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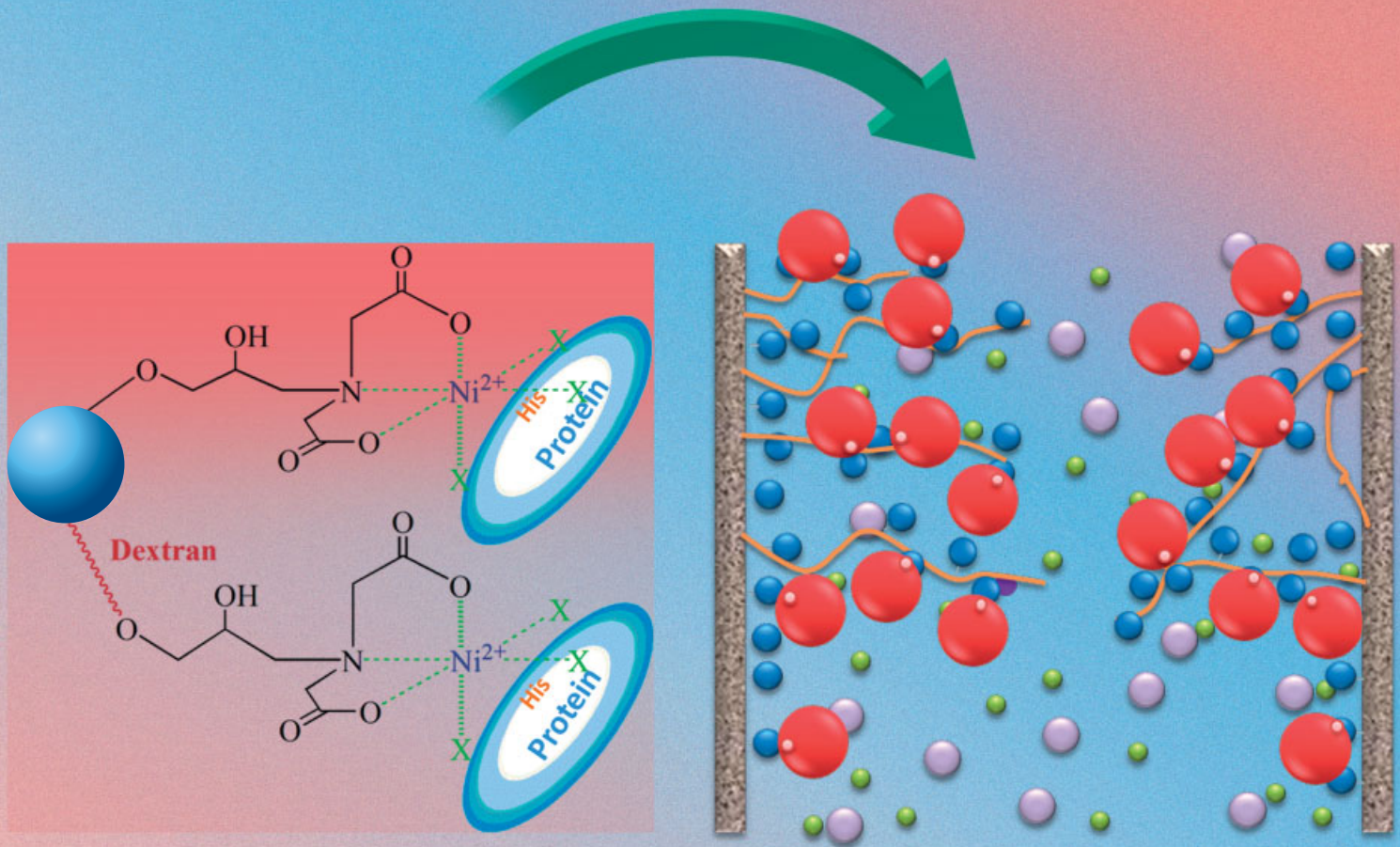

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Research Article

Ultrasound-assisted magnetic dispersive solid-phase microextraction: A novel approach for the rapid and efficient microextraction of naproxen and ibuprofen employing experimental design with high-performance liquid chromatography

A simple, rapid, and sensitive method for the determination of naproxen and ibuprofen in complex biological and water matrices (cow milk, human urine, river, and well water samples) has been developed using ultrasound-assisted magnetic dispersive solid-phase microextraction. Magnetic ethylendiamine-functionalized graphene oxide nanocomposite was synthesized and used as a novel adsorbent for the microextraction process and showed great adsorptive ability toward these analytes. Different parameters affecting the microextraction were optimized with the aid of the experimental design approach. A Plackett–Burman screening design was used to study the main variables affecting the microextraction process, and the Box–Behnken optimization design was used to optimize the previously selected variables for extraction of naproxen and ibuprofen. The optimized technique provides good repeatability (relative standard deviations of the intraday precision 3.1 and 3.3, interday precision of 5.6 and 6.1%), linearity (0.1–500 and 0.3–650 ng/mL), low limits of detection (0.03 and 0.1 ng/mL), and a high enrichment factor (168 and 146) for naproxen and ibuprofen, respectively. The proposed method can be successfully applied in routine analysis for determination of naproxen and ibuprofen in cow milk, human urine, and real water samples.

