



Towards eco-metabolomics: NADES-guided extraction enables semi-quantitative metabolomics for *Melissa officinalis*

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ABSTRACT

In recent years, there has been a growing emphasis on the development of green extraction techniques that minimize environmental impact while maximizing yield of the extracted compounds. To this aim, in this study we investigated the potential of green solvents for extracting bioactive compounds from *Melissa officinalis* (MO) leaves. Specifically, we focus on the application of 20 Natural Deep Eutectic Solvents (NADES) with a relative polarity ranging from 0.34 to 1.29. Their extraction affinity against a set of 11 plant metabolites was predicted using COSMO-RS software and experimentally validated using quantitative LC–HRMS analysis. Subsequently, the same extracts were subjected to non-target metabolomics to uncover the NADES selectivity towards the wide spectrum of MO leaf metabolites. Data preprocessing and feature alignment were performed using MZmine, and aligned features were annotated using SIRIUS+CSI:FingerID.

Overall, 249 and 195, metabolites were annotated in positive and negative ionization ion mode, respectively. Additionally, to have a more accurate view of the different NADES extraction capacity, we adopted a semi-quantitative approach that enables the prediction of concentration for all the annotated metabolites (N = 444).

The results highlighted the selectivity of some NADES in extracting very diverse biochemical classes, providing valuable insights into the composition and concentration of bioactive compounds. Interestingly, thymol-menthol NADES demonstrated the ability to efficiently extract a broad range of bioactive compounds, yielding a metabolome comparable to that obtained with conventional ethanolic. Overall, the entire workflow facilitated the green extraction and annotation of known bioactive molecules that had never been described in MO.

1. Introduction

Plants have long been recognized as a rich source of bioactive compounds with diverse pharmacological properties, making them integral to traditional medicine and modern drug discovery efforts. Among these, *Melissa officinalis* L. (MO), commonly known as lemon balm, stands out for its reputed therapeutic benefits attributed to its bioactive constituents [1]. The extraction of bioactives from MO leaves has garnered considerable interest, driven by the need for efficient and sustainable extraction methods that preserve the integrity of the molecules. In recent years, there has been a growing emphasis on the development of green extraction techniques that minimize environmental impact and energy consumption while maximizing yield of the

extracted compounds. Natural Deep Eutectic Solvents (NADES) have emerged as a promising alternative to conventional solvents, offering advantages such as biodegradability, low toxicity and versatility in extracting a wide range of compounds [2]. NADES are mixtures of two or more compounds, a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), which due to molecular interactions, when mixed in a certain molar ratio, suffer a large depression of their melting point, becoming a liquid at room temperature. NADES have gained significant attention due to their ability to dissolve a wide range of bioactive compounds while being environmentally friendly. These solvents can be tailored to selectively extract specific classes of compounds based on their composition, which is crucial for comprehensive metabolomic studies and natural products discovery. Studies have shown that NADES

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can enhance the extraction efficiency and stability of bioactive compounds compared to conventional solvents [3]. For instance, certain NADES have been optimized to selectively extract highest yields of flavonoids [4] or coumarin [5] or triterpenoids [6], highlighting their potential for future industrial scale applications [7]. However, scaling up the use of NADES from laboratory to industrial scale involves several considerations, including issues with viscosity and pH, as previously described [1,8]. As a result, despite the numerous patents on plant extraction with NADES, only a few are related to their industrial application [9,10,11], with the cosmetic industry being the most active. In this context, MO was selected as vegetable biomass to be extracted because (i) it is cultivated/grown all over the world and (ii) because its medicinal properties are known since ancient time [12], therefore, there are several scientific papers available concerning its metabolites composition. In the present study, the potential of twenty NADES, for the extraction of bioactive compounds from MO leaves, was explored using a non-targeted semi-quantitative metabolomic approach. This allowed for a comprehensive profiling of MO metabolome, revealing a selectivity of some NADES on one hand but also the broad-spectrum extraction efficacy of other NADES. The non-targeted approach also facilitated the annotation of known bioactive molecules that had never been described in MO. This further emphasizes the importance of non-targeted approaches and NADES in plants metabolomics. Through advanced mass spectrometry analysis coupled with machine learning modeling, this research aims to elucidate the behavior and affinity of NADES with different polarity and different physicochemical properties towards bioactive molecules present in MO.

The study aims to assess the efficacy of NADES in selectively extracting biochemical classes from MO leaves, and consequently the production of tailored extracts with diverse biological activities and different secondary metabolites composition.

2. Materials and methods

2.1. Chemicals and reagents

For NADES synthesis, betaine anhydrous (>97 %, CAS 107–43–7) was obtained from TCI (Tokyo, Japan), D-(+)-glucose (CAS 50–99–7) was purchased from Cmd Chemicals (Funchal, Portugal). Choline chloride (≥ 98 %, CAS 67–48–1), glycerol (≥ 99.5 % CAS 56–81–5), DL-proline (99 %, CAS 609–36–9), citric acid (>99.5 %, CAS 77–92–9), ($\pm$)-1,3-butanediol (CAS 107–88–0), urea (CAS 57–13–6), menthol (CAS 1490–04–6), thymol (CAS 89–83–8), decanoic acid (CAS 334–48–5), dodecanoic acid (CAS 143–07–9) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). LC-MS grade methanol and acetonitrile were purchased from Biosolve Chimie (Netherlands); bi-distilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA), formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA) and Tryptophan D5 (CAS 62,595–11–3) from Sigma-Aldrich (St. Louis, Missouri, USA). For target analysis, the following standards were purchased from Sigma-Aldrich Chemie GmbH (Germany): ursolic acid (CAS 77–52–1), D-(+)-galactose (≥ 99 %, CAS 3646–73–9), DL-phenylalanine (CAS 63–91–2), 7-amino-4-methylcoumarin (CAS 26,093–31–2), abietic acid (CAS 514–10–3), jasmonic acid (CAS 77,026–92–7), berberine chloride (CAS 633–65–8), oleic and linoleic acid (CAS 112–80–1; 60–33–3). Fluka Chemie GmbH (Buchs, Switzerland) provided: rutin (CAS 153–18–4), myricetin (CAS 529–44–2) and chlorogenic acid (CAS 327–97–9). Rosmarinic acid (CAS 20,283–92–5) and kaempferol (CAS 520–18–3) were obtained from PhytoLab GmbH & Co. KG (Germany).

2.2. Plant material

Dried biomass of MO leaves was obtained from the European Regenerative Organic Centre (EROC) located in Parma, Italy. Plant material was collected in June 2023 and dried in oven at 40 ± 3 °C for

12 h. Dried leaves from different batches were randomly selected and used for the present study. After drying process, leaves were grinded using a grinder (particle size <800 μ m). After grinding, the plant was stored at room temperature in sealed bags protected from light.

2.3. Synthesis of NADES and plant-based NADES

NADES were synthesized following the procedure previously reported [13]. The selection of NADES components was based on their polarity parameter to cover a wide range of polarity and to assess their efficiency and selectivity. Briefly, HBD and HBA were mixed in the defined molar ratio, heated and stirred until a clear solution was formed. A total of nineteen NADES combination were prepared using the heating and stirring method. Among the nineteen NADES prepared, eleven were binary and eight ternary deep eutectic mixtures. All the NADES were cooled down at room temperature, stored in sealed glass bottle and kept in a dry place at room temperature until further use [13]. Beside conventional NADES, one plant-based (PB) NADES was prepared following the procedure reported by Qader et al. [14]. Briefly, the biomass (MO) was mixed with ChCl in a ratio of 1:10 (w/v). The produced mixture was kept in an oven at 50 °C for 72 h. Then the mixture was left at room temperature in an open vessel for 10 days to extract as much natural product as possible, following the procedure reported by Qader et al. [14]. After 10 days the liquid solvent obtained was filtrated to separate the NADES from the biomass. NADES polarity was measured by solvatochromic dye Nile red [15].

