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Hydrophilicity-Switchable Deep Eutectic Solvent-Based Ultrasound-Assisted Liquid-Phase Microextraction for the Green and Efficient Spectrofluorimetric Determination of Verapamil in Human Plasma

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Abstract

In this study, a hydrophilicity-switchable deep eutectic solvent (HS-DES) was prepared by combining nonanoic acid (C9) with tetrabutylammonium bromide (TBAB) and applied as an efficient extraction medium for the ultrasound-assisted liquid-phase microextraction (UALPME) of verapamil from human plasma samples prior to spectrofluorimetric determination. The hydrophilicity-hydrophobicity transition of the HS-DES was controlled by pH adjustment using NaOH or HCl solutions, enabling reversible phase switching and facilitating effective separation of verapamil from the biological matrix. Key experimental parameters influencing the extraction efficiency were systematically investigated, and further optimization was performed using a central composite design coupled with a desirability function approach. The developed HS-DES-based UALPME (HS-DES-UALPME) method provides a tunable, environmentally friendly, and effective

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strategy for the preconcentration of verapamil from complex biological samples. The method showed wide linear range (200-2000 ng/mL), a good limit of detection (62.16 ng/mL), and extraction recovery (59.55%). To assess the method's sustainability, the AGREE tool was applied, clearly demonstrated the overall greenness of the developed procedure. A comparison of the analytical performance of the method with previously reported methods demonstrated its superiority or, at minimum, its comparability in key analytical parameters.

Keywords: Green analytical chemistry, Hydrophilicity-switchable, deep eutectic solvent, Spectrofluorimetry, Verapamil, Plasma

Introduction

Verapamil, a calcium channel blocker, is widely prescribed for the treatment of cardiovascular disorders such as hypertension, angina pectoris, and arrhythmia. It functions by inhibiting the influx of calcium ions into cardiac muscle cells, thereby reducing myocardial contractility and helping regulate blood pressure. In recent years, various extraction and preconcentration strategies have been developed for the determination of verapamil in biological fluids, including dispersive liquid-liquid microextraction [1], microfluidic devices [2,3] pH-assisted in-line pseudophase microextraction [4], two sequential dispersive solid/liquid phase microextractions [5], and solid-phase extraction [6]. Despite these substantial methodological advancements, the analysis of verapamil in biological matrices continues to pose considerable challenges due to its high lipophilicity, the complex and

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interference-prone nature of biological samples, and the typically low analyte concentrations. These difficulties are further compounded by the need to minimize solvent consumption, reduce hazardous waste, and adopt greener sample-preparation strategies in accordance with contemporary green chemistry principles.

Deep eutectic solvents (DESs) have emerged as a promising class of green solvents with unique advantages such as tunable physicochemical properties, low toxicity, design flexibility, biocompatibility, and ease of preparation [7]. Therefore, they have attracted considerable attention as sustainable alternatives to conventional organic solvents and ionic liquids. DESs are typically formed by combining a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) in specific molar ratios, resulting in a significant depression of the melting point relative to the individual components [8,9]. An advanced subclass of DESs, known as hydrophilicity-switchable DESs (HS-DESs), can reversibly switch between hydrophilic and hydrophobic states in response to external stimuli such as pH variation, temperature change, or CO_2 exposure [10]. Unlike conventional hydrophobic DESs, HS-DESs can reversibly transition between polar and nonpolar states, allowing them to function effectively as both extraction and dispersing solvents. This “smart” behavior simplifies the extraction process, enhances the interfacial contact between the extractant and analyte, and improves overall extraction yield and efficiency [11]. In addition, HS-DESs retain the inherent advantages of green

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solvents, making them ideal candidates for the extraction of bioactive and pharmaceutical compounds.

In recent years, the application of HS-DESs in pharmaceutical analysis has gained significant attention due to their strong affinity for organic molecules and their ability to efficiently preconcentrate trace-level drugs from complex matrices. When switched to a hydrophilic form, they readily mix with aqueous samples and disperse effectively throughout the matrix, improving contact with the target analytes. Conversely, when switched to a hydrophobic state, the solvent selectively extracts hydrophobic or moderately polar drugs from the sample [12,13]. This tunable polarity not only enhances extraction efficiency but also minimizes matrix effects, which is particularly advantageous when dealing with biological fluids such as plasma, urine, or saliva. A few studies demonstrated the successful use of HS-DES-based microextraction techniques for drug extraction, highlighting their potential as greener and more versatile alternatives to traditional organic solvents. Ghane et al. [14] employed a reversible switchable DES for dispersive liquid-liquid microextraction of non-steroidal anti-inflammatory drugs in biological and wastewater samples. Afshar et al. [15] utilized a switchable DES for extracting of three antiepileptic drugs from breast milk. You et al. [16] applied a pH-switchable DES-based homogeneous liquid-liquid microextraction for the extraction and preconcentration of three non-steroidal anti-inflammatory drugs from environmental water. To the best of our knowledge, no prior studies have demonstrated HS-DES-based microextraction targeting

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cardiovascular agents such as verapamil, despite the clear advantages of phase-switching for efficient extraction and recovery. Therefore, a HS-DES-based ultrasound-assisted LPME (HS-DES-UALPME) method was developed as a tunable, environmentally friendly, and highly efficient strategy for extracting and preconcentrating verapamil from complex biological plasma matrices. The HS-DES was prepared by combining nonanoic acid with tetrabutylammonium bromide, and its switching behavior was controlled through pH adjustment. To enhance extraction performance and improve analyte mass-transfer rates, the procedure was coupled with ultrasound irradiation, which promotes uniform dispersion of the solvent within the aqueous sample and accelerates the transfer of verapamil into the HS-DES phase, ultimately resulting in faster extraction and higher recovery [17,18].

Experimental

Materials and chemicals

Tetrabutylammonium bromide (TBAB), tetrapropylammonium bromide (TPAB), octanoic acid (C8), and nonanoic acid (C9), each with a purity of 98%, were purchased from Exir Company (Vienna, Austria) for the preparation of HS-DESs. Verapamil was obtained from Sobhan Pharmaceutical Company (Rasht, Iran). A stock solution of verapamil (1000 mg/L) was prepared by dissolving an appropriate amount of verapamil powder in methanol, and the solution was stored at 4 °C until use. HPLC-grade acetonitrile and methanol were purchased from Daejung Co. (Korea). Hydrochloric acid (HCl, 37%) and sodium hydroxide (NaOH, 99%) were obtained from Merck (Darmstadt,

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Germany), while sodium chloride (NaCl) was supplied by Daejung Co. (Korea). Human plasma samples of blood group O^+ were kindly provided by the Tabriz Regional Blood Transfusion Center (Tabriz, Iran). All methods were carried out in accordance with relevant guidelines and regulations. The experimental protocols involving human plasma samples were approved by the Ethics Committee of Azarbaijan Shahid Madani University. Human plasma samples were obtained from the Regional Blood Transfusion Center and were anonymized prior to use in this study. Written informed consent for blood donation and research use was obtained by the Blood Transfusion Center from all donors and/or their legal guardians.

Instrumentation

An Agilent Cary Eclipse fluorescence spectrometer (USA) was employed to measure the fluorescence intensity of the samples. A quartz microcell with a 1 cm path length and a maximum volume of 750 µL was used to contain the samples during measurement. Hamilton microsyringe (Switzerland) was used for the injection of HS-DESs into the samples. The pH of the aqueous solutions was measured and adjusted using a glass membrane electrode (ELMETRON CP-511, Poland).

Preparation of HS-DESs

HS-DESs were prepared by mixing the HBA and HBD components at different molar ratios and heating the mixtures to 60 °C until a clear and homogeneous liquid was formed. Quaternary ammonium salts, namely TPAB and TBAB, were used as HBAs, while nonanoic acid and octanoic acid served as HBDs.

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The resulting HS-DESs were used directly in the extraction experiments without further purification.

HS-DES-UALPME

Before applying the HS-DES-UALPME method, plasma proteins were first precipitated. For this purpose, 1.6 mL of acetonitrile was added to 0.8 mL of plasma sample spiked with an appropriate amount of analyte. The mixture was vortexed for 10 min and subsequently centrifuged at 6000 rpm for 15 min. The supernatant was transferred to another Falcon tube and the remaining acetonitrile was removed using a gentle flow of N_2 gas, and then the residue was brought to a final volume of 6 mL using an aqueous solution adjusted to pH 10.97. Sodium chloride (4.48% (w/v)) was then added to the solution. Subsequently, 139 µL of HS-DES was slowly added and the mixture was sonicated for 5 min to ensure uniform dispersion in the aqueous phase and to prevent local direct interaction with the subsequently added alkaline solution, thereby enabling a more controlled and reproducible extraction process and inducing a cloudy appearance due to formation of the dispersed phase. For the switching process, 500 µL of NaOH (1 M) was added to the mixture and sonicated for 10.54 min to convert the HS-DES into a hydrophilic phase, resulting in a clear solution. Subsequently, 500 µL of HCl (1 M) was added and the mixture was sonicated for another 5 min, converting the HS-DES back into a hydrophobic phase and rendering the solution cloudy. The mixture was then centrifuged at 5500 rpm for 10 min, after which the HS-DES phase accumulated at the top of the tube. The lower aqueous phase was

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carefully removed using a syringe, and the HS-DES phase was collected from the conical part of the Falcon tube using a microsyringe. The collected HS-DES was diluted to a final volume of 300 µL with methanol. Finally, the fluorescence intensity of verapamil was measured approximately at 310 nm after excitation at 280 nm with slit widths of 5 nm (excitation) and 10 nm (emission) (Fig. 1).