Keywords: Box–Behnken design / Dispersive solid-phase microextraction / Graphene oxide nanocomposites / Plackett–Burman design
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1 Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are used for the relief of a wide variety of pain, fever, swelling, and stiffness. Due to adverse side effects, such as irritation and ulceration of the gastrointestinal mucosa, oral therapy of NSAIDs is often limited [1]. Naproxen and ibuprofen are NSAID derivatives of propionic acid. Oral therapy of naproxen and ibuprofen can cause gastrointestinal problems, such as heartburn, constipation, diarrhea, ulcers, and stomach bleeding.

In recent years, due to the widespread consumption of pharmaceuticals, a new environmental problem has been created. Thus, these pharmaceuticals have entered in almost all environmental matrices such as river water, well water, and wastewater, leading to water pollution. The major sources of these water pollutions are the wastewater of pharmaceutical industries, hospital effluents, and domestic wastewater. Due to trace level concentrations of these water pollutants, it is necessity to develop analytical methods to determine these contaminants with high sensitivity, selectivity, and accuracy [2].

Microextraction procedures are widely used as sample preparation for drug separation from matrices where potentially interfering sample components coexist. It is clear that the choice of an appropriate and selective sample-preparation procedure is essential for accurate determination of these drugs [3–7]. SPME is a sample-preparation method that has been applied to a range of applications such as

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Abbreviations: **FGO**, functionalized graphene oxide; **GO**, graphene oxide; **LOF**, lack of fit; **MFGO**, magnetic-functionalized graphene oxide; **NSAID**, nonsteroidal anti-inflammatory drug; **PF**, preconcentration factor; **UAMDSPME**, ultrasound-assisted magnetic dispersive solid-phase microextraction

environmental, industrial, clinical, forensic, drug and food analysis [8–10]. SPME has several advantages such as simplicity, being solvent-free, and cost effective [9, 11, 12]. However, like other analytical methods, the traditional SPME also has its drawbacks including high time consumption, fiber breakage, stripping of coatings, low operating temperature (generally in the range 240–280°C), instability, and swelling in organic solvents (greatly restricting their use with HPLC) [11, 13]. To overcome these drawbacks, new SPME methods such as dispersive solid-phase microextraction and magnetic solid-phase microextraction have been developed [14–18].

In recent years, the usage of nanoscale materials in SPME as sorbents has shown several advantages in comparison with traditional sorbents. The most advantages of the nanoscale materials in SPME as sorbents are large surface area, unique physical, and suitable chemical properties. The interactions between SPME sorbent and analyte depend on the chemical nature of the surface. Due to large surface area in the nanoscale materials, the percentage of surface atoms in nanomaterials is large compared with bulk objects, therefore reactivity and interaction of nanomaterials as SPME sorbent are more than conventional SPME sorbent. Graphene oxide (GO) is one of the carbon allotropes that have found wide application as sorbents in the SPME methods. Due to unique physical and chemical properties such as mechanical and thermal stability, high adsorption capacity, and 2-D planar structure, GO has great ability to be used as a sorbent material in microextraction techniques [19, 20].

To obtain new adsorbent materials simultaneously possessing the unique properties of GO (large surface area and good mechanical properties) and functional group (high selectivity and interaction with analyte) through combining their individual characteristics, GO can be modified with new functional groups.

To reduce extraction time, a new approach recently considered is dispersive SPE [16–18]. In this method, SPE sorbent in the presence of a surfactant is dispersed in a solvent and forms a stable colloid suspension. This suspension is then injected into the sample solution for extraction of analyte. Due to dispersion of sorbent before injecting into the sample solution, the extraction time is reduced to few seconds.

