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ARTICLE TYPE

Application of of ionic-liquid combined with ultrasonic-assisted dispersive gold nanoparticles for micro-solid phase extraction of unmetabolized pyridoxine and folic acid in biological fluids prior to high-performance liquid chromatography

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In this study, facile and efficient protocol was suggested for gold nanoparticles (Au NPs) synthesis and its subsequent transfer to aqueous solution by application of 1-hexyl-3-methylimidazolium bis (trifluoromethylsulfonylimide) following vigorous shaking via ultrasonic waves. The produced sorbent was applied for micro-solid phase extraction of pyridoxine and folic acid from biological samples. This combination is associated with high extraction efficiency for preconcentration and determination of pyridoxine and folic acid in biological samples. The dependency of extraction efficiency to traditional parameters like extraction time, sample pH, volume of ionic liquid, amount of Au NPs, sample volume, salting effect, desorption time and eluent volume were optimized using experimental design combined with response surface methodology. Under optimized conditions, the suggested method shows high linear range (20-500 ng mL^{-1}) with limits of detection (LODs) of 3.4 and 4.8 ng mL^{-1} for pyridoxine and folic acid, respectively. The precision values (RSDs, n=5) were 2.5 and 3.3% at the concentration level of 50 ng mL^{-1} of both species. while the validated method was successfully used for pyridoxine and folic acid quantification with relative recoveries in acceptable range of 83-104% and RSDs less than 4.1%.

Introduction

B vitamins exist in an extended range of foods. They are water-soluble vitamins and plays an important role in the body. Several neurotransmitters need pyridoxine (B_6 vitamin) for synthesis. It is needed to maintain the health of nerves, skin and red blood cells. Folic acid (vitamin B_9) is essential for numerous bodily functions. Recently, growing time and attention was focused to design novel protocol for the simultaneous determination of vitamins [1-4]. The major part of studies are based on analysis of food matrices, drinks and vitamins supplements [5-7], while scare researches focused on the validation of analytical approaches developed for vitamins in biological fluids [8-10]. These limitations and lengthy sample preparation steps correspond to clinical protocols and lower sensitivity, encourage the researchers for development and design of method for the quantification of pyridoxine and folic acid in biological samples. Various extraction and microextraction techniques such as liquid-liquid extraction (LLE) [11], solid phase extraction (SPE) [12] and dispersive liquid-liquid microextraction (DLLME) [13] have been used for preconcentration of various vitamins. DLLME method is based on the complete dispersion of the extractant solvent into the sample in order to increasing the contact area between the extractant and the solution so that the equilibrium state is achieved in a short time [13]. Conventional DLLME has ternary component-system mode, in which a high density solvent like chlorinated solvents as extractant is dissolved in a disperser solvent that must be miscible with both extractant and sample solution, in order to facilitate homogeneous dispersion. However, chlorinated solvents are toxic and environmentally unfriendly. The limitations of conventional extraction techniques such as application of large volume of pure toxic organic solvents encourage analytical chemists to miniaturized extraction techniques based on green solvents which are usable in wide range of separation and preconcentration procedures [14-16]. Room temperature ionic liquids (RTILs) (known as green solvents) have environmentally friendly properties such as air and moisture stability, extremly low vapor pressure, tunable miscibility in water [17-19] that are most preferable for liquid phase microextraction and solid phase microextraction, which allows more stable droplets compared to traditional organic solvents [20-22]. For example, several techniques have been used based on liquid phase microextraction (LPME) such as single drop microextraction (SDME) using organic solvents as extractants, but high instability of the drop and poor precision levels have been reported as a result of the low viscosity and evaporation of the organic extractant. Therefore, ILs have been proposed for SDME because the advantages such as high viscosity, low vapor pressure and good thermal stability, avoiding evaporation and irreproducible losses of the solvent in HS-SDME, and their immiscibility with water (for some ILs) allows them to be conveniently adopted for DI-SDME [23]. Furthermore, the first application of IL as extractants in HF-LPME was evaluated by Peng et al. in 2007 [24] as the intermediate-extractant solvent after immobilization in the pores of an HF membrane. The first application of IL in DLLME was

suggested by Zhou et al [25] (temperature-controlled IL-DLLME (TC-IL-DLLME). Another modification of LPME, similar to TC-ILDLLME, and termed cold-induced aggregation microextraction (CIAME), was proposed by Gharehbaghi et al [26] using two ILs as extractant solvent and as an additive for enhancement of the extraction procedure.

