Talanta 116 (2013) 1040–1046

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Restricted accessed material-copper(II) ion imprinted polymer solid phase extraction combined with inductively coupled plasma-optical emission spectrometry for the determination of free Cu(II) in urine and serum samples

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ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form
10 August 2013
Accepted 14 August 2013
Available online 23 August 2013

Keywords:
Surface imprinted-emulsion method
Restricted accessed-ion imprinted polymer
Human body fluids
Inductively coupled plasma-optical emission spectrometry (ICP-OES)
Copper

ABSTRACT

A novel restricted accessed material (RAM)—Cu(II) ion imprinted polymer (IIP) was synthesized by the surface imprinted-emulsion method, and possessed a high selectivity to Cu(II) and good macromolecules exclusion property. And a novel method of RAM-IIP packed microcolumn solid phase extraction (SPE) combined with inductively coupled plasma-optical emission spectrometry (ICP-OES) was developed for the determination of trace free Cu(II) in human body fluids. Under the optimized conditions, the adsorption capacity of RAM-IIP for Cu(II) was 15.9 mg g^{-1}. With a preconcentration factor of 30, the limit of detection was 0.17 μg L^{-1}, and the relative standard deviation was 2.2% ($n=7$, $c=1$ μg L^{-1}). The developed method was validated by the analysis of two Certified Reference Materials, and the determined values were in good agreement with the certified values. This method was also successfully applied for the direct analysis of free Cu(II) in human urine and serum samples. While the total Cu can be determined by the proposed method after microwave digestion. The concentrations of free Cu(II) were much lower than that of total Cu, indicating that Cu is mainly coordinated with macromolecules in these biological samples. From this point of view, the developed method exhibits application potential in speciation of free metal ions and metallic complex molecules in biological samples.

1. Introduction

Copper, as an essential element for human beings, plays an important role in carbohydrate and lipid metabolism. It is essential in small amounts for synthesis of hemoglobin and is essential constituent of about thirty enzymes and glycoproteins [1]. However, excessive intake of such metals will endanger the human beings health. In human body fluids, copper exists in various chemical forms such as free copper ions (Cu(II)) and coordinated copper with protein. And there is a dynamic equilibrium between coordinated cooper and free Cu(II). Some disease will affect the dynamic equilibrium and cause the concentration change of coordinated cooper and free Cu(II). For example, Wilson's disease is an inborn error of copper metabolism as the result of serum deficiency of ceruloplasmin. Progression of Wilson's disease leads to a significant increase of free copper ions (Cu^{2+}) circulating in the blood stream. Therefore, it is meaningful to develop analytical methods that can provide fractionation or species information of copper in human body fluids besides total amount. Various detection techniques including inductively coupled plasma mass spectrometry (ICP-MS), flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ETAAS) and inductively coupled plasma optical emission spectrometry (ICP-OES) have been reported for the determination of trace Cu in human body fluids. Among these detection techniques, ICP-OES has gained strong recognition in trace metals analysis due to the advantages of multi-elemental analysis capability, wide dynamic linear range, low limits of detection, and good reproducibility. Despite the aforementioned advantages, direct determination of Cu in biological fluids is limited due to its low level of concentration and matrix interferences in real-world samples. In addition, ICP-OES cannot offer species information. Thus, sample pretreatment techniques are usually required in order to preconcentrate target analyte and separate potentially interfering matrix constituents prior to ICP-OES detection.

Among various sample pretreatment techniques, solid phase extraction (SPE) offers many advantages such as simplicity, no emulsification, low sample/reagent consumption, and easy-to-automate. Thus it is widely used for the preconcentration of trace metal ions [2]. SPE was usually employed for total trace Cu analysis

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http://dx.doi.org/10.1016/j.talanta.2013.08.027

in human body fluids with acid digestion to convert all the copper forms into copper ions, but can hardly be used for the direct analysis of free copper ions in human body fluids due to the interference of matrix constituents. Therefore, it is of significance to develop the SPE method that could directly extract free Cu(II) from human body fluids, in which novel SPE adsorbents with high selectivity for Cu(II) and good exclusion property for sample matrix are expected.

Currently, the adsorbents which could be employed for direct extraction of target analytes from biological fluids include monolithic [3,4], large particle supports and restricted-access materials (RAMs) [5]. Since the first publication about RAMs by Hagestam and Pinkerton [6] in 1985, the researchers have devoted to explore new kinds of RAMs and apply them in various scientific fields. The notable feature of RAMs is that it could directly extract and concentrate target analytes from biological fluids without co-precipitation or digestion. Even though different types of RAMs have various kinds of constructions, their fundamental separation mechanism is the same: the macromolecule could be excluded by the out surface of the RAMs through a combination of size exclusion and conventional hydrophobic or ion exchange interactions, while the small analytes could permeate into the inner surface of the RAMs and could be retained. Owing to its merits, such as fast adsorption dynamics, simple operation and reusable property, RAMs has become an important kind of SPE adsorbents for direct extraction and preconcentration of medicine [7], pesticides [8], hormone [9], peptides [10] and protein [11] from biological samples. However, RAMs only provides the fractination of macromolecules and small molécules, its selectivity for target analytes needs to be improved.

