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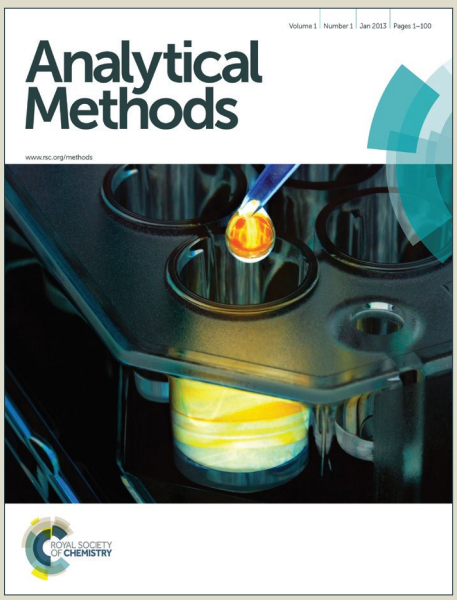

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Application of supramolecular solvent as carrier for ferrofluid based liquid-phase microextraction for spectrofluorimetric determination of levofloxacin in biological samples

***Mojtaba Shamsipur*[a], *Parvin Zohrabi*[b*], *Mahdi Hashemi*[b]**

[a]*Faculty of Chemistry, Razi University, Kermanshah, Iran*

[b]*Faculty of Chemistry, Bu-Ali Sina University, Hamedan, Iran*

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[*]Corresponding author: E-mail address: parvin_zohrabi@yahoo.com (p. zohrabi).

Tel.: +98-81-38228313, fax: +98-81-38272404.

Abstract

In the present study, a novel and environmental friendly liquid-phase microextraction method based on application of supramolecular solvent as a carrier for ferrofluid was proposed for spectrofluorimetric determination of levofloxacin (LEVO). The ferrofluid was composed of oleic acid-coated magnetic particles and supramolecular solvent as the extractant solvent. The supramolecular solvent is water-immiscible nanostructured liquid that was produced from coacervation of decanoic acid aqueous vesicles in the presence of tetrabutyl ammonium (Bu_4N^+). As the ferrofluid can be attracted by a magnet, no special devices and complicated operations were needed for phase separation. The important parameters affecting the microextraction efficiency including pH, volume of extraction solvent, salt effect and extraction time were investigated and optimized. Under optimum conditions, the calibration curve for LEVO determination showed good linearity in the range 1–200.0 ng mL^{-1} (R^2= 0.9987). The repeatability and reproducibility (RSD%) for 20 ng mL^{-1} LEVO were 2.9% and 4.4%, respectively and limit of detection (S/N = 3) was estimated to be 0.2 ng mL^{-1}. The proposed method was successfully applied for the extraction and determination of levofloxacin in biological samples.

Keywords: Ferrofluid, Levofloxacin, Spectrofluorimetric detection, Supramolecular solvent

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1 Introduction

Levofloxacin (LEVO) is a quinolone antimicrobial agent, which exhibits activity against a broad spectrum of Gram-positive and Gram-negative bacteria through inhibition of their DNA gyrase. LEVO is used for treatment of infections of the respiratory and urinary tract, skin and soft tissues [1, 2]. Although LEVO is generally tolerated, in some instances it may cause serious and life-threatening adverse reactions, spontaneous tendon ruptures and irreversible peripheral neuropathy [3, 4]. Due to extensive impact of drug analysis on public health, development of sensitive and reliable methods for determination of drugs in biological and pharmaceutical formulations is of great interest. A number of analytical methods have been developed for the quantitative determination of LEVO in pharmaceutical and biological fluids. Some reported methods are, high performance liquid chromatography (HPLC) [5-8], electrochemical sensor based on molecularly imprinted polymer [9], synchronization-first-derivative fluorescence spectroscopy [10] spectrofluorometry [11] and colorimetry [12].

