Deep eutectic solvent-induced coacervation in micellar solution of alkyl polyglucoside surfactant: supramolecular solvent formation and application in food analysis

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Abstract

The number of deep eutectic solvents were investigated to induce coacervation and cause phase separation in the micellar solution of the alkyl polyglucoside C_8-C_{10}. Three fatty acids were selected as hydrogen bond donors namely hexanoic acid, heptanoic acid, and octanoic acid, while quaternary ammonium salts and a monoterpenoid thymol were used as hydrogen bond acceptors to obtain deep eutectic solvents. The precursors of the deep eutectic solvents could be incorporated into the micelle structure of alkyl polyglucoside C_8-C_{10} and modulate its properties, improving the interaction with the target compounds and promoting phase separation due to the increasing size of the micellar aggregates. The synergy of green surfactant and deep eutectic solvent has been demonstrated in the determination of capsaicinoids as secondary metabolites retaining the pungency of chili peppers and, thus, of spicy foods. Extraction recoveries were above 75% for target analytes. The limits of detection were found to be 1.7 μg g^{-1} for capsaicin and dihydrocapsaicin. The relative recoveries were in the range of 76% – 130%. The research presented is the first example of synergy between alkyl polyglucoside and deep eutectic solvent. The proposed extraction system has the potential to be used in many other analytical tasks, especially in the analysis of solid and heterogeneous samples. Not only fatty acid-based deep eutectic solvents but other based on alcohols, monoterpenoids, etc. can also be used for coacervation. Thus, wide range of already studied deep eutectic solvents could be investigated for this purpose.

Keywords: supramolecular solvents; alkyl polyglucoside; deep eutectic solvent; coacervation; capsaicinoids; surfactant

1. Introduction

Micelle-based extraction techniques have emerged as a powerful tool offering a unique and versatile approach to improving the precision and sensitivity of analytical methods. Micelles, nano-sized assemblies of surfactant molecules, play a pivotal role in the solubilization of target compounds and enable their efficient extraction from the sample matrix [1]. In particular, micelle-based extraction is a useful method for isolating analytes from solid and heterogeneous food samples and is a more environmentally friendly process compared to the use of conventional organic solvents.

As a rule, the features of the micelles depend on the surfactant used for micelle formation. The important property of some surfactants is their clouding behavior, which makes them suitable for the separation and preconcentration of target components [2]. Under the clouding phenomenon, the surfactant solution becomes an opaque suspension that eventually separates into two phases: a surfactant-rich phase, which contains most of the surfactant, and a surfactant-poor phase, which contains mainly water and a surfactant at a concentration close to the critical micelle concentration (CMC). This could be triggered by the temperature, the addition of electrolytes, the change in pH, or the addition of counterions [3]. Recently, it was also shown that deep eutectic solvents (DES) could serve as a modulator of the micellization of surfactants [4,5]. For example, 5% of DES choline chloride (ChCl):urea/ethylene glycol (in a molar ratio of 1:2) in a solution affected the micellization process of the two cationic surfactants, i.e., tetradecyltrimethylammonium bromide and cetyltrimethylammonium bromide, by decreasing the CMC and simultaneously increasing the number of micellar aggregation [5]. Similarly, the influence of DES (ChCl:urea in 1:2 molar ratio) on micellization behavior was observed with the anionic surfactant sodium dodecyl sulfate [4]. This indicates that the micellization process of the surfactants in the DES solutions is strongly favored. Other studies in this field include the investigation of the solubility of DES in cyclohexane in the presence of sodium dodecyl sulfate [6], the aggregation behavior of two cationic surfactants in glycerol-based DES [7], and the morphology modulation of micelles of ionic surfactants in ternary DES [8]. Undoubtedly, such studies on the physicochemical/association behavior of surfactants and their interconnection with DES reveal new opportunities for synergy between surfactants and DES, and can boost their application in various fields [4] for example in chemical analysis. Both surfactants [9,10] and DES [11–13] are widely used in sample preparation prior to instrumental analysis because they have ideal extraction properties for the target analyte and are compatible with frequently used detection tools. In addition, the wide

variety of surfactants (including bio-based ones) and precursors for preparation of DES, as well as their properties, which are in line with the Green Chemistry concept, make them a preferred choice for chemical analysis and an alternative to toxic and volatile organic solvents.

Recent investigations in the field of surfactants have focused on their application as precursors for supramolecular solvents (SUPRAS) [14–16]. The formation of such solvents is also based on the clouding phenomenon, and the extraction of the analyte is provided by the nanostructured surfactant-rich phase. Clouding of surfactants, where a surfactant-rich phase separates from a dilute micellar solution, is, by definition, a coacervation process. This term is usually used in the case of SUPRAS and indicates the phenomenon due to which phase separation is observed [17]. Until now for the phase separation and formation of coacervates based on surfactants or compounds that mimic the properties of surfactants such as alkanols [18], carboxylic acid [19], and primary amines [20,21], the modification of the microenvironment was provided by the addition of polar solvent, changes of pH, ionic strength, temperature, or charge of an outer surface of the micelles. The research carried out so far in the field of the interconnection of surfactant and DES makes it possible to hypothesize the potential application of DES for SUPRAS formation. The DES opens up the possibility of working with components that are in a solid state at ambient conditions but are liquid in the form of DES. Their ability to decrease the CMC and increase the aggregate size could potentially influence the physicochemical properties of the SUPRAS phase. In addition, the DES component could be incorporated into the micelle structure, creating additional binding centers with the analytes, which improves the extraction recovery of the analytes.

In the current research, we have chosen alkyl polyglucoside C_8-C_{10} (caprylyl/capryl glucoside, APG C_8-C_{10}) as a biodegradable and plant-derived surfactant and investigated its ability to proceed the phenomenon of coacervation in the presence of fatty acid-based DES to obtain a SUPRAS phase and allow preconcentration of target analytes [22,23]. The fatty acid-based DES was selected as the initial example to demonstrate its potential as a coacervation agent. Such a scenario was implemented for the analysis of food samples and the determination of the capsaicinoids content in chili sauce samples. The selected analytes (capsaicin and dihydrocapsaicin) belong to the plant alkaloids and are secondary metabolites retaining the pungency of chili peppers and, thus, of spicy foods. [24,25]. Capsaicinoids can be used in therapy due to their analgesic and anti-inflammatory properties as well as their gastroprotective and antitumor properties [26]. In addition, moderate daily consumption of chili pepper sauce has been shown to improve serum glucose and cholesterol levels [27]. However, the potential applications of these compounds are limited by the irritation caused by their pungency [28].

The determination of the capsaicinoid content in spicy foods is a routine procedure aimed to provide a clear understanding of the spiciness of the food and to prevent adverse allergic reactions in consumers [29,30]. Capsaicin and dihydrocapsaicin, which are contained in chili sauce, are usually extracted by organic solvents such as acetone, methanol, ethanol, chloroform, acetonitrile, n-hexane. The extraction step usually takes more than 30 min and requires a large amount of organic solvents [31]. The application of micelle-mediated extraction using alkyl polyglucoside C_8-C_{10} followed by SUPRAS formation induced by DES could be a good alternative to the available extraction methods.