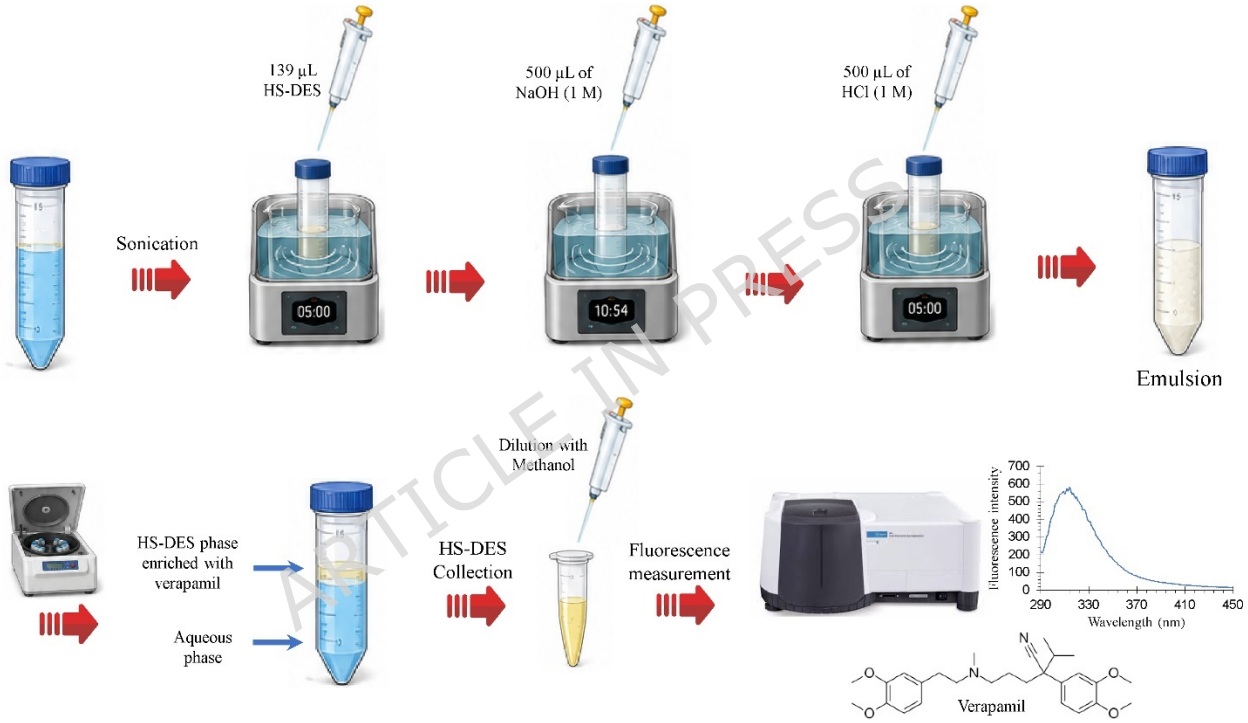


Fig. 1. Schematic representation of the HS-DES-UALPME procedure for extraction and fluorescence determination of verapamil.

Results and discussion

Wavelength selection

To determine the optimal fluorescence wavelengths for verapamil, the emission spectrum was first recorded using an excitation wavelength of 280 nm, which yielded a maximum emission peak at approximately 310 nm. With

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the emission wavelength fixed at 310 nm, the excitation spectrum was then obtained and showed two prominent excitation bands at 230 nm and 280 nm (Fig. S1A). The influence of slit width on fluorescence intensity was further evaluated at an excitation wavelength of 230 nm (Fig. S1B). As expected, increasing both the excitation and emission slit widths enhanced the fluorescence signal; however, excessively wide slits may lead to detector saturation and reduced spectral resolution. To achieve an appropriate balance between signal intensity, signal-to-noise ratio, and detector saturation, slit widths of 5 nm (excitation) and 10 nm (emission) were selected.

Although verapamil exhibits two excitation maxima and the intrinsic fluorescence intensity at 230 nm is higher than at 280 nm, the presence of HS-DES in the final extraction phase results in pronounced quenching at lower excitation wavelengths, particularly around 230 nm [19]. Therefore, despite its lower intrinsic response, the excitation wavelength of 280 nm was chosen because it minimizes matrix-induced quenching and provides more stable and reproducible analytical signals.

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Optimization of switching behavior, HS-DES selection, and solvent conditions

Effect of switching on extraction efficiency

HS-DESs, due to their ability to reversibly transition between hydrophilic and hydrophobic phases, are recognized as suitable candidates for the extraction of drugs from complex biological matrices [20]. In this study, the effect of the

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switching mechanism on extraction efficiency was evaluated. An HS-DESs composed of TBAB and C9 at a 1:3 molar ratio was examined under both non-switching and switching conditions, and the results are shown in Fig. S2. In the non-switch condition, the prepared HS-DESs was added to the sample and emulsified using ultrasonic waves. Subsequently, the HS-DESs was separated from the sample by centrifugation. All other conditions were maintained identical to those of the switch mode. As observed, the extraction efficiency in the switch mode was higher than that in the non-switch mode. These results indicate that the switching mechanism enhances the transfer of the drug into the HS-DES. Previous studies have also shown that HS-DESs, owing to their reversible changes in physical and chemical properties, can effectively extract target compounds from complex matrices [21].

Switching mechanism and optimal acid/base selection

In HS-DES systems composed of a fatty acid and a quaternary ammonium salt, switching can be achieved by adjusting the solution pH. In this process, the addition of acids and bases plays a key role by regulating the ionization state of the components and thereby facilitating phase separation. When the pH is increased above the pK_a of the fatty acid, the carboxylic acid groups become deprotonated (R-COO$^-$), which enhances the solubility of the HS-DES in the aqueous phase. Subsequent addition of acid reprotonates the ionized species, converting them back to their neutral forms (R-COOH). This step is typically accompanied by the appearance of turbidity, indicating the onset of phase separation due to reduced solubility [22].

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HS-DESs can reversibly transition between hydrophilic and hydrophobic states in response to pH changes. Various acids and bases can be employed to adjust the pH; therefore, different acid-base combinations were initially examined to identify the optimal pair (Table S1). Among the nine combinations tested, 1 M NaOH and 1 M HCl provided the most effective hydrophilic-to-hydrophobic phase transition, resulting in good extraction performance. In contrast, other combinations either failed to induce a complete switch or produced a highly viscous system (e.g., NH_3 and HCl). The results also indicated that complete phase transition was achieved using 500 µL each of NaOH and HCl, while lower volumes of NaOH were insufficient to trigger the switch. Accordingly, 500 µL each of the base and acid were used to switch the HS-DES between its hydrophilic and hydrophobic states for subsequent experiments.

Selection of HS-DES

Selecting an appropriate extraction solvent in the HS-DES-UALPME method is critical, as it directly influences both extraction efficiency and solvent-analyte interactions. To extract verapamil from plasma, eight switchable HS-DESs were prepared and evaluated (Fig. 2). Among them, the HS-DES composed of TBAB:C9 at a 1:3 molar ratio showed the highest extraction efficiency, which can be attributed to an optimal balance between hydrophobicity and analyte interaction strength, as well as favorable hydrophobic interactions between the C9 alkyl chain and the lipophilic structure of verapamil. In contrast, the 1:1 and 1:2 ratios exhibited handling

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difficulties caused by high viscosity. Fatty acid chain length also influenced extraction performance, with C8-based HS-DESs showing lower efficiency, likely due to their higher water solubility. To evaluate the performance of C9 alone, two experiments were conducted, one without switching and one with switching. In both cases, the HS-DES demonstrated higher extraction efficiency. Based on these results, the HS-DES mixture of TBAB:C9 at a molar ratio of 1:3 was selected as the optimal system for UALPME of verapamil from biological samples in subsequent experiments.

