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Ultrasound-assisted dispersive solid-liquid microextraction with eutectic solvents for the determination of cannabinoids in different hemp products

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ABSTRACT

The wide range of applications of hemp products, together with the environmental benefits that come from hemp cultivation are driving up the market demand for *Cannabis sativa* L. plant. One of the main restrictions for hemp cultivation and marketing concerns the content of delta-9-tetrahydrocannabidiol (Δ^9-THC), which is known to have psychotomimetic effect. If the recent growing of hemp market is beneficial by an economic and environmental point of view, it is necessary to develop reliable analytical methods for the chemical characterization of hemp products, to guarantee the safety of use for the customers.

This study aimed to develop a simple ultrasound-assisted dispersive solid-liquid microextraction (UA-DSLME) method for the extraction of cannabinoids in hemp products, using eutectic solvents (ESs) as extraction material. Two types of ESs were compared: one prepared with a $[Ch^+][Br^-]$-modified salts as hydrogen bond acceptor and one based on natural terpenoids. The ultrasound-assisted dispersive solid-liquid microextraction method was optimized to be applied for the analysis of aerial parts of hemp collected before flowering, hemp inflorescences and a commercial sample called CBD oil, and proved to be robust and versatile. Under optimal conditions, only 100 μL of ES and 2 mL of water as co-solvent were used in the US-assisted extraction, before the analysis in the UHPLC-PDA system. The developed approach allowed to obtain the same chemical profile of conventional methods, while improving the greenness of the method and the enrichment of the marker analytes. To overcome the strong matrix effect for cannabinoids, a matrix-matched calibration was used. Blank matrices of the samples under study were easily obtained by performing an exhaustive extraction of the marker analytes in the hemp samples. These matrices were successfully used for validation, achieving accuracy values between 82% and 118%.

1. Introduction

Hemp is the common name of "fiber" or "industrial" varieties of *Cannabis sativa* L. plant. The most used classification refers to the content of Δ-9-tetrahydrocannabinol (Δ^9-THC), which is one of the most abundant compounds biosynthesized by the plant. Higher content of Δ^9-THC distinguishes the "drug" chemotypes, because of the well-documented psychotropic effects of this compound and its attractiveness for recreational use.

Cannabidiol (CBD) is the second major cannabinoid in *Cannabis sativa* L. and main marker of hemp varieties. Contrary to Δ^9-THC, CBD does not present psychotomimetic actions, while seems to moderate some of the less desirable effects associated with Δ^9-THC consumption [1–3].

Hemp usage in Europe has a long tradition, mainly for its healing properties in ancient medicines and as a source of fiber for textiles [4,5]. Today, the urgent need for green technologies, energy production and resources have led to a new trend of valorization of hemp and its derived products for several applications. The European Union (EU) is promoting hemp cultivation because of its environmental benefits, such as high carbon storage, erosion prevention, increased biodiversity and low to no pesticide requirement. At the same time, hemp is considered a multipurpose crop for the versatility of applications, ranging from edibles, food supplements (seeds and oil obtained from the seeds), cosmetics, construction materials and textiles [6].

On the other hand, the growing demand and consumption of hemp-

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based products has caused concern regarding the associated health risks, due to the limited data to support the safety of CBD containing products [2]. For this reason, the European Commission included extracts of hemp and derived products containing cannabinoids as "novel foods" [7]. This category is subjected to more stringent regulations and longer approval process, compared to other food products. Moreover, a list of hemp varieties with a certified content of Δ^9-THC lower than 0.3% w/w is annually updated in the EU plant variety database catalog [8].

Another important tool to guarantee the safety of use of hemp products is their quality control, by means of monitoring the chemical composition. Several analytical methods have been described in literature for the analysis of contaminants (such as heavy metals, pesticides, mycotoxins) and endogenous compounds (mainly terpenoids and cannabinoids). The analysis of terpenoids is normally related to their fragrance-conferring properties and used for a more accurate identification and characterization of hemp cultivars [9]. Even if cannabinoids are the most important markers to discriminate between illicit and licit products, the regulation on cannabinoids profiling and quantification in hemp products is still controversial. In many European countries, only Δ^9-THC content must be monitored, while there are no limits for CBD. Because of the increasing of cannabis market, stricter regulations are being introduced, extending the quality control to other cannabinoids, such as cannabinol (CBN) and cannabigerol (CBG) [10], although there are no legal limits for these compounds.

The most common approach for the extraction and analysis of cannabinoids is a solid–liquid extraction with medium or low polarity organic solvents, such as ethanol, methanol or hexane [9,11]. Apart from the consumption of relatively high volume of solvents, these methodologies have a significant impact on the sustainability of the overall analysis process as they often require the use of disposable materials, energy-consuming equipment and instrumentation. More innovative approaches have been developed in recent years, with the aim of simplifying the extraction procedure and reducing the toxicity and amount of extraction materials.

In this regard, eutectic solvents (ESs) are an emerging class of solvents that have found numerous applications as alternative extraction solvents. They are characterized by negligible vapor pressure (compared to traditional organic solvents), low cost of raw materials, and possibility to easily vary their composition. The most peculiar feature of ESs is that they consist of a mixture of two or more (natural) components able to form a hydrogen bonding network, resulting in a lower melting point compared to the starting materials [12].

This study aimed to develop a simple ultrasound-assisted dispersive solid–liquid microextraction method coupled to UHPLC-PDA detection for the analysis of cannabinoids in different hemp products. ESs with different physicochemical features (chemical composition and hydrophobicity) were tested to find the optimal extraction solvent for the enrichment of cannabinoids from complex matrices. The method was optimized to be easy adaptable for the analysis of the aerial parts of *Cannabis sativa* L. collected before flowering, inflorescences, and a commercial sample called CBD oil.

2. Experimental section

2.1. Chemicals, reagents, and materials

LCMS-grade acetonitrile, HPLC-grade acetonitrile and formic acid (>98.0% purity) were supplied by Merck Life Science S.r.l. (Milan, Italy). Deionized water (18.2 MΩ cm) was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Methanol (MeOH) (>99.9% purity) was supplied by Merck Life Science S.r.l. (Milan, Italy). Ethanol (EtOH) absolute was supplied by VWR International Srl (Milan, Italy). For the preparation of ESs, (−)-menthol and thymol were purchased from Merck Life Science S.r.l and linalool from Honeywell Fluka™ (Milan, Italy). Dimethyl-(2-hydroxy)ethyl-hexadecyl ammonium bromide $[N^+_{1\ 1\ 16\ 2(OH)}][Br^-]$ salt was synthesized according to [13]. Potassium bromide (KBr) (>99.5% purity) was supplied by Merck Life Science S.r.l. Individual stock solutions of cannabidiol, CBD CAS 13956-29-1 (Phytolab, Vestenbergsgreuth, Germany) and cannabidiolic acid, CBDA CAS 1244-58-2 (Merck Life Science S.r.l), were prepared in MeOH 100% at 1 mg mL^{-1}. A standard working solution containing all analytes was prepared in MeOH 100 % by dilution of the stock solutions to a concentration of 0.1 mg mL^{-1}. These solutions were kept protected from light and refrigerated at −18 °C.