Thus, the ***hypothesis*** of the research is: *DES could serve as a coacervation agent for the formation of a supramolecular solvent in the micellar solution of an alkyl polyglucoside surfactant and such an extraction system could be suitable for the rapid extraction and preconcentration of target analytes in food samples.*

2. Experimental

2.1 Reagents and solutions

All chemicals and reagents used were of analytical grade. Ultra-pure water was obtained using HydroLab HLP5 (HydroLab, USA) and was used throughout the work. Capsaicin (>95%) (CAS: 404-86-4) was obtained from Sigma-Aldrich, Merck (Saint Louis, USA), dihydrocapsaicin (>98%) (CAS: 19408-84-5) was obtained from Pol-Aura (Olsztyn, Poland). Acetonitrile (CAS: 75-05-8), 2-propanol (CAS: 67-63-0) and methanol (CAS: 67-56-1) were purchased from LiChrosolv, Supelco (Darmstadt, France). Formic acid (FA) (CAS, 64-18-6) was purchased from Avantor Performance Materials (Gliwice, Poland). Thymol (Thy) (CAS, 89-83-8), tetrapropylammonium bromide (TPrABr) (>98%) (CAS, 1941-30-6), choline chloride (ChCl) (CAS: 67-48-1) were obtained from Sigma-Aldrich,Merck (Saint Louis, USA). Hexanoic acid (C6) (CAS: 142-62-1), heptanoic acid (C7) (CAS: 111-14-8), octanoic acid (C8) (CAS: 124-07-2), were obtained from Thermo Scientific (Saint Louis, USA). Plantacare® 810 UP (caprylyl/capryl glucoside (APG C_8-C_{10}, w/w ≥ 50% - ≤ 70% of active surfactant) (CAS: 68515-73-1) was obtained from BASF (Ludwigshafen, Germany).

Stock solutions of capsaicin and dihydrocapsaicin (1.0 g L^{-1}) were prepared by dissolving the appropriate amount of the reagents in methanol. These solutions were stored under dark conditions at 5 °C in a refrigerator and re-prepared every 6 weeks. Working solutions of the analytes were prepared daily by appropriate dilutions of the stock solutions with ultrapure water.

The APG C_8-C_{10} aqueous solutions (w/w, %) were prepared by dissolution of appropriate amount of the reagent in ultrapure water.

2.2 Instrumentation

Chromatographic measurements were performed on the HPLC-UV Agilent Series 1200 HPLC System. The chromatographic separation was achieved with a Phenomenex Kingsorb C18 column (150 mm × 4.6 mm, 5 μm) at 40 °C. The mobile phase of H_2O (A) and acetonitrile with 0.1 % of formic acid (B) at a flow-rate of 0.8 mL min^{-1} was used. The separation of capsaicinoids (capsaicin and dihydrocapsaicin) was performed by gradient elution according to the program: the content of phase B was linearly increased from 45 to 95 % from the 0.5 utill 15 min, kept constant from 15 till 16 min, linearly decreased back to 45 % till 17 min and then kept constant at 45% till 20 min. The detection wavelength was 280 nm. The injection volume was 20 μL.

Centrifugation was performed with the use of Eppendorf Centrifuge 5810R (Eppendorf, USA).

Density meter DMA 4500 (Anton Paar, Austria) was used to determine the density of isotropic solution and SUPRAS phases.

The dynamic viscosity was measured using BROOKFIELD LVDV-II + viscometer (Labo-Plus, Poland) at the temperature of 20℃ and speed of 1.5 rpm.

Shaking of the extraction system was performed with the use of Eppendorf-ThermoMixer C (Eppendorf, USA).

For DES preparation hotplate magnetic stirrer Chemland MS-H280 Pro (Chemland, Poland) was used.

The LEXT OLS4000 3D Laser Measuring Microscope was used to receive images of coacervates (Olympus, Evident Industrial, Japan).

The ultrasonic bath Sonorex (Bandelin, Germany) was used for sample homogenization.

FT-IR measurements were provided with the use of Bruker Tensor 27 spectrometer (Bruker, USA) equipped with an ATR accessory and OPUS software (Bruker, USA).

The ^1H NMR measurements were conducted at a temperature of 20 °C utilizing the Bruker Avance III HD 400 MHz instrument (Bruker, Billerica, MA, USA).

The analysis of variance (ANOVA) model tests were done in Statistica and manually in MS Excel.

2.3 Samples

The spicy sauces with different pungency were purchased in local supermarkets in Gdansk (Poland). The samples were stored in a refrigerator at 5 °C and homogenized before analysis.

To optimize the procedure, an analyte-free model sauce sample was prepared. For this task, a bell pepper was used as it does not contain capsaicinoids but belongs to the genus *Capsicum*, C. *annuum* species. To simulate the composition of pepper sauce, 200 g of bell pepper was cleaned and the pericarp without placenta and seeds were cut into the pot. Then 5 g of salt, sugar and oil as well as a piece of garlic and 5 mL of citric acid solution and 50 mL of water were added. The mixture was heated for 20 min and blended during 10 min to achieve a paste-like consistency. The sauce samples were then stored in the refrigerator at 5 °C.

For the procedure optimization the model sauce sample was fortified with standard capsaicinoids concentration to receive 100 μg g^{-1} of capsaicinoids content.

For added-found analysis, samples were prepared by adding of an appropriate amount of 2.5 g L^{-1} solution of capsaicinoids prepared in methanol to the three real sauce samples with different pungency. The samples were placed in an ultrasonic bath for 30 min to provide homogenization and mixing.

2.4 Deep eutectic solvent preparation

To obtain DES, the hydrogen bond acceptor (thymol, choline chloride, tetrapropylammonium bromide and tetrabutylammonium bromide) and the hydrogen bond donor (hexanoic acid, heptanoic acid and octanoic acid) were mixed in an appropriate molar ratio. The individual DESs were prepared by mixing the components under stirring and heating at 70 °C until a homogeneous and transparent fluid mixture was obtained. The time required to prepare the DES was approximately 30 min under stirring. The DESs obtained were stored at ambient conditions. The composition of the DESs investigated is shown in Table 1.

The DES composition used for the extraction procedure, namely TPrABr:C7:FA in a molar ratio of 1:3:1, was prepared in a manner similar to described above with the mixing of appropriate amount of tetrapropylammonium bromide, heptanoic acid and formic acid.

Table 1. Composition of the used DESs and the calculated solubility of capsaicinoids in DESs

DES (HBA:HBD), molar ratio	Abbreviation	Solubility in DES, g L^{-1}	
		Capsaicin	Dihydrocapsaicin
Thymol:hexanoic acid, ratio 1:1	Thy:C6	6.05	1.89
Thymol:heptanoic acid, ratio 1:1	Thy:C7	5.46	1.72
Thymol:octanoic acid, ratio 1:1	Thy:C8	5.02	1.60
Choline chloride:hexanoic acid, ratio 1:4	ChCl:C6	6.95	2.18
Choline chloride:heptanoic acid, ratio 1:4	ChCl:C7	7.14	2.27
Choline chloride:octanoic acid, ratio 1:4	ChCl:C8	7.14	2.31
Tetrapropylammonium bromide:hexanoic acid, ratio 1:4	TPrABr:C6	5.34	1.73
Tetrapropylammonium bromide:heptanoic acid, ratio 1:4	TPrABr:C7	5.30	1.73
Tetrapropylammonium bromide:octanoic acid, ratio 1:4	TPrABr:C8	5.22	1.73
Tetrabutylammonium bromide:hexanoic acid, ratio 1:4	TBuABr:C6	4.62	1.52
Tetrabutylammonium bromide:heptanoic acid, ratio 1:4	TBuABr:C7	4.61	1.51
Tetrabutylammonium bromide:octanoic acid, ratio 1:4	TBuABr:C8	4.54	1.51

2.5 Extraction procedure

The extraction process comprised of two main stages. Initially, a micellar-mediated extraction was performed by placing 1 g of the real or model sauce sample into a glass tube, followed by the addition of 5 mL of a 10 % solution of APG C_8-C_{10}. The mixture was mixed thoroughly for 12 min to ensure proper solubilization and isolation of the analytes in the micellar solution of APG C_8-C_{10}. The mixture was then centrifuged at 2500 rpm (839×*g*) for 10 min.

In the next stage, 3 mL of the supernatant was transferred to a clean tube and 260 µL of DES (TPrABr:C7:FA, molar ratio 1:3:1) was added to preconcentrate the analytes. Under the influence of the coacervation agent, coacervates spontaneously were formed in the bulk solution. The mixture was mixed again for 12 min and centrifuged at 2500 rpm (839×*g*) for 10 min to accelerate the phase separation.