Molecular imprinted polymers (MIPs), proposed by Wulff [12] and Klotz [13] in 1972, were featured with distinguished virtues of high stability, easy-to-synthesize and high selectivity. They have been widely employed as adsorbent material in SPE for extraction of organic compounds [14], medicine [15], metal ions [16], protein [17] and amine acids [18]. However, non-target protein or humic materials would be adsorbed irreversibly on the employed MIPs at the same time and deteriorate the performance of the MIPs when it was directly used for extraction of target analytes from biological samples or environmental samples. To overcome these problems, the integration of MIPs and RAMs by taking the advantage of their respective virtues is an ideal way. Boos et al. [19] developed multidimensional on-line SPE technique by using RAM in combination with MIP for detection of *R,S*-Tramadol hydrochloride in human plasma. This multidimensional SPE platform was also employed for highly selective on-line analysis of triazines in river water samples [20]. Pawliszyn et al. [21] set up an on-line and automated method for the selective extraction and detection of verapamil and several metabolites in urine, plasma and cell culture by integrating a MIP with RAMs. Moreover, a variety of self-made RAM-MIPs have been applied for the analysis of target analytes in biological and environmental samples. Haginaka et al. [22–27] prepared a series of RAM-MIPs and applied them for the determination of antiepileptics [22], methylthiotriazine herbicides [23], bisphenol A and its halogenated derivatives [24] in river water samples and direct quantitation of bisphenol A [25], β-blockers [26] and 2-arylpropionic acid derivatives [27] in serum or plasma samples. Dong et al. [28] analyzed sulfonamides in bovine milk with column-switching high performance liquid chromatography by using home-made RAM-MIPs. Puoci et al. [29,30] studied the selective recognition/release behaviors of self-prepared RAM-MIPs for caffeine [29] and p-acetaminophenolin [30] in water media. Similar to MIPs, ion imprinted polymers (IIPs) could recognize metal ions and exhibit all the virtues of MIPs. Up to now, several kinds of ion imprinted sorbents of Cd(II) [31], Cu(II) [32], Hg(II) [33], Mn(II) [34], Pb(II) [35] and Ni(II) [36] were synthesized and used for separation and preconcentration of the target metal ions. These ion imprinted materials showed many advantages including high capacity and selectivity, fast mass transfer and binding kinetics to the target metal ion. However, to the best of our knowledge, there is no report on the use of restricted access material-ion imprinted polymer (RAM-IIP) for direct extraction of metal ions in biological fluids.

The aim of this work is to synthesize a RAM-IIP material by the surface imprinted-emulsion method, and to develop a novel method of RAM-IIP SPE-ICP-OES for direct analysis of free Cu(II) in biological fluids without digestion. The method was validated by the analysis of Cu(II) in Certified Reference Materials of GBW09103 human urine and GBW09152 human serum, along with the real-world human urine and serum samples.

2. Experimental

2.1. Apparatus

Ultrasonic oscillator (Shengyuan Instrument Factory, Shanghai, China), 7312-I electric agitator (Master Pattern Factory, Shanghai, China) and vacuum drying oven (DZG-6020, Shanghai Senxin Instrument Factory, Shanghai) were used in the synthesis procedure. The synthesized RAM-IIP was characterized by transmission electron microscope (Hitachi modal X-650, Tokyo, Japan), FT-IR (170SX, NICOLET, USA). Nitrogen sorption experiments were carried out at 77 K using JWBK surface area and pore size analyzer (JWGB Sci. & Tech., Beijing, China).

Intrepid XSP ICP-OES (Thermo, Waltham, MA, USA) with a concentric model nebulizer and a cinnabar model spray chamber was used for the determination of Cu(II), and the optimum operation conditions are summarized in Table 1. The pH values of aqueous solution were adjusted by a Mettler Toledo 320-S pH meter (Mettler Toledo Instruments Co. Ltd., Shanghai, China) supplied with a combined electrode. An HL-2 peristaltic pump (Shanghai Qingpu Huxi Instrument Factory, Shanghai, China) and a self-made poly tetrafluoroethylene (PTFE) micro-column (20 mm × 2.0 mm i.d.) packed with self-prepared RAM-IIP were used in the separation/preconcentration process.

2.2. Standard solution and reagents

High purity deionized water obtained by Repure system (MRM-III-20, Origin of High Purity Technology Co., Ltd., Wuhan, China) was used throughout the experiment. The stock solutions (1 g L^{-1}) of Cu (II), Co(II), Ni(II), Zn(II), Pb(II) and Cd(II) were prepared by dissolving appropriate amounts of $CuSO_4{\cdot}5H_2O$, $Co(NO_3)_2{\cdot}6H_2O$, $NiSO_4{\cdot}$

Table 1
Optimized operating conditions for ICP-OES.

RF generator power (W)	1150
Frequency of RF generator (MHz)	27.12
Coolant gas flow rate (L min^{-1})	14
Auxiliary gas (L min^{-1})	0.5
Plasma gas (L min^{-1})	0.6
Observation height (mm)	15
Max integration times (s)	30
Analytical wavelength (nm)	Cu 324.754; Ni 341.476; Co 238.892; Cd 326.106; Pb 220.353; Zn 213.856

$(NH_4)_2SO_4{\cdot}6H_2O$, $Zn(CH_3COO)_2{\cdot}2H_2O$, $Pb(NO_3)_2$ and $Cd(NO_3)_2$ (Analytical reagent grade, The First Reagent Factory, Shanghai, China) in 1% (v/v) HNO_3 solution, respectively. Working solutions were prepared daily by appropriate dilutions of stock solutions with high purity deionized water. The HNO_3, divinylbenzene (DVB), toluene, methacrylic acid (MAA), sodium dodecyl benzene sulfonate (SDBS), hydrochloric acid, polyethylene glycol (PEG, molecular weight 10,000), azobisisobutyronitrile (AIBN) and all other reagents used were of spec pure grade or analytical reagent grade. Bovine serum albumin (BSA) (97%) was purchased from Beijing biosynthesis biotechnology Co. Ltd. (Beijing, China).