2.2. Samples

Three different samples (Fig. 1) were used in this study: (1) freeze-dried aerial parts (including leaves and stems) of fiber-type *Cannabis sativa* L., (2) seedless dried inflorescences of fiber-type *Cannabis sativa* L. and (3) CBD oil (7% CBD). Sample 1 was kindly provided by the Institute of Science of Food Production, National Research Council (Grugliasco, Italy). The hemp plants were grown in the Western Po Valley (Italy) and the aerial parts were collected before flowering. The harvested samples were immediately freeze-dried and ground in a fine powder to pass a 1 mm screen with a Cyclotec mill (Tecator, Herndon, VA, USA). Sample 2 was purchased from a local Cannabis "light" shop (Cbweed). The inflorescences were labelled as coming from organic farming with Δ^9-THC < 0.5%. Sample 3 was purchased from a local Cannabis "light" shop and labelled as hemp extract in medium chain triglycerides (MCT) from coconut oil. It was stored at −18 °C to prevent degradation. Samples 2 and 3 were purchased in October 2022.

2.3. Preparation of ESs

The ESs tested in the study are reported in Table 1 and were prepared according to the heating and stirring method by mixing the hydrogen bond acceptor and donor (HBA) and (HBD) for 30 min at 60 °C under magnetic stirring, until a homogeneous liquid formed.

2.4. Ultrasound-assisted dispersive solid–liquid microextraction

A similar approach was used for sample treatment of the samples, although some adjustments were made to account for the different physicochemical properties of the three matrices, before the injection in the LC system. The following equipment were employed: a Sonica S3 EP 2400 ultrasonic bath (Soltec, Milan, Italy), a centrifuge, and a vortex mixer (Thermo Fisher Scientific, Rodano, Italy). For the aerial parts, 100 mg of the hemp sample were transferred to a centrifuge tube with 2 mL of KBr 30% w/w aqueous solution and 100 μL of ES (ML or N16, for acronymous, see Table 1). The mixture was then placed in a sonic bath (40 KHz at 25 °C) for 10 min after 30 s of vortexing. Once the extraction was complete, it was subjected to another 30 s of vortexing and centrifuged for 5 min at 2200*g*. Three different phases were formed, starting from the bottom: plant, water, and the ES-rich phase. To allow easy isolation of the latter phase, it was re-suspended in the water layer, and the mixture (without the plant) was transferred in another tube and centrifuged again for 5 min at 2200*g*. At this point, the aqueous phase was removed with a Pasteur pipette, and the remaining upper phase (ES-rich phase) was diluted in 500 μL of MeOH/H_2O (70:30, v/v). The extract was filtered with a 0.20 μm PVDF filter (CPS Analitica, Milan, Italy) prior to injection into the analytical platform.

The same procedure was followed for the inflorescences with few modifications. In this case, 20 mg of plant was used and moisten with 40 μL of EtOH before adding KBr aqueous solution and 100 μL of ES (ML or N16). The mixture was then placed in a sonic bath (40 KHz at 25 °C) for 10 min after 30 s of vortexing. Once the extraction was complete, it was subjected to another 30 s of vortexing and centrifuged for 5 min at 2200*g*. Three different phases were formed, starting from the bottom: plant, water, and the ES-rich phase. This latter was re-suspended in the water layer, and the mixture (without the plant) was transferred in another tube and centrifuged again for 5 min at 2200*g*. Finally, the

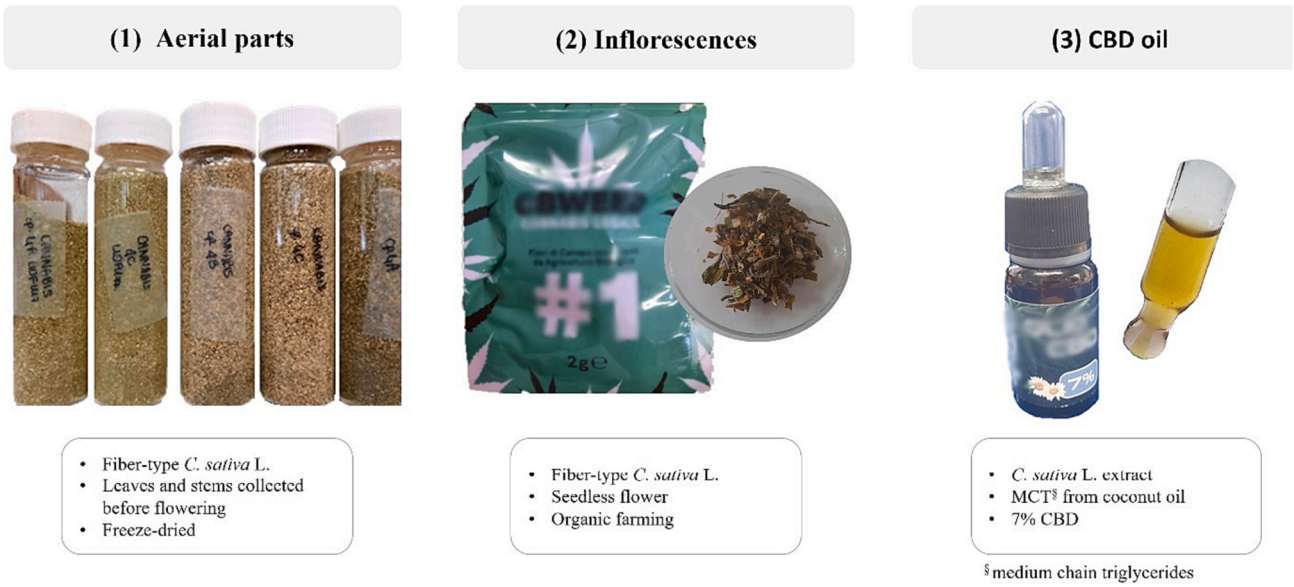
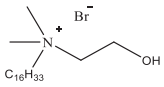
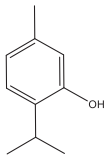
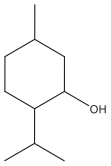
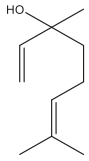


Fig. 1. Hemp products analysed in this study.

Table 1
Properties of the eutectic solvents adopted in this study.

N° (ID)	HBA[a]	Molecular weight (g/mol)[b]	Chemical structures	Commercially available	HBD[c]	Molecular weight (g/mol)[b]	Chemical structures	Commercially available	Molar ratio	Water Solubility (wt%)[d]
1 (N16)	[$N_{1\,1\,16\,(2OH)}^{+}$] [Br^{-}]	394.482		no	Thymol	150.22		yes	1: 2	2.41
2 (ML)	Menthol	156.26[c]		yes	Linalool	154.25		yes	1: 1	0.63

[a] Hydrogen bond acceptor.
[b] Data obtained from Pubchem® 2023.
[c] Hydrogen bond donor.
[d] Water content (wt%) of the ESs measured by Karl Fischer titration, calculated as the difference between the content before and after mixing in water. Data obtained from [13,18].

aqueous phase was removed with a Pasteur pipette, and the remaining upper phase (ES-rich phase) was diluted with 4 mL of EtOH 100% and filtered before the analysis.For CBD oil sample, 100 mg were transferred to a centrifuge tube with 1 mL of KBr 30% w/w aqueous solution and 100 µL of ES (N16). The mixture was then placed in a sonic bath (40 KHz at 25 °C) for 10 min after 30 s of vortexing. Once the extraction was complete, it was subjected to another 30 s of vortexing and centrifuged for 5 min at 2200*g*. The oil and aqueous phase were removed with a Pasteur pipette, and the remaining phase (ES-rich phase) was diluted in 200 µL of EtOH. The extract was filtered with a 0.20 µm PVDF filter prior to analysis. Fig. 2 shows the comparison between the three approaches.