The combination of ultrasound-enhanced dispersive liquid–liquid microextraction and magnetic solid-phase microextraction is introduced for the first time as a novel and efficient method for extraction of naproxen and ibuprofen from complicated matrices. The magnetic-functionalized graphene oxide (MFGO) nanocomposite was synthesized and used as a novel adsorbent for ultrasound-assisted magnetic dispersive solid-phase microextraction (UAMDSPME). For this propose, MFGOs were dispersed in water by a compact hand-held ultrasonic and rapidly injected into the sample solution for extraction. After the extraction, the MFGOs were rapidly separated from the solution under a strong external magnetic field. The analytes absorbed were eluted from the MFGO by the acceptor phase, methanol, under sonication, and was injected into HPLC system for analysis. To optimize the affecting factors in the microextraction method, a multivariate strategy based on the Plackett–Burman screening design (P–B design) and the Box–Behnken optimization design (B–B design) was used for extraction of naproxen and ibuprofen by UAMDSPME.

2 Materials and methods

2.1 Reagents and materials

Naproxen and ibuprofen were purchased from Doctor Abidi (Tehran, Iran) and were of pharmaceutical quality. Standard solution of naproxen and ibuprofen in methanol was made at 100 μg $mL^{-1}$and stored at 4°C before use. HPLC-grade acetonitrile (MeCN) and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals were analytical grade and were obtained from Merck (Darmstadt, Germany).

2.2 Instrumentation

The HPLC system was a Knauer HPLC (Germany, D-14163) consisting of a port sample injection valve with a 20 μL injection loop, a photodiode array detector (S2600), computer system, and software EZ-Chrom Elite with integration capability. The chromatographic separation was carried out at room temperature (22 ± 0.5°C) on a 100/5C_{18} analytical column (6.4 mm × 250 mm, 5.1 μm). The mobile phase, acetonitrile/formic acid–sodium formate buffer (pH 4)/methanol (20:40:40, v/v/v) was delivered at a flow rate of 1.0 mL/min. The photodiode-array detector was set at the wavelength of 225 nm. A compact ultrasonic Hiescher up 100 h (100 W, 30 kHz) was used to disperse magnetic GO nanocomposites.

2.3 Synthesis of functionalized GO

GO nanosheets were synthesized on the basis of previous article [3]. In a typical procedure, concentrated H_2SO_4 (23 mL) was added to a mixture of graphite flakes (0.5 g) and $NaNO_3$ (0.5 g), and the mixture was cooled to 0°C in an ice bath. To keep the temperature of the suspension lower than 20°C, $KMnO_4$ (3.0 g) was gradually added. The suspension was placed in an ultrasonic bath for 20 min at room temperature and was then diluted by 40 mL of deionized water. Finally, a mixture of 3 mL H_2O_2 (30%) and 100 mL deionized water was added dropwise to the suspension to reduce the $KMnO_4$ residual and the color of the suspension changed from dark brown to yellow. The resulting product was washed several times with 5% HCl aqueous solution (250 mL) to remove any metal ion impurities and sulfate ions, and then with distilled water to remove the excess of acid. After filtration, GO was dried under vacuum at 80°C for 24 h.

To convert ester, hydroxyl, and epoxide groups to carboxylic groups, 1 mg GO in 1 mL distilled water was dispersed by sonication for 15 min and 50 mg of NaOH and 50 mg of $ClCH_2COONa$ were added to it. The suspension was

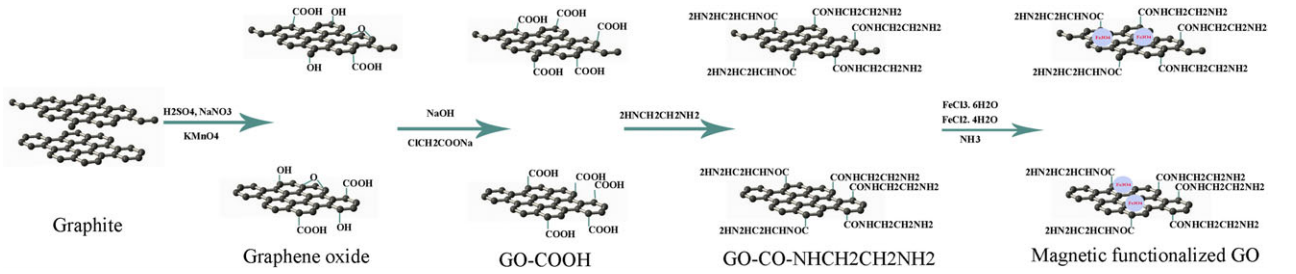

Figure 1. Schematic view of magnetic functionalized graphene oxide synthesis.

placed in ultrasonic bath for 2 h at room temperature and the resulting GO–COOH product was then neutralized by dilute hydrochloric acid and centrifuged until the product was well dispersed in deionized water [21]. For the synthesis of GO–CO–NH–CH_2–CH_2–NH_2, 0.1 g of GO–COOH was immersed in 50 mL of ethylendiamine and refluxed at 100°C for 2 days (48 h). The mixture was then cooled and washed with ethanol several times to remove excessive ethylendiamine. It was then dried under vacuum for 1 h [9].

