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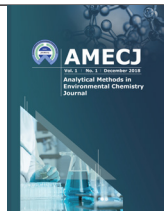

Dispersive solid phase microextraction based on amine-functionalized bimodal mesoporous silica nanoparticles for separation and determination of calcium ions in chronic kidney disease

Sara Davari [a], Farnaz Hosseini [a], and Hamid Shirkhanloo [b,*]

[a] *Islamic Azad University of Pharmaceutical Sciences (IAUPS), Medical Nano Technology Tehran, Iran*

[b] *Research Institute of Petroleum Industry (RIPI), West Entrance Blvd., Olympic Village, P.O. Box: 14857-33111, Tehran, Iran*

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ABSTRACT

The ultrasound assisted- dispersive solid phase microextraction method (USA-SPME) was used for in-vitro study on separation/extraction of calcium ions in human blood of chronic kidney disease (CKD). In this procedure, amine-functionalized bimodal mesoporous silica nanoparticle (NH_2-UVM_7) as a solid phase was used for in-vitro separation/extraction of calcium from blood/serum samples. Moreover, a mixture of NH_2-UVM_7 with ionic liquid and acetone (S/IL/Ac) was added to serum/blood sample containing of Ca (II) at pH of 7.3. After ultrasonic bath and centrifuging, NH_2-UVM_7/IL settled down in bottom of tube, which was extracted Ca (II) ions by binding to amine group ($[Ca]^{2+} \rightarrow : NH_2 - UVM_7$). The concentration of Ca (II) was determined by flame atomic absorption spectrometry (F-AAS, N_2O, C_2H_2) after back extraction remained adsorbent in IL by 0.5 mL of HNO_3 (0.5 M). The results showed us, the NH_2-UVM_7 is a powerful adsorbent for decreasing and controlling of high level calcium concentration in human body and can be used for in vivo study on decreasing calcium concentration in hypercalcemia patient with CKD. The capacity absorption of NH_2- UVM_7 in blood and water samples was obtained 258.5 mg g^{-1} and 267.2 mg g^{-1} at room temperature (25°C). The characterization of NH_2-UVM_7 (SEM, TEM, FTIR and XRD) and comparisons between proposed method and previous methods showed us, the NH_2-UVM_7 as effectiveness sorbent for decreasing calcium concentration level in blood of hypercalcemia patients. Validation of methodology was confirmed using standard reference material (NIST, SRM). Finally, the LOD and %RSD was obtained 3.0 mg L^{-1} and 3.6, respectively.

1. Introduction

Calcium is essential element for bones and teeth in body. It is also important role in heart function, blood clotting, and muscle functioning. Calcium levels increase in patients with kidney disease. Raised calcium levels cause headaches, nausea, sore eyes, aching teeth, itchy skin, and confusion. Calcium (Ca) as a mineral has important role in human body such as; bones, teeth, and nerves. The kidneys keep calcium at normal levels in blood. Also, the vitamin D is important factor for calcium balance in blood serum and kidneys help to activate vitamin D. Chronic kidney disease (CKD) caused to renal failure and hypercalcemia in human. (Normal range: 84–102 mg/L or 2.2–2.5 mmol/L). Hypercalcemia has a positive chronotropic effect on decreasing of heart rate and a positive inotropic effect on increasing of contractility [1, 2]. In CKD, the kidneys are not able to keep the levels of calcium at healthy levels, start to failure and increase parathyroid hormone. So, it is very important that blood calcium level determined

* E-mail: hamidshirkhanloo@gmail.com

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correctly. In parathyroid surgery for removal of glands, blood calcium and phosphate levels must be checked [3-7]. Different techniques, including spectrophotometry, flame atomic absorption spectrometry (F-AAS), inductively coupled plasma (ICP), inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), and other spectrometry methods were used for determination calcium in human biological samples [8-12]. In recent years, many methods have been used for sample preparation in biological samples, such as microwave digestion coupled with ICP-MS, liquid liquid microextraction (LLME), micro solid phase extraction (MSPE) based on nanomaterials, and ionic liquid-solid phase extraction (IL-SPE) for improving of metal extraction[13-16].