2.3. Synthesis of restricted access-ion imprinted polymer

0.3 mL emulsion reagent Span 80 was dissolved in the mixture of 10 mL DVB and 3 mL toluene with stirring to form a uniform solution A; 0.3 mL MAA was mixed with 10 mL 1.28 g L^{-1} $CuSO_4$ aqueous solution to form solution B; then solution B was added into solution A with ultrasonification for 5 min and mixture C was obtained. 6.97 g SDBS was added into 125 mL 0.01 mol L^{-1} $MgSO_4$ aqueous solution with stirring and the pH was adjusted to pH 5 with acetic acid–sodium acetate buffer solution, after that the solution was poured into three-neck round bottom flask with stirring under nitrogen atmosphere, refluxed at 70 °C. Hereafter, solution C and 100 mL 3.5 mmol L^{-1} PEG aqueous solution were added into three-neck round bottom flask, then 0.15 g AIBN was added and the reaction was kept at 70 °C under nitrogen atmosphere for 4 h. Afterward, 0.05 g AIBN was added and the reaction lasted for another 12 h. The product was filtered and cleaned repetitively with 1 mol L^{-1} HCl until no Cu could be detected by ICP-OES in the eluent. The product was rinsed with high purity water until free from HCl (tested by pH test strips) and finally dried in vacuum drying oven for use.

For comparison, the restricted access material-non-ion imprinted polymer (RAM-non-IIP) was also prepared using an identical procedure without the addition of $CuSO_4$.

2.4. Micro-column preparation

A total of 20 mg of RAM-IIP was filled into a PTFE micro-column (20 mm × 2.0 mm i.d.) plugged with a small portion of skimmed cotton at both ends. Before use, 1.0 mol L^{-1} HNO_3 and high purity water were passed through the column in sequence for cleaning. Then, the column was conditioned to the desired pH with 0.1 mol L^{-1} NH_4NO_3 buffer solution.

2.5. The exclusion property of macromolecules

The column was sequentially preconditioned by water, HCl (0.07 mol L^{-1}), water, MeOH/water (50/50), water, and finally 25 mmol L^{-1} phosphate buffer (pH 7.4) before use. BSA was selected as the representative macromolecule to test the exclusion property of the prepared RAM-IIP sorbent for the macromolecules, and the adsorption experiment was performed by loading the column with 2.0 mL 1 g L^{-1} BSA in 25 mmol L^{-1} phosphate buffer solution (pH 7.4). The effluent and original BSA standard solution were subjected to UV–vis spectrophotometric analysis, respectively, and the experiment was repeated three times. The absorption spectra of BSA in the effluent and original BSA standard solution were compared to evaluate the protein exclusion property of the prepared RAM-IIP.

2.6. Selectivity studies

Competitive adsorption of Cu(II)/Ni(II), Cu(II)/Co(II), Cu(II)/Cd(II), Cu(II)/Pb(II) and Cu(II)/Zn(II) from their binary mixture at different concentrations was investigated by using self-prepared RAM-IIP and RAM-non-IIP as SPE adsorbents and ICP-OES as detection technique. The extraction percentage (E (%)), the distribution ratio (D), the selectivity coefficient (S) and the relative selectivity coefficient (S_r) were calculated by the following equations:

$$E(\%) = (C_0 - C_e)/C_0 \times 100$$

$$D = (C_0 - C_e)/C_e$$

$$S = D_{Cu}/D_m$$

$$S_r = S_{IIP}/S_{non-IIP}$$

C_0 and C_e are the initial, equilibrium concentrations of metal ions in aqueous solution, respectively. D_{Cu} and D_m are the distribution ratios of Cu(II) and competitive ions (Cd(II), Pb(II), Ni(II), Co(II) and Zn(II)), respectively. S_{IIP} and $S_{non\text{-}IIP}$ are the selectivity coefficients for RAM-IIP and RAM-non-IIP material, respectively.

2.7. Sample preparation

Urine samples were supplied by healthy volunteers in our lab and were immediately analyzed after adjusted to pH=5 with 0.01 mol L^{-1} HNO_3 and 0.01 mol L^{-1} $NH_3{\cdot}H_2O$. Serum samples were provided by Zhongnan Hospital of Wuhan University, Wuhan, China. After ten-fold dilution with high purity deionized water and pH adjustment with 0.01 mol L^{-1} HNO_3 and 0.01 mol L^{-1} $NH_3{\cdot}H_2O$ to pH=5, the serum samples were immediately subjected to SPE pretreatment and subsequent ICP-OES determination. High purity water without any analytes addition was employed as the blank and subjected to the same procedure described above.