2.4 Synthesis of magnetic-functionalized GO nanocomposite by coprecipitation

To synthesize MFGO, 0.3 g of functionalized graphene oxide (FGO) was dispersed in 150 mL water by using ultrasonic bath irradiation for 30 min. The solution of $FeCl_3 \cdot 6H_2O$ (0.825 g) and $FeCl_2 \cdot 4H_2O$ (0.322 g) in 25 mL of water was added dropwise to the FGO suspension at room temperature under nitrogen flow with vigorous stirring. Ammonia solution (28%) was then added dropwise until the pH of mixture reached 10 and a black precipitate was formed after stirring for about 45 min. The precipitate was centrifuged, washed with ethanol several times, and was finally dried [4]. Supporting Information Figs. S1 and S2 show chemical synthesis procedure and the FTIR spectra of FGO and MFGO, respectively.

2.5 Ultrasound-assisted magnetic dispersive SPME

To carry out the extractions procedure, 15.0 mL of naproxen and ibuprofen standard solutions were placed in a 20 mL conical test tube. The pH of solutions was adjusted at 6.7 by adding 1 mL of sodium dihydrogen phosphate/disodium hydrogen phosphate buffer.

A mixture of MFGOs (20 mg) and deionized water (1 mL) was poured into a conical test tube and ultrasonically treated for 5 min to disperse MFGOs. The resultant suspension was rapidly injected by an autosampler to the naproxen and ibuprofen standard solutions, followed by shaking for 30 s. A cloudy mixture was formed as a result of dispersion of fine particles of MFGOs in the bulk of aqueous sample, and naproxen and ibuprofen were extracted into these fine particles. After the extraction, the MFGOs were rapidly separated from the solution under a strong external magnetic field. After discarding the supernatant solution, the absorbed analytes onto the MFGOs were eluted with 1.0 mL of methanol under sonication for 180 s. To analyze naproxen and ibuprofen, 20 μL of the supernatant methanol phase were injected into the HPLC system.

2.6 Real samples preparation

Cow milk was collected from a farm on the outskirts of Mashhad, Iran. The cow milk (10 mL) was spiked with 5 and 25 ng/mL of naproxen and ibuprofen, respectively, in 20 mL conical test tubes and was diluted with distilled water to a volume of 15 mL. The mixture was acidified at pH ~2 with 6 M HCl and then centrifuged at room temperature for 15 min at 8000 rpm. The supernatant was carefully transferred to a new conical test tube and stored at 4°C.

Human urine samples were collected from healthy volunteers in our laboratory and stored in polypropylene tubes at 20°C. Well and river water samples were collected form outskirts of Mashhad and Golestan river (Mashhad, Iran), respectively. To remove any particulate matter, all the human urine and real water samples were centrifuged at 8000 rpm for 5 min and then stored at 4°C before analysis.

2.7 Calculation of percentage of extraction recovery and preconcentration factor

The percentage of extraction recovery, ER%, was used to obtain the optimized extraction conditions. The ER%, as analytical response, was calculated using the following Eq. (1):

$$\mathrm{ER\%} = \left(C_0 \times V_0 / C_{aq} \times V_{aq}\right) \times 100 \quad (1)$$

where C_0 and C_{aq} are the concentration of the naproxen and ibuprofen in the acceptor phase, methanol, and the aqueous sample, respectively. V_0 and V_{aq} are volumes of the acceptor phase, methanol, and aqueous sample, respectively.

The preconcentration factor (PF) is defined as the ratio of the analyte concentration in the acceptor phase, methanol, and aqueous sample. PF was calculated according to the following Eq. (2):