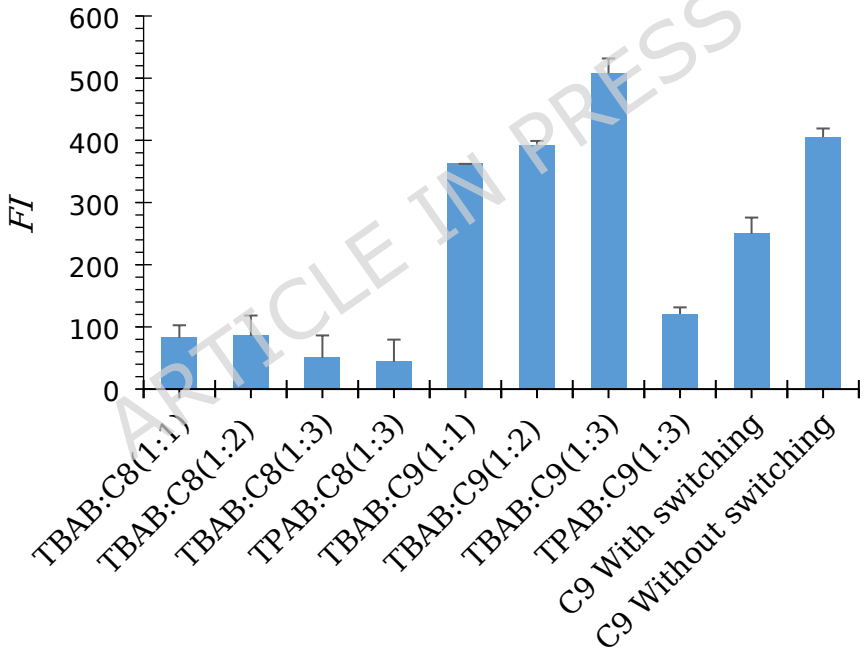


Fig. 2. Effect of HS-DES composition on the extraction efficiency of verapamil. *Extraction conditions*: sample volume, 6 mL; pH, 11; HS-DES volume, 150 µL; sonication time, 10 min; NaCl concentration, 10% (w/v); NaOH (1 M), 500 µL; HCl (1 M), 500 µL; centrifugation time, 10.5 min; drug concentration, 2 µg/mL; final volume, 300 µL; solvent, acetonitrile.

Solvent for adjusting HS-DES volume in fluorescence analysis

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The required volume for fluorescence measurements in this study was 300 µL; however, the collected HS-DES phase was insufficient and required dilution with a suitable solvent. In fluorescence-based methods, selecting an appropriate solvent is critical because it directly influences fluorescence intensity and affects phase compatibility. Solvent selection was based on two criteria: minimal fluorescence quenching and complete miscibility with both the HS-DES and water to avoid cloudiness. Methanol, ethanol, acetone, and acetonitrile were evaluated (Fig. S3). Acetone caused significant fluorescence quenching, while acetonitrile increased the viscosity of the HS-DES phase. Ethanol provided moderate performance. In contrast, methanol exhibited the least fluorescence quenching, produced the highest fluorescence intensity, and showed complete miscibility with both the HS-DES and water, resulting in a clear and stable solution. Based on these results, methanol was selected as the optimal solvent for adjusting the volume of the HS-DES phase.

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Multivariate optimization

To optimize the remaining parameters including pH (A), HS-DES volume (B), NaCl concentration (C), and sonication time (D) a central composite design (CCD) combined with a desirability function was employed. Each factor was investigated at five levels selected on the basis of preliminary experiments. The HS-DES volume was varied between 80 and 200 µL to ensure sufficient collected phase and compatibility with the final 300 µL volume required for fluorescence measurement. Volumes below 80 µL were difficult to separate,

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whereas volumes above 200 µL reduced fluorescence intensity due to the increased proportion of HS-DES in the final extract [19]. The pH range (9-11) was chosen with respect to the pK_a value of verapamil (8.75) [23], ensuring that the analyte remained predominantly in its neutral form and thereby favoring its transfer into the HS-DES phase. The maximum salt concentration examined was 12% (w/v), as higher levels significantly increased viscosity and produced a semi-gel structure in the HS-DES. The coded and uncoded factor levels are presented in Table 1. The CCD comprised 30 randomized experiments (16 factorial points, 8 axial points, and 6 center points) and used an axial distance of α = 2 to produce a rotatable design.

Table 1. Coded and uncoded values of the experimental variables used in the CCD.

Factor	-2	-1	0	1	2
A: pH	9	9.5	10	10.5	11
B: HS-DES volume (µL)	80	110	140	170	200
C: NaCl (%, w/v)	0	3	6	9	12
D: Sonication time (min)	1	4	7	10	13

For statistical analysis, the model coefficients were estimated using regression analysis and the model was validated through analysis of variance (ANOVA). The results showed that the quadratic model was statistically significant ($F = 24.65$, $P < 0.0001$), with a high coefficient of determination ($R^2 = 0.9583$), indicating that it explained more than 95% of the variation in

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the response (Table S2). Moreover, the lack-of-fit test (F = 0.93, P = 0.5687) confirmed that the model adequately fit the experimental data and provided reliable predictions.

Based on the ANOVA results, a Pareto chart was constructed to visualize the relative effects of the studied factors on the response. According to the Pareto chart in Fig. 3, all factors exhibited positive effects. Among them, pH and HS-DES volume had significant effects, whereas NaCl concentration and sonication time showed non-significant effects. As shown in Fig. 3, the pH of the sample solution had the greatest influence on the response, as indicated by the longest bar among the factors studied, followed by the HS-DES volume. The positive effect of pH can be attributed to the deprotonation of verapamil ($pK_a \approx 8.75$), which favors its non-ionized form and consequently enhances its affinity and transfer into the hydrophobic HS-DES phase. Since the final phase volume was maintained at 300 µL in all experiments, no signal reduction due to dilution occured; thus the extraction efficiency increased with higher HS-DES volumes [19].

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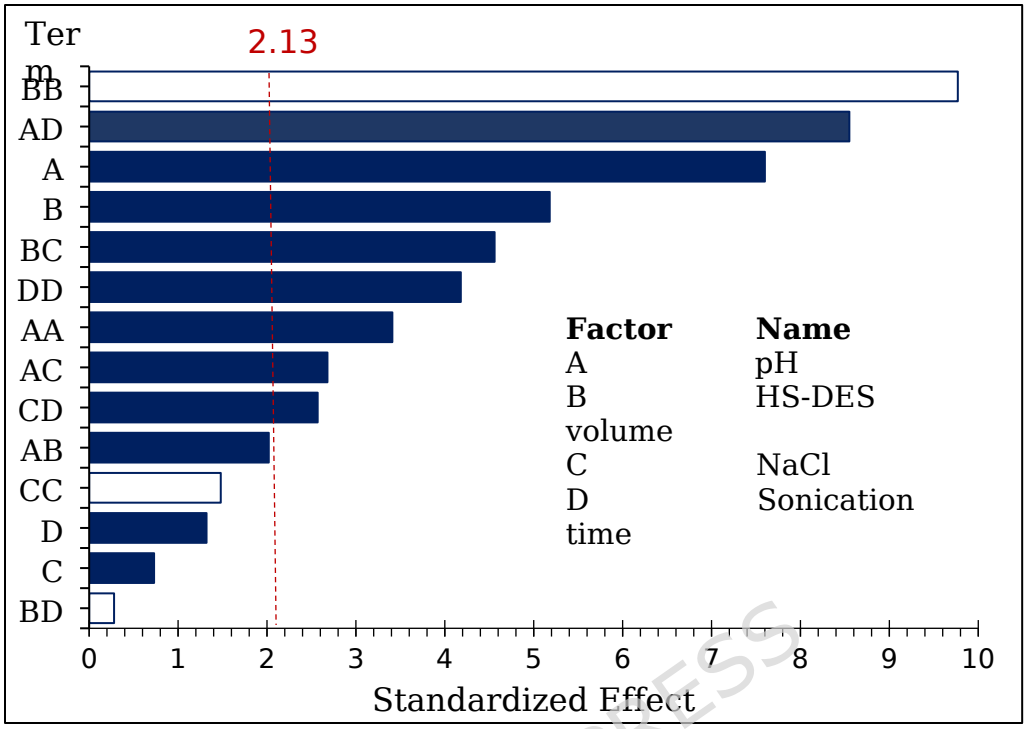


Fig. 3. Pareto chart. Solid bars represent positive effects, hollow bars represent negative effects, and the dashed line denotes the 95% confidence level.

NaCl concentration showed a positive but statistically non-significant effect. Low salt levels resulted in poor phase separation, whereas moderate amounts improved separation and increased the collected HS-DES volume. At higher concentrations, however, increased viscosity and density of the medium likely reduced ultrasonic cavitation efficiency and slowed analyte diffusion, offsetting the beneficial effects. Sonication time also showed a positive but non-significant effect on the response. In general, in ultrasound-assisted microextraction methods, longer sonication times reduce the size of HS-DES droplets, increases their surface area, and consequently enhances extraction efficiency. However, in this extraction, sonication had an non-significant

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effect, likely due to the HS-DES switching process, which already increases the effective surface area for analyte transport by altering the solvent polarity.

