



In-vitro extraction and separation of copper ions from human blood samples based on amoxicillin/clavulanic acid by ultrasound assisted-dispersive centrifuge liquid-liquid micro extraction

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ABSTRACT

The low concentration of copper (Cu^{2+}) can be effected on the central nervous system (CNS) and caused to multiple sclerosis (MS). Although many *antibiotics* can treat the bacterial infections but some of *antibiotics* decrease essential metal concentrations in human body and must be controlled by determining. In this study, in-vitro extraction of copper (Cu^{2+}) with amoxicillin/clavulanic acid (AMOXC) has been studied due to interacting with metals. By procedure, Cu^{2+} ions were separated from blood samples by *ultrasound assisted-dispersive centrifuge liquid-liquid micro extraction* (USA-DC-LLME). The mixture of AMOXC (0.01 g), ionic liquid ([BMIM][PF₆]) and acetone injected to 10 ml of serum blood sample at human pH=7.2. After extraction, the concentration of Cu^{2+} ions was determined by flame atomic absorption spectrometry (F-AAS). The LOD, enrichment factor (EF), linear range (LR) and working range (WR) were obtained $6 \mu\text{g L}^{-1}$, 9.92, 0.018-0.5 mg L^{-1} and 0.02-2.58 mg L^{-1} , respectively (RSD<1.1%). The validation of technology was confirmed by ICP and spiking samples.

1. Introduction

Copper (Cu) acts as a main co-factor in humans and enter into cuproenzymes that catalyze electron transfer reactions (ETR) required for cellular respiration, iron oxidation, neurotransmitter biosynthesis, antioxidant, peptide amylation. The Cu intake has been associated with toxic effects in humans include influenza-like syndrome, hemolysis and kidney failure due to Cu sulfate intake [1]. Cu exists in blood plasma in Cu bound to caeruloplasmin (65–71%) and convert the iron

Fe^{2+} into Fe^{3+} which was bonded to transferrin [2]. Copper enter to biochemical reactions and human physiology. In biological matrix such as plasma, copper complex to proteins as caeruloplasmin and albumin or amino acids [3]. The defect of central nervous system caused to multiple sclerosis (MS) disease in human due to demyelination of nerve fibers of the brain and spinal cord. Based on previous studies, the genetic, immunological and environment factors have main source for creating MS in humans with disability. But, the environment factors such as heavy metals such as copper, zinc, cadmium and lead has significant role in MS [5, 6]. The deadline of time exposure of heavy metals in

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human blood samples depended to kind of heavy metals and chorionic/acute exposure (cadmium was seen in blood up to 1 hour as acute exposure). In chorionic exposure the concentration of heavy metals can be followed in nail or hair. Copper used for many products as an essential element in nature and copper has concentration between 80-180 $\mu\text{g dL}^{-1}$ in blood of human adults (Mean= 1.5 mg L^{-1}). Copper exposure in industry occurs primarily through inhaled particulates and according to studies, copper can be a cause of the pathogenesis of MS [6]. The value of copper (Cu^{2+}) effect on the central nervous system (CNS) and reducing of their concentration caused to numerous CNS disorders such as multiple sclerosis (MS), Wilson disease, Alzheimer and Parkinson's diseases [7-9]. Also, some of antibiotics caused to decrease essential metals such as Zn^{2+} and Cu^{2+} in human body. So, in present of antibiotics, determination of Zn^{2+} and Cu^{2+} in human matrixes is very important. Some receptors such as β -ketoenol-bipyridine and ketoenol-pyrazole were functioned on silica hybrid adsorbent and other sorbents [10, 11]. For determination of copper the different analytical techniques such as, ET-AAS [12, 13], ion selective electrode, Square wave adsorptive anodic stripping voltammetric (SW-ASV) [14] and flame atomic absorption spectrometry (F-AAS) [15] were used in different samples such as blood, waters and tissues(fish)[16]. High interferences in human blood are main problems for determination copper by instruments. So, sample preparation must be used before analysis by instruments. The different procedures such as, cloud point extraction (CPE)[9], liquid-liquid extraction (LLE) [15], solid-phase extraction (SPE) in human biological fluid (blood, serum and urine) [17,18-22] and dispersive liquid-liquid microextraction (D-LLME) based on ionic liquids (IL) [23,24,15] were used before Cu analysis. Recently, a applied dispersive liquid-liquid microextraction was used for separation/extraction of ions from liquid samples. In this study, USA-DC-LLME based on AMOXC was used for efficient extraction of Cu ions in human biological samples before determination by F-AAS. The hydrophobic ionic liquid was used for simple separating of Cu-

AMOXC from blood samples.

