Simultaneously determination of copper and zinc in human serum and urine samples based on amoxicillin drug by dispersive ionic liquid- liquid microextraction coupled to flame atomic absorption spectrometry

Kian Azami [a,*] and Seyed Mojtaba Mostafavi [b]

[a] *Department of Pharmacology and Toxicology, Pharmaceutical Sciences Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-6451, Tehran, Iran*
[b] *Department of Chemistry, Iranian-Australian Community of Science, Hobart, Tasmania, Australia*

ABSTRACT
In this work the effect of amoxicillin on copper and zinc (Cu and Zn) deficiency was evaluated by determining of Cu and Zn concentration in human serum and urine samples. By dispersive ionic liquid cloud point extraction procedure (DIL-CPE), 0.03 g of pure amoxicillin drug was added to mixture of 0.1 g of hydrophobic ionic liquid and 0.2 mL acetone which was injected to 2 mL of serum or urine samples which was diluted with DW up to 10 mL. The cloudy solution was shacked for 7 min and Cu and Zn ions was extracted based on sulfur group on amoxicillin ligand at pH of 7 by DIL-CPE. Then, the solution centrifuged and after back extraction with I mL of nitric acid (0.2 M), the remained solution was determined by flame atomic absorption spectrometry (F-AAS). The enrichment factor (EF), LOD and linear range (LR) for copper and zinc was obtained (9.92; 9.81), (28.5 µg L^{-1}; 15.2 µg L^{-1}) and (100 -505 µg L^{-1}; 41- 153 µg L^{-1}), respectively. The results showed us, the concentration of the Cu and Zn ions can be decreased by increasing amoxicillin drug dosage in human body. The mean value for serum copper/zinc ratio was obtained 1.11 ± 0.28. The DIL-LME method was validated by ICP-MS analysis and spike of real samples for Zn and Cu ions in serum and urine samples.

1. Introduction

Copper and zinc are essential ions for the human body. Copper has different forms such as CuS, CuS_2, $CuFeS_2$ and $CuSO_4.5H_2O$ in environment. The high concentrations of Cu and Zn more than 2 ppm in human blood are toxic and the range between essentiality limit and toxicity form is very small. Copper and zinc has normal ranges between 0.8-1.6 mg L^{-1} in different ages for the human serum and urine samples [1]. Zinc is used as cofactor for many enzymes in the human body. Zinc effect on cell structure of human body, the structure of protein, the gene expression, the immune system, and the growth in children. Zinc deficiency cause to many diseases such as, diarrhea, a compromised immune system, night blindness, hair loss, and the taste alterations [1-

*Corresponding Author: Kian Azami
Email: Kianazami@yahoo.com
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2]. Total zinc is about 40 mg per day for adults over 20 years. Copper helps to transportation of iron, energy production, the pigmentation of skin, hair, and eyes. Copper acts as an antioxidant for defending of cell damage which was caused by free radicals. Copper deficiency cause to anemia, low white blood cell count, loss of myelin, multiple sclerosis (MS), the loss of pigmentation, the impaired growth and osteoporosis [3]. The copper intake from food/water or supplements has 10 mg per day and over limit accumulates in the liver. Based on previous studies, a ratio of 8-15 mg of zinc for every 1 mg of copper reported. However, this ratio seems to be more important for human bodies mechanism [1-4]. Zinc is absorbed in the small intestine by a carrier-mediated mechanism. Under normal physiologic conditions, transport processes of uptake are not saturated. The mean value for serum copper was normal range (17.47 ± 3.31 μmol L^{-1}; 111.32 μg dL^{-1}), and the mean value for serum zinc was at the lower edge of the normal value (12.24 ± 1.04 μmol L^{-1}; 80.01 μg dL^{-1}), while the mean value for serum copper/zinc ratio was 1.44 ± 0.31 ranging from 0.65 to 2.67 [5-7]. Copper and zinc concentration in liquid phase can be determined directly by inductively-coupled plasma atomic emission spectrometry (ICP-AES) [8] or electrothermal atomic absorption spectrometry (ET-AAS) [9] with low detection limit (LOD). The conventional flame atomic absorption spectrometry (F-AAS) [10] was used in many laboratories and had low interferences ions as compared to ICP-AES or ETAAS. As difficulty matrix in human blood or serum patients a sample preparation is require for preconcentration/separation/extraction of ions from samples before determination. Many procedures for metal determination in water and human matrix was used with different analytical techniques and reagents [11,12]. The Liquid–liquid extraction by using salophen as an complex reagents [13], the sandwich supported liquid membrane [14], the modified carbon based solid phase extraction [15], the solid-phase extraction on MWCNTs - D2EHPA-TOPO [16], dispersive liquid–liquid microextraction of copper (II) by oxinate chelate [17] and dispersive liquid-liquid microextraction-slotted quartz tube-flame atomic absorption spectrometry [18] are well-known procedures for preconcentration and separation of trace copper or zinc from different matrix.

In this work, the pure amoxicillin antibiotic drug was used as chelating agent for copper and zinc (Cu/Zn) extraction in human serum and urine samples by DIL-CPE procedure at optimized pH. The Cu and Zn deficiency was evaluated by determining its concentration in human serum/ urine samples by F-AAS. The Cu and Zn ions can be separated from liquid phase by hydrophobic ionic liquid as green solvent.