The sample preparation step plays an essential role in analytical procedures of biological samples due to low concentration of interested analytes and complex matrixes of these samples. In recent years, attention has been paid to the use of alternative solvents, mainly supercritical fluids [13-15], ionic liquids [16, 17] and more recently supramolecular solvents (SUPRASs) [18-20] in analytical extractions. Supramolecular solvents are water immiscible liquids produced by the sequential self-assembly of amphiphilic molecules at two scales; molecular and nano [21, 22]. They are generated through self-assembly processes, induced by changes in the environmental conditions of aqueous or hydroorganic solutions of the amphiphile (e.g. pH modification, salt addition, presence of a non-solvent for the amphiphile, etc.). Self-assembly causes the spontaneous separation of an amphiphile-rich liquid phase from the bulk solution [23]. They have the capability to provide different types of interactions (i.e. ionic, hydrogen bonding, π-cation and hydrophobic) with analytes. These interactions are important for extraction of analytes from water phase to supramolecular solvent phase and for increase extraction efficiency [19, 24].

A second major asset of SUPRASs is the high concentration of amphiphiles they contain. This characteristic results in a high number of binding sites for analytes and permits to achieve high extraction efficiencies using low extractant volumes, which is requisite in microextractions

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[23]. Moreover, the solvent is produced from environmentally friendly and inexpensive biosurfactants and the coacervation occurs rapidly at room temperature [26].

Generally, the convenient separation and retrieval of extracting solvent after extraction is of crucial importance in the microextraction methods. Most of these methods require different processing steps, such as centrifugation, refrigeration to freeze the extracting solvent, manually retrieving it to let it thaw, and use of special equipment and additional materials such as surfactants [26-30]. Very recently, ferrofluid is suggested as extracting solvent to overcome these drawbacks. The ferrofluids are homogeneous suspensions of single-domain magnetic nanoparticles in an appropriate carrier liquid. The magnetic nanoparticles need to be stabilized in the carrier liquid because they tend to agglomerate due to Van der Waals forces. In order to prevent particles from agglomeration, the Fe_3O_4 nanoparticles are coated by an appropriate material such as surfactant.

The volume fraction of the magnetic material is typically only a few percent. Magnetic suspension has both magnetic and fluid properties [31, 32]. The volume fraction of the magnetic material in ferrofluid is typically only a few percent [33, 34]. Since the amount of magnetic nanoparticles in ferrofluids is low (3-10%), many properties of the ferrofluid, such as density, vapor pressure and chemical properties of the liquid are the same as the base fluid [35]. Simple separation of ferrofluid using an externally applied magnetic field leads to attractive applications in liquid phase microextraction. Recently, application of different organic solvents and ionic liquids as carrier has been reported in the microextraction based on ferrofluid [35-38].

The aim of this study was to use SUPRAS as carrier for ferrofluid base microextraction coupled to spectrofluorimetry for the determination of levofloxacin in human plasma and urine samples. All the experimental parameters affecting the extraction were investigated in details and the analytical characteristics of the method were evaluated.

2 Experimental

2.1 Reagents and materials

All reagents used were of analytical grade. Levofloxacin and tetrabutylammonium hydroxide (Bu_4NOH, 40% w/v in water) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Decanoic acid was purchased from Fluka (Buchs, Switzerland). Other reagents including ferric

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chloride ($FeCl_3 \cdot 6H2O$), ferrous chloride ($FeCl_2 \cdot 4H_2O$), ammonium hydroxide (28%), oleic acid (OA) and methanol were purchased from Merck (Darmstadt, Germany). Stock solution of LEVO (1000 μg mL^{-1}) was prepared in methanol and stored in freezer at 4^o C. The working solutions were prepared daily by an appropriate dilution of the stock solution with double distilled de-ionized water.

2.2 Apparatus

Fluorescence measurements were performed using a Varian Cary Eclipse spectrofluorometer equipped with a xenon flash lamp. Samples were excited at 290 nm, and emission spectra were measured at a wavelength range of 400 to 650 nm. All measurements were performed in a 10 mm optical path quartz microcell. UV–Vis spectra were collected by an Agilent 8453 diode array spectrophotometer. A centrifuge (Hettich, EBA 20, Tuttlingen, Germany) was used for centrifuging.

