this preliminary screening will be used to set the variables to be used in a design of experiment (DoE), with the objective of optimizing the yield for all the different products observed. Preliminary results point out that the reactions outcome strongly depends on the chemical conditions, with solvent being the key parameter. Indeed, in MTBE with either 1.2 or 5.1 BF3OEt2 equivalents Δ9-tetrahydrocannabinol (Δ9-THC) was efficiently obtained, while in both toluene and α,α,α–trifluorotoluene a mixture of Δ8-tetrahydrocannabinol (Δ8-THC, the main product) and Δ4(8)-tetrahydrocannabinol (Δ4(8)-iso-THC) was observed. Finally, when performing the reaction in acetonitrile, different products were detected, with the relative abundance depending on both the concentration of the starting CBD and the amount of BF3OEt2 employed. The observable products include Δ8-THC, Δ8-iso-THC and Δ4(8)-iso-THC alongside with an adduct with solvent (Ritter reaction). The isolation and full characterization of the products by means of preparative experiments is currently ongoing (Marzullo et al, J. Nat. Prod. 2020, 83, 2894–2901; Capucciati et al, J. Nat. Prod. 2024, 87, 869–875). Compounds obtained were fully characterized for their spectroscopic behaviour and added to the GC-MS library (as intended in milestone 2.2 and 3.2).
4 - *Development of a general synthesis of pre-cannabinoid terpenyl esters and other derivatives*	Period (two months): **3 – 11** (1 apr. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIUPO	The presence of ortho-para di-oxygenation within the cannabinoid structure makes the carboxylate group chemistry idiosyncratic, rationalizing the failure of all common esterification procedures based on alcohol or acyl activation. General and strategic synthesis of the terpene ester of pre-cannabinoids has been achieved involving CBDA and CBGA (the most important non-psychotropic cannabinoids) with mono- and sesqui-terpenoids and carbodiimides in the presence of Bronsted acids. Other minor derivatives with different sizes in the aliphatic side chain (propyl, butyl, hexyl, heptyl) have been obtained from CBG in the presence of alumina.

Milestone: 3 - Chemical analysis of extracts and building a compound data library

1 - *Full chemical characterization of the extracts and pure compounds obtained*	Period (two months): **3 – 11** (1 apr. 24 – 30 set. 25)

Research Units Involved	Activities and Results
UNIMORE UNIPV	Using previously optimized HPLC and GC methods, it was possible to obtain a detailed and comprehensive chemical characterization of extracts from industrial hemp biomass and other hemp waste materials.

Chemical characterization of cannabinoids

UHPLC-HRMS analysis allowed for a full characterization of the residual cannabinoids present in the waste biomasses. Several cannabinoids were found in both hemp biomasses, even if they are considered as a production waste. However, even if they are qualitatively rich in cannabinoids, a quantitative analysis must be performed to fully characterize them. Quantitative analysis data, obtained by HPLC-UV, are shown in Table 1. Cannabidiol (CBD) was the most abundant compound in biomass from SFE, followed by its biosynthetic precursor cannabidiolic acid (CBDA).

Table 1. Amount of cannabinoids in hemp biomasses. Data expressed ad mg/g ± SD

Compound	Biom_EtOH	Biom_SFE
CBDV	< LOQ	0.1 [a]
CBDB	0.1 [a]	0.2 ± 0.1
CBDA	0.3 [a]	3.8 ± 0.1
CBGA	< LOQ	0.4 [a]
CBG	< LOQ	0.2 [a]
CBD	0.3 [a]	6.5 ± 0.3
CBCV	< LOQ	< LOQ
CBN	0.1 [a]	0.7 [a]
Δ^9-THC	< LOQ	0.6 [a]
Δ^8-THC	< LOQ	< LOQ
CBC	-	0.7 [a]
Δ^9-THCA	< LOQ	0.2 [a]

[a] SD < 0.05

Chemical characterization of polyphenols

UHPLC-HRMS analysis allowed us to fully characterize the residual polyphenols present in the waste biomasses. All the polyphenols were characterized thanks to the fragmentation pattern of the hemp polyphenols identified in a previous work. Biom_SFE resulted to be richer in polyphenols than Biom_EtOH. In addition to cannflavins, which are the typical isoprenoid flavones of hemp, hydroxycinnamic acid amides were found. In particular, in both waste biomasses N-trans-feruloyltyramine, CFL-B and CFL-A were identified.

HPLC-UV was applied to quantify CFL-B and CFL-A in the biomasses. Table 2 shows the obtained quantitative results. Cannflavins were found to be present in a very low amount, and CFL-A was the only one over the limit of quantification (LOQ) in Biom_SFE only.

Table 2. Amount of polyphenols in hemp biomasses. Data expressed ad mg/g ± SD

Compound	Biom_EtOH	Biom_SFE
DEM-B	< LOQ	< LOQ
CFL-B	< LOQ	< LOQ
DEM-A	< LOQ	< LOQ
CFL-A	< LOQ	0.1 [a]

[a] SD < 0.05

Chemical characterization of terpenes

GC-MS analysis allowed us to see that both waste biomasses were free of terpenes. This is however a substantially expected result, since for the extraction of CBD and subsequent purification, the plant material is decarboxylated under heating, with the consequent elimination of volatile compounds.

Chemical characterization of cannabinoids in hemp roots and hurds

Nine samples were analyzed in this work by the UHPLC-HRMS instrument, encompassing five from roots and four from hurds (canapulo) extracts both obtained as waste material from hemp, to assess their chemical profile. The compounds detected in roots extracts using the negative mode acquisition were cannabinolic acid (CBNA), cannabinodiolic acid (CBNDA), cannabichromenic acid (CBCA), cannabidiolic acid (CBDA), (-)-trans-Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA), cannabigerolic acid (CBGA), cannabielsoic acid (CBEA), cannabinovarinic

acid (CBNVA) and cannabidivarinic acid (CBDVA). CBNA, CBGA were identified in all root extracts while CBNDA, CBDA, CBNVA and CBDVA were detected in all samples with the only exception of RO16A. In addition, CBCA and Δ^9-THCA were found in RO15B while CBEA was not identified in sole RO15B. Indeed, the main compounds detected in hurd extracts at the same analysis condition were: CBNDA, CBDA, CBGA, CBEA and CBDVA as compounds identified in all samples. Furthermore, other compounds were detected, namely: CBNA and CBNVA in HU17A, HU18D and HU19B and CBCA in HU17A respectively. Plus, cannabidibutolic acid (CBDA-C4) was found in HU17A, HU18A and HU19B.