2. Experimental
2.1. *Apparatus and Reagents*
A flame atomic absorption spectrometer model, with an air-acetylene flame, was used for copper (II) and zinc (II) determination in human serum and urine samples (Shimadzu, F-AAS, model 680, Tokyo, Japan). Copper based on wavelength 324.7 nm, slit 0.5 nm and lamp current 3.0 mA (1-5 mg L^{-1}) were selected. Zinc lamp with wavelength 213.9 nm, slit 0.5 nm and current 5.0 mA (0.4-1.5 mg L^{-1}) were used. A pH meter with glass electrode was adjusted the pH of human samples (Metrohm, E-632). For validation, the electrothermal atomic absorption spectrophotometer (ET-AAS, GBC 932) and ICP-MS in real samples as certified reference material (CRM) was used for determination of copper (II) and zinc (II) in serum and urine samples. The calibration curve of copper (II) and zinc (II) with injecting 20 μL of standard solution to graphite tube were used. All the reagents with analytical grade were used. Deionized-distilled water (DW, Millipore, USA) was prepared for experimental run. The copper (II) and zinc (II) solutions were prepared by appropriate diluting a 1000 μg L^{-1} of Cu and Zn solution (Merck) with DW. The pure amoxicillin **(Fig.1)**, acetone and ionic liquid purchased from Sigma Alderich (Switzerland) and Merck Company (Germany). Buffer solutions were prepared by standard methods. The pKa1 = 2.68 carboxyl, (pKa2 = 7.49 amine, and

pKa3 = 8.49) for amoxicillin (CAS N:26787-78-0) was considered. 1-Methyl-3-octylimidazolium hexafluorophosphate (CAS Number: 304680-36-2; $C_{12}H_{23}F_6N_2P$; [MOIM][PF$_6$]) was purchased from Sigma, Germany. The pH of the samples was adjusted up to 7 with a phosphate buffer (HPO$_4$/ H$_2$PO$_4$; 0.2 M).
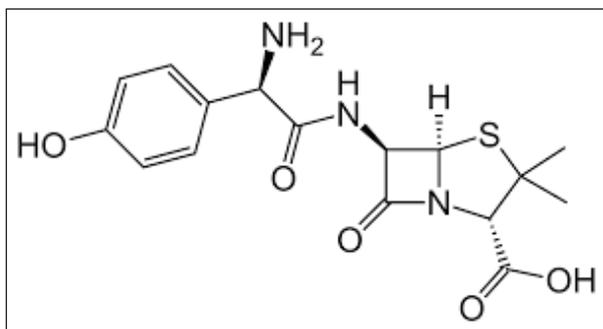

Fig. 1. The schema of amoxicillin

2.2. Characterizations

Amoxicillin, as organic compounds with the penicillin core structure was used as ligand in this study. Amoxicillin is structurally characterized by a penam ring (C_5H_7NOS) bearing two methyl groups and an amide group. Fourier transform infrared (FT-IR) spectra were recorded from KBr pellets using a spectrophotometer FTIR Shimadzu (Kyoto, Japan). For amoxicillin, the C-O stretching vibrations show intense IR absorptions, due to the considerable change in the molecular dipole moment produced by this vibration mode **(Fig. 2)**. Powder X-ray diffraction (XRD) was conducted on a X-ray diffractometer. X-ray diffraction analysis of pure amoxicillin and the optimized formulation was done by X-ray powder diffractometer (PW 3040/ 60 Xpert PRO, Panlytical, Netherlands). The X-ray diffraction patterns were recorded using Cu Kα radiations (λ=1.5405980Å), a current of 30 ma, and a voltage of 50 Kv. The samples were analyzed over 5–35 (2θ range) with a scan step size of 0.02s and 0.5s per step **(Fig. 3)**. Scanning electron microscopy (SEM) images were obtained using a Tescan Mira-3 Field Emission Scanning Electron Microscope (FE-SEM). The external and internal morphology

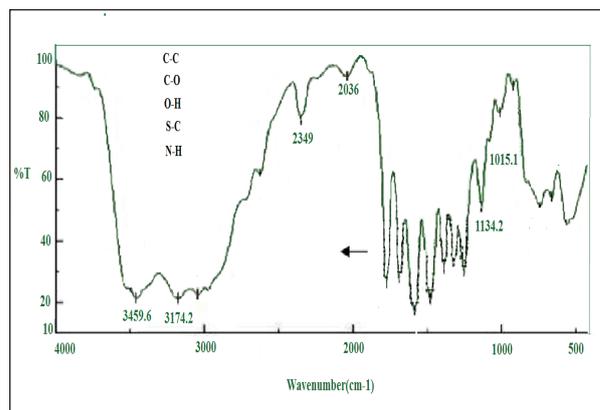of the amoxicillin was studied by scanning electron microscopy **(Fig. 4)**.

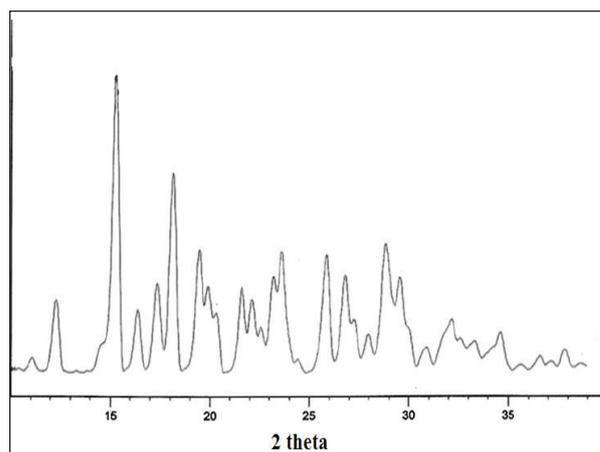**Fig. 2.** FT-IR spectra of pure amoxicillin

