



Experimental design of magnetic ionic liquid ultrasound-assisted dispersive liquid-liquid microextraction for the determination of 5-HMF in honey samples

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ARTICLE INFO

Keywords:

Magnetic ionic liquid
Multivariate optimization
5-HMF
Dispersive liquid-liquid microextraction
Honey
Food analysis
Food composition

ABSTRACT

A green, sensitive, and accurate ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) procedure using a magnetic ionic liquid (magnetic-IL) was optimized for the preconcentration and extraction of 5-hydroxymethylfurfural (5-HMF) in honey samples collected from different regions of Turkey prior to analysis by UV–VIS spectrophotometer. A multivariate optimization strategy was employed to optimize microextraction factors (pH, $[C_{8mim}]FeCl_4$ magnetic-IL amount, ultrasound time, dispersing agent volume) affecting the magnetic-IL UA-DLLME. Since the separation of the phases was achieved with a neodymium magnet, centrifugation step was not necessary. Under optimal experimental conditions, linear range of 3–600 $\mu g L^{-1}$, limit detection of 1.0 $\mu g L^{-1}$ and an enrichment factor of 175 were obtained. The magnetic-IL UA-DLLME procedure was successfully applied for the analysis of 5-HMF in the honey samples, and the extraction recovery were in the range of 92–103%. To evaluate the precision of the magnetic-IL UA-DLLME procedure, relative standard deviations from the inter-day assay and intra-day assay ranged from 3.9% to 5.1% and 3.3–4.2%, respectively. This study reports the first application of magnetic-IL with the UA-DLLME technique based on multivariate optimization for the determination of 5-HMF in different honey samples.

1. Introduction

5-hydroxymethylfurfural (5-HMF), which is seen as a quality criterion, is formed by chemical reactions that occur especially due to the storage of honey products at unsuitable temperatures and the heat treatment applied during their production (Shapla et al., 2018). High 5-HMF concentrations in honey indicate that the honey has been overheated, has poor storage conditions and is old honey (Khalil et al., 2010). 5-HMF emerges as an intermediate product during the decomposition of hexose in an acidic environment or during the Maillard reaction (Lee et al., 2019; Nemati et al., 2021). It has been reported that high concentrations of 5-HMF, in addition to its toxic effect, are irritating to the upper respiratory tract, eyes, skin and mucous membranes (Lee et al., 2019). Therefore, World Health Organization (WHO) and the European Union (Council Directive 2001/110, 2001) reported the maximum concentration of 5-HMF in honey as 40 $mg kg^{-1}$. Therefore, it is important to develop fast, green and selective analytical methods to determine the amounts of 5-HMF in honey samples.

Various analytical techniques such as capillary electrophoresis-ultraviolet (CE-UV) (Wu et al., 2018), ultra-performance liquid

chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) (Feng et al., 2019), ultra-high performance liquid chromatography coupled to photodiode array (UHPLC-PDA) (Pernica et al., 2019), liquid chromatography coupled with high-resolution mass spectrometry (SPE-LC-MS) (Imperiale et al., 2022) and gas chromatography-mass spectrometry (GC-MS) (Khani et al., 2021) have been utilized for the determination of 5-HMF in real samples. Although these techniques are sensitive and selective, they have significant disadvantages such as complex technology, expensive equipment, expert user requirement, and time-consuming determination step (Veríssimo et al., 2017). Although the UV–VIS spectrophotometer has low selectivity and low sensitivity compared to these techniques, its simplicity, fast measurement capacity, and easy access are its important advantages. At trace analysis, the important problem that arises in all techniques is the matrix effect and low detection capacity (Doğan et al., 2020).

To overcome these problems, an effective separation and preconcentration procedure should be applied prior to the determination of 5-HMF in honey samples. These procedures including ultrasonic-assisted cloud point extraction (Gürkan, & Altunay, 2015), headspace-solid-phase microextraction (Veríssimo et al., 2017),

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<https://doi.org/10.1016/j.jfca.2022.104817>

Received 11 March 2022; Received in revised form 18 July 2022; Accepted 8 August 2022

Available online 10 August 2022

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salting-out-assisted liquid–liquid extraction (Chen et al., 2019) and cold-induced-homogeneous liquid–liquid extraction method combined with deep eutectic solvent-based dispersive liquid–liquid microextraction (Khani et al., 2021) are one of the most effective ways to provide precise and accurate chemical analysis. In this step, samples are subjected to an appropriate procedure that ensures compatibility with the analytical technique, ensures appropriate concentrations of analytes, and removes interfering compounds.

Efficiency of the separation and preconcentration procedures depends on the extraction solvent to be used. Until now, different extraction solvents such as organic solvents (Kocúrová et al., 2012), surfactants (Saraji, & Bidgoli, 2010), ionic liquids (Rykowska et al., 2018), and deep eutectic solvents (Li, & Row, 2019) have been used in dispersive liquid-liquid microextraction (DLLME) studies. In recent years, the use of magnetic ionic liquids (magnetic-ILs) has become popular in DLLME studies (Clark et al., 2016). Since centrifugation and heating steps are not required in DLLME studies using magnetic-ILs, they are simpler, more environmentally friendly, and especially do not require much energy compared to other extraction solvents (Yu, et al., 2016). The magnetic-ILs-based extraction solvents not only have unique properties (their negligible vapor pressure at room temperature, high thermal stability, and variable viscosity) unique to conventional ionic liquid, but also respond to an external magnetic field (Yu et al., 2016). In addition, magnetic-ILs are generally not miscible with hydrophobic solvents, so they are promising as extraction solvents of DLLME for lipophilic matrices.