2.3. Real samples preparation

The urine samples were collected from different volunteers. The samples were centrifuged at 3000 rpm for 10 min at room temperature and the supernatant was diluted with de-ionized water. For plasma analysis 2 mL of acetonitrile and 2-3 drop of trichloroacetic acid (as a deproteinizer) were added to a 1 mL plasma sample and then the mixture was centrifuged at 3000 rpm for 10 min. After appropriate dilution, the obtained solution was analyzed by the described procedure.

2.4 Supramolecular solvent production

The following procedure was routinely used to prepare the supramolecular solvent [21]. Decanoic acid (5.15 g) was mixed with tetrabutyl ammonium hydroxide (3.9 g) in 100 mL distilled water at pH 7. The mixture was magnetically stirred for 5 min at 1300 rpm to dissolve the decanoic acid, and then the mixture was centrifuged at 3500 rpm for 10 min to accelerate phase separation. The prepared SUPRAS is less dense than water and consequently remains at the top of the solution. Then, it was withdrawn using a 10 mL syringe, and was used for further experiments.

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2.5 Preparation of the oleic acid -coated Fe_3O_4 NPs

The preparation of the oleic acid coated Fe_3O_4 NPs was based on the method reported by Shen et al. [39]. Briefly, $FeCl_3$ $6H_2O$ (5.84 g) and $FeCl_2$ $4H_2O$ (2.15 g) were dissolved in 150 mL de-ionized water under nitrogen atmosphere with vigorous stirring at 80° C. Then, a solution of 0.1 mL oleic acid in 5 mL acetone is added to the heated salt solution. A black suspension is formed upon the addition of 20 mL of 28 % aqueous ammonia. Further additions of oleic acid are made in five 0.2 mL (undiluted) volumes over 5 min intervals. The extremely high affinity between iron oxide and carboxylic acid group of the surfactant makes not only the orientation of surfactant at the particle/surfactant interface favorable but also the chemisorption reaction between them [39]. The crystal growth is allowed to proceed for 30 min at 80°C with constant stirring. The suspension is then cooled to room temperature. The suspended particles are flocculated by acetone and are washed five times with 10 mL of acetone-methanol mixture (1:1 v/v) to remove the excess oleic acid. Then, the prepared suspension (OA-Fe_3O_4) was collected by magnetic decantation and dried under vacuum for 24 h.

The morphological features of OA-Fe_3O_4 were observed by SEM. The typical SEM image of OA-Fe_3O_4 was shown in Fig. 1. The SEM analysis showed that the OA-coated magnetic particles are spherical in shape, narrowly distributed and well dispersed, with a particle size of ca. 24 nm.

2.6 Preparation of ferrofluid

The oleic acid-coated magnetic particles (10 mg) and supramolecular solvent (2 mL) were mixed in a vial with ultrasound assisted (40 Hz, 100 W) for 10 min at room temperature. After that, a magnet was held next to the bottom of the vial and then the insoluble matter (undispersed coated magnetic particles) was discarded. In this way, a low density dark brown magnetic fluid was obtained. This magnetic fluid was stable for about one month.

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2.7 Extraction procedure

A 30 mL aliquot of the analyte solution containing of 3% NaCl was placed in a 50 mL centrifuge tube. Then 70 μL of the magnetic fluid was added to the glass tube and mixture was vigorously shaken using a vortex agitator for 5 min. Then, a strong magnet was placed next to the side of the centrifuge tube to attract and isolate the extraction solvents and the sample solution was discarded simply by decanting it. Thereafter, the magnet was removed and 200 μL of methanol as precipitation reagent was introduced to the vial. Finally, the magnet was again placed at the bottom of the centrifuge tube in order to attract and isolate the magnetic nanoparticles. When, all of the MNPs were settled, the organic phase was easily collected into an eppendorf tube and 200 μL of the collected organic solvent was transferred to quartz microcell using a syringe for the subsequent spectrofluorimetric detection.