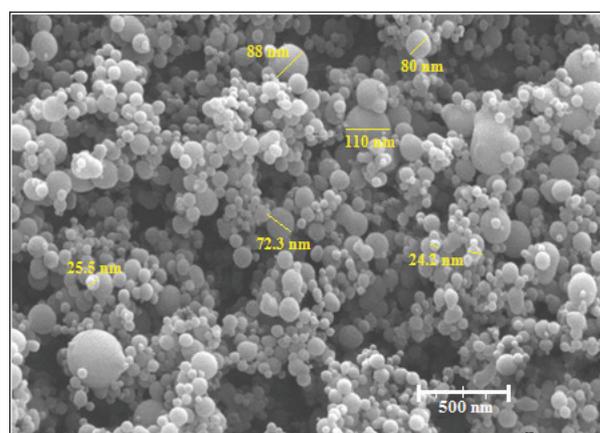**Fig. 3.** XRD spectra of pure amoxicillin

Fig. 4. FE-SEM for pure amoxicillin

2.3. Sample Preparation

Serum potentially contains elements and proteins which were produced in the human body. Sample preparation helps to reduce time, errors and interferences in analytical chemistry. The sample collection and handling have improved the sensitivity, selectivity, and reproducibility of Cu/Zn analysis in serum samples. For increasing accuracy and precision of results, the human serum samples were prepared. First, the glass laboratories placed in mixture of sulfuric acid and nitric acid (ultra pure grade; 0.5 molar: 0.5 molar) for 24 hours and washed for ten times with DW. The Cu and Zn concentrations in serum have an important limit concentration (~0.8-1.5 mg L^{-1}) and So, the human serum sampling, storage and analysis must be carefully done. In addition, 10 mL of the human serum were prepared from personnel of multiple Sclerosis patients in Iran (MS; 25 Men, 25 women, 25-55 age), based on the world medical association declaration of Helsinki (WMADH). Clean and sterilized syringes with plastic needles were purchased for Merck, Germany for serum blood sampling. The human biological samples were maintained frozen in refrigerator (below -4°C). For long-term storage of serum samples, we placed samples at −20, −80 °C or using liquid nitrogen. Urine samples were prepared and storage based on standard method in human samples.

2.4. Extraction Copper and Zinc Procedure

As shown in **Figure 5**, the copper and zinc were simultaneously extracted based on pure amoxicillin by DIL-CPE procedure. Firstly, 0.1 g of hydrophobic ionic liquid [MOIM][PF$_6$] and 0.2 mL acetone was mixed together and then 0.03 g of pure amoxicillin as a antibiotic drug was added. The mixture was injected to diluted serum or urine sample (2:10) by 2 mL of syringe with PVC needle. The cloudy solution was obtained and shacked for 7 min by shaker accessory. Then, the Cu or Zn ions was extracted from serum or urine samples based on sulfur bonding of amoxicillin at pH of 7 by DIL-CPE procedure (Cu─:S:─Zn). After centrifuging for 3 minute (3500 rpm), ionic liquid separated from serum sample in end of conical tube. The Cu or Zn ions were simply back extracted with 0.5 mL of nitric acid (0.2 M) and diluted with DW up to 1 mL. Finally, the remained solution was determined by flame atomic absorption spectrometry (F-AAS). The recovery of proposed method based on pure amoxicillin antibiotic drug was achieved for Cu and Zn extraction by the recovery equation (mean of RSD% < 2.2; more than 95%). The C_p is the primary concentrations of Cu or Zn in sample and C_F is the final concentration of Cu or Zn by DIL-CPE/F-AAS procedure (n=10, Eq. A).

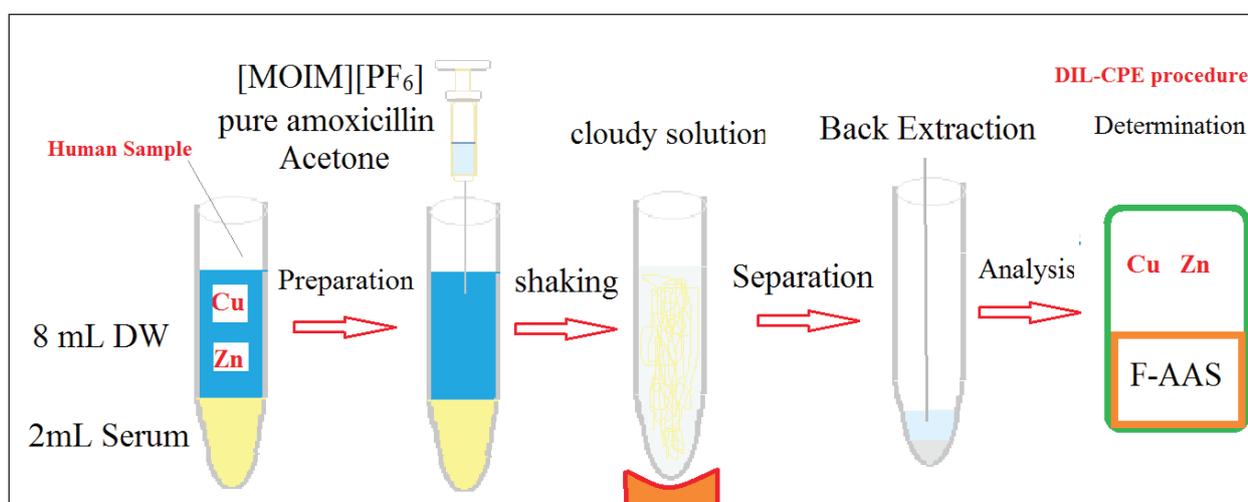$$Recovery \% = (C_p - C_F)/C_p \times 100$$

(EQ.A)

Fig. 5. Simultaneously extracted of Cu and Zn in serum and urine samples based on amoxicillin by DIL-CPE procedure

3. Results and Discussion

The DIL-CPE procedure provides novel and interesting approach based on amoxicillin drug for extraction of copper and zinc from human serum and urine samples. In order to obtain favorite separation and quantitative extraction of Cu and Zn ions with high sensitivity and precision, the analytical parameters of proposed DIL-CPE method must be optimized.