As regards the positive acquisition mode, the single compound that was identified in all roots extracts was cannabidiol (CBD) while cannabichromene (CBC) and (-)-Δ^9-trans-tetrahydrocannabinol (Δ^9-THC) were identified only in RO16A. For what concern hurds extracts, cannabinol (CBN), CBD and cannabidivarin (CBDV) were detected in all sample while cannabinodiol (CBND) in HU17A and HU18A and Δ9-THC in HU17A and HU18D. Furthermore, CBC was evaluated only in HU18D sample. However, the compounds detected were putatively identified according to exact mass and mass fragmentation compared with the standards reported in the literature [Berman et al., 2018; Caroli et al., 2023; Citti et al., 2018; Brighenti et al., 2024].

The quantitative analysis was carried out at HPLC-UV/Vis instrument. Experimental analysis performed, showed that the amount of each target compound investigated were below the limit of quantification (LOQ) value. Consequently, it was not possible to estimate their amount in the samples.

Chemical characterization of polyphenols in hemp roots and hurds

UHPLC-HRMS analysis allowed us to identify the polyphenols contained in root and hurd extracts, by matching the obtained fragmentation patterns and tR with the ones already described in literature [Caroli et al., 2023]. Coumaric acid, hydroxygallic acid, N-coumaroyltyramine, N-cis-ferulotyramine and N-trans-ferulotyramine were found in all root and hurd samples. Coumaric acid and hydroxygallic acid were identified in negative mode exclusively as phenolic acid better ionize in negative mode [Caroli et al., 2023], while N-coumaroyltyramine, N-cis-ferulotyramine and N-trans-ferulotyramine were identified in both positive and negative mode. However, dihydroferulic acid was identified in positive mode in all samples and in negative mode only in hurd extracts. Apigenine was evaluated in positive and negative acquisition in RO15D and RO16A, HU17A, HU18A, HU18D and HU19B. In addition, the analysis performed on hurd extracts indicated the presence of Diosmetin/chrysoeriol and cannflavines (CFL-A, CFL-B and CFL-C) in all samples both in negative and positive ionization. Finally, hydroxymatairesinol/nortrachelogenin was found in all hurd samples in positive ionization.

As previously described for cannabinoids in the same samples, also polyphenols occurred below the LOD or LOQ value.

Chemical characterization of cannabinoids in hemp aerial parts

The extract obtained from cannabis aerial parts was analyzed by a UHPLC-HRMS instrument in order to identify the phytocannabinoids profile; each analysis was performed in duplicate. According to the literature, neutral cannabinoids were analyzed in positive ion mode while cannabinoic acids in negative mode as neutral and acidic cannabinoids better ionize in positive and negative mode, respectively [Durante et al., 2022].

The main cannabinoids identified were the ones belonging to CBD-type subclasses, namely cannabidiol (CBD), cannabidivarin (CBDV), cannabidibutol (CBD-C4 or CBDB), cannabidihexol (CBDH) and cannabidiphorol (CBDP). The identification of all these compounds was performed by comparison of tR [Berman et al., 2018], fragmentation pattern of the respective cannabinoids (CBs) standards [Brighenti et al., 2024] with the only exception of CBDH and CBDP. Regarding CBDH and CBDP, they were tentatively identified by comparing their retention times (tR) [Berman et al., 2018] and exact masses with those reported in the literature [Linciano et al., 2020; Citti et al., 2019], since no fragmentation data were recorded under the parameters set in the mass analyzer, likely due to the very low amounts of CBDH and CBDP present in the analyzed matrix. In addition, other compounds were detected including cannabinol (CBN), cannabichromene (CBC), (-)-Δ^9-trans-tetrahydrocannabinol (Δ9-THC), cannabigerol (CBG), and Δ^9 -tetrahydrocannabivarin (Δ^9-THCV). CBG, Δ^9-THC, and CBC were identified by comparison with tR [Berman et al., 2018] and mass fragmentation patterns of CBs standards [Durante et al., 2022], while CBN was putatively identified based on tR, exact mass, and mass fragmentation data compared with the corresponding data reported in the literature [Berman et al., 2018; Durante et al., 2022]. Additionally, Δ9-THCV was tentatively identified by comparing its tR [Berman et al., 2018] and mass fragmentation data with those reported in the literature [Citti et al., 2019]. Conversely to neutral cannabinoids, acidic cannabinoids were analyzed in the negative ion mode [Durante et al., 2022] and identified by comparison of tR, exact mass and mass fragmentation data reported in the literature [Berman et al., 2018]. Many acids were identified including cannabinolic acid (CBNA), cannabinodiolic acid (CBNDA), cannabichromenic acid (CBCA), cannabidiolic acid (CBDA), (-)-Δ^9-trans-tetrahydrocannabinolic acid (Δ^9-THCA), cannabigerolic acid (CBGA), cannabielsoic acid (CBEA), cannabivarinic acid (CBNVA), cannabidivarinic acid (CBDVA), (-)-Δ9-tetrahydrocannabidivarinic acid (Δ^9-THCVA) and cannabidibutolic acid (CBDA-C4 or CBDBA). Table 3 shows the obtained quantitative results.

Table 3. Amounts of cannabinoids in hemp aerial parts. Data expressed as mg/g± SD

Compound	Extract
CBDV	0.1*
CBDB	7.3±0.8
CBDA	10.1±1.2
CBGA	0.7±0.1
CBG	0.2*
CBD	1.9±0.4
Δ^9-THC	0.2*
CBC	0.3*
Δ^9-THCA	0.6±0.1
CBCA	0.8*

SD<0.05

Characterization of polyphenols in hemp aerial parts

The extract obtained from cannabis aerial parts was analyzed by a UHPLC-HRMS instrument both in positive and negative ion mode in order to identify the polyphenols profile. The detected polyphenols were hydroxygallic acid, N-coumaroyltyramine, N-cis-feruloyltyramine, N-trans-feruloyltyramine, apigenin, diosmetin/chrysoeriol, hydroxymatairesinol/nortrachelogenin, CFL-A, CFL-B and CFL-C. DEM-B and DEM-A were also identified. No quantitative analysis was carried out for polyphenols since their signals were far below the low limit of quantification (LOQ).

The activities have been then developed performing a voltammetric method for the determination of CBD and THC in hemps and hemps extracts, discriminating from the two cannabinoids in order to identify psychotropic and non-psychotropic hemp. The methods would rely on the oxidation of the phenolic core of the target analytes in water/ethanol mixture (20:89), which gave rise to a voltammetric wave (in DPV) centred at +0.5 V vs Ag/AgCl/NaCl

3M. Several attempt were done to distinguish between the voltammetric wave of CBD and THC, also exploiting the possibility of making voltammetric measurements at platinum or gold microelectrodes, at which due to radial diffusion the kinetic aspect of the electron transfer would be lesser hampered. However, no positive results were obtained. As fouling effects were also observed at classical Pt or Au electrode, which impair the reproducibility and the results obtained, macroscopic glassy carbon and palladium electrodes were used as well, accomplished by a careful choice of the supporting eletctrolyte/solvent. Among them, acetonitrile, DMSO and DMF were tested, with lithium perchlorate or tetrabutylammonium salts (perchlorate and hexafulorophospate) as supporting electrolytes, but no better results were obtained. In particular, fouling effects precluded the possibility of developing an analytical method, as the voltammetric signal does not increase proportionally with the amount of analyte present in the solution and poor linearity is observed.