Nowadays, IL-SPE has efficient recovery for metal extraction in blood samples. In addition, many carbonaceous materials such as activated carbons [17], natural Adsorbents [18], fullerenes [19], carbon nanotubes [20], and graphene [21, 22] have used for extraction/separation due to their unique properties, such as nano particle size, high surface area, and adsorption capacity [23].

The mesoporous silicate nanoparticles (MSNPs) have been used for a large reactants inside the pores. The properties of MSNPs have simply accessed to sulfur/amine/carboxylate functional groups on surface structure. The Nano mesoporous silica have high surface area and physical adsorption as compared to MSM. The properties of MSNPs have been investigated in metal extraction/separation in biological and water samples by biotechnology. In addition, MSNPs as adsorbents have large surface area and high adsorption capacity for removal of metals from human body such as urine, blood, and plasma. The bimodal of mesoporous silica nanoparticles (UVM_7) are an interesting material which can be considered as an special sorbent for extraction of metals in blood samples[24-28]. In this work, a new applied method based on NH_2-UVM_7 as a nano adsorbent was used for calcium extraction/separation in human blood samples by USA-SPME. To the best of our knowledge, there are no reports on decreasing calcium concentration level in patient with renal failure and hypercalcemia.

2. Experimental

2.1. Reagents and Instrumental

The experiments were performed using a GBC-932 flame atomic absorption spectrometer equipped with an auto-sampler instrument (F-AAS, Dandenong, Victoria, Australia). A hollow cathode lamp of calcium operated at a current of 15 mA and a wavelength of 239.9 nm with a spectral band width of 0.5 nm and deuterium background corrector was applied (100-760 mg L^{-1}). Chemical interferences were seen for air acetylene for calcium determination. For improving of interferences strontium/lanthanum (2000 mgL^{-1}) was added to solution samples. All analytical grade of reagents such as HNO3, Hcl, H2SO43, NaOH, buffers, lanthanum solution (0.5 %), tetraethyl ortho-silicate, triethanolamine , cetyltrimethylammonium bromide and triethoxysililpropylamine were purchased from Merck Company (Germany). In a 1000 mL volumetric flask, add 50 mL deionized water to 1.249 g anhydrous calcium carbonate ($CaCO_3$). Dissolve by adding dropwise 10 mL concentrated hydrochloric acid (HCl). Dilute to 1 liter with deionized water. This standard stock solution is 1000 mg Ca^{2+}/L.

2.2. Synthesis of NH_2-UVM_7

The general procedure for synthesis of bimodal mesoporous silica nanoparticle (UVM_7) is the atrane route, in which the presence of the polyalcohol is the key to balancing the hydrolysis and condensation reaction rates. In a typical synthesis, TEOS (tetraethyl ortho-silicate) was added to predetermine amounts of $TEAH^3$ (triethanolamine). The solution was heated up to 140 °C under

vigorous stirring. After cooling down to 90 °C, CTAB (cetyltrimethylammonium bromide) was added to this solution. For the functionalization of calcined UVM_7 with amine groups, 1.2 g of triethoxysililpropylamine ($C_9H_{23}NO_3Si$) and 2 g of calcined UVM_7 were added to appropriate amount of toluene and refluxed for 24 h at 80 °C [14]. The amine-functionalized bimodal mesoporous silica nanoparticle (NH_2-UVM_7) was used for extraction calcium ions from blood and serum samples.