Multivariate optimization approaches have been used frequently in microextraction studies (Altunay, 2021). These approaches including full factorial design, central composite, Box-Behnken and Doehlert matrix have significant advantages as they are economical, require few experiments, and allow simultaneous optimization of more than one variable strategy (Altunay et al., 2021).

The objective of this study was to develop a magnetic ionic ultrasound-assisted dispersive liquid-liquid microextraction (magnetic-IL UA-DLLME) combined with UV–VIS spectrophotometer for the extraction and quantitation of 5-HMF in honey samples collected from different regions of Turkey. The main parameters were optimized by Box–Behnken design (BBD). Mathematical model and response surfaces based on the BBD demonstrated the detailed effect of variables on recovery of 5-HMF. To the best of our knowledge, this study reports the first application of magnetic-IL with the UA-DLLME technique based on multivariate optimization for the determination of 5-HMF in different honey samples.

2. Materials and methods

2.1. Reagents and solutions

All chemicals and reagents were of analytical reagent and supplied by Merck (Darmstadt, Germany) and Sigma-Aldrich (Milwaukee, WI, USA). A 100 mg L⁻¹ stock solution of 5-HMF was prepared by dissolving appropriate amount of 5-HMF (sigma) in 1000 mL of the water and stored at 4 °C. The working/calibration solutions were prepared by proper dilution of the stock solution with the water. In the optimization step, acetate, borate, phosphate and citrate buffer solutions were prepared to adjust the pH of the solutions. 1-octyl-3-methylimidazolium chloride ([C₈mim]Cl) and CYPHOS® IL 101 (trihexyl(tetradecyl)phosphonium chloride) were purchased from Merck. FeCl₃·0.6 H₂O and FeCl₂·0.4 H₂O were purchased from Sigma, and were used for the preparation of magnetic-ILs. Analytical grade chloroform, ethanol and methanol were used in experimental studies.

2.2. Sample preparation

Pine honey-1, flower honey-1, and chestnut honey-1 were collected from Artvin/Turkey in August 2020. Linden honey, pine honey-2 and

flower honey-2 were collected from Rize/Turkey in September 2020. Flower honey-3 were collected from Sivas/Turkey in July 2020. Chestnut honey-2 was collected from Bursa/Turkey in June 2020. Oak honey was collected from Kırklareli/Turkey in September 2019. While 200 g were taken from twenty (20) samples of Pine honey-1 and Chestnut honey-1, 100 g were taken from ten (10) samples of Flower honey-1, Linden honey-2, Pine honey-2, Flower honey-2, Flower honey-3 and Chestnut honey-2. Also, 50 g were taken from five (5) samples of Oak honey. All samples were collected in topaz glass bottles and stored at 4 °C in the dark until sample preparation. Honey samples were collected from different regions of Turkey. Honey samples collected from Rize and Artvin represent the Eastern Black Sea region, honey samples collected from Sivas represent the Central Anatolia region, honey samples collected from Kırklareli represent the Tarakya region, and honey samples collected from Bursa represent the Southern Marmara region.

The collected honey samples were prepared according to the following procedure (Gürkan & Altunay, 2015). First, 2 g of the honey samples were carefully weighed and transferred into a 100 mL beaker. Then, 2.0 mL of 2.0 mol L⁻¹ HClO₄, potassium hexacyanoferrate (2 mL of 0.25 mol L⁻¹) and zinc acetate (2 mL of 0.25 mol L⁻¹) were added to the beaker, respectively. Then, the final volume was made up to 100 mL with water. Afterwards, the obtained mixture was placed on the heating plate with magnetic stirrer and heated at 45 °C for 15 min. The mixture cooled to room temperature was centrifuged at 1792 rcf for 10 min and the supernatant was finally filtered using a membrane filter and made ready for the application of the magnetic-IL UA-DLLME procedure.

2.3. Instrumentation

An UV-1800 Shimadzu dual-beam spectrophotometer (Tokyo, Japan) with a 300 µL quartz microcell was used for the spectrophotometric analysis of 5-HMF. A Hettich Universal-320 model centrifuge (London, England) was used in the sample preparation step. The pH of all solutions was adjusted by a digital pH meter (Selecta 2001 model, Sartorius, North America) supplied with a combined electrode. A rotary evaporator BUCHI R-200 (BÜCHI Labortechnik AG, Flawil, Switzerland) was used for the preparation of the magnetic ILs. A SK5210LHC Kudos Model ultrasonic bath (Shanghai, China) was used to acquire micro-sized extraction drops of magnetic IL. Ultra-pure water was obtained by a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). A neodymium magnet (1.17 T magnetic field) was utilized to separate the magnetic IL from the sample solution. Design-Expert® software, version 12.0.1. (Stat-Ease Inc., Minneapolis, USA), was applied for ANOVA analysis, experimental design, and multivariate optimization.

2.4. Preparation of magnetic-IL

In this study, two different magnetic ILs were prepared in the light of previously reported studies. The [P_{6,6,6,14}] FeCl₄ magnetic-IL was prepared by the following experimental steps (Del Sesto et al., 2008). First, an equimolar concentration of FeCl₂·0.4 H₂O and 50% (w/v) CYPHOS IL 101 solution dissolved in chloroform was carefully added to a 100 mL beaker. In the second step, the resulting mixture was kept at room temperature under constant temperature for 24 h to complete the reaction. In the final step, a rotary evaporator was used to evaporate excess chloroform and remove from the solution. The obtained magnetic IL ([P_{6,6,6,14}]FeCl₄) was dried in an oven at 50 °C.