2.5. Conventional methods

For every matrix, conventional extractions were used as reference methods for the phytochemical characterization of the samples and for comparison purposes. For the aerial parts, a MeOH-based solid–liquid extraction was previously optimized by our group [14]. Briefly, 5 mL of MeOH 100 % was added to 100 mg of sample and an US-assisted extraction was performed for 10 min at 40 KHz, at 25 °C. The liquid phase was then submitted to centrifugation at 2200*g* for 10 min. The US extraction procedure was repeated twice on the same plant matrix. After centrifugation, the supernatant was collected and filtered with filter paper (12 cm in diameter) and the solvent completely evaporated at 40 °C in a rotary evaporator. The dried extract was submitted to solid-phase extraction (SPE) to eliminate the chlorophylls, which could damage the chromatographic column, in particular at high concentrations. For this purpose, the extract was reconstituted with 1.5 mL of MeOH/water (40:60, v/v) and eluted with 8 mL of MeOH/water (85:15, v/v) through Agilent Bond Elut C18 cartridge (bed mass 500 mg, volume 1 mL) (previously activated with 4 mL of MeOH and 4 mL of water). The obtained extract was dried with a gentle nitrogen stream, diluted to 5

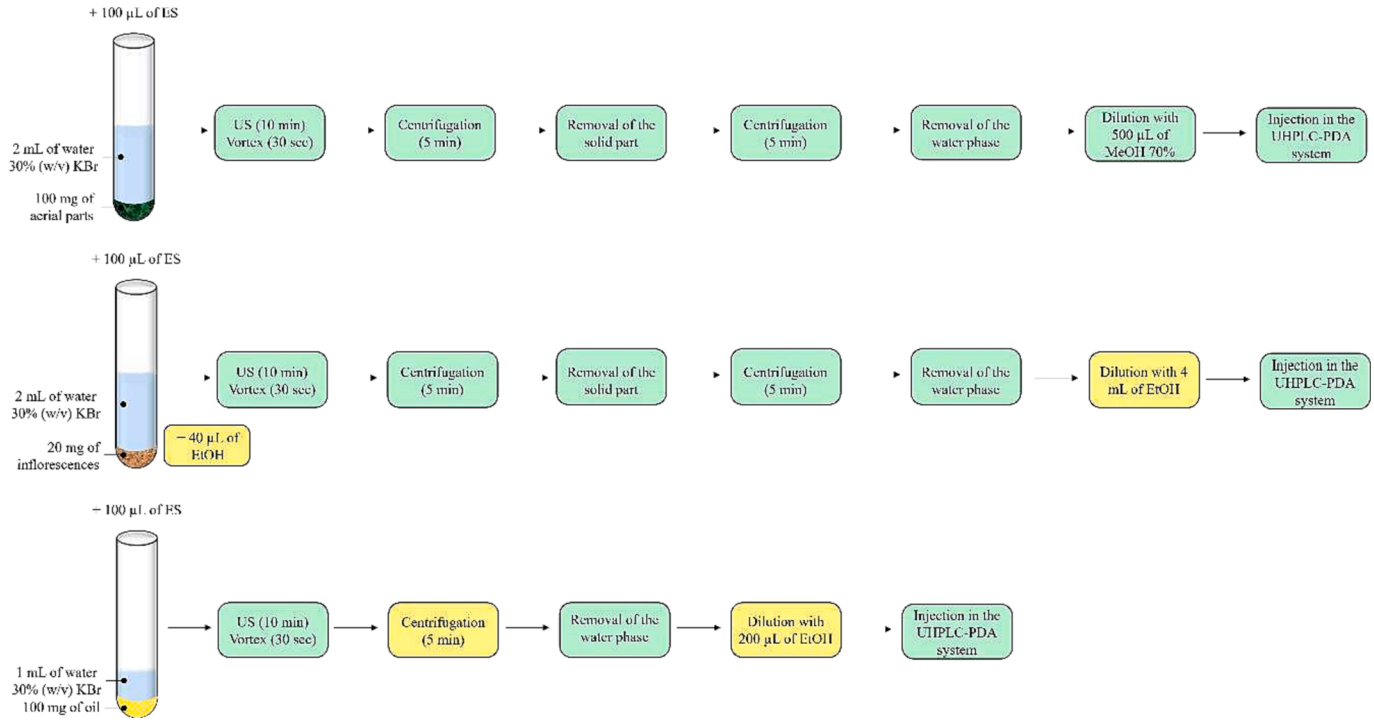


Fig. 2. Ultrasound-assisted dispersive solid–liquid microextraction method optimized for the analysis of different hemp products (aerial parts, inflorescences, and CBD oil). The differences between the procedures are highlighted in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mg/mL with MeOH/water (85:15, v/v) and finally filtered (0.20 µm, polyvinylidene fluoride, PVDF) before the injection in the LC instrument. For the inflorescences, the same MeOH-based solid–liquid extraction was used.

For CBD oil, the procedure was modeled on the method reported by Madej *et al.* [15], with some modifications. After the addition of 200 µL of MeOH 100% to 100 mg of oil, the sample was vortexed for 30 s and submitted to sonication for five minutes. To separate the solvent from the oil layer, the mixture was kept for 1 h in a freezer at − 18 °C. The liquid layer of MeOH was then filtered (0.20 µm, PVDF) before the analysis.

2.6. UHPLC-PDA and UHPLC-PDA-MS/MS system and operating conditions

For the phytochemical characterization of the samples, a Shimadzu Nexera × 2 UHPLC system was used, and it was equipped with an SPD-M20A photodiode array detector (PDA) in series with a Shimadzu LCMS-8040 triple quadrupole system with an electrospray ionization (ESI) source (Shimadzu, Dusseldorf Germany). Mass spectrometer operative conditions were as follows: heat block temperature, 200 °C; desolvation line (DL) temperature, 230 °C; nebulizer gas (N_2) flow rate, 3 L min^{-1}; and drying gas (N_2) flow rate, 15 L min^{-1}. Full scan mass spectra were acquired from 50 to 2000 *m/z*, both in positive and in negative scan modes, with an event time of 0.5 s. When pseudomolecular ions $[M + H]^+$ in ESI^+ or $[M - H]^-$ in ESI^- were identified, they were subjected to collision (collision energy, −35.0 V for ESI^+ and 35.0 V for ESI^-) in product ion scan mode with an event time of 0.2 s. All other analyses were carried out with a Shimadzu UHPLC XR chromatograph equipped with a SPD-M20A photodiode array detector (Shimadzu, Dusseldorf, Germany). An Ascentis Express C18 column (15 cm × 2.1 mm, 2.7 µm, Supelco, Bellefonte, USA) was used for both analytical platforms. The separation of analytes was achieved at 30 °C, using a binary mobile phase composed of water/formic acid (99.9:0.1, v/v) as mobile phase A and acetonitrile/formic acid (99.9:0.1 v/v) as mobile phase B. For the analysis of the aerial parts (sample 1) the gradient program was as follows: 0–2 min 15% B, 2–52 min 15–86% B, 52–55 min 86% B, at a constant flow rate of 0.25 mL·min^{-1}. The total analysis time including pre- and post-running was 67 min. For the analysis of the inflorescences and oil (sample 2 and 3) the gradient program was as follows: 0–2 min 30% B, 2–42 min 30–86% B, 42–44 min 86% B, at a constant flow rate of 0.25 mL·min^{-1}. The total analysis time including pre- and post-running was 56 min. UV spectra were acquired in the 220–450 nm wavelength range, and the resulting chromatograms were registered at the λ max of the identified peaks for quantitative analysis (270 nm for cannabinoids). All UHPLC data were processed using LabSolution software (Shimadzu, Dusseldorf Germany).

