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Development of a dispersive solid-liquid microextraction method using natural eutectic solvents for a greener extraction of phytochemicals from fiber-type *Cannabis* sp.

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Abstract

The use of plant materials as natural resources has become fundamental for several applications, in perspective of a sustainable growth. *Cannabis* sp. crop perfectly fits this purpose, thanks to its rich phytocomplex, that can be exploited in pharmaceutical and cosmetic industry. At the same time, the increasing demand for innovative technologies, energy saving and greener solvents makes necessary the development of new extraction methods to obtain the metabolites of interest. In this study, a dispersive solid-liquid microextraction (DSLME) method, using natural eutectic solvents (ESs), was first optimized and validated for the extraction of the non-volatile fraction of the aerial parts of hemp collected before flowering. The DSLME was directly carried out on the freeze-dried powder of the plant, without the need of a pre-extraction with harmful organic solvents. The optimized method is fast and easy to perform compared to conventional procedures, and only requires 100 µL of ES and 2 mL of water. The ES easily formed the dispersion in water after 30 seconds of vortex, and no dispersive solvent was added. After 10 minutes of sonication, the enriched phase was simply recollected after centrifugation and solubilized in 500 µL of methanol/water (70:30, v/v) before the injection in the UHPLC-PDA system. Several natural compounds (menthol, linalool, terpinen-4-ol, eugenol, carvacrol, thymol) were combined and the influence of the two components of the ES, on the performance of extraction, was also evaluated. The proposed solvents showed a higher enrichment of cannabinoid compounds compared to the more polar flavonoids, thanks to the hydrophobic features of the proposed ESs.

Keywords: *Cannabis sativa* L., non-psychotomimetic cannabinoids, flavonoids, microextraction, eutectic solvent, UHPLC-PDA

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Abbreviation: DSLME, dispersive solid-liquid microextraction; ESs, eutectic solvents; UHPLC, ultra high performance liquid chormatography; PDA, photo-diode array detector; THC, tetrahydrocannabinol; CBD, cannabidiol; DESs, deep eutectic solvents; HBD, hydrogen bond donor; HBA, hydrogen bond acceptor; SLE, solid-liquid extraction; MeOH, methanol; CBDA, cannabidiolic acid; SPE, solid-phase extraction; PVDF, polyvinylidene fluoride; RP, reverse phase; FTIR, fourier transform infrared spectroscopy; ATR, attenuated total reflectance; NMR, nuclear magnetic resonance; US, ultrasound; DLLME, dispersive liquid-liquid microextraction; LLE, liquid-liquid extraction; LC, liquid chromatography; SD, standard deviation; HDESs, hydrophobic deep eutectic solvents; S/N, signal to noise ratio; DMSO, dimethyl sulfoxide; ANOVA, analysis of variance.

1. Introduction

Cannabis sativa L. crop is now the subject of renewed interest in many fields and applications for its unique characteristics. It is important to distinguish between the different chemotypes due to the presence of several varieties and modern hybrids which may differ in morphology, metabolites content and uses (Fike, 2016; Jin et al., 2021). However, the classification and taxonomy of this plant is often challenging and then confusing, due to high variability within the genus (Giupponi et al., 2020). The main classification seems to refer to drug- (cannabis) and fiber- (hemp) types, based on the content of tetrahydrocannabidiol (THC) and cannabidiol (CBD), the main representatives of phytocannabinoids class. While the first chemotype is of interest for recreational use and in medical field because of the psycho activity of THC, hemp represents an interesting multifunctional crop for industrial purposes for the low content in psychotomimetic chemicals. The exploitation of hemp has its roots in the past, when was used to obtain fiber from stalks and oil from "seeds" (Fike, 2016; Rehman et al., 2021). Nowadays, the range of applications is increased including textile, cosmetic, nutraceutical, chemical and energy, among others. The great value of industrial hemp lies in its relatively easy cultivation and maintenance, strong and durable fiber but also in the rich phytocomplex which includes several chemical classes. While cannabinoids are the most studied, more than 500 phytochemicals were isolated, including phenolic compounds, terpenes, fatty acids and steroids (Flores-Sanchez and Verpoorte, 2008). Due to the complex phytochemistry of *C. sativa* and the variability of its composition, it is important to have reliable and fast techniques of analysis to characterize the plant, differentiate strains and ensure safety of use. From a chemical point of view, the inflorescences are the most studied part of the plant for its high content in glandular trichomes which are the tissues with the highest biosynthesis of cannabinoids (Flores-Sanchez and Verpoorte, 2008). However, the inflorescences represent only a by-products of fiber production and Westerhuis and colleagues (Westerhuis et al., 2019) had reported that to obtain high quality fiber for textiles, hemp should be harvested before flowering. Moreover, although

the inflorescences represent a rich source of bioactive compounds, their use is usually controversial for the high content in CBD and derivatives. In fact, cannabis flowers as such or extracts of inflorescences are not allowed as cosmetic ingredients according to European legislation (CosIng database, 2022). For these reasons, more studies should be focused on the other parts of the plant, in particular, the stalk and leaves as the main products for industrial applications. In this sense, preliminary *in-vitro* studies on these parts of hemp plant, demonstrated an antioxidant and potential inhibition of tyrosinase enzyme activity, involved in skin-aging processes, supporting a possible use in cosmetic products (Mastellone et al., 2022). The valorization of plants and derived products is fundamental in view of a sustainable development and growth and industry aims to improve sustainability policies and implement new technologies (Li and Chemat, 2019). *C. sativa* perfectly fits this purpose being a multifunctional crop that can be exploited for many applications. Also, the scientific community is moving in this direction and new protocols were proposed in recent years. In particular, according to the principles of Green Chemistry (Anastas, 1999), the main goal is to reduce the use of toxic solvents, the energy consumption and the production of waste. For this purpose, new methodologies and extraction phases were developed and proposed for several applications, including *C. sativa* samples (Fiorito et al., 2022). In this context, deep eutectic solvents (DESs) arise as greener alternative to conventional solvents, thanks to their easy preparation and low cost of raw materials. They are composed by two or more components to form a hydrogen bonding network which is the key point for the formation of the DES. Different natural compounds were used as hydrogen bond donor (HBD) or acceptor (HBA) such as aminoacids, polyols, terpenes and fatty acids. Moreover, according to the nature of the HBA and HBD, hydrophilic or hydrophobic DESs can be prepared (Farooq et al., 2020). Thanks to these features, DESs were largely employed as extraction solvents of various bioactive compounds from plant samples and in microextraction approaches for their ability to selectively

enrich the compounds on interest (Cai et al., 2019; Hikmawanti et al., 2021; Křížek et al., 2018; Mastellone et al., 2021).

In this study, a reliable and sustainable method was used to extract different phytochemicals from fiber-type hemp samples, collected before flowering, mainly composed by stems and leaves. The proposed method is based on a dispersive solid-liquid microextraction (DSLME) method using different natural eutectic solvents (ESs) in order to reduce the consume of harmful solvents that are normally employed as extraction media in industry and laboratory routine. The quantification of the target analytes was carried out by high performance liquid chromatography coupled to PDA detector (UHPLC-PDA). The performance of the DSLME was carefully optimized and compared to a conventional solid-liquid extraction (SLE) method with MeOH to ensure the reliability and efficacy of the extraction for real applications.

2. Materials and Methods

2.1 Plant Material

The dried plant samples (fiber-type *Cannabis sativa* L.) were 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 (mainly stem and leaves) were collected before flowering (Mastellone et al., 2022). The harvested samples were immediately submitted to freeze-drying (using a lyophilizer), and subsequently grounded in a fine powder to pass a 1 mm screen with a Cyclotec mill (Tecator, Herndon, VA, USA). Finally, they were re-frigerated at 4 °C to prevent degradation.

2.2 Chemical and reagents

HPLC-grade acetonitrile, methanol (MeOH) (>99.9% purity), acetone (>99% purity), formic acid (>98% purity) and cannabidiolic acid (CBDA) were supplied by Merck Life Science S.r.l. (Milan, Italy). Luteolin-7-*O*-glucuronide and apigenin-7-*O*-glucuronide were supplied by Phytolab (Vestenbergsgreuth, Germany). De-ionized water (18.2 MΩ cm) was obtained from a

Milli-Q purification system (Millipore, Bedford, MA, USA). Individual standard solution of CBDA was prepared by dissolving it in methanol at 1 mg mL^{-1} and then diluted at different concentrations to obtain the calibration curve. This solution was kept protected from light and refrigerated at 4 °C. For the preparation of the natural eutectic solvents, (-)-menthol, linalool, thymol, carvacrol, terpinen-4-ol and eugenol were purchased from Merck Life Science S.r.l. (Milan, Italy). For ^{1}H NMR analysis, dimethyl sulfoxide (DMSO) was procured from Cambridge Isotope Laboratories (Andover, MA, USA).