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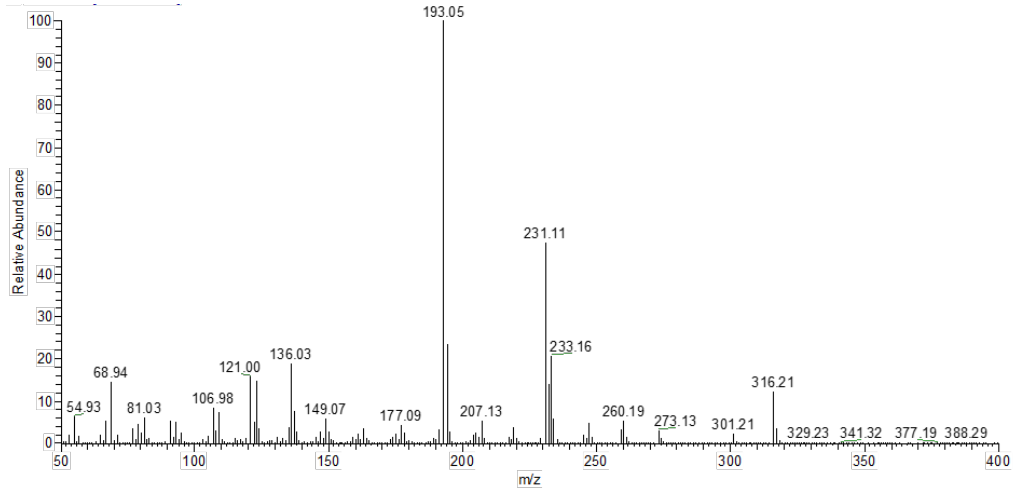
2 - *Building a library with all chromatographic and mass spectrometry data of isolated compounds*	Period (two months): **2 - 11** (30 gen. 24 - 30 set. 25)

Research Units Involved	**Activities and Results**
UNIMORE UNIPV	In relation to the activities previously described, characterization analyses are also underway by the other RUs. At the moment the compounds have been characterized for their spectral and chromatographic behavior as reported below. The in-depth and further definition activities are underway.  Figure 1. MS fragmentation pattern of compound 2.

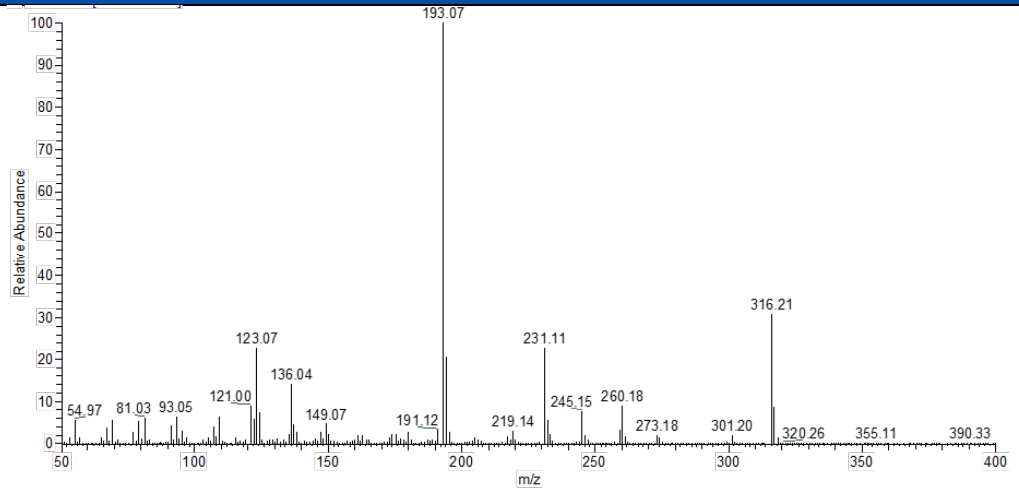


Figure 2. MS fragmentation pattern of compound 3.

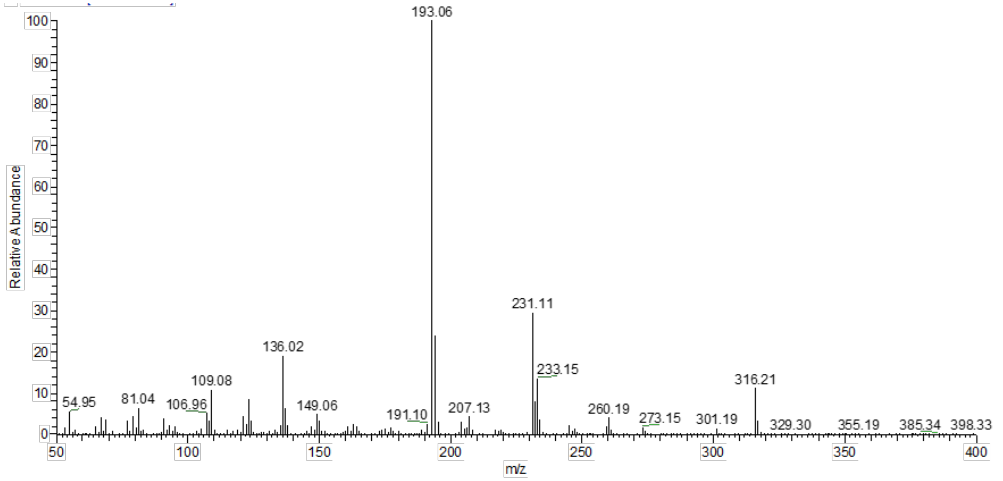


Figure 3. MS fragmentation pattern of compound 4.

Analyses are underway to define the structure of the molecules corresponding to the fragmentation patterns.
As already reported in the previous reporting and re-proposed for greater clarity in this context, the new compounds isolated during the activities described in milestone 3.3 have been characterized for their spectral and chromatographic behavior. In detail, several derivatives of cannabigeol have been identified and characterized (described in detail in 3.2).