3 Results and discussion

In this study, a new liquid–liquid microextraction method based on the application of supramolecular solvent as carrier for ferrofluid was used as a separation and pre-concentration method for spectrofluorimetric determination of LEVO in biological samples. The intensity of the fluorescence peak was used to assess the extraction efficiency under various conditions (the wavelengths of 290 and 484 nm were used as maximum excitation and emission wavelengths). To reach high extraction efficiency the procedure conditions such as pH, the salt concentration, volume of extraction solvent and extraction time were optimized. A univariate approach was employed to optimize influential factors in this method and all results were average of three replicate measurements.

3.1 Effect of the extraction solvent volume

The effect of extracting solvent volume on the extraction of LEVO was investigated in the range of 50–200 μL. The results are shown in Fig. 2A. As can be seen, fluorescence intensity of LEVO increased with increasing the volume of extracting solvent up to 70 μL and then decreased with further increases in solvent volume due to dilution effects. Therefore, the volume of 70 μL was selected as an optimum solvent volume for further studies.

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3.2 Effect of sample pH

The pH of the sample solution plays an important role in this procedure due to its effect on the state and solubility of the analyte in aqueous phase. The effect of sample pH on the extraction recovery of LEVO was investigated in the range of 3.0-11.0 (Fig.2B). As can be seen from Fig. 2B, the highest extraction efficiency was achieved in the interval of 7.0-8.0. This may be explained by considering that, LEVO is an amphoteric drug and exists as cation in acidic pH, as zwitterion in neutral pH, or as anion in basic pH. Among these entities, zwitterion possesses zero net charge and therefore it extracts better in vesicular phase. Thus the pH of 7.5 was selected for further studies because zwitterionic form is predominant at this pH value.

3.3 Effect of salt addition

The influence of salt addition on the extraction efficiency was investigated by adding different amounts of sodium chloride (0–5%, w/v). Generally, addition of salt can decrease the solubility of analyte in the aqueous phase and improve the extraction efficiency due to salting-out effect. On the other hand, salt addition can increase the ionic strength of the sample and changes the physical properties of the Nernst diffusion film; so it can reduce the rate of diffusion of the analyte into the extraction phase and reduces the extraction efficiency. The results (Fig.2C) revealed that the extraction efficiency increased with increasing of NaCl concentration up to 3% and then remained constant. Therefore, a concentration of 3% NaCl was used in further studies.

3.4 Effect of extraction time

Extraction time is a crucial parameter that affects extraction recovery, because it influences the partition of the target analyte between the donor phase and acceptor phase. The influence of the extraction time was evaluated in the range of 1–8 min at the constant experimental conditions. The results in Fig. 2D, show that the signal intensity increased by increasing the extraction time up to 5 min and then remained constant up to 8 min. Based on this observation; the extraction time of 5 min was chosen as the optimum value.

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3.5 Analytical characteristics

Under the selected experimental conditions, a linear calibration graph was obtained over the concentration range 1–200 ng mL^{-1} of LEVO with the correlation coefficient (R^2) of 0.9987. The limit of detection, defined as LOD = $3S_b/m$ (where S_b and m are standard deviation of the blank and slope of the calibration curve, respectively), was found to be 0.2 ng mL^{-1}. The precision of the method was evaluated through investigation the repeatability and reproducibility (expressed as RSD%). The repeatability was evaluated over five replicates spiked at concentration level 20 ng mL^{-1} of LEVO within one day (n = 5). The reproducibility was evaluated over five daily replicates, spiked at same level per work day, over a period of three days (n = 15). The repeatability and reproducibility were 2.9% and 4.4%, respectively. The enrichment factor (EF) was defined as the ratio of the final analyte concentration in the collected phase to the analyte concentration in the aqueous sample where the analyte concentration in the collected phase was calculated from the direct calibration graph (0.1–10 mg L^{-1}) of LEVO standard solution. The enrichment factor of 85.0 was found for LEVO determination. Comparison of the analytical performances achieved by purposed method and other methods for determination of LEVO is presented in Table 1. The presented method has distinct advantages in terms of low limit of detection, better RSD, ease of operation and simplicity.