2.3 Instrumentation and Equipment

A Radwag analytical balance (Radom, Poland) with a minimum readability of 10 mg was used to weight reagents, standards, and samples. An ultrasonic bath (Soltec, Sonica S3 EP 2400), a centrifuge (Remi group, Mumbai, India), a vortex (Thermo Fisher Scientific, Rodano, Italy) a rotary evaporator (Phoenix instruments, Garbsen, Germany) and a Visiprep™ SPE vacuum manifold (Merck, Milan, Italy) were employed for the reference extraction procedure. Solid phase extraction (SPE) cartridges packed with C18 silica sorbent (Agilent Technologies, Wilmington, DE, USA) were used for the purification of the extracts from chlorophylls. All samples were filtered with polyvinylidene fluoride (PVDF) syringe filters (0.20 μm) from CPS Analitica (Milan, Italy) before the injection in the LC system.

Quantitative analyses were carried out with a Shimadzu UHPLC XR chromatograph equipped with a photodiode array detector SPD-M20A (Shimadzu, Dusseldorf, Germany) using an Ascentis Express RP Amide column (10 cm × 2.1 mm, 2.7 μm, Supelco, Bellefonte, USA). Mobile phase A was water/formic acid (99.9:0.1, v/v) and mobile phase B was acetonitrile/formic acid (99.9:0.1 v/v). The gradient program was as follows: 0 to 3 min 5% B, 3 to 20 min 5% to 15% B, 20 to 30 min 15% to 25% B, 30 to 42 min 25% to 75% B, 42 to 52 min 75% to 100% B, 52 to 53 min 100% B with a flow-rate of 0,4mL/min. The total analysis time including pre- and post-running was 65 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. The calibration curve and the analytical performances of the method are reported in Table S1. Analyses were done in triplicate and the analytical performances were measured in terms of repeatability.

All HPLC data were processed using LabSolution software (Shimadzu, Dusseldorf Germany). For the characterization of the eutectic solvents a FTIR-ATR spectrometer (PerkinElmer, Waltham, Massachusetts, USA) and a Bruker (Massachusetts, USA) 600 MHz NMR spectrometer were used. Karl Fischer Coulometer Metrohm (Herisau, Switzerland) was employed to measure the water content of the ESs.

2.4 Preparation of the ES

The ESs were prepared following the heat and stirring method (Farooq et al., 2020). Briefly, the HBA and HBD were mixed at 1:1 molar ratio (see Table 1) under magnetic stirring for 30 minutes at 40 °C. After obtaining a homogeneous solvent, they were dried and store in a desiccator to prevent moisture absorption from the atmosphere.

Table 1. List of the ESs prepared in this study.

ID	Component 1	Component 2	Molar ratio	Method of preparation
ML	Menthol	Linalool	1:1	Heat and stirring
MTe	Menthol	Terpinen-4-ol	1:1	Heat and stirring
MTh	Menthol	Thymol	1:1	Heat and stirring
MC	Menthol	Carvacrol	1:1	Heat and stirring
LTe	Linalool	Terpinen-4-ol	1:1	Heat and stirring
LTh	Linalool	Thymol	1:1	Heat and stirring
LC	Linalool	Carvacrol	1:1	Heat and stirring
LE	Linalool	Eugenol	1:1	Heat and stirring
TeTy	Terpinen-4-ol	Thymol	1:1	Heat and stirring
TeC	Terpinen-4-ol	Carvacrol	1:1	Heat and stirring

2.5 Karl Fischer titration

The same amount (0.5 g) of ES and water was weighted in an Eppendorf tube and mixed manually with a spatula. The two phases were separated by centrifugation at 4000 rpm for 2

minutes and a small amount of the ES phase was injected in Karl Fischer coulometer. The same measurement was carried out before mixing the ES with water.

2.6 DSLME using natural ESs

A scheme of the optimized procedure is showed in Figure 1A. 100 mg of hemp were transferred in a centrifuge tube with 2 mL of water and 100 µL of the ES. After 30 seconds of vortex, the mixture was transferred in the bath sonicator (40 KHz at 25 °C) for 10 minutes. Once the extraction was completed, it was submitted to other 30 seconds of vortex and centrifuged for 5 minutes at 4000 rpm. As shown in Figure 1B, three different phases formed, starting from the bottom: (1) the plant, (2) water, and (3) the ES-rich phase. To allow an easy isolation of this latter, it was re-suspended in water (2) and the mixture (without the plant) was transferred in another tube and centrifuged again for 5 minutes at 4000 rpm. 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). Before the injection in the UHPLC-PDA, the extract was filtered with 0.20 µm, PVDF filter. The same method was used for the extraction of the reference standard compounds (luteolin-7-*O*-glucuronide, apigenin-7-*O*-glucuronide and CBDA), see section 3.5.

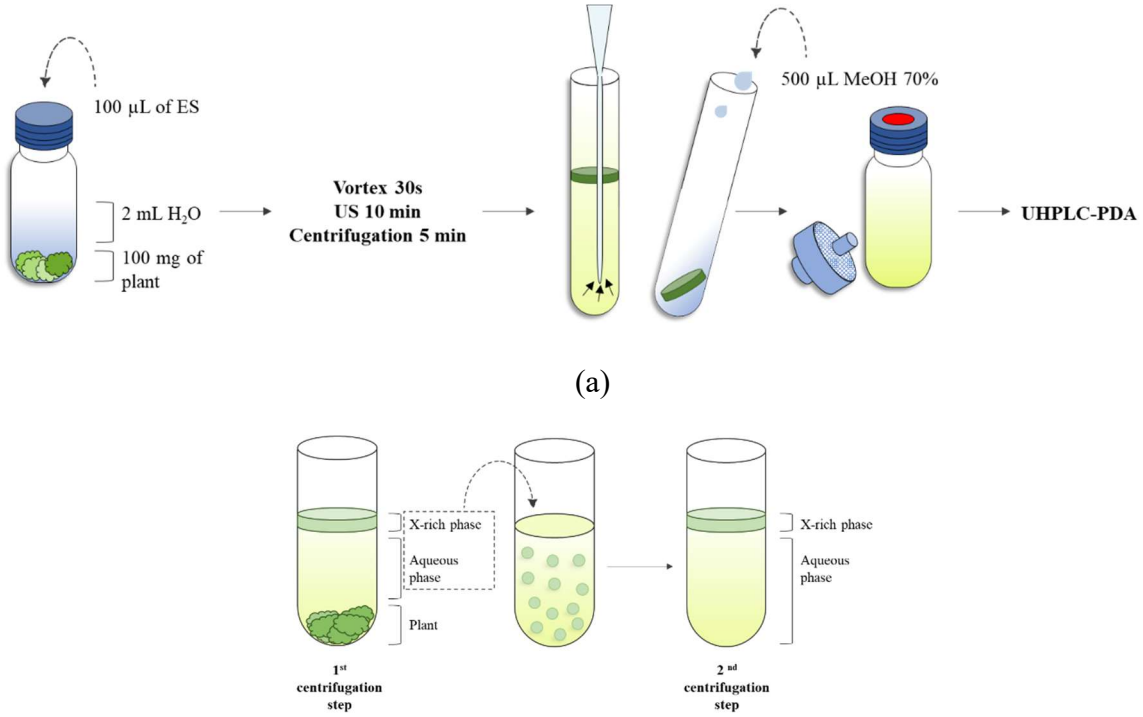

Figure 1. (a) Optimized DSLME method (b) focus on centrifugation step.

2.7 Reference SLE method

The reference method was previously optimized by our group and was used for comparison purpose. One extract was prepared without pre-concentrate the analytes, while for the second one, the solvent was evaporated to obtain a higher sensitivity, as reported in (Mastellone et al., 2022). Briefly, 5 mL of MeOH was added to 100 mg of sample and an US-assisted extraction was performed for 10 minutes at 40 KHz, at 25 °C. The liquid phase was then submitted to centrifugation at 4000 rpm for 10 minutes. The US extraction procedure was repeat twice on the same plant matrix to obtain an exhaustive extraction of the target analytes. After centrifugation, the supernatant was collected and filtered with filter paper (12 cm in diameter) prior to the injection in the HPLC.