Other magnetic IL was prepared according to the following procedure (Wang et al., 2012). First, equimolar concentrations of [C₈mim]Cl and FeCl₃·0.6 H₂O were added in a 100 mL beaker including methanol. The resulting mixture was left to stand under a nitrogen atmosphere for about 4 h. Afterwards, the excess methanol was removed by evaporation. The obtained product was washed with water twice to remove the excess FeCl₃ in the prepared magnetic IL. Finally, the obtained magnetic IL ([C₈mim]FeCl₄) was dried in an oven at 90 °C for 48 h.

Table 1
ANOVA analysis for the magnetic-IL UA-DLLME procedure of 5-HMF.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3968.66	14	283.48	187.14	< 0.0001	significant
X ₁	589.40	1	589.40	389.10	< 0.0001	
X ₂	266.02	1	266.02	175.62	< 0.0001	
X ₃	338.14	1	338.14	223.23	< 0.0001	
X ₄	168.00	1	168.00	110.91	< 0.0001	
X ₁ X ₂	34.81	1	34.81	22.98	0.0003	
X ₁ X ₃	0.1600	1	0.1600	0.1056	0.7500	
X ₁ X ₄	12.60	1	12.60	8.32	0.0120	
X ₂ X ₃	420.25	1	420.25	277.43	< 0.0001	
X ₂ X ₄	1139.06	1	1139.06	751.96	< 0.0001	
X ₃ X ₄	5.06	1	5.06	3.34	0.0889	
X ₁ ²	112.88	1	112.88	74.52	< 0.0001	
X ₂ ²	787.96	1	787.96	520.18	< 0.0001	
X ₃ ²	4.72	1	4.72	3.12	0.0992	
X ₄ ²	22.89	1	22.89	15.11	0.0016	
Residual	21.21	14	1.51			
Lack of Fit	14.93	10	1.49	0.9525	0.5706	not significant
Pure Error	6.27	4	1.57			
Cor Total	3989.87	28				
C.V.%	1.71	Adjusted R ²	0.9894	R ²	0.9947	

2.5. Optimization strategy

Application of multivariate optimization approach allows the analyst to select the best experimental conditions. Therefore, Box-Behnken design (BBD) was applied to determine optimum values of four factors such as pH (X₁), [C_{8mim}]FeCl₄ volume (X₂), methanol volume (X₃) and sonication time (X₄). The operating range was chosen as X₁ (3–8), X₂ (150–600 µL), X₃ (100–300 µL) and X₄ (3–15 min), respectively. The coded, unit and levels of these parameters in the BBD were given in Table S1. A total of 29 experiments were conducted, 5 of which were central point experiments. Each experimental run was performed in triplicate. The experimental results were analyzed using second-order regression. The necessary data for a quadratic polynomial are presented in the equation below (Bezerra et al., 2008).

$$y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i < j \leq k} b_{ij} x_i x_j + \varepsilon \quad (1)$$

Where y is response, x_i was parameters, k was parameter number, b₀ was constant, b_i, b_{ij} and b_{ii} were regression parameters for the effects of linear, interaction and quadratic coefficients, respectively, and ε was residue. In addition, the terms X_iX_j and X_i² represent the interaction and quadratic terms, respectively (Mäkelä, 2017).

2.6. Magnetic-IL UA-DLLME procedure

The experimental steps of the magnetic-IL UA-DLLME procedure were summarized below. First, an aliquot of 10 mL sample solution was added in a conical centrifuge tube. Second, the pH of the mixture was adjusted to 6.6 using citrate buffer solution. Three, 540 µL of [C_{8mim}] [FeCl₄] and 280 µL methanol (as dispersing solvent) were spiked to the mixture obtained. Four, the tube was placed in an ultrasonic bath and sonicated for 13 min at room temperature. Five, a neodymium magnet was held at the bottom of the tube to attract the analyte-enriched magnetic-IL nanoparticles. Then, the supernatant aqueous solution was drained by decantation. Six, the remaining phase was poured into micro-cuvettes and the final volume was made up to 300 µL with acetonitrile. The absorbance measurements were made at 284 nm using a UV-vis spectrophotometer. All studies were performed against aqueous standards and blank solutions. The experimental steps were presented in Fig. S1. Obtained UV-spectra were presented in Fig. S2.

2.7. Reference method

Reference white method (AOAC, 1990) is based on measuring the absorbance of a clarified aqueous honey solution against a reference solution of the same honey in which the 5-HMF chromophore is destroyed by the bisulfite salt at a wavelength of 284 nm. The experimental steps of the method are summarized below. First, 5 g of honey sample were transferred to a 50 mL bottle, and were dissolved by adding 25 mL of the water. Then, 0.5 mL of Carrez-I and 0.5 mL of Carrez-II solutions were added to the resulting mixture, and mixed thoroughly, and the final volume of the mixture was completed to 50 mL. The mixture was carefully filtered and the first 10 mL of filtrate was discarded. 5 mL aliquots were transferred to two test tubes. To a tube was added 5 mL of distilled water (sample solution); 5 mL of 0.2% sodium bisulfite solution was added to the second tube (reference solution). Both sample tubes were thoroughly mixed with a vortex mixer. Then, the absorbance values at 284 nm were read by using a spectrophotometer. The 5-HMF in honey was then calculated, using the following equation (AOAC, 1990):

$$5\text{-HMF (mg/100 g of honey)} = [(A_{284} - A_{336}) \times \text{Factor}] / W \quad (2)$$

Where, W = weight of sample,
Factor = (126 × 100 × 1000 × 100)/(16,830 × 1000) = 74.87.
126 = molecular weight of HMF.
16,830 = molar absorptivity of HMF at 284 nm.