All factor interactions, except for the interaction between HS-DES volume and sonication time, exhibited positive effects on the response. Among these, the interactions between pH and sonication time and between HS-DES volume and NaCl concentration were statistically significant at the 95% confidence level. The significant interaction between HS-DES volume and salt concentration likely reflect the increased volume of the collected HS-DES phase at higher salt concentrations. In addition, the quadratic term of HS-DES volume exhibited a significant negative effect on the response. This indicates that extraction efficiency initially increases with HS-DES volume up to a certain level, after which it declines. This reduction can be ascribed to the higher proportion of HS-DES in the final phase, leading to lower fluorescence intensity [8]. In contrast, the quadratic terms of pH and sonication time exhibited significant positive effects on the response. Based on these findings, and considering only the statistically significant parameters, the regression equation is expressed as:

$$FI = 308.20 + 36.16\times A + 24.65\times B + 11.76\times AB + 15.62\times AC + 49.86\times AD + 26.60\times BC + 14.97\times CD + 15.19\times A^2 - 43.4802\times B^2 + 18.6221\times D^2 \quad (1)$$

The desirability function (DF) approach was used to identify the optimal conditions based on the experimental design results. In this method, the predicted response is transformed into a desirability value ranging from 0

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(undesirable) to 1 (fully desirable). For a single response, the desirability directly reflects the performance of that response, with values closer to 1 indicating more favorable outcomes. By maximizing the desirability, the optimal factor levels can be determined, ensuring the highest achievable response under the experimental conditions. The optimization criteria for all studied factors, along with the lower and upper limits of fluorescence intensity, are presented in Table S3. The target for each factor was set within the selected range, allowing the optimal points to fall anywhere within the experimental domain. The importance levels for the factors were set to the software default of 3, while the response was assigned the highest importance value of 5, with the target set to maximize fluorescence intensity. Using the DF and the predefined goals, the software predicted the optimal extraction conditions, resulting in a fluorescence intensity of 553.1 units for verapamil. The corresponding optimal values were determined as follows: pH = 10.97, HS-DES volume = 139 µL, NaCl concentration = 4.48% (w/v), and sonication time = 10.54 min. Finally, to confirm the reliability of these optimal conditions, validation experiments were conducted in triplicate. The obtained results showed close agreement with the predicted values generated by the DF-based optimization.

Furthermore, the response surface plots in Fig. 4 clearly illustrate the main effects and interactions among the factors on overall desirability. An increase in pH produced a linear rise in desirability, confirming its critical influence on improving the response. The HS-DES volume exhibited a quadratic trend,

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with the highest desirability observed at intermediate levels. The non-significant effect of NaCl concentration on the response is also evident from the plots. Additionally, the curvature and inflection points observed in some graphs indicate the presence of significant interactions among the factors.

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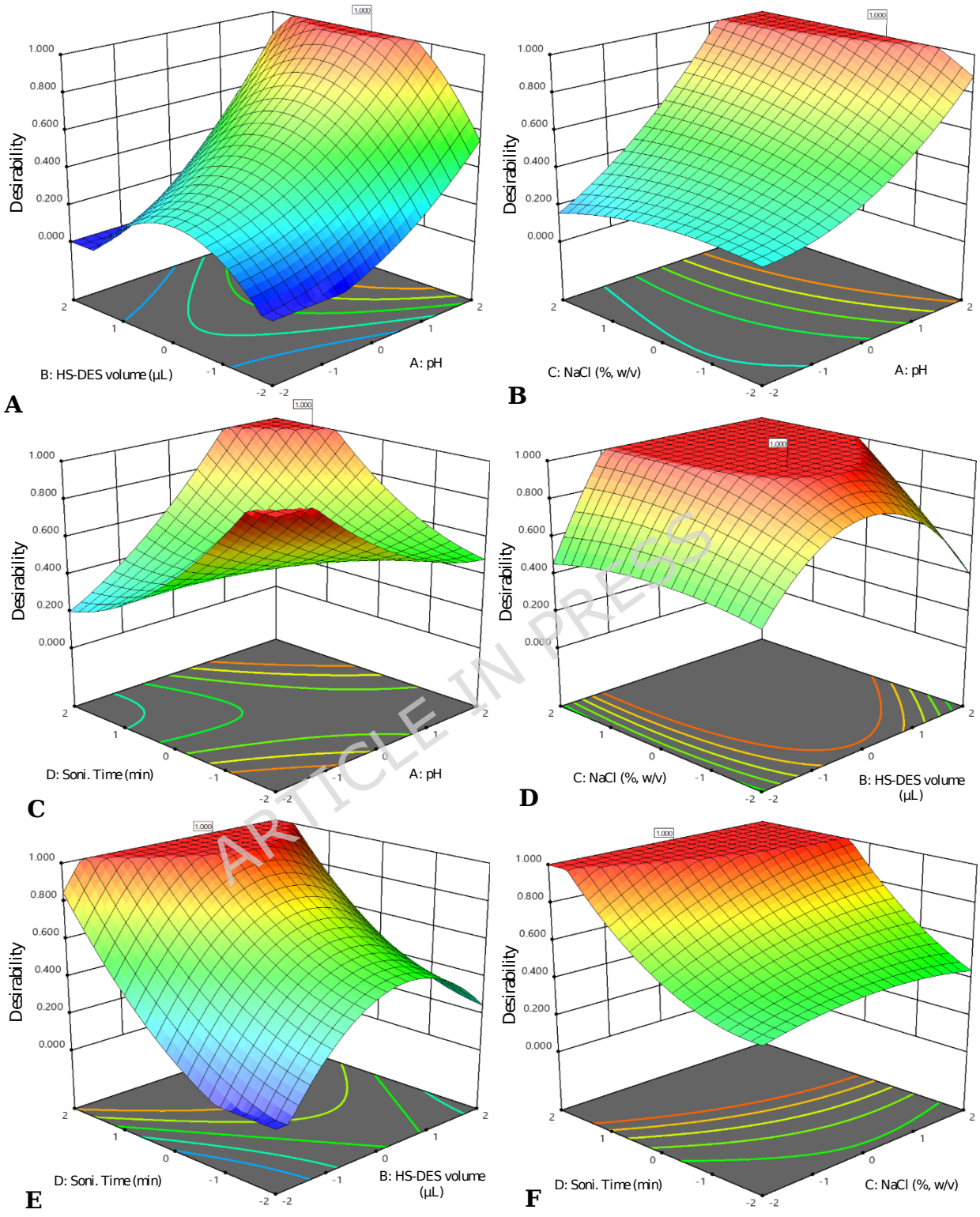


Fig. 4. Response surface plots for two factors based on the DF, with all other factors fixed at their optimum values.

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Validation

To evaluate the analytical performance of the method, several key parameters were examined, including the calibration curve, coefficient of determination (R^2), limit of detection (LOD), limit of quantification (LOQ), extraction recovery (ER), and method precision. For construction of the calibration curve, 0.8 mL of drug-free plasma was spiked at seven concentration levels, followed by sample preparation and extraction as described in the experimental section. The resulting calibration curve, covering concentrations from 200 to 2000 ng/mL, showed a strong linear relationship between fluorescence intensity and analyte concentration, supported by a high coefficient of determination ($R^2 = 0.9921$). This level of linearity confirms the method's suitability for accurate quantification of verapamil across the studied concentration range (Fig. 5). The LOD and LOQ values were calculated using the following equations:

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$$\text{LOD or LOQ} = \frac{F \times SD}{b} \quad (2)$$

where F is a constant with values of 3.3 and 10 for LOD and LOQ, respectively. SD represents the standard deviation of the blank, and b is the slope of the calibration curve. The calculated results are summarized in Table 2. Precision was assessed through intra-day and inter-day experiments, each performed in three replicates. The resulting RSD (%) values demonstrated excellent repeatability and method stability. The ER was calculated based on a plasma sample volume of 0.8 mL and a final extract volume of 300 µL,

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resulting in an ER value of 59.55%. This ER value is consistent with previous reports on DESs and HS-DESs [24-26].