The same procedure was repeated for the second extract but, after filtration, the solvent was completely evaporated at 40 °C in a rotary evaporator. At this point, the dried extract was submitted to SPE procedure 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 a C18 cartridge (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 mg/mL with MeOH/water (85:15, v/v) and finally filtered (0.20 μm, PVDF) before the injection in the LC instrument.

2.8 Statistical Analysis

Experimental designs and ANOVA statistical analysis were carried out by using Statgraphics® 18–X64 software. Excel software (Microsoft Office, v.2016) was employed for the remaining calculations.

3 Results and discussion

3.1 Preparation of the eutectic solvents

A set of different natural HBA and HBD were tested for the preparation of the ESs and after some preliminary tests, the combination menthol : linalool (1:1) resulted to be the most promising. For this reason, similar compounds were selected as acceptor and donor and the resulting combinations are reported in Table 1. The heat and stirring method was selected to be easy and fast to perform.

In order to minimize possible degradations caused by the exposure of the compounds at high temperatures for prolonged time (Farooq et al., 2020), 30 minutes and 40 °C were selected as optimum conditions to obtain homogeneous and stable solvents. After their preparation, the ESs were followed up at frequent intervals to monitor the formation of crystals, signal of instability. All the solvents showed to be stable even up to a month. To prevent evaporation of the components or moisture absorption they were sealed with parafilm and store in a desiccator.

3.2 Optimization of the dispersive solid liquid microextraction method

The combination menthol : linalool (1 : 1) was selected for preliminary tests and optimization of the extraction method for the analysis of the aerial parts of fiber-type *Cannabis sativa* L. DSLME method was developed with the aim of avoiding any pre-treatment of the solid sample. In fact, plant matrices, when dispersive liquid-liquid microextraction (DLLME) is applied, usually are previously subjected to liquid-liquid extraction (LLE) with solvent (Mastellone et al., 2021). In this case, water was simply used as co-solvent to promote the dispersion of the ES in the hemp sample. The volume and the pH of water, the sample amount and the ultrasound (US) time were the parameters considered in the optimization of the extraction method (Table S2). A screening test was carried out to determine which of these factors significantly affected the extraction performances. A full two-level factorial design (2^n, with n = 4 variables) was used for this preliminary study, which comprises sixteen experiments combining the minimum and maximum values considered for each factor. Moreover, three replicates of the central point (intermediate value for each variable) were carried out, in order to monitor the reproducibility of the method. The volume of water was evaluated in a range to have enough solvent to completely cover the sample and to not exceed the volume of the test tube and therefore the low and high limits were set at 1 and 4 mL, respectively. Regarding the sample amount, a minimum value of 50 mg and a maximum value of 100 mg were set. The limits for the extraction time were selected as 5 and 20 minutes, being common times reported in the literature for plant extraction based on US (Yusoff et al., 2022). Also, the pH of water was taken into account, considering the presence of acid cannabinoids in the hemp sample, as reported by a previous study on the same matrix (Mastellone et al., 2022).

With the aim of decreasing the ionization and solubility of these compounds in the water phase, an acid environment (pH = 3) was compared to the neutral condition (pH = 7). Once the experiments were performed, the extraction efficiency was evaluated in term of the sum of the peaks area of the target analytes. The Pareto chart and interactions plot reported in Figure S1A-

B showed that none of the variables considered had a significant influence on the extraction of the target analytes.

For this reason, the optimum conditions were chosen in order to simplify as much as possible the procedure. The volume of water (at a neutral pH) was set at 2 mL to completely cover the sample powder and allow an easy dispersion and separation of the ES. To easily compare the proposed DSLME method with a conventional SLE (section 2.7) already reported for the same hemp sample by our group, the same amount of plant (100 mg) was used. Eventually, the US time was set at 10 minutes to reduce the time of analysis but also allow the cell disruption and the release of the target analytes.

In addition, the effect of vortex time was considered in the optimization study, being the fundamental step to disperse the ES in the sample. A minimum time of 30 seconds and a maximum of 2 minutes were tested, taking into account for the higher value, the operator's health. In this case, an analysis of variance (ANOVA) test was carried out and no significance difference (F-ratio 0.28, P-value 0.60) in term of the sum of the peaks area of the target analytes was found between the two conditions and therefore 30 seconds was chosen as optimum time. In Figure S1C, the results are shown graphically in the Box-and-Whisker plot.

The optimum conditions (Figure 1A) were compared *versus* the same procedure without the US and the vortex steps to investigate the contribution of each step of the method to the extraction of the compounds of interest. Moreover, one extraction was performed by adding the ES after the US step. As reported in Figure S2, the best results were obtained with the optimized method, for all the compounds. This suggested that (1) as already reported for solid plant sample, US was fundamental to break the cell walls with the consequent release of the target analytes (Toma et al., 2001); (2) the vortex step helped to form a fine droplet of the ES in the sample, thus increasing the area of contact between the analytes and the extraction solvent; (3) the ES did not only pre-concentrate the analytes but also helped their extraction during the US step. In fact, as reported for ionic liquids (Kumari et al., 2020; Swatloski et al., 2002), also

DESs seem to be able to interact with the hydroxylic groups of cellulose, one of the main components of the plant cell wall. This interaction helps the disruption of the hydrogen bondings between the polymers thus favouring the releasing of the metabolites from the cells (Zhang et al., 2020).

Before the injection in the LC system, the analytes in the ES-rich phase were diluted in a small amount (500 µL) of MeOH/ H_2O (70/30, v/v). Also, the percentage of water in the hydro alcoholic diluting solution was optimized to make the final solution compatible with the chromatographic initial solvent composition and to avoid the interference of chlorophylls in the final extract.

3.3 Preliminary screening using different natural eutectic solvent and comparison with the conventional SLE method

Once the DSLME method was optimized, all the prepared solvents (Table 1) were tested as extraction media to analyse the phytocomplex of the aerial parts of fiber-type *Cannabis sativa* L. Each extraction was performed in triplicate and the results expressed as the mean area together with the standard deviation (SD). The results are reported in Figure 2. The chromatogram in Figure 3 showed that the main class of compounds present in the sample were flavonoids glycosides and the more apolar acid cannabinoids. The profile of ML as pure solvent presented some peaks at the wavelength of interest, however they did not interfere with the target analytes.

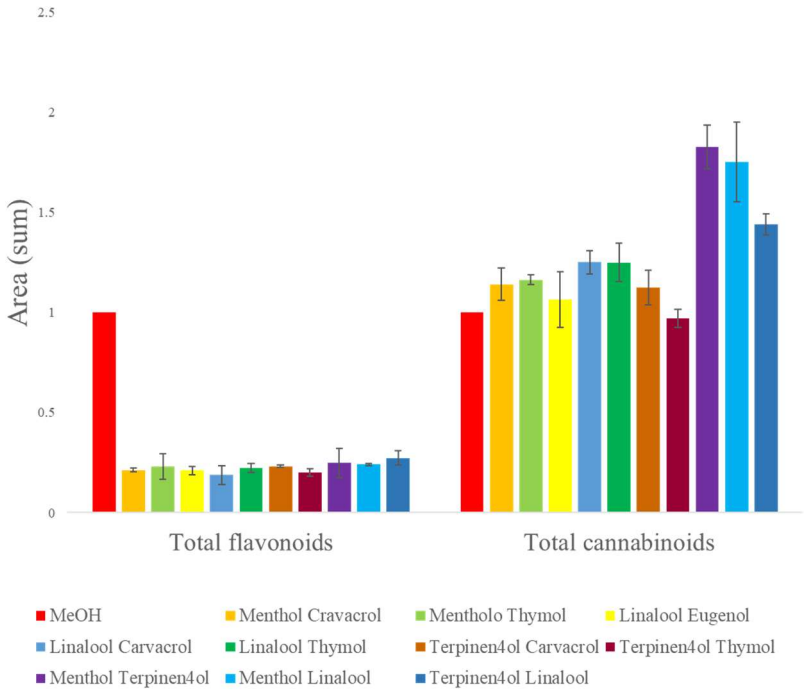

Figure 2. Comparison of the extraction performances (in term of peaks area) between the conventional SLE and the proposed DSLME, for flavonoids and acid cannabinoids. The red bar (MeOH) represents the SLE method.