2. Experimental

2.1. Instrumental

The spectra GBC 906 double beam atomic absorption spectrophotometer was used for copper determination (FAAS, GBC, Model; Plus 906, Australia). The air-acetylene as fuel gas based on background correction was selected for F-AAS. The light of HCL adjusted on burner by vertical and horizontal positions. The Avanta software of spectra was used for collecting data. The copper hollow cathode lamp (HCL, slit=0.7) with wavelength of 327.4 nm and current of 4.0 mA was adjusted in vertical and horizontal position. For copper analysis, the limit of detection, working range was obtained 0.06 mg L^{-1} and 0.2-24.0 mgL^{-1} , respectively by F-AAS. All samples as minimum volume were used by auto-sampler injector (0.5-5 mL). The pH values of the solutions were measured by a digital pH meter (Metrohm 744), especially in human samples. All samples were shaken with Thermo accessory as mixer (250 rpm, USA) and centrifuged with Thermo fisher (1000-4500 rpm, USA).

2.2. Reagents

The ultra-trace reagents with analytical grade such as; HNO_3 , HCl , NaOH , copper salt and acetone solutions were purchased from Merck (Darmstadt, Germany). The pure AMOXC as amoxicillin trihydrate: potassium clavulanate (4:1) was purchased from sigma, Germany (Product N: SMB00607, Batch N: 128M4800V, 75% AMOX and less than 15% clavulanic acid and less than 15% water) (Fig.1). The Cu(II) stock standard solution was prepared as nitrate salt of 1000 mg L^{-1} (ppm) solution in 1 % HNO_3 . The standard solutions were prepared daily by dilution of the standard solution of Cu(II) by ultra-pure water(UPW). High purity distilled water had already prepared from Millipore (Bedford, USA). 1-Ethyl-3-methylimidazolium acetate (CAS N: 143314-17-4), 1-Ethyl-3-methylimidazolium tetrafluoroborate (CAS N: 143314-16-3), 1-Ethyl-3-methylimidazolium hexafluorophosphate (CAS N: 155371-19-0), and 1-butyl-2,3-dimethylimidazolium

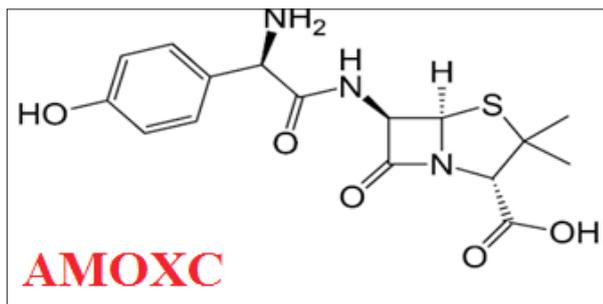


Fig. 1. Schema of amoxicillin trihydrate: potassium clavulanate

hexafluorophosphate ([BDMIM] [PF₆]) as a hydrophobic ionic liquids were selected for separation AMOXC from blood sample. The T-X100 with analytical grade were purchased from Merck Co., Darmstadt, Germany. The pH was adjusted with buffer solutions (Merck, Germany). Sodium phosphate and citric acid was used for phosphate citrate buffer for PH=2.0–7.5.

2.3. Sample preparation

The glasses were washed with a HNO₃ solution (0.5M) for at least 24 h and thoroughly rinsed 10 times with ultra-pure water. For sampling, 20 ml of samples of blood were collected from subjects and control peoples (N=30, men, 30-55 age). 10 mL of blood sample mixed with heparin (pure, 20 micro liter) and storage in low temperature. Then, 10 mL of blood samples were stay at the room temperature for 30 minutes to coagulate and then centrifuged at 3000 rpm for 5 minutes. Next, serums were separated by a sampler. Plasma was also prepared by procedure by centrifuging process. The human blood/serum samples were maintained at -20 °C up to 72 h. In

humans with low sample volume of blood, it diluted up to 20 mL and then was used. The Ethical Committee of Azad University (SN.IAU.SRB.940522664) confirmed the human sample method.