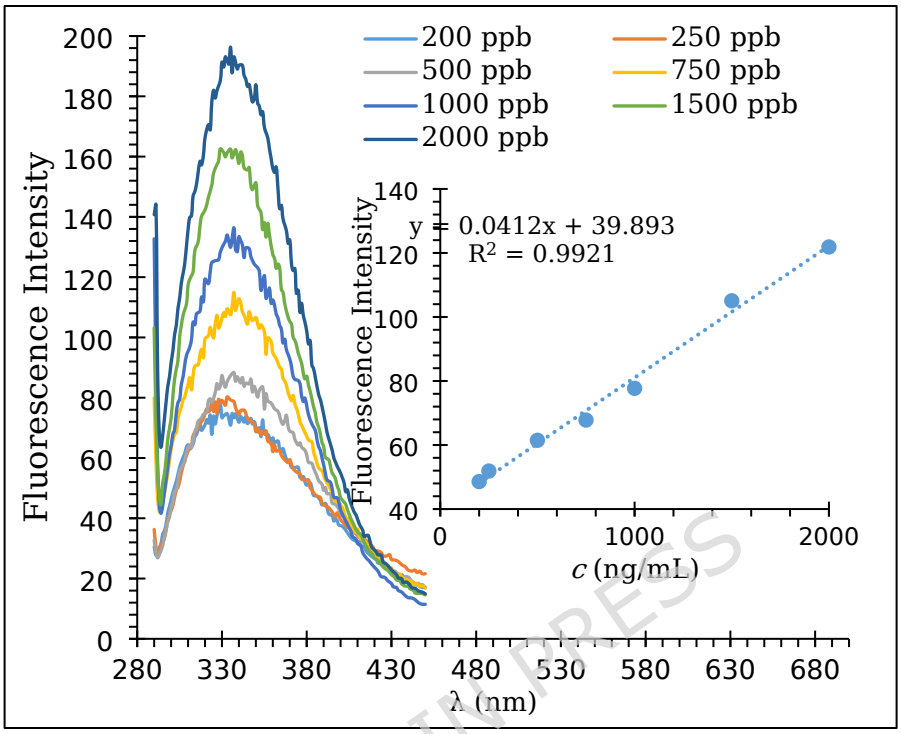


Fig. 5. Calibration curve for different concentrations of verapamil in plasma samples. *Extraction conditions*: plasma volume, 0.8 mL; sample volume, 6 mL; pH, 10.97; HS-DES volume, 139 µL; sonication time, 10.54 min; NaCl concentration, 4.48% (w/v); NaOH (1 M), 500 µL; HCl (1 M), 500 µL; centrifugation time, 10 min; final volume, 300 µL; solvent, methanol.

Table 2. Analytical performance parameters established for the determination of verapamil in plasma samples.

Linear range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	RSD% (n=3)		ER(%)
				Intra-day	Inter-day	
200-2000	0.9921	62.16	188.35	4.6	5.7	59.55

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The influence of potential interfering species and co-administered drugs on the analytical performance of the method was assessed. Each interfering species was added to the sample solution at concentrations 100-, 50-, or 10-fold higher than that of the target analyte. The percentage change in signal intensity was then calculated by comparing the signal obtained in the presence of the interfering species with that obtained in their absence (Table 3). As shown, most of the evaluated drugs exhibited negligible interference on the analytical response even at concentrations 50-fold higher than that of verapamil. Despite structural similarity, the limited interference observed for most co-administered drugs highlights the selectivity of the HS-DES system toward verapamil. Only amlodipine produced a significant effect at the 50-fold level; however, its interference was markedly reduced when present at only 10-fold excess. In addition, the potential interference from two common biological cations calcium and magnesium was examined using their chloride salts. The results indicated that even at 100-fold excess, the interference caused by these ions remained acceptable and below 10%.

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Table 3. Influence of co-administered drugs and selected biological cations on the extraction efficiency of verapamil (250 ng/mL).

Drug	**Ratio**[a]	**(ΔF%)**[b]
Phenobarbital	50	4.87
Metoprolol	50	9.23
Amlodipine	50	-75.82
Amlodipine	10	-4.87
Losartan	50	9.04
Aripiprazole	50	-7.04
Ca^{2+}	100	9.77
Mg^{2+}	100	2.03

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[a] Interferent-to-analyte ratio
[b] Percent change in fluorescence intensity

To evaluate the environmental sustainability of the method, the AGREE (Analytical GREEnness) tool was applied (Fig. 6). The method achieved an overall AGREE score of 0.64, indicating a satisfactory level of greenness and demonstrating its suitability as a more sustainable analytical approach. This score reflects the incorporation of several environmentally favorable features, including reduced reagent consumption, minimized waste generation, and the use of a green solvent.

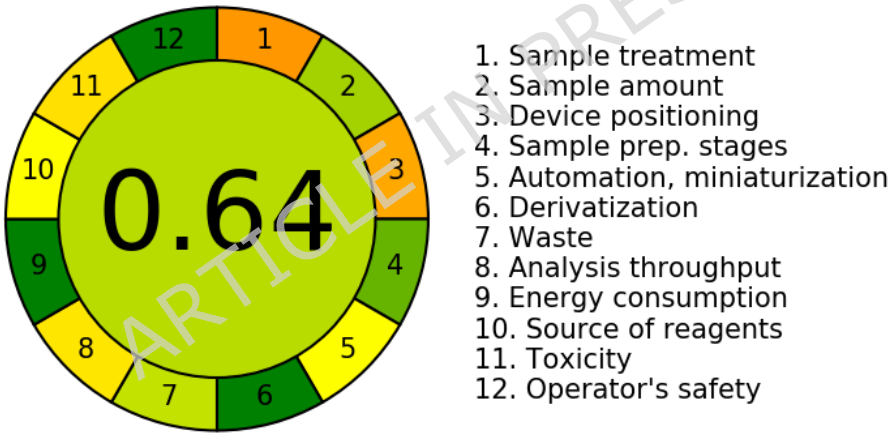


Fig. 6. Greenness assessment of the HS-DES-UALPME method using the AGREE metric, yielding an overall score of 0.64.

A comparative summary of the analytical performance of the method with previously reported approaches is presented in Table 4. As shown, in most cases the method exhibits superior performance for the evaluated parameters, while in the remaining cases it provides comparable results or differs only slightly in favor of the alternative methods. In addition, the

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method demonstrates a robust environmental profile, as reflected by its AGREE score of 0.64 and its reduced reagent use, minimal waste production, and reliance on a green solvent, further emphasizing its suitability as a sustainable alternative to conventional analytical approaches.

Table 4. Comparative analytical performance of the proposed method and previously reported approaches.

Method	Sample	Linear range (ng/mL)	LOD (ng/mL)	ER%	Ref.
Deproteinization/HPLC-UV	Rat plasma	500-10000	100	-	[27]
DLLME/HPLC-UV[a]	Exhaled Breath	70-800	45	-	[1]
MLC-FL[b]	Urine and serum	100-2000	18.5 and 20.2	-	[28]
SPE/UHPLC-PDA-FLD[c]	Plasma	40-1250	40 (LLOQ)	84	[6]
In-tube SPME/HPLC-UV[d]	Plasma and Urine	100-800	52	-	[29]
Microfluidic device /HPLC-DAD	Urine	350-5000	110	58	[2]
pH-assisted PPME/CZE-UV[e]	River water and urine	300-20000	100	-	[4]
Microfluidic method /HPLC-DAD	Urine	450-10000	140	-	[3]
HS-DES-UALPME	Plasma	200-2000	62.16	59.55	This work

[a] Dispersive liquid-liquid microextraction/ high performance liquid chromatography- ultraviolet
[b] Micellar liquid chromatography and fluorescence detection
[c] Solid phase extraction**/**ultra-high performance liquid chromatography**–**photodiode array**–**fluorescence detector
[d] In-tube solid-phase microextraction
[e] pH-assisted in-line pseudophase microextraction/capillary zone electrophoresis

Plasma samples Analysis

The determination of verapamil in human plasma was performed using the standard addition method to verify the accuracy and precision of the HS-DES-UALPME procedure. Three concentration levels of the analyte were spiked into plasma samples and analyzed. The results demonstrated that the method

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could reliably quantified verapamil within the complex plasma matrix with satisfactory accuracy and precision. As shown in Table 5, the relative recovery values were close to the theoretical value of 100%, indicating that external factors, particularly matrix effects, had minimal influence on the measurements. The satisfactory relative recoveries demonstrate the applicability of the method for routine analysis and potential therapeutic drug monitoring of verapamil in plasma.

Table 5. Relative recovery values obtained for verapamil in spiked plasma samples.

Added (ng/mL)	Found[a] (ng/mL)	RR (%)[b]	RSD% (n=3)
250	260.4	104.1	5.0
500	517.7	103.5	3.3
800	824.3	103.0	2.3

[a] Average of three replicates
[b] Relative recovery

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Conclusion

In this study, an HS-DES-UALPME method was successfully developed for the extraction and determination of verapamil in human plasma. The TBAB:C9-based HS-DES showed efficient pH-responsive phase switching behavior, enabling effective extraction and isolation of verapamil from the complex plasma matrix. Experimental variables were optimized using CCD combined with the DF, resulting in satisfactory analytical performance. Under optimized conditions, the method provided a wide linear range, satisfactory sensitivity, good precision, and acceptable extraction recovery.