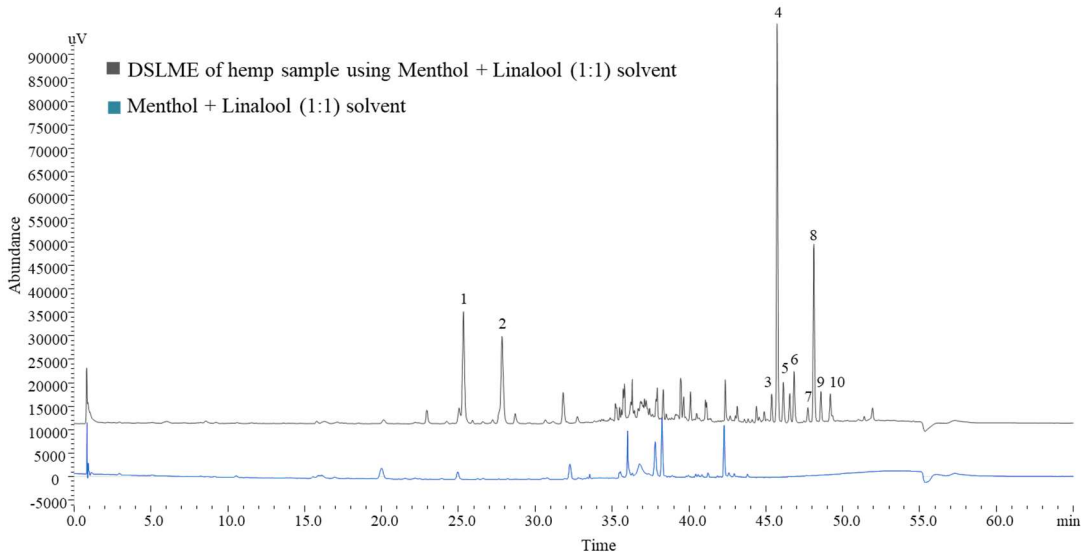

Figure 3. Chromatographic profile at 270 nm of the hemp extract after DSLME with ML and of the ML as pure solvent. Peaks identification: (1) luteolin-7-*O*-glucuronide, (2) apigenin-7-*O*-glucuronide, (3) cannabinoid A, (4) varinic acid derivative A, (5) varinic acid derivative B, (6) varinic acid derivative C, (7) cannabinolic acid, (8) Δ^9-tetrahydrocannabinolic acid, (9) cannabinoid B (10)

cannabichromenic acid. The identification data for the target compounds refers to a previous work on the same hemp samples, published by our group (Mastellone et al., 2022).

The SLE with MeOH as extraction solvent was more effective, compared to the ES, in the extraction of flavonoids which are more polar, while an opposite behavior could be observed for the acid cannabinoids. This can be explained by the hydrophobic characteristics of the ES which resulted in a higher interaction with the less polar cannabinoids. This is particularly true for three combinations: ML, MTe and LTe. In general, the presence of aromatic groups in the structure of one of the components (thymol, carvacrol, eugenol) did not seem to help the extraction. In fact, as reported in Table S3, the compounds forming the three ESs (ML, MTe and LTe) with the best extraction performances, do not present aromatic rings in their structure. Apart from the differences in the extraction efficiency of both methods, it is important to also consider the sustainability of the two approaches. The SLE required the use of a larger volume of methanol (15 mL), long evaporation time to concentrate the final extract and a purification step with SPE to eliminate chlorophylls prior to injection in the HPLC. The proposed DSLME is faster, comprises fewer steps and the use of a small volume (100 µL) of a greener solvent, thus being less tedious and easy to perform. Eventually, it could be a greener alternative for a selective and good enrichment of acid cannabinoids, without the need of evaporating the solvent by using nitrogen or energy. Recently, a metric tool to measure the analytical greenness of sample preparation methods was proposed by Wojnowski et al (Wojnowski et al., 2022).The software was used to compare the two extraction methods and the pictograms obtained are reported in Figure S3 of the supplementary material. As expected, the DSLME is characterized by a higher overall score, that indicates a higher sample preparation greenness performance. The description of the parameters considered in the metric, together with the values assigned for the two methods are also reported in the supplementary material.

3.4 Characterization of the eutectic mixture

The three solvents that showed the best extraction performances (section 3.3) were subjected to a deeper characterization through FTIR, NMR and hydrophobicity measurement.

3.4.1 FTIR analysis

ML, MTe and LTe were characterized by FTIR spectroscopy to monitor the correct formation of hydrogen bonding between the two components. In particular, the IR spectra of the single components were compared with that of the solvent, to monitor the shift of the band corresponding to the hydroxylic group.

Figure S4 of the supplementary material shows the representative FTIR spectra of the three solvents and their components. Regarding the single compounds, the menthol spectra showed the representative band of the hydroxyl group at 3248 cm^{-1}, linalool at 3381 cm^{-1} and terpinen-4-ol at 3463 cm^{-1}. For ML, when the solvent is formed, the band of the hydroxyl group of menthol shifts to a higher wavenumber than that of pure menthol and is slightly different from that of linalool. In the fingerprinting region (between 1500 and 600 cm^{-1}) a difference between the spectra of linalool and the solvent (e.g. 1000 cm^{-1}) can also be observed, suggesting the formation of new interactions. A similar behavior was observed with MTe, where menthol represents the acceptor of the hydrogen bonding, while a smaller shift of the hydroxylic band is reported for LTe.

The stability of the ES during the extraction process was then investigated by comparing the FTIR spectra before and after performing the DSLME. This investigation was fundamental to understand whether the ES was responsible of the extraction and not only one of the components since, as reported by (Cao and Su, 2021), the presence of water could break the hydrogen bonding of ESs, compromising their structure. Adopting the optimized method, the extraction was simulated without the plant and the ES-rich phase isolated and submitted to FTIR analysis. As reported in Figure S5, the profile of ML (use as reference during the

optimization) before and after the extraction were comparable, confirming that the interaction and enrichment of the target analytes was due to the ES.

3.4.2 NMR analysis

The formation of the ES and the molar ratio between the HBA and HBD was also verified by ^{1}H NMR. A mixture of the ES and water (in the same amount) was prepared, and the water layer was also analyzed by ^{1}H NMR. All spectra are reported in the Figure S6 of supplementary material. Recently, some concerns about the hydrophobicity of hydrophobic deep eutectic solvents (HDES) arose in the scientific community (Cao and Su, 2021). In fact, it was demonstrated that in some cases, only one of the components of the solvent is hydrophobic and the other partly solubilizes when the DES is put in contact with water. The NMR spectra of the water layer, clearly showed that both the components of the ES tested are characterized by a low solubility in water. These results were also supported by the water content percentage, measured by Karl Fischer Titration (see the following section).

3.4.3 Karl Fischer titration

As report by Florindo and colleagues (Florindo et al., 2019), the hydrophobicity of the DES can be evaluated by measuring their solubility in water. Karl Fischer titration is a well-established method to determine trace amounts of water in samples. In literature, the initial water contents of HDESs after preparation are in the range between 0.004 and 0.8 weight (wt) %, while, when mixed with water, they vary from 0.523 to 6.938 wt% in HDESs. In order to test the hydrophobicity of our solvents, the water content was measured before and after mixing the ES with the same amount of water. As reported in Table 3, all the ES tested in this study were within the accepted values for conventional HDESs.

Table 3. % of water in selected ES, measured by Karl Fischer titration.

ES component 1	ES component 2	H_2O content before partitioning	H_2O content after partitioning
Menthol	Linalool	0.044%±0.005	0.671%±0.005
Menthol	Terpinen-4-ol	0.079%±0.002	0.270%±0.013
Linalool	Terpinen-4-ol	0.105%±0.008	0.674%±0.027

3.5 Validation of the proposed method

The combination menthol : linalool (1:1) was selected as reference extraction solvent for the validation of the proposed DSLME method for the extraction of the non-volatile compounds from hemp aerial parts. The presence of a solid matrix adds more challenges in the validation and quantification of the target analytes, compared to a liquid sample. In fact, the analytes are subjected to different partitions: plant-aqueous phase and aqueous phase-ES. To study the behavior of the analytes in a simpler system, some preliminary tests were carried out with the commercial standard of luteolin-7-*O*-glucuronide, apigenin-7-*O*-glucuronide and CBDA, by performing the extraction without the plant. This latter was used as the reference standard for all the acid cannabinoids due to the similar physicochemical features. In agreement with the results obtained in section 3.3, CBDA was successfully extracted in the ES-rich phase, while the two flavonoids were poorly recovered (Figure S7). The selectivity of the method for this class of compounds was therefore confirmed and the validation study was focused on the acid cannabinoids.