2.4. Sample preparation

The screening of NADES systems for the extraction of bioactives was carried out using maceration technique as reported in the European Pharmacopoeia [16]. 100 mg of dried leaves were placed into polypropylene microcentrifuge tubes and each solvent was added in a ratio of 1:10. The maceration process, carried out at 25 °C lasted for 24 h with constant stirring at 400 rpm. After 24 h samples were centrifuged for 40 mins at 25 °C and then the exhausted MO leaves were separated from the fluid extract. Extraction was performed in triplicate (23 extraction solvents (20 NADES and 3 conventional solvents) x 3 replicates = 69 samples) and samples stored at 4 °C until further analysis. AGREEprep score was used to assess the greenness of the sample preparation obtained using conventional solvents (ethanol) versus NADES [17]. Samples for injection have been prepared the same day of the analysis. Fluid extracts have been further diluted 1:40 in optima grade methanol and added with the internal standard (Tryptophan D5) to obtain a 1 ppm concentration in vial. The sample extracts underwent targeted and non-targeted analysis. The targeted analysis aimed to i) quantify the extraction efficiency of the different NADES over a set of 11 metabolites and ii) to assess the accuracy of COSMO-RS (Conductor-like Screening Model for Real Solvents, Software for Chemistry and Materials BV, Amsterdam, The Netherlands) in predicting the extraction efficiency. This allowed for precise quantification and identification of known compounds, ensuring accurate monitoring of specific metabolites. On the other hand, the non-targeted approach enabled the comprehensive characterization of the MO metabolome, providing a broader understanding of sample's biochemical diversity. This dual strategy ensured a thorough evaluation of the extract, enhancing the reliability and scope of the study.

2.5. Targeted analysis

An extensive literature review was conducted concerning the known MO metabolites and their concentrations, resulting in 212 metabolites belonging to different biochemical classes. Among those, we selected 11 representative metabolites from 8 biochemical classes covering a range of XLogP [18] from -2.6 to 7.3 (see Table S1). These 11 metabolites were used to optimize the chromatographic separation, to check the

recovery with the 23 solvents and to perform the semi-quantitative analysis. The chromatographic conditions used allowed to obtain a good separation of the 11 metabolites representatives of different biochemical classes, as depicted in **Fig. S1**. For accurate quantification and validation, calibration curve of chlorogenic acid, rutin, jasmonic acid, rosmarinic acid, phenylalanine, abietic acid, 7-amino-4-methylcoumarin, kaempferol, ursolic acid, D-galactose and linoleic acid were constructed to quantify the analytes in the different samples. Ranges of the calibration lines have been selected after injection of the different samples to cover the broad range of concentration expected in the diverse extracts. The regression coefficient (R^2) value of the calibration curves, the concentration, the XLogP of analytes, the Limit of Quantification (LOQ) and Limit of Detection (LOD) are reported for completeness in the **Table S1**. The same metabolites were also used as calibrants for semi-quantification model validation (as described in section semi-quantitative metabolomics), in order to find a linear regression between predicted ionization efficiencies and measurement specific response factors [19].

2.6. UHPLC—HESI-HRMS analysis

The data acquisition was performed using Agilent 1290 UHPLC system coupled to a Thermo Fisher Q-Exactive Focus Quadrupole-Orbitrap Mass Spectrometer equipped with an electrospray ionization (ESI) interface. The 69 samples were injected (5 μ L) and chromatographically separated using a reversed-phase C18 HSS T3 Acquity column 2.1 $\times$ 100 mm, 1.8 μ m particle size (Waters, Milford, MA, USA). A gradient profile was applied using water (eluent A) and acetonitrile (eluent B), both acidified with 0.1 % formic acid as mobile phases. A multi-step elution dual-mode gradient was developed. After an isocratic step of 2 min (0.0 to 2.0 min, 5 % B; 0.40 mL/min) the B% increased up to 99 % B in 11.5 min and then held for 3.5 min (until 15.0 min). Afterwards, in 1 min the initial conditions were restored to 5 % B. The total run time was 21 min including 5 min of re-equilibration. The column was maintained at 40 °C and a flow rate of 0.4 mL/min used. The settings of the interface, HESI source, were configured to operate effectively in both negative and positive ion modes, and the parameters were set as follows: sheath gas flow rate 30 mL/min, aux gas flow rate at 15 mL/min and sweep gas flow rate at 33 mL/min, spray voltage 3.2 kV, capillary temperature 320 °C, S-lens RF level 47.0, aux gas heater temp 0 °C. The mass spectra were acquired in DDA (Data Dependent Analysis) by full scan MS1 and MS2 in positive and negative ionization mode in a scan range from 100 to 1000 m/z. The full MS resolution was 70,000 FWHM, AGC target 3e6, maximum IT 200 ms and with a profile spectrum data type. The dd/MS² analysis was performed with a resolution of 17,500 FWHM with three different collision energy: 15,30,45 eV, AGC target 2e5, maximum IT 50 ms, loop count of 3, minimum AGC target of 8.00e3, intensity threshold 1.6e5, apex trigger 2 to 15 s, dynamic exclusion of 4.0 s, charge exclusion 2–8, and a profile spectrum data file.

2.7. Computational screening of NADES

To assess the interactions between different NADES and plant metabolites, σ -profiles were generated. For this purpose, the COSMO-RS software, version 2022.103, within the Amsterdam Density Functional (ADF) package was utilized [20]. A universal force field (UFF) was employed to pre-optimize the molecular geometry of both the extracted compounds and the NADES constituents, using the density functional GGA (Generalized Gradient Approximation) with the Triple Zeta Plus (TZP) basis set. The σ -profile, which represents a histogram of the charge density distribution on the molecular surface, was used to identify the affinity of a component in various solvents. The process involves two primary steps. First, the COSMO model creates a virtual conductor environment, allowing the polarization charge density on the surface to reach its optimal energetic state. Subsequently, a statistical thermodynamic calculation determines the interaction energy between pairs of

surface segments, taking into account electrostatics and hydrogen bonding. This step yields the surface composition function, or σ -profile, as well as the chemical potential of the surface segment, referred to as the σ -potential [20].

2.8. Non-targeted metabolomics workflow

The 69 samples that were screened for the targeted analysis also underwent non-targeted analysis. By using the same set of samples, chromatographic and High-Resolution Mass Spectrometry (HRMS) conditions, the study provides a robust framework for understanding the full metabolome profile of the extracts. In order to measure performance and system stability, Quality Control samples (QC) were also prepared and injected during the analyses. QC were obtained by mixing equal volumes (10 μ L) of all samples extracts and internal standard following the same procedure as for the other samples.

2.9. Multivariate data analysis

MetaboAnalyst was employed as a tool for conducting multivariate analysis, with the primary aim of confirming the absence of any anomalies in the instrumental analysis process, thus enhancing the overall accuracy and reliability of the data collected. The data set was normalized using the internal standard (Tryptophan D5), and then PCA (Principal Component Analysis) was performed for both target and non-target analysis. Raw data were centered and Pareto scaled prior to PCA analysis to assess the quality of the data and to visualize trend within the different NADES solvent. MetaboAnalyst was also used to build **Fig. 2**, which reports the Variable Importance in Projection (VIP) scores calculated by the MetaboAnalyst Partial Least Squares- Discriminant Analysis (PLS-DA) module [21]. R-studio [22] was employed as a tool for conducting multivariate analysis in parallel to MetaboAnalyst. Concentrations obtained from the target analysis were uploaded and the matrix was used for multivariate analysis in order to graphically visualize the capacity and the selectivity of solvents to extract the metabolites of interest based on their XLogP (metabolites) and solvent polarity (NADES).