3. Results and discussion

3.1. Preliminary selection of magnetic-ILs

In order to provide high recovery and easy phase separation, it is important to select the appropriate magnetic-IL prior to the multivariate optimization strategy. A 25 µg of 5-HMF were added to model solution during optimization studies. In this context, two magnetic-IL prepared including [C_{8mim}]FeCl₄ and [P_{6,6,6,14}] FeCl₄ were investigated for recovery of 5-HMF. As a result of three repetitive studies, the recovery of 5-HMF was 90.2% with a 2.2% of relative standard deviation when the magnetic-[C_{8mim}]FeCl₄ was used, while the recovery of 5-HMF was 62.4% with a 3.5% of relative standard deviation when the magnetic-[P_{6,6,6,14}] FeCl₄ was used. In the light of these results, magnetic-[C_{8mim}] FeCl₄ was chosen as the extraction solvent for the BBD step.

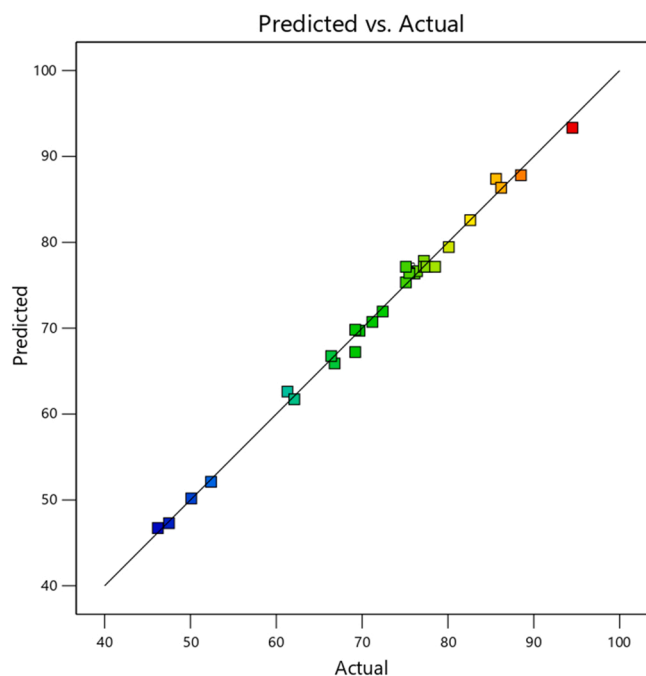


Fig. 1. The predicted response and the actual response.

3.2. Multivariate optimization

3.2.1. ANOVA analysis

The levels of the microextraction parameters and the recovery for 5-HMF were presented in Table S2. Using the results in Table S2, analysis of variance (ANOVA) was performed to investigate the significance of the effects of optimized variables on the magnetic-IL UA-DLLME procedure. In statistical evaluation, the model equation and related terms are considered significant if the p-values are less than 0.05 (95% confidence level). In this context, when the ANOVA data in Table 1 were evaluated, it can be seen that the p-value of all variables in the established model is less than < 0.0001 , which means that all linear variables are significant for the model. Additionally, the binary and quadratic interactions that are meaningless to the model are $X_1 X_3$ (p-value: 0.7500), $X_3 X_4$ (p-value: 0.0889) and X_3^2 (p-value: 0.0992), respectively.

Goodness of fit for the quadratic equation was expressed by the coefficients of determination (R^2 , adjusted- R^2). Here, R^2 (0.9947) denotes the average amount of variance around the established model. Adjusted- R^2 (0.9894) is R^2 for the number of terms in the model, and if these studied factors do not contribute significantly to the model, it decreases as the number of variables in the model increases. The high R^2 values obtained as a result of the studies mean that we can use the model to analyze and optimize the effects of microextraction parameters on the recovery of 5-HMF. Moreover, these obtained data confirm the accuracy and reliability of the established model.

After the optimization step, the effect of the studied parameters on the recovery of 5-HMF can be explained by the following quadratic equation.

$$\text{Recovery(\%)} = +77.16 + 7.01 X_1 - 4.71 X_2 + 5.31 X_3 - 3.74 X_4 + 2.95 X_1 X_2 + 0.20 X_1 X_3 - 177 X_1 X_4 + 10.25 X_2 X_3 + 16.88 X_2 X_4 + 1.12 X_3 X_4 - 4.17 X_1^2 - 11.02 X_2^2 + 0.8533 X_3^2 + 1.88 X_4^2.$$

In addition, the harmony between the recovery values obtained as a result of the experimental study and the recovery values predicted by the model is clearly seen in Fig. 1.

3.2.2. 3D response surfaces

3D response surfaces were plotted to obtain more details of the influence of relevant optimized factors on the recovery of 5-HMF. While

keeping the other factors constant at their central levels, these graphs allow to evaluate the relationship between the response and levels of two factors at the same time. The 3D response surfaces were presented in Fig. 2 represent the relationship between recovery and the four factors including pH, $[C_{8mim}]FeCl_4$ volume, methanol volume and sonication time. Fig. 2a describes the 3D response surface for the effect of pH and $[C_{8mim}]FeCl_4$ volume on recovery of 5-HMF. The recovery of 5-HMF increased with increasing $[C_{8mim}]FeCl_4$ volume from 350 μL to 600 μL and pH from 5.0 to 8.0. However, with a further decrease in $[C_{8mim}]FeCl_4$ volume from 350 to 150 μL and pH from 5.0 to 3.0, the recovery of 5-HMF declined. In particular, the decrease in the recovery of 5-HMF at low pHs can be attributed to a decrease in its activity due to the fact that the magnetic-IL does not protonate through its anionic groups (Fiorentini et al., 2019).