Table S1 reports several quality analytical figures of merit of the method, including the λ selected for quantification, the linearity range, the calibration sensitivity (evaluated as the calibration slope), determination coefficient (R^2), limit of detection (LOD) and limit of quantification (LOD) of CBDA. In order to evaluate a possible carry-over effect for the analytes between consecutive analyses, a blank sample (MeOH 70%) was injected occasionally and randomly, confirming the absence of residual peaks for all the analytes of interest. The LODs

were experimentally determined by decreasing the concentration of the analyte in the extraction phase 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 and LOQ values were 0.20 mg L^{-1} and 0.25 mg L^{-1}, respectively.

Studies on intra-day repeatability, intermediate precision and enrichment factor were carried out to prove the improvement and applicability of the proposed method in the analysis of acid cannabinoids in real hemp samples. The quantification data obtained from the previous study (after assuring an exhaustive extraction of the compounds from the sample) (Mastellone et al., 2022) were used as a reference value to calculate the enrichment of the analytes from the plant. As reported in Table 4, the RSD values (intra and inter-day) for all the compounds are lower than 15%, showing a good reproducibility of the method. The enrichment factor (EF) of the proposed method was compared to the one obtained by performing the MeOH extraction, followed by evaporation of the solvent to concentrate the extract. The proposed DSLME with ES allowed to obtain comparable EF to the ones of SLE with MeOH. It is however important to highlight that in the conventional approach the extraction was repeated three times on the same matrix and the pre-concentration of the extract required the evaporation of the solvent with consumption of toxic organic solvents, nitrogen and energy for the rotary evaporator.

Table 4. Quantification data for cannabinoids, expressed as μg g^{-1} of hemp (±SD), precision data and enrichment factor for DSLME and the reference SLE.

Compound	μg g^{-1} of plant found with DSLME	Precision (*n*=3)		EF	
		Intra-day RSD %	Inter-day RSD %	DSLME	SLE[a]
Cannabinoid A	27.46 ± 3.04	9.16	4.94	5	7
Varinic acid A	245.77 ± 35.20	12.00	4.06	4	6
Varinic acid B	21.26 ± 2.06	8.13	1.24	8	7
Varinic acid C	43.29 ± 5.00	10.23	4.18	5	7
CBNA	18.54 ± 1.65	7.95	3.36	6	6
THCA	119.39 ± 17.01	11.74	2.81	4	5
Cannabinoid B	25.50 ± 2.59	10.39	3.89	6	6

CBCA	26.95 ± 3.41	11.96	1.93	5	5

[a] quantitation data obtained with an exhaustive SLE (Mastellone et al., 2022)

The main features of the proposed extraction method were compared to similar applications for the extraction of non-volatile phytochemicals from *Cannabis sativa* L. plant, reported in literature in recent years. Most of the studies focused on similar approaches, using US-assisted extraction or maceration in organic solvents, such as methanol, ethanol and acetone (Cicaloni et al., 2022; Jin et al., 2020; Pellati et al., 2018). More innovative methodologies are employed for the analysis of cannabinoids in non-plant origin samples, for forensic and clinical applications (Ahmad et al., 2021; Birk et al., 2021; Morisue Sartore et al., 2022; Tomai et al., 2021) (e.g blood, hair, oral fluid, urine). As reported in Table S4, the DSLME developed in the present study, demonstrates to meet the requirements in terms of novelty, sustainability, and reliability of the results.

4. Conclusions

In this study, an innovative dispersive solid liquid microextraction using natural eutectic solvents as extraction phase was proposed for the extraction of phytochemicals from fiber-type hemp matrices. In order to obtain optimal performance in term of sustainability but also efficiency, the DSLME method was optimized and validated, demonstrating to be faster, greener and easier to apply compared to a conventional SLE with MeOH, with comparable results in terms of extraction performances. The majority of the investigated ESs demonstrated to be more selective for cannabinoids compared to flavonoids enabling to efficiently extract this class of compounds directly from plant materials without using organic solvents. The tunability, stability, easy preparation, low cost and green features of the tested solvents make them good candidates not only for analytical application but also for industrial purposes, where easy to handle processes are required. Further advances may be directed to study the interaction of these eutectic solvents with other bioactive compounds with similar chemical characteristics

to cannabinoids, together with modifications of ES components to change the solvent selectivity.

Acknowledgements

The authors thank Francesco Gai from the National Research Council (Italy) for providing the *Cannabis sativa* L. samples and Dr. Jared Anderson from Iowa State University for allowing to carry out NMR and Karl Fischer experiments. This research was funded by the 'Ricerca Locale' (Ex60%2021) project of the University of Turin, (Italy). This article is based upon work from the Sample Preparation Study Group and Network supported by the Division of Analytical Chemistry of the European Chemical Society.

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Supplementary materials

Development of a dispersive solid-liquid microextraction method using natural eutectic solvents for a greener extraction of phytochemicals from fiber-type *Cannabis* sp.

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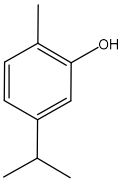
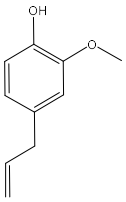
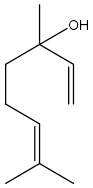
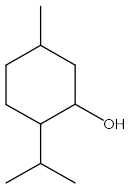
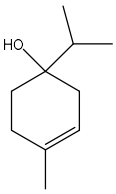
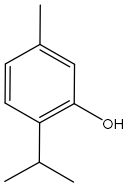
Table S1. Figure of merits of the UHPLC-UV method for CBDA.

Compound	Λ max (nm)	Linear Range (mg L^{-1})	Slope ± SD	$S_{x/y}$	R^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
CBDA	270	0.5 - 50	13592.37 ± 43.68	0.89	0.999	0.20	0.25

Table S2. Variables considered in the experimental design (2^n screening study), for the optimization of the extraction method.

Variable	Low value	High value
A. Volume of H_2O (mL)	1	4
B. Sample amount (mg)	50	100
C. US time (min)	5	20
D. pH of aqueous phase	3	7

Table S3. Molecular weight and chemical structure of the compounds used to prepare the ESs.

Compound	Molecular weight ($g\ mol^{-1}$)	Chemical structure
Carvacrol	150.22	
Eugenol	164.20	
Linalool	154.25	
Menthol	156.27	
Terpinen-4-ol	154.25	
Thymol	150.22	

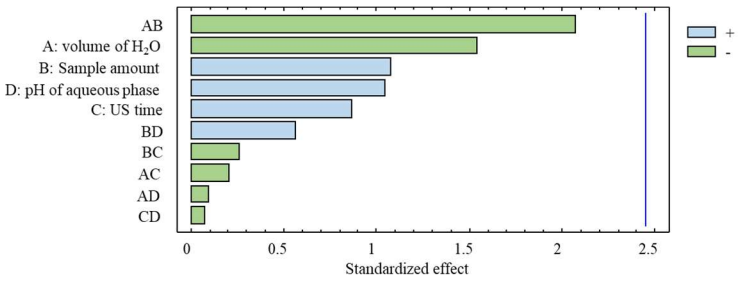
Sample	Target analytes	Pre-treatment of the sample	Type of solvent	Volume of solvent	Extraction time	Analytical platform	LOQ	Ref
Reference studies								
Leaves, stem	Flavonoids, cannabinoids	SLE[a]	MeOH and acetone	15 mL	30 min, US[b]	UHPLC-PDA/MS	nr[c]	Mastellone et al., 2022
Inflorescences	Flavonoids, cannabinoids, terpenoids	SLE	EtOH 70%	5 mL	30 min, US	UPLC-MS	nr	Cicaloni et al., 2022
Inflorescences, leaves, stem bark, and roots	Flavonoids (1) cannabinoids (2)	SLE	EtOH, water, HCl 25:10:4, MeOH (1) MeOH (2)	5 mL, 50 mL (1) 20 mL (2)	135 min, water bath (2) 20 min, US (2)	HPLC-PDA/MS	nr	Jin et al. 2021
Inflorescences	Flavonoids (1), cannabinoids (2)	SLE	*n*-Hexane, acetone (1) EtOH (2)	25 mL, 25 mL (1) 25 mL (2)	45 min (1) 45 min (2)	HPLC-PDA/MS	1.3-2.5 mg L^{-1}	Pellati et al., 2018
This work								
Leaves, stem	Flavonoids, cannabinoids	DSLME	Hydrophobic NADES	100 µL	10 minutes, US	UHPLC-PDA	0.25 mg L^{-1}	This work

Table S4. Main features of representative extraction methods from *Cannabis sativa* L. samples, for the analysis of phytochemicals by liquid chromatography.