For microwave digestion, 1 mL urine and 1 mL serum were transferred into different PTFE vessels and 4 mL concentrated HNO_3, 2 mL H_2O_2 were added, respectively. The microwave digestion (WX-3000 microwave accelerated system, EU Chemical Instruments Co. Ltd., Shanghai, China) was performed according to the following heating programs: 150 °C at 15 atm for 4 min and 180 °C at 25 atm for 4 min. After digestion, clear solution could be obtained. Then it was heated to very small volume to remove the superfluous acid on the ECH-1 temperature control heating panel (Sineo 235 Microwave Chemistry Technology Co. Ltd., Shanghai, China), the residue was dissolved with high purity deionized water. After adjustment of the pH to 5.0 with 0.01 mol L^{-1} HNO_3 and 0.01 mol L^{-1} $NH_3{\cdot}H_2O$, the solution was made up with high purity deionized water to 15 mL and was immediately subjected to SPE pretreatment and subsequent ICP-OES determination. 0.3 mL GBW09103 human urine and 0.3 mL GBW09152 human serum were transferred into different PTFE vessels and 4 mL concentrated HNO_3, 2 mL H_2O_2 were added, respectively. The samples were digested and analyzed according to the procedure mentioned above. The blank samples with the same amount of acid and H_2O_2 were subjected to the same procedure except for adding no analytes.

2.8. General procedure

In separation/preconcentration step, the sample was drawn through the column by a peristaltic pump, and then high purity water was passed through the column in order to clean it. In elution step, the eluent was propelled through the column and the eluting solution was then introduced into the ICP-OES for determination.

3. Results and discussion

3.1. Characterization of restricted access-ion imprinted polymer

The self-prepared RAM-IIP was characterized by FT-IR, TEM and low-temperature nitrogen adsorption/desorption measurements.

The FT-IR spectra of the self-prepared RAM-IIP are shown in Fig. 1. It can be seen that the adsorption bands at 3438 cm^{-1} was the stretching frequency of O–H. The adsorption bands at 2939 cm^{-1} and 1109 cm^{-1} were ascribed to the stretching frequency of $-CH_2$ and C–O, respectively. The characteristic adsorption bands of C=O appeared at 1725 cm^{-1}.

Fig. 2 shows the TEM images of the self-prepared RAM-IIP materials. It can be seen that the prepared RAM-IIP materials were spherical particles with an average diameter of 30 nm.

The pore structure parameters of RAM-IIP were investigated by nitrogen adsorption/desorption experiments. The results demonstrated that the Brunauer–Emmertt–Teller (BET) surface area of the self-prepared material was 86.2 $m^2 g^{-1}$, the Barrett–Joyner–Halenda (BJH) adsorption average pore size and average pore volume were 6.29 nm and 0.17 $cm^3 g^{-1}$, respectively.

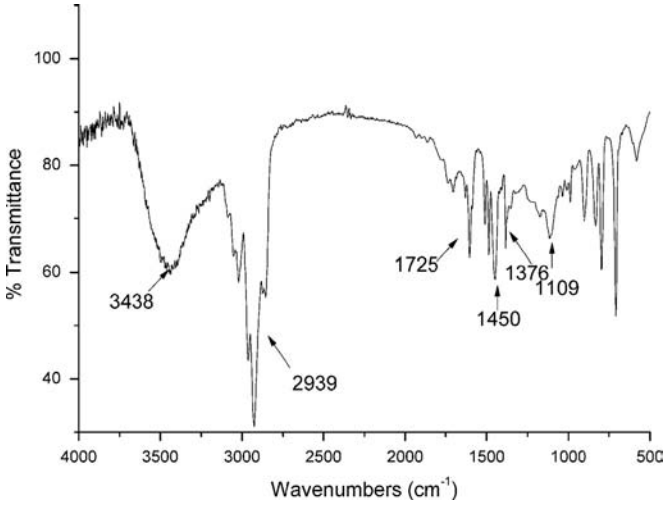

Fig. 1. IR spectroscopy of restricted access-ion imprinted polymer.

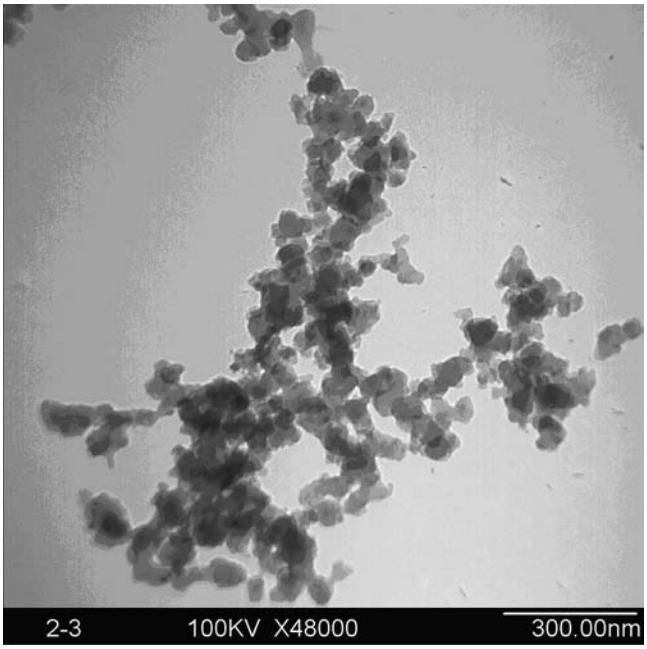

Fig. 2. TEM images of restricted access-ion imprinted polymer.