$$\mathrm{PF} = C_0 / C_{aq} \quad (2)$$

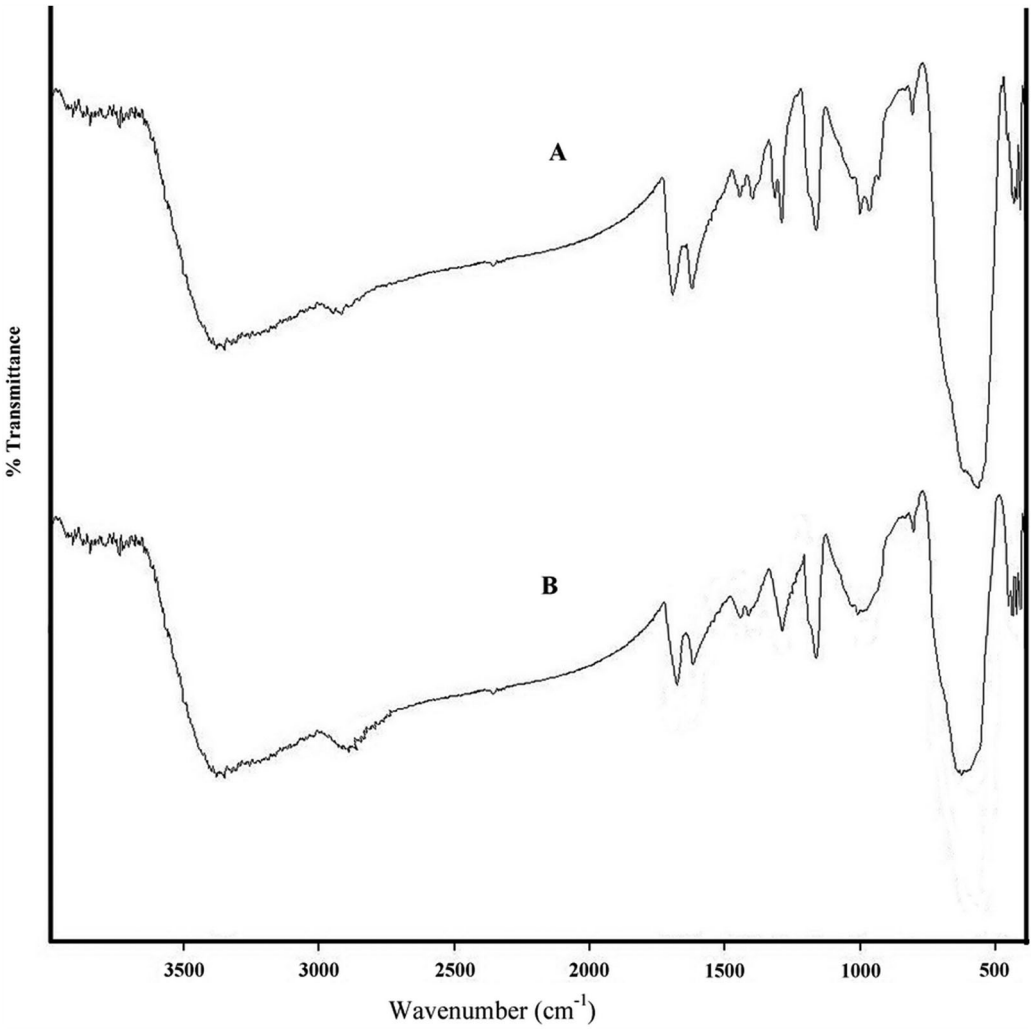

Figure 2. FTIR spectra of functionalized graphene oxide (A) and magnetic functionalized graphene oxide (B).

To obtain ER% and PF, C_{aq} was the concentration of the naproxen and ibuprofen in the sample solutions before microextraction and C_0 was determined from a calibration curve using direct injection of standard solutions.

3 Results and discussion

3.1 Optimization of the microextraction procedure

Several factors which may affect the microextraction performance, such as volume of donor phase, pH, extraction time, desorption time, the type of desorption solvent, and stirring rate were optimized. An affecting factor in microextraction is the type of desorption solvent. Organic solvents (acetonitrile, ethanol, 2-propanol, and methanol) are investigated as desorption solvents (Supporting Information Fig. S3). The results showed that ER% is greatest for methanol. Therefore, methanol as a desorption solvent was selected for further studies. A P–B design was used for screening of the variables that significantly affects the microextraction process. After determining the significant variables, B–B was employed to derive the corresponding response surface equation and to investigate the interaction among these variables. The experimental design matrix and data analysis were performed using MINITAB version 14 software.

3.2 Screening design

To reduce the number of significant factors affecting the microextraction efficiency, P–B design was used to examine for the microextraction procedure. Based on the preliminary experiments, seven factors including donor phase volume, pH of sample solution, extraction time, desorption time, desorption solvent volume, amount of sorbent, and shaking rate at two levels, were selected. A matrix of the P–B design was developed consisting of 15 experiments, 12 runs, and 3 center points, and carried out randomly to eliminate the effects of extraneous or nuisance