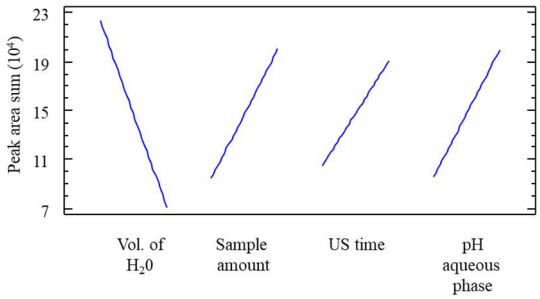
[a] Solid liquid extraction

[b] Ultrasound

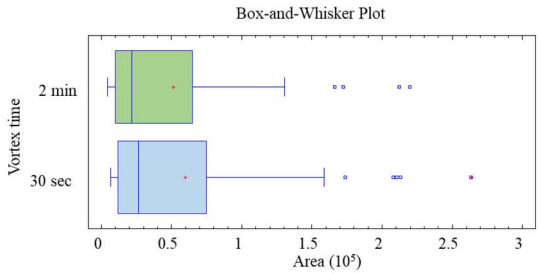
[c] Not reported



(a)



(b)



(c)

Figure S1. (a) Pareto chart of the 2^n screening study, (b) interactions plot of the 2^n screening study and (c) box-and-Whisker Plot.

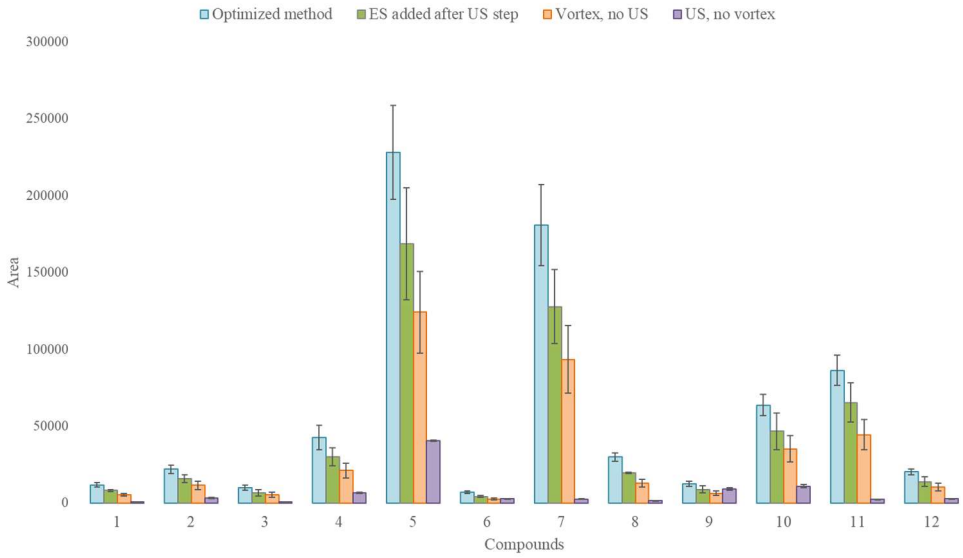
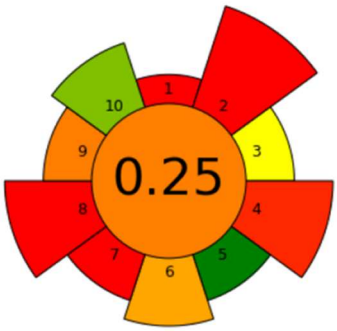
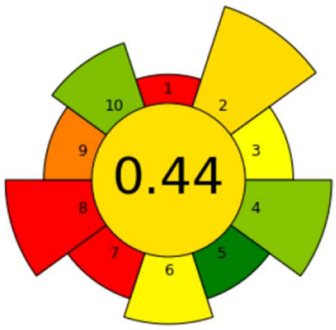


Figure S2. Comparison of the extraction performances (in term of peaks area) between the optimized DSLME method (blue), the addition of the ES after US step (green), the extraction without the US step (red) and without the vortex step (violet). For peaks identification, see Figure 3.



#	Criterion	Score	Weight
1.	Sample preparation placement Sample preparation placement: Ex situ	0.0	1
2.	Hazardous materials Mass [g] or volume [mL] of problematic materials: 30	0.0	5
3.	Sustainability and renewability of materials Materials are not sustainable or renewable and are used several times	0.5	2
4.	Waste Mass [g] or volume [mL] of waste: 30.1	0.08	4
5.	Size economy of the sample Mass [g] or volume [mL] of the sample: 0.1	1.0	2
6.	Sample throughput Hourly sample throughput: 4	0.33	3
7.	Integration and automation No. of sample prep. steps: 6 steps or more; degree if automation: Manual systems	0.0	2
8.	Energy consumption Approximate energy consumption per analysis [W]: 3000	0.0	4
9.	Post-sample preparation configuration for analysis Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10.	Operator's safety No. of distinct hazards: 1 hazard	0.75	3

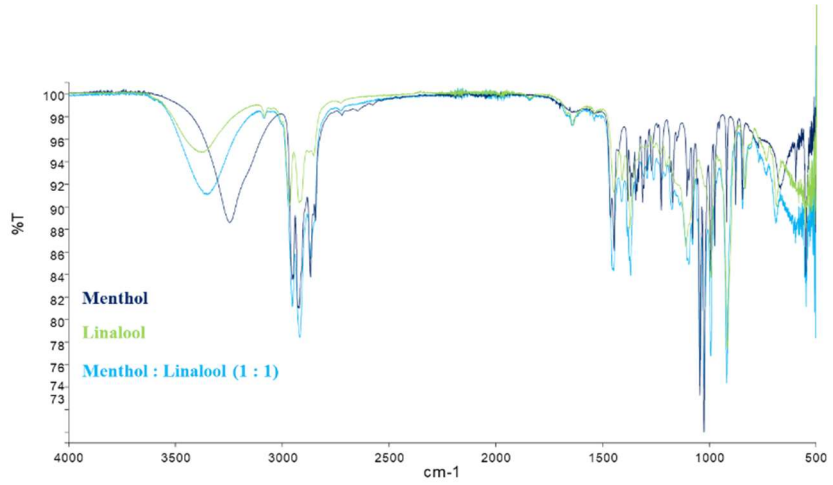
(a)



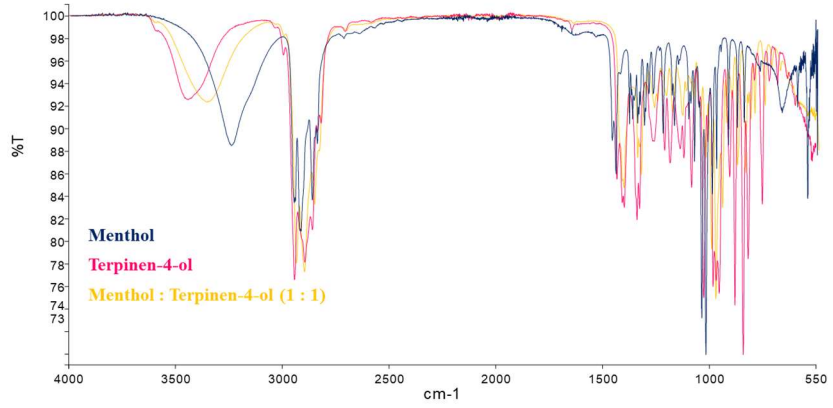
#	Criterion	Score	Weight
1.	Sample preparation placement Sample preparation placement: Ex situ	0.0	1
2.	Hazardous materials Mass [g] or volume [mL] of problematic materials: 0.5	0.43	5
3.	Sustainability and renewability of materials 50-75% of reagents and materials are sustainable or renewable and can only be used once	0.5	2
4.	Waste Mass [g] or volume [mL] of waste: 0.5	0.74	4
5.	Size economy of the sample Mass [g] or volume [mL] of the sample: 0.1	1.0	2
6.	Sample throughput Hourly sample throughput: 8	0.49	3
7.	Integration and automation No. of sample prep. steps: 6 steps or more; degree if automation: Manual systems	0.0	2
8.	Energy consumption Approximate energy consumption per analysis [W]: 600	0.0	4
9.	Post-sample preparation configuration for analysis Liquid chromatography, gas chromatography with quadrupole detection, etc.	0.25	2
10.	Operator's safety No. of distinct hazards: 1 hazard	0.75	3