Fig. 2b explains the 3D response surface for the effect of $[C_{8mim}]FeCl_4$ volume and methanol volume on recovery of 5-HMF. The best recovery for 5-HMF was obtained at 260 μL $[C_{8mim}]FeCl_4$ amount and 230 μL of methanol. With further increases in $[C_{8mim}]FeCl_4$ volume (420–600 μL) and methanol volume (250–300 μL), the recovery of 5-HMF decreased sharply. The decrease in recovery with increasing methanol volume may be due to the relatively long alkyl chain of $[C_{8mim}]FeCl_4$, which makes it difficult to collect them from the aqueous matrix by external magnetism (Wang et al., 2018).

Fig. 2c shows the 3D response surface for the influence of $[C_{8mim}]FeCl_4$ volume and sonication time on the recovery of 5-HMF. The recovery of 5-HMF gradually decreased when $[C_{8mim}]FeCl_4$ volume and sonication time were in the range of 420–600 μL and 5–9 min, respectively. Here, the purpose of sonication is to effectively disperse the magnetic ionic liquid into the sample solution. In this way, phase separation is facilitated by increasing the interaction of 5-HMF in the sample solution with the extraction solvent. The results show that low sonication times are sufficient for this event to occur. In addition, the decrease in the recovery of 5-HMF with the increase of sonication time can be attributed to the radical formation of water molecules due to the acoustic cavitation (Altunay, & Tuzen, 2021).

Fig. 2d describes the 3D response surface for the influence of sonication time and methanol volume on recovery of 5-HMF. The recovery of 5-HMF increased with decreasing methanol volume from 250 μL to 100 μL and sonication from 9 to 3. Here, sonication was applied to increase the dispersion of methanol added as a dispersive solvent to the sample solution. In this way, it is seen that low methanol volumes and low sonication times are sufficient for quantitative recovery of 5-HMF. The main reason for achieving quantitative recovery in the low operating range in two variables is that both variables have an indirect dispersive property. Accordingly, optimum analytical results were obtained at low values due to the resulting synergistic effect.

3.2.3. Optimum conditions

Experimental studies were carried out to determine the optimum values of the studied factors. In this context, the required ranges for the quantitative recovery of 5-HMF of the variables studied with the Design-Expert® software were determined. Three alternative experimental conditions were studied in the solutions of the Design-Expert® software. As a result of the study, the closest value to the recovery estimated by the model was obtained at pH:6.6, $[C_{8mim}]FeCl_4$ volume: 540 μL , methanol volume:280 μL , and sonication time: 13 min conditions. The experimental recovery obtained (92.4%) using these optimum values showed a statistically high agreement with the value estimated (93.8%) by the BBD. For these reasons, the above-mentioned conditions were chosen as optimum value for the variables for the determination of analytical parameters, validation studies and analysis of real samples.

3.3. Method performance characteristics

The analytical figures of merit of the magnetic-IL UA-DLLME procedure obtained using the optimized conditions were investigated for