Mixed hemimicelle solid-phase extraction methods (MHSPE) is based on loading of ionic surfactants on metal oxides surfaces to form hemimicelles and admicelles (monolayer and bilayer respectively) at the surfactants concentration less than critical micelle concentration. The surfactant hydrophobic groups in hemimicelle region and polar (mainly ionic) groups of the surfactants in admicelle region make favor the nonpolar or polar analytes extraction. The MHSPE method benefit from remarks including high extraction yield and high breakthrough volume and easy elution of analytes, while it suffer from laborious and time-consuming procedure, particularly for large-volume samples. A new kind of IL describe the formation of micelles in aqueous solution and replace as surfactants, while supply all advantages [27-29]. Au NPs generally stabilized with surfactant to prevent their agglomeration [30].

In this study, ionic liquid composed of imidazolium unit and hydrophobic group used as stabilizing agent for the mono dispersion of Au NPs in an aqueous medium. As a result, application of ionic liquid combined with ultrasonic assisted dispersive Au NPs for micro-solid phase extraction (μSPE) posses unique advantages such as: (i) high density of ionic liquid facilitate settling extraction phase after microextraction; (ii) cationic unit of ionic liquid interact with gold nanoparticle (containing delocalized electron) and lead to mono dispersion of sorbent and increasing the extraction efficiency of analytes (iii) the amphiphilic nature of ionic liquid lead to improvment in stability of Au NPs following micelle formation and high extraction efficiency of pyridoxine (B_6 vitamin) and folic acid (B_9 vitamin). To our knowledge, there is not any report for usage of ionic liquid in combination with Au NPs for microextraction and preconcentration of vitamins from biological samples. So, the suggested method with distinguished properties like rapid, easy, affordable and accessible can be used for determining target analytes in various real biological samples. A rapid microextraction method based on IL for selective vitamin B determination is presented in this work. Due to the low solvent consumption and the selection of IL as organic phase, the developed method is in good agreement with green chemistry principles. Compared with conventional methods, the method presents many advantages such as low viscosity, quick phase separation and high extraction efficiency. The proposed method is simpler and faster, compared to conventional solid-phase extraction (SPE) (which usually includes a number of laborious steps, such as sorbent conditioning, rinsing the sample, washing and elution of the analytes). The suggested method indicate that the proposed procedure is simple, fast, interference-free, selective and environment-friendly, and it can be used for B_6 and B_9 selective preconcentration and determination in biological samples. This technique combined with HPLC-UV shows a good limit of detection and a wide calibration range with a reduced amount of sample, IL and sorbent, while using low run and analysis in biological samples against other reports that used food samples. The method detection limit is comparable to, or better than, others extraction methods prior HPLC–UV analysis reported for B_6 and B_9 determination.

Experimental

Reagents and solutions

All chemicals such as chlorauric acid hydrated ($HAuCl_4.4H_2O$), orthophosphoric acid (85%, w/w), acetic acid, sodium hydroxide, hydrochloric acid, aceton, starch, ethanol and sodium chloride were purchased from Merck (Darmstadt, Germany). Analytical-grade1-Hexyl-3-methylimidazolium hexafluorophosphate, Hexyl-3-butylimidazolium hexafluorophosphate, 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonylimide) were purchased from IoLiTec Company (Heilbronn, Germany). Acetonitrile, water and methanol (HPLC-grade), pyridoxine hydrochloride and folic acid were supplied by Sigma-Aldrich (St. Louis, USA).

Apparatus

The chromatographic measurements were carried out with an Agilent technologies (Agilent, Wilmington, USA) 1100 HPLC system equipped with Standard Micro Auto Sampler (model G1313A), Micro Vacuum Degasser (model G1379A), Quaternary Pump (model G1311A), Series Multiple Wavelength Detector (model G13658) and a Zorbax SB-C8 (250 mm×4.6 mm, 5 µm) (Agilent) column. The mobile phase for the determination of B_6 and B_9 vitamins was of acetonitrile-acetate buffer (40:60, V/V) and passed through column with flow rate of 1 mL min^{-1}.

The chromatographic calculations were performed using a Chemstation data handling system. An ultrasonic bath with heating system (Tecno-GAZ SPA, Italy) at 40 kHz of frequency and 500 W of power was used for the ultrasound-assisted extraction. A Hermle Labortechnik GmbH centrifuge (model Z206A, Germany) was used to accelerate the phase separation. The morphology of synthesized nanoparticles was followed by scanning electron microscopy (SEM, Hitachi, Japan) under an acceleration voltage of 30 KV. X-ray diffraction (XRD) pattern was recorded by an automated Philips X'Pert X-ray diffractometer (Philips, Holland, Netherland,40 kV and 40 mA).

Synthesis of gold nanoparticles

The gold nanoparticles (Au NPs) were synthesized according to previous reports [31,32]. Initially, 1 mL aliquot of 0.1 mol L^{-1} $HauCl_4.4H_2O$ was added into 50 mL of aqueous solution containing 0.2% W/V of starch at the ultrasonic bath. After about 20 min, the mixture was heated at 70 ℃ for 6 h and then filtered and washed several times with distilled water.