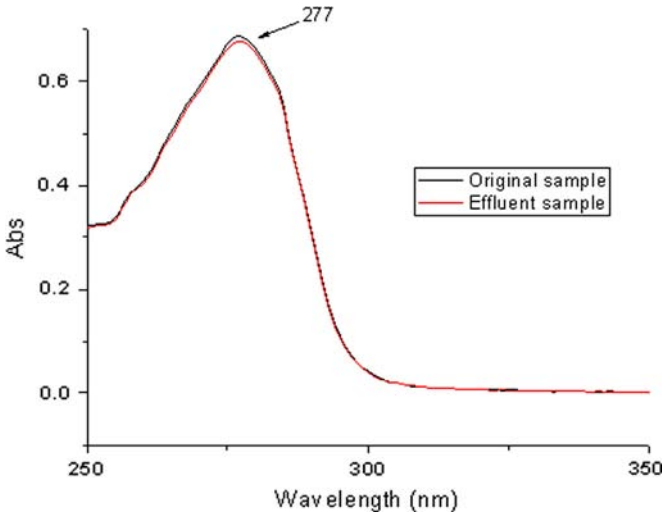

Fig. 3. Protein binding experiment.

3.2. Exclusion property of macromolecules

BSA was selected as the model protein to investigate the macromolecule exclusion property of the prepared RAM-IIP and the UV–vis spectrophotometric analytical results are shown in Fig. 3. As can be seen, the UV spectra obtained from original and effluent BSA solution were coincident well for full scan of wavelength from 200–600 nm. At the maximum UV absorption wavelength of 277 nm, the absorbance (Abs) for original and effluent BSA solution was 0.676 ± 0.003 and 0.684 ± 0.002, respectively. All these results demonstrated that the prepared RAM-IIP materials had good exclusion property for macromolecule.

3.3. Selectivity studies

Table 2 summarizes the extraction percentage (E (%)), distribution ratio (D), selectivity coefficient (S) and relative selectivity coefficient (S_r) of Cu(II), Co(II), Ni(II), Cd(II), Pb(II) and Zn(II) on RAM-non-IIP and RAM-IIP materials at different concentration levels. It can be seen that the relative selectivity coefficient (S_r) was obtained in the range of 0.2–4.3 when the concentration of the studied ions was lower than 0.5 mg L^{-1}, indicating there was no obvious distinction for the adsorption selectivity between RAM-non-IIP and RAM-IIP material. However, when the concentration of the studied ions were higher than 0.5 mg L^{-1}, RAM-IIP material shows the adsorption selectivity to the target Cu(II) rather than other metal ions. The results can be attributed to the non-covalent-surface imprinted synthesized method and adsorption capacity of the self-made polymer. The interaction between template ion and function monomer was mainly electrostatic attraction. Cu(II) and the other metal ions could be adsorbed quantitatively by electrostatic attraction on both RAM-non-IIP and RAM-IIP material at concentration lower than 0.5 mg L^{-1} because the amounts of the studied ions were lower than adsorption capacity of both RAM-non-IIP and RAM-IIP materials and no competition effect occurred. At concentration higher than 0.5 mg L^{-1}, there was competition effect between Cu(II) and the other metal ions due to the limited adsorption capacity, but Cu(II) would preferentially occupy the adsorption sites in the RAM-IIP very rapidly. Hence, the selectivity of the RAM-IIP for Cu(II) was improved at concentration higher than 0.5 mg L^{-1}.

Table 2
Percentage extraction, distribution ratios, selectivity coefficients and relative selectivity coefficients of restricted access-non-imprinted and restricted access-ion imprinted polymer.