The SUPRAS phase coalesced at the bottom of the vial containing the analytes was withdrawn (400 µL) into a chromatographic vial and dissolved in 2-propanol (100 µL) to make the solution clear and less viscous prior to HPLC-UV analysis. A schematic representation of the procedure is shown in Fig. 1.

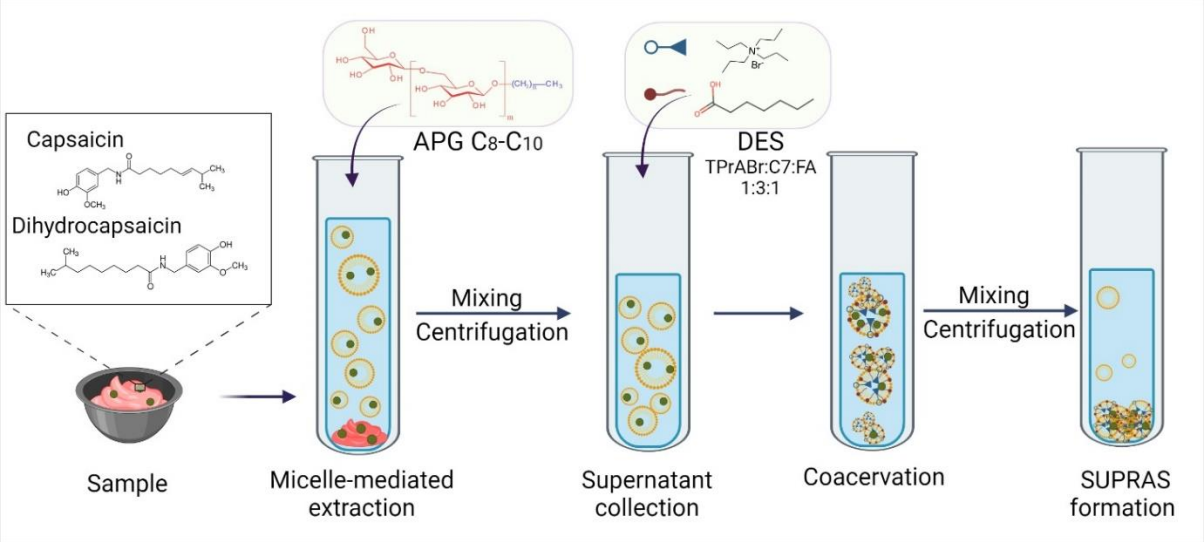

Figure 1. The schematic representation of the extraction procedure

2.6 Solubility calculation

To choose the DES with a strong affinity for capsaicin and dihydrocapsaicin, the Conductor-like Screening Model for Real Solvents (COSMO-RS) was employed using a previously described methodology [32,33]. The ADF COSMO-RS program from SCM in the Netherlands was used for all computational analyses [34,35]. Gas-phase geometry optimization was conducted to assess the compatibility and energetics of 12 DES indicated in Table 1. Optimization was performed using the continuum solvation COSMO model at the BVP86/TZVP level of theory. A vibrational frequency analysis was performed to ensure that the DES configurations achieved a global energy minimum. All computations were carried out under the following conditions: a pressure of 101325 Pa and a temperature of 20 °C. The affinity of DES for capsaicinoids was determined by calculating solubility. To estimate the solubility (*xi*, g L^{-1}) of capsaicin and dihydrocapsaicin in various DES, equation (1) was used.

$$\log(x_i) = log \cdot \left[\frac{\exp(\mu_i^c - \mu_i^{DES} - \Delta G_{fus})}{RT}\right] \quad (1)$$

where: μ_i^c – chemical potential of capsaicinoids (J·mol^{-1});

μ_i^{DES} – chemical potential of capsaicinoids at infinite dilution in the DES (J·mol^{-1});

ΔG_{fus} – Gibbs free energy of fusion change of capsaicinoids (J·mol^{-1});

R – universal gas constant (8.314 J·mol^{-1}·K^{-1});

T – temperature (K).

3. Results and discussion

3.1 Theoretical consideration

APG has been proposed as an alternative green and bio-based surfactant for the extraction process [36,37]. This type of surfactant belongs to the non-ionic surfactants, but its properties are different from the conventional non-ionic surfactants of the Triton X family. APG shows normal cloud point, but the clouding behavior depends upon the changes in the chemical structure. The APG implementation as precursors for the formation of SUPRAS was associated with the application of two types of coacervation agents, such as alkyl carboxylic acids [38] and primary amines [39]. Both stimuli trigger the coacervation phenomenon, as the acid or amine molecules could be incorporated into the structures of the APG-based micelles and induce their aggregation. In addition, primary alcohols were also proposed as a coacervation agent in APG-based micellar solution [40].

The hypothesis of using DES as a coacervation agent was based on the consideration that DES serves as a modulator of micellization and that micelles grow by incorporating DES precursors into the structure of the micelles. Since DESs are tailor-made solvents, it can be assumed that they also influence SUPRAS properties such as surface charge, polarity, viscosity and density.

The primary idea behind the type of DES that could be used for the initial investigation of DES as coacervation agent and testing the general hypothesis of its application for the formation of SUPRAS stemmed from our previous study in which alkyl carboxylic acid induced coacervation in the micellar solution of APG surfactant [38]. Another fact was that the fatty acid-based DESs are well investigated and therefore it is possible to predict the mechanism of their behavior in micellar solution of APG C_8-C_{10}. Therefore, fatty acid-based DESs were investigated as possible precursors for SUPRAS formation. Since in analytical practice, extraction recovery is a crucial parameter influencing the choice of extraction

solvents, the computational calculations were conducted before the experimental part to predict which DES might be more suitable for a given analytical task. Moreover, computational prediction makes the investigation less time-consuming and helps to focus only on the solvents that could theoretically be best suited for the isolation of the target compounds.

3.2 Selection of a suitable fatty acid-based DES

In the current study, three fatty acids were selected as HBDs, namely hexanoic acid, heptanoic acid and octanoic acid. Quaternary ammonium salts such as choline chloride, tetrapropylammonium bromide, tetrabutylammonium bromide and a monoterpenoid thymol were used as HBAs to obtain hydrophobic DES. The composition of the investigated DES is presented above in Table 1. Capsaicinoids (capsaicin and dihydrocapsaicin) are hydrophobic compounds with a log P value of 3.66 for capsaicin and 3.98 for dihydrocapsaicin [41]. Computational calculations indicate that the affinity of the studied DESs to capsaicinoids decreased depending on the HBA used: ChCl > Thy > TPrABr > TBuABr (Table 1). To clarify this phenomenon and gain a better understanding of the interaction between various DES and capsaicinoids, 3D surface charge densities were used to compute the charge distribution and σ-profile [42,43]. The σ-profile results facilitate the prediction of non-covalent interactions, such as H-bonding, electrostatic interactions, and polar-inducing interactions, between DES and capsaicinoids. The σ-profile is a tool used to estimate the polarity of a substance. This is a straightforward method based on the presence of visible peaks on a chart. The peaks in the chart indicate whether a substance has a low or high polarity. Substances with low polarity had narrow visible peaks, whereas those with high polarity had broad visible peaks [44]. In this study, the σ-profile charts were divided into three regions: HBDs, HBAs, and non-polar. Hydrogen bond donors have a σ value less than -0.0084 e/Å^2, hydrogen bond acceptors have a σ value greater than 0.0084 e/Å^2, and substances with σ values between -0.0084 e/Å^2 and 0.0084 e/Å^2 are considered non-polar [45,46]. To clarify, the unit "e" stands for elementary charge, which is the charge of a proton (approximately 1.602×10^{-19} coulombs). The unit "Å²" refers to square angstroms, a measure of area. Thus, σ (e/Å²) represents the charge density on the molecular surface. P(σ) denotes the probability distribution of the screening charge density σ. In other words, P(σ) describes how frequently different values of σ occur on the surface of the molecule. The σ-profile is essentially a histogram that shows this distribution.

