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Behaviour of deep eutectic solvent based on terpenoid and long-chain alcohol during dispersive liquid-liquid microextraction: Determination of zearalenone in cereal samples

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ABSTRACT

In recent years, the numerous articles have been devoted to the application of hydrophobic deep eutectic solvents based on natural terpenoids and long-chain alcohols as extraction solvents in dispersive liquid–liquid microextraction. Typically, the microextraction procedure assumes injection of deep eutectic solvent solution in a dispersive solvent (polar organic solvent) into aqueous phase. The decomposition of deep eutectic solvent in the extraction system can be observed due to the partial dissolution of its precursors in aqueous phase containing the dispersive solvent. In this work, the process of dispersive liquid–liquid microextraction with the use of deep eutectic solvents based on terpenoids and long-chain alcohols was investigated. The extraction systems were applied to the mycotoxin (zearalenone) determination in cereal samples as a model analytical task. The solvent based on DL-menthol and 1-hexanol provided high extraction recovery (93 ± 4) % and satisfactory enrichment factor (15.8 ± 0.7). The limit of detection evaluated from the signal-to-noise ratio was 2 μg/kg. The stability of the deep eutectic solvent in acetonitrile–water mixture was studied using gas chromatography-flame ionization detection and Karl Fisher method. It was shown that the formation of terpenoid-rich phase (60 % (m/m) of menthol) took place during the dispersive liquid–liquid microextraction, and the deep eutectic solvent based on terpenoid and long-chain alcohol cannot be considered as the extraction solvent.

1. Introduction

The current trend in Green Chemistry is development and application of environmentally-friendly solvents in green and sustainable technologies. In this regard, deep eutectic solvents (DESs) are of rapidly growing interest as an attractive and safe alternative to typically used toxic organic solvents [1,2]. Various DESs based usually on quaternary ammonium salts, alcohols, natural organic acids, amides, urea, saccharides and terpenoids have been introduced in literature as green extraction solvents in liquid–liquid extraction (LLE) [3,4]. Obviously, the stability of DESs in presence of water is of great importance in various modes of LLE. This topic was mostly covered for hydrophilic and quasi-hydrophobic DESs in several reviews [5–7] and research papers [8–11]. On the one hand, water can be helpful in tailoring extraction capability of hydrophilic DESs. On the other hand, destruction of hydrogen bonds between hydrophilic DES components can occur at high water content. Since hydrophilic DESs are water-miscible, they cannot be used for separation of analytes from aqueous samples. Quasi-hydrophobic DESs are used for LLE, but it should be considered that disintegration of such DES can take place due to partial or complete dissolution of its components in aqueous medium leading to dramatic change in DES composition. For example, it was found that quasi-hydrophobic DESs based on quaternary ammonium salts (tetrabutylammonium bromide and tetraoctylammonium bromide) and fatty acids (hexanoic, heptanoic, octanoic, nonanoic and decanoic acids) are unstable upon contact with aqueous solution, which resulted in separation of the organic phase containing high water content [10]. In fact, extract phase cannot be considered as initial two-component DES in these conditions, because resulted mixture of the components has a very different composition in comparison to the initial DES phase. In contrast, hydrophobic DESs are suitable for contact with aqueous phase.

In recent years, the numerous articles have been devoted to dispersive liquid–liquid microextraction (DLLME) based on hydrophobic DESs [12–16]. Natural terpenoids (DL-menthol and thymol), long-chain alcohols, and fatty acids are frequently used as hydrogen bond donors and acceptors for synthesis of hydrophobic DESs [17,18]. Usually, DLLME procedures assume injection of

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DES solution in a dispersive solvent (polar organic solvent) into aqueous phase. For instance, DES composed of L-menthol and butylated hydroxytoluene at a molar ratio of 3:1 was dissolved in ethanol, and the resulted solution was rapidly injected into a wine sample leading to cloudy state of the extraction system followed by phase separation and determination of pesticides by high-performance liquid chromatography-mass spectrometry [16]. However, sample pretreatment of solid matrices involving DLLME is carried out differently. Typically, analytes preconcentration from solid samples is conducted to increase enrichment factors (EFs) and obtain less limits of detection (LODs) by their extraction into relatively large volume of a polar organic solvent. In this case obtained extract can act as the dispersive solvent. Appropriate DES is dissolved into the obtained extract followed by the DLLME [13,19,20]. For example, pesticides were extracted from cereals using acetonitrile, then DES based on thymol and octanoic acid at a molar ratio of 1:4 was added to the extract, and resulted DES solution was introduced into water, which was followed by separation of the extract and its analysis by high-performance liquid chromatography with UV–vis detection [13]. Despite considerable hydrophobicity of precursors of the DES, they can be soluble in obtained mixture of dispersive solvent and water. Partial dissolution of the precursors in dispersive solvent–water mixture can lead to DES decomposition. In spite of the fact that solubility of hydrophobic DES precursors in aqueous phase in the presence of the dispersive solvent can be increased, the DES phase composition after phase separation as well as an extraction mechanism during the DLLME have not been investigated.