3.1. Optimization of pH

The pH of human serum or urine is main factor for efficient extraction of Cu and Zn ions by DIL-CPE procedure. The retention of Cu and Zn ions by amoxicillin ligand has been investigated at different pH from 2 to 11 with buffer solutions containing (100 µg L^{-1}, 500 µg L^{-1}) and (50 µg L^{-1}, 150 µg L^{-1}) for copper and zinc as LLOQ and ULOQ concentrations. It showed that amoxicillin ligand (pH$_{PZC}$ = 5.6), the extraction efficiencies of Cu (II) and Zn(II) were improved with the increase of pH values

more than 6 and the quantitative extraction were obtained at pH 6-8 and then the recoveries were reduced at pH more than 9. Consequently, the Cu and Zn ions quantitative extracted at pH 7 **(Fig.6)**. Due to serum pH, the extraction mechanism of Cu (II) and Zn(II) ions based on amoxicillin ligand is mainly depended on the electrostatic attractions of deprotonated sulfur groups of amoxicillin ligand with the positively charged Cu^{2+} and Zn^{2+} cations. At low pH (pH< pH$_{PZC}$), the amoxicillin ligand have positively charged (SH$_2^+$) as a protonation system. So, the extraction efficiency can be decreased due to the electrostatic repulsion between the Cu^{2+} and Zn^{2+} cations and positively charge of ligand. In addition, by increasing pH, the sulfur groups in amoxicillin ligand becomes negatively charged (R-S:$^{2-}$....Cu/Zn) and so the electrostatic attraction between negatively charged sulfur groups and positively charged Cu^{2+} and Zn^{2+} cations increased. In more pH (pH>9) the extraction were decreased due to the
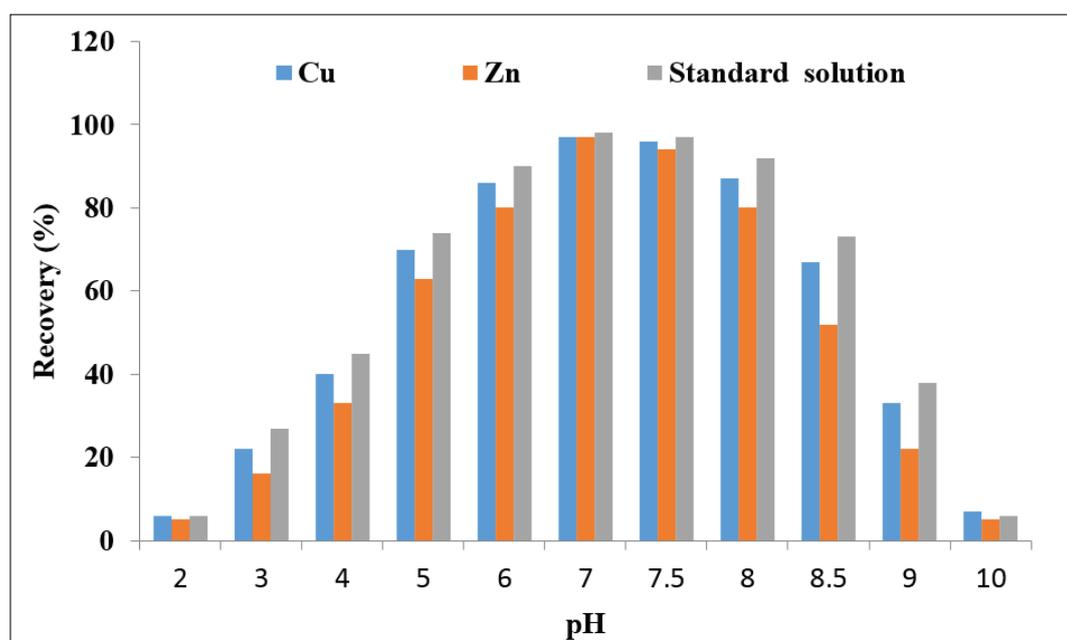

Fig. 6. The pH effect on simultaneously copper and zinc extraction with pure amoxicillin by DIL-CPE procedure

formation of hydroxyl complexes of Cu (II) and Zn(II) ions.

3.2. Effect of amoxicillin dose

Different amounts of amoxicillin ligand (AMOX-L) in the range of 5 to 50 mg were tested on the recoveries of Cu (II) and Zn(II) ions for the presented DIL-CPE procedure. The results were shown in **Figure 7**. It was found that 30 mg and 25 mg of AMOX-L was sufficient for quantitative recoveries of copper and zinc ions in serum and urine samples, respectively. So, 30 mg of AMOX-L was used as optimum amount of AMOX-L for further works. Higher amount of AMOX-L had no significant effect on the extraction of Cu (II) and Zn(II) ions. Due to capture of Cu (II) and Zn(II) on surface of AMOX-L and the metal concentration in solution, it came to equilibrium with each other. Eventually, 30 mg of AMOX-L was used as ligand for further work.