The non-polar region exhibited the most pronounced peaks for all studied DES and analytes, which can be attributed to the presence of hydrocarbon groups such as -CH_2-, =CH-, =C=, and -CH_3. As the length of the hydrocarbon chain in the HBA and HBD structures increased, the intensity of the peak in the nonpolar region increased correspondingly. However,

the nonpolar part of the DES structure plays a minor role in the formation of strong hydrogen bonds. The other two HBA and HBD regions indicate the ability to form strong non-covalent bonds with the analytes. The study revealed that minor peaks in the hydrogen bond donor and acceptor regions were observed for all DES, which were attributed to the presence of active groups such as –OH and –COOH in the polar regions. These active groups can serve dual functions as HBD and HBA. The higher-intensity peaks in the positive region primarily indicate their role as excellent HBA, while, to a lesser extent, they also act as HBDs. These characteristics are essential for efficient extraction of capsaicin and dihydrocapsaicin. Both capsaicinoids contain four active groups that can form strong hydrogen bonds with the polar groups of DES, including –OH, =NH, –O–, and =O. Therefore, owing to the presence of additional hydroxyl groups in the structure of Thy and ChCl, which have a higher ability to form strong non-covalent bonds, greater solubility of capsaicinoids can be observed.

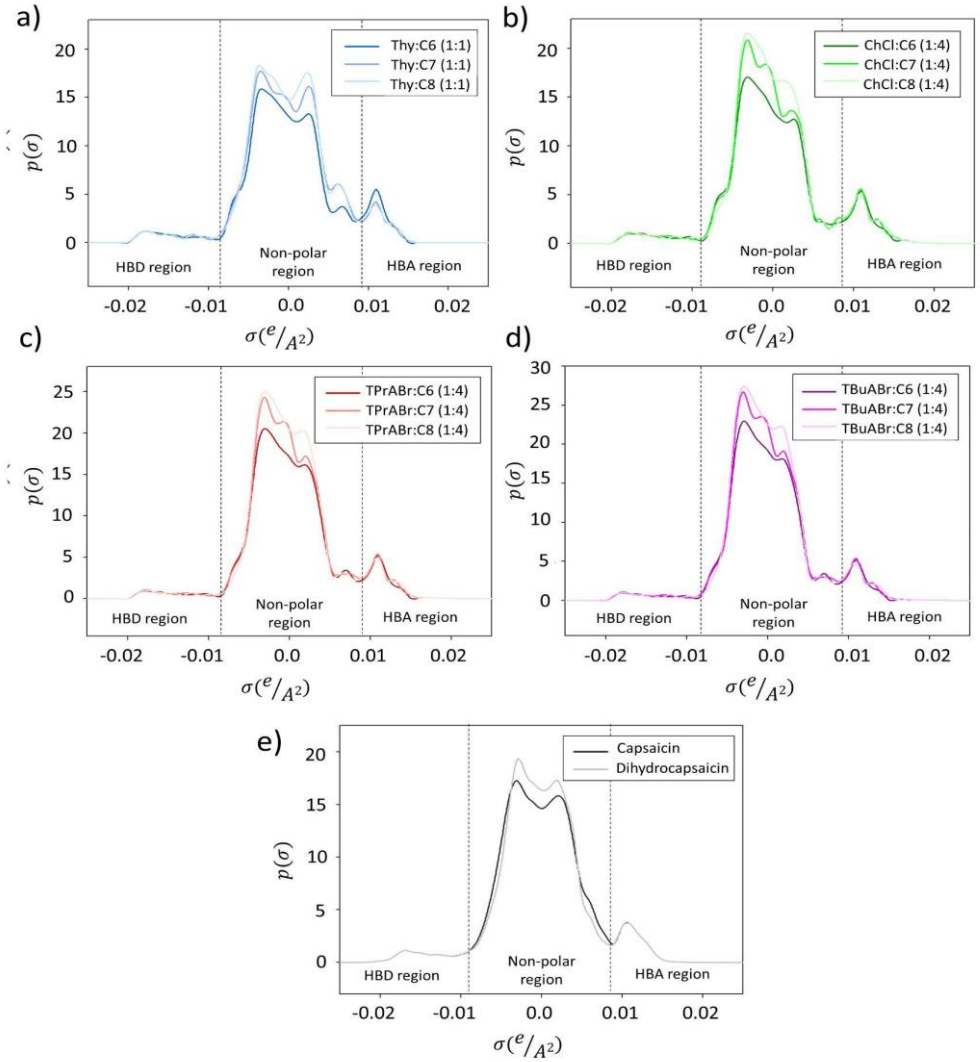

Figure 2. The σ-profiles of a) DES obtained by combining Thy and alkyl carboxylic acids in 1:1 molar ratio; b) DES consist of ChCl and alkyl carboxylic acids in 1:4 molar ratio; c) DES

consist of TPrABr and alkyl carboxylic acids in 1:4 molar ratio; d) DES consist of TBuABr and alkyl carboxylic acids in 1:4 molar ratio; e) capsaicin and dihydrocapsaicin.

Therefore, for the verification of the studied DESs as coacervation agents for SUPRAS formation in the aqueous solution of APG C_8-C_{10}, three DESs of each HBA type were initially selected, which have the highest calculated solubility of capsaicinoids in: ChCl:C7, TPrABr:C6, and Thy:C6. The DES with TBuABr as HBA was not tested due to its low solubility of capsaicinoids. In each case, the preliminary investigation was performed as follows: into 1 mL of the 10% solution of APG C_8-C_{10}, the studied DESs were carefully added under the stirring at ambient condition in increments of 20 µL until coacervation occurred. At least 80±10 µL of DES ChCl:C7, 100±10 µL of TPrABr:C6 and only 40±5 µL of Thy:C6 were required for coacervation. In all cases, the formation of coacervates was observed, which coalesced at the bottom of the vial. However, the different volumes required for the different DES type show that the DES precursors have a significant influence on the coacervation process. Thus, the volumes of DES required for the same HBD (C6) differ more than twice (for Thy and TPrABr).

Microscopic images were captured to confirm coacervates formation (Figure 3). This proves that the proposed DESs can be used as coacervation agents, however, the amounts of particular DES added required for coacervation were different.

