

## COSMO-RS in prescreening of Natural Eutectic Solvents for phenolic extraction from *Teucrium chamaedrys*

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### ABSTRACT

Natural Eutectic Solvents (NES) are a promising class of environmentally friendly liquids that offer an alternative to conventional organic solvents. To investigate their potential for extracting phenolic compounds from plant materials, we employed the COSMO-RS (COnductor like Screening MOdel for Real Solvents) computational method. Nineteen NES solvents were prepared to determine the most selective and effective solvent for extracting phenolic compounds from *Teucrium chamaedrys* using both theoretical and experimental evaluation. Screening of extraction efficiency was performed by quantification of individual compounds using ultra-high-performance liquid chromatography with a diode array detector and a triple-quadrupole mass spectrometer (UHPLC-DAD-MS/MS), as well as spectrophotometric assays (total phenolic content, total flavonoid content, and radical scavenging activity). In this paper, we propose a new approach to model NES properties using COSMO-RS. While the majority of previous studies have modeled these solvents as individual or pseudo-component complexes between hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA), we have developed a ternary HBD:HBA:water supramolecular complex model to represent NES solution structures more accurately. This model provides insights into the intermolecular interactions driving the extraction process and predicts extraction efficiencies that agree with experimental data. Two eutectic mixtures (choline chloride:succinic acid in molar ratio 1:1 and 20% of water (w/w) and choline chloride:glycerol in molar ratio 1:1 and 20% of water (w/w)) showed strong affinity towards phenolic compounds. Overall, our findings suggest that the ternary complex model is a more appropriate approach for modeling NES properties.

### 1. Introduction

Natural Eutectic Solvents (NES) have gained recognition as a promising green alternative to conventional extraction solvents [1]. NES are composed of natural compounds, such as amino acids, carboxylic acids, alcohols, sugars, and amines, which form a hydrogen bond network. This results in desirable physicochemical properties, including low volatility, liquid state below 0 °C, adjustable viscosity, broad polarities, and the ability to dissolve a wide range of compounds. However, the formation of the hydrogen bond network also leads to high viscosity, which limits mass transfer and can hinder their use as extraction solvents. To overcome this problem two approaches can be applied: adding water or increasing temperature. The polarity of NES can be fine-tuned

by simply changing the combination of HBA (Hydrogen-Bond Acceptor) and HBD (Hydrogen-Bond Donor), molar ratios of compounds, or adding water, which provides the possibility to design NES solvent to target specific compounds. Tailorable properties and natural origin of NES make them less toxic, environmentally friendly and diverse media compared to conventional solvents [2,3].

Computational models can provide valuable insight into the underlying mechanism of the extraction process, aiding in the design and improvement of the extraction process. The use of computational models in the prediction of NES solvent extraction helps to ensure that the extraction process is efficient, economically viable, and environmentally sustainable. COSMO-RS (COnductor like Screening MOdel for Real Solvents) is a computational model that predicts various

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thermodynamic and transport properties of liquids, including NES. This model uses quantum mechanics and statistical thermodynamics to simulate the behavior of molecules in solution, providing predictions of properties such as solubility, partitioning, and viscosity. The use of COSMO-RS in predicting the properties of NES is particularly useful for the optimization of solvent extraction processes, as it allows for the assessment of the performance of different solvents under various conditions without the need for expensive and time-consuming experimental trials.

*Teucrium chamaedrys* L. is a medicinal herb used in traditional medicine in the Balkans due to its wide range of pharmacological effects and it is applied in the treatment of digestive disorders, coughs, asthma, abscesses, conjunctivitis, and cellulite. Previous studies showed that *T. chamaedrys* has strong biological activity and revealed that it is rich in phenolic compounds, especially flavonoids suggesting that *T. chamaedrys* is promising plant source of antioxidant compounds [4–9].

The aim of this study was to evaluate the efficient NES for the extraction of phenolic compounds from *T. chamaedrys* and to correlate experimental data with theoretical predictions. This goal will be achieved by: (i) evaluation of extraction efficiency by quantification of individual compounds, (ii) application of COSMO-RS for quantum chemical simulation, by calculation of the activity coefficients at infinite dilution and the  $\sigma$ -profiles, in order to understand the molecular interaction between solute and different solvents, (iii) correlation of thermodynamic activity coefficient at infinite dilution and  $\sigma$ -profiles with experimentally obtained results of extraction efficiency. This article is, also, a rare example of the examination of the impact of water in COSMO-RS predictions of extraction efficiency.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Aluminum chloride and maleic acid were ordered from Acros Organics (Geel, Belgium). Urea and ascorbic acid were purchased from Betahem (Belgrade, Serbia). Folin-Ciocalteu reagent was provided from Carlo Erba reagents (Milan, Italy). Lactic acid and tartaric acid were bought from ICN Biomedicals Inc. (Aurora, OH, USA). Succinic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Choline chloride, L-proline, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), methanol, sodium carbonate, sodium nitrite, trolox, gallic acid, protocatechuic acid, syringic acid, chlorogenic acid, *p*-hydroxybenzoic acid, aesculetin, caffeic acid, isoorientin, rutin, vitexin, isoquercetin, *p*-coumaric acid, quercitrin, astragalin, rosmarinic acid, luteolin, quercetin, naringenin, kaempferol, hispidulin, and rutin were provided from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). Glycerol was purchased from Zorka Pharma (Šabac, Serbia). All solvents and chemicals were analytical purity grade.

### 2.2. Extraction of phenolic compounds from *T. chamaedrys* with methanol and NES

*T. chamaedrys* was bought in a local shop in Belgrade, Serbia. The sample was cut into small pieces and milled for 5 min into a powder using a coffee grinder (Bosch Coffee Electric Grinder TSM6A017C). Raw-starting plant material was stored in sealed containers at room temperature in the dark. A total of 500 mg of ground raw-starting material was dissolved with 5 mL of methanol and extracted by reflux method at a temperature of 50 °C for 45 min. The resulting extract was filtered using qualitative filter paper to remove ground plant material and methanol was evaporated under reduced pressure at 40 °C. Obtained extract was re-dissolved in 0.5 mL of methanol and diluted to match the solid/liquid ratio of NES extracts.

The composition of studied NES is listed in Table 1. A total of 4 g of HBA was weighted in Erlenmeyer flasks and a certain mass of HBD was

**Table 1**

The composition of the studied NES and their abbreviations.

Abbreviation	Eutectic mixture	Molar ratio	Water content, %
E1	L-proline: Maleic acid	1:1	20
E2	Choline chloride: Lactic acid	1:1	30
E3	Choline chloride: Tartaric acid	1:1	20
E4	Choline chloride: Tartaric acid	1:1	30
E5	Choline chloride: Tartaric acid	1:1	40
E6	Choline chloride: Tartaric acid	1:1	50
E7	Choline chloride: Urea	1:2	20
E8	Choline chloride: Urea	1:2	30
E9	Choline chloride: Urea	1:2	40
E10	Choline chloride: Urea	1:2	50
E11	Choline chloride: Succinic acid	1:1	20
E12	Choline chloride: Succinic acid	1:1	30
E13	Choline chloride: Succinic acid	1:1	40
E14	Choline chloride: Succinic acid	1:1	50
E15	Choline chloride: Glycerol	1:1	20
E16	Glycerol: Urea	1:1	20
E17	Glycerol: Urea	2:1	20
E18	Glycerol: Lactic acid	1:1	20
E19	Glycerol: L-Ascorbic acid	1:1	20

added. Mass of HBD was recalculated so that the compounds forming the eutectic mixture are in a certain molar ratio (1:1 or 1:2). Selected compounds were heated to 80 °C and constantly stirred on a magnetic stirrer for 30 min. In the eutectic mixture, a certain mass of water was added (20–50% w/w), and stirring continued for 30 min. The extraction of phenolic compounds was performed by the reflux method. 500 mg of ground plant sample was mixed with 5 mL of previously prepared NES mixtures and stirred in Erlenmeyer flasks at a magnetic stirrer for 45 min at a temperature of 50 °C. The resulting mixture was centrifuged at 10 000 rpm for 15 min and the supernatant was purified using solid-phase extraction (SPE). Reversed-phase sorbents (Cartridge Bond Elut C18, Agilent Technologies) are conditioned with 5 mL methanol followed by 5 mL of water. Then 3 mL of sample was loaded on cartridge and 10 mL of water was used for washing of non-adsorbed compounds. For elution of phenolic compounds 3 mL of methanol was used. The obtained extracts were stored in dark glass vials at –20 °C prior to analysis.