The aims of this work were to study behavior and stability of hydrophobic DES based on terpenoid and long-chain alcohol in dispersive solvent–water mixture during DLLME as well as to reveal the role of each DES component in sample pretreatment process. To solve model and relevant analytical task, the extraction systems were applied to the mycotoxin (zearalenone, Fig. S1) separation from solid cereal samples, employing formation of the extract phase during the DLLME for analyte preconcentration before determination by high-performance liquid chromatography with fluorescence detection (HPLC-FLD). Zearalenone is one of the most common mycotoxins produced by *Fusarium fungi* [21].

2. Materials and methods

2.1. Reagents and solutions

All chemicals and reagents were of analytical grade. Ultra-pure water from a Millipore Milli-Q RG system (Millipore, USA) was used throughout the work. Thymol and DL-menthol were obtained from Sigma-Aldrich (USA). Isopropyl alcohol, 1-hexanol, 1-heptanol, 1-octanol, 1-decanol, K_2HPO_4, citric acid, sodium chloride and sucrose were purchased from Vecton (Russia). Methanol and acetonitrile were obtained from J.T. Baker Chemical Company (USA).

A certified reference material (CRM 7944–2001, Imid, Russia) was used as stock zearalenone solution (100 g/L) in benzene and stored in a freezer at −18 °C. Working zearalenone solutions were prepared daily by appropriate dilutions with methanol.

To obtain DESs natural terpenoid (DL-menthol or thymol) as hydrogen bond acceptor and long-chain alcohol as hydrogen bond donor were added in a certain molar ratio to a flask and heated up to 60 °C during 5 min under mixing.

2.2. Instrumentation and chromatographic analysis conditions

A Shimadzu LC-20 Prominence liquid chromatograph equipped with RF-20A fluorescence detector (Shimadzu Corporation, Kyoto, Japan) and a Gemini C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, USA) was used for chromatographic analysis. Chromatographic measurements for proposed and reference procedures were performed in an isocratic elution mode. The mobile phase, consisted of ultra-pure water and acetonitrile (50:50, v/v), was pumped at flow-rate of 1.0 mL/min. The oven temperature was set at 25 °C. The volume of sample loop was 20 μL. The fluorescence excitation and emission wavelengths were 274 and 460 nm, respectively. The duration of chromatographic analysis was 20 min.

A gas chromatography Chromatek-Crystal 5000 system (Chromatek, Russia) with flame ionization detection (GC-FID) was used for the determination of acetonitrile, DL-menthol and 1-hexanol. Separations were carried out on an Agilent J&W HP-5 capillary column (10 m × 0.53 mm i.d. × 2.65 μm). The oven temperature was programmed as follows: initial temperature equal to 50 °C was held for 1 min, then raised to 150 °C at a rate of 20 °C/min, and held at 150 °C for 1 min. The total analysis time was 7 min. The injection volume was 0.5 μL, the flow rate of nitrogen was 2.7 mL/min, the temperature of the injector and the flame ionization detector was 200 °C.

The water concentration in extract phase was determined by the Karl-Fisher method using an 831 KF Coulometer (Metrohm, Switzerland). An ultrasonic bath (Sapphire, Russia, 130 W, 35 kHz) was used for the separation of zearalenone from solid food samples.

2.3. Samples

Wheat, corn flakes, wheat and corn flour, dry *Saccharomyces cerevisiae* yeast, unrefined sunflower oil were purchased from local supermarkets (St. Petersburg, Russia). The wheat and corn flakes samples were milled separately using a laboratory grinder and a porcelain mortar with pestle until all sample particles can pass a 1 mm laboratory stainless steel sieve. The homogenized samples were stored in polypropylene tubes in a dry place at room temperature for 5 days. Each sample was analyzed according to the developed procedure. In all cases analyte content was lower than LOD so these samples were considered as blank samples.

Bread sample was prepared in the laboratory using the following procedure adopted from [22]. Firstly, (3.20 ± 0.05) g of blank wheat flour, (3.20 ± 0.05) g of blank corn flour, (0.110 ± 0.002) g of sucrose, (0.050 ± 0.001) of sodium chloride and (0.070 ± 0.001) g of dry *Saccharomyces cerevisiae* yeast were thoroughly mixed in a porcelain crucible using stainless steel spatula. Then, (3.20 ± 0 .05) g of water and (0.100 ± 0.002) g of unrefined sunflower oil were added to the solid mixture and mixed until homogeneous viscoelastic material (dough) was formed. The crucible was covered with a filter paper and put into a water bath thermostated at (40.0 ± 1.0) °C for the fermentation during 40 min. Afterwards, the filter paper was removed, and the crucible was placed into a muffle furnace to perform bread baking at 230 °C during 20 min. Then, the crucible was cooled to room temperature, and the bread was taken out. Finally, the sample was crushed using the laboratory grinder and the porcelain mortar with pestle until all sample particles can pass the 1 mm laboratory stainless steel sieve.

Spiked bread samples were obtained by weighing (0.500 ± 0.0 05) g of homogenized laboratory-made bread sample in 4 mL polypropylene vial with subsequent addition of 10 μL of certified reference material (CRM 7944–2001) diluted with methanol to the required concentration of zearalenone. Then, evaporation of methanol was carried out in a water bath thermostated at (40.0 ± 1.0) °C during 20 min followed by cooling to room temperature and closing vial cap. Spiked bread samples were stored in a dry place at room temperature for 2 days.