Conc. (mg L^{-1})	Element		E (%)		D		S		S_r
			Non IIP	IIP	Non IIP	IIP	Non IIP	IP	
0.1	Cu/Zn	Cu	91	94	10.1	15.7			
		Zn	90	92	9.0	11.5	1.1	1.4	1.2
	Cu/Co	Cu	96.8	90.5	30.3	9.5			
		Co	99	96.2	99.0	25.3	0.3	0.4	1.2
	Cu/Ni	Cu	97	94.8	32.3	18.2			
		Ni	93	90	13.3	9.0	2.4	2.0	0.8
	Cu/Pb	Cu	96	96	24.0	24.0			
		Pb	93	94	13.3	15.7	1.8	1.5	0.8
	Cu/Cd	Cu	99.4	96.3	165.7	26.0			
		Cd	94.4	92.2	16.9	11.8	9.8	2.2	0.2
0.2	Cu/Zn	Cu	91.5	96.6	10.8	28.4			
		Zn	88.3	92.6	7.5	12.5	1.4	2.3	1.6
	Cu/Co	Cu	98.8	97.5	82.3	39.0			
		Co	99.6	99.4	249.0	165.7	0.3	0.2	0.7
	Cu/Ni	Cu	98.2	97.2	54.6	34.7			
		Ni	96	88.9	24.0	8.0	2.3	4.3	1.9
	Cu/Pb	Cu	93	95	13.3	19.0			
		Pb	72	75.8	2.6	3.1	5.2	6.1	1.2
	Cu/Cd	Cu	94.6	94.5	17.5	17.2			
		Cd	98.9	98.92	89.9	91.6	0.2	0.2	1.0
0.5	Cu/Zn	Cu	94	95.8	15.7	22.8			
		Zn	81.4	91.8	4.4	11.2	3.6	2.0	0.6
	Cu/Co	Cu	99.1	98.3	110.1	57.8			
		Co	99	97.2	99.0	34.7	1.1	1.7	1.5
	Cu/Ni	Cu	95.1	93.7	19.4	14.9			
		Ni	97.2	93	34.7	13.3	0.6	1.1	2.0
	Cu/Pb	Cu	98	99.5	49.0	199.0			
		Pb	82	81	4.6	4.3	10.8	46.7	4.3
	Cu/Cd	Cu	99.2	96.6	124.0	28.4			
		Cd	95.4	89.3	20.7	8.3	6.0	3.4	0.6
1	Cu/Zn	Cu	97.2	98.3	34.7	57.8			
		Zn	88.9	86	8.0	6.1	4.3	9.4	2.2
	Cu/Co	Cu	97.4	95.4	37.5	20.7			
		Co	97.3	94.3	36.0	16.5	1.0	1.3	1.2
	Cu/Ni	Cu	97.7	95.6	42.5	21.7			
		Ni	94.2	88.7	16.2	7.8	2.6	2.8	1.1
	Cu/Pb	Cu	99.3	99.4	141.9	165.7			
		Pb	89.2	90.8	8.3	9.9	17.2	16.8	1.0
	Cu/Cd	Cu	97.2	99.3	34.7	141.9			
		Cd	85	87	5.7	6.7	6.1	21.2	3.5
2	Cu/Zn	Cu	99.3	99.7	141.9	332.3			
		Zn	96.5	95	27.6	19.0	5.1	17.5	3.4
	Cu/Co	Cu	96.7	97.3	29.3	36.0			
		Co	96.5	71	27.6	2.4	1.1	14.7	13.8
	Cu/Ni	Cu	99.2	98.3	124.0	57.8			
		Ni	97.9	93.1	46.6	13.5	2.7	4.3	1.6
	Cu/Pb	Cu	96.7	98.8	29.3	82.3			
		Pb	95.4	96	20.7	24.0	1.4	3.4	2.4
	Cu/Cd	Cu	98.2	99.1	54.6	110.1			
		Cd	83	81.4	4.9	4.4	11.2	25.2	2.3
5	Cu/Zn	Cu	99.7	99.9	332.3	999.0			
		Zn	86	83	6.1	4.9	54.1	204.6	3.8
	Cu/Co	Cu	87.4	96.7	6.9	29.3			
		Co	66.4	52	2.0	1.1	3.5	27.0	7.7
	Cu/Ni	Cu	92.7	94.6	12.7	17.5			
		Ni	81.7	66.3	4.5	2.0	2.8	8.9	3.1
	Cu/Pb	Cu	99	91.1	99.0	10.2			
		Pb	98.8	72.5	82.3	2.6	1.2	3.9	3.2
	Cu/Cd	Cu	96.3	97.2	26.0	34.7			
		Cd	87.6	72.7	7.1	2.7	3.7	13.0	3.5

3.4. Optimization of the extraction conditions

3.4.1. Effect of pH

The effect of pH (in the range of 2–8 adjusted by 0.01 mol L^{-1} HNO_3 and 0.01 mol L^{-1} $NH_3{\cdot}H_2O$) on the adsorption percentage of Cu(II) was investigated and the results are given in Fig. 4. As can be seen, the adsorption percentage of Cu(II) was increased with the increase of pH from 2 to 3 and maintained constant in the pH range of 3–6. However, the adsorption percentage was decreased with the increase of pH from 6 to 8. Thus, pH 5 was selected for the separation and preconcentration of Cu(II), and the microcolumn was pre-conditioned to pH 5 with 0.1 mol L^{-1} NH_4NO_3 buffer solution (pH 5).

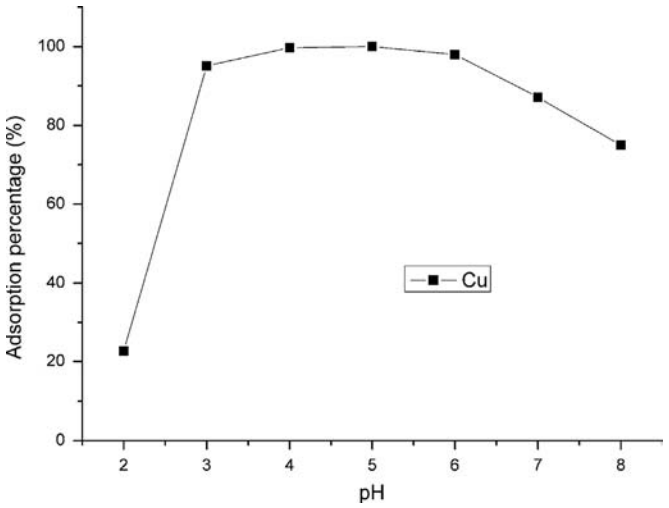

Fig. 4. Effect of pH on the adsorption percentage of Cu on restricted access-ion imprinted polymer

3.4.2. Optimization of elution conditions

As can be seen from Fig. 4, the adsorption of Cu(II) was negligible at pH < 2. Thereby HNO_3 was used as eluent and the effect of HNO_3 concentration in the range of 0.1–0.5 mol L^{-1} on the elution of Cu(II) was investigated. The experimental results indicated that the HNO_3 concentration had no obvious effect on the recovery of Cu(II) in the tested concentration range of 0.1–0.5 mol L^{-1}. For further experiments, 0.4 mol L^{-1} HNO_3 was selected as the eluent.