2.10. Data pre-processing and features alignment

Data acquired in the .RAW Thermo format, were converted to .mzML using Proteowizard's MSConvertGUI (64-bit) tool [23] and then imported to MZmine 4.0.3 an open source software [24], to perform data a pre-processing workflow using Processing wizard (or ADAP Automated Data Analysis Pipeline) including EIC (Extracted Ion Chromatogram) construction, chromatographic peak detection, spectral deconvolution and alignment with the following parameters: (1) UHPLC chromatogram builder= retention time 0.20 –19.00 min, max peaks in chromatogram 15, MS1 level minimum consecutive scans 5, approximate feature FWHM 0.050 mins, RT tolerance (intra-sample), RT tolerance (sample-to-sample) 0.100 mins. (2) Orbitrap: ion mode (positive or negative), factor of lowest signal (with centroid data), noise threshold MS1 noise level 5.0E3, MS2 noise level 1.0E3, minimum feature height 1.0E5, m/z tolerance (scan-to-scan) 0.0020 m/z or 10.000 ppm, m/z tolerance (intra-sample) 0.0015 m/z or 3.000 ppm, m/z tolerance (sample-to-sample) 0.0015 m/z or 5.000 ppm. (3) Smoothing= Savitzky Golay; (4) Local minimum feature resolver= minimum relative feature height 25 %, chromatographic threshold 75 %, Minimum search range RT/Mobility 0.050, minimum absolute height 5E4, peak duration 0.10–3.00 min; (5) Isotopic filtering= retention time tolerance 0.020 s, m/z tolerance 0.0010–5.000 ppm, monotonic shape no, maximum charge 2, representative isotope, most intense; (6) Isotopic peaks finder= m/z tolerance 0.0010–5.000 ppm, maximum charge of isotope m/z 1; Join aligner= 0.0015–5.000 ppm, weight for m/z 3, retention time tolerance 0,200 min, weight for RT 1, (7) Feature list rows filter= Minimum aligned features 1, retention time filter 2.00–15.00 min,

Chromatographic FWHM (Full Width at Half Maximum) 0.00–6.00, never remove feature with MS2, on; (8) Peak finder= intensity tolerance 25 %, m/z tolerance 0.002–5.000 ppm, retention time tolerance 0.200 min, minimum scans 4 . Features with retention time below 0.5 min above 18.0 min were excluded. The resultant feature list was exported to a formats compatible with SIRIUS (.csv and .mgf with a metadata file).

2.11. Metabolites annotation

SIRIUS 5 [25] was used for the computation of detected features; MZmine 4.0.3 SIRIUS's output was uploaded on this open source tool, and then all the detected features were computed both in positive and negative ionizations mode with the following parameters: mass accuracy 5 ppm (Orbitrap), all possible adducts; ZODIAC [26] (Identification of molecular formulas)=use 2 step approach, edge thresholds 0.95, min local connections 10; CSI:FingerID [27] (predicts molecular fingerprint from MS/MS and fragmentation tree for each compound)= all fallbacks adducts, search molecular structure in all databases, tag lipids, off; CANOPUS on (predicts compounds categories for each compound individually predicted based on its predicted molecular fingerprint) [28].

The identification level of the annotated metabolites is reported following the Chemical Analysis Working Group (CAWG) criteria, including level I (comparison against an authentic standard) and level II (putatively identified molecule) annotations [29]. For the annotation, both spectral matching (comparison with identified spectra, in Biocycl, Pubchem, HMDB, COCONUT, CHEBI, KEGG, Natural Products, Super-Natural) and literature search were used.

2.12. Semi-quantitative metabolomics

The semi-quantification of the annotated metabolites from the non-targeted analysis was performed using MS2Quant [19], a machine learning model that enables prediction of concentration from fragmentation (MS^2) spectra of detected chemicals. The set of metabolites used for the targeted analysis were used as calibrants for concentration prediction and for model validation. Phenylalanine, 7-amino-4-methylcoumarin, kaempferol, ursolic acid and abietic acid were used as calibrants for positive ionization mode while chlorogenic acid, rutin, rosmarinic and jasmonic acid for negative ionization. SIRIUS 5 +CSI:FingerID, was used to annotate the structure of the tentatively identify molecules and to export the descriptor, SMILES, needed to run MS2Quant model. MS2Quant package has been tested with R version 4.1.1 and 4.2.2, as well as with SIRIUS versions 4.9.15 to 5.6.2.

Table 1
Nomenclature, composition, synthesis and visual aspect of NADES.

	Component 1	Component 2	Water	Molar ratio	Lab synthesis	Visual aspect
BG	betaine	glycerol		2:1	heating and stirring 80 °C	transparent viscous liquid
BGH	betaine	glycerol	yes	2:1:10.2		transparent liquid
BBG	betaine	1,3-butanediol		1:3	heating and stirring 80 °C	transparent viscous liquid
BBGH	betaine	1,3-butanediol	yes	1:3:10.6		transparent liquid
GCAH	glucose	citric acid	yes	1:1:3	heating and stirring 80 °C	pale yellow viscous liquid
GCAH	glucose	citric acid	yes	1:1:6		pale yellow liquid
UG	urea	glycerin		1:3	heating and stirring 50 °C	transparent viscous liquid
UGH	urea	glycerin	yes	1:3:2.5		transparent viscous liquid
BLA	betaine	lactic acid		1:1	heating and stirring 80 °C	transparent viscous liquid
BLAH	betaine	lactic acid	yes	1:1:3.5		transparent liquid
PCAH	proline	citric acid	yes	1:1:1	heating and stirring 70 °C	pale yellow viscous liquid
PCAH	proline	citric acid	yes	1:1:4.4		pale yellow liquid
BH	betaine	water		1:1	heating and stirring 80 °C	transparent liquid
BH	betaine	water		1:5		transparent liquid
BH	betaine	water		1:10		transparent liquid
TM	thymol	menthol		1:1	heating and stirring 70 °C	transparent liquid
OAM	oleic acid	menthol		2:1		pale yellow liquid
DAM	dodecanoic acid	menthol		1:4	heating and stirring 70 °C	transparent liquid
DAT	decanoic acid	thymol		1:2		transparent liquid

3. Result and discussion

3.1. NADES preparation

Twenty NADES with different composition and molar ratios were synthesized. The formation of the liquid mixture was defined from direct observation. The mixtures able to form clear liquids were identified and listed in Table 1. During the synthesis, mixtures gradually formed liquids when mixed and heated, they appeared as clear, homogenous liquids and remained stable without re-crystallization upon cooling.

3.2. Target analysis: extraction efficiency of nades vs conventional solvents

MO leaves were extracted with 19 NADES, 1 PB NADES and 3 conventional solvents to evaluate the extraction efficiency under the same experimental settings (1:10 sample-to-solvent ratio, maceration for 24 h under constant stirring). Within the 11 metabolites used, we were able to quantify only 10 of them as listed in Table S2. Jasmonic acid is not included in Table S2 because was <LOQ in all samples. At first, we demonstrated the reproducibility of the sample extraction. As depicted in the PCA score scatter plot in Fig. 1 the three extraction replicates per sample are tight clustered together. Moreover, the PCA plots reveal different metabolomic composition for MO extracts depending on the NADES used as extraction solvent. Fig. 1 (panel A) displays the separation in the score plot of non-polar NADES (blue), conventional extraction (purple) and polar NADES (red) extracts. This figure shows the NADES extraction efficiency as a function of their polarity parameter. PCA score plot and biplot (2 components explained 57 % of variance) reported in Fig. 1, were built using the results obtained from the targeted analysis. Results of target analysis and polarity parameter of solvents are reported for completeness in Table S2.