2.4. Extraction procedure

At first (0.500 ± 0.005) g of homogenized wheat, corn flakes or bread sample was placed into 4 mL polypropylene vial with a

cap (Fig. 1). Then, 1.5 mL of DES (DL-menthol:1-hexanol = 2:1, mol/-mol) solution in acetonitrile (50 g/L) was added, and the vial was closed and shaken during 1 min for initial wetting of the sample. Separation of zearalenone into a liquid phase was carried out under ultrasonication at 75 °C during 15 min followed by centrifugation of the obtained suspension at 8000*g* during 3 min. Afterwards, 1.0 mL of supernatant was injected into 4 mL polypropylene vial containing 2.0 mL of ultra-pure water followed by the mixture shaking during 1 min for the DLLME. The phase separation was achieved by centrifugation (8000*g*) during 3 min. Finally, 30 μL of terpenoid-rich phase was aspirated from the top, diluted twice with methanol and analyzed by HPLC-FLD.

3. Results and discussion

3.1. Theoretical considerations and preliminary studies

To achieve high selectivity and sensitivity, the sample preparation strategy included two stages for analyte separation from the solid food sample and its preconcentration (Fig. 1). The first one was ultrasound-assisted solid–liquid extraction of zearalenone from the solid sample into the DES solution in a dispersive solvent. The mass transfer of the analyte into an organic medium can be accelerated by fragmentation, sonocapillary effect and local shear stress ensured by ultrasonic irradiation [23]. The second preconcentration stage involved the injection of the obtained supernatant into an aqueous phase for the DLLME. Formation of the extract phase microdroplets resulted in analyte preconcentration. In this case, the separation of the analyte into the formed phase can be triggered by its possible affinity to the DES components rather than to the mixture of water and the dispersive solvent.

Initially, various hydrophobic DESs based on terpenoids (DL-menthol and thymol) and long-chain alcohols (1-hexanol, 1-heptanol, 1-octanol, and 1-decanol) were studied for zearalenone preconcentration by the DLLME and its determination by HPLC-FLD to reveal more effective extraction system. The DES solution in acetonitrile (molar ratio of terpenoid to long-chain alcohol 1:1, 1.0 mL, 100 g/L) containing analyte (9 μg/L) was injected into 2.0 mL of ultra-pure water. Acetonitrile as the dispersive solvent was dissolved in aqueous phase, and extraction solvent microdroplets were generated. In all cases formation of extract phase was observed. After phase separation the obtained extracts were analyzed by HPLC-FLD.

Thymol had comparable retention time with that of zearalenone along with notable fluorescence intensity at optimal conditions for analyte. DL-menthol and long-chain alcohols did not cause any interference effects on the HPLC-FLD determination of zearalenone. As can be seen from the obtained data (Fig. 2 A), DESs based on 1-hexanol, 1-heptanol or 1-octanol demonstrated high and close to each other extraction recoveries (ERs), while 1-decanol resulted in less ER due to its high hydrophobicity. The DES based on DL-menthol and 1-hexanol was selected for further experiments, because this solvent was less viscous and more compatible with the mobile phase used for the HPLC-FLD.

A molar ratio between DES components (DL-menthol and 1-hexanol) was investigated in the range from 1:2 to 3:1 due to its influence on the hydrophobicity and viscosity of the eutectic mixture (Fig. 2 B). All studied DES were in liquid state at room temperature. It was found that ER increased at molar ratio varied from 1:2 to 2:1 and then became constant. Apparently, menthol promoted the mass transfer of the analyte into the organic phase by the strong hydrophobic interactions between the lactone ring of zearalenone and the cyclohexane ring of menthol. Almost quantitative extraction was observed for molar ratios equal to 2:1 and 3:1. All subsequent studies were carried out for DES with 2:1 molar ratio having lower viscosity and better compatibility with the mobile phase.

3.2. Investigation of appropriate DLLME condition

3.2.1. Effect of dispersive solvent nature

The nature of dispersive solvent, which enables the dispersion of fine droplets of extractant in an aqueous medium, is of great importance. It should be miscible both with DES and water but at the same time not too disruptive in relation to interactions between zearalenone and DES components. In this study-three dispersive polar solvents (methanol, acetonitrile and isopropanol) were studied for this purpose. One mL of DES solution in each dispersive solvent (100 g/L) containing zearalenone (9 μg/L) was injected into aqueous phase (2.0 mL). The extraction phases were