The effect of elution flow rate on the recovery of Cu(II) was examined with the flow rate varying in the range of 0.2–2.5 mL min^{-1}. It was found that Cu could be recovered quantitatively when the elution flow rate was in the range of 0.2–1.5 mL min^{-1}, while the recovery was decreased with a further increase of the elution flow rate. Additionally, the effect of elution volume on the recovery of Cu(II) was also investigated. It was found that the recovery of Cu(II) was increased with the increase of elution volume from 0.1 to 0.5 mL and maintained constant with further increase of elution volume from 0.5 to 1 mL. In the subsequent experiment, 0.5 mL 0.4 mol L^{-1} HNO_3 was used to recover Cu(II) at a flow rate of 1 mL min^{-1}.

3.4.3. Effects of sample flow rate and sample volume

The effect of sample flow rate in the range of 0.2–2.5 mL min^{-1} on the recovery of Cu(II) was examined. The results indicate that the sample flow rate varying in the range of 0.2–2.5 mL min^{-1} had no remarkable effect on the adsorption of Cu(II). For further experiments, a sample flow rate of 2 mL min^{-1} was applied.

In order to increase the preconcentration factor as possible, large volume of sample solution is favorable. For this purpose, by fixing the mass of Cu(II) constant, the effect of sample volume in the range of 1–100 mL on the recovery of Cu(II) was investigated. The recovery of Cu(II) remained almost constant when the sample volume increased from 1 to 45 mL, and then decreased with

further increasing the sample volume from 50 to 100 mL. Considering the limited amount of biological samples available, sample volume of 15 mL was used in subsequent experiment.

3.4.4. Effects of coexisting ions

Under the optimum conditions, the effect of common coexisting ions in urine and serum on the preconcentration and determination of Cu(II) were examined. For this purpose, different concentrations of K^+, Na^+, Ca^{2+}, Mg^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Br^-, SO_4^{2-}, SiO_3^{2-}, PO_4^{3-}, $C_2O_4^{2-}$, F^-, uric acid, citric acid were added in 0.1 mg L^{-1} of Cu(II) solution, respectively. The obtained sample solutions were subjected to the general procedure. The tolerance limit was defined as the largest amount of coexisting ions making the recovery of Cu(II) maintaining in the range of 90–110%. The experimental results show that the tolerance amount of coexistence ions are 3000 mg L^{-1} for K^+ and Na^+, 500 mg L^{-1} for Ca^{2+} and Mg^{2+}, 5 mg L^{-1} for Fe^{3+}, 50 mg L^{-1} for Br^-, SO_4^{2-}, SiO_3^{2-} and PO_4^{3-}, 10 mg L^{-1} for $C_2O_4^{2-}$, 20 mg L^{-1} for F^-, 200 mg L^{-1} for uric acid, 80 mg L^{-1} for citric acid, 1 mg $L^{-1}$for Co^{2+} and Ni^{2+}, indicating that the prepared RAM-IIP materials have an excellent selectivity for Cu(II) under the optimal conditions and are suitable for the analysis of Cu(II) in samples with complicated matrix.

3.5. Adsorption capacity and regeneration of RAM-IIP

Adsorption capacity is an important factor to evaluate the performance of the solid phase extraction material, and it determines how much SPE adsorbent is required to quantitatively concentrate target analytes from a given solution. Based on the method recommended by Maquieira et al. [37], the adsorption capacity of the self-prepared RAM-IIP for Cu(II) was investigated and the results are listed in Table 3. For comparison, Table 3 also gives the adsorption capacity data of Cu(II) which was obtained by other ion imprinted materials. As can be seen, the adsorption capacity of RAM-IIP for Cu was higher than those reported in Refs. [39,40,42,43] but was lower than those reported in Refs. [38,41].

Regeneration is another important characteristic to evaluate the performance of the adsorption material. It was found that the self-prepared RAM-IIP packed microcolumn could be regenerated by passing through 0.5 mL 0.4 mol L^{-1} HNO_3, which means that the desorption process is the regeneration of the column at the same time. By performing the adsorption and desorption repeatedly, it was found that the self-prepared RAM-IIP packed microcolumn could be reused for more than 35 times without obvious loss of recovery of Cu(II) indicating that the material has good stability under acidic conditions.

3.6. Analytical performance

Under the optimized operating conditions, the analytical performance of the developed method was evaluated and the results are shown in Table 4. With a preconcentration factor of 30, the limit of detection (LOD, evaluated as the concentration corresponding to three times the standard deviation of 11 runs of the blank solution) obtained by RAM-IIP SPE-ICP-OES was 0.17 μg L^{-1} for Cu(II). The relative standard deviation (RSD, $n=7$, $c=1$ μg L^{-1}) was calculated to be 2.2%. Table 5 is the comparison of the LOD obtained by this method with that obtained by other similar approaches. As can be seen, the LOD of the developed method was better than those reported in Refs. 38,39,41–43, but inferior to Ref. [40]. Compared with the reported SPE methods just for total copper determination after acid digestion, the proposed method allows for the determination of free copper ions directly and total copper after acid digestion, and the amount of the coordinated copper could been obtained by subtracting free copper ions from the total copper. So the proposed method can provide the fractionation information of free copper ions and coordinated copper besides total amount information.

Table 3
Comparison of adsorption capacities for Cu(II)-imprinted material (mg g^{-1}).