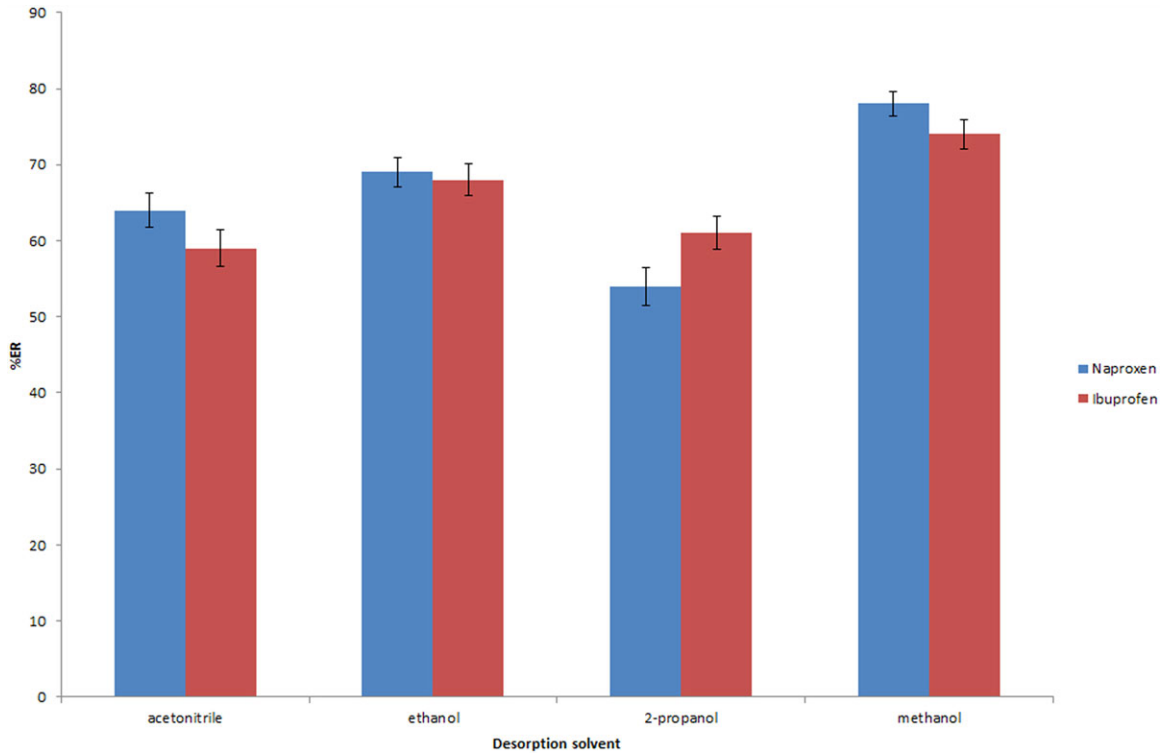

Figure 3. Effect of type of desorption solvent on the extraction.

Table 1. Linearity, repeatability, detection limits, and preconcentration factor of the proposed analytical procedure

Analytes	Linear dynamic range (ng/mL)	R^2	%RSD ($n = 5$)		LOD (ng/ mL) ($n = 5$)	LOQ (ng/ mL) ($n = 5$)	Preconcentration factor
			Interday	Intraday			
Naproxen	0.1–500	0.9979	5.6	3.1	0.03	0.1	168
Ibuprofen	0.3–650	0.9968	6.1	3.3	0.1	0.3	146

Table 2. Assay of naproxen and ibuprofen in cow milk, real water, and human urine samples by means of the proposed method ($n = 3$)

Analytes		Naproxen			Ibuprofen		
Spiked value (ng/mL)		0	5	20	0	5	20
Well water	Found (ng/mL)	Nd	4.76 ± 0.08	19.32 ± 0.06	Nd	4.69 ± 0.09	18.93 ± 0.07
	Recovery percentage	—	95.2	96.6	—	93.8	94.7
River water	Found (ng/mL)	Nd	4.83 ± 0.06	19.11 ± 0.05	Nd	4.79 ± 0.08	19.36 ± 0.06
	Recovery percentage	—	96.6	95.6	—	95.8	96.8
Cow milk	Found (ng/ mL)	Nd	5.23 ± 0.08	18.98 ± 0.07	Nd	4.71 ± 0.08	19.05 ± 0.05
	Recovery percentage	—	104.6	94.9	—	94.2	95.3
Human urine	Found (ng/ mL)	Nd	4.32 ± 0.09	21.98 ± 0.08	Nd	4.43 ± 0.09	18.03 ± 0.09
	Recovery percentage	—	86.4	109.9	—	88.6	90.2

Nd, not detected.