2.3. Human Sample preparation

For sample preparation of blood/serum samples, only 0.2 mL of samples diluted with DW up to 10 mL and used as real sample. The people of this study selected in two groups: the biological samples from normal men (control groups, 20 N) and renal failure with hypercalcemia as a subject men (n=20). The subject and control groups was selected from men which was matched from people of the same age. For sampling, all glass tubes were washed with a 1.0 mol L^{-1} of HNO_3 solution for at least 24 h and thoroughly rinsed 15 times with ultrapure water before we use. The calcium concentrations in healthy human such as, whole blood / serum have a range from 8.4 to 10.2 mg dL^{-1}. Even minor contamination at any stage of sampling, sample storage and handling, or analysis has the potential to affect the accuracy of the results. In this study, only 0.2 mL of blood/serum samples were collected from dialysis patients and healthy matched controls which were aged between 30 to 60 years. Separate and disposable sterilized plastic syringes were used for human blood sampling. Based on world medical association declaration of Helsinki and recommendations guiding physicians in biomedical research and human Laboratory, the sample storage and blood/urine sampling was prepared based on principles of Helsinki law and absolutely protect the life and health of the human subject. [29]. For analysis of whole blood samples, 10 µL of pure heparin liquid (free Ca, Germany) is added to 10 mL of sample by auto sampler and used 0.2 mL for proposed procedure. By proposed method, the analysis of blood samples can be obtained with minimum of sample (0.2 mL) which was diluted by DW up to 10 mL(DF=50). The human blood/urine sample was maintained at –20 °C in a cleaned glass tube without any reagents.

2.4. Characterizations of NH_2-UVM_7

The SEM was performed to illustrate the morphology and particle size distribution of the calcined NH_2- UVM_7. TEM image also illustrates