The score plot in Fig. 1 (panel A) explains the different extraction capability of solvents according to their polarity, as evidenced by the arrangement of samples in the PCs coordinates. In the upper-left quadrant apolar NADES (blue-purple) cluster together, in the upper-center area of the plot conventional solvents grouping together according to their different polarity parameter (PC2) while in the right quadrant the polar NADES cluster together, positive along the PC1 axis and negative along the PC2. Organic acid-based (BLA, PCAH, GCAH) and sugar-based NADES (GCAH) cluster together according to their similar polarity and their similar extraction capacity, while polyalcohol-based NADES (BG, BGG, UG, UGH, BBG, BBGH), which are less polar than organic acid-based NADES, cluster together in the vicinity of water. Conventional solvents, like ethanol and hydroalcoholic solution (purple) cluster in the

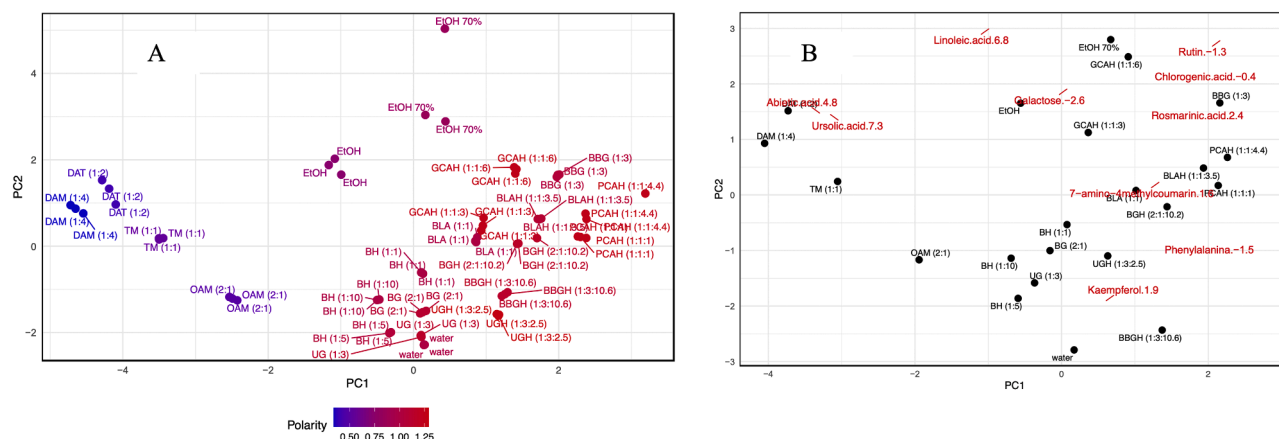


Fig. 1. PCA score plot and biplot of target analysis. Panel A) Score plot clustering NADES according to their extraction efficiency, color-code: polarity parameter; panel B) biplot to graphically visualize extraction selectivity. Vector for easiness of interpretation is reported with its XLogP.

upper center of the PCA plot space since they have medium polarity (positive on both PC1 and PC2), and they are traditionally used as extraction solvents to achieve high extraction yield for a broad spectrum of compounds [30]. In order to explain the differences between the samples in the PCA, the loading plot was inspected. The biplot represented in Fig. 1 (panel B), shows the overlay of more lipophilic compounds, such as terpenes (ursolic (XLogP 7.3) and abietic acid (XLogP 4.8)) with the apolar NADES (TM, DAT, DAM) explaining the most efficient in terms of extraction yield of these two molecules. Interestingly, these two variables (ursolic and abietic acid) lay close one each other and so they are positive correlated. Ursolic and abietic acid were found to be in nearly opposite projections of phenylalanine (XLogP -1.5); this behavior is due to their XLogP, terpenes with an XLogP up to

4.8 while phenylalanine, which is a water-soluble amino acid has a XLogP of -1.5, indicating different solubility of compounds in NADES. Flavonoids and polyphenols like chlorogenic acid, rutin and kaempferol cluster together with small angles in between, indicating similar behavior. 7-amino-4-methylcoumarin overlays with the cluster of organic acid-based NADES, which are in fact the only solvents that have been able to efficiently extract this coumarin. Similar behavior can be seen for galactose, which is found in the vicinity of the cluster of the sugar-based NADES, according to “similia similibus solvuntur”. Finally, in the vicinity of the ethanol projection lays linoleic acid (XLogP 6.8), which cluster with terpenes while it negative correlates with more polar analytes.

The solvent capacity of NADES toward target biomolecules varies

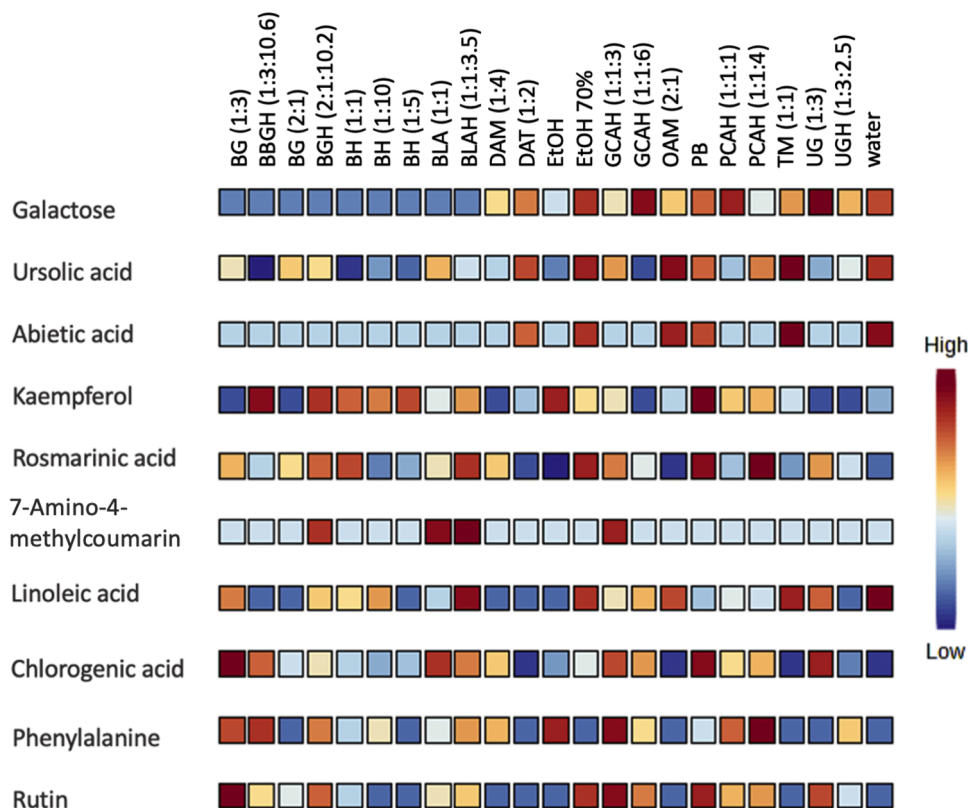


Fig. 2. VIP scores in PLS-DA analysis calculated for ten metabolites. The color of the squares is indicative of the analyte concentration in the various samples, blue <LOQ, light blue medium-low yield, orange to red high extraction yield.