Sorbent	Cu	Ref.
Poly(EGDMA-MAH/Cu(II)) [a] microbeads	48	[38]
Salicylic acid–formaldehyde–PAR[b]–Cu(II)	0.31	[39]
Cu(II)-imprinted polymethacrylic microbeads	2.39	[40]
Cu(II)-imprinted polymer	29.8	[41]
Stoichiometric Cu(II)-imprinted chelating resin	4.8	[42]
Cu(II)-imprinted styrene–divinylbenzene beads	9.55	[43]
Restricted access-Cu(II) imprinted polymer	15.9	This work

[a] Poly(ethylene glycol dimethacrylate–methacryloylamidohistidine/Cu(II)).
[b] 4-(2-Pyridylazo) resorcinol.

Table 4
Analytical performance of the developed method.

	Cu
Linear range (μg L^{-1})	1–100
R	0.9952
LOD (μg L^{-1})	0.17
RSD (%) ($n=7$, $c=1$ μg L^{-1})	2.2
Preconcentration factor	30
Reused times	40

3.7. Sample analysis

In order to validate the accuracy of the proposed method, the Certified Reference Material of GBW09103 human urine and GBW09152 human serum were analyzed and the analytical results along with the Student t-test results are given in Table 6. As can be seen, the t-test value for GBW09103 human urine and GBW09152 human serum were all smaller than 4.30 ($t_{0.05,\,2}=4.30$), indicating that there is no distinctive difference between the determined value and certified value. In other words, the determined values were in good agreement with the certified values.

The proposed method was applied to the direct determination of free Cu(II) in urine and tenfold diluted serum samples and the analytical results along with the recoveries for the spiked samples are given in Table 7. It can be seen that the recoveries for the spiked samples were between 90% and 108%. The total amount of Cu in the same human urine and serum samples was also determined by the same method after microwave digestion, and the analytical results are also listed in Table 7. As can be seen, the determined values of total Cu obtained after digestion were much higher than the free Cu(II) obtained by direct RAM-IIP SPE-ICP-OES analysis, indicating that Cu(II) in serum and urine were mainly coordinated with macromolecules. In this context, this method is useful for the medical diagnosis of the disease that will cause the disorder of copper equilibrium in organisms such as Wilson's disease, and may provide a new possibility for the speciation of free/coordinated metals in biological samples.

4. Conclusions

A novel restricted access material-Cu ion imprinted polymer (RAM-IIP) was synthesized by surface imprinted-emulsion method and a novel method of RAM-IIP packed microcolumn SPE combined with ICP-OES was developed for the direct determination of trace free Cu(II) in human body fluids. The self-prepared RAM-IIP was demonstrated to possess high selectivity to Cu(II) and good macromolecules exclusion property. The proposed RAM-IIP

Table 5
Comparison of LOD ($\mu g\ L^{-1}$) for Cu(II) obtained by this work and other SPE-based methods.

Analytical technique	Sorbent	LOD	Ref.
SPE-FAAS	poly(EGDMA-MAH/Cu(II)) microbeads	0.4	[38]
SPE-GTA-AAS	Salicylic acid–formaldehyde–PAR–Cu(II)	0.9	[39]
SPE-ETAAS	Cu(II)-imprinted polymethacrylic microbeads	0.064	[40]
SPE-ICP-OES	Cu(II)-imprinted polymer	0.32	[41]
SPE-ICP-OES	Stoichiometric Cu(II)-imprinted chelating resin	0.5	[42]
SPE-FAAS	Cu(II)-imprinted styrene–divinylbenzene beads	1.07	[43]
SPE-ICP-OES	Restricted access-Cu(II) imprinted polymer	0.17	This work

Table 6
Analytical results for Cu in certified reference material of GBW09103 human urine and GBW09152 human serum (mean ± SD, $n=3$).

Certified reference material	Element	Found	Certified	t-test[a]
GBW09103 human urine ($mg\ L^{-1}$)	Cu	0.457 ± 0.03	0.45 ± 0.04	0.40
GBW09152 human serum ($mg\ kg^{-1}$)	Cu	0.975 ± 0.09	1.085 ± 0.044	2.11

[a] $t_{0.05,\ 2}=4.30$.

Table 7
Analytical results of Cu in human urine and serum samples ($\mu g\ L^{-1}$, mean ± SD, $n=3$).

Sample		Spiked	Without digestion		With digestion
			Measured	Recovery (%)	Measured
Urine	Sample 1	0	n.d.[a]	–	18.9 ± 1.5
		1	1.08 ± 0.07	108	–
	Sample 2	0	n.d.	–	20.7 ± 2
		1	0.9 ± 0.08	90	–
Serum		0	3.7 ± 0.3	–	927 ± 83
		3	6.6 ± 0.2	96.7	–

[a] Not detected.

SPE-ICP-OES method was successfully applied to the direct analysis of free Cu(II) and the determination of total Cu after microwave digestion in urine and diluted serum samples and showed a good application potential for trace metal ions analysis in biological fluids. Additionally, this method may provide a new possibility for the speciation of free/coordinated metals in biological samples.

Acknowledgment

Financial supports from the National Nature Science Foundation of China (No. 20975076, 21175102, 21205090) and Science Fund for Creative Research Groups of NSFC (No. 20621502, 20921062) and SRFDP (20110141110010) are gratefully acknowledged.

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