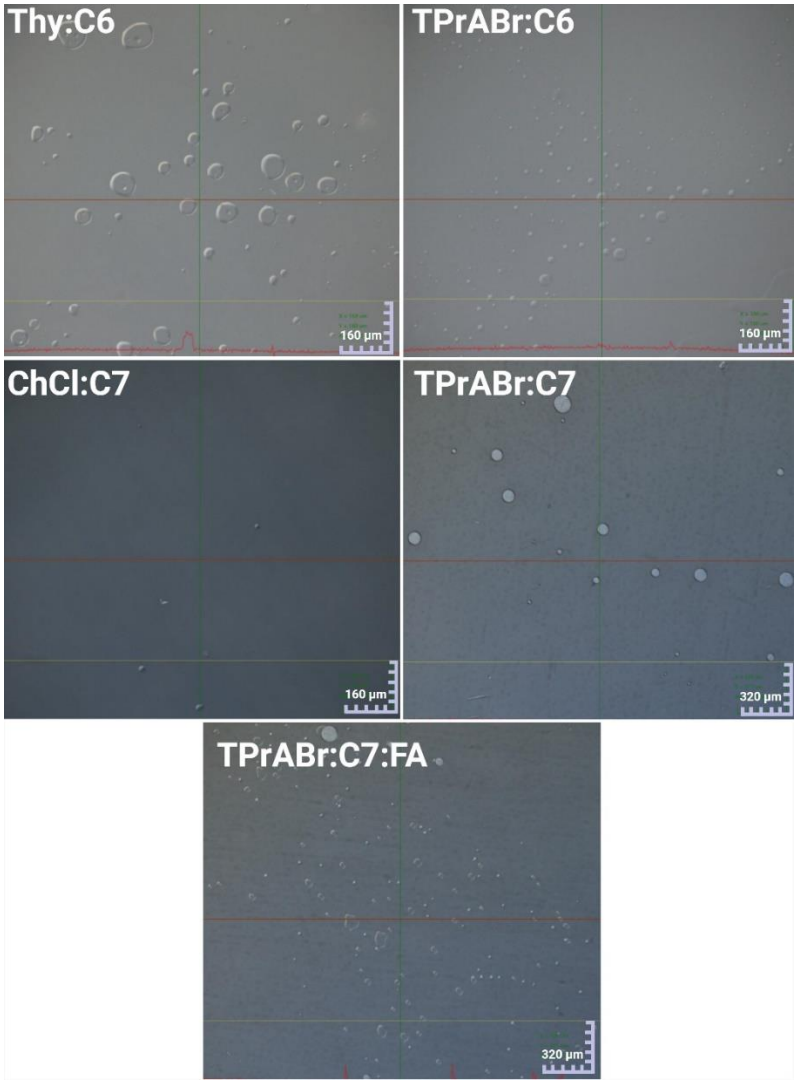

Figure 3. The optical microscope images of coacervates formed after addition particular DES ($C_{APG\ C8\text{-}C10}$ = 10 %)

In the next step, similar experiments were performed with the 10 mg L^{-1} solution of capsaicinoids diluted with 10% solution of APG C_8-C_{10} and the addition of the volumes of each DES required for the coacervation. After 15 s of shaking and centrifugation for 5 min at 2500 rpm (839 *g*), the SUPRAS phase was withdrawn, diluted 1:1 in 2-propanol and HPLC-UV analysis was performed. The preliminary conclusions were as follows. The ChCl:C7 was observed to crystallize at ambient conditions, which made it less convenient for analysis. The Thy:C6 demonstrated good stability under ambient conditions. However, since thymol is incorporated into APG C_8-C_{10} micelles and becomes one of the components of SUPRAS, it causes an interference effect during HPLC-UV analysis of the analytes. Specifically, the retention time of thymol overlaps with that of the capsaicinoids. In addition, the same absorption wavelength precludes the possibility of analytes determination. However, this DES

could be used for other analytes as it allows SUPRAS formation. Next, TPrABr:C6 showed good stability at ambient conditions and no strong absorbance at 280 nm, however, the peaks of capsaicinoids were tailed, having low resolution. To proceed with the experiments, the TPrABr:C7 was checked in a similar way as described above and showed satisfactory conditions for the formation of SUPRAS as well as for the chromatographic separation of capsaicinoids. Therefore, it was selected as the optimal DES for further studies. Since the viscosity of TPrABr:C7 was quite high (dynamic viscosity value 216 mPas s^{-1}) and the repeatability of the results was between 20-25%, the attempt was made to decrease the viscosity. In the literature it was indicated that the viscosity could decreased with the addition of water [47,48] or solvents like formic acid [49]. Thus, two different compositions of TPrABr:C7:FA at a ratio 1:1:1 and 1:3:1 were checked. The measurement of dynamic viscosity was performed and it was found that for 1:3:1 composition the viscosity was 87 mPas s^{-1} while for 1:1:1 was 218 mPas s^{-1}. Thus the ternary mixture namely TPrABr:C7:FA at a 1:3:1 molar ratio was chosen as a suitable DES composition.

In the next step the ternary diagram was prepared to understand the mechanism of SUPRAS formation. To achieve this, an aqueous solution of APG C_8-C_{10} within the concentration range between 0.5 to 15 % was prepared. The DES TPrABr:C7:FA was then added to this solution until the coacervation occurred (Fig. 4). It was found that for low APG C_8-C_{10} concentration (0.5 and 1.0%) the coacervation did not proceed.

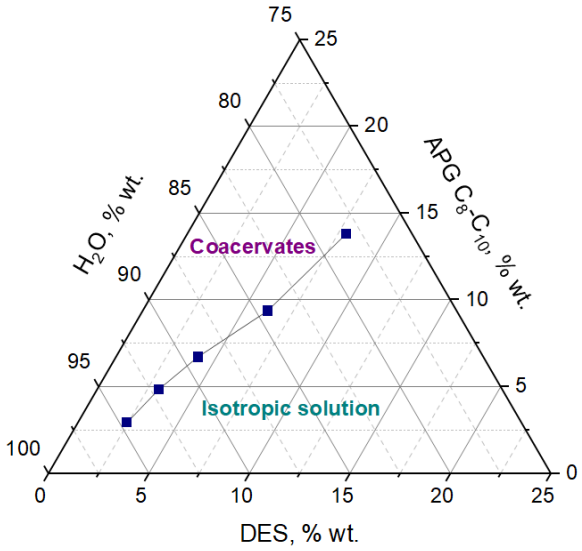

Figure 4. The phase diagrams for systems: APG C_8-C_{10}-DES-water (t=25 °C)

Obviously, at a lower concentration of APG C_8-C_{10}, a lesser amount of DES is required to trigger coacervation, and this amount increases proportionally with increasing surfactant concentration. The mechanism of interaction of APG C_8-C_{10} solution with DES and the

stimulation of the coacervation phenomenon is associated with the processes described below. Initially, the decomposition of the DES occurs in the alkaline medium of APG C_8-C_{10}. The components of DES, especially heptanoic acid, are ionized in the alkaline medium and further neutralized when the pH of the extraction medium decreases. Consequently, the acid molecules are incorporated into the micelles and enlarge them. This forced the micelles to aggregate and separate into the water-immiscible, surfactant-rich phase. In addition, TPrABr could also be incorporated into the micelles. The incorporation of heptanoic acid and $TPrA^+$ into the APG C_8-C_{10} micelles has been confirmed using ^{1}H NMR spectroscopy (Fig. 5). The spectra for SUPRAS clearly displays characteristic peaks corresponding to both the $TPrA^+$ ion and heptanoic acid, providing strong evidence of incorporation of the DES components into the APG C_8-C_{10} micelles.

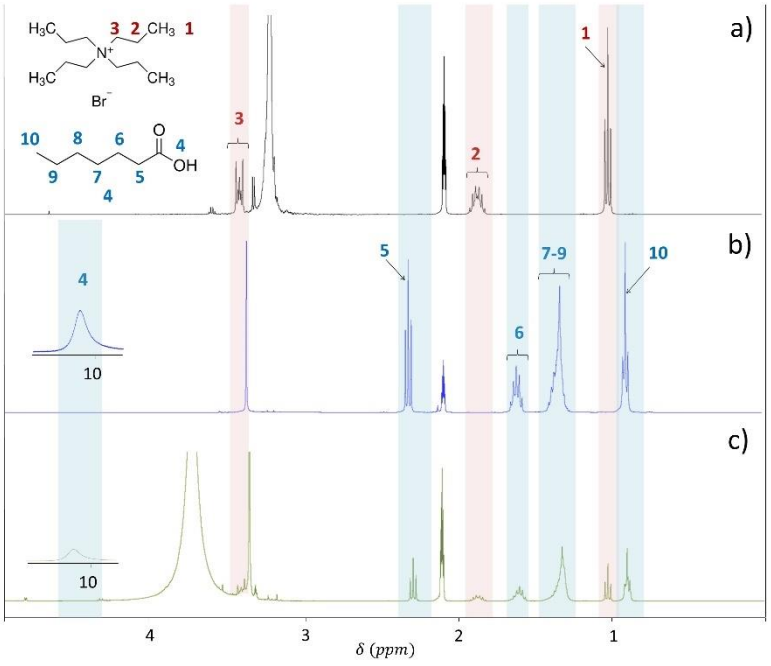

Figure 5. ^{1}H NMR spectra of (a) TPrABr; (b) heptanoic acid; (c) SUPRAS obtained using fatty acid-based DES TPrABr:C7:FA

At the same time, bromide ions potentially might be able to screen the charge of the micelles when combined with ammonium ions. Formic acid only has an influence on the pH of the extraction medium. The measured FT-IR spectra (ESM Fig. 1) show possible incorporation of DES components into the APG C_8-C_{10} micelles since the FT-IR spectrum for SUPRAS has characteristic peaks belonging to C=O (1705 cm^{-1}) and C-H (2800-3000 cm^{-1}) which were not observed for APG C_8-C_{10}.