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In addition, the method involved low solvent consumption and short extraction time, supporting its simplicity and environmentally friendly nature. The AGREE evaluation further confirmed its compliance with green analytical chemistry principles. Comparison with previously reported methods demonstrated that the proposed HS-DES-UALPME approach offers competitive analytical performance together with reduced consumption of hazardous organic solvents and improved environmental sustainability. Therefore, the developed method represents a simple, efficient, and green alternative for the determination of verapamil in biological samples.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

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Not applicable.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work the authors used ChatGPT in order to assist with grammar checking and language refinement to improve the readability and language of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the published article.

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References:

1 Pourkarim, F. *et al.* Determination of Verapamil in Exhaled Breath Condensate by Using Microextraction and Liquid Chromatography. *Current Pharmaceutical Analysis* **15**, 535-541, doi:https://doi.org/10.2174/1573412914666180717125434 (2019).

2 Martín, A., Santigosa, E. & Ramos-Payán, M. A green microfluidic method for the simultaneous extraction of polar and non-polar basic compounds in biological samples. *Microchemical Journal* **189**, 108553, doi:https://doi.org/10.1016/j.microc.2023.108553 (2023).

3 Santigosa, E., Pedersen-Bjergaard, S., Giménez-Gómez, P., Muñoz, M. & Ramos-Payán, M. A rapid and versatile microfluidic method for the simultaneous extraction of polar and non-polar basic pharmaceuticals from human urine. *Analytica Chimica Acta* **1208**, 339829, doi:https://doi.org/10.1016/j.aca.2022.339829 (2022).

4 Adel Ahmed, M. & Quirino, J. P. pH-assisted in-line pseudophase microextraction and separation with tridodecylmethylammonium chloride admicelles in open-tubular capillary-based separations. *Microchemical Journal* **182**, 107871, doi:https://doi.org/10.1016/j.microc.2022.107871 (2022).

5 Hemmati, M., Rajabi, M. & Asghari, A. Efficient and clean pre-concentration of ultra-trace calcium channel blockers from biological matrices via a hyphenated procedure of two sequential dispersive solid/liquid phase

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microextractions. *Analytica Chimica Acta* **960**, 138-150, doi:https://doi.org/10.1016/j.aca.2017.01.001 (2017).

6 González Mendia, O. *et al.* Efficient Method Development and Validation for the Determination of Cardiovascular Drugs in Human Plasma by SPE–UHPLC–PDA–FLD. *Chromatographia* **80**, 605-615, doi:https://doi.org/10.1007/s10337-017-3274-6 (2017).

7 Erbas, Z. & Soylak, M. Simple Deep Eutectic Solvent-Based Liquid Phase Microextraction for Sudan IV from Food, Water, and Cosmetics. *Analytical Letters* **58**, 2946-2958, doi:10.1080/00032719.2024.2447940 (2025).

8 Fathi Majd, S., Heidari, H. & Razmi, H. A natural hydrophobic deep eutectic solvent-based microextraction method integrated with a spectrofluorimetric technique for the determination of daclatasvir as a direct-acting hepatitis C antiviral drug in plasma samples. *Microchemical Journal* **192**, 108949, doi:https://doi.org/10.1016/j.microc.2023.108949 (2023).

9 Soylak, M., Çoban, A. N. & Ahmed, H. E. H. A novel deep eutectic solvent based on oleic acid and betaine hydrochloride for liquid-phase microextraction of Sudan IV from water and food samples. *Journal of the Iranian Chemical Society* **22**, 1125-1137, doi:10.1007/s13738-025-03212-0 (2025).

10 Yan, Y. *et al.* Development of switchable deep eutectic solvents: Applications in extraction of natural products. *Chinese Chemical*

ARTICLE IN PRESS

Letters **36**, 110845, doi:https://doi.org/10.1016/j.cclet.2025.110845 (2025).

11 Wang, Y. *et al.* A switchable deep eutectic solvent for the homogeneous liquid-liquid microextraction of flavonoids from “Scutellariae Radix”. *Journal of Chromatography A* **1688**, 463712, doi:https://doi.org/10.1016/j.chroma.2022.463712 (2023).

12 Wei, S., Wu, Y. & Xi, J. CO2-responsive switchable hydrophilic solvent as a novel extractant for selective extraction and separation of natural bioactive ingredients: A comprehensive review. *Food Chemistry* **463**, 141170, doi:https://doi.org/10.1016/j.foodchem.2024.141170 (2025).

13 Zhang, J. *et al.* Responsive switchable deep eutectic solvents: A review. *Chinese Chemical Letters* **34**, 107750, doi:https://doi.org/10.1016/j.cclet.2022.107750 (2023).

14 Ghane, M., Ezoddin, M., Mohadesi, A., Abdi, k. & Ali Karimi, M. Reversible switchable deep eutectic solvent for dispersive liquid–liquid microextraction of non-steroidal anti-inflammatory drugs in biological and wastewater samples combined with high-performance liquid chromatography. *Microchemical Journal* **203**, 110915, doi:https://doi.org/10.1016/j.microc.2024.110915 (2024).

15 Khosrowshahi, E. M. *et al.* pH-induced Homogeneous liquid–liquid Microextraction Method based on new Switchable Deep Eutectic Solvent for the Extraction of Three Antiepileptic Drugs from Breast

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ARTICLE IN PRESS

Milk. *Bioanalysis* **13**, 1087-1099, doi:https://doi.org/10.4155/bio-2021-0067 (2021).

16 You, H., Huang, D., Chen, L., Liu, Y. & Li, H. pH-induced deep eutectic solvents-based homogeneous liquid-liquid microextraction for the extraction and preconcentration of three nonsteroidal anti-inflammatory drugs from environmental water. *Microchemical Journal* **219**, 116120, doi:https://doi.org/10.1016/j.microc.2025.116120 (2025).

17 Wei, P., Liu, J., Zhao, J. & Zhao, L. Strategies for green extraction of Dioscin from Dioscorea nipponica Makino by ultrasound-assisted switchable natural deep eutectic solvents. *Microchemical Journal* **215**, 114308, doi:https://doi.org/10.1016/j.microc.2025.114308 (2025).

18 Babu Balaraman, H., Viswanathan, G., Muniasamy, R., Gayatri, T. & Kumar Rathnasamy, S. Sustainable valorization of papaya peels for thrombolytic cysteine protease isolation by ultrasound assisted disruptive liquid phase microextraction with task specific switchable natural deep eutectic solvents. *Microchemical Journal* **175**, 107118, doi:https://doi.org/10.1016/j.microc.2021.107118 (2022).

19 Heidari, H. & Hashemi, A. Ultrasound-assisted emulsification-microextraction of propranolol in plasma using a natural hydrophobic deep eutectic solvent for fluorescence detection: A green analytical approach. *Sustainable Chemistry and Pharmacy* **44**, 101977, doi:https://doi.org/10.1016/j.scp.2025.101977 (2025).

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20 Jain, R. *et al.* Advances in green sample preparation methods for bioanalytical laboratories focusing on drug analysis. *Bioanalysis* **17**, 489-508 (2025).

21 Carasek, E., Bernardi, G., Morelli, D. & Merib, J. Sustainable green solvents for microextraction techniques: Recent developments and applications. *Journal of Chromatography A* **1640**, 461944, doi:https://doi.org/10.1016/j.chroma.2021.461944 (2021).

22 Lu, Y., Wang, X., Gu, H. & Gao, M. Morphological transformation assisted switchable deep eutectic solvents combined with HPLC-DAD for the detection of six UV-filters in surface and bathing waters. *Microchemical Journal* **169**, 106626, doi:https://doi.org/10.1016/j.microc.2021.106626 (2021).

23 Hasegawa, J., Fujita, T., Hayashi, Y., Iwamoto, K. & Watanabe, J. pKa Determination of Verapamil by Liquid-Liquid Partition. *Journal of Pharmaceutical Sciences* **73**, 442-445, doi:https://doi.org/10.1002/jps.2600730405 (1984).

24 Yang, L., Yang, X., Wang, E., Lu, B. & Duan, L. Development of CO2-switchable deep eutectic solvent-based liquid-liquid microextraction approach: Application in urinary excretion and tissue distribution studies. *Analytica Chimica Acta* **1307**, 342620, doi:https://doi.org/10.1016/j.aca.2024.342620 (2024).

25 Niu, J. *et al.* Switchable deep eutectic solvent as green and efficient media for liquid-phase microextraction of phenoxyacetic acid herbicides in

ARTICLE IN PRESS

water and food matrices. *Food Chemistry* **442**, 138433, doi:https://doi.org/10.1016/j.foodchem.2024.138433 (2024).

26 Jouyban, A., Farajzadeh, M. A. & Afshar Mogaddam, M. R. In matrix formation of deep eutectic solvent used in liquid phase extraction coupled with solidification of organic droplets dispersive liquid-liquid microextraction; application in determination of some pesticides in milk samples. *Talanta* **206**, 120169, doi:https://doi.org/10.1016/j.talanta.2019.120169 (2020).