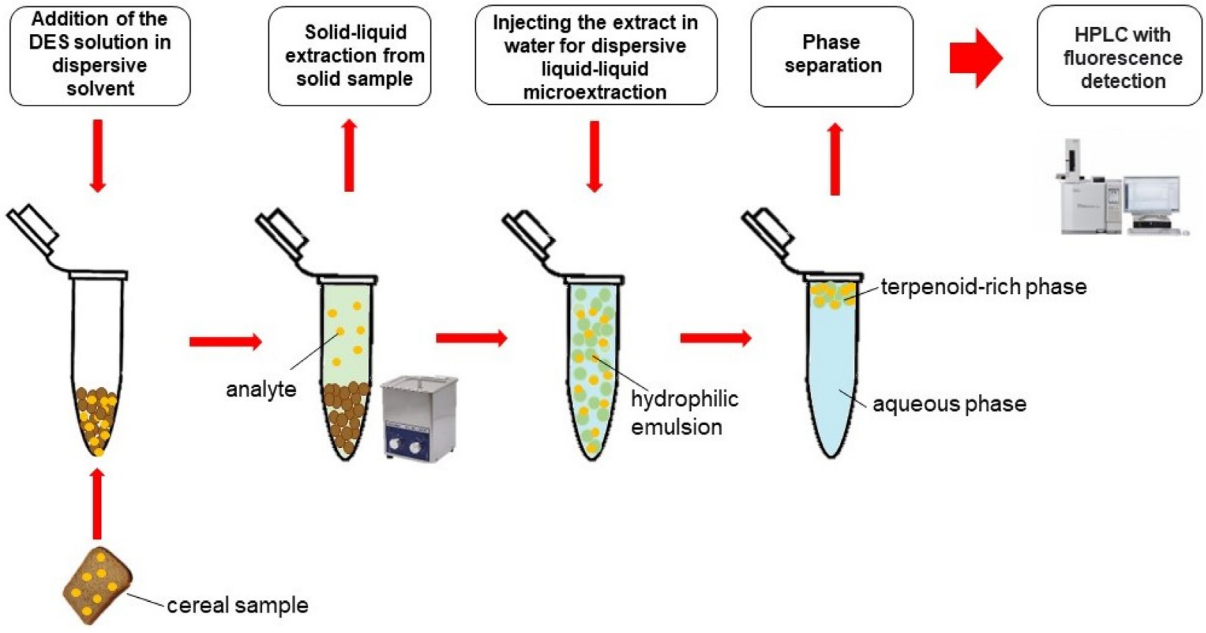

Fig. 1. Graphical representation of ultrasound-assisted solid–liquid extraction followed by DLLME with the use of DES.