greatly, as shown in Fig. 2 (VIP score) and Fig. 3 (histogram charts), where the results obtained from the target analysis are shown graphically. In Fig. 2, the colors of the squares indicate the relative abundance of each analyte in the samples under study. Ranked first in VIP plot, galactose (representative of the class of sugars and carbohydrates) is extracted with a significant difference between the 23 solvents. Indeed, the quantification of galactose was reported exclusively in sugar and urea-based NADES. Following the rule of like dissolves like, phenylalanine, a polar, water-soluble compound, was extracted mainly by water based and polar NADES. Sugar-based NADES resulted overall in a higher yield for the sugar under study and the same behavior for amino acid-based NADES, which shown better extraction capacity towards phenylalanine. On the contrary, rutin (XLogP -1.3), chlorogenic acid (XLogP 0.4) and kaempferol (XLogP 1.9) belong to the class of flavonoids and polyphenols, were extracted with all the NADES investigated in a concentration range of rutin > chlorogenic acid > kaempferol, in accordance with what previously reported by Petrisor et al. [1]. All polar NADES successfully extracted the molecules of interest, whereas for the apolar NADES, it was only possible to quantify kaempferol, as highlighted in Fig. 3 panel A, which is indeed the most apolar of the three representatives selected for this biochemical class. In addition, MO leaves are expected to be rich in coumarins and usually extracted using alcoholic maceration or ultrasound assisted extraction [31]. Our result (Fig. 2) are in agreement with those reported by Aryati et al. [5] and Wang et al. [32] which shown that coumarins (7-amino-4-methylcoumarin) can be better extracted using organic acid-based NADES. The highest extraction yield of coumarin was obtained with BLA, BLAH, GCAH; with BLAH being the best one, as represented in Fig. 3 (panel B). Ursolic acid, a triterpenoid with relevant biological activities, is also expected to be highly present in MO leaves, with a concentration varying between 4000 and 10,000 $\mu\text{g/g}$ [1]. The highest extraction yield was obtained when using apolar NADES, in accordance with what previously reported by Silva et al. [6] and ethanol, while the other NADES were able to extract the triterpenoid with lower efficiency, as shown by the different colors of the squares and in panel C Fig. 3. Similar trend was found for linoleic acid and abietic acid widely present

in aromatic plants like MO. Overall the profile of extracted compound is consistent among the four synthesized apolar NADES. Finally, rosmarinic acid, one of the most abundant phenolic acid in lemon balm, was detected in all samples, especially in the PB NADES, at high concentrations, Fig. 3 (panel D). This result is quite interesting, as it suggests that rosmarinic acid, present in significant concentrations within the MO leaves, may act as HBD in the formation of the NADES system. In fact, PB NADES is composed solely of MO leaves and choline chloride as HBA. The structure of rosmarinic acid, with its numerous free -OH groups, likely plays a key role in forming the hydrogen bonds required for the eutectic system. This hypothesis aligns with previous findings by Qader et al. [14].

3.3. Screening of NADES-molecule affinity via COSMO-RS

The extracted compounds were analyzed using their σ -profiles, which show how charge density is distributed across the molecular surface. In these profiles, the region that donates hydrogen bonds is found at values $< -0.085 \text{ e}/\text{\AA}^2$, while the region that accepts hydrogen bonds is above $+0.085 \text{ e}/\text{\AA}^2$. Values between -0.085 and $+0.085 \text{ e}/\text{\AA}^2$ indicate that the molecular surface is too non-polar to effectively participate in hydrogen bonding [33]. From the extraction results, it was observed that flavonoids like chlorogenic acid and rutin had the highest yields when using polar NADES. Kaempferol was extracted by both non-polar and polar NADES. The σ -profiles for these flavonoids, including chlorogenic acid, kaempferol, and rutin, showed similar patterns with peaks in the non-polar regions (Fig. 4, panel A). Smaller peaks were also noted in both HBD and HBA regions for all three compounds. For kaempferol, the peaks in the HBA and HBD regions were less intense than those for chlorogenic acid and rutin. This suggests that kaempferol's lower content of polar groups allows it to be extracted by both types of solvents, but with lower efficiency. In contrast, chlorogenic acid and rutin were only effectively extracted by polar NADES due to their stronger HBA signals. The NADES made from proline, citric acid, and water (PCAH) extracted the most phenylalanine. Its σ -profile is mainly found in the non-polar region, featuring two major peaks and one minor

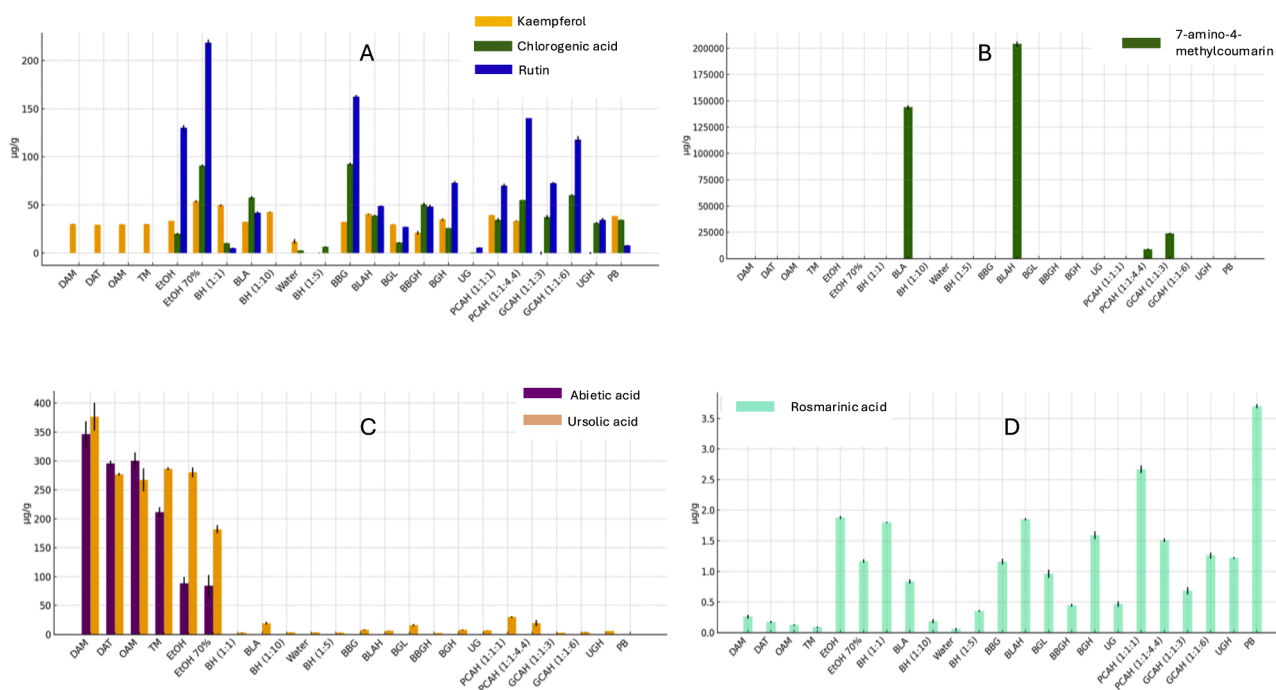


Fig. 3. Histogram charts representing the efficiency of NADES in the extraction of selected MO metabolites. In the X axis NADES and conventional solvents are ordered according to their polarity parameter. Panel (A) extraction trend of kaempferol, rutin and chlorogenic acid, panel (B) 7-amino-4methylcoumarin, panel (C) abietic and ursolic acid, panel (D) rosmarinic acid. Data are reported as mean $\pm$ SD for three replicates.