27 Lau-Cam, C. A. & Piemontese, D. Simplified reversed-phase HPLC method with spectrophotometric detection for the assay of verapamil in rat plasma1Presented at the Analysis and Pharmaceutical Quality Section of the Eleventh Annual American Association of Pharmaceutical Scientists Meeting, October 1996, Seattle, Washington, USA.1. *Journal of Pharmaceutical and Biomedical Analysis* **16**, 1029-1035, doi:https://doi.org/10.1016/S0731-7085(97)00117-9 (1998).

28 Rambla-Alegre, M., Gil-Agustí, M. T., Capella-Peiró, M. E., Carda-Broch, S. & Esteve-Romero, J. S. Direct determination of verapamil in urine and serum samples by micellar liquid chromatography and fluorescence detection. *Journal of Chromatography B* **839**, 89-94, doi:https://doi.org/10.1016/j.jchromb.2006.03.054 (2006).

29 Walles, M. *et al.* Verapamil drug metabolism studies by automated in-tube solid phase microextraction. *Journal of Pharmaceutical and Biomedical*

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Analysis **30**, 307-319, doi:https://doi.org/10.1016/S0731-7085(02)00267-4 (2002).

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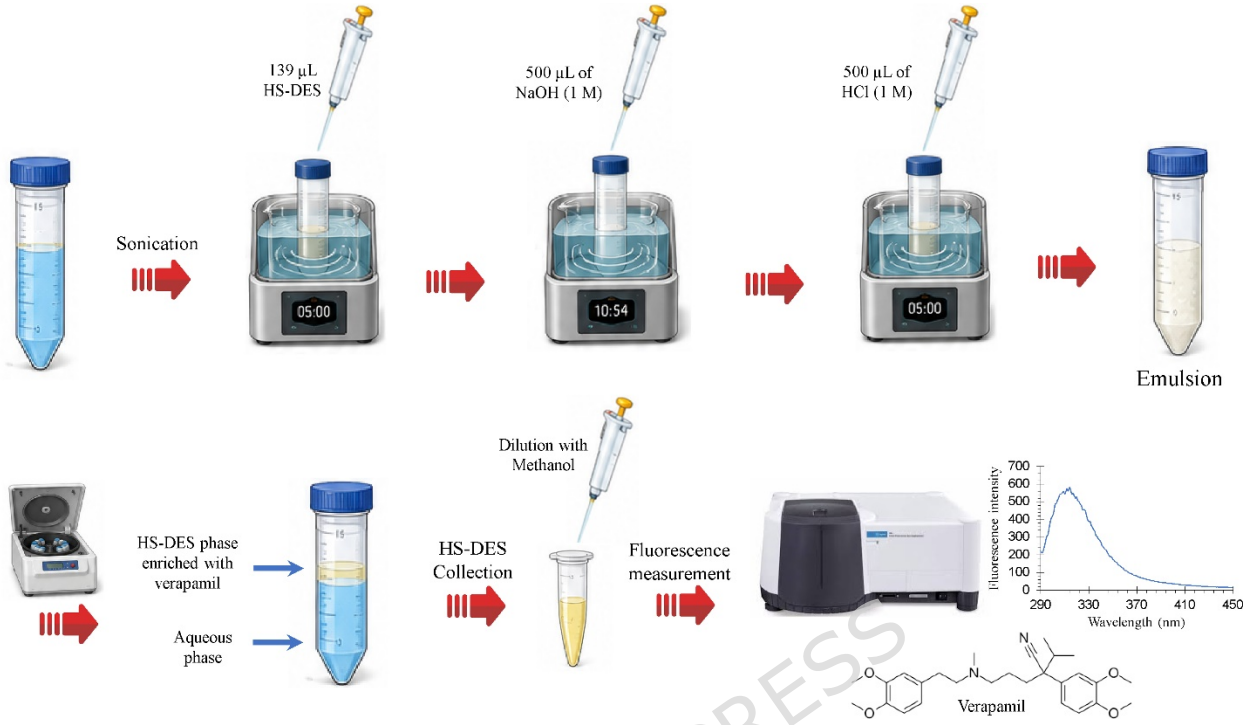


Fig. 1

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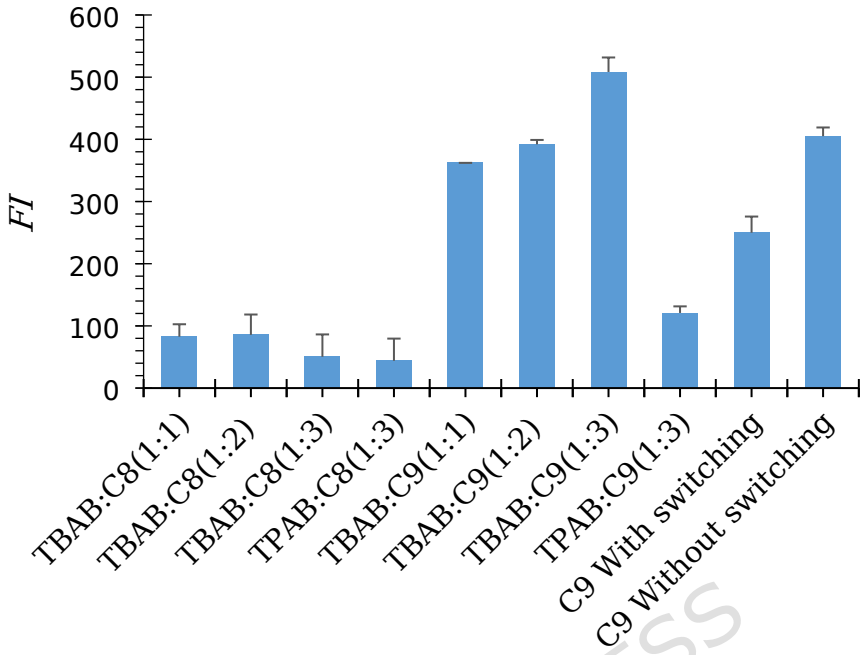


Fig. 2

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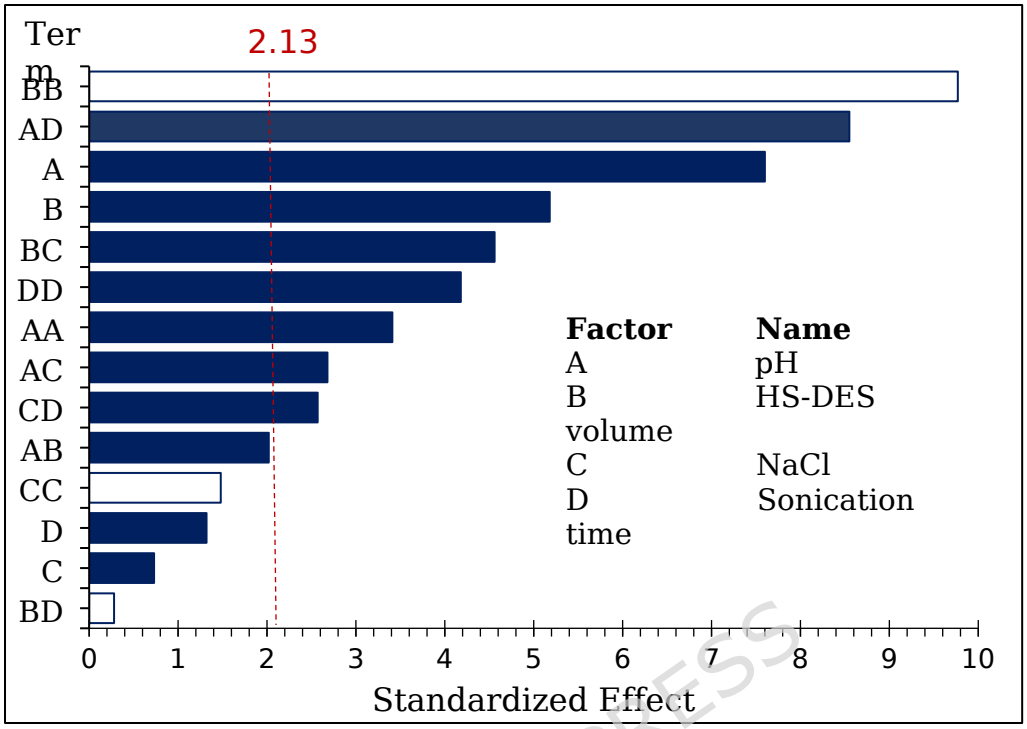