Microextraction procedure

μSPE technique was performed as follows: 8 mg of Au NPs and 100 μL of 1-hexyl-3-methylimidazolium bis (trifluoromethylsulfonylimide) were placed into a 15 mL screw cap glass tube with conical bottom. Then, 10 mL of aqueous sample containing analytes (200 ng mL^{-1} of each compound) at pH 5 in the absence of any electrolyte were added. The insertion of tube in ultrasonic bath (for 10 min) lead to complete dispersion of ionic liquid and Au NPs in solution. Then, the mixture was then centrifuged for 5 min at 3500 rpm. The Au NPs and ionic liquid were sedimented at the bottom of the tube and aliquot was decanted. Finally, the elution of the target analytes was accomplished using 100 μL of methanol following insertion of sedimented phase in ultrasonic bath for 10 min and subsequently was centrifuged again. In the next step, 25 μL of this solution was injected into HPLC-UV system for analysis.

Results and discussion

Characterization of gold nanoparticles

SEM analysis of Au NPs show morphology and spherical structure of sorbent (Fig. S1A). The high surface area correspond to this nanoparticle and presence of porous with different size suitable for sorption and microextraction. The crystal phase of Au NPs was studied by X-ray diffraction (XRD) analysis (Fig. S1B) and it was revealed that peaks correspond to (111), (200), (220) and (311) planes of the standard face-centered cubic phase of gold nanoparticles.

Choice of material

Critical problem for using the nanoparticles in extraction process is the agglomeration of sorbent in aqueous medium. Most of developed methods utilize thiols as capping agents in order to improve nanoparticle stability. Thiolated modifiers provide enhanced stabilization through the sterical interaction between surface layers and/or their charge repulsion [30]. Ionic liquids (ILs) have emerged as a new class of sorbent coatings in SPME. ILs as porogens are used instead of organic molecules in microextraction systems and its cation and anion portions of the IL have pore structure [33,34]. However, the application of ILs in micro-solid-phase extraction is promising field. This optimization followed the following principles of IL selection (1) on the basis of various ionic liquid with various anion and cation; (2) ILs must be liquid under the experimental conditions and (3) formed hydrophobic ILs have a greater density than water for easy sedimentation after extraction process.

In this work, ionic liquid was used as stabilizing agent for the preparation of ionic liquid (IL)-coated Au NPs and also as the extraction phase for micro solid-phase and preconcentration of pyridoxine and folic acid from biological samples. Due to the high surface area and excellent adsorption capacity of Au NPs after modification with IL, satisfactory extraction recoveries were achieved with only 8 mg Au NPs, 100 μL IL in 10 mL solution at pH 5 and 10 min for extraction time. The suggested method benefit from advantages correspond to both IL and solid phase. To our knowledge, this is the first report on ultrasonic assisted dispersive gold nanoparticles modified with ionic liquid based μSPE for the preconcentration of pyridoxine and folic acid from biological fluids. The effect of ILs and Au-NPs on the adsorption of analytes, amounts of ionic liquid (0–200 μL) and Au-NPs (0–15 mg) were investigated by central composite design. The adsorbed analytes were determined using high performance liquid chromatography (HPLC-UV) analysis. As well, it can be seen that, the extraction efficiencies obtained with suggested sorbent were much better in comparison with the use of IL or Au NPs alone (Fig. 1).