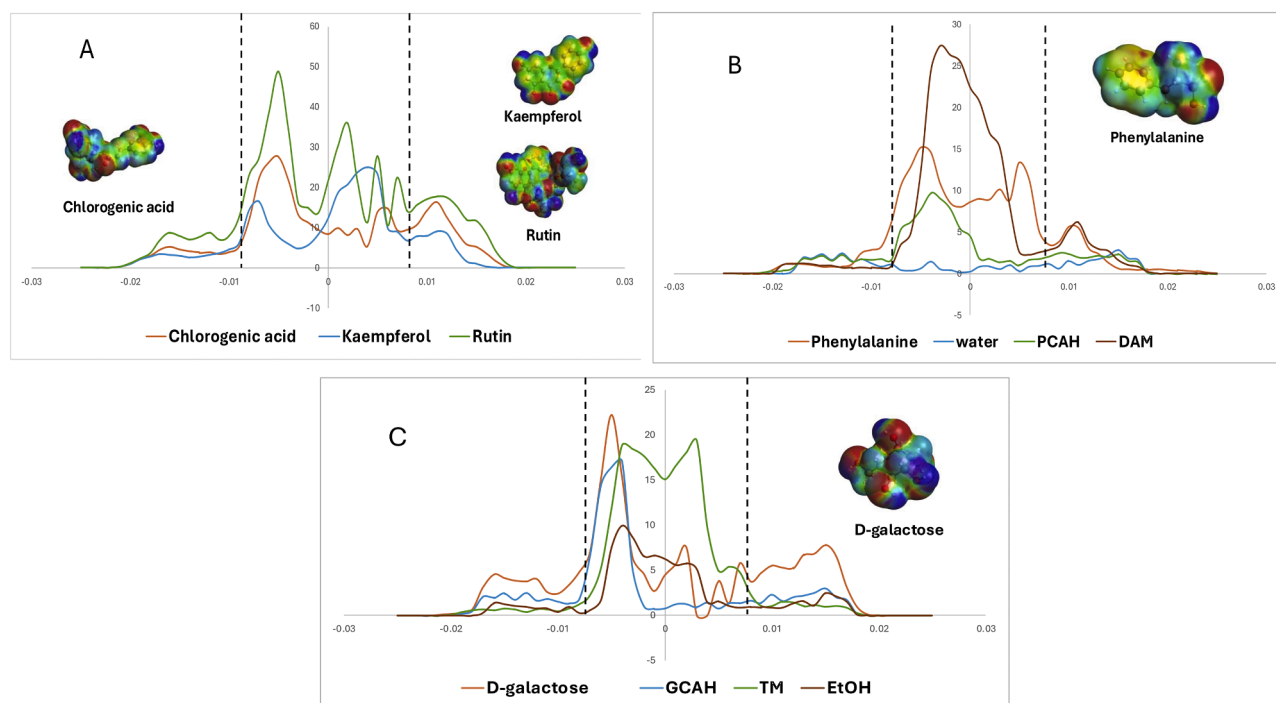


Fig. 4. σ -profile of selected metabolites. (A) σ -profile of flavonoids chlorogenic acid, kaempferol and rutin; (B) σ -profile of phenylalanine and water, PCAH, DAM; (C) σ -profile of D-galactose, GCAH, TM, EtOH.

peak (Fig. 4, panel B). This indicates that phenylalanine is a non-polar molecule, primarily because of its benzene ring and carbon chain. There is also a small peak in the HBA region, attributed to its amine and carboxylic acid groups. This shows that phenylalanine can dissolve well in polar solvents with HBD groups. PCAH displayed a σ -profile with most peaks in the polar region, and two minor peaks in the HBD region, while no peaks were found in the HBA region. For DAM, a major peak was observed in the non-polar region, along with a minor peak in the HBA region, resembling the trend of the HBA peak from phenylalanine. PCAH is preferred for extraction because it can interact well with the HBA groups of phenylalanine and the HBD groups of PCAH. Since both DAM and phenylalanine have HBA groups and are found in the non-polar region, they repel each other, leading to lower extraction efficiency of phenylalanine with DAM compared to PCAH. NADES GCAH (1:1:6) extracted the most D-galactose. Its σ -profile showed one large peak and three smaller peaks in the non-polar region, along with two broader peaks in both the HBD and HBA regions (Fig. 4, panel C). The polar peaks are due to the hydroxyl groups, while the non-polar peak results from the carbon in D-galactose. This indicates that D-galactose can dissolve in solvents that have both polar and non-polar characteristics. GCAH (1:1:6) displayed a large peak in the non-polar region that matched the charge density of D-galactose. Several smaller peaks were observed in the HBD region, and D-galactose showed a strong peak in the HBA region. This suggests that there are complementary interactions between the NADES and D-galactose in both the HBA and HBD regions, as both compounds have similar charge densities in the non-polar region. NADES TM showed two major peaks in the non-polar region and no peaks in either the HBD or HBA regions, which resulted in lower extraction efficiency due to the absence of HBA and HBD interactions compared to GCAH.

3.4. Non-target analysis: qualitative metabolome annotation

The target analysis and the computational screening conducted using COSMO-RS provided important information on the selectivity and extraction efficiency of various NADES with respect to 11 target

metabolites, each representative of a distinct biochemical classes found in MO leaves. Following the validation of the LC-MS method, and after gaining both predictive and experimental understanding of the behavior of the different NADES, the workflow was implemented with non-targeted analysis to broaden the metabolite coverage. Initially, a qualitative approach was employed, after which, a semi-quantitative approach was applied. The resulting UHPLC–HRMS raw datasets, acquired both in positive and in negative ionization modes, underwent several data processing steps, like features alignment, data pre-processing, and filtering steps. Overall, 2851 and 2659 features were aligned in positive and negative mode respectively, using MZmine. At first, unsupervised models were employed, specifically PCA, to reduce the dimensionality of our dataset while maximizing the retention of its inherent variability. The PCA score plots (depicted in Fig. S2) reveals different metabolomic composition for MO extracts, due to a distinctive extraction efficiency of the different solvents. Afterwards, the aligned datasets (HESI positive and negative) were independently analyzed using SIRUS 5 CSI:FingerID. Overall, the annotation process yielded a total of 444 annotated features. Within the 444 annotated metabolites, 18 were identified in both polarities, including flavonoids, phenolic acids and terpenoids. From the 444, 11 were identified at level I indicating the highest confidence level of identification and 433 were categorized as level II denoting a reliable matching with existing libraries. All the annotated features are reported and uploaded to Zenodo (<https://doi.org/10.5281/zenodo.14801806>, MO_full_compounds_annotation). The 444 annotated metabolites were grouped into biochemical classes, this categorization, carried out via SIRUS5+CSI:FingerID (CANOPUS-ClassyFire), offers a comprehensive snapshot of the chemical complexity within the analyzed compounds under different ionization modes. As an example, some chemical classes such as alkaloids, carbohydrates and glycolipids were mostly identified via positive ionization mode while phenolic acids, flavonoids and organic compounds exhibited a better ionization in negative mode (see MO_full_compounds_annotation, <https://doi.org/10.5281/zenodo.14801806>). From a qualitative point of view, MO annotated metabolome includes primary metabolites such as sugars (glucose, erlose, melitose and galactose)

organic acids such as tartaric acid, amino acids (proline, phenylalanine, histidine) and nucleotides which were detected and annotated in positive ionization ion mode. Lipids like glycerolipids (DG 34:2, DG 34:1, DG 33:2, DG 24:2, TG 33:0, TG 26:0) fatty acids and derivatives (verneolate, hydroxyoctadecenedioic acid, linoleic acid and coriolic acid) were also annotated in our MO leaves extracts. With respect to secondary (or specialized) metabolites most of the phenolic acids and flavonoids were detected and annotated in negative ionization ion mode, these include luteolin, luteolin-7 glucuronide, rutin, kaempferol, chlorogenic acid and isochlorogenic acid, ethyl caffeate, salviaflaside and lithospermic acid. Another relevant bioactive class of secondary metabolites annotated in MO extracts were terpenoids. Within this biochemical class, we identified ursolic, ursonic, betulonic acid and arjungenin and diterpenoids like rosmanol and carnosol. From a qualitative standpoint, the use of NADES as extraction solvents, coupled with a non-targeted approach for the identification of extracted metabolites, enabled the identification of several bioactive compounds that had not previously been described in extracts of MO (as shown in Fig. 5). Indeed, the chemical space of MO metabolome, built with the SMILES codes of the MO metabolites, highlight the newly annotated MO metabolites in orange. This outcome was achieved because the use of 23 different solvents (NADES and conventional ones), each with distinct physicochemical properties, facilitated the extraction of metabolites that are not typically recovered

with conventional solvents such as ethanol, water, or hydroalcoholic solutions. Furthermore, the annotation of metabolites in a non-targeted experiment is based on MS/MS matches with online databases. In our case, through the application of SIRIUS 5 + CSI:FingerID and natural product databases such as Biocycl, HMDB, COCONUT, CHEBI, KEGG, Natural Products, SuperNatural we were able to annotate a substantial number of metabolites.