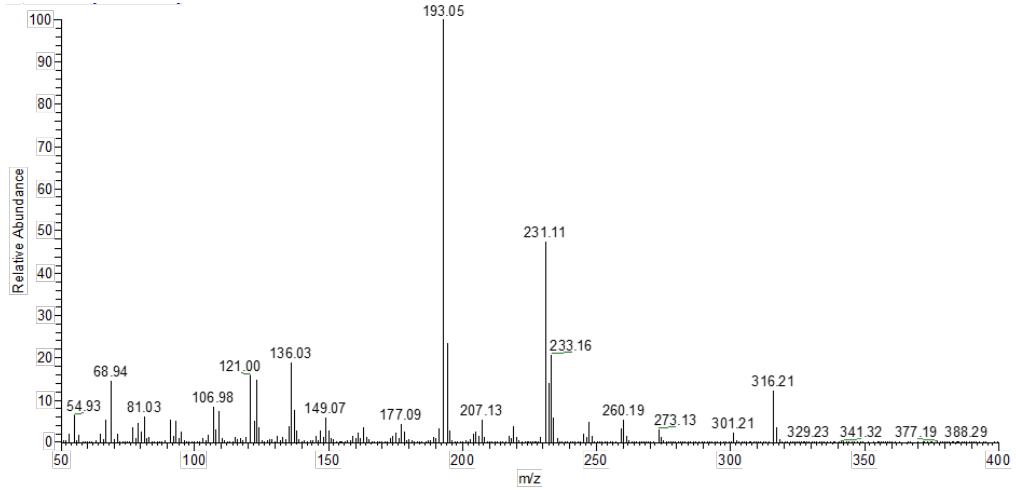


Figure 1. MS fragmentation pattern of compound 2.

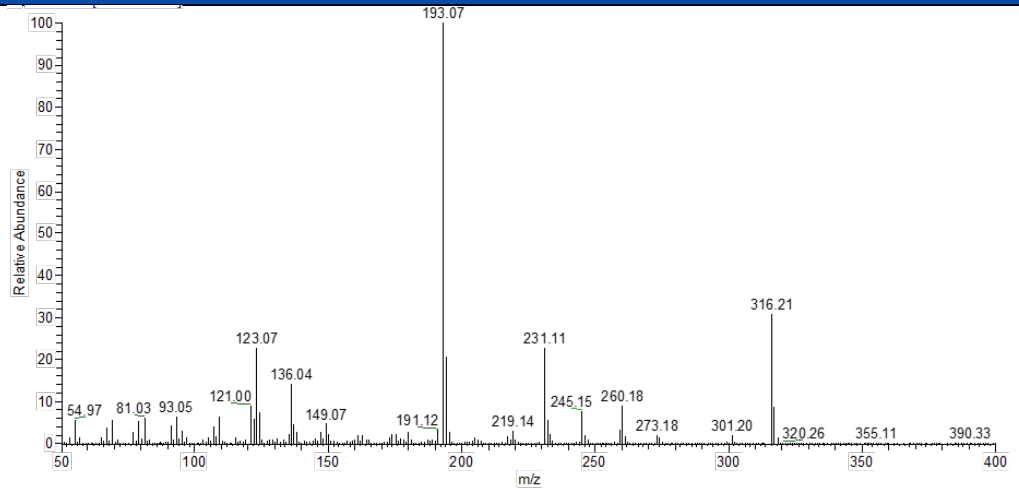


Figure 2. MS fragmentation pattern of compound 3.

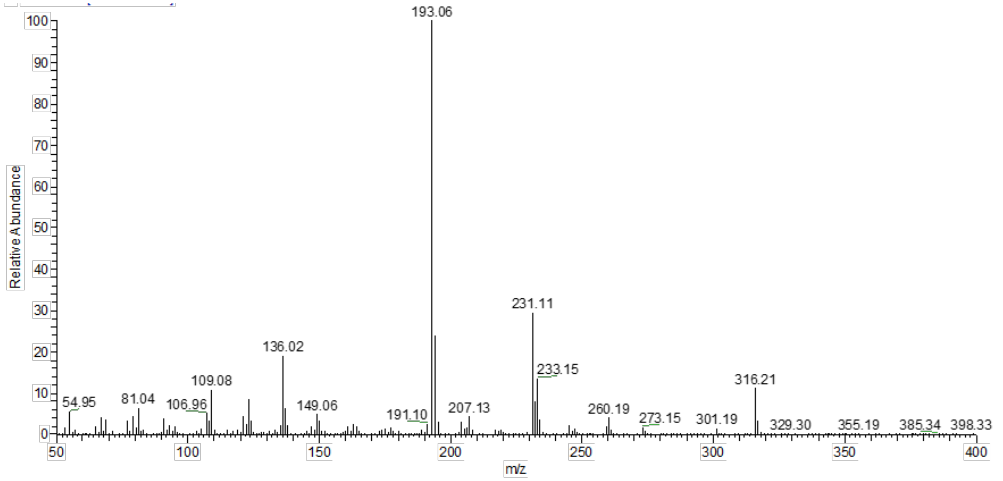
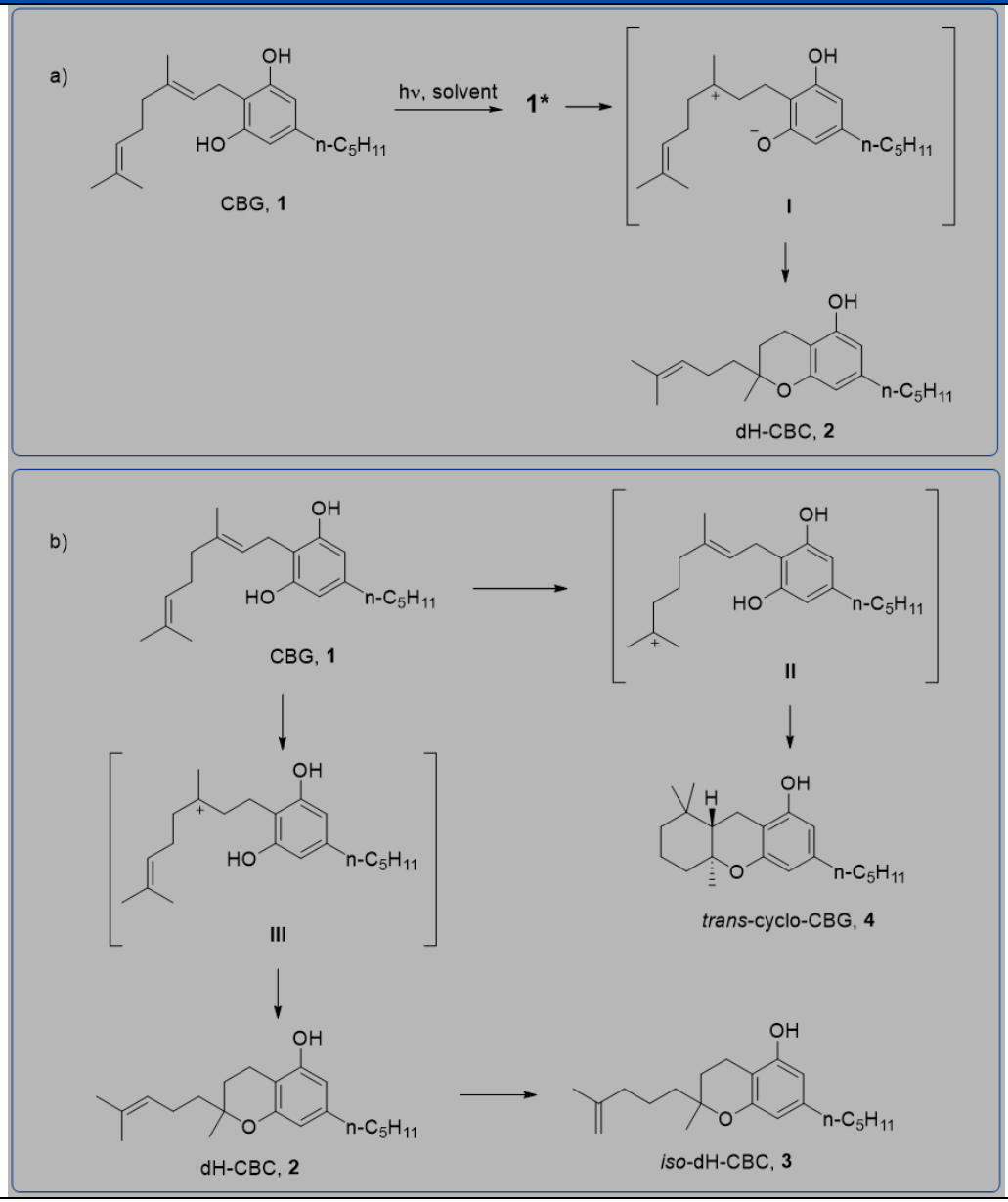