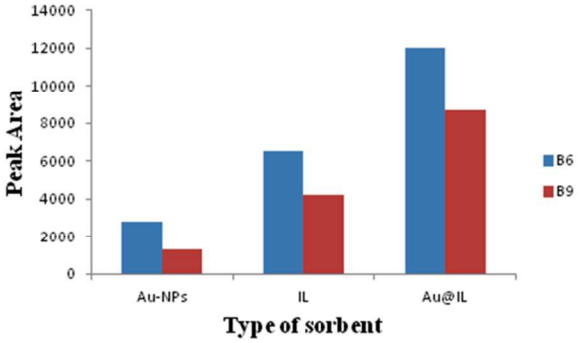


Fig. 1. Comparison of Au-NPs, IL and Au-NPs@IL

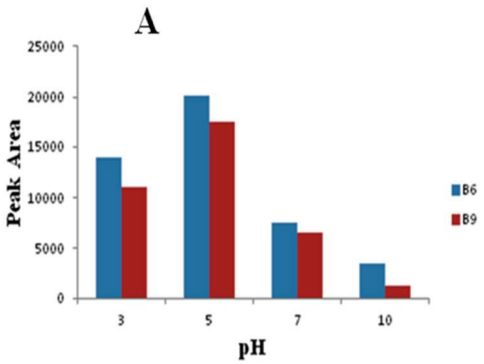
Adsorption mechanism

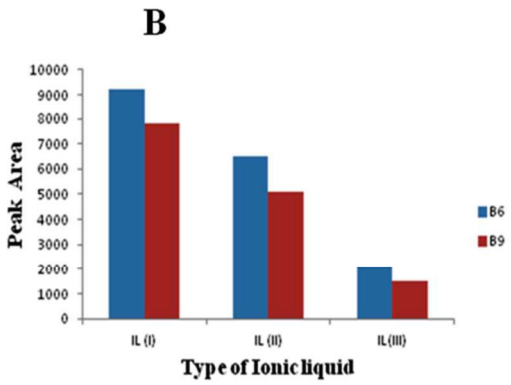
In the absence of ILs, the analytes hardly adsorbed onto the Au NPs surface. In the presence of IL, the adsorption amounts of analytes increased remarkably with increasing IL amount up to 100 μL. Electrostatic attraction between the cationic portion of ionic liquid and the oppositely charged group on the metal NPs leads to the formation of shell of ionic liquid on the surface of sorbent. The high adsorption of analytes is due to the presence of hydrophobic interaction of analytes with the carbon chain of ionic liquid. An imidazole ring of ionic liquid can lead to $\pi - \pi$ interactions with the pyridoxine and folic acid. The hydrophobic interaction between the tails of ionic liquid hydrocarbon chains lead to enhance in analytes extraction [17, 28, 33,34].

Optimization of procedure

To ascertain the full detail of the optimization approach, pH , type of IL and eluent have primarily optimized by univariate method. The influence of pH as one of the most important factors on pyridoxine and folic acid extraction efficiency were investigated in the range of 3.0–10.0. It can be influenced the

adsorption behavior of target analytes and the change of charge density on the Au-NPs surface. The results (Fig. 2A) show pyridoxine and folic acid effectively extracted in pH 5.0. This can be attributed to the fact that in pH 5, Au NPs surface was negatively charged and the $[C_6MIM]^+$ was adsorbed onto the Au-NPs surface. At pH 3.0, the reactive sites of analytes are protonated while the increase in pH leads to their deprotonation and achievement of neutral association (pK_a value of pyridoxine and folic acid are 5.5 and 8.2) that accelerate analyte mass transfer to extraction phase. In pH above 5.0, analytes are not in the neutral form and lead to reduction the extraction efficiencies. Therefore, pH 5.0 selected as optimum point for subsequent experiments. The type of ionic liquid strongly affect on extraction efficiency depend on physicochemical properties of the ionic liquid. IL are selected according to their good and reasonable properties such as high density (for easy separation of sedimentary phase from aqueous sample after extraction process) and capability to interact with analytes (creation of hydrophobic interaction with analytes). Therefore, hydrophobic ILs based on imidazolium cation and PF_6^- or $N(SO_2CF_3)_2^-$ anion [16] are good candidate for extraction process. The IL with different carbon chain lengths, may have different hydrophobic interactions with the target analytes. In this method, ILs such as 1-hexyl-3 methylimidazolium hexaflourophosphate, 1-butyl-3-methylimidazolium hexaflurophosphate and 1-hexyl-3-methylimidazolium bis (trifluoromethylsulfonylimide) $[C_6MIM][N(SO_2CF_3)_2]$ were selected for microextraction procedure. A higher peak area signal following application of $[C_6MIM][N(SO_2CF_3)_2]$ as extraction solvent is related to the lower solubility in water and higher density of $[C_6MIM][N(SO_2CF_3)_2]$. So, $[C_6MIM][N(SO_2CF_3)_2]$ was selected for subsequent experiments (Fig. 2B). The eluent significantly affect the extraction of the target analytes. In this regard, elution power of organic solvents including methanol, acetone and acetonitrile were studied and the respective results are presented in Fig. 2C. Under the same extraction conditions, methanol provided the highest extraction efficiency due to highest desorption ability for pyridoxine and folic acid. Therefore, methanol was used as the eluent solvent in the following experiments.





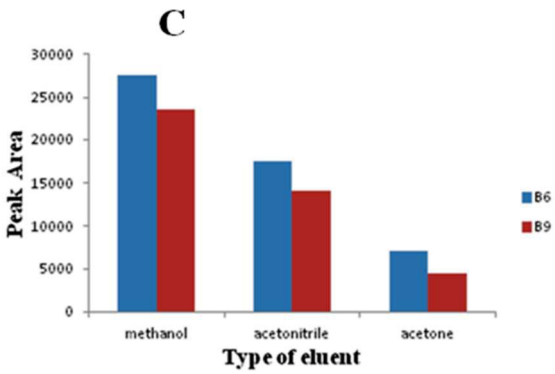


Fig. 2. (A) Effect of pH on extraction efficiency; (B) Effect of type of Ionic liquid on extraction efficiency; IL (I): 1-Hexyl-3-methylimidazolium bis(trifluoromethylsulfonylimide); IL (II): 1-Hexyl-3-methylimidazolium hexafluorophosphate ; IL (III): 1-Hexyl-3-butylimidazolium hexafluorophosphate; (C) Effect of various eluent on extraction efficiency.