2.4. Procedure

By USA-DC-LLME procedure, the 10 mg of pure AMOXC powder (15 micro molar conc.) mixed with ionic liquid ([BMIM][PF₆], IL, 0.1 g) in present of 500 micro liter of acetone/ethanol, then, the mixture was injected to 10 ml of serum blood sample at human pH=7.2 (Fig.2). After 5 min shaking time, the hydrophobic ionic liquid [BMIM][PF₆] was separated by centrifuging at 3500 rpm for 4 min in conical glass tube. The upper liquid phase separated by auto-sampler accessory (1-100 mL) and remained phase (IL-AMOXC/Cu²⁺) were back-extracted from IL phase by decreasing pH up to 1 (HNO₃, 0.5 M, 0.5 mL). Finally the concentration of Cu²⁺ ions was simply determined by F-AAS after dilution with DW up to 1 mL.

3. Results and discussion

For extraction copper from blood samples all parameters (pH, sample volume, amount of AMOXC and IL) must be optimized. The serum, blood, urine samples was prepared from patients with copper disease such as multiple sclerosis. After tuning pH with favorite buffers, the sample determines by ultrasound assisted-dispersive centrifuge liquid-liquid micro extraction (USA-DC-LLME) coupled to F-AAS.

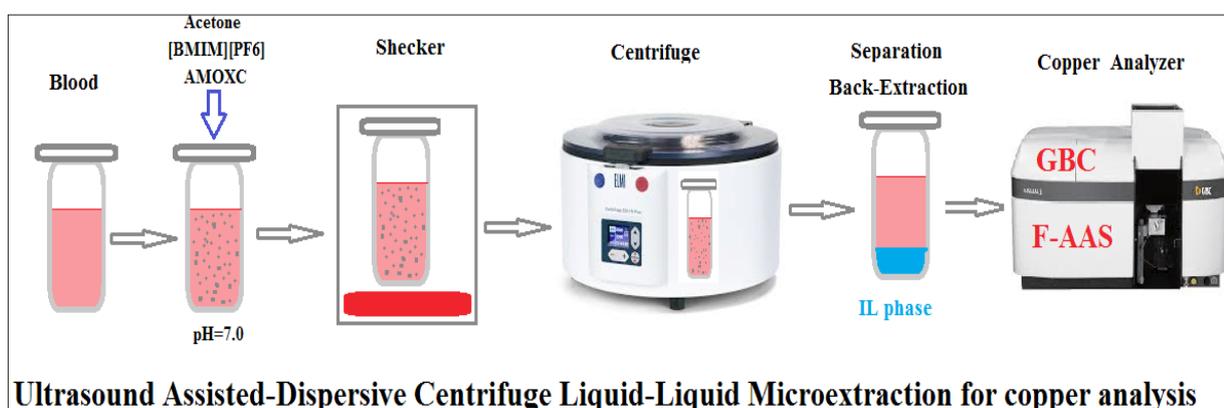


Fig. 2. The copper extraction from blood samples based on AMOXC by USA-DC-LLME

3.1. PH effect

The effect of sample pH on complexation of Cu(II) ions on AMOXC was studied for 0.2 mg L⁻¹ and 2.0 mg L⁻¹ of Cu(II) (lower limit of quantification; LLOQ and upper limit of quantification; ULOQ) from different pH 1-12. The complexation of AMOXC-Cu was depended on the pH of solutions and function group of ligand. The results showed, the maximum extraction of Cu(II) was achieved in human pH (7.0), and the recovery values for Cu(II) were below 5% in acidic or basic pH. The extraction mechanism of Cu(II) ions with AMOXC was highly referred to pH and complex formation between Cu(II) ions and dative sulfur bonding of AMOXC (Fig. 3). The sulfur groups can be deprotonated (:S-R) at wide range of pH from 6 to 9. The extraction efficiency of Cu(II) at pH values below 6 and upper of 8 cannot occurred due to similarity charge or precipitation of hydroxyl copper (Cu(OH)₂).

3.2. Sample volume effect

The sample volume in one of the important factors which must be optimized for Cu extraction from blood samples by AMOXC. As evaluation extraction efficiency, different volumes between 1.0-30 mL of blood and serum samples was examined and optimizes in pH=7. The results showed us that the maximum recovery for copper extraction based on AMOXC was achieved for less than 12 mL for human blood samples and less than 17 mL of

standard solutions. Therefore, 10 mL and 15 mL of sample volume was selected as optimum point for copper extraction for blood and water samples, respectively by USA-DC-LLME procedure (Fig.4).