3.6 Interference study

The possible interference effects of some species which may exist in biological samples and pharmaceutical formulations were investigated. Interference study was carried out by the addition of different concentration of potential interferents to the sample solution containing 50 ng mL^{-1} of LEVO. The tolerable amount of each interferent was taken as LEVO: interferent ratio that resulted in error not exceeding ±5%. The results (Table 2) showed that 200 fold execs of Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^- , NO_3^-, CO_3^{2-} and 1000 fold excess of glucose, fructose, sucrose, ascorbic acid and dextrose had no interference on the performance of the method. Tolerable ratios are greater than those found in biological samples [11], so no interference from these species is expected.

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3.7 Analysis of real samples

The analytical applicability of the proposed method was investigated in human plasma and urine samples. Plasma and urine samples obtained from healthy volunteers with no drug consumption. Recovery studies were carried out by spiking the blank samples obtained from healthy volunteers with different amount of LEVO. Results (Table 3) showed that recovery values were in the range of 94.0-106.0 %. Acceptable recoveries demonstrated that the matrix of biological samples had no effect on the performance of the presented method.

4 Conclusions

In this research, a new liquid–liquid microextraction method has been developed for pre-concentration and determination of levofloxacin in biological samples based on the application of supramolecular solvent as carrier for ferrofluid. The nanostructured ferrofluid was composed of oleic acid-coated magnetic particles and adsorbed supramolecular solvent. The SUPRAS based on DeA–Bu_4NDe vesicles constitutes a suitable alternative to toxic organic solvents. The ferrofluid was convenient for the use of low-density extraction solvents such as SUPRASs without any special device and complicated operation. The presented microextraction method lacks tedious steps of conventional microextraction methods, such as centrifugation, refrigeration and thawing of organic solvent. Also, the developed method offers outstanding advantages such as simplicity of the extraction, excellent enrichment in a short extraction time, good repeatability and reproducibility, and environmentally friendly.

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Figure Captions

Fig. 1. The SEM images of OA-Fe_3O_4

Fig. 2. Effect of experimental parameters on extraction of levofloxacin (A) extraction solvent volume, (B) pH of the sample, (C) salt concentration and (D) extraction time; other conditions: LEVO: 20 $ngmL^{-1}$, sample volume: 30 ml, desorption solvent volume, 200 µL of MeOH, extraction time 6min. error bar represent the standard deviation at the experiments

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Table 1. Comparison of diverse methods for the determination of LEVO

Method	LOD[a] (ng mL^{-1})	LR[b] (ng mL^{-1})	RSD%	Ref.
DLLME –NACE– UV [c]	5.74	133–1600	6.8	40
DLLME– UHPLC–DAD	4.26	27–1000	–	41
Enhanced spectrofluorimetry	30	15-1000	0.8	11
MIP/G-Au modified electrode[d]	190	360-36000	–	9
Spectrofluorimetry	17	50-1000	–	42
LLME–Spectrofluorimetry	0.2	1-200	2.9	This work

[a] Limit of detection.

[b] Linear range.

[c]Dispersive liquid–liquid microextraction- nonaqueous capillary electrophoresis

[d]Molecularly imprinted polypyrrole‑graphene‑gold nanoparticles modified electrode

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Table 2. Effect of interfering species on the determination of levofluxacine

Species	**Tolerance limit (w/w)**
Na^+, K^+, Ca^{2+}, Mg^{2+} Cl^-, CO_3^{2-}, NO_3^-	200
glucose, fructose, sucrose, ascorbic acid, dextrose	1000

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Table 3. Results of the LEVO determination in the spiked biological samples.