2.7. Matrix-matched standard curves

Matrix-matching standard curves were prepared by spiking each blank samples with an appropriate volume of working standard solution. For the aerial parts and inflorescences, the blank samples were obtained by treating 1 g of matrix with 5 mL of MeOH, followed by 5 mL of water. The ultrasound extraction was repeated five times with both the solvents to exhaustively removed the target analytes. For CBD oil, a pure coconut oil, purchased from a local herbal shop, was employed as blank sample.

3. Results and discussions

3.1. Phytochemical characterization of the hemp samples

The qualitative characterization of the three samples was performed by UHPLC-PDA-ESI-MS/MS metabolite analysis, in order to identify the marker compounds to monitor in this study. The characterization focused on the specialized non-volatile metabolites. For this step, the reference methods (see Section 2.5) were used to extract all the compounds of interest from the matrices. For the aerial parts (collected before flowering), qualitative data were obtained from a previous study by our group, in which flavonoids and non-psychotomimetic

cannabinoids were identified [14]. For the inflorescences and the CBD oil a similar chromatographic profile was obtained, and mainly cannabinoids (CBDA and CBD as the most abundant) were found. The identification of CBDA and CBD was confirmed by the injection of authentic commercial reference standards. As reported by [16], the content of cannabinoids in *C. sativa* L. is lower before flowering, while it normally increases approaching the flowering period. On the contrary, the polyphenols are more abundant before flowering. According to the label, the CBD oil was composed of a hemp extract obtained with food grade solvent. Due to the high content of CBD in hemp inflorescences of the plant, this extract was probably obtained from the flowers, justifying the similar profile of the oil (sample 3) and inflorescences (sample 2). The information regarding the identification of the marker analytes under study are reported in Table S1.

The chromatographic profiles at 254 nm of the three samples after conventional extraction, are illustrated in Fig. S1.

3.2. Chromatographic method

Two chromatographic methods were used to achieve the optimal analytical separation of the marker analytes in the three complex samples. The presence of both more polar (flavonoids) and less polar (cannabinoids) compounds in the extract of the aerial parts required the optimization of a longer chromatographic method, starting with a higher percentage of water (85% instead of 70%) as mobile phase. Moreover, 5 μL of aerial parts and CBD oil extract samples were injected in the UHPLC system, while the injection volume for the inflorescences was decreased to 3 μL of sample because of the high signal of CBDA which saturated the detector, even after dilution of the extract. The same injection volume was used in the validation of the method (Section 3.7).

Mix solutions of the target compounds (CBD and CBDA) were injected at increasing concentration, using the two adopted injection volumes, for the calibration curves preparation. Table S2 shows several quality analytical parameters of the calibrations obtained by UHPLC-PDA. Calibration curves were linear with determination coefficients (R^2) higher than 0.992. The LODs were experimentally determined by decreasing the concentration of the analyte until a signal-to-noise ratio (S/N) of 3 was obtained. The LOQs were estimated as S/N of 10 and experimentally verified by injecting the standard compound at the predicted concentration. The LOD ranged between 0.03 μg mL^{-1} and 1 μg mL^{-1}, and the LOQ between 0.1 μg mL^{-1} and 4 μg mL^{-1}.

3.3. Choice of the extraction solvent

Eutectic solvents (ESs) were selected as extraction materials for their desirable properties such as low cost of raw materials, low vapor pressure (compared to conventional organic solvents) and easy preparation [17]. Moreover, the possibility to easily vary their composition allows to overcome some limits of conventional solvents which are normally characterized by a low chemical tunability. From previous studies [13,18], we selected two types of ESs, which have demonstrated good extraction performances for the analysis of the aerial parts of fiber-type *Cannabis sativa* L.. Table 1 reports several properties of the ESs used in this study. Further information about the characterization and properties of the ESs are reported in [13,18]. The first solvent (N16) is formed by a [Ch^+][Br^-]-modified salts as HBA and thymol as HBD, at 1:2 M ratio. The HBA was synthetized by the authors, and it is characterized by a long alkyl chain with 16 carbon atoms as one of the substituents of the ammonium group. The second mixture (ML) is a natural eutectic solvent formed by menthol and linalool at a 1:1 M ratio. Apart from the different chemical structure of the components forming the two ESs, they also distinguish for the different hydrophobicity, higher for the natural ESs. The hydrophobicity was calculated by Karl Fischer titration, in the previous studies, measuring the water content after preparation and after mixing the solvent in water [13,18].

3.4. Application of the extraction method on different hemp products

An ultrasound-assisted dispersive solid–liquid microextraction (UA-DSLME) method using ESs was used for the analysis of cannabinoids in the three different hemp products. This method was selected as an alternative of conventional extraction approaches for the simplicity and speed of the procedure while also reducing the amount of solvent to improve the enrichment of analytes in the final extract. The UA-DSLME was optimized in a previous work [18] for the extraction of polar and less polar compounds from the aerial part (leaves and stems) of the plant collected before flowering. The optimized UA-DSLME was then tested on hemp inflorescences and CBD oil to evaluate the applicability and versatility of the method on samples with different features. Compared to leaves and stems, inflorescences are characterized by an oily texture because of the high content of resins which are produced by glandular trichomes, mainly present in female flowers [19]. The presence of these resins made the sample extremely hydrophobic, preventing the plant to enter completely in contact with the aqueous solution, used in the UA-DSLME method. During the extraction, the KBr aqueous solution acts as co-solvent, favoring the transfer of the analytes from the sample to the ES phase. For this reason, the inflorescences were moistened with few microliters of EtOH 100% before adding 2 mL of KBr 30% w/w aqueous solution and 100 μL of ES. Moreover, the amount of sample to extract (100 mg for the aerial parts) was reduced to 20 mg because of the high content of cannabinoids which saturated the signal of the UHPLC-PDA system. For the same reason, once collected, the ES rich phase was diluted with 4 mL of EtOH 100%, instead of 500 μL of MeOH 70%. In the case of CBD oil, only one centrifugation step was necessary to isolate the ES rich phase which was then diluted with 200 μL of EtOH 100%, prior to injection in the UHPLC system, because of the lower content in marker analytes. In the case of the inflorescences and CBD oil, the final extracts were diluted in EtOH which presents a lower toxicity compared to MeOH. Regarding the aerial parts, MeOH was used to avoid the back-extraction of chlorophylls, which is more abundant in the herbaceous parts of the plants. When the UA-DSLME was performed with ML for the analysis of CBD oil, it was not possible to separate the ML phase from the oil, after the centrifugation step. Contrary to N16, ML is more hydrophobic and easily solubilized in the oil phase. For this reason, the extraction was performed only with the [Ch^+][Br^-]-based ES.