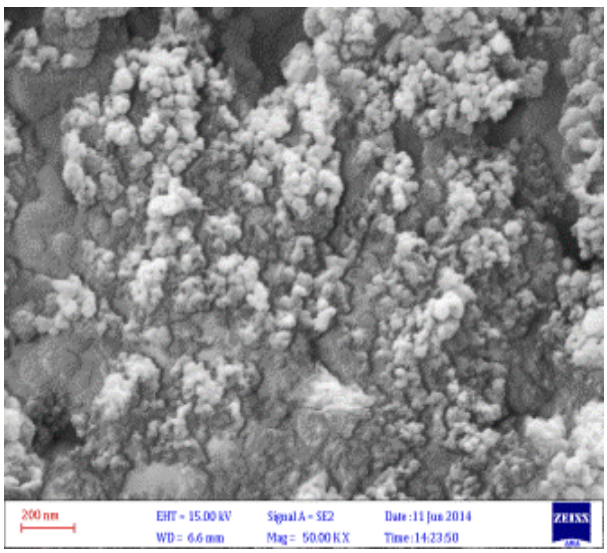

Fig. 1a. SEM of NH_2-UVM_7

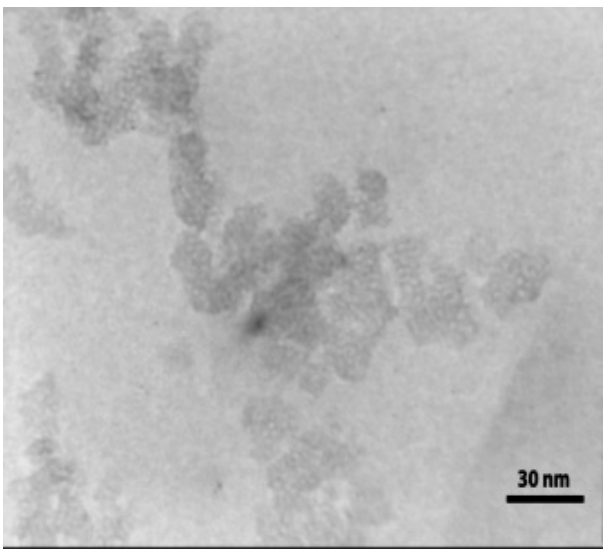

Fig. 1b. TEM of NH_2-UVM_7

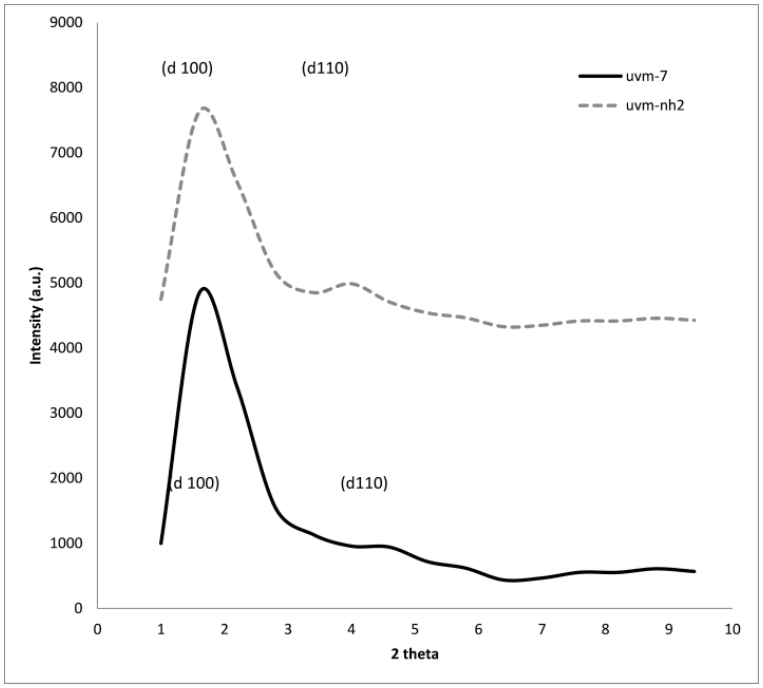

Fig. 2. XRD of UVM_7 and NH_2-UVM_7

pore structure of NH_2- UVM_7 (Fig 1a and 1b). XRD patterns of calcined UVM_7 and NH_2-UVM_7 are shown in figure 2. There are three resolved diffraction peaks in XRD patterns of NH_2-UVM_7 and UVM7, which can be indexed as the (100), (110), (200) and (210) reflections associated with hexagonal symmetry (Fig.2). The nitrogen adsorption-desorption isotherms of UVM_7 and NH_2-UVM_7 were determined and displayed. The corresponding isotherm of both samples displays two distinct regions at medium and high relative pressure which can be attributed to the presence of bimodal pore system. The first is related to the presence of small mesopores (IUPAC clacification), and the second is related to the large mesopores (Fig.3).

2.5. General procedure

In this procedure, 10 mL of standard solution and human blood /serum sample containing calcium ions was used for extraction/separation of calcium. The pH was adjusted to 7.5 with buffer solutions. The amine group of NH_2-UVM_7 (5 mg) as a complexing agent was dispersed in 1-Butyl-4-methylpyridinium hexafluorophosphate [BMPy] [PF_6] (IL/Ac, 0.2 mL) and injected to human serum samples for separation/extraction of Ca ions. The solution place in ultrasound bath for 5 min and Ca^{2+} were complexed and efficient preconcentrated/ extraction by amine group of NH_2-UVM_7 at optimized pH. After shaking, the sample was centrifuged for 5 min and S/IL/Ac settled down in bottom of tube, which was extracted Ca (II) ions by binding to amine group ($[Ca]^{2+} \rightarrow : NH_2 - UVM_7$). Finally, the settled phase was back extracted by 0.5 mL of HNO3 (0.5 M), diluted up to 1 mL with DW and determined by F-AAS. In addition in 1-Butyl-4-methylpyridinium hexafluorophosphate [BMPy] [PF_6] (IL/Ac, 0.2 mL) can be extracted calcium from blood samples up to 6.8% (Fig.4). Extraction conditions of calcium with proposed method was shown in table 1.

4. Results and Discussions:

4.1. Effect of pH

In this work, the influence of sample pH on absorption of Ca (II) has been investigated using different pH from 2 to 12 for 10-75 mg L^{-1} of calcium standard and 0.2 mL of blood samples.