Fig. 3

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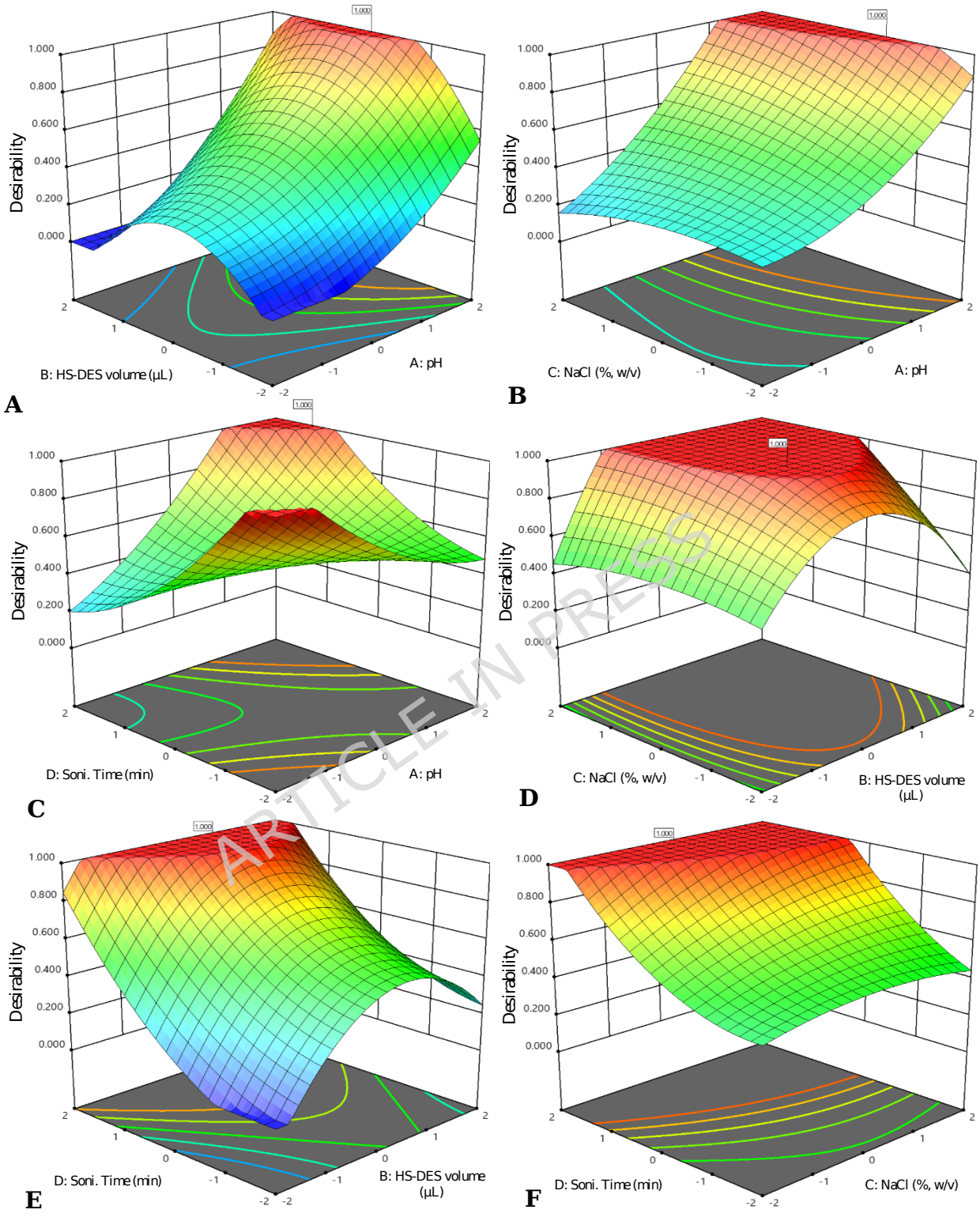


Fig. 4

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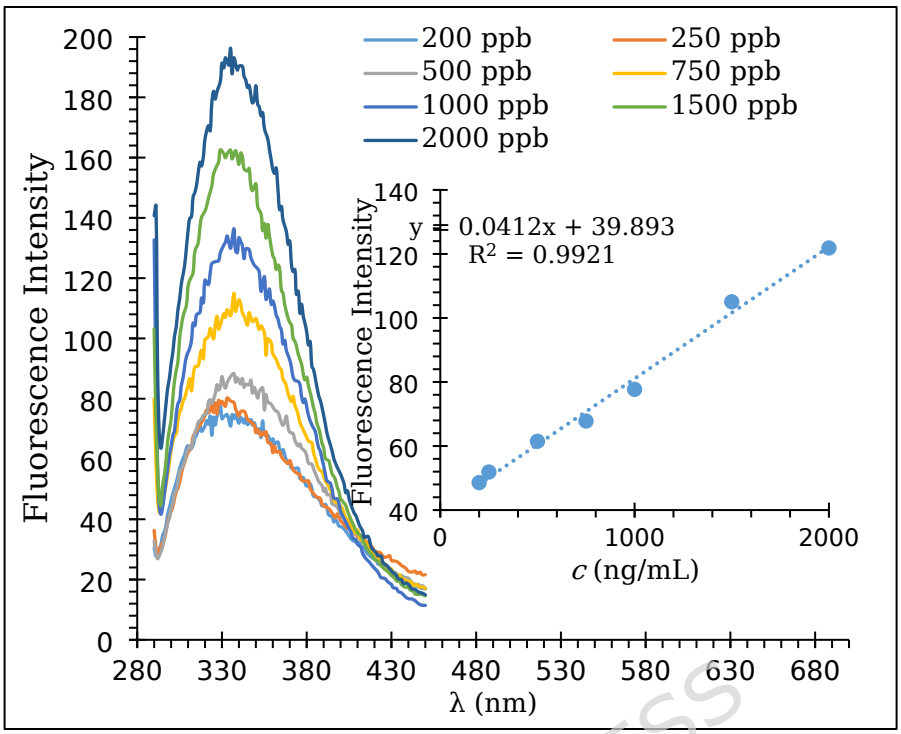


Fig. 5

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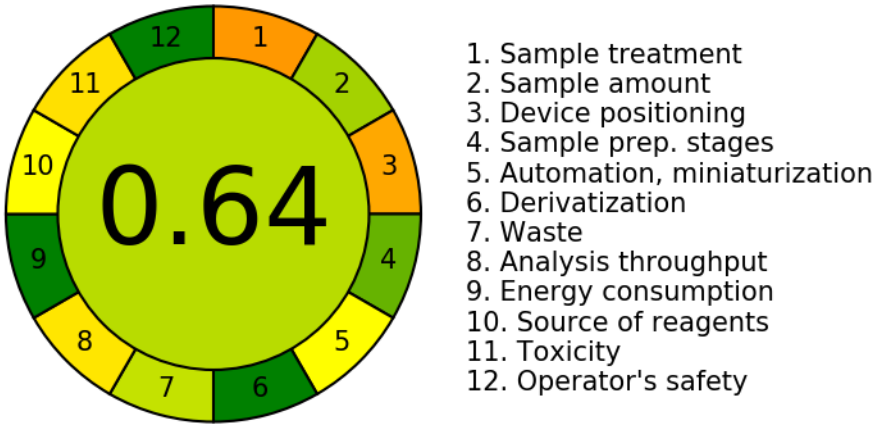


Fig. 6

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Table 1. Coded and uncoded values of the experimental variables used in the CCD.

Factor	-2	-1	0	1	2
A: pH	9	9.5	10	10.5	11
B: HS-DES volume (µL)	80	110	140	170	200
C: NaCl (%, w/v)	0	3	6	9	12
D: Sonication time (min)	1	4	7	10	13

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Table 2. Analytical performance parameters established for the determination of verapamil in plasma samples.

Linear range (ng/mL)	R^2	LOD (ng/mL)	LOQ (ng/mL)	RSD% (n=3)		ER(%)
				Intra-day	Inter-day	
200-2000	0.9921	62.16	188.35	4.6	5.7	59.55

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Table 3. Influence of co-administered drugs and selected biological cations on the extraction efficiency of verapamil (250 ng/mL).

Drug	**Ratio**[a]	**(ΔF%)**[b]
Phenobarbital	50	4.87
Metoprolol	50	9.23
Amlodipine	50	-75.82
Amlodipine	10	-4.87
Losartan	50	9.04
Aripiprazole	50	-7.04
Ca^{2+}	100	9.77
Mg^{2+}	100	2.03

[a] Interferent-to-analyte ratio
[b] Percent change in fluorescence intensity

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[c] Solid phase extraction**/**ultra-high performance liquid chromatography**-**photodiode array**-**fluorescence detector
[d] In-tube solid-phase microextraction
[e] pH-assisted in-line pseudophase microextraction/capillary zone electrophoresis

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Table 5. Relative recovery values obtained for verapamil in spiked plasma samples.

Added (ng/mL)	Found[a] (ng/mL)	RR (%)[b]	RSD% (n=3)
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800	824.3	103.0	2.3

[a] Average of three replicates
[b] Relative recovery

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