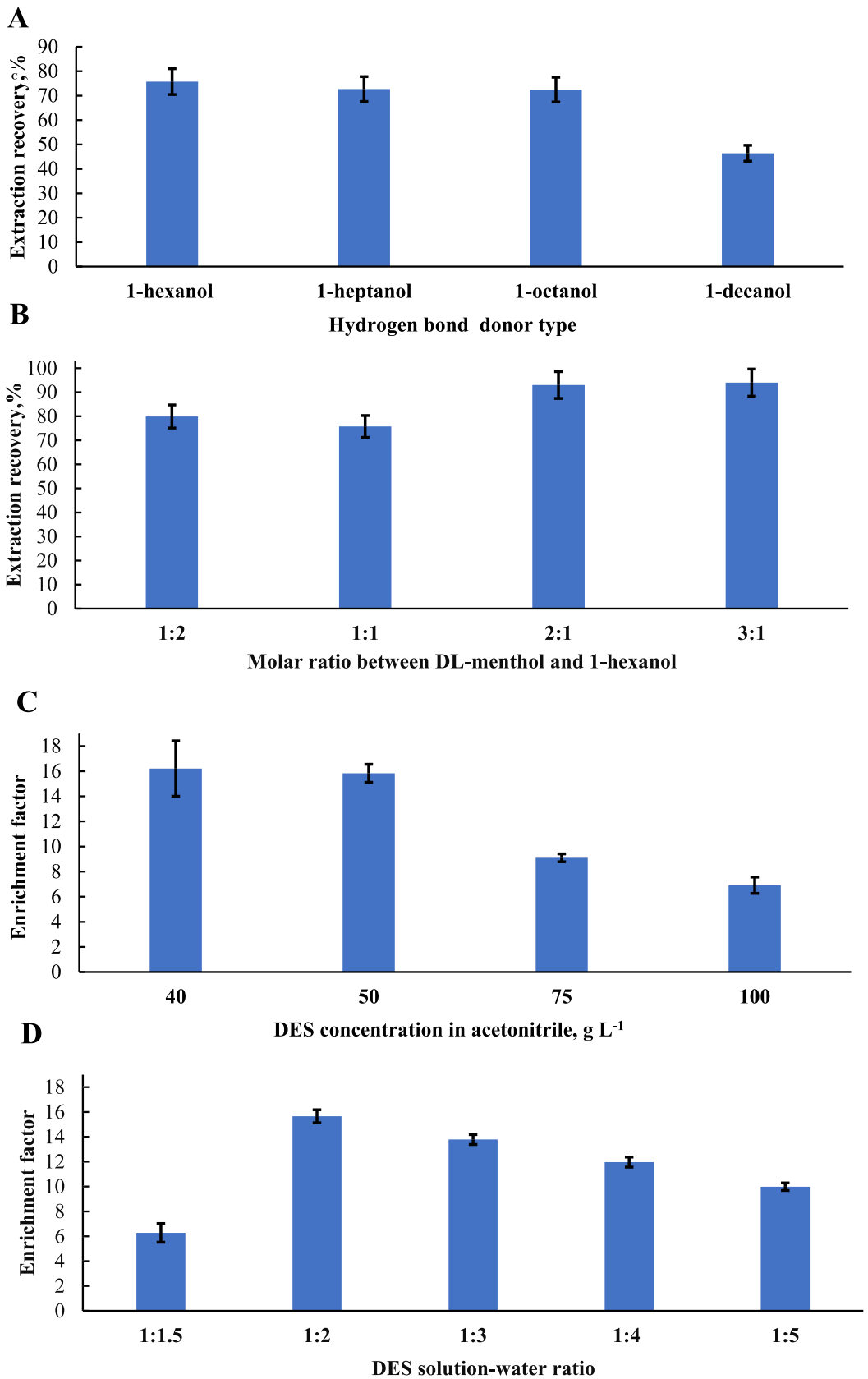

Fig. 2. Investigation of appropriate experimental conditions for DLLME of zearalenone (zearalenone concentration 9 μg/L): **A.** Effect of hydrogen bond donor type on the extraction recovery (molar ratio of DL-menthol to long-chain alcohol 1:1, DES concentration in acetonitrile 100 g/L, volume ratio between DES solution and aqueous phase 1:2); **B.** Effect of molar ratio of DES components on the extraction recovery (hydrogen bond donor 1-hexanol; DES concentration in acetonitrile 100 g/L, volume ratio between DES solution and aqueous phase 1:2); **C.** Effect of DES concentration in acetonitrile on the enrichment factor (molar ratio of DL-menthol to 1-hexanol 2:1, volume ratio between DES solution and aqueous phase 1:2); **D.** Effect of volume ratio between DES solution in acetonitrile and aqueous phase on the enrichment factor (molar ratio of DL-menthol to 1-hexanol 2:1, DES mass in acetonitrile 50 mg).

successfully separated in every experiment, however, a poor chromatographic peak shape and a shift in retention time were observed in case of methanol, while other solvents did not affect the HPLC-FLD determination of zearalenone. Acetonitrile was chosen as the dispersive solvent because it provided the lower volume of the separated organic phase and, as a result, higher EF (Fig. S2). It can be explained by the higher solubility of isopropanol in extraction phase resulting in the higher extract phase volume.

3.2.2. Effect of DES concentration in dispersive solvent

The concentration of DES in acetonitrile affects the extract volume and, consequently, EF. This parameter was varied from 25 to 100 g/L (Fig. 2 C). On the one hand, at concentrations between 25 and 50 g/L too low volumes of DES phase were separated, which was not enough for analysis or led to poor reproducibility of results. On the other hand, high concentrations of DES cause decrease in peak area values due to extract dilution. The highest EF was achieved at DES concentration of 50 g/L, which was considered as optimal.

3.2.3. Effect of phases ratio

Another parameter, that needs to be addressed to reach higher EF, is volume ratio between DES solution in acetonitrile (extract from the solid sample) and aqueous phase. The compromise should be found between minimal dilution of the extract and possibility to implement DLLME. The presence of high amount of acetonitrile in aqueous phase can lead to more pronounced dissolution of DL-menthol and 1-hexanol. Moreover, the dispersive solvent can decrease distribution coefficient of analyte. The volume ratio between DES solution and aqueous phase was studied in the range from 1:1.5 to 1:5. DES mass in acetonitrile was kept constant (50 mg) equivalent to 1 mL of 50 g/L DES solution as found to be optimal previously. As shown in Fig. 2 D, the EF increased until optimal volume ratio of 1:2 was reached. At the ratio of 1:1.5 a high acetonitrile content negatively affected analyte distribution between phases resulting in less efficient extraction. The decrease of EF at higher aqueous phase content was associated with dilution effect. Thus, 1.0 mL of DES solution in acetonitrile (extract from the solid sample) and 2.0 mL of aqueous phase (phase ratio 1:2, v/v) were chosen for further experiments.

3.2.4. Effect of pH

Food samples can contain natural organic acids, which can change pH of aqueous phase during DLLME and behavior of analyte in microextraction process. Zearalenone (Fig. S1) is a macrolide comprising a fourteen-membered lactone fused to 1,3-dihydroxybenzene. As a weak acid (pKa = 7.62 [24]), it can exist in molecular or anionic form depending on pH. In alkaline medium anionic form is predominant due to deprotonation of hydroxy groups, but in neutral or acidic medium it exists in molecular form. The effect of pH on the DLLME of zearalenone was studied. The pH of the aqueous phase was varied from 2 to 8. It was found that pH is not affected extraction performance in the studied range (Fig. S3), because analyte remained mainly in molecular form.

3.3. Stability of DES

Menthol-based hydrophobic DESs are stable in contact with the aqueous phase at different phase ratio [12,25]. However, acetonitrile enhances solubility of hydrophobic substances in aqueous phase and DES. In this case, DES components ratio can be changed during the DLLME due to their different solubility in acetonitrile–water mixture. To investigate behavior of DES in acetonitrile–water (1:2, v/v) mixture, extract and aqueous phase after the DLLME were analyzed by GC-FID and the Karl-Fisher method. It was found that the formation of terpenoid-rich phase took place during the DLLME. According to obtained results (Fig. 3) the extract phase contained 59 ± 6 % (m/m) of menthol, 26.1 ± 1.9 % (m/m) of acetonitrile, 7.90 ± 0.08 % (m/m) of water and 5.9 ± 0.5 % (m/m) of 1-hexanol. Meanwhile, aqueous phase contained 78 ± 4 % (m/m) of water, 21.4 ± 1.0 % (m/m) of acetonitrile, 0.56 ± 0.03 % (m/m) of menthol and 0.31 ± 0.01 % (m/m) of 1-hexanol. Thus, DES based on terpenoid and long-chain alcohol cannot be considered as the extraction solvent in the DLLME.