(b)

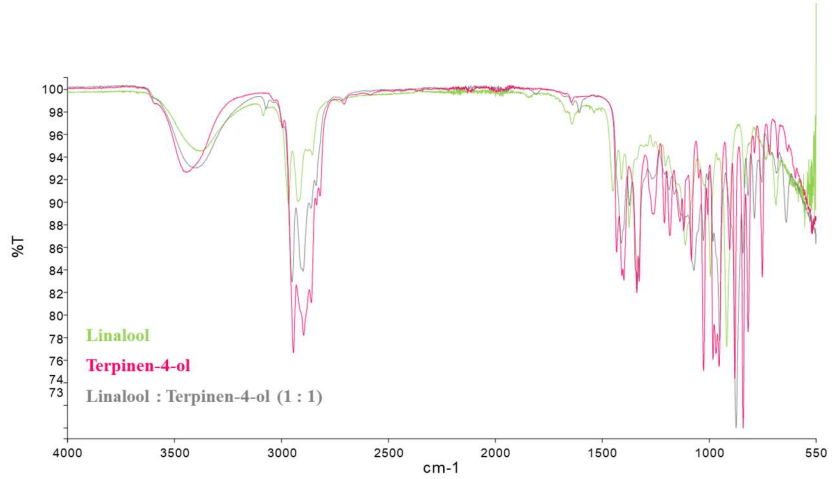
Figure S3. Pictograms and reports obtained with AGREEprep metric (Wojnowski et al., 2022), for (a) SLE and (b) DSLME.



(a)



(b)



(c)

Figure S4. FTIR spectra of (a) menthol, linalool and ML, (b) menthol, terpinene-4-ol and MTe and (c) linalool, terpinene-4-ol and LTe.

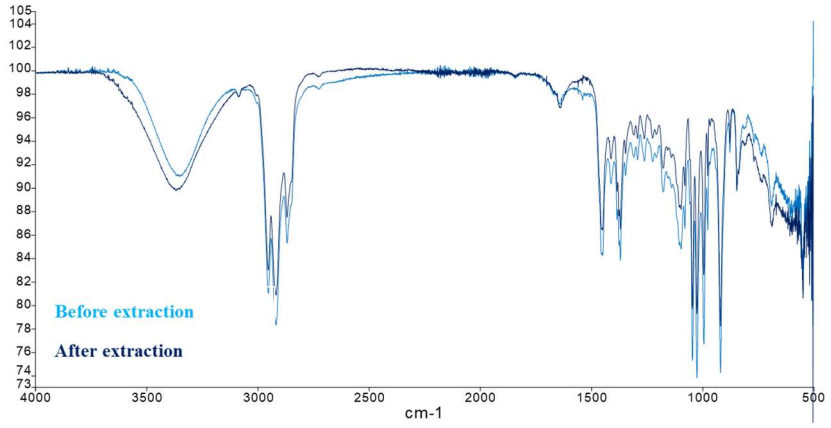
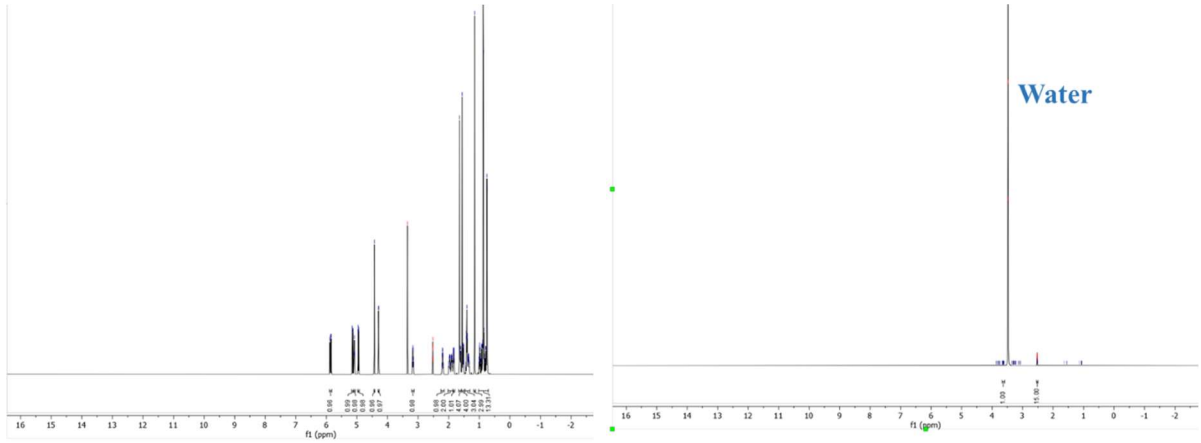
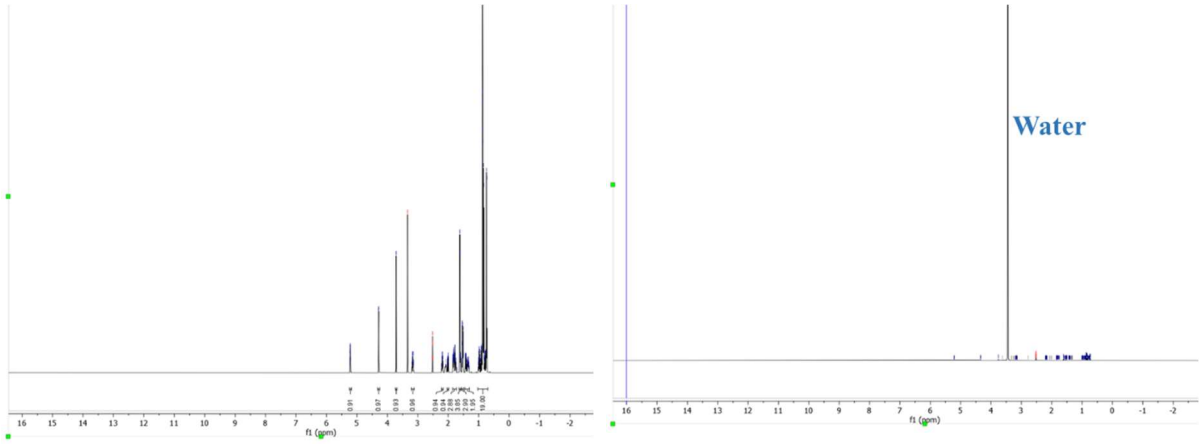


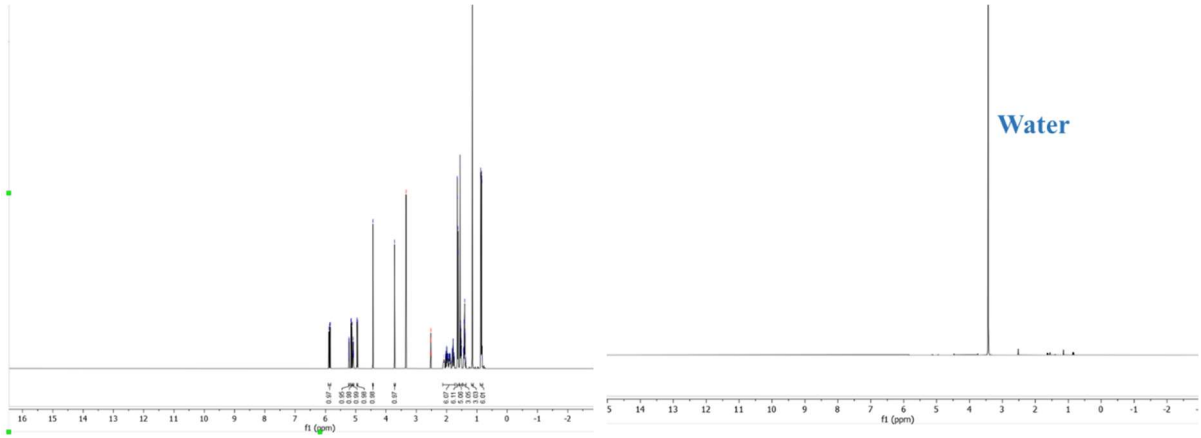
Figure S5. FTIR spectra of ML as pure solvent and after the DSLME process.