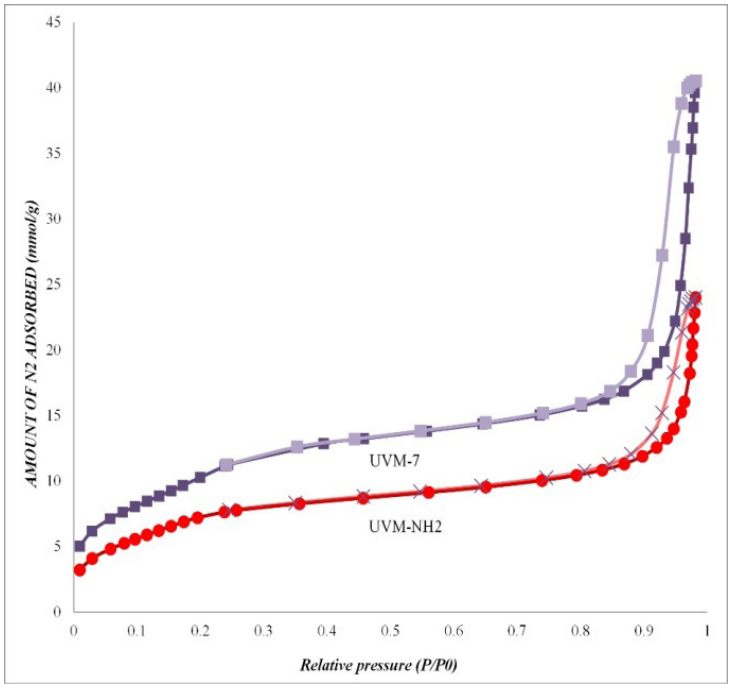

Fig. 3. The isotherms of UVM_7 and NH_2-UVM_7

Table 1. Extraction conditions of calcium with proposed method

Parameter	Value
Working pH	7.50
Amount of NH_2-UVM_7	5.00 mg
Sample volume of blood and serum	0.20 mL
Volume of sample injection	1.00 mL
working range (blood)	9.80-75.90 mg L^{-1}
Linear range (Urine)	10- 50 mg L^{-1}
Intra-day precision (RSD %, n=10)	3.60
Inter-day precision (RSD %, n=10)	4.20
Limit of detection of blood (LOD)	3.00 mg L^{-1}
Preconcentration factor blood (PF)	10.20
Buffer concentration	0.03 mol L^{-1}
Volume and concentration of back-extraction solvent (HNO_3)	500 µL and 0.50 mol L^{-1}
Correlation coefficient	$R^2 = 0.9995$
Ionic liquid/acetone	0.20 mL

The buffer were used for adjusting between pH=7 to 7.7. The complexation was strongly conditioned by the pH of solutions and subsequently affects extraction efficiency of the complex. The result shows that the highest extraction efficiency for Ca (II) was achieved from pH 7.5 (Fig. 5).

4.2. Effect of sample volume

Sample volume one of the most important parameters to be studied. The effect of sample volume was examined in the range of 1-50 mL for 10-50 mg L^{-1} of Ca (II). Quantitative extraction was observed between 1 - 15 mL. At higher