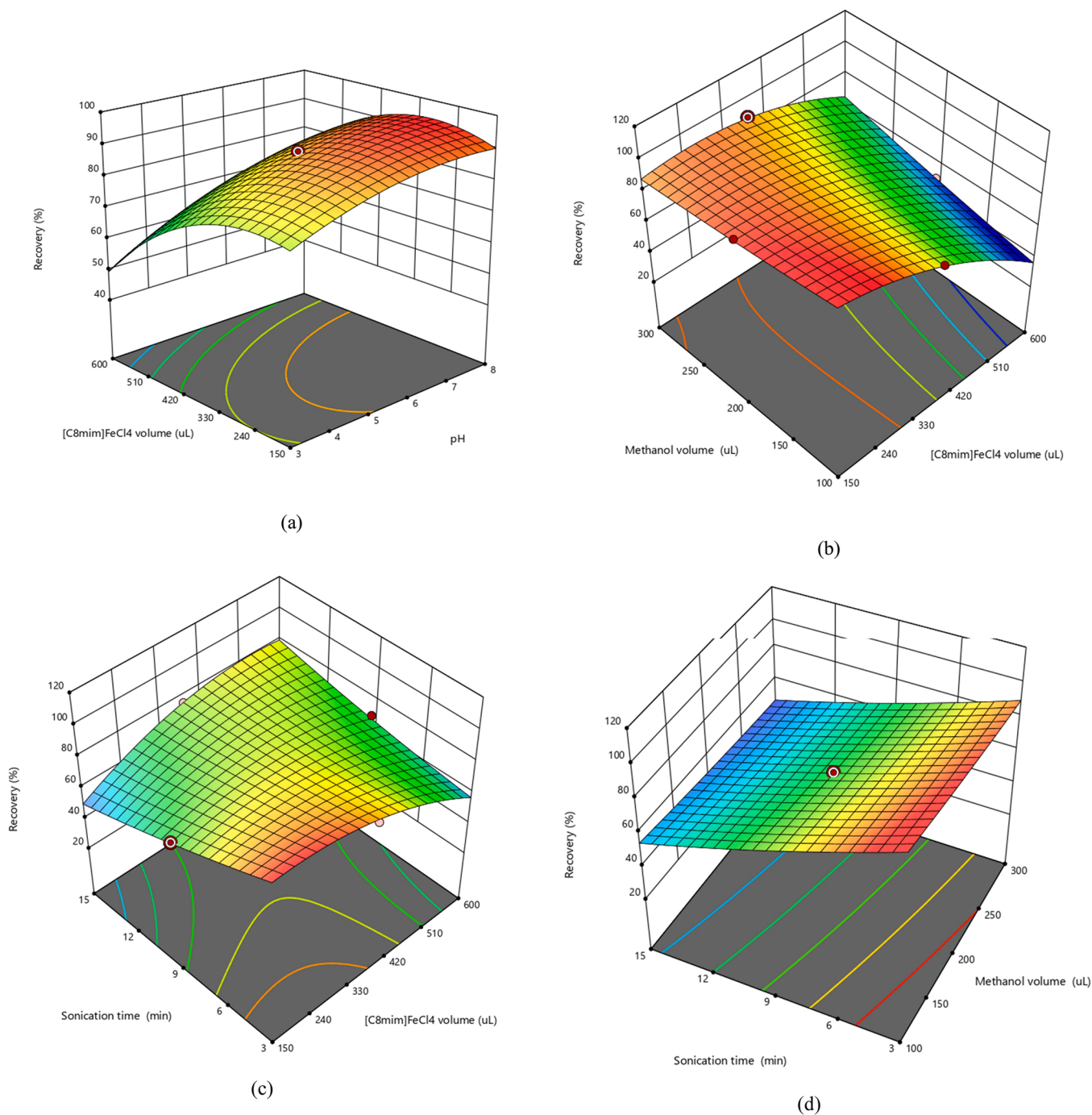


Fig. 2. Response surfaces (in recovery) for the Box-Behnken design of the 5-HMF. (a) $[C_{8mim}][FeCl_4]$ volume-pH; (b) $[C_{8mim}][FeCl_4]$ volume-Methanol; (c) $[C_{8mim}][FeCl_4]$ volume- sonication time; (d) Methanol volume-sonication time.

regression equation, linear range, coefficients of determination (R^2), limit of detection (LOD), limit of quantification (LOQ), relative standard deviation (RSD), enrichment factor (EF) and extraction recovery. The LOD and LOQ, which are determined as $3Sd/m$ and $10Sd/m$, respectively (Sd and m were standard deviation of the blank, and slope of a calibration graph, respectively). The LOD and LOQ were calculated as 1.0 and $3.0 \mu\text{g L}^{-1}$, respectively. Linear range was $3\text{--}600 \mu\text{g L}^{-1}$ with a R^2 of 0.9991 . The RSD and extraction recovery ($75 \mu\text{g L}^{-1}$ of 5-HMF) for three replicated analysis were 1.8% and $96 \pm 2\%$, respectively. The EF (175) was calculated according to the following equation-1.

$$EF = C_{\text{final}}/C_0 \quad (3)$$

Where C_{final} was the amount of 5-HMF in final phase after the magnetic-IL UA-DLLME procedure, and C_0 was the initial amount of 5-HMF in aqueous sample before the magnetic-IL UA-DLLME procedure. The comprehensive analytical data was presented in Table 2.

3.4. Selectivity

Since the multivariate optimization step is performed using model solutions, it is necessary to investigate the selectivity of the magnetic-IL UA-DLLME procedure for the 5-HMF before the analysis of real samples. In this context, the selectivity of the optimized conditions for 5-HMF was investigated by the following study. The chemical species in Table 3

Table 2
Analytical characteristics of the presented analytical method.

Analytical characteristics	After presented analytical method	Before presented analytical method
Regression equation	A = $(75 \pm 1) \times 10^{-3}$ [5-HMF] - $(14 \pm 3) \times 10^{-4}$	A = $(44 \pm 2) \times 10^{-4}$ [5-HMF] + $(12 \pm 1) \times 10^{-4}$
Linear range ($\mu\text{g L}^{-1}$)	3–600	250–1000
R ²	0.9991	0.9984
LOD ($\mu\text{g L}^{-1}$)	1.0	75.8
LOQ ($\mu\text{g L}^{-1}$)	3.0	1000
RSD (N = 3, 75 $\mu\text{g L}^{-1}$ of 5-HMF)	1.8	–
Extraction recovery	96 \pm 2	–
EF	175	–

LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; EF: Enrichment factor

Table 3
Selectivity of optimized conditions for 100 $\mu\text{g L}^{-1}$ of 5-HMF in the presence of interference ions.

Interference ions	Extraction recovery (%)	RSD (%)	*Tolerance limit
K ⁺	97	2.0	2000
Na ⁺	97	2.5	2000
Zn ⁺²	98	2.2	2000
Mg ⁺²	97	2.3	2000
Proline	99	2.4	2000
Cu ⁺²	98	2.9	1500
Sucrose	96	2.6	1500
p ⁺³	96	2.1	1000
Maltose	95	2.4	1000
2-nitrobenzaldehyde	97	2.8	1000
p-Dimethylaminobenzaldehyde	97	2.5	500
Glucose	95	2.7	500
2-chlorobenzaldehyde	94	3.3	500
Furfural	94	3.1	250
4-hydroxy-2,5-dimethyl-3(2 H)-furanone	93	3.2	250

* [Interference ions amount]/ [5-HMF amount]

Table 4
Recovery, intra-day and, inter-day studies.