Sample	Added (ng mL^{-1})	Found (ng mL^{-1})	Recovery (%)
Urine[a]	-	ND[b]	-
	10.0	10.6 ± 0.5[c]	106.0
	50.0	47.0 ± 4.0	94.0
	100.0	96.0 ± 4.0	96.0
Human serum[a]	-	ND[b]	-
	10.0	9.4 ± 0.9	94.0
	50.0	51.0 ± 3.0	102.0
	100.0	95.0 ± 5.0	95.0

[a] Drug free samples from healthy volunteers

[b] Not detected

[c] mean ± SD (n=3)

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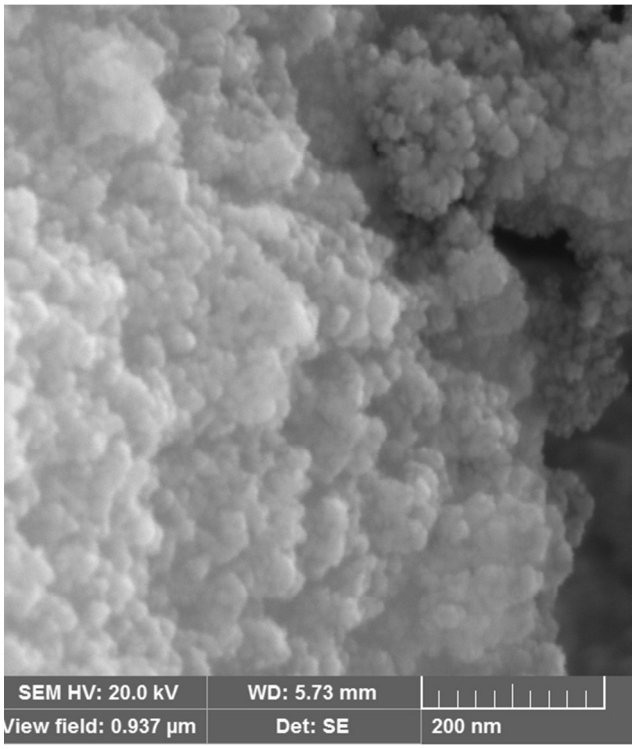

Fig. 1.

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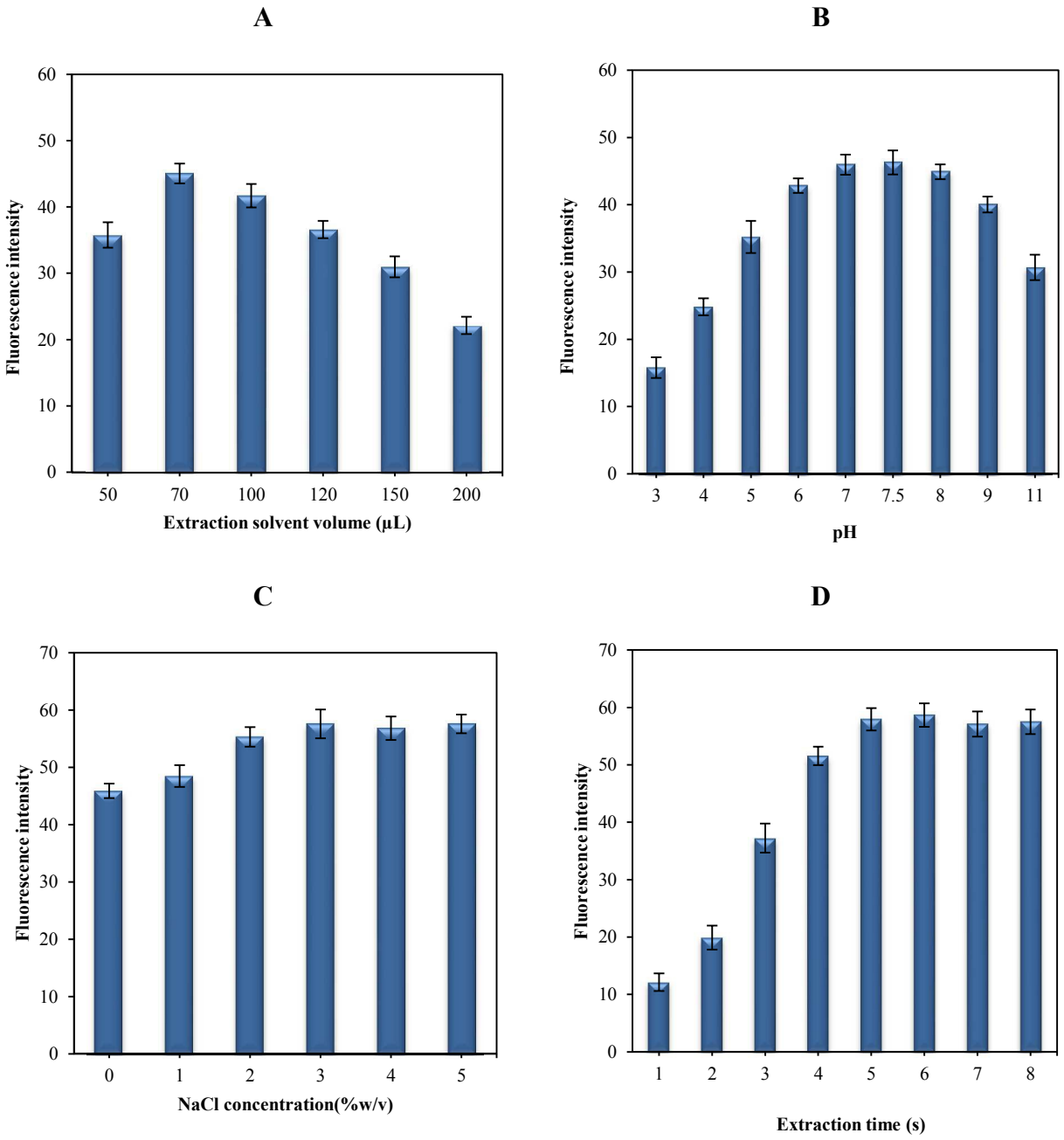