variables [22, 23]. Their levels and the corresponding symbols are shown in Supporting Information Table S1. Each run was carried out in triplicates and the mean of the ER% was used as its response. Analysis of variance test was conducted to determine the main effects using the *t*-test with a 95% probability. The ER% of the experimental design analyzed by standardized Pareto chart is given in Fig. 1. A Pareto chart is a type of bar chart used to determine the magnitude and the importance of an effect. The bar length on the plot displays the absolute value of these effects. If the bar length of each parameters on the pareto chart is greater than the critical t- value (reference line), these parameters are

Table 3. Comparison of the proposed method with other reported methods for naproxen and ibuprofen determination

Method	LOD (ng/mL)		Linear dynamic range (ng/mL)		References
	Naproxen	Ibuprofen	Naproxen	Ibuprofen	
SSPE–LC–UV[a)]	0.8	9	0.02–250	0.2–750	[4]
CITP	4	2	40–200	40–200	[6]
FI[b)]	0.9	0.2	5–20	0.4–2.4	[6]
SPME–LC–UV[c)]	0.03	—	0.2–20	—	[25]
CHF–LPME–GC–FID[d)]	1–2	1–2	5.0–500	2.5–500	[26]
SPME–HPLC	0.03	0.1	0.1–500	0.3–650	This method

a) Supramolecular SPE with LC and photometric detection.
b) Fluorescence spectroscopy.
c) SPME with LC and photometric detection.
d) Continuous hollow fiber liquid-phase microextraction with GC and flame ionization detector.

considered to be statistically significant. According to Fig. 1, the pH of sample solution, shaking rate, and desorption solvent volume were the most significant variables for both analytes and were evaluated in the B–B for further assessment. The pH of sample solution was the most significant factor having a positive effect on the ER% and the other two factors, shaking rate, and desorption solvent volume, were the next most important significant variables having a positive and negative effects on the ER%, respectively. Comparison between the three significant factors of pH, shaking rate, and desorption solvent volume on microextraction of naproxen shows that the effect of pH is much more than other factors, but in the extraction of ibuprofen, the effect of three factors is much closer. Other variables, the donor phase volume, extraction time, desorption time, and amount of sorbent, had no significant effects on the ER% and were fixed at 15.0 mL, 30 s, 180 s, and 20 mg, respectively.

3.3 Optimization design

The next step in the present research was the optimization of the factors selected from the first screening design. To perform the optimization, response surface design, such as B–B and central composite, can be used. In the present study, a B–B design was applied to optimize the three factors (pH of sample solution, shaking rate, and desorption solvent volume) selected from the first screening design. The examined levels of the factors are given in Supporting Information Table S2. In the current study, a matrix of the B–B design was developed consisting of 15 experiments, 12 runs, and 3 center points, and performed randomly, for the same reasons as mentioned for the P–B design (Supporting Information Table S2).

Supporting Information Table S3 presents the results of the analysis of variance for naproxen and ibuprofen, respectively that allowed a quadratic model fit to the data and permitted the response to be modeled by fitting a second-order polynomial with a p value of 0.05. The lack of fit (LOF) is the variation of the data around the fitted model. LOF tests are sufficient to evaluate the fit of model because the effects of the additional higher-order terms are removed from the error. If the p value is higher than the selected α level, this indicates that the model fits accurately to the data. As shown in Supporting Information Table S3, the p values of the LOF were 0.384 and 0.275 for extraction of naproxen and ibuprofen, respectively, indicating that these models well fit the responses for extraction of both analytes. R^2 indicates how well polynomial model equation fits response variables. In general, the R^2 increases this polynomial model equation to be better fitted to response variables. R^2 would always increase when a new term is added which may be due to chance alone. Adjusted R^2 is a modification of R^2 that adjusts for the number of terms in a model. R^2 gives the percentage of explained variation when all independent variables in the model affect the dependent variable, whereas the adjusted R^2 gives the percentage of explained variation by only those independent variables that in reality affect the dependent variable. Therefore, the adjusted R^2 is better than the R^2 because the adjusted R^2 will increase only if the new term improves the model more than it would be expected by chance [24]. The adjusted R^2 values were higher than 0.95 for the areas, which were statistically acceptable at $p < 0.05$ levels indicating a good relationship between the experimental data and the fitted model.

The B–B design allowed a quadratic model fit to the data and permitted the response to be modeled by fitting a second-order polynomial, which can be expressed in the following Eq. (3):

$$\mathrm{ER\%} = \beta_0 + \beta_1 F_1 + \beta_3 F_3 + \beta_6 F_6 + \beta_{11} {F_1}^2 + \beta_{33} {F_3}^2 + \beta_{66} {F_6}^2 + \beta_{13} F_1 F_3 + \beta_{16} F_1 F_6 + \beta_{36} F_3 F_6 \quad (3)$$

where β_0 is the intercept, and β_1 to β_{36} represent the regression coefficients. The results obtained from the B–B design, namely, the regression coefficients, the Student's t distribution, and p values, are listed in Supporting Information Table S4. The R^2 indicates how well the polynomial model equation fits to response variables. The R^2 values are 98.51 and 98.66 for extraction of naproxen and ibuprofen, respectively. These results indicated that the polynomial model

equations are well fit to response variables and less than 1.5% of the total variations were not explained by the model.