### 2.3. Quantification of phenolic compounds

Quantification of phenolics in NES extracts was performed using Dionex Ultimate 3000 UHPLC system (ThermoFisher Scientific) configured with a diode array detector (DAD) and a triple quadrupole mass spectrometer (TSQ Quantum Access Max, ThermoFisher Scientific). An analytical Hypersil gold C18 column (50 × 2.1 mm) with 1.9  $\mu$ m particle size (ThermoFisher Scientific) was used. The mobile phase consisting of 0.2% acetic acid in water (A) and LC-MS grade acetonitrile (B) was applied with the flow rate of 0.4 mL/min in the gradient elution, as previously described [10]. The injection volume was 10  $\mu$ L. Settings of a triple quadrupole mass spectrometer (qqqMS), equipped with a heated electrospray ionization (HESI) source, were as follows: vaporizer temperature 450 °C, spray voltage 4000 V, sheet gas (N2) pressure 50 AU, ion sweep gas pressure 0 AU and auxiliary gas pressure at 20 AU, capillary temperature at 320 °C, skimmer offset 0 V. The mass spectrometer was operated in negative ion mode, collision energy was 30 eV. Phenolic compounds were quantified by the external standard quantification procedure. Working standard solutions were prepared by dissolving the stock solution (1000 mg/L) of a pure compound to obtain

concentration 100 mg/L. Dilution of the solution with mobile phase yielded working solutions at concentrations of 0.025, 0.050, 0.100, 0.250, 0.500, 0.750 and 1.000 mg/L. Calibration curves were obtained by plotting the peak areas of the standards against their concentration. Calibration curves revealed good linearity, with  $R^2$  values exceeding 0.9998. The phenolic compounds were identified by direct comparison with commercial standards. The total amounts of each compound were evaluated by calculation of the peak areas.

#### 2.4. Determination of total phenolic content (TPC)

TPC in samples was determined using the Folin–Ciocalteu method. The spectrophotometric measurements were performed on a GBC UV–Visible Cintra 6 spectrophotometer. All the extracts were diluted to fit the calibration range and gallic acid was used as standard in the range of 20–120 mg L<sup>-1</sup>. A 0.5 mL of the extracts and 0.5 mL ultrapure water were mixed with 2.5 mL of 10% v/v Folin–Ciocalteu reagent and incubated for 5 min. Subsequently 7.5% w/w sodium carbonate (2.0 mL) was added. Absorbance was measured at 765 nm after incubation of mixture for 2 h at room temperature. TPC values were expressed as g gallic acid equivalent (GAE) per kg of dry sample. All measurements were done in duplicate, and the results were expressed as mean values  $\pm$  standard deviation (SD).

#### 2.5. Determination of total flavonoid content (TFC)

The total flavonoid content in samples was determined using aluminum chloride method, while rutin in the range of 20–200 mg L<sup>-1</sup> was used as standard. 0.3 mL of extracts were mixed with 3.4 mL of 30% (v/v) methanol, 0.15 mL of 0.5 mol/L sodium nitrite, and 0.15 mL of 0.3 mol/L aluminum-chloride. After incubation for 5 min at room temperature, 1.0 mL of 1 mol/L sodium hydroxide was added. The absorbance was measured at 506 nm and TFC values were expressed as g rutin equivalent (RUE) per kg of dry sample.

#### 2.6. Determination of the radical-scavenging activity (RSA)

Radical scavenging activity was determined using the DPPH radical solution. 0.1 mL of the extracts was mixed with 4 mL of methanol solution of DPPH (79  $\mu$ mol/L). The mixture was incubated for 1 h at room temperature and absorbance was measured at 517 nm. Trolox was used as standard in the range of 100–600  $\mu$ mol/L. RSA was calculated as a percentage of DPPH discoloration using the equation (where  $A_{DPPH}$  is the absorbance of methanol solution of DPPH,  $A_{sample}$  is the absorbance of samples):

$$RSA(\%) = \frac{(A_{DPPH} - A_{sample})}{A_{DPPH}} \times 100$$

The results were expressed as millimoles of Trolox equivalents per kg of dry sample.

#### 2.7. COSMO-RS modeling

The COSMO-RS (Conductor-like Screening Model for Real Solvents) framework is widely utilized for the calculation of surface charge densities ( $\sigma$ -profiles) in NES. The determination of  $\sigma$ -profiles plays a crucial role in understanding the solvation behavior and thermodynamic properties of NES. In COSMO-RS, there are two commonly employed methods for calculating  $\sigma$ -profiles: the individual approach and the pseudo-component approach. The individual approach involves summarizing the  $\sigma$ -profiles of HBD and HBA constituents in a specified molar ratio, whereas the pseudo-component approach considers the hydrogen-bonded, supramolecular complex between HBD and HBA.

Despite the common practice of adding 10–50% water by weight to NES to reduce viscosity and adjust properties, the impact of water is rarely considered in COSMO-RS predictions of extraction efficiencies

[11]. In order to calculate the affinity coefficients of the six most abundant phenolics (chlorogenic acid, quercetin, hispidulin, caffeic acid, syringic acid, and luteolin) in all NES, we optimized the structure of HBD:HBA:water supramolecular complexes listed in Table 1. The first step was to optimize the geometry of the HBD:HBA complex in a specific molar ratio. The structures of phenolics and individual NES constituents were downloaded from PubChem, and the initial conformation was obtained by MMFF94s force field [12]. The geometry was then refined using the PM7 semiempirical quantum-chemical model [13]. Ten HBD:HBA complexes were optimized for each NES, and the lowest-energy one was selected for further analysis. Subsequently, water molecules were added to the optimized complex by placing a water box (side 10 Å) around it, and out of the approximately 15 added water molecules, the three closest to the hydrogen-bonded atoms in the HBD:HBA complex were retained. The structure files (in.mol2 format) of all HBD:HBA:water supramolecular complexes can be found in the Supplementary information.

Based on the calculated  $\sigma$ -profiles, the COSMO-RS method enables the determination of (pseudo)chemical potential in both liquid ( $\mu_i^{solv}$ ) and gaseous ( $\mu_i^{gas}$ ) phase. Additionally, it defines the parameter  $\mu_i^{pure}$ , which characterizes the pseudochemical potential of a compound  $i$  within a liquid solution. Using these parameters, the activity coefficient of compound  $i$  can be calculated as follows:

$$\gamma_i = e^{\frac{\mu_i^{pure} - \mu_i^{solv}}{RT}}$$

The activity coefficients at infinite dilution ( $\gamma^\infty$ ) were determined using the Amsterdam Density Functional (ADF) COSMO-RS implementation in the ADF2022.101 program [14] (<https://www.scm.com/product/cosmo-rs/>), accessed on February 2nd, 2023). To assess the surface charge densities and  $\sigma$ -potentials of phenolics and NES, we performed single-point calculations on geometries optimized with the PM7 model. The calculations utilized ADF with a small core TZP basis set, the Becke-Perdew (GGA:BP86) functional, and the relativistic scalar ZORA method for calculations in the gas phase.

#### 2.8. Statistical analysis

Descriptive statistics and paired *t*-test were performed using Analysis ToolPak, Excel for Microsoft 365.