Figure 3. MS fragmentation pattern of compound 4.

	The formulas corresponding to the numbers given in the captions of the figures above are given below (they also refer to Figure 1 in Milestone 3.3).


a)
OH
HO
n-C5H11
CBG, 1
hν, solvent
1*
OH
O
n-C5H11
I
OH
O
n-C5H11
dH-CBC, 2
b)
OH
HO
n-C5H11
CBG, 1
OH
HO
n-C5H11
II
OH
HO
n-C5H11
III
H
OH
O
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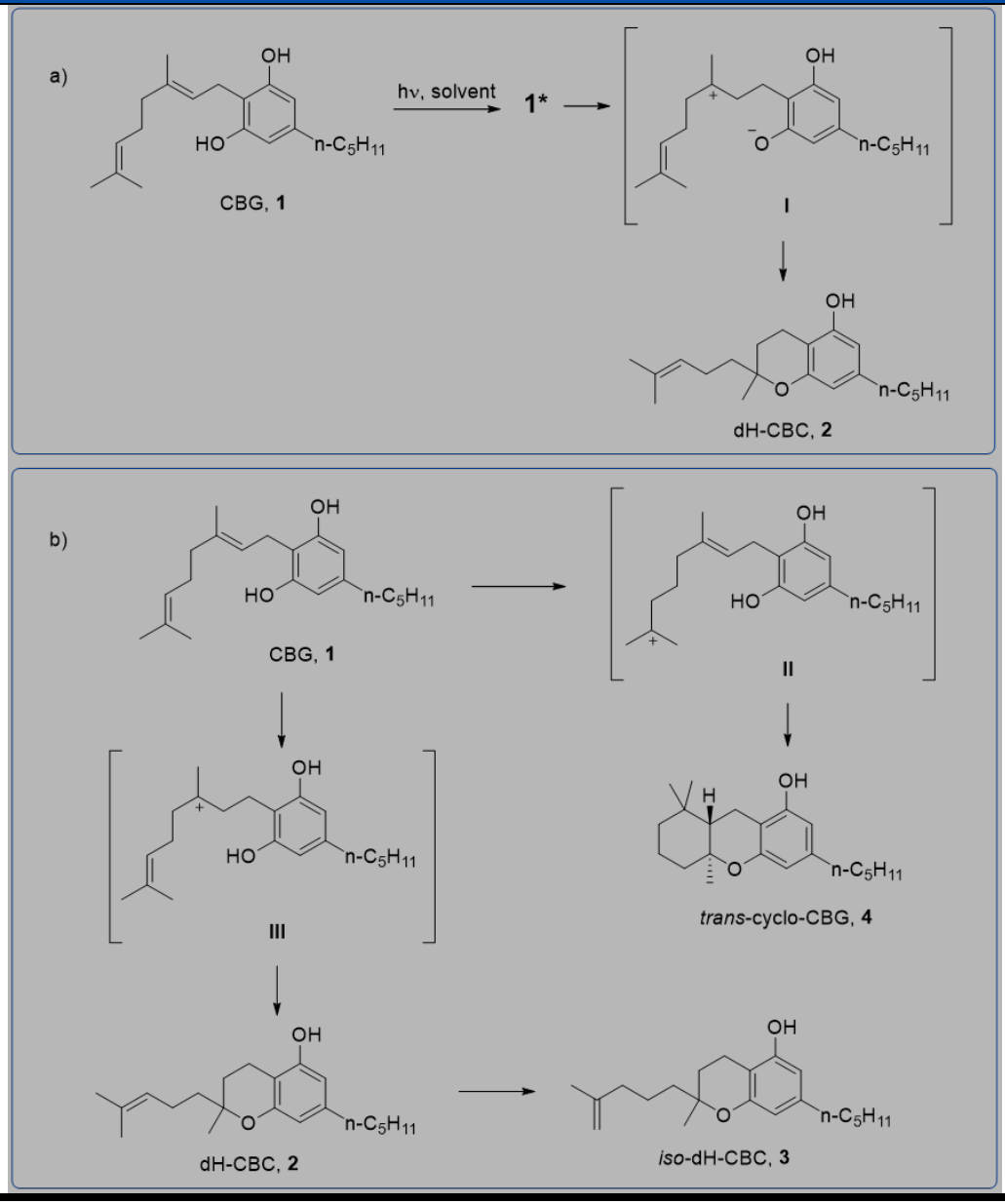
trans-cyclo-CBG, 4
OH
O
n-C5H11
dH-CBC, 2
OH
O
n-C5H11
iso-dH-CBC, 3