The regression coefficients (Supporting Information Table S4) show that the pH of the sample solution is highly significant when compared with shaking rate and desorption solvent volume. The results show that interaction between two deferent main factors are lower than the two same main factors. These regression coefficients are positive values for main factors and negative values for interaction effects with the exception of the effect of the interaction between the pH of sample solution and shaking rate in the extraction of ibuprofen that has a positive value.

The optimization plot (Fig. 2) indicates the predicted conditions for the optimum point and the desirability of the prediction. The influence of each factor in the response is shown by individual plot in this figure. These figures can be used to evaluate the changes in the level of each variable that simultaneously affects the response (ER%) and the overall desirability of the responses. According to the overall results of the optimization study the following experimental conditions were chosen: pH of sample solution, 6.7; shaking rate, 700 rpm; desorption solvent volume, 1.0 mL.

3.4 Method validation

Under the optimized experimental conditions, the method was validated to evaluate its practical applicability by figures of merit such as linear dynamic range, LOD, LOQ, precision, and accuracy which are summarized in Table 1. The calibration graphs were linear in the concentration range of 0.1–500 and 0.3–650 ng/mL for naproxen and ibuprofen, respectively. High preconcentration factors of 168 and 146 were obtained for naproxen and ibuprofen by the proposed extraction method, respectively. To evaluate the precision of the method, five similar experiments were carried out for the spiked samples at the concentration level of 10 ng/mL of naproxen and ibuprofen on the same day and three consecutive days. The percent RSD of intraday precision and interday precision is listed in Table 1 and shows good reproducibility and precision for the proposed method.

3.5 Real samples analysis

To test the feasibility of the proposed method, several real samples such as cow milk, river water, well water, and human urine samples were investigated and the mean extraction recoveries were calculated for three analysis. All samples were centrifuged at 5000 rpm for 5 min and then filtered through a 0.45 μm filter before analysis. Several aliquots of naproxen and ibuprofen standard solutions were spiked into the cow milk, real water, and human urine samples at the concentrations of 5 and 25 ng/mL of naproxen and ibuprofen, respectively, and determined according to the method described in Section 2.5. The results summarized in Table 2 indicate that naproxen and ibuprofen can be quantitatively recovered from real samples by the proposed procedure. These results demonstrate the applicability of the proposed procedure for naproxen and ibuprofen determination in cow milk, real water, and human urine samples. Figure 3 shows the chromatogram obtained by UAMDSPME under the optimal conditions for spiked river water samples taken from Golestan river (Mashhad, Iran) containing 5 ng/mL of naproxen and ibuprofen. No significant interfering peaks were observed at the retention position of analytes.

3.6 Comparison to other methods

A comparison of the proposed method with other reported preconcentration methods is given in Table 3. These results reveal that the method has many advantages such as suitable dynamic range, low LOD, and LOQ and high preconcentration factor with low sample consumption, free from solvent, and cost effective. Moreover, the proposed method indicates that the MFGO has great ability to be used as a sorbent material in microextraction techniques. These characteristics demonstrate that the method is very suitable for analysis of naproxen and ibuprofen for cow milk, real water, and human urine samples.

4 Conclusion

A novel UAMDSPME method using MFGOs as a new adsorbent by HPLC–DAD (diode array detector) was developed for rapid determination of naproxen and ibuprofen from cow milk, real water, and human urine samples. The MFGO was synthesized and used as a novel adsorbent for UAMDSPME. This novel adsorbent showed great adsorptive ability toward these analytes. A multivariate strategy was first used as a P–B design to study the main variables that affect the microextraction process and second the B–B design to optimize the previous selected variables for extraction of naproxen and ibuprofen by UAMDSPME. The microextraction method has the advantages of simplicity of operation, high speed, low sorbent, and sample consumption, inexpensive, and environmentally friendly. The resulting optimized procedure allowed quantification of trace levels of naproxen and ibuprofen using UAMDSPME combined with HPLC–DAD. The application of the method to real samples confirmed that this procedure is suitable for quantitative determination of naproxen and ibuprofen in cow milk, real water, and human urine samples for routine laboratory analysis.

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The authors have declared no conflict of interest.

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