### 3. Results and discussion

#### 3.1. Chemical constituents of NES

The extraction efficiency depends on polarity, viscosity, and dissolving ability of solvents. High solubility of phytochemicals in NES is related to dipole–dipole and hydrogen bonding interactions. The various components of NES cause different abilities of solvent and the yield of extracted plant metabolites. Numerous studies confirmed that the extraction of plant bioactive compounds using optimized NES and extraction process conditions resulted in better extraction efficiency compared to conventional solvents [15–18]. In a current study, nineteen NES mixtures prepared from primary plant metabolites (Table 1) were tested for extraction efficiency of phenolic compounds from *T. chamaedrys*. According to previous reports [19,20], the selection of NES constituents was based on their ability to extract phenolics. Hydrogen-bond acceptors such as choline chloride, L-proline, urea, and hydrogen-bond donors such as tartaric, succinic, maleic, lactic, L-ascorbic acids, urea, and glycerol were used for NES preparation. The extraction efficiency of NES towards phenolics was compared with methanol as a conventional solvent.

The polarity of a solvent is the most affecting feature on the extraction of bioactive compounds from plant material. The polarities of phenolic compounds, which are relatively hydrophilic, are governed by the presence of one or more hydroxyl groups. Considering the

hydrophilic nature of most phenolics, choline chloride-based NES in combination with three organic acids were selected for extraction. Lactic, tartaric and succinic acids were chosen as HBD since they have different numbers of carboxyl and hydroxyl groups that result in various intra- and intermolecular hydrogen bond interactions. Furthermore, urea and glycerol were also used for preparation of NES as affordable and readily available compounds that have been widely used in the NES studies. It was shown that an increase in the ratio of polyol components in NES leads to increased diffusion and mass transfer and therefore enhanced extraction efficiency [21].

The high viscosity affected by hydrogen bonding and van der Waals interactions is the main disadvantage of NES. It can reduce diffusion coefficients of analytes, leading to low mass transfer and long extraction times. The effectiveness of the extraction could be improved by adding water to the NES and increasing the temperature. Previous studies investigated [22,23] the influence of adding water on supramolecular structures and physical properties of NES. The results confirmed that diluting of NES led to breaking hydrogen bonds between constituents of NES, which resulted in significant decrease in viscosity and density, and increase in conductivity and water activity. Also, water dilution has a

great effect on the polarity of NES, which further changes solubility of targeted compounds. For medium polarity compounds, addition of water improves solubility that can result in higher extraction yields, while in the case of less polar compounds influence is more complex since water content is correlated with the viscosity. Small addition of water contributes to better extraction by lowering viscosity, but extensive addition can strongly decrease solubility of relatively hydrophobic molecules. Since phenolics have a wide range of polarities, changing water content can influence not only extraction yields, but also composition of obtained extracts.

Therefore, besides investigation of different HBA and HBD on efficiency in the extraction of phenolic compounds, water content was also evaluated in this study. The content of added water was ranging from 20% to 50% (*w/w*) as it was previously marked as optimal share able to improve the viscosity of the NES components. Water was added to three different systems: choline chloride: tartaric acid = 1:1, choline chloride: urea = 1:2, and choline chloride: succinic acid = 1:1. Attention must be paid, however, to the changes of the interactions between the target compounds and NES caused by the higher content of water [22]. Higher water content is recommended only in cases when the mixture is too

**Table 2**

Phenolic content of different NES extracts of *T. chamaedrys* expressed as mg/kg of dried sample. Compounds present in a higher amount than in methanol are highlighted in bold font.

Phenolic compounds (mg/kg)*										
Extracts	PCA	SA	CGA	PHBA	AE	CA	ISO	RUT	VIT	IQUE
E1	2.7 ± 0.4	11.1 ± 0.2	58.0 ± 1.0	16.4 ± 0.4	7.4 ± 1.8	10.47 ± 0.02	0.21 ± 0.08	6.3 ± 0.1	0.27 ± 0.09	10.42 ± 1.4
E2	3.9 ± 0.2	5.9 ± 1.1	57.82 ± 0.08	27.3 ± 0.6	4.8 ± 0.2	7.5 ± 1.0	<b>0.36 ± 0.08</b>	<b>7.7 ± 0.6</b>	<b>0.35 ± 0.02</b>	<b>16.4 ± 0.2</b>
E3	3.6 ± 0.2	9.6 ± 0.6	<b>120.3 ± 0.9</b>	29.5 ± 1.3	<b>13.7 ± 1.5</b>	<b>17.4 ± 1.0</b>	<b>0.25 ± 0.03</b>	6.5 ± 0.7	<b>0.31 ± 0.04</b>	10.8 ± 1.7
E4	4.82 ± 0.03	13.1 ± 0.6	104.0 ± 0.8	22.1 ± 0.4	<b>21.8 ± 1.5</b>	<b>29.1 ± 0.8</b>	<b>0.28 ± 0.06</b>	6.1 ± 0.8	<b>0.29 ± 0.07</b>	9.2 ± 2.7
E5	3.6 ± 0.7	6.8 ± 0.5	<b>141.1 ± 0.7</b>	<b>43.4 ± 0.6</b>	<b>15.2 ± 0.5</b>	<b>20.7 ± 0.4</b>	<b>0.32 ± 0.02</b>	6.1 ± 0.6	<b>0.31 ± 0.03</b>	<b>12.6 ± 0.9</b>
E6	4.4 ± 0.4	7.0 ± 0.5	<b>141.44 ± 8.82</b>	27.6 ± 0.5	<b>17.1 ± 1.4</b>	<b>22.8 ± 0.5</b>	<b>0.45 ± 0.09</b>	<b>6.8 ± 0.2</b>	<b>0.34 ± 0.07</b>	10.9 ± 0.6
E7	3.2 ± 0.7	10.0 ± 0.3	19.5 ± 1.0	<b>81.8 ± 2.0</b>	7.6 ± 0.2	11.8 ± 1.3	<b>0.24 ± 0.02</b>	<b>10.5 ± 0.2</b>	<b>0.57 ± 0.03</b>	3.2 ± 0.2
E8	2.7 ± 0.3	6.50 ± 0.05	83.7 ± 0.6	23.1 ± 0.9	2.0 ± 0.8	3.7 ± 0.9	<b>0.25 ± 0.04</b>	<b>8.2 ± 1.0</b>	<b>0.37 ± 0.05</b>	10.2 ± 0.2
E9	2.3 ± 0.2	9.8 ± 0.7	5.0 ± 0.8	37.9 ± 0.5	6.59 ± 0.01	6.6 ± 2.2	0.08 ± 0.03	6.2 ± 0.3	0.16 ± 0.09	0.37 ± 0.05
E10	<b>7.4 ± 0.5</b>	9.2 ± 1.0	<b>137.2 ± 1.7</b>	<b>41.1 ± 1.3</b>	5.8 ± 1.1	9.5 ± 0.3	<b>0.4 ± 0.2</b>	<b>9.0 ± 0.2</b>	<b>0.57 ± 0.10</b>	<b>12.3 ± 1.7</b>
E11	3.8 ± 0.3	<b>216.0 ± 2.0</b>	30.9 ± 0.8	32.8 ± 1.4	<b>54.8 ± 0.5</b>	<b>100.1 ± 5.3</b>	0.23 ± 0.08	1.4 ± 0.8	0.073 ± 0.02	0.22 ± 0.06
E12	2.2 ± 0.6	5.4 ± 0.5	39.9 ± 0.9	15.8 ± 0.8	3.3 ± 0.4	5.2 ± 0.6	0.12 ± 0.07	5.3 ± 0.4	0.14 ± 0.05	5.8 ± 0.8
E13	<b>12.8 ± 0.4</b>	<b>20.5 ± 0.7</b>	95.70 ± 0.05	24.1 ± 1.0	<b>45.2 ± 0.3</b>	<b>77.8 ± 0.6</b>	<b>0.41 ± 0.09</b>	<b>8.8 ± 0.4</b>	0.27 ± 0.10	8.6 ± 0.4
E14	2.8 ± 0.6	3.5 ± 0.1	<b>122.1 ± 1.4</b>	35.7 ± 0.5	7.7 ± 0.8	14.8 ± 1.3	<b>0.38 ± 0.07</b>	<b>7.1 ± 0.7</b>	<b>0.33 ± 0.02</b>	<b>13.3 ± 1.2</b>
E15	6.3 ± 0.3	72.2 ± 0.5	22.4 ± 0.5	2.1 ± 0.7	3.6 ± 0.4	<b>0.44 ± 0.04</b>	6.1 ± 0.2	<b>0.30 ± 0.04</b>	<b>13.5 ± 0.3</b>	
E16	4.0 ± 0.6	3.7 ± 0.2	27.20 ± 0.08	16.1 ± 0.6	1.7 ± 0.6	1.9 ± 0.6	<b>0.38 ± 0.05</b>	<b>7.4 ± 0.6</b>	<b>0.43 ± 0.07</b>	<b>12.4 ± 1.6</b>
E17	3.0 ± 0.7	5.4 ± 0.9	1.34 ± 0.07	36.3 ± 2.0	6.0 ± 0.2	5.6 ± 1.1	0.09 ± 0.07	<b>10.1 ± 0.4</b>	0.14 ± 0.02	0.74 ± 0.08
E18	2.0 ± 0.5	7.8 ± 0.7	37.9 ± 0.4	9.1 ± 0.5	4.1 ± 0.3	6.2 ± 0.9	<b>0.29 ± 0.10</b>	<b>7.6 ± 0.4</b>	<b>0.37 ± 0.04</b>	13.1 ± 2.1
E19	2.7 ± 0.4	2.51 ± 0.08	72.8 ± 1.0	15.7 ± 1.8	6.3 ± 1.0	9.5 ± 0.4	<b>0.35 ± 0.08</b>	<b>7.2 ± 0.2</b>	0.24 ± 0.07	12.4 ± 1.8
MeOH	5.5 ± 0.7	19.3 ± 0.5	108.3 ± 1.5	40.0 ± 1.5	9.9 ± 0.4	15.4 ± 0.1	0.23 ± 0.09	6.6 ± 0.6	0.29 ± 0.07	11.2 ± 0.7