Analytical studies	Spiked levels		
	Low (10 $\mu\text{g L}^{-1}$)	Middle (200 $\mu\text{g L}^{-1}$)	High (500 $\mu\text{g L}^{-1}$)
Inter-day assay (N = 3)	5.1	4.4	3.9
Intra-day assay (N = 3 \times 3)	4.2	3.8	3.3
Recovery (%)	95 \pm 4	97 \pm 4	98 \pm 2

were added to the model solutions at different concentrations. Then the proposed method was applied to the resulting mixture. Extraction recovery, RSD and tolerable limits were determined for each studied ion. The tolerable limit for the studied ions is in the range of 2000–250. Also, the RSD and extraction recovery values varied in the range of 2.0–3.3% and 99–93%, respectively. The quantitative results obtained showed that the magnetic-IL UA-DLLME procedure has high selectivity for 5-HMF.

3.5. Precision

The precision of the magnetic-IL UA-DLLME procedure was evaluated by adding three different concentrations of standard 5-HMF to honey samples by intraday and interday studies. The added 5-HMF concentrations were selected as low (10 $\mu\text{g L}^{-1}$), middle (200 $\mu\text{g L}^{-1}$) and high (500 $\mu\text{g L}^{-1}$) concentrations for the linear range obtained. The intraday study was investigated by three replicate measurements of the

Table 5
Determination of 5-hydroxymethylfurfural in different honey samples at spiked level (N = 3. P = 0.95; tk = 2.78; F=19).

Samples	Presented analytical method			Reference method	t-value	F-value
	Spiked μg	Found $\mu\text{g kg}^{-1}$	Recovery (%)			
Pine honey-1	–	25.7 \pm 0.3	–	25.3 \pm 0.4	1.40	2.37
	50	73.7 \pm 1.2	96	74.8 \pm 1.4	1.04	3.65
	200	221.7 \pm 2.4	98	218.1 \pm 2.0	1.99	2.18
Flower honey-1	–	67.1 \pm 1.1	–	68.2 \pm 1.3	1.11	3.91
	50	114.6 \pm 1.8	95	117.4 \pm 1.6	2.03	4.24
	200	265.1 \pm 2.3	99	263.7 \pm 2.0	1.14	3.17
Chestnut honey-1	–	8.9 \pm 0.1	–	8.7 \pm 0.2	1.64	2.89
	50	60.4 \pm 0.9	103	61.5 \pm 0.7	1.67	3.07
	200	212.9 \pm 1.8	102	214.6 \pm 1.6	1.52	2.95
Pine honey-2	–	124.3 \pm 2.7	–	127.3 \pm 2.5	1.42	3.76
	50	170.8 \pm 3.1	93	173.4 \pm 3.3	1.20	2.15
	200	316.3 \pm 4.2	96	319.1 \pm 4.4	0.81	1.56
Flower honey-2	–	33.1 \pm 0.8	–	34.2 \pm 0.6	1.93	3.34
	50	85.1 \pm 1.1	104	86.8 \pm 1.3	1.74	3.27
	200	237.1 \pm 2.7	102	234.7 \pm 2.5	1.13	2.95
Flower honey-3	–	51.6 \pm 1.5	–	53.5 \pm 1.7	1.46	3.06
	50	97.6 \pm 2.0	92	99.6 \pm 2.3	1.14	2.53
	200	245.6 \pm 3.4	97	251.4 \pm 3.0	2.23	4.75
Linden honey	–	23.4 \pm 0.4	–	23.1 \pm 0.6	0.75	1.62
	50	71.4 \pm 1.2	96	72.7 \pm 1.4	1.23	2.33
	200	221.4 \pm 3.5	99	218.1 \pm 3.3	1.19	1.96
Chestnut honey-2	–	11.9 \pm 0.2	–	11.6 \pm 0.3	1.48	2.17
	50	62.9 \pm 1.0	102	62.1 \pm 1.2	0.90	1.66
	200	213.9 \pm 2.6	101	211.2 \pm 2.3	1.35	3.14
Oak honey	–	48.3 \pm 0.7	–	49.0 \pm 0.5	1.43	3.47
	50	95.8 \pm 1.3	95	97.4 \pm 1.4	1.46	3.58
	200	242.3 \pm 3.1	97	245.5 \pm 2.9	1.31	2.75

added 5-HMF concentrations in one day, while the interday study was investigated by triplicate measurements of the same concentrations on three consecutive days. The RSDs for intraday and interday studies were in the range of 3.3–4.2% and 3.9–5.1%, respectively. For these studies, recovery was obtained in the range of 95 \pm 4–98 \pm 2%. Analytical data was presented in Table 4.

3.6. Robustness

The following experiments were carried out to evaluate the robustness of the magnetic-IL UA-DLLME procedure. These experimental studies included evaluating changes in recovery produced by small variations of the four factors optimized in the multivariate optimization

Table 6

A comparison between the presented analytical method with some other published studies for determination of 5-HMF.