With the above mentioned few modifications (see Fig. 2 for details), the developed UA-DSLME method resulted to be a versatile and robust approach for the analysis of hemp samples with different physico-chemical properties.

The chromatographic profiles obtained at 254 nm, for the three hemp products, are shown in Fig. 3. As reported in our previous study [13], N16 ES allowed to increase the extraction of the more polar flavonoid compounds while maintaining a similar extraction efficiency of cannabinoids compared to the reference ML hydrophobic solvent. As for the inflorescences, the extraction of both the acidic and neutral forms of CBD was improved with N16 solvent. The hydrophobic properties of [Ch^+][Br^-]-based HDESs, together with hydrophilic domains (hydroxyl group and charge on the ammonium group) seem to facilitate the interaction with both polar and less polar compounds.

Apart from the extraction performance of the ESs, their compatibility with reverse phase liquid chromatography was also investigated since chromatographic techniques are fundamental for the analysis and study of plant metabolome to separate and identify the analytes of interest. The single components of the ESs (menthol, linalool, N16 and thymol) were injected to verify that the signal of the solvent (if present) did not interfere with the one of the target analytes. Only thymol, which features an aromatic ring in its structure, absorbed at the wavelengths of analysis, however its signal did not interfere the signal of the analytes, as shown in the chromatograms reported in Fig. 3. Because of the high concentration of thymol, blank injections of MeOH 100% were regularly repeated within the analysis of the extracts to detect potential carryover.

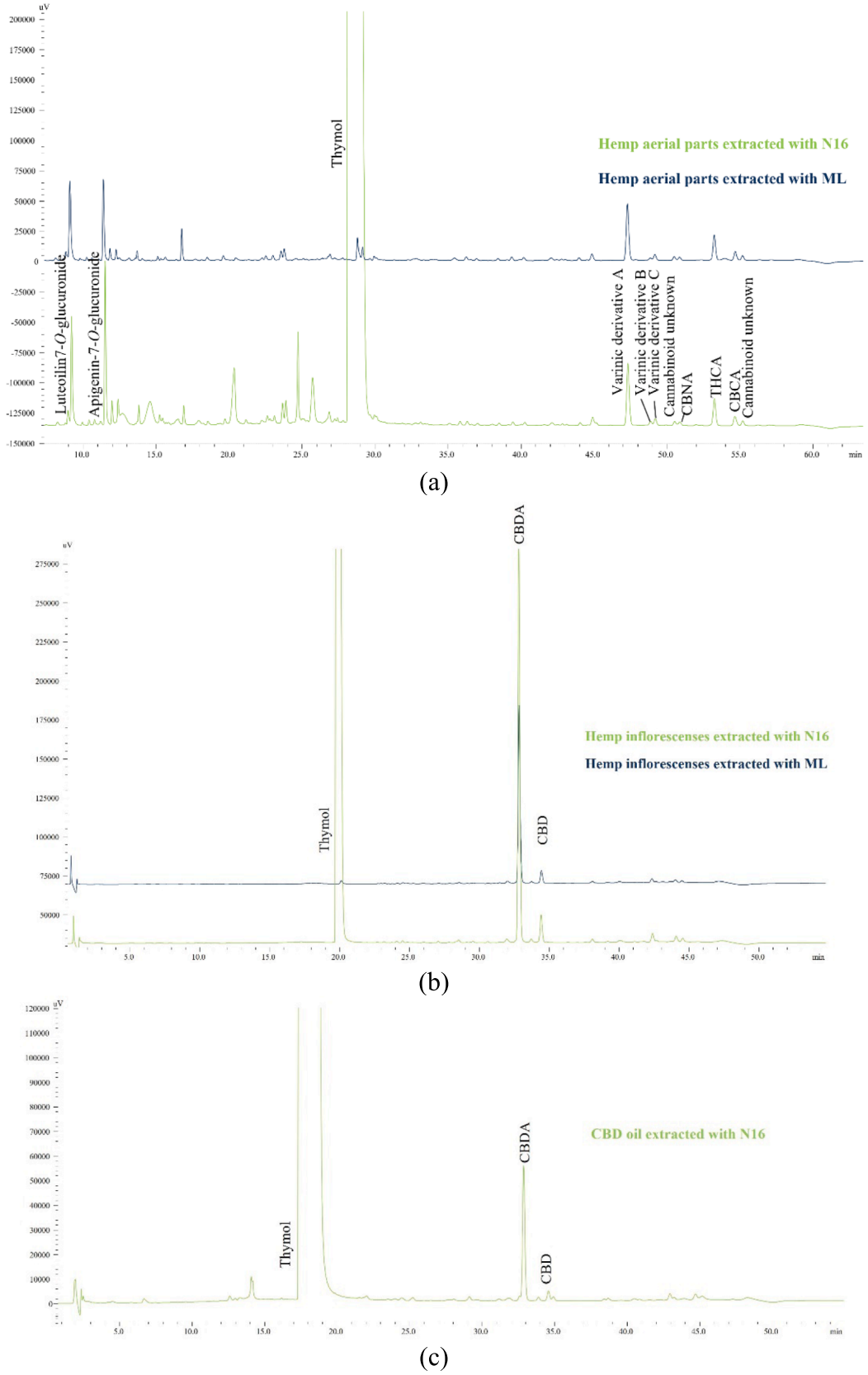


Fig. 3. Chromatographic profiles at 254 nm, obtained after the UA-DSLME coupled to UHPLC-PDA of (a) hemp aerial parts (b) hemp inflorescences and (c) CBD oil. For name abbreviation, see Table S1.

3.5. Evaluation of matrix effect (ME)

Matrix effect is known to affect the efficiency of chromatographic separation and the extraction efficiency of marker analytes, especially in the analysis of complex matrices [20,21]. Considering the complexity of the hemp samples under study, the contribution of matrix effect in the analysis of cannabinoids, was evaluated in the validation of the UA-DSLME approach developed. To reduce matrix effect during calibration, different strategies are available, among the main: (1) standard addition, (2) isotope labeled internal standard, and (3) matrix-matched calibration. The first approach is used when a blank matrix is not available, however in the case of analytes at high concentration (e.g. CBDA in the inflorescences) high amount of standard are necessary to build the calibration curve. The use of stable isotope labeled internal standards (SIL-ISs) is considered a valid approach to overcome ME, but the main disadvantages is the high cost and stability of SIL-ISs during sample preparation (deuterium can be exchanged with hydrogen) [20]. To develop an affordable and easy applicable validation method, matrix-matched calibration was selected. In this case, the marker analytes are spiked to a blank sample and a linear calibration curve is built. The main disadvantage when studying specialized metabolites in plants is to find a matrix exactly similar to the sample and free of the analytes under study. To overcome this problem, an exhaustive extraction of the marker analytes was performed on the aerial parts and inflorescences of the plant, in order to obtain a matrix with similar characteristics to the real samples, avoiding the use of artificial matrices (made to simulate the authentic matrices in terms of composition, salts content, analyte solubility…), which can be tedious and challenging to prepare.