3.4. Role of DES for zearalenone separation

In this research, the separation of zearalenone from solid bread samples (100 μg/kg, 0.5 g) was performed in two solvents: pure acetonitrile (1) or DES solution in acetonitrile (50 g/L) (2). After zearalenone separation into acetonitrile the DES (50 mg) was added into the extract (1.0 mL). All extracts were injected in 2.0 mL of ultra-pure water to perform the DLLME for analyte preconcentration. It was found that pure acetonitrile provided lower extraction recovery (ER 66 %) than DES solution in acetonitrile (ER 93 %) in the same conditions. DES components can be engaged in hydrophobic and van der Waals interactions with the analyte, may form hydrogen bonds between carboxyl- and hydroxyl groups in their structure with ester, ketone and hydroxyl groups of zearalenone. Thus, intermolecular interactions between DES components and the analyte are favourable for its separation from solid sample, additionally speed up the process and improve its efficiency.

3.5. Investigation of appropriate solid–liquid extraction condition

The sample mass is a significant parameter to achieve the low LOD. The sample mass was investigated in the range from 0.1 to 0.5 g. The higher sample mass cannot be used, because 1.5 mL of DES solution in acetonitrile was not enough to wet the sample. It was found that the peak area values gradually increased with growing sample mass (Fig. 4 A), so the maximum value (0.5 g) was chosen as optimal.

The extraction kinetics is strongly affected by temperature of the mixture and viscosity of the solvent, especially, when analyte should be separated from the solid sample. The temperature of the ultrasonic bath was varied from 25 to 75 °C to find favorable conditions for zearalenone extraction. The higher temperature promoted fast acetonitrile evaporation. The mass transfer of analyte from the sample to DES solution in acetonitrile was speeded up at higher temperatures (Fig. 4 B) due to more effective penetration of the solvent into the sample particles. The highest temperature (75 °C) was selected for future experiments.

The extraction time should be chosen focused on the relative recovery value and analysis throughput. It was found that the

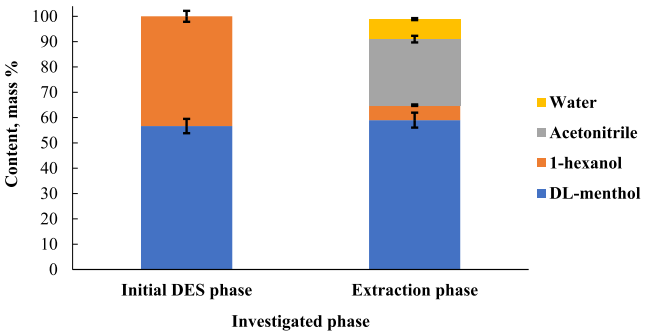

Fig. 3. Composition of initial DES (DL-menthol and 1-hexanol) and extraction phases after DLLME (molar ratio of DL-menthol to 1-hexanol 2:1, DES concentration in acetonitrile 50 g/L, volume ratio between DES solution and aqueous phase 1:2).

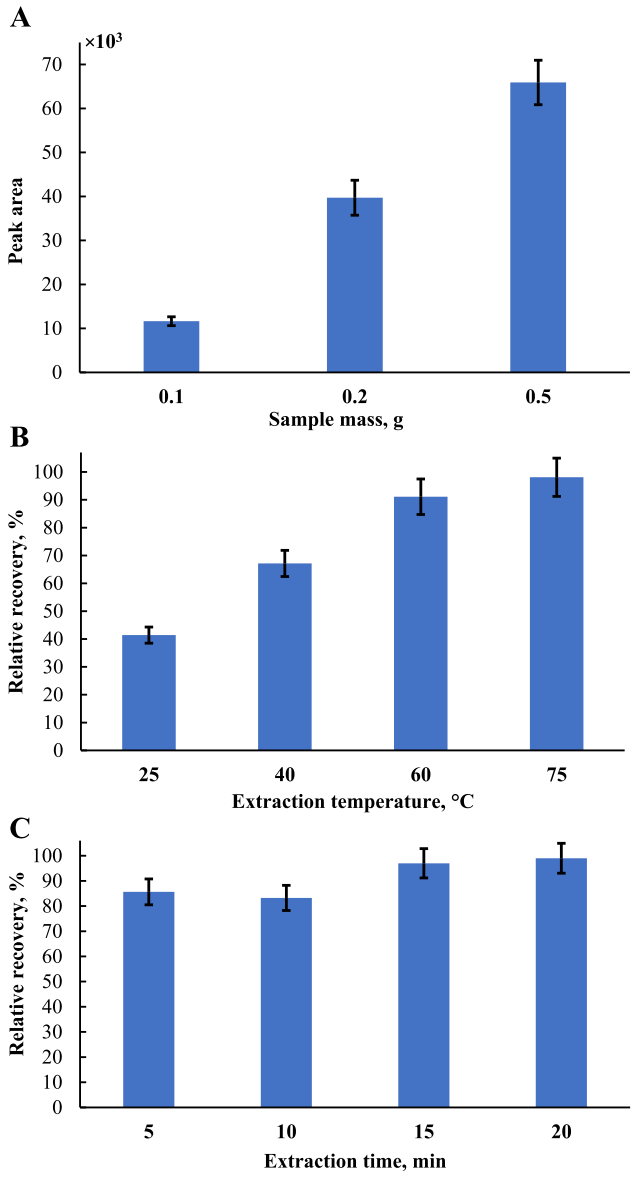

Fig. 4. Investigation of appropriate experimental conditions for ultrasound-assisted solid–liquid extraction of zearalenone from bread sample (zearalenone content 100 μg/kg): **A.** Effect of sample mass on the peak area (extraction temperature 25 °C, extraction time 15 min); **B**. Effect of extraction temperature on the relative recovery (sample mass 0.5 g, extraction time 15 min); **C.** Effect of extraction time on the relative recovery (sample mass 0.5 g, extraction temperature 75 °C).

extraction time of 15 min provided satisfactory relative recovery (Fig. 4 C).