3.5. Semi-quantification validation

A key distinct aspect of this work was moving beyond qualitative metabolomics by employing a predictive model to semi-quantify all the annotated metabolites and to assess the efficiency and selectivity of the solvents under study. MS2Quant, a software designated to assist the quantification of metabolites from MS/MS data was used for the semi-quantification of $[M + H]^+$ and $[M-H]^-$ annotated metabolites from SIRUS+CSI:Finger ID. First, we validate MS2Quant model, in order to assess precision and accuracy. 7-Amino-4-methylcoumarin, ursolic acid, abietic acid, kaempferol and phenylalanine were used as calibrants for model validation in positive ionization, while chlorogenic acid, rutin and rosmarinic acid were used for validation of the model in negative ionization mode. Model validation resulted in prediction error of 0.0008, calculated as shown in **Formula S1** and a Root Mean Square

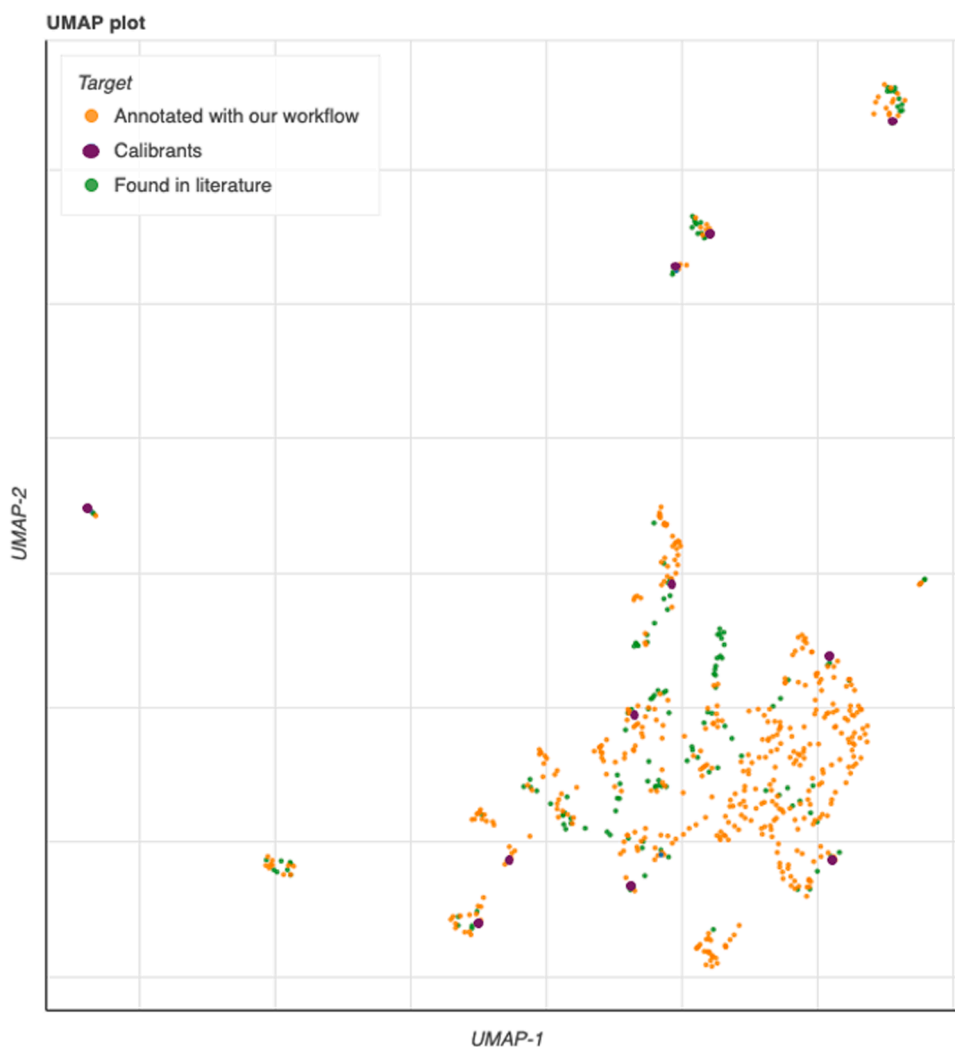


Fig. 5. Chemical space visualization of the MO metabolome. Dots in the chemical space represent: (i) orange dots: 444 metabolites annotated using our workflow (ii) green dots: 212 metabolites already described for MO leaves and (iii) purple dots: calibrants used as metabolites for targeted analysis, for model optimization (LC-MS) and validation (MS2Quant). Uniform Manifold Approximation and Projection (UMAP) was built using ChemPlot web application [34], with tailored UMAP as dimensionality reduction method.

Error (RMSE) of 0.0007, for positive ionization mode and RMSE of 0.0011 and MPE of 0.0009 for negative mode. Prediction errors were calculated for each datapoint and shown in box plot **Fig. S3**. After validation, MS2Quant was used to predict the concentration of all metabolites annotated in positive and negative ionization ion mode as $[M + H]^+$ and $[M - H]^-$ ions, respectively.

3.6. Non-target analysis: semi-quantification using MS2Quant

LC—HESI-HRMS is a powerful and widely utilized technique in mass spectrometry; however, accurate quantification typically necessitates numerous analytical standards, which can be both costly and time-consuming. As the primary objective of this study was to evaluate the efficacy and selectivity of NADES in extracting different plant metabolites, our focus was on obtaining quantitative data. After conducting qualitative annotation and model validation, we utilized MS2Quant to estimate the molar concentrations of the identified metabolites. This approach allowed us to achieve precise quantification results without relying extensively on a large number of standards. Among the 444 metabolites annotated, 198 were annotated as $[M + H]^+$ and 187 as $[M - H]^-$

$H]^-$ and semi-quantified using MS2Quant. MO_MS2Quant_semi quant table uploaded on Zenodo, <https://doi.org/10.5281/zenodo.14801806>, reports the molar concentration for the 385 semi-quantified metabolites within the 23 samples, grouped in HESI positive and negative.

To effectively illustrate the most interesting patterns related to extraction efficiency and selectivity, chord diagrams were generated for 4 of the samples under study and reported in **Fig. 6**. Ethanol (**Fig. 6**, panel B), was chosen and applied in this study to achieve a high extraction yield. It is regarded as an optimal solvent for extracting plant metabolites due to its medium polarity (E_N^{NR} 0.74) which allows the extraction and solubility of a wide range of metabolites from different biochemical classes. The results from the semi-quantification confirmed this, as evidenced by the width and variation in the color bands displayed in the second chord diagram (**Fig. 6**, panel B). Similarly, we demonstrate that TM (1:1) **Fig. 6**, (panel A) with a polarity E_N^{NR} 0.55 was able to effectively extract a broad range of biochemical classes with a yield (width of bands) similar to that of ethanol, with only minor variations in the proportions of each class. This indicates that both solvents are effective for broad-spectrum extractions and can be replace one another. However, their differences lie in toxicity and cost. As