1(a) 1(b)



2(a) 2(b)

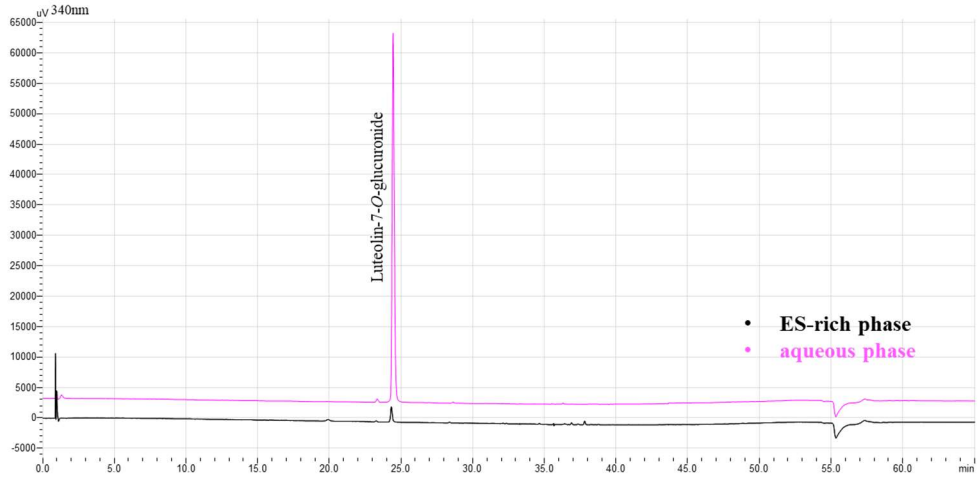


3(a) 3(b)

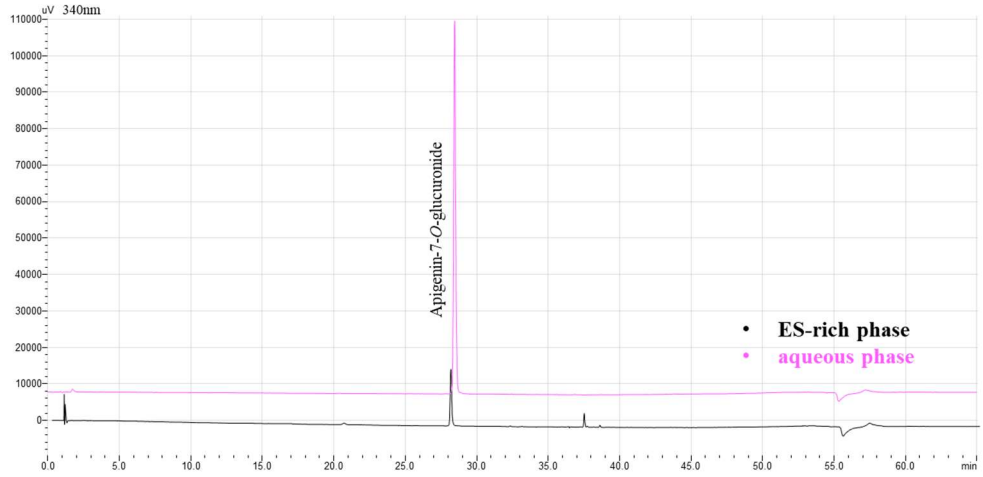
Figure S6. ^{1}H NMR spectra of 1(a) ML and 1(b) its water layer, 2(a) MTe and 2(b) its water layer and 3(a) LTe and 3(b) its water layer.

ML: ^{1}H NMR (600 MHz, DMSO) δ 5.86 (dd, J = 17.3, 10.7 Hz, 1H), 5.14 (dd, J = 17.4, 2.0 Hz, 1H), 5.08 (t, J = 6.7 Hz, 1H), 4.95 (dd, J = 10.8, 2.0 Hz, 1H), 4.43 (s, 1H), 4.29 (d, J = 5.6 Hz, 1H), 3.17 (tt, J = 10.2, 4.9 Hz, 1H), 2.20 (pd, J = 7.0, 2.6 Hz, 1H), 2.02 – 1.80 (m, 3H), 1.68 – 1.59 (m, 4H), 1.59 – 1.48 (m, 4H), 1.46 – 1.31 (m, 3H), 1.15 (s, 3H), 1.02 – 0.72 (m, 13H).

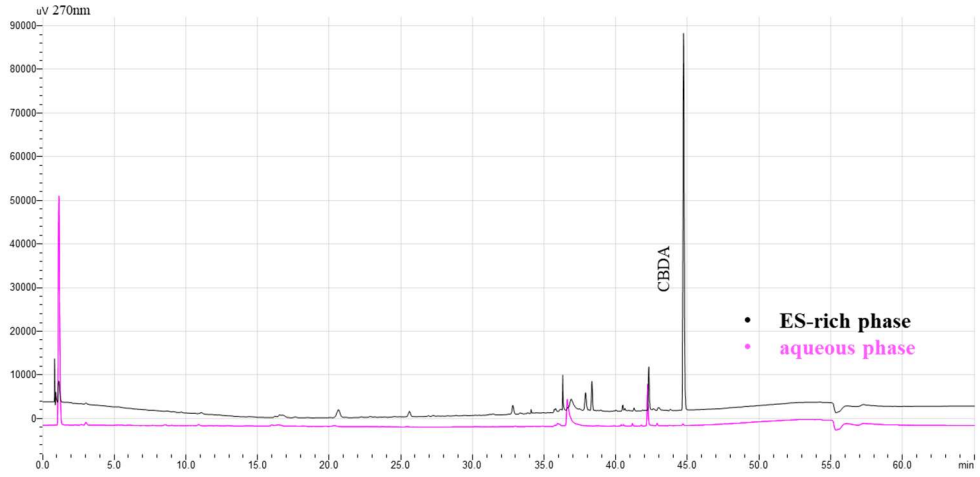
MTe: ^{1}H NMR (600 MHz, DMSO) δ 5.21 (ddt, J = 4.4, 3.0, 1.6 Hz, 1H), 4.29 (d, J = 5.6 Hz, 1H), 3.71 (s, 1H), 3.16 (tdd, J = 10.1, 5.6, 4.2 Hz, 1H), 2.19 (hd, J = 7.0, 2.7 Hz, 1H), 2.01 (dp, J = 17.6, 2.6 Hz, 1H), 1.87 – 1.70 (m, 3H), 1.65 – 1.59 (m, 4H), 1.59 – 1.49 (m, 3H), 1.46 – 1.37 (m, 1H), 1.37 – 1.29 (m, 1H), 0.99 – 0.71 (m, 20H). LTe: ^{1}H NMR (600 MHz, DMSO) δ 5.86 (dd, J = 17.3, 10.7 Hz, 1H), 5.22 (tp, J = 3.1, 1.4 Hz, 1H), 5.14 (dd, J = 17.3, 2.0 Hz, 1H), 5.08 (tp, J = 7.2, 1.4 Hz, 1H), 4.95 (dd, J = 10.7, 2.0 Hz, 1H), 4.43 (s, 1H), 3.71 (s, 1H), 2.13 – 1.73 (m, 6H), 1.67 – 1.59 (m, 6H), 1.58 – 1.48 (m, 5H), 1.46 – 1.34 (m, 3H), 1.15 (s, 3H), 0.85 (dd, J = 14.0, 6.9 Hz, 6H).



(a)



(b)



(c)

Figure S7. Comparison of the chromatographic profile of (a) luteolin-7-*O*-glucuronide, (b) apigenin-7-*O*-glucuronide and (c) CBDA in the ES-rich phase *versus* the aqueous layer, after performing the DSLME.