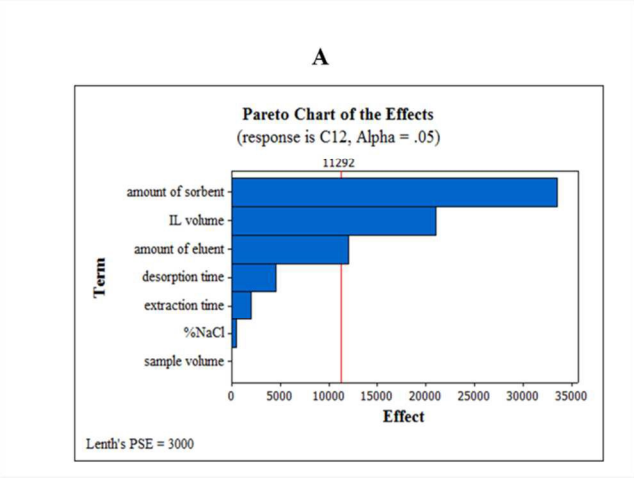
Screening of the effective parameters using fractional factorial design

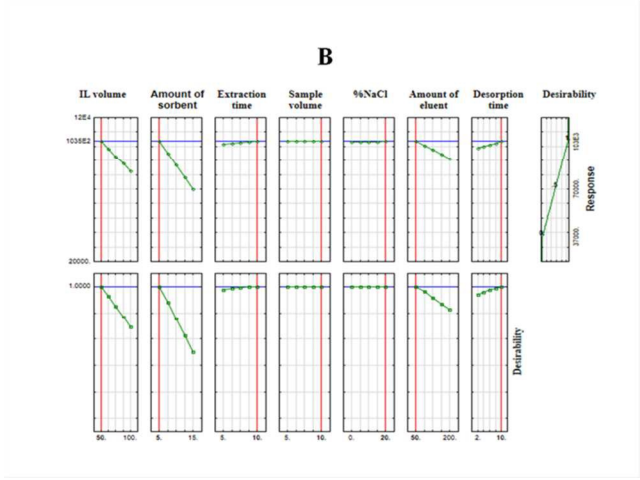
Recently, experimental design methods have been extensively applied for optimization of extraction efficiency. Fractional factorial design [35, 36] have been mainly used for screening the main variables and their interaction following analysis of variance (ANOVA). In this approach, the adequacy of the regression model is judged according to lack-of-fit test. ANOVA can compare the treatment-induced variation (change in the combination of variable levels) with the random errors inherent in the response measurements [37]. This comparison is executed from the F value and provides information about the mean-square of model and residual error. The high extraction efficiency simply obtained by optimization of effects of several experimental parameters, such as the amount of $[C_6MIM][N(SO_2CF_3)_2]$ and Au NPs, the ultrasound extraction time, %NaCl, eluent and sample volume. The Pareto plot (Fig. 3A) strongly support that factors including the amount of sorbent, eluent volume and ionic liquid volume are the most important factors affecting the extraction

efficiency. So, three important factors were optimized using central composite design in combination with response surface methodology in the next step. In this study, ultrasonic agitation was vital to adequately disperse the ionic liquid and Au-NPs into the sample solution. The extraction efficiency was progressed following exposure to ultrasonic waves at different time and maximum extraction efficiency was achieved after 10 min sonication (Fig. 3B). In the extraction process, the desorption time significantly influence on the extraction efficiency of analytes (Fig. 3C). The desorption process should equilibrate for sufficient time to achieve satisfactory extraction efficiency. As a result, 10 min was an efficient time for desorption of analytes, therefore selected as optimum value for further experiments. In two liquid phase systems, the enrichment factor can be improved by increasing the volume ratio of acceptor to donor phases according to the following equation [16]:

$$ER\% = \left(\frac{C_f \times V_f}{C_{aq} \times V_{aq}}\right) \times 100 = EF \times \frac{V_f}{V_{aq}} \quad (1)$$

Where C_f and C_{aq} are the initial and final concentration of analytes in organic and aqueous phase, respectively. V_f/V_{aq} is the volume ratio of the organic phase to the aqueous sample solution. At higher volume of sample, a significant increase was observed that is probably due to change of extraction efficiency according to equation 1 significantly affect the enrichment factor. Therefore, it is reasonable that higher sample volume lead to higher extraction efficiency [16]. So, 10 mL of sample volume was used to obtain high EF.