The potential interaction between capsaicinoids and SUPRAS is primarily attributed to their hydrophobic properties rather than ionization, as the pH of the SUPRAS phase tends to be acidic, correlating with the pKa of heptanoic acid. Hydrophobic interaction are favored by the alkyl chain of heptanoic acid, TPrA ion as well as the APG C_8-C_{10}. However, interactions via hydrogen bonds are also possible due to OH- functional groups in the chemical structure of APG and the carboxyl group of heptanoic acid.

3.3 Optimization of microextraction parameters

3.3.1 Investigation of appropriate microextraction conditions

Micelle-mediated extraction is a convenient way to isolate target analytes from solid food samples, as the extraction is performed under mild conditions with low environmental impact and without the use of toxic organic solvents. This extraction process comprises of two steps: micelle-mediated isolation of the target analytes into the colloidal solution of the surfactant, followed by preconcentration by inducing phase separation. Therefore, it is important to optimize the concentration of the APG C_8-C_{10} solution and the volume of the DES. However, the volume of DES added was strictly dependent on the APG C_8-C_{10} concentration. Therefore, these two parameters were optimized simultaneously, which had an additional effect on the obtained SUPRAS phase. For this purpose, different concentrations of APG C_8-C_{10} solutions in the range between 3 and 15 % were prepared. Further, 1 g of the model sample was taken and 5 mL of APG C_8-C_{10} at a certain concentration was added having the alkaline medium (pH=12.0). Capsaicinoids were isolated in the aqueous micellar solution through two possible interactions: ionization under alkaline conditions (pKa of capsaicin 9.93; pKa of dihydrocapsaicin 9.72) and incorporation of the analytes into the micelle structure due to hydrophobic interactions. This step was performed by shaking the extraction mixture for 5 min followed by centrifugation (5 min at 2500 rpm (829 *g*)). Then 3 mL of the supernatant was withdrawn and the required amount of DES was added. In this step, the extraction mixture was shaken for 5 min and centrifuged again for 5 min at 2500 rpm (829 *g*). The resulting clean SUPRAS phase was withdrawn and diluted 1:1 with 2-propanol. It was found that the highest analytical signal was observed for 5 % APG C_8-C_{10} solutions, but the repeatability was not satisfactory. As the APG C_8-C_{10} concentration increased, the analytical signal decreased due to the dilution effect as a larger volume of SUPRAS phase was obtained, but the repeatability increased. An APG C_8-C_{10} concentration of 10 % was chosen as optimal for the analysis as it ensures both – repeatability and convenience of phase withdrawing. In this case, the volume of DES added was 260 µL to 3 mL of supernatant. A further 400 µL of SUPRAS phase was withdrawn for analysis.

A design of experiments (DOE) was performed to optimize other important extraction parameters such as extraction time (micellar-mediated isolation), preconcentration time (SUPRAS phase) and sample amount. A spiked model sample was used for this purpose. Three variables were treated simultaneously to optimize the extraction conditions and the experimental values are shown in ESM Table 1. The conditions presented in ESM Table 1 were used to perform a Box-Behnken experimental design with five repetitions in the central point of the experimental plan. The points of the experimental design shown in ESM Table 2 were performed in random order. ESM Table 2 also shows the responses of the system in the form of signals of capsaicin and dihydrocapsaicin (peak areas). Since the signals have similar values, it was decided not to apply Derringer's desirability function but to use simple additions to obtain the final value to be optimized. Consequently, the SUM peak area of both analytes was applied to obtain the response functions shown in Figure 6.

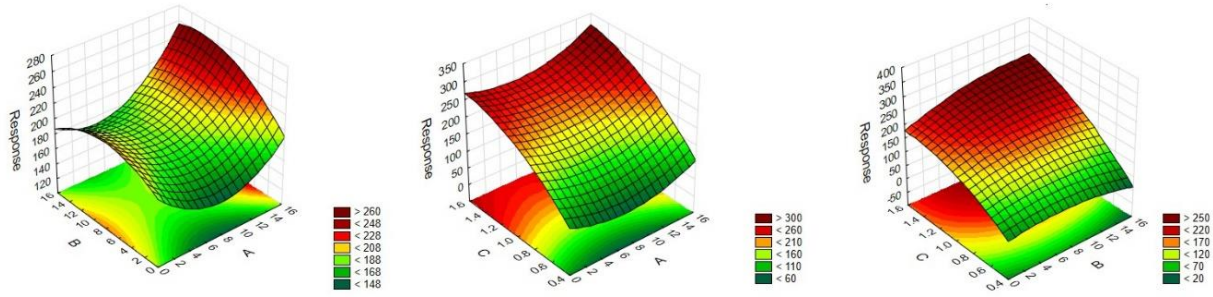

Figure 6. Response functions obtained for pairs of parameters A – extraction time [min]; B - preconcentration time [min]; C – sample amount [g].

Based on the response functions, critical values were calculated and used as optimal extraction parameters in the study. It was found that the optimal conditions are as follows: extraction time (micelle-mediated isolation) – 14 min, preconcentration time – 14 min, sample amount – 1.5 g. However, since the response hardly changed near the optimal 14 min for both preconcentration and extraction time, it was decided to use 12 min. Similarly, despite the optimal sample amount of 1.5 g, it was decided to use 1.0 g because the observed matrix effects were lower.

The model has R^2 value equal to 0.79, what is typical value for this type of optimization. The ANOVA results presented in ESM Table 3 show that the most crucial effect is carried by sample amount parameter, extraction time is less significant. R^2 adjusted is 0.74, what is in good agreement with R^2 value, showing acceptable adjustment of model with experimental

data. R^2 predicted equals to 0.48, what proves that the model has the prediction capability. F-values are 4.18, 2.14 and 41 and p-values are 0. 027, 0.13 and <0.0001 for A, B and C variables, respectively. Adequate precision is 25.07, meets the requirement of S/N ratio >4. The precocentration time plays the least important role and consequently it has potential to be reduced with a little worse response of the system. The equation (2) for the response function is:

$$\text{Response} = -8.89 \times A + 0.697 \times A^2 + 8.71 \times B - 0.419 \times B^2 + 370 \times C\ 103 \times C^2 - 96.5 \quad (2)$$

In the next step the investigation was provided to compare extraction properties of pure solvents, as well as SUPRASs obtained after heptanoic acid-induced coacervation and DES-induce coacervation. Specifically, to spiked model sample 5 mL of pure heptanoic acid, DES (TPrABr:C7:FA in a ratio of 1:3:1), or 10 % aqueous solution of APG C_8-C_{10} were added. Another two experiments with the use of 10 % aqueous solution of APG C_8-C_{10} were used for SUPRAS formation. The isolation of the analytes proceeded for 12 min, followed by centrifugation. After, for pure solvents the supernatant was withdrawn and used for HPLC-UV analysis. For the SUPRAS systems, after centrifugation, the supernatant was transferred into a clean vial, and heptanoic acid or DES TPrABr:C7:FA in a ratio of 1:3:1 was added to the solution. The system was mixed for 12 min, centrifuged again, and the bottom phase was withdrawn for HPLC-UV analysis. In the case of pure solvents, where preconcentration of analytes does not occur, heptanoic acid demonstrated a 15% higher extraction efficiency compared to APG C_8-C_{10} and DES for the selected analytes. For the SUPRAS, the system in which coacervation was induced by heptanoic acid exhibited almost 20% higher extraction efficiency compared to the DES-induced coacervation. These results are consistent with COSMO-RS calculations, where the solubility of capsaicinoids in pure heptanoic acid was determined to be 6.67 for capsaicin and 2.10 for dihydrocapsaicin in compare to the DES based on TPrABr and heptanoic acid (Table 1). Nevertheless, the obtained results should not be seen as a limitation of the proposed DES-induced coacervation. Instead, they should serve as a motivation for further investigation in this field to better understand such systems and explore their potential applications in various areas. In addition, these findings highlight the necessity of conducting experimental trials rather than relying solely on computational predictions. Although the DES based on choline chloride showed the highest affinity in COSMO-RS (even compared to the pure component), practical challenges were encountered in handling this DES. A compromise must therefore be found between the effectiveness of the extraction, compatibility with equipment, and ease of handling.