Phenolic compounds (mg/kg)\*

Extracts	PCOA	QR	AST	RA	LUT	QUE	NAR	KMP	HPD
E1	2.5 ± 0.4	11.8 ± 0.2	5.4 ± 0.3	5.7 ± 0.8	7.2	<b>11.3 ± 0.7</b>	3.8 ± 0.5	2.3 ± 0.6	0.68 ± 0.02
E2	2.3 ± 0.9	<b>27.7 ± 0.6</b>	<b>10.8 ± 1.0</b>	<b>10.1 ± 0.6</b>	<b>9.8 ± 0.1</b>	<b>10.4 ± 0.2</b>	<b>7.9 ± 0.3</b>	2.4 ± 0.9	0.63 ± 0.02
E3	3.0 ± 0.3	15.2 ± 0.5	8.1 ± 0.8	<b>8.7 ± 0.8</b>	4.3 ± 0.2	<b>16.6 ± 0.3</b>	4.3 ± 0.8	2.9 ± 0.2	0.37 ± 0.02
E4	<b>3.4 ± 0.9</b>	11.1 ± 0.3	6.4 ± 0.2	<b>8.8 ± 0.2</b>	4.8 ± 0.3	<b>30.2 ± 0.4</b>	3.9 ± 0.5	2.9 ± 0.3	0.32 ± 0.04
E5	<b>4.06 ± 0.02</b>	16.1 ± 0.7	9.6 ± 0.1	<b>10.0 ± 0.1</b>	4.3 ± 0.8	<b>13.8 ± 0.9</b>	4.8 ± 0.2	2.1 ± 0.2	0.23 ± 0.08
E6	<b>5.4 ± 0.6</b>	12.9 ± 0.7	8.3 ± 0.5	<b>10.2 ± 0.1</b>	4.0 ± 0.9	<b>16.6 ± 0.5</b>	4.7 ± 0.2	<b>3.3 ± 0.2</b>	0.24 ± 0.05
E7	0.7 ± 0.5	8.2 ± 0.5	4.1 ± 0.6	6.1 ± 0.8	<b>6.6 ± 0.3</b>	6.2 ± 0.6	1.0 ± 0.4	2.4 ± 0.1	<b>7.84 ± 0.01</b>
E8	2.51 ± 0.05	15.4 ± 0.7	7.8 ± 0.5	<b>8.3 ± 0.5</b>	4.7 ± 0.1	6.1 ± 0.3	4.8 ± 0.3	2.5 ± 0.2	0.51 ± 0.05
E9	0.3 ± 0.2	1.1 ± 0.6	0.44 ± 0.07	3.4 ± 0.4	<b>13.9 ± 0.4</b>	6.3 ± 0.8	0.08 ± 0.02	<b>3.7 ± 0.4</b>	<b>34.8 ± 0.2</b>
E10	<b>4.6 ± 0.4</b>	13.1 ± 0.5	5.3 ± 0.2	<b>8.3 ± 0.4</b>	<b>8.4 ± 0.8</b>	6.2 ± 0.7	5.5 ± 0.8	<b>3.5 ± 0.2</b>	1.04 ± 0.09
E11	<b>3.8 ± 0.4</b>	0.50 ± 0.06	0.27 ± 0.05	2.6 ± 0.6	<b>44.5 ± 0.9</b>	<b>36.75 ± 0.09</b>	2.0 ± 0.9	<b>16.3 ± 0.1</b>	<b>113.5 ± 0.4</b>
E12	1.5 ± 0.2	4.9 ± 0.3	2.8 ± 0.1	2.1 ± 0.6	2.0 ± 0.2	<b>7.72 ± 0.03</b>	1.4 ± 0.1	1.9 ± 0.1	0.50 ± 0.03
E13	6.2 ± 0.1	8.6 ± 0.6	5.7 ± 0.4	<b>8.3 ± 0.6</b>	11.8 ± 0.6	<b>33.7 ± 0.6</b>	4.88 ± 0.09	<b>5.3 ± 0.1</b>	<b>3.37 ± 0.05</b>
E14	2.3 ± 0.2	15.1 ± 0.5	8.4 ± 0.4	<b>8.2 ± 0.9</b>	4.10 ± 0.06	<b>8.8 ± 0.3</b>	4.8 ± 0.4	2.956 ± 0.3	0.30 ± 0.06
E15	2.0 ± 0.8	19.3 ± 0.8	8.7 ± 0.4	<b>8.0 ± 0.8</b>	<b>7.2 ± 0.1</b>	6.19 ± 0.08	<b>8.7 ± 0.7</b>	<b>3.1 ± 0.2</b>	1.19 ± 0.07
E16	1.3 ± 0.5	16.47 ± 0.08	7.1 ± 1.0	<b>9.5 ± 0.5</b>	<b>8.3 ± 0.3</b>	6.1 ± 0.2	6.7 ± 0.1	2.7 ± 0.6	0.99 ± 0.07
E17	4.1 ± 0.1	2.0 ± 0.5	1.7 ± 0.8	3.1 ± 0.3	1.3 ± 0.4	6.3 ± 0.7	0.20 ± 0.07	1.9 ± 0.1	0.42 ± 0.04
E18	3.1 ± 0.5	16.1 ± 0.3	9.2 ± 0.9	8.8 ± 0.7	<b>8.72 ± 0.08</b>	<b>8.9 ± 0.2</b>	6.3 ± 0.3	2.3 ± 0.4	0.69 ± 0.02
E19	1.6 ± 0.4	19.1 ± 0.3	9.9 ± 0.7	11.5 ± 0.2	5.7 ± 0.8	<b>8.7 ± 0.1</b>	6.2 ± 0.3	2.1 ± 0.2	0.98 ± 0.02
MeOH	3.12 ± 0.02	21.56 ± 0.01	10.3 ± 0.2	7.6 ± 0.2	6.4 ± 0.2	7.460 ± 0.01	7.9 ± 0.1	3.0 ± 0.7	1.51 ± 0.2

\* Means of triplicate analyses ± standard deviation; Abbreviations: Protocatechuic acid (PCA), Syringic acid (SA)), Chlorogenic acid (CGA), *p*-Hydroxybenzoic acid (PHBA), Aesculetin (AE), Caffeic acid (CA), Isoorientin (ISO), Rutin (RUT), Vitexin (VIT), Isoquerceatin (IQUE), *p*-Coumaric acid (PCOA), Quercitrin (QR), Kaempferol (AST), Rosmarinic acid (RA), Luteolin (LUT), Quercetin (QUE), Naringenin (NAR), Kaempferol (KMP), Hispidulin (HPD).

viscous for extraction, particularly for plant materials in powder form [24].