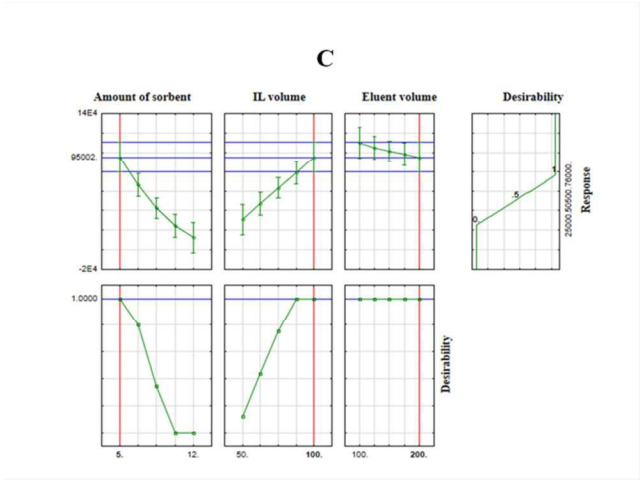


Fig 3. (A) Pareto plot; (B) optimum plot of fractional factorial design; (C) optimum plot of central composite design.

Central composite design

Central composite design (CCD) in combination with response surface methodology (RSM) are mathematical and statistical techniques which allow the determination and evaluation of the relative significance and interaction of all variables on the process [38-40]. ANOVA help the researchers to attain useful information about content of influence (as sole or pair situation) and also to fit the experimental results to the polynomial model equation that judged according to the determination coefficient (R^2). F-test was used to estimate the statistical significance of all terms in the polynomial equation within 95% confidence interval. The critical factors and the nature of the response surface in the experiment is used for optimization of variables. The efficiency of the model was checked using ANOVA according Fisher's statistical analysis. The regression coefficients in the response surface model for the linear, quadratic and interaction effects of the variables are shown along with F- and P-value (Table 1). A p-

value less than 0.05 confirm significant contribution of each variable on response at 95% confidence level. The polynomial model equation suitably was confirmed by high and reasonable value of the coefficient of determination (R^2 = 0.975 and adjusted R^2 = 0.940). This model which consists of three main effects, couple of three factor interactions and three curvature effects is shown in following equation:

$$Y = 36286.3 - 10511.6\ X_1 + 8974.5\ X_2 - 2527.3\ X_3 + 4197.1\ X_1^2 - 115.4\ X_2^2 + 447.1\ X_3^2 - 8375.0\ X_1 X_2 - 625.0\ X_1 X_3 - 1875\ X_2 X_3 \quad (2)$$

On the basis of these calculations, maximum peak area was obtained at optimum conditions set as: 100 μL of $[C_6MIM][N(SO_2CF_3)_2]$, 10 min ultrasonic time and 10 min desorption time for agitating of the 10 mL sample solution in pH 5 in the presence of 8 mg of Au NPs without addition of salt and 100 μL of methanol as eluent (Fig. 3 B and C).

Table 1- Analysis of variance (ANOVA) of the quadratic response surface model.

Source	Degree of freedom	Sum of square adjusted	Mean square adjusted	F	P
interaction	3	592375000	197458333	17.62	0.001
Residual Error	8	89657060	11207133		
Lack of fit	5	79657060	15931412	4.78	0.114
Pure Error	3	10000000	3333333		
Total	19	3571750000			

The low extraction efficiency of analytes in the absence of $[C_6MIM][N(SO_2CF_3)_2]$ confirm the critical role of ionic liquid in the extraction of target analytes. The effect of ionic liquid volume (Fig. 4A) reveal that the extraction efficiency has negative correlation with the volume of $[C_6MIM][N(SO_2CF_3)_2]$, that may be related to the remarkable enrichment ability of IL. In higher volume of IL (above 100 μL), according to equation 1, lead to reduction of enrichment factor, therefore, 100 μL of IL selected as optimum point for further experiments. The nanosized sorbent have greater surface areas, and then satisfactory results may be achieved with lesser amounts of nanosized sorbent. Fig. 4B shows that only 8 mg of Au NP are enough for the extraction of analytes under the optimal conditions. The higher values of peak area is probably due to increase in the rate of sorption which emerged from higher surface area and the availability of more active sorption site [31,32]. At value higher than 8 mg, all the Au NPs may not separate effectively in the same duration, which leads to a decrease in recoveries. Therefore, 8 mg Au NPs was used in further experiments. As can be seen (Fig. 4C), extraction efficiency was increased in the acceptor phase volume of 100 μL. The reason for this effect might be that a small volume of methanol cannot effectively elute the analytes, while large volume due to subsequent dilution lead to decrease in the concentration of the analytes in the organic phase [16]. Therefore, 100 μL of methanol was chosen as the optimum volume of the eluent for subsequent experiments.