3.3.2 Validation

The following validation parameters should be evaluated for quantitative procedures: linearity and sensitivity, precision, recoveries, and accuracy.

Under the optimized extraction conditions, the calibration curves for capsaicin and dihydrocapsaicin determination were constructed from seven data points using the standard solution of analytes (Table 2). The matrix-match calibration curve was used. The extraction recovery (ER, %) was calculated as follows: ER (%) = (Csupras·Vsupras) / (Cinitial·Vaq), where Co is the analyte concentration in the SUPRAS phase, Vsupras and Vaq are the volumes of the SUPRAS and aqueous phases of capsaicinoids solution, respectively, and Cinitial is the initial concentration of the analytes.

Table 2. Analytical performance of the proposed procedure for determination of capsaicinoids

Parameters	**Analytical parameters**	
	Capsaicin	**Dihydrocapsaicin**
Linear range, μg g^{-1}	5.0 – 500	5.0 – 500
Intercept	1.3858	-3.4831
Slope	2144.2	2089.6
Determination coefficient	0.9999	0.9999
Limit of quantification, μg g^{-1}	5.0	5.0
Limit of detection, μg g^{-1}	1.7	1.7
CV, %		
Intra-day (C = 5.0 or 500 μg g^{-1})	10.1/6.5	11.2/6.1
Inter-day (C = 5.0 or 500 μg g^{-1})	11.3/10.5	12.8/10.5
Extraction recovery, %	79.2±6.1	80.3±5.2

The *sensitivity* was characterized by the LODs. It was measured by standard IUPAC method as 3·standard deviation of the blank (3s). The LODs values were 1.7 μg g^{-1} for both analytes. A non-spiked model sample was used as a blank.

The *precision* of the procedure was evaluated with regard to its repeatability. The repeatability of the developed procedure was determined by analyzing of 5 replicates of the model sample at two concentration levels for all analytes. The intra-day repeatability was less than 12% at C_{min} and less than 7% at C_{max}. The inter-day repeatability was less than 13 and 11 %, respectively.

Relative recovery values were determined within the range of 0.050 to 0.20 mg g^{-1} of capsaicinoids. The investigation was performed for 3 replicates of three spicy sauces samples with different pungency. Both analytes were found in all analyzed samples. It is shown that relative recovery (RR,%) values, calculate as RR, %=(Cfound-Creal)/Cadded for analytes were

between 76 and 130 % (Table 3). The difference in RR could be attributed to the matrix composition of the sample, which may affect the volume of the SUPRAS phase.

Table 3. Relative recoveries of capsaicinoids from sauce samples (n=3)

Sample	Analyte	Added, μg g^{-1}	Found, μg g^{-1}	Relative recovery, %
Sauce sample 1	Capsaicin	0	38.0±1.0	-
	Dihydrocapsaicin		17.0±1.0	-
	Capsaicin	50	96.0±9.0	117±18
	Dihydrocapsaicin		76.6±6.1	76±12
	Capsaicin	100	152±7.0	114.2±6.6
	Dihydrocapsaicin		135.9±5.0	98.0±1.0
	Capsaicin	200	289±13	125.6±6.3
	Dihydrocapsaicin		282±15	122.1±3.9
Sauce sample 2	Capsaicin	0	82.0±4.9	-
	Dihydrocapsaicin		43.8±2.4	-
	Capsaicin	50	145±12	126±23
	Dihydrocapsaicin		103±11	119±21
	Capsaicin	100	206±16	123±16
	Dihydrocapsaicin		172±11	127±10
	Capsaicin	200	342±29	130±15
	Dihydrocapsaicin		303±12	129.6±6.1
Sauce sample 3	Capsaicin	0	57.2±5.9	-
	Dihydrocapsaicin		19.5±1.7	-
	Capsaicin	50	113±14	110±27
	Dihydrocapsaicin		82±12	125±23
	Capsaicin	100	186.9±9.1	124.5±2.0
	Dihydrocapsaicin		148.2±6.1	128.7±6.1
	Capsaicin	200	317±10	129.7±5.2
	Dihydrocapsaicin		280±15	130±10

To survey the *accuracy*, the added-found method was applied by performing the developed procedure on spiked foodstuffs at 50, 100 and 200 μg g^{-1} levels of capsaicinoids. The results obtained showed that the proposed procedure could be used for food analysis.

In addition, the reference procedure was used to analyse three other chili sauce samples, and the results were compared with those obtained using the developed method. As a reference

procedure the isolation of capsaicinoids from spicy sauce (1g) into methanol (5 mL) has been used, based on the [50]. The analytical results (Table 4) are in good agreement with the results obtained using reference method. *F*-values ≤ 19 indicate insignificant difference in precision between both methods at the 95% confidence level. *t*-values ≤ 2.78 indicate insignificant difference between the results obtained using these methods.

Table 4. Results for the determination of capsaicinoids in spicy sauce samples (n = 3, P = 0.95, $F_{cr.}$ = 19, $t_{cr.}$ = 2.78).

Sample	Analyte	Developed method, μg g^{-1}	Reference method, μg g^{-1}	F-test	t-test
Sauce sample 4	Capsaicin	33.7±1.4	36.5±2.4	2.94	2.19
	Dihydrocapsaicin	16.5±1.4	19.1±1.2	1.47	2.95
Sauce sample 5	Capsaicin	51.2±2.2	49.0±2.0	1.29	1.67
	Dihydrocapsaicin	20.4±1.1	22.3±1.0	1.19	2.78
Sauce sample 6	Capsaicin	41.5±1.0	41.8±2.9	11.19	0.20
	Dihydrocapsaicin	41.1±1.0	38.0±2.6	8.67	2.35

The developed method was compared with other methods proposed for the determination of capsaicinoids in spicy sauce samples (Table 5). As can be seen the extraction process in most methods is time and labor-consuming as requires heating, sonication, centrifugation, filtration and dilution. In each case, organic solvents are used and the procedure takes a long time. However, in case of solid-phase microextraction the procedure involves application of water instead of organic solvent and require 30 min for extraction [51]. In the developed method, the extraction is carried out within 12 min into the micelles of bio-based surfactants, making the method green with low environmental footprint. The use of DES as a coacervation agent provide the preconcentration of analytes and improves the LOQs of the method.

3.3.3 Real sample analysis

Samples of chili pepper sauces of different brands labeled as "spicy" were analyzed. These sauces were mainly made from chili peppers, bell peppers and tomatoes, with the addition of citric or acetic acid, potassium sorbate, sodium chloride, xanthan gum, starch and sugar. It was found that the most of samples contain both capsaicinoids, however, in one sample thought it was labeled as "spicy" no capsaicinoids were detected. To recalculate the results into the traditionally used for spicy product Scoville Hot Unite (SHU), capsaicin and dihydrocapsaicin contents in μg g^{-1} were summarized and multiplied by 16. It should be mentioned that in case of elimination of matrix effect it is necessary to dilute samples with APG C_8-C_{10} solution to remain the same composition of the extraction system. The results of

real sample analysis are presented in Table 6 and example of chromatograms of real sample analysis are presented in ESM Fig. 2. The calculated SHU results are consistent with our organoleptic test, in which we have ordered the sauces according to their hotness.