3.3. AMOXC effect

The amount of ligand is very important for copper extraction in blood samples, so, the concentration of AMOXC in human samples must be studied for high extraction. For this purpose, the vary concentration between 1-50 micro molar concentration of AMOXC was prepared and used for evaluation of copper extraction in blood/standard samples by USA-DC-LLME procedure. The experimental results showed, the high extraction of copper was obtained with more than 15 micro molar of AMOXC in 10 mL of samples (~ 10 mg pure powder) by USA-DC-LLME procedure (Fig. 5). Also, the extraction efficiency more than 97% was made by 12 micro molar of AMOXC concentration in 10 mL of water samples. First, 10 mg of pure AMOXC powder mixed with ethanol/acetone (0.5 mL) and then added to 0.1 g of IL in 2 mL syringe. After shacking, the mixture injected to 10 mL of blood samples. The extraction efficacy was decreased about 74.6% when the mixture added step by step without shacking.

3.4. Ionic liquid effect

Separation process for AMOXC-Cu from liquid samples (blood, standard solution) is very difficult.

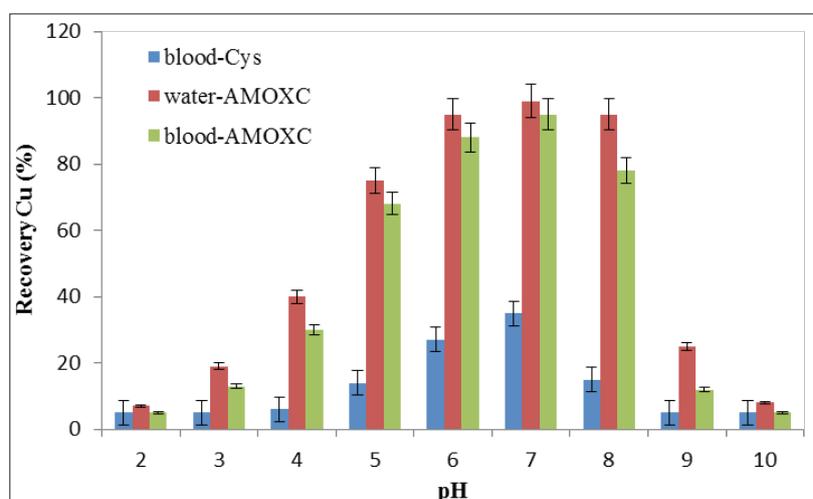


Fig. 3. The effect of pH on copper extraction based on AMOXC by USA-DC-LLME procedure

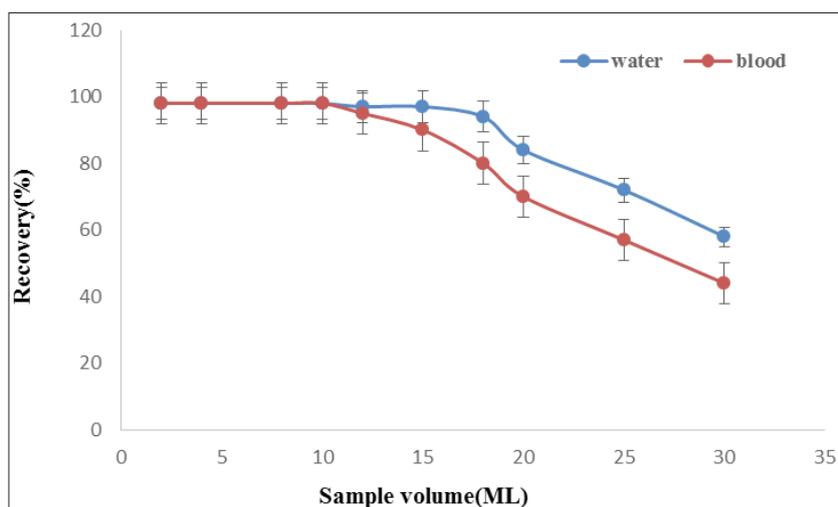


Fig. 4. The effect of sample volume on copper extraction based on AMOXC by USA-DC-LLME procedure

The different techniques with filter, centrifuging and organic solvents was used for separation ligands from liquid phase. Recently, the benign solvents as green solvents (IL) was introduced in many papers. In this study we used different hydrophobic ionic liquids for separation processes in blood samples. The different volumes or gram (0.05-0.3 g) of 1-Ethyl-3-methylimidazolium acetate, 1-Ethyl-3-methylimidazolium tetrafluoroborate, 1-Ethyl-3-methylimidazolium hexafluorophosphate, and 1-butyl-2,3-dimethylimidazolium hexafluorophosphate [BDMIM][PF₆] as a hydrophobic ionic liquids were selected for separation AMOXC from blood sample. Based on results, 0.1 g of [BDMIM][PF₆] has more extraction efficiency as compared to others (Fig.