3.3. Effect of the IL amount

In the presented DIL-CPE method, 1-Methyl-3-octylimidazolium hexafluorophosphate ($C_{12}H_{23}F_6N_2P$; [MOIM][PF_6]) as hydrophobic ionic liquid was used as green extraction solvent in order to separate the Cu (II) and Zn(II) ions which was complex with AMOX-L as a coordination complex ions in the serum and urine samples.

The effect of [MOIM][PF_6] amounts on the extraction efficiencies of presented method was studied within the range of 0.05-0.25 g for 10 mL of standard solution, serum and urine samples containing 30 mg of AMOX-L, copper values (100 µg L^{-1}, 500 µg L^{-1}) and zinc values (50 µg L^{-1}, 150 µg L^{-1}) as LLOQ and ULOQ concentrations. The results showed that the quantitative recoveries were obtained with 0.08 g of [MOIM][PF_6]. Therefore, in order to achieve a suitable preconcentration, 0.1 g of IL was chosen as optimum leading to a final IL for serum and urine samples **(Fig.8)**. Moreover, the effect of IL for
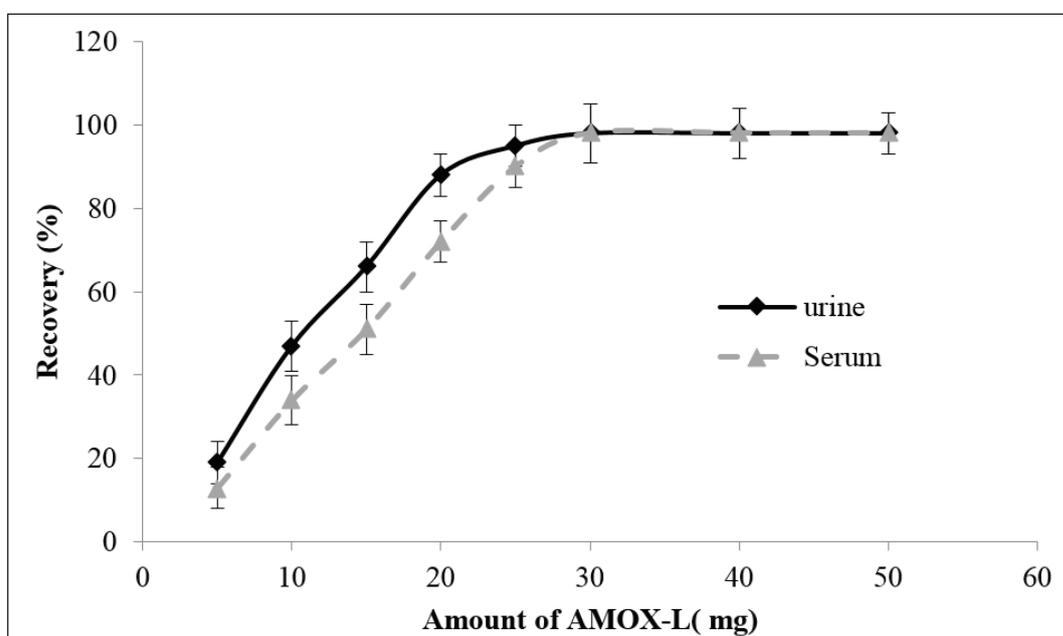
Fig. 7. The effect of AMOX-L on simultaneously copper and zinc extraction by DIL-CPE procedure
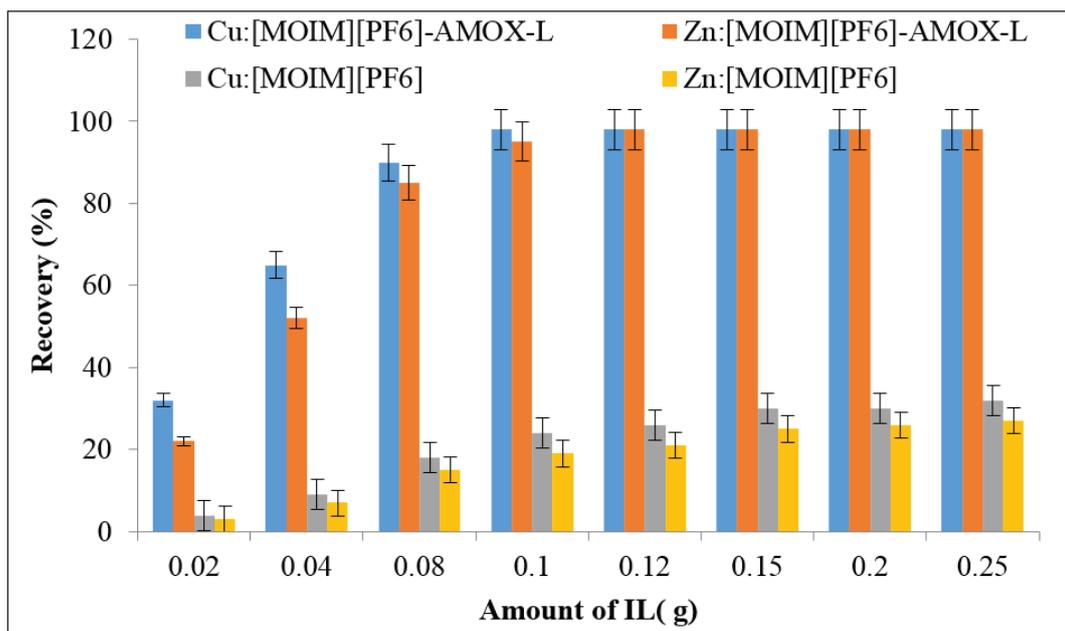
Fig. 8. The effect of [MOIM][PF$_6$] on simultaneously copper and zinc extraction by DIL-CPE procedure