CBD oil is normally prepared by mixing CBD (usually in form of extract or inflorescences) with a carrier oil [22]. According to the label of the CBD oil used in this study, the hemp extract rich of CBDA and CBD was solubilized in fractionated coconut oil. While in the case of aerial parts and inflorescences, the exhausted blank matrix was employed for the validation of the method, pure coconut oil was used for CBD oil. The comparison of the chromatographic profile between the blank and the real samples is shown in Fig. S2.

For each sample, increasing concentrations (n = 4) of CBDA (for aerial parts, where only cannabinoid acids were present) and CBDA and CBD (inflorescences and oil) were spiked to the blank matrix in order to build a calibration curve. The following ranges were used: 1.5 – 6 $\mu g \cdot mL^{-1}$ for CBDA in the aerial parts, 0.8 – 4 $\mu g \cdot mL^{-1}$ for CBDA and CBD in the inflorescences, and 0.25 – 4 $\mu g \cdot mL^{-1}$ for CBDA and 2 – 6 $\mu g \cdot mL^{-1}$ for CBD in the oil. The same calibration curve was built without the sample (blank), adding the standard mixture in water. For both the approaches, after the addition of the standard mix, the UA-DSLME with the two ESs (ML and N16) was performed. The matrix effect (ME) of the three hemp extracts was calculated as the slope of the calibration curve built on the blank matrix (k_1) versus the one without the sample (k) using the following equation [23]:

$$ME = \left(1 - \frac{k_1}{k}\right) \times 100$$

Table S3 reports the ME value for the aerial parts, inflorescences and CBD oil after performing the UADSLME with ML and N16. The ME ranged from 26.56 % to 83.27% with higher values for the oil. These results highlighted the important contribution of the matrix effect that could not be overlooked for all the tested samples. For this reason, the calibration curves obtained in the blank matrix were used to measure the analytical performances of the method.

3.6. Analytical performance of the UA-DSLME method

As described in the previous section, the method was validated using a matrix-matched calibration, performing the entire UA-DSLME-UHPLC-PDA method on blank matrices. CBDA and CBD were used as reference standards for the validation and quantification of cannabinoid acids and neutral, respectively, due to the similar physicochemical features. The neutral form usually presents two UV absorbtion maxima at 210 and 270 nm, while the acid form three UV absorbtion maxima at 220, 270 and 305 nm. Table 2 reports several quality analytical figures of merit of the method, the linearity range, the calibration sensitivity (evaluated as the calibration slope), determination coefficient (R^2), limit of quantification (LOQ), accuracy, enrichment factor (EF) and intra-day RSD. The λ selected for quantification of both CBDA and CBD was 270 nm. The LOQs were estimated as S/N of 10 and experimentally verified by injecting the standard compound at the predicted concentration. LOQ values ranged from 0.1 $\mu g\ mL^{-1}$ and 1 $\mu g\ mL^{-1}$, depending on the matrix and solvent employed. The intra-day repeatability of the method was evaluated in terms of RSD (%) after performing three independent experiments within the same day. The intra-day RSD values were lower than 10%, showing a good precision of the method. The EF was calculated at 2 $\mu g \cdot mL^{-1}$ concentration of the standards mix and varied between 2 and 20. The accuracy of the method was calculated by testing the capacity of the matrix-matched calibration curves to predict a specific concentration in the real samples. The values ranged from 82% to 118%, showing a good accuracy of the developed method.

Fig. 4 shows the quantification data, expressed as mg of cannabinoids in mL of extract of aerial parts, inflorescences, and CBD oil. The data were obtained performing the UA-DSLME method with both ML and N16 solvent. As already observed in the comparison of the chromatographic profile, N16 improved the enrichment of cannabinoids from the inflorescences (0.714 mg mL^{-1} for CBDA and 0.357 mg mL^{-1} for CBD with N16 versus 0.224 mg mL^{-1} for CBDA and 0.174 mg mL^{-1} for CBD with ML) while no relevant difference between the two solvent was found in the extraction of cannabinoids from the aerial parts. Because of the impossibility to perform the extraction of CBD oil with ML (see paragraph 3.5), quantification results were obtained only for N16. These results highlighted that the characteristics of the samples under study highly affected the overall analytical method, in terms of sample preparation and analytical performance. Even in the case of inflorescences and aerial parts samples, where the physicochemical differences are less evident (compared to CBD oil), the extraction performance of cannabinoids varied according to the solvent used.

3.7. Comparison with other extraction methods

When developing new extraction methods, it is fundamental to compare the procedure and the analytical performance with reference methods reported in literature. Table 3 provides several examples of extraction methods developed for the analysis of cannabinoids in the aerial parts, inflorescences of *C. sativa* L. and CBD oil, together with the methods reported in this study. Organic solvents, such as EtOH or MeOH, are normally employed for the extraction of cannabinoids, independently from the matrix under study. Hydrophilic DESs have also been successfully applied for the analysis of cannabinoids in hemp leaves. In the case of CBD oil, the most common approach is the dilution of the oil before the injection into the analytical platform. The direct injection of oil samples in the LC system can lead to some problems, depending on the viscosity and complexity of the carrier oil used to prepare CBD oil. The dilution of the sample can help to solve this drawback, but it can affect the enrichment of marker analytes and the limit of detection of the method. For most of the methods reported, external calibration is used for quantification. As shown by this study, matrix effect highly influences the response of marker compounds and it should be taken into consideration during the validation of the method.

3.8. Measurement of greenness of the method using AGREEprep, BAGI and SPMS metrics

Several green metrics have been developed in recent years to "measure" objectively the greenness of analytical procedures. Every

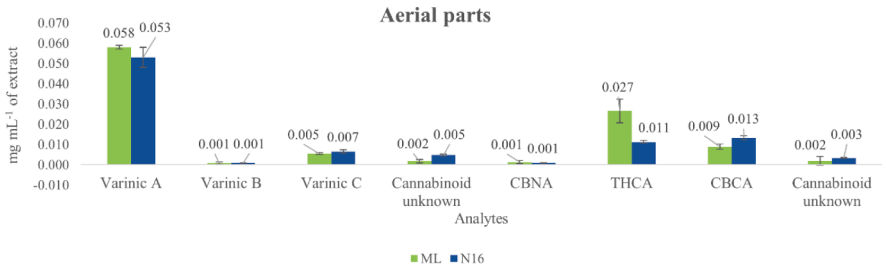
Table 2
Analytical performance of the overall UA-DSLME method for the determination of cannabinoids using ESs.