Analytical method	Linear range ($\mu\text{g L}^{-1}$)	Extraction solvent	LOD ($\mu\text{g L}^{-1}$)	RSD (%)	Extraction time (min)	EF	References
UALLME-CE-UV	30–100	Trichloromethane	30	3.74	8	–	(Wu et al., 2018)
CPE-spectrophotometer	2–200	Triton X-114	0.75	4.2	22	37.5	(Baş and Gürkan, 2021)
DSPE-LC-MS/MS	2.5–12.5	Acetonitrile	1.1	7.4	15	–	(Huang et al., 2019)
UACPE-FAAS	4–240	Triton X-45	1.27	6.5	18	70	(Gürkan, 2021)
SALLE- HPLC-DAD	–	Acetonitrile: H ₂ O	27	2.92	6	–	(Chen et al., 2019)
UPLC-PDA	16.1–21.8		5.2	< 10	4	–	(Pernica et al., 2021)
Magnetic-IL UA-DLLME	3–600	[C _{8mim}][FeCl ₄]	1	1.8	13	175	Presented analytical method

UALLME-CE-UV: ultrasonic assisted liquid–liquid micro-extraction coupled with capillary electrophoresis-ultraviolet; CPE: Cloud point extraction; DSPE-LC-MS/MS: Dispersive Solid-Phase Extraction Sorbent Followed by Liquid Chromatography–Tandem Mass Spectrometry; UACPE-FAAS: Ultrasound-assisted-cloud point extraction-followed by flame atomic absorption spectrometry; SALLE- HPLC-DAD: Salting-out-assisted liquid–liquid extraction coupled with high-performance liquid chromatography with diode-array detection; UPLC-PDA: Ultra-high performance liquid chromatography coupled to photodiode array

strategy. In this context, three experimental studies were carried out in which the level of only one factor was changed at a time for each optimized parameter. The ranges tested for each factor were the following: pH (6.0–7.2), [C_{8mim}][FeCl₄] volume (480–600 mL), dispersing solvent volume (240–320 μL), and sonication time (11–16 min). The mean difference and standard deviation of the method were calculated separately for each parameter from the results obtained in this study. From the studies obtained, it was found that there was no significant difference and the RSDs varied in the range of 1.3–2.4%. These results showed that the magnetic-IL UA-DLLME procedure had good robustness. The experimental plan and the results obtained were presented in Table S3.

3.7. Analysis of honey samples

Applicability of the magnetic-IL UA-DLLME procedure was tested for the determination of 5-HMF in honey samples collected from different regions of Turkey. In addition, the same samples collected were analyzed in parallel with the independent reference method. In this way, the accuracy and reliability of the results obtained using the magnetic-IL UA-DLLME procedure were evaluated. All honeys were spiked with 50 and 200 μg of 5-HMF. The results show that the recoveries for the added samples were in the acceptable range (92–104%). F-value (1.56–4.75) and t-value (0.75–2.23) were calculated to evaluate whether there was a significant difference between the results obtained by the two methods. From the results in Table 5, all F and t values obtained from triplicate measurements at 95% confidence level were lower for critical t-value (2.78) and critical F-value (19). These obtained results confirm the accuracy of the results found by the magnetic-IL UA-DLLME procedure. The European Union (EU Directive 110/2001) has set the maximum allowable level of HMF in honey as 40 mg kg^{-1} with the following exceptions: 80 mg kg^{-1} for honey from countries with tropical temperatures and 15 mg kg^{-1} for honey with low enzymatic levels, respectively (Spano et al., 2006). The amounts of 5-HMF in the analyzed honey samples were within the legal limits. The highest 5-HMF content was determined in pine honey-2 ($124.3 \pm 2.7 \mu\text{g kg}^{-1}$), flower honey-1 ($67.1 \pm 1.1 \mu\text{g kg}^{-1}$) and flower honey-3 ($33.1 \pm 0.8 \mu\text{g kg}^{-1}$), respectively. The lowest amount of 5-HMF was found in chestnut honey-1 collected ($8.9 \pm 0.1 \mu\text{g kg}^{-1}$) from Artvin/Turkey.

3.8. Comparison of the optimized procedure with other procedures

To evaluate the effectiveness of the magnetic-IL UA-DLLME procedure, analytical parameters including linear range, LOD, RSD, extraction solvent, extraction time and EF were compared to other analytical methods. LOD and RSD value of the magnetic-IL UA-DLLME procedure was lower than that of all analytical methods compared. Compared to the similar spectrophotometric technique, the EF value of the optimized method was higher and the extraction time was quite low. On the other hand, the optimized method exhibited a wider linear range compared to the techniques that are expensive and require expert users.

Comprehensive comparison data are given in Table 6.

4. Conclusions

In this work, an accurate and selective ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) procedure using a magnetic ionic liquid (magnetic-IL) was optimized for the preconcentration and extraction of 5-HMF in honey samples collected from different botanical and geographical origins in Turkey prior to analysis by UV–VIS spectrophotometer. The main parameters were optimized by Box–Behnken design (BBD). Mathematical model and response surfaces based on the BBD demonstrated the detailed effect of variables on recovery of 5-HMF. The [C_{8mim}][FeCl₄] magnetic IL has been shown to be a useful alternative solvent for the extraction and determination of 5-HMF at trace levels. The magnetic-IL UA-DLLME procedure is a fast and simple extraction technique with large EF and low LOD. The instrumentation cost can be expressed as quite low. In addition, the magnetic-IL UA-DLLME procedure has some advantages including high efficiency and low organic solvent-consumption. The good intraday and interday precision obtained allows satisfactory quantification. This study reports the first application of magnetic-IL with the UA-DLLME procedure based on multivariate optimization for the determination of 5-HMF in different honey samples. As a result, the magnetic-IL UA-DLLME procedure was developed and optimized in this research article has excellent prospects for the extraction and quantification of trace levels of 5-HMF in honey samples.

CRedit authorship contribution statement

Nail Altunay: Supervision, Investigation, Validation, Writing – original draft, Writing – review & editing.

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.

Data availability

The authors do not have permission to share data.

Acknowledgments

Dr. N. Altunay thanks to Sivas Cumhuriyet University Scientific Research Projects Commission for partial supports.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the

online version at [doi:10.1016/j.jfca.2022.104817](https://doi.org/10.1016/j.jfca.2022.104817).

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