6). The 1-Ethyl-3-methylimidazolium acetate and 1-Ethyl-3-methylimidazolium tetrafluoroborate had lower recovery because of solubility in liquid phase and missed of IL. The 1-Ethyl-3-methylimidazolium hexafluorophosphate as hydrophobic IL can be separated copper-AMOXC from blood samples but has lower recovery (85%) as compared to [BDMIM][PF₆] with (97.8%).

3.5. Interference ions study

Many ions such as Zn²⁺, Mn²⁺, Na⁺, K⁺, HCO₃⁻, SO₃⁻, Fe²⁺, Co²⁺, exist in water or blood samples and can be effected on complexations of copper with AMOXC in presence of interference ions. The complexation copper depended on power of ligand and competition

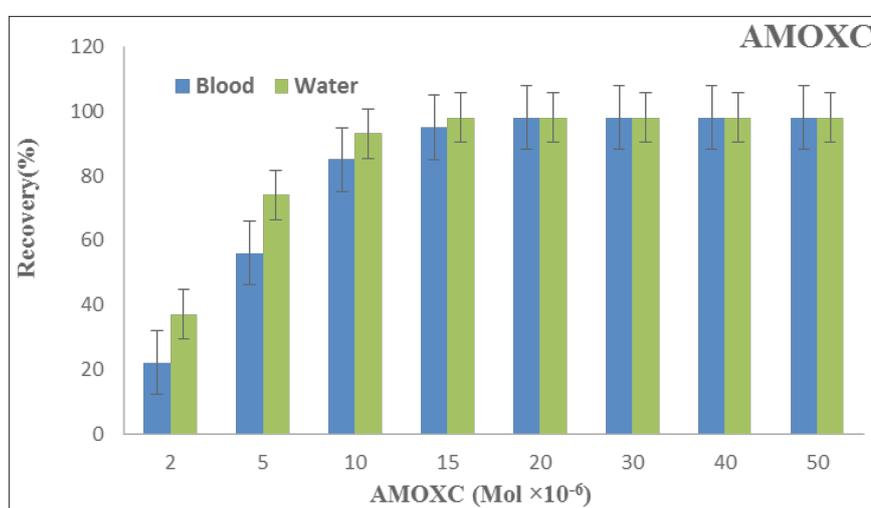


Fig. 5. The effect of AMOXC concentration by USA-DC-LLME procedure

of ions in different pH. So, in optimized pH many ions had no effect on extraction process, but some of them may be done. Therefore the effect of important ions which was bonding with amino acids or proteins was studied. The recovery of copper extraction based on AMOXC in presence of different concentration of 1-4 mg L⁻¹ of cations and anions was evaluated by USA-DC-LLME procedure. As selectivity of determination of F-AAS, the concentration of other ions can't reported. So, the recovery was checked as effect of interference ions. The results showed, the interference ions had no effect on complexation processes of copper – AMOXC by USA-DC-LLME procedure (Table 1).

3.6. Validation procedure

The methodology of copper extraction must be validated by different technologies. By spiking of blood, serum, urine, plasma samples, the favorite

recovery more than 95% was achieved for LLOQ and ULOQ range. So, the USA-DC-LLME procedure was validated for copper complexation based on AMOXC (Table 2). In addition, ET-AAS coupled with microwave digestion was used for evaluation of purposed procedure after dilution samples up to 100 mL(1:10) (Table 3). As intra-day and inter-day studies, the mean copper concentration based on AMOXC in subjects and control groups (N=30, men, 30-55 age) was checked by USA-DC-LLME procedure. The results showed no significance difference between subjects and control groups with favorite *p*-value (Table 4). The mean concentration of copper in control groups were a little higher than Cu blood subjects. The regression analysis and t-test were achieved between Cu in subject and control groups. There were a correlation ($0.45 < r < 0.5$) between blood of subject and control groups. (*p*-value < 0.001).