3 - *Characterization of the photochemical and thermal stability of the isolated compounds*	Period (two months): **2 - 11** (30 gen. 24 - 30 set. 25)
Research Units Involved	**Activities and Results**
UNIPV	To investigate the photochemical stability of the active principles in the plant matrix and to shed light on the anecdotal reports of intoxication from the numerous attempts to convert CBD to THC, and given how little is known about the appropriate storage conditions and degradation patterns of Cannabis, we investigated the degradation rate of several cannabinoids in their native plant matrix, also with the aid of a DoE approach. We conducted a systematic characterization of the photochemical behavior of Δ9-THC and CBD in three different cannabis chemotypes: chemotype I (c-I, Δ9-THC 2.50% w/w), II (c-II, CBD:Δ9-THC 5.82% w/w:3.19% w/w) and III (c-III, CBD 3.02% w/w), by an extraction procedure combined with GC-MS analysis. Taking advantage of the interconversion of cannabinoids among themselves and the possible photochemical conversion of the main ones into less known, but still active, derivatives, we compared the photochemical degradation products of CBD and Δ9-THC with our large GC-MS library composed of 22 cannabinoids (including THD and HHC isomers). While light exposure leads to a substantial degradation of all cannabinoids without favoring the formation of any kind of cannabinoid artefacts, the atmosphere used for storage seems to be the key factor when it comes to cannabinoid integrity. Further analyses are ongoing to better define aspects of stability. The presence of oxygen dramatically increases degradation rates due to the oxidation reactions and the formation of non-cannabinoid oxidation products, critically reducing shelf-life. CBD shows a better resistance to light exposure, followed by CBN; out of the three cannabinoids considered in this work, Δ9-THC seems to be the most light-sensitive cannabinoid: proof can be found mainly in the change of CBD:Δ9-THC:CBN ratio during c-II irradiation. The influence of the different parameters (light intensity, exposure time, extraction time with acetone) was tentatively investigated with a 23 DoE approach, that allows to evaluate not only the influence of any single variable but also their mutual interaction in the degradation/extraction process. Moreover, the light driven and acid-catalyzed cyclization of cannabigerol (CBG) in organic solvents has been investigated in detail. The obtained results pointed out the presence of different reaction paths in the photochemical and the acid-catalyzed conditions, with a remarkable selectivity toward the formation of a 2,2-substituted-benzochromane derivative in the first case.Summing up, the photochemically induced acidity of the phenol moieties in CBG plays a key role in its selective conversion to chromane (CBCa), and the efficiency observed for the process depends on the nature of the examined solvent. On contrast, when treated with a strong acid, two competitive pathways can be observed, to form 2 and trans-cyclo-cannabigerol (trans-cyclo CGG, along with minor amount of

	iso-chromane (iso-CBCa). In this case, the reaction leads to a complete disappearance of the substrate only when toluene is employed as the reaction medium The isolated compounds were characterized for their retention index and fragmentation patterns, and added to the GC MS cannabinoids library.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HO
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CBG, 1
hν, solvent
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O
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I
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O
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dH-CBC, 2
b)
OH
HO
n-C5H11
CBG, 1
OH
HO
n-C5H11
II
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n-C5H11
dH-CBC, 2
OH
O
n-C5H11
iso-dH-CBC, 3

Scheme 1. Reactivity of CBG observed in the present investigation. Scheme a) photochemical reactivity; scheme b) thermal reactivity.

Solvent	**(nm), ε ($M^{-1}cm^{-1}$)**	**(nm)**	[a]
EtOH	210, 29213 274, 752	302	0.038
MeOH	210, 28648 274, 668	301	0.038
MeCN	207, 44571 272, 918	300	0.057
Toluene	-	-	0.035

Table 1. Photophysics of CBG in three different solvents. a4-Chloroanisole has been used as a reference.

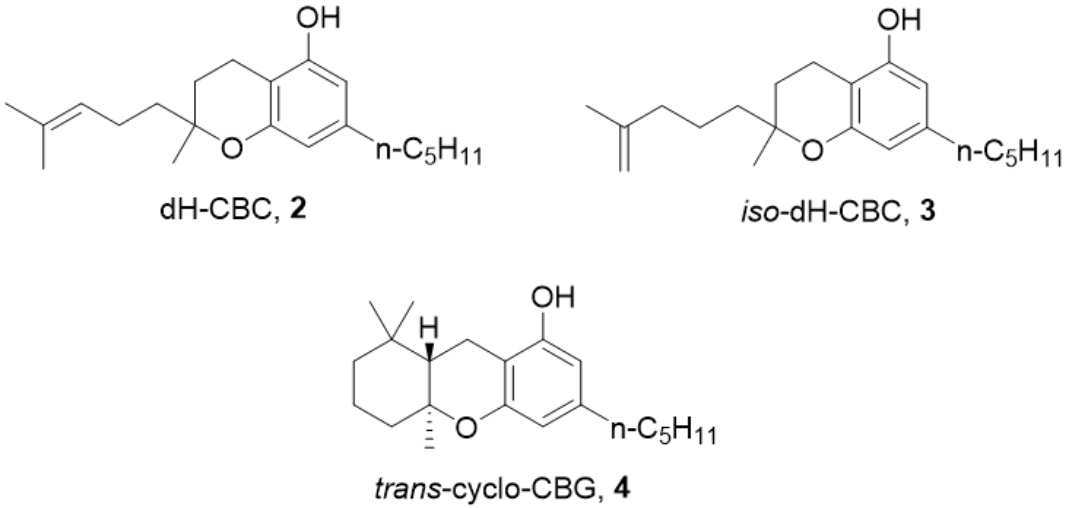

Figure 1 Structures of the product which can be obtained by CBG irradiation or acid treatment.

Milestone: 4 - Evaluation of the bioactivity of hemp by-products extracts and isolated compounds

1 - *Determination of the antioxidant activity of extracts and isolated compounds*	Period (two months): **4 – 11** (1 giu. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIMORE UNIFE	*Antioxidant activity* Antioxidant activity of two synthetized cannabinoids (Milestone 2.3-2.4) named A and B were performed through DPPH and ABTS assays. *Materials and methods* DPPH assay The assay was performed on purified molecules temporarily named as "A" and "B" (Milestone 2.3-2.4), following the procedure ideated by Brand-Williams et al. in 1995 [doi:10.1016/S0023-6438(95)80008-5], with further optimizing

modifications [Guerrini et al., 2023 - doi:10.3390/antibiotics12010177]. DPPH (2.2-Diphenyl-l-pict3,1-hydrazyl) is a stable free radical that has a deep purple color and an absorption peak at 515 nm, both of which disappear upon reduction by an antiradical compound. The decolorization is therefore an index of the antioxidant activity of a sample. For these analyses, a solution of DPPH 0.208 mM in EtOH was prepared, along with Trolox, a synthetic antioxidant used as positive control, at 0,04 mg/mL in EtOH. Sample A (10,4 mg) was solubilized in 3 mL EtOH and then diluted 1:3 (final concentration 1,16 mg/mL), while sample B (15,0 mg) was solubilized in 4 mL EtOH and then diluted 1:4 (final concentration 0,94 mg/mL). Reduction of absorption was measured with a microplate reader at 515 nm and IC_{50} was calculated, i.e. the sample concentration needed to reduce half of the radical DPPH.