Considering the temperature, it was shown that viscosity noticeably decreased with its change from room to 50 °C [25,26]. Some authors used even higher temperatures [27,21], but it should be also taken into account that some phytochemicals are sensitive to elevated temperatures. Considering the above-mentioned facts, the extraction temperature was set to be 50 °C.

### 3.2. Screening of extraction efficiency of NES

The efficiency of NES solvent to extract phenolic compounds from *T. chamaedrys* was evaluated by UHPLC-DAD-MS/MS, and spectrophotometric tests (TPC, TFC, RSA).

#### 3.2.1. Quantification of individual phenolic compounds by UHPLC-DAD-MS/MS

Nineteen phenolic compounds were identified in NES extracts (Table 2). The most abundant group and the main compounds were phenolic acids (protocatechuic, syringic, chlorogenic, *p*-hydroxybenzoic, caffeic, rosmarinic and *p*-coumaric acids), flavone (luteolin, isoquercetin, hispidulin, astragalin), flavonol (quercetin, kaempferol), flavanon (naringenin), and glycosides (quercitrin and rutin).

Choline chloride:tartaric acid (1:1) mixtures with 20–50% water content (E3–E6) were excellent extraction media for targeting aesculetin and quercetin, with E4 extracting these compounds 2.2 and 4.2 times, respectively, more than methanol. Also, these eutectic systems extracted chlorogenic, rosmarinic and caffeic acids in higher concentrations compared to methanol. The extraction process of main phenolics was improved as the water content in NES system increased, while no regular trends could be observed among the data with addition of water in systems. Choline chloride:urea (1:2) with different water content (E7–E10), showed varied phenolic profiles. Mixture E7 extracted *p*-hydroxybenzoic acid, vitexin and hispidulin in higher amounts compared to methanol, E9 extracted luteolin and hispidulin, while E10 targeted isoorientin and vitexin. These results demonstrate that water content greatly influences NES properties and extraction performances, highlighting a need for closer understanding of interaction in deep eutectic solvents and inclusion of water molecules in theoretical studies. Eutectic solvents prepared with choline chloride and succinic acid in 1:1 M ratio

(E11–E14) showed similar behavior and diverse extraction performance depending on water content (20–50% w/w). NES E11 and E13 extracted higher amounts of aesculetin, caffeic acid, luteolin, quercetin, kaempferol and hispidulin, E12 showed poor extractability, while E14 targeted chlorogenic acid. Eutectic mixture E11 extracted syringic acid and hispidulin in noticeable higher yields compared to methanol, 11 and 75 times more, respectively. Glycerol and urea were mixed in molar ratios 1:1 and 2:1 and obtained mixtures showed different extraction efficiency, highlighting that ratio of components has an impact on performance.

The phenolic profiles of *T. chamaedrys* obtained by methanol and each NES separately, were compared using a paired *t*-test (Table S1). Two extraction solvents were tested by applying both to the same set of phenolic compounds, which contained different amounts of analyte, by looking at the difference between each pair of results. Significantly lower amounts of phenolic compounds were extracted by NES E1, E8, E12 and E15 compared to methanol, making them less efficient extraction media than organic solvent. No statistically significant differences between methanol and other NES were observed, indicating equal extraction efficiency, but the low toxicity and biodegradability of NES give them an advantage over conventional solvent (Table 2 and Table S1).

#### 3.2.2. TPC, TFC and RSA as extract quality parameters

TPC, TFC and RSA of 19 NES extracts of *T. chamaedrys* were determined and compared with methanol as a referent extract (Fig. 1 and Table S2). NES extracts of *T. chamaedrys* are rich in phenolics, especially in flavonoids, TPC values were in the range 9.2–94 g of GAE per kg DW, while TFC values were ranged from 24.23 g to 180.3 g of rutin equivalent per kg DW. Four NES extracts, E2 (choline chloride:lactic acid 1:1, 30% water), E10 (choline chloride: urea 1:2, 50% water), E15 (choline chloride: glycerol 1:1, 20% water) and E16 (glycerol: urea 1:1, 20% water) have higher TPC and TFC content than methanol extract, and the highest value was found in eutectic mixture E15. The presence of one liquid compound (glycerol, lactic acid etc.) or water in NES mixture, could lower the viscosity. Eutectic solvents E15 and E16 have glycerol and E2 has lactic acid, while E10 contains 50% of water, which contributes to lowering viscosity of prepared NES and increasing the extraction efficiency. The RSA values were in the range 18–734 mmol trolox equivalent per kg DW, with 5 NES extracts with higher values