Fig. 2.

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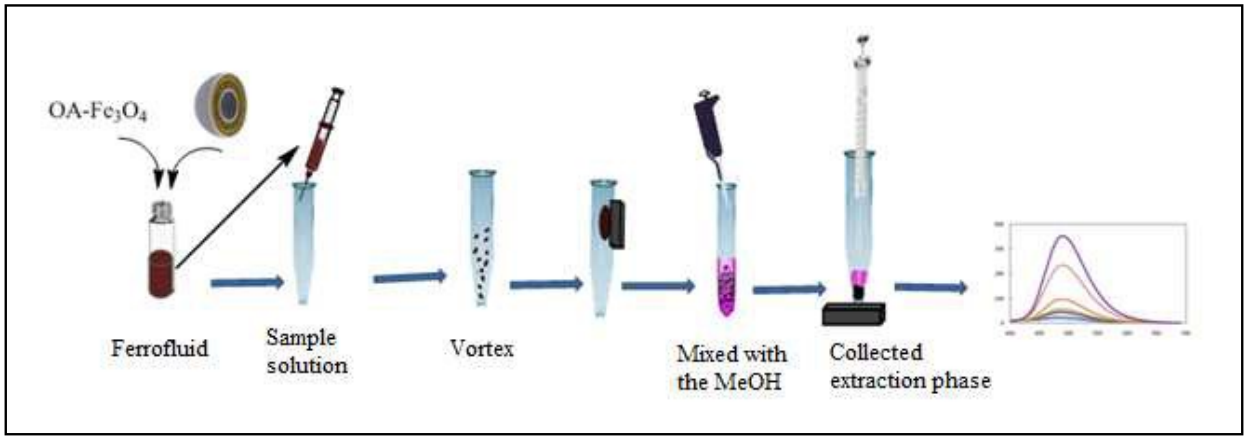
Graphical Abstract



The proposed method focuses on the development of the supramolecular application as carrier for ferrofluid base microextraction coupled to spectrofluorimetry for the determination of levofloxacin in biological samples. This method lacks tedious steps of conventional microextraction methods such as, centrifugation, refrigeration and thawing of organic solvent.

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