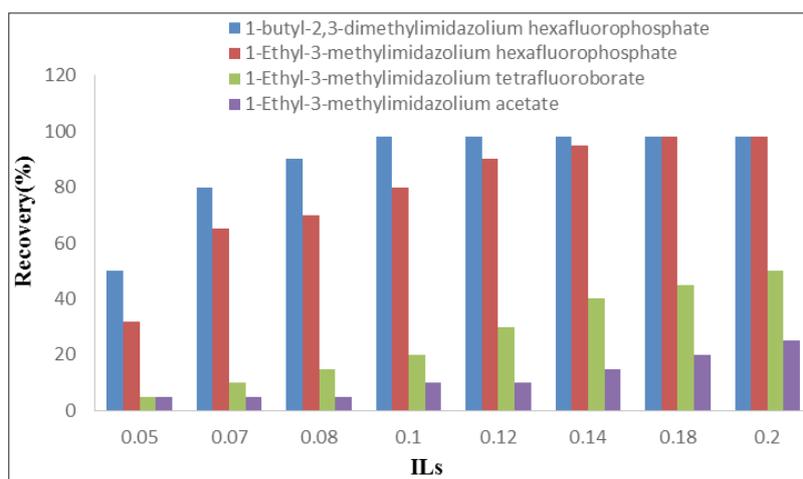


Fig. 6. The effect of different ILs on copper separation based on AMOXC by USA-DC-LLME procedure

Table 1. Effect of interfering ions on the extraction recovery of Cu (II) ions by USA-DC-LLME procedure

Foreign Ions	Concentration ratio ($C_{\text{interferent ion}}/C_{\text{Cu}^{2+}}$)		Recovery (%)	
	Standard	Blood	Standard	Blood
K ⁺ , Na ⁺ , Li ⁺ , Cl ⁻ , F ⁻ , Mg ²⁺ , Ca ²⁺	1200	1000	97.5	96.8
Co ²⁺ , Ni ²⁺	900	700	98.2	97.3
Pb ²⁺ , Ag ⁺	600	500	97.6	97.9
Zn ²⁺ , Mn ²⁺	700	600	95.5	98.4
Fe ²⁺ , V ³⁺ , As ³⁺ , Mo ³⁺	850	800	97.0	96.8
Cd ²⁺ , Al ³⁺	800	550	98.2	97.3
Hg ²⁺	1000	900	96.5	95.7
CO ₃ ²⁻ , SO ₃ ²⁻	950	600	99.1	98.5

4. Conclusions

A simple Pharmacology and biology method based on USA-DC-LLME was used for evaluating of AMOXC for copper extraction in human blood samples. The results demonstrated that AMOXC can decreased copper concentration in blood samples in efficient time. On the other hands amino acids and proteins was also complexed with copper and caused to decrease copper extraction by AMOXC. So, the AMOXC competed with amino acids and proteins for extraction copper and other metals at pH 7.2. In addition, *the antibiotics such as AMOXC* can treat the bacterial infections but they can decrease essential metals (Cu) in biological matrixes and cause disease in humans. By purposed procedure, the copper extracted with AMOXC at pH=7 and determined by F-AAS. The 1-butyl-2,3-dimethylimidazolium hexafluorophosphate ([BDMIM][PF₆]) as hydrophobic IL was used for separation of Cu from liquid phase. In optimized conditions, the low LOD and RSD% values as well

as good working ranges (0.02-2.58 mg L⁻¹) and high recoveries caused to consider as a recent innovative procedure.

5. Acknowledgements

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Table 2. Spiking of human samples based on AMOXC for copper extraction by USA-DC-LLME coupled to F-AAS

Sample	Added(mg L ⁻¹)	*Found(mg L ⁻¹)	Recovery (%)
Blood	-----	0.884 ±0.043	-----
	0.8	1.675±0.072	98.8
Serum	-----	1.226±0.058	-----
	1.2	2.384±0.094	96.5
Urine	-----	0.431± 0.022	-----
	0.4	0.838±0.039	101.7
Plasma	-----	0.362±0.024	-----
	0.3	0.657±0.028	98.3

Table 3. Comparing of ETAAS/digestion with USA-DC-LLME procedure coupled to F-AAS

Sample	Added	ET-AAS	USA-DC-LLME	Recovery (%)	Recovery (%)
		Microwave	F-AAS	ET-AAS	F-AAS
Blood	-----	1.002±0.055	0.985±0.048	-----	-----
	1.0	1.977±0.112	1.981±0.094	97.5	99.6
Serum	-----	1.212±0.063	1.197±0.053	-----	-----
	1.2	2.423±0.124	2.376±0.103	100.9	98.3

Table 4. The mean copper concentration based on AMOXC in subjects and control groups (N=30) by USA-DC-LLME procedure

Sample	Control group (30n)		Patient group (30n)		r
	Intra-day	Inter-day	Intra- day	Inter day	
Blood	1.34±0.05	1.27±0.06	1.16±0.04	1.19±0.05	0.58
Serum	1.41±0.06	1.46±0.07	1.25±0.05	1.26±0.06	0.61

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