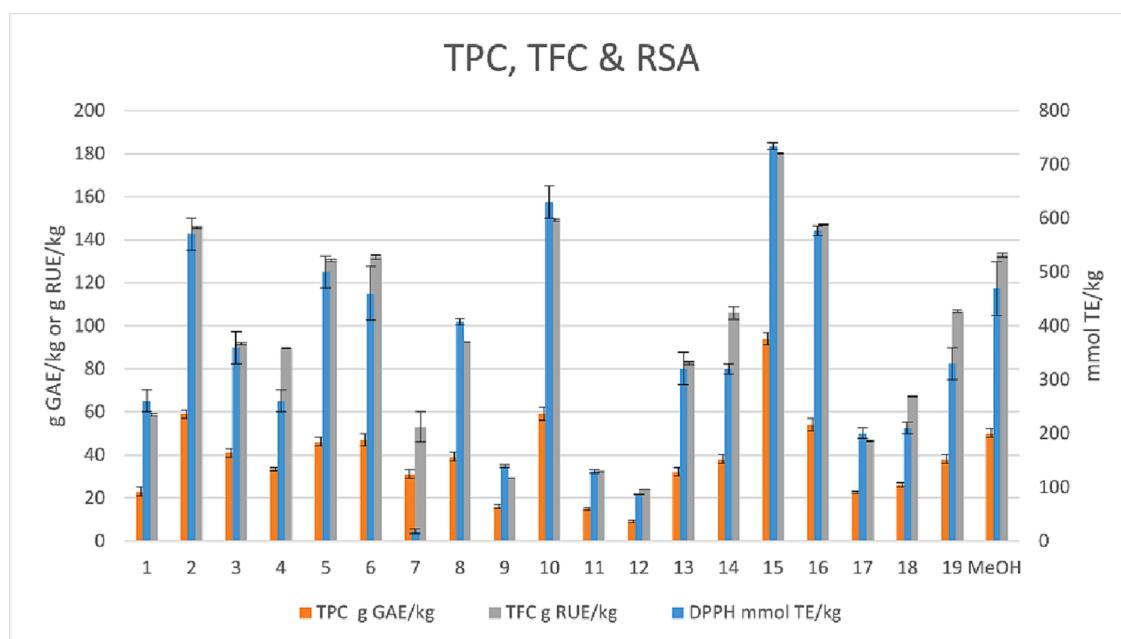


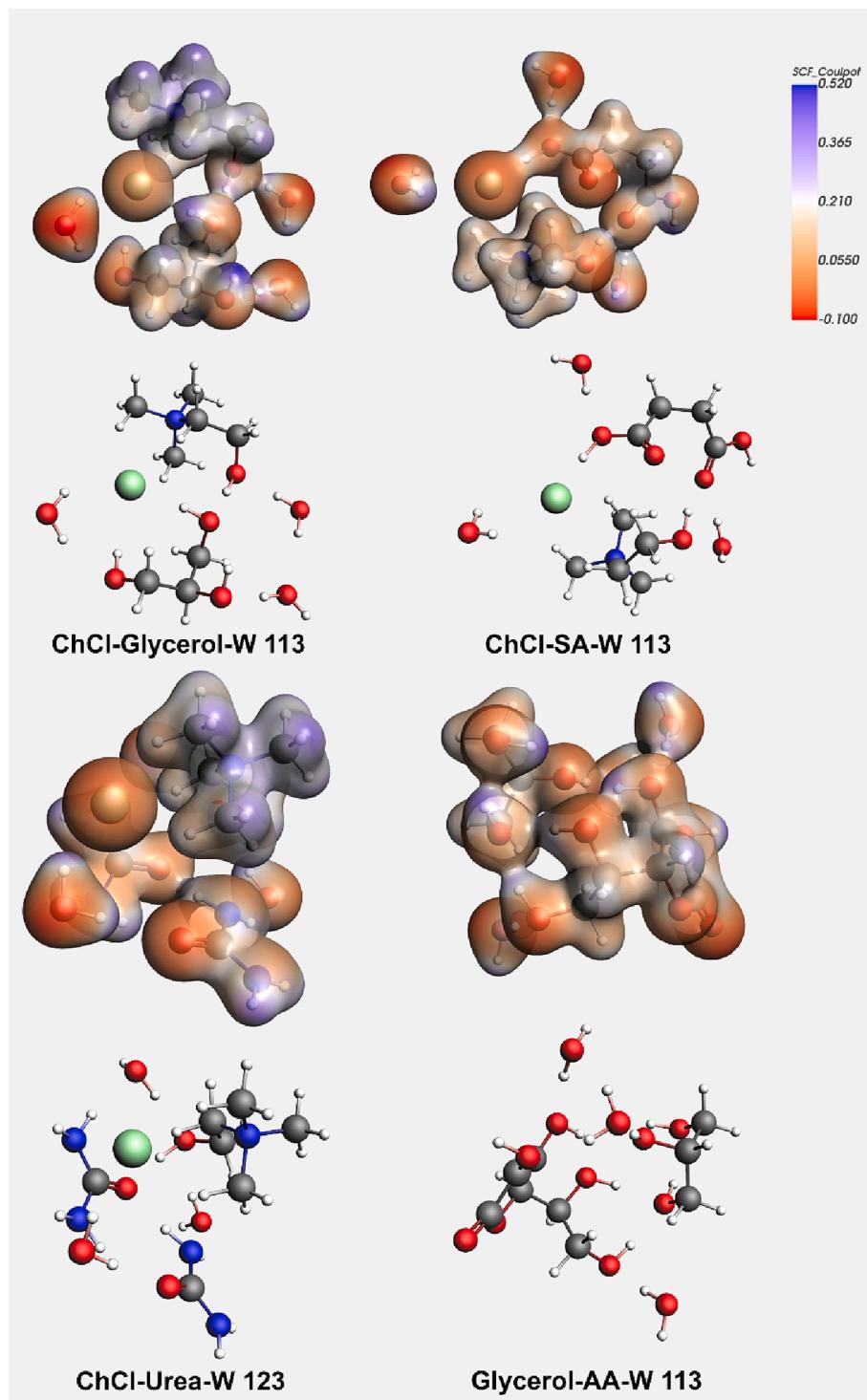
Fig. 1. RSA, TPC and TFC in different NES extracts of *T. chamaedrys*.

compared to methanol (E2, E5, E10, E15 and E16). The majority of extracts of *T. chamaedrys* showed strong antioxidant activity, which is correlated with high phenolics content.

The spectrophotometric assays of *T. chamaedrys* (presented as results of TPC, TFC and RSA tests), obtained by methanol and each NES separately, were compared using a paired *t*-test (Table S2). Two extraction solvents were tested by applying both to the same set of values (results of TPC, TFC and RSA), which contained different values, by looking at the difference between each pair of results. No statistically significant differences between methanol and all NES were observed, indicating equal

extraction efficiency (Table S2 and Table S3). Good Pearson's correlation was observed between RSA and TPC values ( $r = 0.916$ ), RSA and TFC values ( $r = 0.953$ ), and between TPC and TFC values ( $r = 0.943$ ), suggesting that phenolics, especially flavonoids were the main compounds responsible for the antioxidant activity of *T. chamaedrys* extracts.

Increase in the percentage of water content in NES leads to a more efficient extraction, primarily due to a decrease in viscosity, resulting in rise of the values of the TPC, TFC and RSA values. However, the rising trend in results is not uniform and equal for three studied systems (choline chloride: tartaric acid = 1:1, choline chloride: urea = 1:2, and



**Fig. 2.** 3D structures and surface charge densities (in  $e/\text{\AA}^2$ ) of selected NES supramolecular complexes.

choline chloride: succinic acid = 1:1) since structure of HBD has greater influence. In the case of NES that have choline chloride as HBA and 20% of water, but different HBD lactic, succinic, tartaric acids, urea or glycerol (E2, E3, E11, E7 and E15, respectively), obtained results of spectrophotometric tests illustrate influence of HBD. The TPC results obtained for all extracts were lower compared to previously reported ( $243.65 \pm 3.46$  mg GAE/g and  $169.50 \pm 0.25$  mg GAE/g) [4,5], while TFC values were higher than reported by the same authors ( $9.75 \pm 0.25$  mg RE/g and  $61.80 \pm 0.51$  mg RE/g). The results of spectrophotometric assays demonstrated the impact of HBD structure and water content on both the properties of NES and their extraction performance. These findings emphasize the importance of gaining a deeper understanding of the interactions between NES and targeted compounds, as well as predicting the behavior of the system.

### 3.3. Theoretical evaluation of extraction efficiency by COSMO-RS

Experimental evaluation of the extraction performances of a large number of NES is a long-term process, further complicated by the fact that the best solvent cannot be unambiguously determined based solely on  $\log P$  value or the “like dissolves like” rule. In that sense, the solvent screening is usually performed theoretically in order to better understand the molecular interactions between NES and the target compounds, and then experimentally validated [28]. As it was mentioned previously, the influence of HBA and HBD structures on the extraction efficiency is mainly utilized by the COSMO-based thermodynamic model [29–31].

#### 3.3.1. Structure and surface charge density of NES

Previous COSMO-RS studies on the extraction efficiency of NES have either considered these solvents as a stoichiometric mixture of individual constituents [32–35] or as pseudo-compounds formed through hydrogen bonding between HBD and HBA [36,37]. The  $\sigma$  profiles and  $\sigma$  potentials calculated in the presence of water can provide valuable insights for more realistic modeling of intermolecular interactions and improved predictions of extraction efficiency. The optimized geometries and surface charge densities of the HBD:HBA:water supramolecular complexes are depicted in Fig. 2. The results demonstrate that the hydrogen bonding between NES constituents is preserved in the presence of three water molecules, enabling an accurate representation of the NES solution structure. The regions with negative surface charges are predominantly found near water oxygen atoms, while positively charged surface segments are situated near N atoms in ChCl or urea-based NES. These segments will interact with oppositely charged

surface segments when combined with phenolics, as illustrated in Fig. 3.

#### 3.3.2. $\sigma$ -Profile and $\sigma$ -potential analysis

In the COSMO-RS theory, the interaction between solvent and solute is characterized by their screening charge densities,  $\sigma$  and  $\sigma'$ . Histograms that show the screening charge density distributions for a given molecule are called  $\sigma$ -profiles. If the solvent and solute have complementary  $\sigma$ -profiles, they tend to have high solubility and extractability. The strong interactions between their interacting surface segments lead to a favorable chemical potential,  $\mu(\sigma)$ . The  $\sigma$ -profile is typically divided into three parts that reflect the hydrogen-bond accepting, nonpolar, and hydrogen-bond donating regions, making it a useful tool for comparing the polarity of different molecules.

The results presented in Fig. 4 show that phenolics exhibit a significant portion of their  $\sigma$  surface in the non-polar region, emphasizing the crucial role of hydrophobic and Van der Waals interactions in the extraction process. Additionally, their polar regions exhibit more prominent peaks in the HBA region. Two NES, choline chloride:glycerol:water (1:1:3) and choline chloride:succinic acid:water (1:1:3), exhibit a strong peak centered around  $-0.007 \text{ e}/\text{\AA}^2$  that grants these solvents both HBD and hydrophobic properties. This peak originates from cholinium cation and is highly complementary with the  $\sigma$  features of target phenolics from *T. chamaedrys*.