3.6. Validation

The validation of the procedure included evaluation of linearity, LOD, limit of quantification (LOQ), precision, selectivity and extraction performance (Table 1). All experiments were performed under optimized conditions using working solutions of zearalenone in methanol prepared from certified reference material (CRM 7944–2001) or spiked bread sample. According to [26] samples for validation of the procedure can be prepared by spiking typical materials with certified reference materials of suitable purity and stability or its solutions.

The calibration plot (concentration *versus* peak area) was constructed from six data points using the working zearalenone solutions. It was found to be linear by the least-squares method for analyte content from 5 to 500 μg/kg. Correlation coefficient, which measures how strong a linear relationship is between two variables, was equal to 0.999, that indicated excellent linearity.

LOQ and LOD were evaluated from the chromatographic signal-to-noise ratio and corresponded to the concentrations obtained from the calibration plot for peak area values equal to 3 and 10 standard deviations for blank samples, respectively. The LOD of 2 μg/kg was achieved, while the LOQ was estimated to be 5 μg/kg.

The precision of the procedure was determined at intra-day and inter-day levels as relative standard deviation (RSD) of analyte concentration. In first case five replicates of the fortified samples were analyzed during the day, while in the second one the same samples were assessed on different days. The RSD values for intra-day and inter-day precision were varied from 5 to 10 % and from 6 to 12 %, respectively.

The selectivity was investigated by studying the ability of the proposed technique to measure the analyte of interest in samples containing usually presented interference components. Blank bread samples were subjected to ultrasound-assisted extraction and dispersive liquid–liquid microextraction steps followed by HPLC-FLD analysis. No peaks were observed at retention time of the analyte, which indicated sufficient selectivity of the technique.

ER and EF are the main parameters of extraction performance. ER of (93 ± 4) % was achieved along with EF equal to 15.8 ± 0.7.

3.7. Application to analysis of real samples

The maximum residue limits (MRLs) for zearalenone in wheat, corn and derived products, established in European Union, fall into the range from 50 to 100 μg/kg [27], while in Russian Federation regulations are less stringent and permit from 50 to 1000 μg/kg of the substance depending on kind of cereal or related product [28]. The developed procedure was applied to the HPLC-FLD zearalenone determination in bread, wheat, and cornflakes samples at three different levels (50, 100 and 200 % of MRL, established in European Union). The typical chromatograms obtained for the sample (bread) after only solid–liquid extraction and after the whole extraction process are presented in Fig. S4. Spiked bread, wheat, and cornflakes samples were made by addition of zearalenone solution in methanol to blank samples and evaporation of the solvent.

Sample preparation and analysis according to the reference procedure were adopted from [29]: at first, 0.63 of ammonium sulfate, 1.26 mL of ultra-pure water and 1.25 mL of acetonitrile was added to 0.5 g of homogenized wheat, corn flakes or bread sample. After mixing during 1 min the suspension was placed in the ultrasonic bath for extraction of zearalenone during 10 min at 40 °C. Then, tube with a mixture was centrifuged at 5000*g* during 5 min, and 0.5 mL of the top phase, 300 μL of 1-dodecanol and 2 mL of ultra-pure water were added into a clean centrifuge tube. After vigorous mixing of the mixture during 1 min for DLLME the tube was centrifuged at 5000*g* during 5 min and placed in a refrigerator for solidification of the top phase at 4 °C during 10 min. Afterwards,

Table 1
Validated features of the proposed procedure.

Parameter	Value
Linear range, μg	5–500
Correlation coefficient	0.999
Limit of detection, μg	2
Limit of quantification, μg	5
Enrichment factor	15.8 ± 0.7
Extraction recovery, %	93 ± 4
Relative standard deviation (n = 5), %:	
Intra-day (C = 25 or 250 μg)	5/10
Inter-day (C = 25 or 250 μg)	6/12

Table 2
Determination of zearalenone in bread, corn flakes, and wheat (n = 3, P = 0.95, $t_{cr.}$ = 2.78, $F_{cr.}$ = 19.00).

Sample	Added value, μg/kg	Found value, μg/kg		*t*-test	F-test	Relative recovery, %
		Proposed procedure	Reference procedure [28]			
Bread	25	22 ± 4	20 ± 4	1.53	1.00	88
	50	51 ± 11	45 ± 4	2.22	7.56	102
	100	108 ± 11	100 ± 13	2.03	1.40	108
Corn flakes	25	28 ± 4	26 ± 5	1.35	1.56	112
	50	53 ± 11	46 ± 7	2.32	2.47	106
	100	100 ± 9	99 ± 4	0.44	5.06	100
Wheat	50	33 ± 7	39 ± 7	2.62	1.00	66
	100	97 ± 11	100 ± 15	0.70	1.86	97
	200	167 ± 12	175 ± 15	1.80	1.56	84

Table 3
Comparison of the presented procedure with the existing ones for the chromatographic determination of zearalenone in cereals.