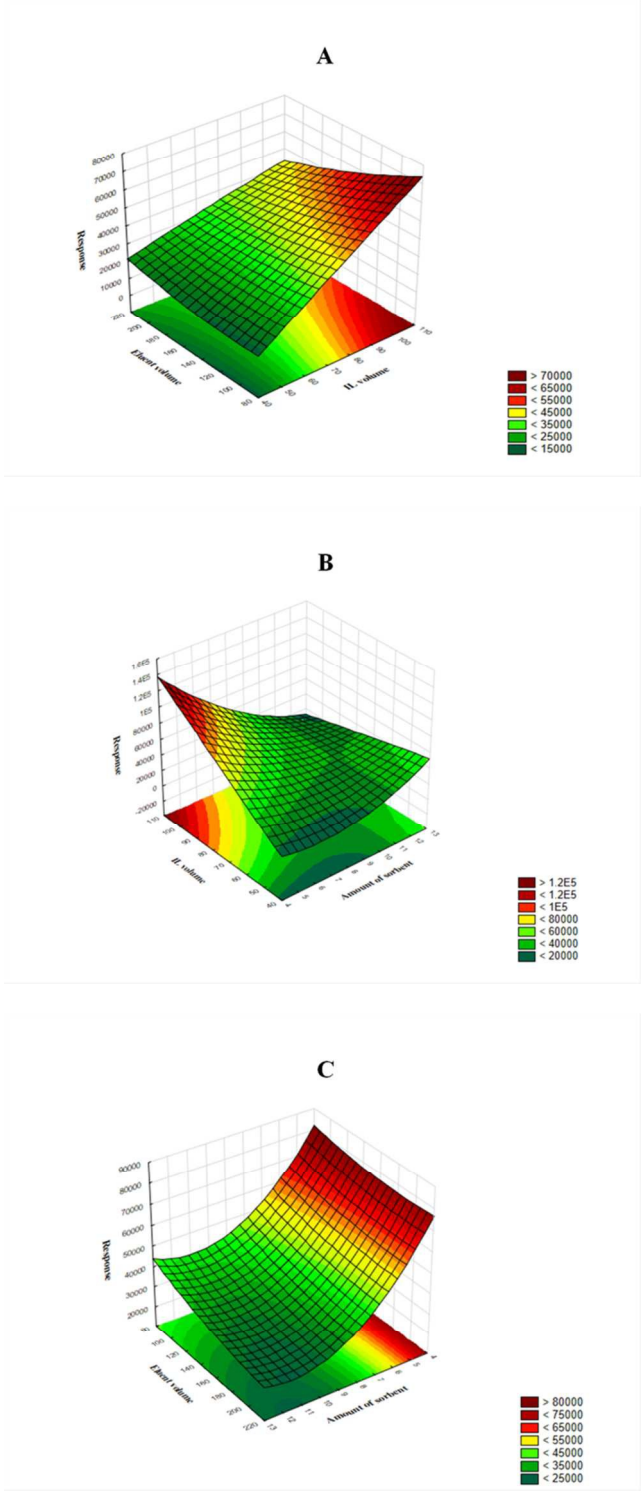


Fig. 4. Response surface plot for central composite design of (A) Eluent volume-Ionic liquid volume ; (B) Ionic liquid volume –Amount of sorbent ; (C) Eluent volume- Amount of sorbent.

Evaluation of the method

In order to evaluate the performances of the present method, dynamic linear range, limits of detection (LODs) and quantification (LOQs) were studied (Table 2). As it was seen, the present method exhibits satisfactory linear response in the concentration range of 20–500 ng mL^{-1} with good correlation coefficients (r) > 0.999. The LODs and LOQs are in the range of 3.4–4.8 ng $mL^{-1}$and 11.6-16.1 ng mL^{-1} for pyridoxine and folic acid, respectively. The intra- and inter-day precision of the present method at two level concentration viz 50 and 200 ng mL^{-1} were examined (i.e. five replicate in a day) and the inter-day RSDs were obtained by conducting the same experiments at consecutive five days. It was found that intra and inter-day RSDs were in the range of 1.8–3.3% and 3.2–4.9%, respectively, that indicate the acceptable precision.

Table 2. Analytical performance of microextraction method.