4 Conclusion

In the current investigation, the influence of DES on the clouding behavior of an aqueous solution of APG C_8-C_{10} was demonstrated for the first time. It was found that the fatty acid-based DESs can induce phase separation and the formation of SUPRAS, but a different amount of certain DES is required to induce coacervation, depending on the alkyl carboxylic acid used to prepare the DES. In addition, the concentration of APG C_8-C_{10} has an effect on the amount of DES added and the volume of SUPRAS phase obtained.

Compare to previously developed SUPRAS were only alkyl carboxylic acid [38,52,53] was used for coacervation, the fatty acid-based DESs using as coacervation agent have several advantages: (i) such SUPRAS have more interaction sites, as not only acid but also other components can be incorporated into the surfactant micelles; (ii) they may potentially modulate the properties of SUPRAS in different ways, depending on the DES precursors, thus, affecting the polarity and hydrophobicity of the received SUPRAS; (iii) more possibilities for SUPRAS tailoring, as a wide range of fatty acid-based DES could be prepared, while a single acid with several homologues is limited; (iv) components that are solid in their pure state (monoterpenoids, ammonium salts) could be prepared as a single liquid DES mixture.

Computational prediction was used to understand which type of DES might be most suitable for a given analytical task from the point of view of extraction recovery of target analytes, as well as to understand the established intermolecular interactions within the micelles core.

The synergy between green surfactants and fatty acid-based DES was used for the determination of capsaicinoids in spicy sauce samples. Micellar-mediated extraction using a solution of APG C_8-C_{10} offers the possibility of effective and rapid isolation of analytes without the use of harmful organic solvents. The application of DES as a modifier of APG C_8-C_{10} micelles shows the possibility of stimulating the coacervation phenomenon and the formation of SUPRAS in which the analytes are preconcentrated. The composition of the sample matrix can have an influence on the volume of the SUPRAS phase obtained. Therefore, if it is necessary to eliminate the interfering matrix effect, the sample should be diluted with the same surfactant solution in order to obtain the same conditions for the coacervation process.

The proposed extraction system has the potential to be used in many other analytical tasks, especially in the analysis of solid and heterogeneous samples. The application of fatty

acid-based DES is the first example of the synergy between APG and DES, but other types of DES based on alcohols, monoterpenoids, etc. have also a potential to be used for coacervation inducing. For instance, alcohol-based DES could provide increased solubility for more polar analytes, while monoterpenoid-based DES may offer additional selectivity due to their aromatic properties. These variations could improve the selectivity, sensitivity, and efficiency of the extraction process, offering solutions to challenges faced in complex sample matrices.

A promising area of research is the comprehensive investigation of such multicomponent SUPRAS based on a variety of DES and surfactants. Future studies should focus on exploring these diverse DES compositions and SUPRAS structure-property relationships. We believe that such eco-friendly systems hold significant potential for a wide range of analytical applications and beyond.

Table 5. Comparisons of the methods used for capsaicinoids determination in chili sauce samples

Detection technique	Extraction procedure	Extraction solvent used	Linear range	LOQ	LOD	Recovery, %	Ref
GC-MS	Direct immersion SPME into sample sauce	Water	0.1 - 1.7 μg mL^{-1}	[c]0.069 μg mL^{-1} [d]0.108 μg mL^{-1}	[c]0.014 μg mL^{-1} [d]0.022 μg mL^{-1}	[c]36.1 - 88 [d]19.7 - 86	[51]
MISER-HPLC-MS	Extraction with methanol and periodically agitated within 1 h, centrifugation	Methanol	0.04 - 100 μg mL^{-1}	Not indicated	Not indicated	Not indicated	[50]
HPCL-FLD	Sonification with ethanol during 1 h, stirring during 2 h via a magnetic stirrer, centrifugation, evaporation, reconstitution	Ethanol	0.2 - 200 μg mL^{-1}	[c]0.0099 μg mL^{-1} [d]0.0133 μg mL^{-1}	[c]0.0029 μg mL^{-1} [d]0.0040 μg mL^{-1}	[c]88.08 - 93.64 [d]84.15 - 94.78	[26]
Voltammetry	Sonification with ethanol for 0.5 h. The sample solution was left on the bench overnight	Ethanol	0.1 - 100 μM	Not indicated	[c]0.1 μM	[c]97 - 107.6	[54]
HPLC-UV	Heating in methanol at 95°C for 1 hr, centrifugation	Methanol	0.313 - 80 μg mL^{-1}	Not indicated	Not indicated	[c]86.1 - 96.7 [d]86.1 - 100.9	[55]
Differential pulse and square wave voltammetry	Sonification with ethanol for 60 min and filtering. Dilution in supporting electrolyte	Ethanol	0.20 - 8.0 mmol L^{-1} (DPV) 0.080 - 6.0 mmol L^{-1} (SWV)	Not indicated	[c]0.085 mmol L^{-1} (DPV) [c]0.028 mmol L^{-1} (SWV)	[c]93 - 110	[56]
DART-MS	Sonification with acetonitrile followed by centrifugation, and filtering	Acetonitrile	[c]11.54 - 95.91 μg mL^{-1} [d]6.64 - 76.95 μg mL^{-1}	[c]0.0859 μg mL^{-1} [d]0.2494 μg mL^{-1}	[c]0.0234 μg mL^{-1} [d]0.0510 μg mL^{-1}	[c]86.5 - 88.8 [d]85.45 - 89.20	[57]
HPLC-FLD	Heating with ethanol at 90°C. for 6h, dilution, and filtering	Ethanol	0.2 - 10.0 μg kg^{-1}	[c]0.163 μg kg^{-1} [d]0.160 μg kg^{-1}	[c]0.054 μg kg^{-1} [d]0.053 μg kg^{-1}	[c]91.1 - 94.8 [d]91.4 - 97.0	[58]
HPLC-UV	Micellar mediated extraction followed by preconcentration into SUPRAS	APG C_8-C_{10}/DES	5.0 – 500 μg g^{-1} (0.1 - 100 μg mL^{-1})	[cd]1.7 μg g^{-1} [cd](0.033 μg mL^{-1})	[cd]1.7 μg g^{-1} [cd](0.033 μg mL^{-1})	[cd]76 - 130	This Work

[c] – Capsaicin; [d] – dihydrocapsaicin.
DPV – differential pulse voltammetry; SWV – square wave voltammetry; GC-MS – gas chromatography with mass spectrometric detection; HPLC-UV – high performance liquid chromatographic with ultra violet detection; HPLC-FLD – high performance liquid chromatographic with fluorometric detection; MISER – Multiple injections in a single experimental run;
DART-MS – direct analysis in real time mass spectrometry.

Table 6. Analysis of real spicy sauce samples (n=3)

Sample No.	Capsaicin, µg g^{-1}	Dihydrocapsaicin, µg g^{-1}	Scoville Hot Unite, SHU
Sample 1	740±15	408.0±6.5	18367
Sample 2	318±15	154.0±8.5	7553
Sample 3	37.68±0.45	16.57±0.17	868
Sample 4	3.39±0.32	<LOQ	57
Sample 5	<LOQ	<LOQ	-
Sample 6	17.6±6.0	15.0±4.4	520
Sample 7	9.7±3.8	<LOQ	154
Sample 8	82.2±4.9	44.0±2.4	2016
Sample 9	57.2±5.9	19.5±1.7	1227
Sample 10	6.2±1.1	14.7±1.1	335
Sample 11	44.4±1.9	44.0±3.1	1414
Sample 12	27.7±2.6	13.95±0.68	666
Sample 13	148±11	93.2±4.2	3858

Declarations of interest

The authors declare that they have no competing interests.

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