ABTS assay

The assay was performed following the method first proposed by Miller et al. in 1993 [doi:10.1042/cs0840407], with further optimizing modifications [Guerrini et al., 2023 - doi:10.3390/antibiotics12010177]. Similarly to DPPH, free cationic radical $ABTS^{\cdot}+$ (has a deep blue/green color and an absorption peak at 734 nm. Upon reaction with an antioxidant sample, the decolorization and decreasing of absorption at 734 nm can be measured in order to calculate the IC_{50}, i.e. the sample concentration needed to reduce half of the radical ABTS.

For this analysis, first was prepared an ABTS solution (38,5 mg in 10 mL H2O), then 500 µL of $K_2S_2O_8$ (69,5 mg in 5 mL H_2O) were added as an oxidising agent; the solution was allowed to react overnight, to oxidize ABTS to $ABTS^{\cdot}+$. As positive control, a solution of Trolox 0,4 mg/mL EtOH was prepared. Sample A (10,4 mg) was solubilized in 3 mL EtOH and then diluted 1:3 (final concentration 1,16 mg/mL), while sample B (15,0 mg) was solubilized in 4 mL EtOH and then diluted 1:3 (final concentration 1,25 mg/mL). Reduction of absorption was measured with a microplate reader at 515 nm and IC_{50} was calculated.

Results and discussion

The results are reported below (Table 1).

Sample	DPPH	ABTS
	IC_{50} µg/mL	
A	422,50±13,17	3,31±0,16
B	368,93±24,80	4,86±0,21
Trolox	7,10±0,12	2,59±0,20

	Table 1: Antioxidant activity, measured with DPPH and ABTS assays and expressed in IC_{50}, of samples A and B. With DPPH, both samples showed an IC_{50} around 400 µg/mL, which indicates a weak antioxidant activity when compared to Trolox: both A and B, to reduce half of the radical DPPH, require a concentration more than 50 times higher than Trolox. In other words, they have less than 2% of Trolox's activity. On the other hand, both samples showed a higher ability to reduce free radical ABTS: their IC_{50} is less than twice higher than Trolox. In particular, A has 78,25% of Trolox's activity and B has 53,29%. This is probably given by the different redox mechanisms involved in the two assays, in addition to the fact that $DPPH^{\cdot}$ is more stable than $ABTS^{\cdot}+$.
2 - *Determination of the neuroprotective activity of extracts and isolated compounds*	Period (two months): **5 – 11** (1 ago. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIMORE	Neuroprotective activities will be performed on the pure compounds that are currently in preparation. The bioactivities test will be performed in the following period on the extracts and pure compounds collected by the activities still in progress.
3 - *Determination of the antiproliferative activity of extracts and isolated compounds*	Period (two months): **5 – 11** (1 ago. 24 –30 set. 25)
Research Units Involved	**Activities and Results**
UNIMORE	Antiproliferative activities will be performed on the pure compounds that are currently in preparation. The bioactivities test will be performed in the following period on the extracts and pure compounds collected by the activities still in progress.
4 - *Evaluation of the antimicrobial activity of extracts and isolated compounds against human and plant pathogens*	Period (two months): **5 – 11** (1 ago. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIFE	Antimicrobial activities of the extracts have been preliminarily performed on *Staphylococcus aureus* taken as a sentinel microorganism for the more targeted development of further evaluations on a panel of other microorganisms. **Antimicrobial activities performed (Materials and methods)** Antimicrobial activity of different extracts was tested so far against a Gram + bacteria, *Staphylococcus aureus*. It was evaluated through determination of the minimal inhibitory concentration (MIC) following CLSI's standard method of dilutions in microplate (Cockerill and Clinical and Laboratory Standards Institute 2012).

Briefly, the microdilution tests were performed in sterile round-bottomed 96-well microplates, keeping in mind that each microplate can contain three samples at most.
200 µL of sample in Mueller Hinton Broth (MHB) with 1% DMSO were added in the first well of each column (line A). 100 µL of MHB with 1% DMSO were added in all the wells of lines B-G. Then serial dilutions 1:2 were performed from each well to the next: 100 µL of line A well were passed to line B on the same column and so on until line G. From line G wells, 100 µL were taken and discarded. This way all line A-G wells contained 100 µL of sample at decreasing concentrations.
Half of line H wells were used as blank (200 µL of MHB) and the other half as negative control (200 µL of *S. aureus* in MHB with 1% DMSO). As a positive control, a solution of 0,4 mg/mL of chloramphenicol in MHB with a 1% DMSO was tested with the same method next to the samples in each plate.
In each well of lines A-G, 100 µL of *S. aureus* in MHB at a concentration of $1*10^6$ UFC/mL (previously determined by bacterial count) were added, this way every well of the microplate contained a total of 200 µL. The plates were incubated at 35°C for 24 hours.
MIC was determined by visual reading.
To improve the reading, 20 µL of TTC (2,3,5-triphenyl tetrazolium chloride) 0.4% (w/v) were added to each well (Veiga et al. 2019). TTC is colourless in water, but in the presence of metabolically active bacteria it's reduced to red-coloured formazan, proportionally to the quantity of living cells. This way, MIC corresponds to the lowest concentration that doesn't show a red colour in the well. Analysing the wells with a spectrophotometer, the percentage inhibition can be determined and the IC_{50} (sample concentration that can halve bacterial growth) can be calculated.

Results

Sample	Highest concentration evaluated (mg/mL)	MIC (µg/mL)
Shives (UPO) UAE	2,5	>2500
Shives (WLH) UAE	2,5	>2500
Roots (UPO) UAE	1	>1000
Aerial parts (WLH) UAE	0,5	125,00
Pure molecule "A" (UniPv)	0,5	>500
Pure molecule "B" (UniPv)	0,5	>500
Cannabispiranol A (UPO)	0,5	>500
DM43A (UPO)	0,5	>500
ACA60A (UPO)	0,5	125,00
ACA61A (UPO)	0,5	15,63
ACA61B (UPO)	0,5	<7,81

Table 1: Antimicrobial activity expressed as minimal inhibitory concentration (MIC) of different samples against *S. aureus*.