Analytical method	Sample	Sample pretreatment	Time of sample treatment	Sample amount, g	Linear range, μg/kg	LOD, μg/kg	ref.
HPLC-MS/MS	Cookies	Extraction in a mixture of acetonitrile and water (86:14, v/v) with an addition of sodium chloride, defatting with *n*-hexane	67	10	6 – 80	0.3	[34]
HPLC-UV	Rice, corn flakes and soybean	Extraction in methanol, dilution with acetate buffer solution, purification on solid-phase extraction C18 cartridge	>20	10	–	–	[31]
HPLC-FLD	Wheat	Extraction into a mixture of phosphate buffer solution and methanol (20:80, v/v), purification on immunoaffinity column, evaporation	>45	5	12.5 – 250	7	[32]
HPLC-FLD	Corn, wheat, rice, barley, rye	Pressurized liquid extraction into a mixture of acetonitrile and methanol, filtration, evaporation	13	5	–	6	[33]
HPTLC-FLD	Wheat, malt	Extraction in a mixture of acetonitrile and water (90:10, v/v) with an addition of sodium chloride, filtration, dilution with water, purification on immunoaffinity column, evaporation	–	20	10 – 65	3	[37]
HPLC-FLD	Bread, wheat and corn flakes	Ultrasound-assisted solid–liquid extraction followed by dispersive liquid–liquid microextraction with the use of DES	19	0.5	5 – 500	2	This work

HPLC-MS/MS – high-performance liquid chromatography with tandem mass-spectrometry detection, HPLC-UV – high-performance liquid chromatography with ultraviolet detection, HPLC-FLD – high-performance liquid chromatography with fluorescence detection, HPTLC-FLD – high-performance thin layer chromatography with fluorescence detection.

the solidified top phase was transferred into another tube, kept at room temperature until thawed, diluted twice with methanol and analyzed by HPLC-FLD according to *Section 2.2*.

The results, obtained by the developed and reference techniques, were compared using *t*-test and *F*-tests to evaluate the accuracy of the developed approach (Table 2). *t*-test was applied to estimate how close obtained mean values are to each other. *F*-test was employed to compare RSDs. *t*-values $\leq$ 2.78 indicated an insignificant difference between the results obtained using these procedures. *F*-values $\leq$ 19.00 indicated an insignificant difference in precision between both procedures at the 95 % confidence level.

The relative recoveries were varied from 88 to 108 %, from 100 to 112 % and from 66 to 97 % for bread, cornflakes, and wheat samples, respectively. In accordance with [30] acceptable relative recovery values for 100 μg/kg and higher contents should be in the range from 80 to 110 %, while in case of smaller concentrations relative recovery from 60 to 115 % is permissible. Therefore, the developed procedure proved its applicability for the analysis of bread, wheat and cornflakes at MRL levels and below.

3.8. Comparison of techniques for zearalenone determination

The most frequently used techniques for the sensitive determination of zearalenone based on high-performance liquid chromatography with ultraviolet [31], fluorescence [32,33] or mass-spectrometric [34] detection are presented in Table 3. Considering the complexity of foods matrices, the developed analytical techniques include sample preparation procedures based on analyte separation into polar (acetonitrile [34], methanol [31], ethyl acetate [35]) or non-polar (chloroform [36]) organic solvents and aqueous-organic mixtures (methanol with phosphate buffer solution [32]). Developed procedures include time-consuming and laborious procedures of extract evaporation for preconcentration [32,33] or extract purification using defatting [34], immunoaffinity columns [32,37], and solid-phase extraction cartridges [31]. In the proposed procedure preconcentration of the analyte is achieved faster and easily without mentioned evaporation and sorption/elution stages.

4. Conclusion

In this study, the experimental investigation of behavior and stability of hydrophobic DES based on terpenoid and long-chain alcohol in DLLME process was carried out for the first time. It was shown that the extraction phase after the DLLME was primarily comprised of terpenoid. In the presence of the dispersive solvent formation of the DES in aqueous phase was not observed. From this point of view DES based on terpenoid and long-chain alcohol cannot be considered as extraction solvent in the DLLME, and numerous articles contain wrong conception about the DLLME using these DESs.

However, it was revealed in this work, that the presence of DES components in the dispersive solvent played a key role for effective analyte separation from solid food sample. Possible intermolecular interactions between DES components and the analyte are favour-

able for its separation from solid sample, additionally speed up the process and improve its efficiency.

Studied extraction system was successfully applied to zearalenone separation from cereal samples followed by its sensitive determination. The obtained results are useful for future developments in this area and aimed at drawing the attention of the researchers to the stability issues with hydrophobic DESs in polar solvent–water mixtures.

CRediT authorship contribution statement

Aleksei Pochivalov: Conceptualization, Methodology, Writing – original draft, Investigation. **Kseniia Pavlova:** Formal analysis, Visualization, Investigation, Data curation, Writing – original draft. **Sergey Garmonov:** Conceptualization, Supervision. **Andrey Bulatov:** Conceptualization, Supervision, Writing – review & editing.

Data availability

The authors are unable or have chosen not to specify which data has been used.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2022.120231.

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