Compound	RSD (%) (n=5)	Dynamic linear range (ng mL^{-1})	LOQ (ng mL^{-1}) (n=5)	LOD (ng mL^{-1}) (n=5)	Intraday RSD (%)	Inter-day RSD (%)
Pyridoxine	2.8	20-500	11.6	3.4	1.8	3.2
Folic acid	3.9	20-500	16.1	4.8	2.9	3.8

Table 3. Comparison of the proposed method with other methods obtained for the extraction and determination of pyridoxine and folic acid.

Extraction method	Sample volume	Real sample	LOD (ng mL^{-1})	LOQ (ng mL^{-1})	DLR (ng mL^{-1})	RR (RSD) %	Detection system	Refs
SPE-SB-cartridge	10 mL	vitamin-fortified fruit juices and fruit drinks	40	60	-	78-93 (2.3)	HPLC-DAD	41
Determination of analytes without any application of extraction	10 mL	pharmaceutical preparations	21.17-30.48	76.95-101.1	-	59-89 (2.3-2.7)	HPLC-DAD	42
DLLME			0.9	3	3-150	98-114 (2.12)	HPLC-UV	43
UA-DIL-μSPE	10 mL	Sour Cherry Juice	3.4-4.8	11.6-16.1	20-500	83-104 (below 4.1)	HPLC-UV	Present work

The present method was further compared with the reported methods for the preconcentration/determination of analytes in various media [41-43]. The LODs and RSDs obtained by the present method are similar to other reported methods (Table 3). Therefore, it can be concluded that the present method is suitable for the determination of pyridoxine and folic acid in biological samples. However, other reported methods for the determination of target analytes have disadvantages such as time consuming, insensitivity, difficult operation, matrix effect, low accuracy, toxic and expensive solvents and etc. To overcome these limitations in the determination and extraction of pyridoxine and folic acid, application of of ionic-liquid combined with ultrasonic-assisted dispersive gold nanoparticles is the best option. The method was successfully applied to the determination of vitamins in biological samples.

Analysis of real samples

The proposed method was used for separation/preconcentration and subsequent determination of pyridoxine and folic acid in biological samples (urine, plasma and saliva). The samples were prepared according to the procedure extensively described elsewhere [10,44]. A small portion of eluent was injected into HPLC and their analytes content were analyzed. Due to the absence of any target analytes in the above mentioned real samples, standard addition method was applied following spiking known concentration of analytes to real samples. Fig. S2 illustrates the chromatograms of the plasma sample. The reasonable and acceptable relative recoveries (RR, %) in the range of 83-104% and RSD% (n=5) lower than 3.9% for biological samples confirm the applicability of under study method for real sample analysis (Table 4).

Table 4. Results obtained from analysis of biological samples.

		Urine sample (n=5)	Plasma sample (n=5)	Saliva sample (n=5)
Analytes	Spiked (ng mL^{-1})	RR (RSD) %	RR (RSD) %	RR (RSD) %
Pyridoxine	50	96 (2.7)	84 (3.5)	95 (2.9)
	200		89 (3.1)	104 (2.5)
Folic acid	50	87 (3.5)	83 (3.9)	91 (3.4)
	200	89 (3.1)	88 (4.1)	94 (2.9)

Selectivity study

The selectivity study was performed by analysis of 10 mL of sample solution containing 100 ng mL^{-1} of each target analytes and interferences (B_1, B_2 and B_{12} vitamins) at 100 fold concentration were added to the analytes and subsequently the recommended procedure was carried out. It was observed that suggested method has maximum relative recoveries for pyridoxine (92%) and folic acid (88%).

In addition, high extraction selectivity of Au-NPs@[C_6MIM] for the determination of pyridoxine and folic acid is due to the high

enrichment factor of Au-NPs@[C_6MIM] (130 and 98 for B_6 and B_9 vitamins) in comparison with the enrichment factor of IL (70 and 53 for B_6 and B_9 vitamins) or Au-NPs (35 and 20 for B_6 and B_9 vitamins) alone. This remarkable increase in enrichment factor may be due to the existence of ionic liquid on the surface of Au NPs. The imidazole ring can lead to π - π interaction with functional groups of analytes and enhance the adsorption of target analytes. The hydrophobic functional group on the surface imidazole ring of ionic liquid lead to hydrophobic interaction with analytes. The aromatic π system of analytes can interact with the free electron of Au NPs and increase enrichment factor values [17,28,33,34].

Conclusions

It can be concluded that ionic liquid not only acts as a stabilizing or protecting agent but also as organic phase in order to extraction from aqueous medium. Such a combination does not only provide effective stabilization but also allows the preconcentration of analytes using Au NPs. In the present study, a simple, reliable, environmentally friendly and rapid procedure was developed for the determination of pyridoxine and folic acid. The proposed method is fast based on use of only a small amount of sorbent , ionic liquid and possible higher good recoveries. It could be considered that this method is very promising for the extraction of analytes form complex matrices following optimization of extraction parameters.

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