Cu and Zn extraction with the same experiments were evaluated without and AMOX-L. Based on results, the efficient extraction for Cu and Zn ions were obtained less than 24% and 19% for urine and serum as complexation Cu and Zn with amino acids such as cysteine (Cys) and proteins (Cu/Zn….Pr /Cys). Therefore the IL had low effect on Cu and Zn extraction at optimum conditions. In addition, by increasing the ultrasonication time up to 60 min, the almost 27% and 31% of Cu and Zn ions extracted by 0.1 g of IL without any AMOX-L. These results confirm the critical role of AMOX-L as complex agent for Cu and Zn extraction.

3.4. Effect of eluents

The elution solutions were optimized in order to obtain the maximum back-extraction Cu and Zn ions from IL with the minimum concentration and volume of the elution solution. By DIL-CPE method, the different elution solutions were selected with high recovery. The coordination of Cu and Zn cations with AMOX-L was dissociated and ions released into the aqueous phase in acidic pH. For evaluation of the type, the concentration and the volume of acid

solutions for back extraction ions from ligand, 1000 μL of different mineral acids solutions such as HCl, H$_3$PO$_4$, HNO$_3$ and H$_2$SO$_4$ (0.1-0.5 mol L^{-1}) were examined by DIL-CPE procedure. The results showed that 0.2 mol L^{-1} HNO$_3$ (0.5 mL) quantitatively back-extracted Cu and Zn from ligand/IL **(Fig. 9)**.

3.5. Effect of sample volume

As shown in **Figure 10**, the effect of sample volume on the extraction of copper and zinc in serum and urine samples were evaluated and optimized by different volumes from 1- 25 mL containing copper values (100 μg L^{-1}, 500 μg L^{-1}) and zinc values (50 μg L^{-1}, 150 μg L^{-1}) as LLOQ and ULOQ concentrations by DIL-CPE procedure. As shown in Fig. 10, satisfactory recoveries were obtained between 2-10 mL for urine and serum samples. In addition, the high sample volume caused to significantly decrease the extraction Cu and Zn in human samples. Therefore, 10 mL of human samples was used for further investigation.
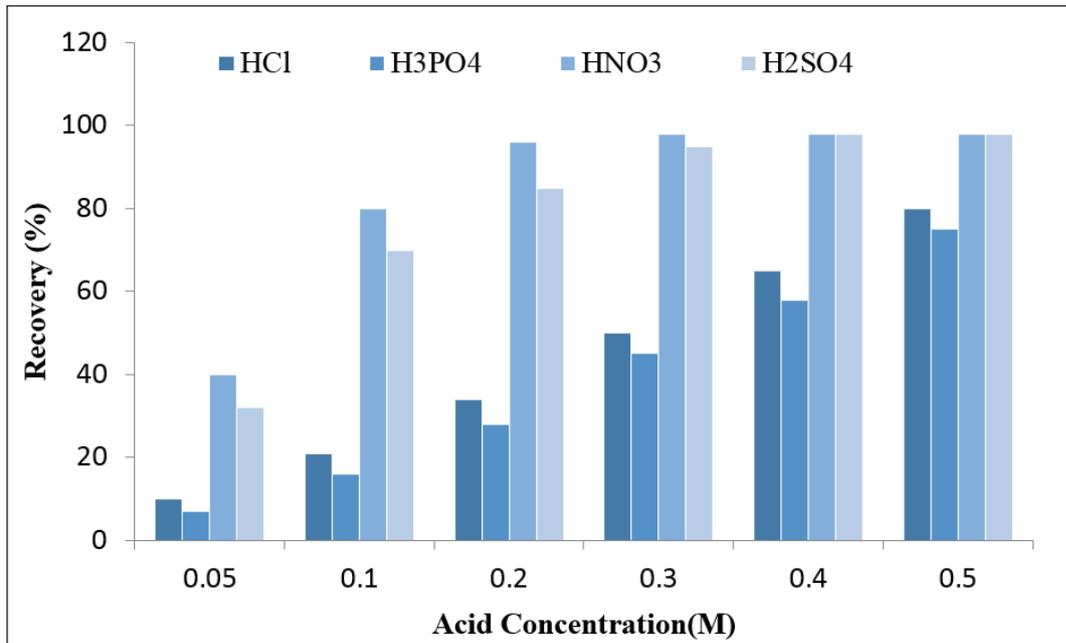
3.6. Effect of time