Compound	Working range ($\mu g \cdot mL^{-1}$)	(Slope ± SD[a]) $\cdot 10^{-3}$	R^2 [b]	$S_{y/x}$ [c] %	LOQ[d] ($\mu g \cdot mL^{-1}$)	EF[e]	Accuracy[f]	RSD[g]
Aerial parts								
Eutectic solvent ML								
CBDA	1.5–6	9.87	0.992	5.93	1	5	108	4.59
Eutectic solvent N16								
CBDA	1.5–6	7.73	0.995	5.21	0.6	6	116	2.43
Inflorescences								
Eutectic solvent ML								
CBDA	0.8–4	4.58	0.998	3.35	0.3	4	99	5.71
CBD	0.8–4	0.48	0.999	0.07	0.5	20	82	3.38
Eutectic solvent N16								
CBDA	0.8–4	3.35	0.992	7.84	0.3	5	92	1.96
CBD	0.8–4	0.39	0.992	6.88	0.5	20	91	4.69
Oil								
Eutectic solvent N16								
CBDA	0.25–4	36.05	0.993	8.96	0.1	3	118	5.26
CBD	2–6	0.95	0.998	2.67	1	2	104	8.21

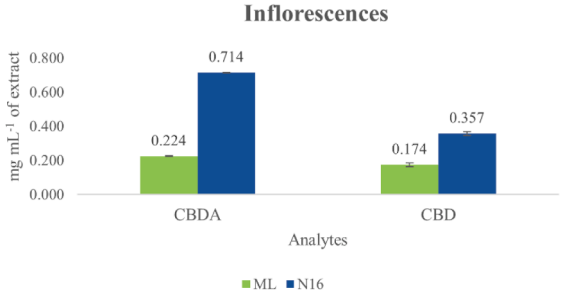
[a] Standard deviation of the slope.
[b] Determination coefficient.
[c] Standard deviation of the residuals in percent (or error of the estimate).
[d] Limit of quantification, were estimated as S/N of 10 and experimentally verified.
[e] Enrichment factor, calculated on a standard concentration of 2 $\mu g \cdot mL^{-1}$.
[f] Accuracy, calculated on a standard concentration of 3 $\mu g \cdot mL^{-1}$ for oil and inflorescences and 5 $\mu g \cdot mL^{-1}$ for aerial parts.
[g] Intra-day relative standard deviation (n = 3).

metrics consider different aspects of the analytical method and it is important to select the most suitable metric according to the procedure employed and the information that the user want to obtain [24]. In this study, sample preparation step was the fundamental and most critical part of the overall method. In fact, the complexity of the matrices under study required a sample pre-treatment before the analysis in the UHPLC-PDA system. Sample preparation is known to have a significant impact on the sustainability of the overall analysis process as they often require the use of disposable materials, energy-consuming equipment and instrumentation, and extraction methods that employ harmful solvents. The UA-DSLME method developed aimed to improve several of the listed aspects, anyway sample preparation was still necessary. AGREEprep metric is the modified version of AGREE tool and was developed to measure the impact of sample preparation [25]. For this reason, AGREEprep was selected to compare the greenness of sample preparation methods used in this study. Fig. S3 shows the comparison of the pictograms obtained for the UA-DSLME on the aerial parts, inflorescences and CBD oil, using N16 and ML solvents and the one obtained for the reference MeOH extractions (see Section 2.5 for the procedure). The energy consumption was measured with a Zhurui PR10 power meter plug (Zhurui, China). The overall outcome gives by the metric goes from 0 to 1, where 1 means that the criteria of green sample preparation (GSP) [26] are completely fulfilled. As shown in Fig. S3, the UA-DSLME approaches have similar scores (around 0.5), meaning that the principles of GSP are partially satisfied. Low values are attributed to *ex situ* sample preparation (criterion 1), due to the impossibility to integrate this step with sampling. Moreover, the developed UA-DSLME is a manual procedure with no degree of automation (criterion 7). In fact, specific and customized tools would be necessary to fully-automated this method. The use of HPLC is also considered a negative aspect due to the high consumption of energy and solvent (criterion 9). On the other hand, the use of a low volume (100 μL) of natural solvents is positively considered by the metric. The few modifications applied for the UA-DSLME on inflorescences allowed to obtain slight improvements to the greenness, due to the low sample amount required (20 mg) and the replacement of MeOH with EtOH (lower number of pictograms in the material safety data sheet) in the final dilution. Regarding CBD oil, only one centrifugation step was necessary to separate the sample from the ES rich phase, simplifying and reducing the number of sample preparation steps to perform. In general, the use of N16 ES slightly reduced the greenness of the method, due to the synthetic process necessary to obtain the $[Ch^+][Br^-]$-modified salt. However, as previously discussed, N16 can improve the extraction of some marker analytes, thanks to its peculiar structure, justifying its application. In this regard, several authors have highlighted that in the development of analytical methods, the greenness of a methodology should not be at the expense of its functionality [27,28]. For all the three hemp samples, the use of UA-DSLME improved the final score of the method, compared to the reference one. Regarding the aerial parts and the inflorescences, the main drawbacks of the reference method are the use of quite high volume (15 mL) of MeOH and the need to purify the extract by SPE, making tedious and time-consuming the entire process. The low score obtained for the MeOH extraction of CBD oil is due to the high energy consumption necessary to freeze the oil and separate the MeOH layer. Moreover, this approach has a low sample throughput, because each sample require 1 h of freezing before being analyzed.

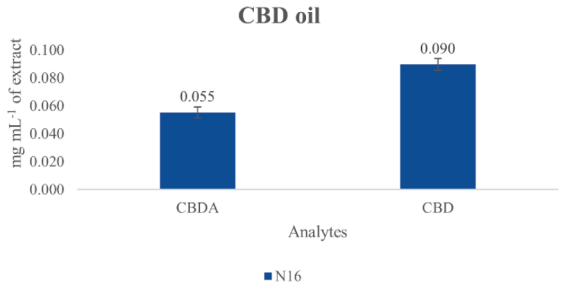
More recently, other metrics, namely Blue Applicability Grade Index (BAGI) [29] and Sample Preparation Metric of Sustainability (SPMS) [30] have also been introduced. The first focuses on measuring of the productivity and practical efficiency of the method, a parameter already introduced by Novak *et al.* with the concept of White Analytical Chemistry [27]. The second aims to exclusively evaluate, in term of sustainability, the sample preparation step, without considering the sampling and the instrumental technique, parameters normally covered by other metrics. As for AGREEprep, the two metrics were applied to the UA-DSLME method on the aerial parts, inflorescences and CBD oil, using N16 and ML solvents and to the MeOH reference extractions. The pictograms are reported in Figs. S4 and S5. The criteria evaluated from every metric are also reported in the supplementary materials. Table 4 reports the final scores and the color code obtained for each method by the three metric tools. In terms of greenness, a similar ranking was obtained by AGREEprep and SPMS, where the UA-DSLME method with natural ESs was classified with the highest score. The methods developed in this study mainly differ for the sample preparation step, while the sampling and the instrumental part are similar. Indeed, the use of a chromatographic technique reduces the "absolute" greenness of the



(a)



(b)



(c)

Fig. 4. Quantification data for cannabinoids, expressed as mg of compound in 1 mL of extract, obtained after performing the UA-DSLME with ML and N16 solvents (n = 3).

developed methods when evaluated with AGREEprep (about 50% of the maximum score in contrast to 65–80% obtained with SPMS). Moreover, it can be observed that no difference can be seen for the UA-DSLME methods applied to solid samples (aerial parts and inflorescences) with the SPMS metric, showing that the improvements due to further miniaturization and the replacement of MeOH by EtOH are not recognized. The classification given by BAGI was less effective to distinguish between the different methods, because the criteria considered by this metric are more focused on the productivity (e.g multi-element analysis, degree of automation) and less on the greenness of the method (e.g nature of the extractant, operator safety). For instance, the extraction of compounds from different chemical classes (i.e flavonoids and cannabinoids) in the aerial parts, was positively evaluated by BAGI. Because the aim of this study was to improve the sustainability of a method applicable for the determination of phytochemicals in different hemp matrices, AGREEprep and SPMS gave a better understanding of the strengths and weaknesses of the developed approaches. Because several green metrics have been proposed so far, depending on the application, it is important to select the best tool to obtain feedback in line with the scope of the analysis.