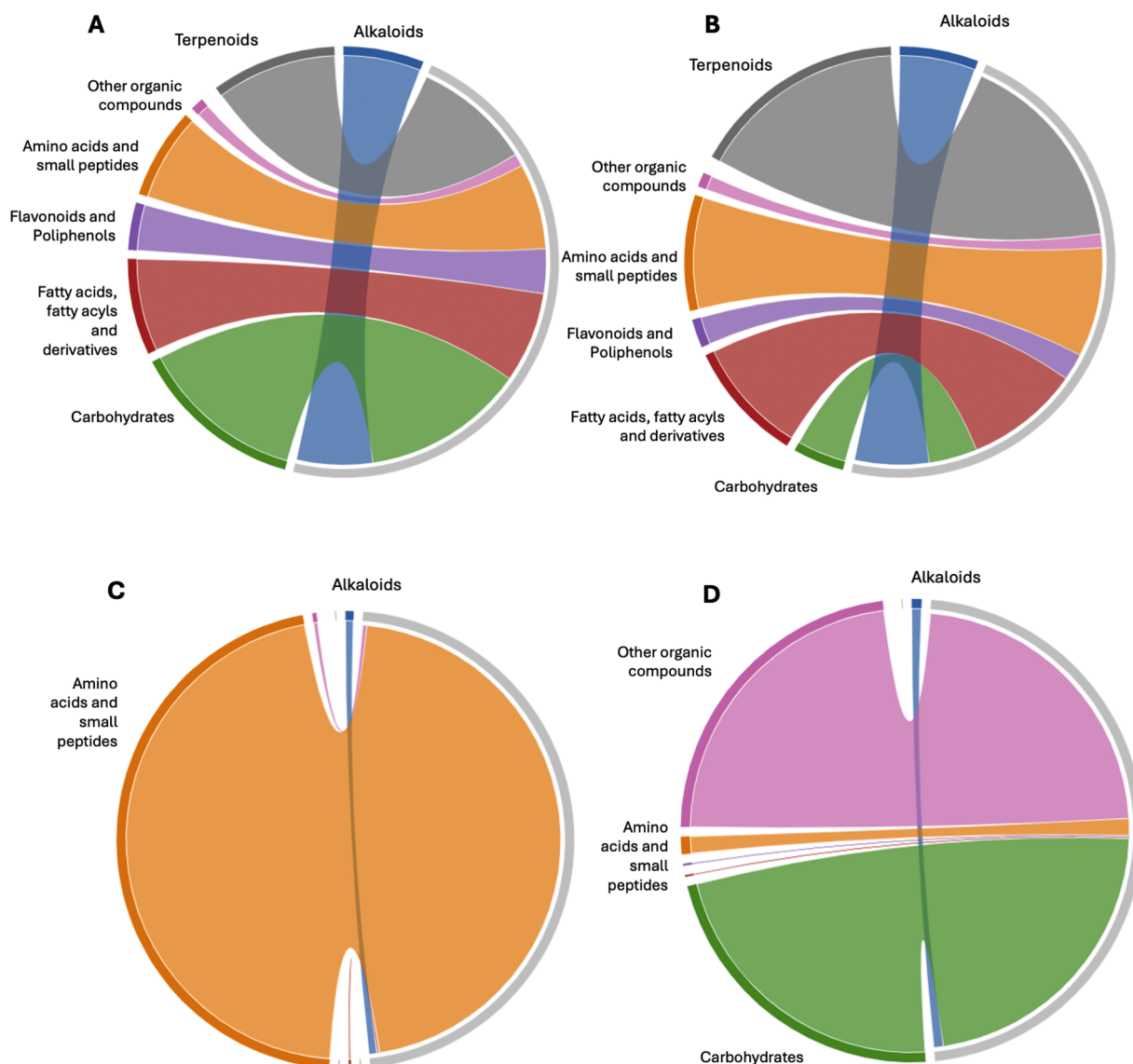


Fig. 6. Graphical representation of the extraction selectivity and efficiency of 4 different solvents (panel A: TM, panel B: EtOH, panel C: PCAH (1:1:4:4), panel D: GCAH (1:1:3)). Chord graphs are built using the molar concentration obtained from semi-quantification with MS2Quant (MO_MS2Quant_semi quant uploaded in Zenodo, <https://doi.org/10.5281/zenodo.14801806>).

highlighted in **Fig. S4**, moving from ethanol to NADES extraction improved significantly the greenness of the sample preparation step, resulting in a green score of 0.77, compared to 0.52 for ethanol extraction. NADES TM, being composed of natural, biocompatible compounds, is less toxic overall. Thymol and menthol have a lower environmental impact and reduced human health risks compared to ethanol, though they can still cause mild skin irritation at higher concentrations [35]. Ethanol, on the other hand, is widely available and cheaper due to its mass production, making it a cost-effective option for industrial applications. However, ethanol carries higher risks in terms of human toxicity, especially with ingestion or inhalation, and can be more drying and irritating to the skin [36]. TM is more expensive due to the higher cost of thymol and menthol, and while it is an emerging and environmentally friendly solvent, it is more suited for high-value applications in industrial field [35]. Overall, **Fig. 6**, panel A and B demonstrates that both solvents perform similarly in terms of extraction efficiency (number of colored bands and their width), but the choice between them depends on balancing cost, toxicity, and the specific application requirements. In contrast, **Fig. 6** panel C and D, demonstrate the advantage of using NADES to create tailored extracts, enriched in specific biochemical classes. **Fig. 6**, panel C shows the distinctive selectivity of NADES PCAH (1:1:4.4) composed of proline, citric acid and water in extracting amino acids and small peptides. A possible explanation is that citric acid, the HBD in this NADES, is a tricarboxylic acid, making it highly capable of forming hydrogen bonds and interactions with amino acids [37]. The acidic environment created by citric acid (pH= 3.7) can help to protonate amino acids, enhancing their solubility. Additionally, proline can also interact with hydrophobic parts of the amino acids through van der Waals forces, making it a perfect candidate for the extraction of these biochemical class [38]. Lastly, the NADES GCAH (1:1:3) **Fig. 6** panel D represents a highly effective medium for extracting carbohydrates and other organic compounds. The possible reasons are (i) the principle of like dissolves like that explains why substances with similar chemical properties tend to dissolve in each other [39], (ii) the polarity of the solvent (E_N^{NR} 1.24) plays a crucial role in dissolving organic compounds (e.g. organic acids like aconitate and tartaric acid) and carbohydrates [40], (iii) the acidic environment (pH 2.3) helps in disrupting cell wall structures and facilitate the release of intracellular sugars and organic compounds [41].

4. Conclusion

Our study demonstrated the applicability of green solvent extraction, like NADES (with a broad polarity range) to effectively extract bioactives from MO. The prediction of solute affinity against solvents using COSMO-RS provided an adequate preliminary assessment of NADES interactions with different metabolites, which were later confirmed through targeted analysis. The results revealed that hydrophobic NADES can be used to create products rich in terpenoids, which are suitable for antimicrobial formulation. Amino-acid-based NADES proved optimal for extracting small peptides and amino acids, while sugar-based NADES excelled in extracting carbohydrates and organic compounds, making them suitable for food supplements or cosmetics. Organic acid-based NADES demonstrated high selectivity in extracting coumarins. Overall, our workflow which incorporates NADES for green sample preparation and the application of a non-targeted semi-quantitative metabolomic approach allowed for an in-depth investigation of MO leaves metabolome and demonstrated the potential of NADES for producing high value extracts. We believe that this research not only advances our knowledge of NADES applications in plant metabolite extraction but also sets the stage for further exploration of sustainable and efficient green extraction technologies in various fields.

CRedit authorship contribution statement

Chiara Spaggiari: Writing – review & editing, Writing – original

draft, Investigation, Formal analysis, Conceptualization. **Isa Sara Aimee Hiemstra**: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Antoinette Kazbar**: Writing – review & editing, Writing – original draft, Supervision, Resources. **Gabriele Costantino**: Writing – review & editing, Resources. **Laura Righetti**: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Conceptualization.

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.

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Supplementary materials

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Data availability

<https://doi.org/10.5281/zenodo.12721470> (The MZmine batch files have been uploaded to Zenodo for accessibility and reference:)

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