Fig. 9. The effect of eluents on back-extraction of copper and zinc extraction
by DIL-CPE procedure
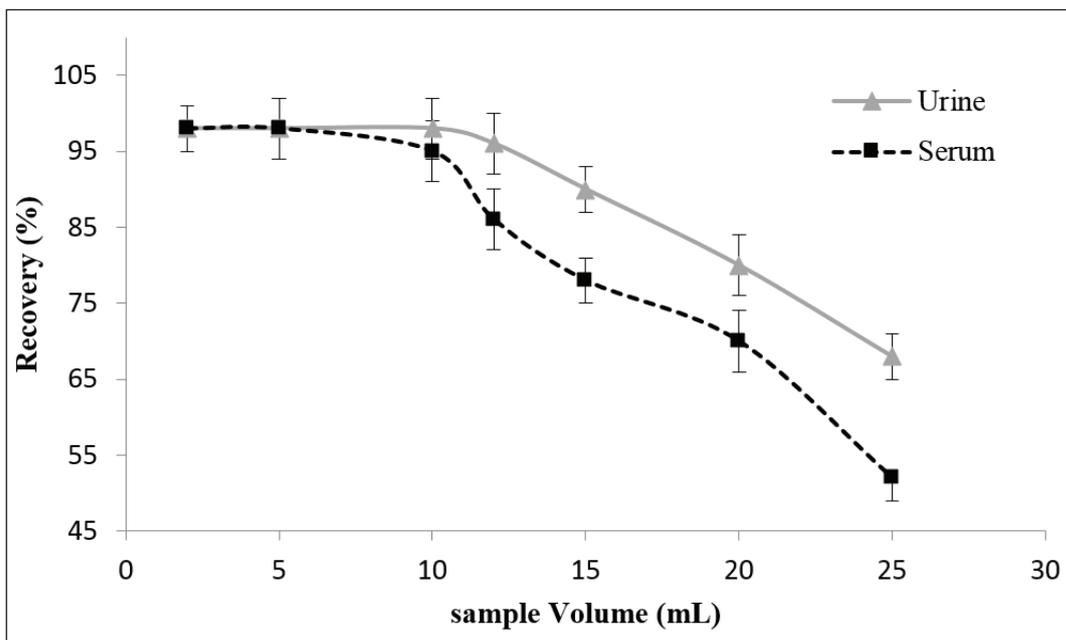
Fig. 10. The effect of sample volume on extraction of copper and zinc by DIL-CPE procedure

Dispersion is main factors for DIL-CPE procedure and allows to perfectly contacting of the Cu and Zn cations with AMOX-L as complex agent. Due to the favorite dispersion of the ligand into the liquid phase, the recovery of extraction phase increased. The effect of the ultrasonication time on the DIL-CPE procedure based on AMOX-L was studied within the range of 1–10 min. The results showed, by increasing time up to 2.5 min, the relative extraction increased and after this time remained constant. So, the time of 2.5 min selected for Cu and Zn extraction for further studies. On the other hand, after complex ions with AMOX-L, the centrifugation was needed to accelerate the separation IL from liquid phase.

Therefore, different times for centrifuging were examined between 1-5 min at 3500 rpm. The result showed that 2.0 minutes is sufficient to perfect separation phase.

3.7. Interference study

For evaluating of the analytical application in real samples, the important interference of coexisting ions effected on copper and zinc extraction in serum and urine samples were studied by the DIL-CPE procedure. For this proposed, the different amounts of the interfering ions added to 10 mL of liquid solution containing 500 µg L^{-1} of copper and 150 µg L^{-1} of zinc. The results showed, the most of the coexisting cations and anions had no effect on the extraction of Cu and Zn ions under optimum conditions (SD of recovery $< \pm 5\%$). In fact, the tolerable concentration ratio of interference of coexisting ions (M) per Cu and Zn (M/Zn^{2+} or M/Cu^{2+}) for Hg^{2+}, Ag$^+$ and Au^{3+} was less than 50 and 30 for zinc and copper, respectively. This ratio was almost 100-200 for Ni^{2+}, Pb^{2+} and Co^{2+} ions. The results showed that the AMOX-L have favorite ligand for Cu and Zn extraction despite the high concentrations of the coexisting ions **(Table 1)**.
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3.8. Analytical figures of merit

The analytical characteristics for Cu and Zn extraction in human serum and urine samples were

Table 1. The effect of interferences ions on extraction of Cu(II) and Zn (II) in serum samples by DIL-CPE procedure

Interfering Ions in serum (M)	Mean ratio $(C_M /C_{Cu(II)})$	Recovery (%)
	Cu(II)	Cu(II)
V^{3+}, Fe^{3+}	800	97.2
Cd^{2+}, Mn^{2+}	600	98.1
I$^-$, Br$^-$, F$^-$, Cl$^-$	1200	99.3
Na$^+$, K$^+$	1000	98.4
Ca^{2+}, Mg^{2+}	750	97.1
CO$_3^{2-}$, PO$_4^{3-}$, NO$_3^-$	900	97.7
Ni^{2+}, Co^{2+}	100	96.9
Pb^{2+}	700	98.2
Hg^{2+}, Ag$^+$, Au^{3+}	30	97.3

Interfering Ions in serum (M)	Mean ratio $(C_M /C_{Zn(II)})$	Recovery (%)
	Zn(II)	Zn(II)
V^{3+}, Fe^{3+}	900	98.7
Cd^{2+}, Mn^{2+}	750	97.6
I$^-$, Br$^-$, F$^-$, Cl$^-$	1100	98.8
Na$^+$, K$^+$	900	98.2
Ca^{2+}, Mg^{2+}	950	96.6
CO$_3^{2-}$, PO$_4^{3-}$, NO$_3^-$	1100	98.7
Ni^{2+}, Co^{2+}	200	97.5
Pb^{2+}	800	96.7
Hg^{2+}, Ag$^+$, Au^{3+}	50	98.0

studied by the purposed DIL-CPE procedure. The intra-day analytical performance was shown in **Table 2** for the multiple sclerosis patients (50) and healthy peoples (50). Under the optimal conditions, the linearity for the Cu (II) and Zn(II) concentration ranges between 100 -505 μg L^{-1} and 41- 153 μg L^{-1}, respectively as a lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) with mean correlation coefficient of R^2=0.9997 for Intra-day analysis. The LOD and LOQ are as an analytical signal three times higher than the background noise and three times higher than LOD, respectively. The precision of the AMOX-L/DIL-CPE procedure showed by the relative standard deviation (RSD %) for ten replicate determination containing 100 μg L^{-1} of Cu and Zn concentration which was obtained lower than