4. Conclusions

The determination of the chemical composition of established and emerging hemp products is of utmost importance for regulatory authorities, to guarantee the efficacy and safety in the use of these products. The main challenges of these analyses are related to the complex phytochemical profile of *Cannabis sativa* L. and the diversification of hemp products, that also includes forms of cannabis available on the illicit drug market.

In this study, a simple ultrasound-assisted dispersive solid–liquid microextraction method was developed, using two different type of eutectic solvents, one prepared with a [Ch^+][Br^-]-modified salts as HBA and one based on natural compounds. The UA-DSLME method was tested for the analysis of cannabinoids in three different hemp products: the aerial parts of *Cannabis sativa* L. collected before flowering, the inflorescences of the plant and the CBD oil, and it resulted to be easily adaptable to matrices with different physicochemical properties.

The validation of the procedure showed that the matrix effect played a fundamental role in the extraction efficiency of the marker analytes for the three samples, therefore a matrix-matched calibration was employed to evaluate the analytical performance of the methodology.

Table 3
Analytical methods adopted for the determination of cannabinoids in different hemp products.

Sample (g)	Analytes	Sample treatment	Extraction material (mL)	Analytical platform	Quantitative method	LOD[a] of CBD	Ref
Aerial parts (no flowers)							
Leaves and stems (0.1)	8 cannabinoids	UA-DSLME	ESs (0.1)	UHPLC-PDA	Matrix-matched calibration	0.3–0.1 $\mu g \cdot mL^{-1*}$	This work
Leaves and stems (0.1)	12 cannabinoids	Solid-liquid extraction	MeOH (15)	UHPLC-PDA-MS	External calibration	/	[14]
Leaves and stem barks (0.4)	14 cannabinoids	Solid-liquid extraction	MeOH (20)	HPLC-UV-MS	External calibration	0.0004–0.004 $\mu g \cdot mL^{-1}$	[31]
Leaves (0.2)	CBD	Solid-liquid extraction	Hydrophilic DESs[b] (5)	HPLC-UV	External calibration	/	[32]
Inflorescences							
Fiber-type cannabis (0.02)	CBDA, CBD	UA-DSLME	ESs (0.1)	HPLC-PDA	Matrix-matched calibration	0.4 $\mu g \cdot mL^{-1}$	This work
Fiber-type cannabis (0.25)	4 cannabinoids	Solid-liquid extraction (DM[c], UAE[d], MAE[e], SFE[f])	EtOH (10)	HPLC-PDA-MS/MS	External calibration	0.7 $\mu g \cdot mL^{-1}$	[33]
Fiber-type cannabis (0.05)	5 cannabinoids	Solid-liquid extraction	MeOH (5)	HPLC-UV	External calibration	/	[34]
Fiber-type cannabis (0.4)	CBDA, CBD	Solid-liquid extraction (DM, UAE, MAE)	EtOH (20)	UPLC-PDA	External calibration	0.07 $\mu g \cdot mL^{-1}$	[35]
Oil							
MCT[g] from coconut oil (0.1)	CBDA, CBD	UA-DSLME	ESs (0.1)	HPLC-PDA	Matrix-matched calibration	1 $\mu g \cdot mL^{-1}$	This work
Different carrier oil (/)	CBD, THC	Dilution	MeOH/water (/)	HPLC-PDA	External calibration	0.25 $\mu g \cdot mL^{-1}$	[36]
Different carrier oil (0.01)	11 cannabinoids	Dilution	Isopropanol/MeOH (0.8)	HPLC-PDA	External calibration	/	[37]
Hemp leaves oil extract (0.1)	CBDA, CBD	Liquid liquid extraction	ACN (0.5) H_2O (5)	HPLC-PDA	Matrix-matched calibration	1.94 $\mu L \cdot mL^{-1}$	[15]

[a] Limit of detection.
[b] HBA: Choline chloride, betaine, HBD: D-sorbitol, urea, oxalic acid, benzoic acid, citric acid, ethyl tartrate, zinc chloride, lactic acid, glycerol, salicylic acid, succinic acid, mannitol, acetamide.
[c] Dynamic maceration.
[d] Ultrasound-assisted extraction.
[e] Microwave-assisted extraction.
[f] Supercritical fluid extraction.
[g] Medium chain triglycerides.

Table 4
Total scores of the analytical methods used in this study, obtained by AGREEprep, BAGI and SPMS metrics.

Method	Sample	Extraction material	AGREEPrep[a]	BAGI[b]	SPMS[c]
Developed methods					
UA-DSLME	Aerial parts	ML	0.49	65	7.89
		N16	0.44	65	6.42
UA-DSLME	Inflorescences	ML	0.57	62.5	7.89
		N16	0.46	62.5	6.42
UA-DSLME	Oil	N16	0.53	62.5	6.63
Reference methods					
SLE	Aerial parts	MeOH	0.21	60	3.47
SLE	Inflorescences	MeOH	0.21	57.5	3.47
SLE	Oil	MeOH	0.38	57.5	5.47

[a]Global score: from 1 if the criteria is completely fulfilled to 0 if not fulfilled, color code: from green (completely fulfilled) to red (not fulfilled) [25].
[b]Global score: from 100 if the criteria is completely fulfilled to 25 if not fulfilled, color code: dark blue for high, blue for medium, light blue for low, and white for no compliance with the set criteria [29].
[c]Global score: from 10 if the criteria is completely fulfilled to 1 if not fulfilled, color code: green for successful, yellow for acceptable, orange for tolerable, and red for inadequate [30].

The ES formed by the [Ch^+][Br^-]-modified salt showed better extraction performance for all the hemp products tested, but slightly lower sustainability (a synthetic reaction is necessary) compared to the natural ES. These results highlighted the importance to balance and consider both these aspects (greenness and functionality) in the development of analytical method, to obtain truly innovative, efficient, and reliable methodologies.

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CRediT authorship contribution statement

Giulia Mastellone: Investigation, Conceptualization, Writing – original draft, Writing – review & editing. **Arianna Marengo:** Validation, Writing – review & editing. **Barbara Sgorbini:** Validation, Writing – review & editing. **Patrizia Rubiolo:** Validation, Funding acquisition, Writing – review & editing. **Jared L. Anderson:** Resources, Validation, Writing – review & editing. **Cecilia Cagliero:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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