The  $\sigma$ -potential analysis was performed to evaluate the thermodynamic properties and to compare the polarity and hydrogen bonding ability of NES and target phenolics. As shown in Fig. 5, the entire screening charge density range is separated into three areas: the electronic basicity region ( $\sigma < -0.0082 \text{ e}/\text{\AA}^2$ ), the nonpolar region ( $-0.0082 \text{ e}/\text{\AA}^2 \leq \sigma \leq 0.0082 \text{ e}/\text{\AA}^2$ ), and the electronic acidity region ( $\sigma > 0.0082 \text{ e}/\text{\AA}^2$ ). The  $\sigma$ -potential of a solvent in the electronic basicity region indicates its HBA strength, while that in the electronic acidity region represents its HBD strength. The strength of HBA and HBD is proportional to the magnitude of the  $\sigma$ -potential, with a lower  $\sigma$ -potential indicating a stronger ability.

The results indicate that the high HBA affinity of hispidulin, quercetin, caffeic acid, and chlorogenic acid can be balanced by the high HBD affinity of two NES, which is significantly stronger compared to that of water and methanol. Therefore, the interactions that govern the extraction mechanism are hydrogen bonding between the polar  $-OH$  groups of phenolics as HBDs and cholinium cations as HBAs. However, it is important to note that non-polar interactions also play a significant role in the mechanism.

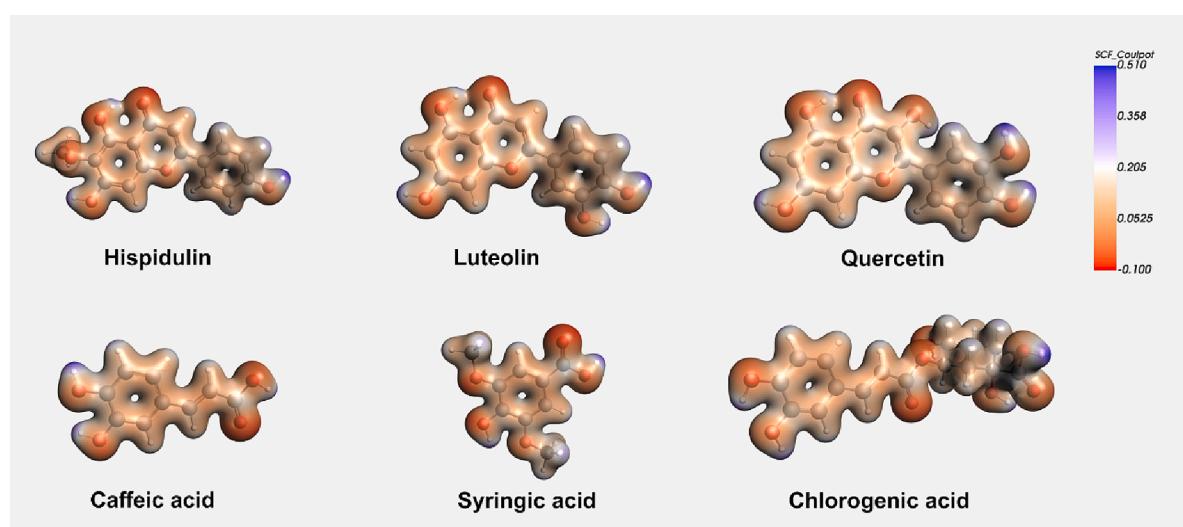
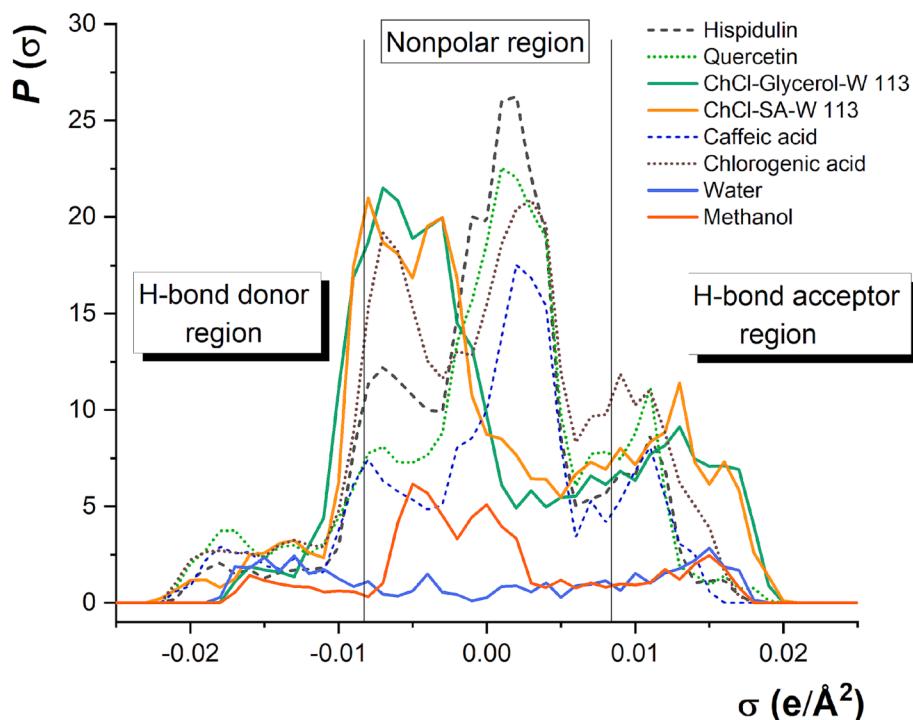
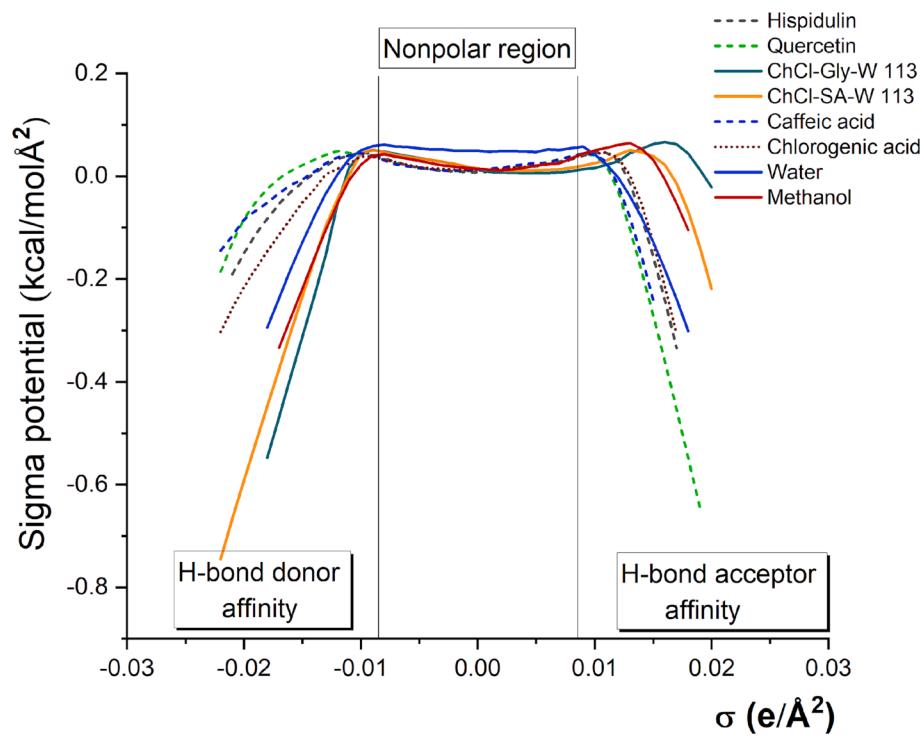


Fig. 3. Surface charge densities of six most abundant phenolics from *T. chamaedrys*.



**Fig. 4.** The  $\sigma$ -profiles of four solvents (two NES, water, and methanol) and four solutes (hispidulin, quercetin, caffeic acid, and chlorogenic acid).