Sample	Highest concentration evaluated (mg/mL)	MIC (µg/mL)	IC_{50} (µg/mL)
DOE 3	0,1	12,50	6,87
DOE 6	0,1	50,00	19,95
DOE 8	0,1	12,50	6,32
DOE 17	0,1	50,00	14,08
CAF	0,4	20	3,42

Table 2: Antimicrobial activity expressed as minimal inhibitory concentration (MIC) of DOE aerial parts SFE extracts against *S. aureus*.

All tabulated results can be found in MS 4.4 UniFe Supplementary Material (Tables 1 and 2).
While pure compounds and shives and roots extracts didn't show inhibition of bacterial growth at the highest concentration tested, aerial parts and extracts provided by UPO showed much more promising results, with MICs between 125-2,81 µg/mL.
DOE extracts (SFE on aerial parts provided by WLH) are currently being evaluated as well against *S. aureus* and results obtained so far are in line with other extracts on aerial parts: they are all showing MICs below 50 µg/mL and IC_{50} below 20 µg/mL. These are very interesting results, especially if compared to chloramphenicol used as positive control, which gave a MIC of 20 µg/mL and an IC50 of 3,44

	References • Cockerill FR, Clinical and Laboratory Standards Institute, editors. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard - ninth edition. Wayne, Pa: CLSI. • Veiga A, Toledo MDGT, Rossa LS, Mengarda M, Stofella NCF, Oliveira LJ, Gonçalves AG, Murakami FS. 2019. Colorimetric microdilution assay: Validation of a standard method for determination of MIC, IC50%, and IC90% of antimicrobial compounds. Journal of Microbiological Methods. 162:50–61. https://doi.org/10.1016/j.mimet.2019.05.003
5 - *Determination of the safety profile and bioavailability of the isolated compounds*	Period (two months): **7 – 11** (1 dic. 24 – 30 set. 25)
Research Units Involved	**Activities and Results**
UNIMORE	Antiproliferative activities will be performed on the pure compounds that are currently in preparation. The bioactivities test will be performed in the following period on the extracts and pure compounds collected by the activities still in progress.

Milestone: 5 - Project management and coordination

1 - *Project meetings*	Period (two months): **1 – 12** (30 nov. 24 – 29 nov. 25)
Research Units Involved	**Activities and Results**
UNIFE UNIMORE UNIPV UNIUPO	- November 30, 2023: Kick-off meeting (Google Meet) - frequent communications via whatsapp group and email; - 8 January 2024: Informal sharing of management (administrative) information through WhatsApp PRIN-PNRR group (see Kick-off meeting minute) with the RUs about: 1) website activation: need to wait for indications if a sub-contract will be possible); 2) bi-monthly reporting on timesheet: need to wait for ministerial platform activation; 3) activation of research grants will be possible until 31 July 2024. - January 12, 2024 (Google Meet): 1) from UNIUPO: first considerations/compositional evaluations about extracts potentially obtainable from hemp (7 kg) and roots (730 g) to better drive the following extraction strategies; 2) UNIUPO will send hemp roots and inner woody parts of hemp stems (approximately 200 g/UR) to UNIPV and UNIFE after chopping for further extraction tests as starting activities; 3) UNIMORE will analyze the first extracts. UNIFE note: it is important to plan reasonably in advance the purchase of consumable material to avoid administrative problems - January 26, 2024 (Google Meet): meeting to decide whether or not to proceed with the Linnea company based on the MTA document (Material Transfer Agreement).

	- May 13, 2024 (Google Meet): coordination meeting regarding the activities of each RU during the first quarter; forecast of the involvement of the Whole Lotta Hemp company of Parma for the supply of by-products; coordination for the activities of the following months. - October 10, 2024 (Google Meet): coordination meeting regarding the activities of each RU during the first four-month period; forecast of the involvement of the Whole Lotta Hemp company of Parma for the supply of by-products; coordination for the activities of the following months.
2 - *Mid-term report*	Period (two months): **6 – 6** (1 ott. 24 – 30 nov. 24)
Research Units Involved	**Activities and Results**
UNIFE	The present document represent the target of the MS 5.2
3 - *Final report*	Period (two months): **11 – 12** (1 ago. 25 – 29 nov. 25)
Research Units Involved	**Activities and Results**
UNIFE	This activity will be performed in the last four-month period of the project.

Milestone: 6 - Dissemination and technological transfer

1 - *NOrCa website construction and management, and social media dissemination*	Period (two months): **2 – 12** (31 gen. 24 – 29 nov. 25)
Research Units Involved	**Activities and Results**
UNIMORE UNIPV UNIUPO UNIFE	– February 2 and 9, 2024. Meeting with the IT Office of UNIFE for the set up of the NorCa website. – Collection of information from each RU for the website construction. – URL of the NorCa website: https://norca.unife.it/it (temporarily available and under construction) – Congress Communication (UNIPV): ACS Spring 2024 – GC-MS as a straightforward approach to evaluate cannabinoids stability in smokable Cannabis preparations. Antonella Profumo, Arianna Bini, Daniele Merli. March 17-21 2024 New Orleans, LA (USA) – (UNIPV) – Paper publication (UNIPV): Arianna Bini, Sofia Salerno, Stefano Protti, Federica Pollastro, Antonella Profumo, Luca Morini, Daniele Merli1,4 Photodegradation of cannabidiol (CBD) and Δ9-THC in cannabis plant material. Photochemical & Photobiological Sciences, 2024, https://doi.org/10.1007/s43630-024-00589-4 – Public Engagement event/Seminar (UNIUPO): Prof.ssa Federica Pollastro, Cannabis e Canapa. UTEG Galliate, 8 maggio 2024. – Paper publication (UNIPV): Stefano Protti, Arianna Bini, Mariella Mella, and Daniele Merli Photochemical Vs Thermal Acid Catalysed Cyclization of Cannabigerol (CBG): An Unexpected Selectivity ChemPhotoChem 2024,

	e202400157 https://doi.org/10.1002/cptc.202400157 – – Poster presentation at the International Conference on the Science & Practice of medical Cannabis (London, UK, 29-30 may, 2024 - https://ct-cann2024.com/). – Set up of the website currently operative. NOrCa website: https://norca.unife.it/it
2 - *Mid-term stakeholder workshop*	Period (two months): **6 – 8** (1 ott. 24 – 30 mar. 25)
Research Units Involved	**Activities and Results**
UNIMORE UNIPV UNIUPO UNIFE	This activity will be performed in the following four-month period of the project.
3 - Final event for the dissemination of NOrCa results, free public seminar for the community	Period (two months): **11 – 12** (1 ago. 25 – 29 nov. 25)
Research Units Involved	**Activities and Results**
UNIMORE UNIPV UNIUPO UNIFE	This activity will be planned during the last four-month period of the project.