2.2%. The enrichment factor was calculated based on calibration curve and curve fitting rule (tga=m$_1$/m$_2$). According to proposed method, a favorite linear ranges and satisfactory EF were achieved for determination of Cu(II) and Zn (II) ions in human samples. The mean value for serum copper/zinc ratio in MS patients was obtained 1.11 ± 0.28 which was lower than normal range as 1.44 ± 0.31.

3.9. Analysis of real and certified samples

The DIL-CPE procedure was used for determination of copper and zinc in serum and urine samples by AMOX-L ligand. The validation of method was obtained based on spiking samples by known concentration of Cu (II), and Zn(II). The efficient recovery was achieved by spiking samples, which confirms the accuracy of the

Table 2. Determination of copper and zinc in serum by DIL-CPE procedure (Mean intra-day and inter –day analysis for 50 MS and 50 healthy peoples (HP); μgL^{-1})

Serum Sample	[a] Mean of MS (n=50)		[a] Mean of HP (n=50)		✚ Data Subject	
	Intra-day	Inter day	Intra-day	Inter day	r	P value
Copper	965.4 ± 51.6	972.1 ± 58.7	1154.5 ± 62.6	1168.7 ± 71.4	0.107	<0.001
Zinc	658.4 ± 29.5	666.2 ± 33.8	875.3 ± 44.6	861.9 ± 48.8	0.123	<0.001

✳Mean of three determinations of samples ± confidence interval (P = 0.95, n =10)

✚Correlations are based on Pearson coefficients (r). Statistical significance will be observed if P < 0.001

Table 3. Validation of developed the DIL-CPE procedure based on AMOX-L for Cu and Zn determination by spiking real samples (μgL^{-1})

Sample	Added Cu	Added Zn	Found Cu*	Found Zn*	Recovery(%)Cu	Recovery(%)Zn
Serum	------	------	235.5 ± 11.2	189.6 ± 9.3	------	------
	200	200	429.6 ± 20.8	380.4 ± 18.7	97.1	95.4
Urine	------	------	197.3 ± 10.1	201.2 ± 9.4	------	------
	200	200	394.8 ± 17.6	404.7 ± 18.9	98.7	101.7
Serum	------	------	252.7 ± 11.2	168.8 ± 8.3	------	------
	250	150	499.8 ± 23.6	315.5 ± 14.7	98.8	97.8
Urine	------	------	171.4 ± 8.1	142.3 ± 6.6	------	------
	150	150	324.2 ± 13.5	288.7 ± 14.2	101.8	97.6

* Mean of three determinations of samples ± confidence interval (P = 0.95, n =10)

All samples (2 mL) diluted with DW up to 10 mL(Dilution factor: DF=5)

Table 4. Validation of methodology based on ICP-MS analysis in real samples
and compared to the DIL-CPE procedure (µgL⁻¹)

Sample	Added(µgL⁻¹)	*Found(ICP-MS)	*Found(DIL-CPE)	Recovery
Serum(Cu)	------	302.2 ± 7.2	297.5 ± 14.2	98.4
	200	------	492.8 ± 22.4	97.6
Serum (Zn)	------	182.3± 4.2	178.9 ± 8.2	98.1
	150	------	334.2 ± 15.4	103.5
Urine (Cu)	------	261.5 ± 6.8	257.2 ± 11.7	98.3
	250	------	497.9 ± 24.6	96.3
Urine (Zn)	------	192.3 ± 4.5	200.6 ± 9.5	104.3
	200	------	399.8 ± 17.4	99.6

* Mean of three determinations of samples ± confidence interval (P = 0.95, n =10)
All samples (2 mL) diluted with DW up to 10 mL(DF=5)

DIL-CPE procedure **(Table 3)**. The recoveries of spiked samples for Cu and Zn were ranged from 96 to 105%, which demonstrated that the DIL-CPE procedure was satisfactory for determination copper and zinc in urine and serum samples. On the other hand, the certified standard reference materials (CRM) were prepared in serum and urine sample by ICP-MS and used for the validation methodology **(Table 4)**.

4. Conclusions
A simple, rapid, reliable and sensitive method was developed for separation and extraction of Cu (II) and Zn(II) in serum and urine samples based on AMOX-L by the DIL-CPE procedure. The [MOIM][PF$_6$] ionic liquid as a trapping phase was used for separating of Cu/Zn-loaded AMOX-L from liquid phase. IL helps to reducing the sample preparation and separation time for extraction process. Using a small amount of AMOX-L with high extraction recovery, good precision, minimal acid elution (500 µL) and green solvent caused to make the efficient extraction based on environmentally friendly for determining of Cu and Zn in urine and serum samples. Also, the low LOD and RSD% values as well as the quantitative recoveries (more than 95%) were obtained in optimized conditions. Therefore, the developed method based on AMOX-L can be used as favorite sample preparation in human biological samples in short time. As obtained results, the amoxicillin

can be affected on copper and zinc deficiency in human body at human pH when the patients used it by over dosage for many times.

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