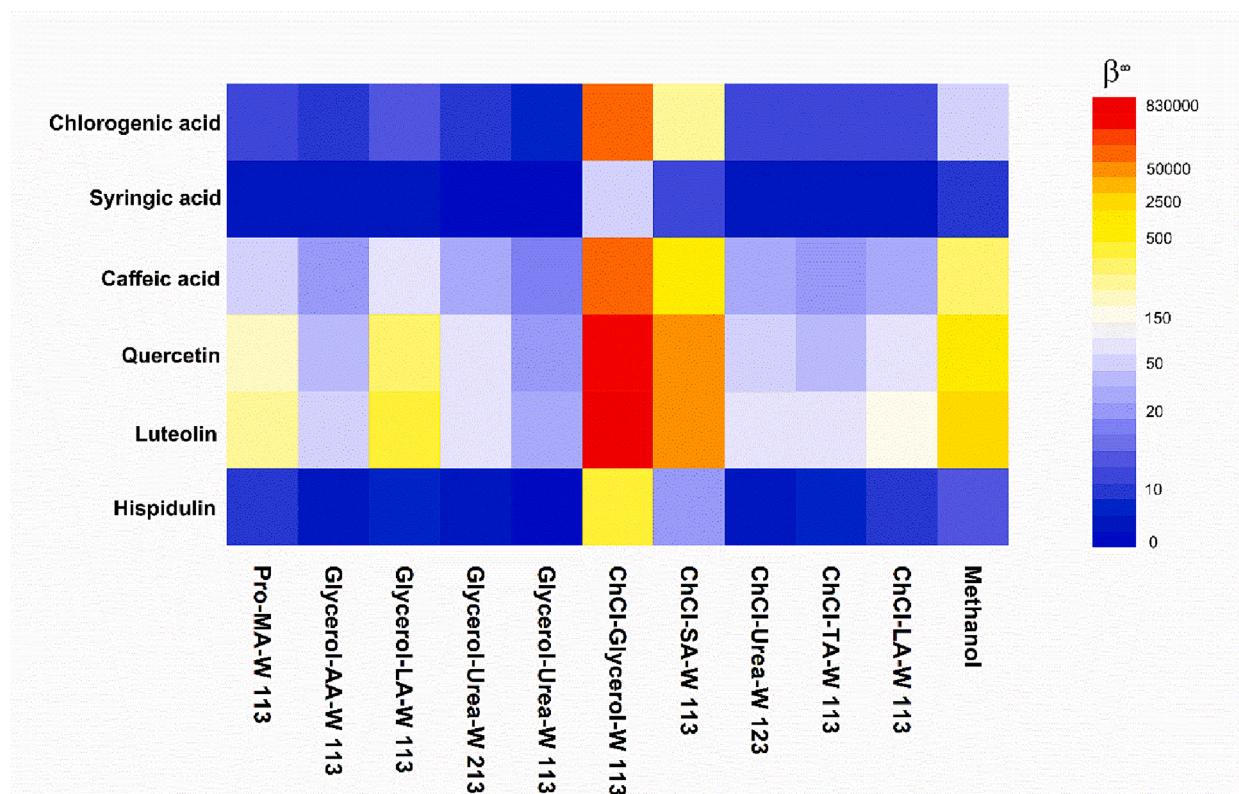


**Fig. 5.** Sigma potentials,  $\mu(\sigma)$  of four phenolics and four solvents.

### 3.3.3. Activity coefficients at infinite dilution calculated by COSMO-RS

The COSMO-RS is widely used for predicting macroscopic properties such as activity coefficients, distribution coefficients, and solubilities. By determining the sigma potentials of all species in a solution, the activity coefficients can be calculated by solving a nonlinear equation that considers the interactions between species and the influence of the solvent.

The calculated activity coefficients at infinite dilution ( $\gamma^\infty$ ) were transformed into the solvent-solute affinity parameter  $\beta^\infty$ , which is proportional to the solubility of the compound [35]. The  $\beta^\infty$  values for the six most abundant phenolics in *T. chamaedrys* in ten NES:water mixtures and methanol as a reference solvent are displayed in Fig. 6 and listed in Table S4. The results indicate that choline chloride:glycerol:water (1:1:3) and choline chloride:succinic acid:water (1:1:3) have a



**Fig. 6.** The solvent–solute affinity parameter  $\beta_{\infty}$  for six solutes in ten NES and methanol. The x-axis label indicates the mol ratio of HBD, HBA, and water in each NES. Higher values for  $\beta_{\infty}$  represent better mutual affinity and therefore higher extractability. The abbreviations Pro, MA, ChCl, TA, SA, LA, AA, and W denote L-proline, maleic acid, choline chloride, tartaric acid, succinic acid, lactic acid, L-ascorbic acid and water, respectively, respectively.

greater affinity than methanol for all the solutes. The solubility parameters of all NES are significantly higher compared to water, ranging from 100 to approximately 1,000,000 times greater (as seen in Table S5).

### 3.3.4. Significance of COSMO-RS for natural eutectic solvent optimization

The results of COSMO-based analysis used for better understanding of the influence of HBA and HBD structures on the extraction efficiency, sometimes showed an opposite trend compared to experimental results [38]. It is usually occurred in case of solvent–solute combinations in which non-polar interactions play an important role [39]. Namely, physical parameters such as the viscosity and temperature largely affect the efficiency of NES extraction, beside interactions between solvent and solute modeled by COSMO-RS. However, the predictions made using COSMO-RS for the extraction efficiency of phenolics from *T. chamaedrys* showed good qualitative correlations with the results of spectrophotometric tests (RSA, TPC, and TFC values) (Fig. 1 and Table S1). Among the NES tested, ChCl-glycerol 1:1, 20% water (E15), which corresponds to a molar ratio of 1:1:3, showed the best performance. The results of UPLC-DAD-MS/MS analysis (Table 2) also indicated that the highest concentrations of five out of six modeled solutes (excluding chlorogenic acid) were found in NES composed of ChCl-SA 1:1 and 20% water. These results confirm the efficacy of the proposed framework for COSMO-RS modeling in facilitating the rational selection of NES for targeted extractions.

## 4. Conclusion

In conclusion, this study demonstrated the potential of nineteen different NES for extracting phenolic compounds from *Teucrium chamaedrys*. Both experimental and theoretical evaluations were conducted to determine the extraction efficiency of each NES. The results showed

that choline chloride: succinic acid in a molar ratio 1:1 with 20% of water (*w/w*) had the highest extraction performance in terms of quantified phenolics, while choline chloride: glycerol in a molar ratio 1:1 and with 20% of water (*w/w*) was the most efficient in terms of antioxidant activity, total phenolics, and flavonoid content. The study also revealed that the structure of HBA and HBD and water content significantly influenced the extraction process. The COSMO-RS methodology was employed to predict the extractability of phenolics in various NES, and the results were consistent with the experimentally obtained data. Choline chloride: glycerol 1:1 and choline chloride: succinic acids 1:1 were found to have greater affinity for all the solutes compared to methanol. The success of COSMO-RS model based on ternary HBD:HBA: water complexes in predicting NES behavior suggests its potential as a prescreening tool for the extraction of bioactive compounds. This is a rare example of the examination of the impact of water in COSMO-RS predictions of extraction efficiencies.

This study is another example of NES's capability to replace harsh organic solvents in isolation of bioactive compounds. Additionally, it is a bright example of synergism between experimental and theoretical evaluation assessments that are necessary to determine the optimal NES mixture for efficient extraction of phenolics. The methodology described herein could be applied to other natural products and groups of bioactive compounds with equal efficacy.

## CRediT authorship contribution statement

**Mila Lazović:** Investigation, Methodology, Software, Validation, Writing – original draft. **Ilija Cvijetić:** Methodology, Software, Validation, Writing – original draft. **Milica Jankov:** Investigation, Methodology. **Dušanka Milojković-Opsenica:** Writing – review & editing. **Jelena Trifković:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Petar Ristivojević:** Conceptualization,

Methodology, Supervision, Writing – review & editing, Visualization.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Petar Ristivojevic reports financial support was provided by Ministry of Science, Technological Development and Innovation of Republic of Serbia.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2023.122649>.

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