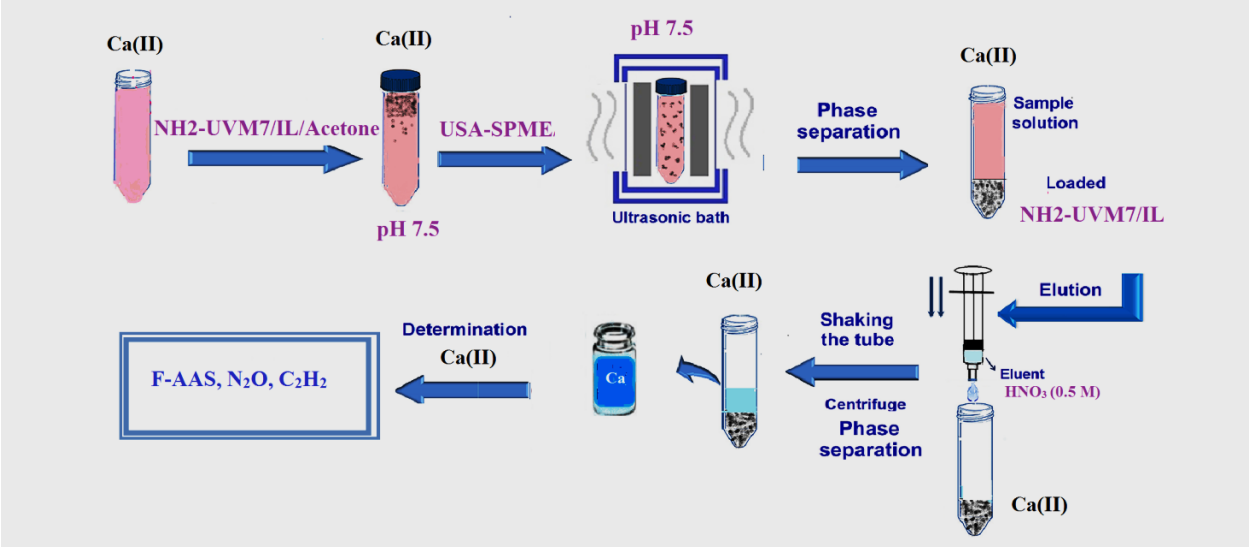

Fig. 4. The procedure of extraction/separation of calcium by USA-SPME

volumes the recoveries are decreased. Therefore, a sample volume of 10 mL was selected for further experiments of USA-SPME in standard and blood samples (Fig. 6). As a consequence, the volume required to back extraction of Ca (II) ions from NH_2-UVM_7 depends on the strength of Ca (II) retention and amount of NH_2-UVM_7 were used in USA-SPME.

4.3. Effect of amount of adsorbent

In optimized conditions, 0.2 mL of blood samples, pH of 7.5 for 10 mL of sample volume, the effect of amount of sorbent was evaluated. It was observed that extraction efficiency of the system was remarkably affected by NH_2-UVM_7 amount in blood samples, so it was examined within the range of 1–15 mg. Quantitative extraction was observed at higher than 4 mg by USA-SPME. Therefore, in order to achieve a suitable preconcentration, 5 mg of NH_2-UVM_7 was chosen as optimum leading to a final adsorbent (Fig. 7). Because of high surface of nano-adsorbent (S/V) a very little amount of NH_2-UVM_7 were used.

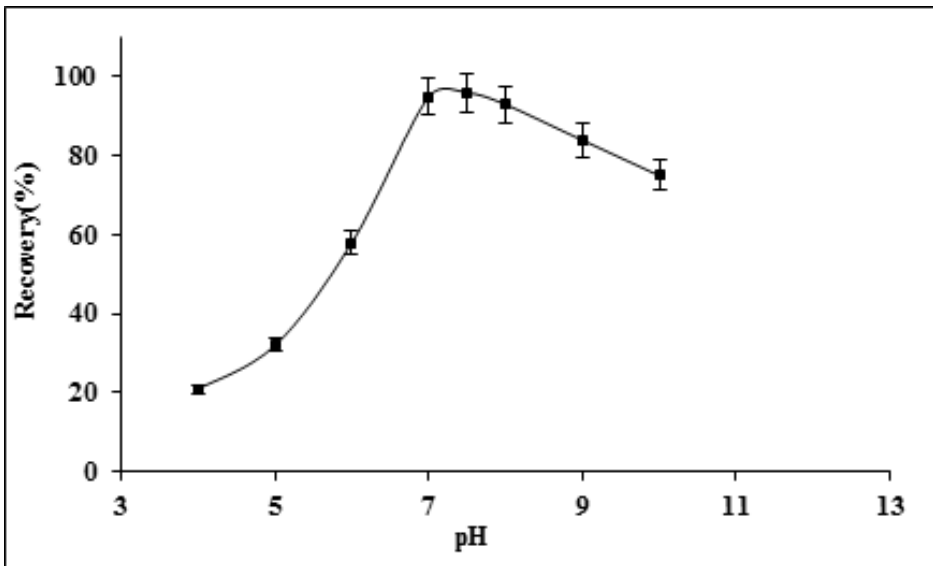

Fig. 5. The influence of sample pH on absorption of Ca (II) by USA-SPME

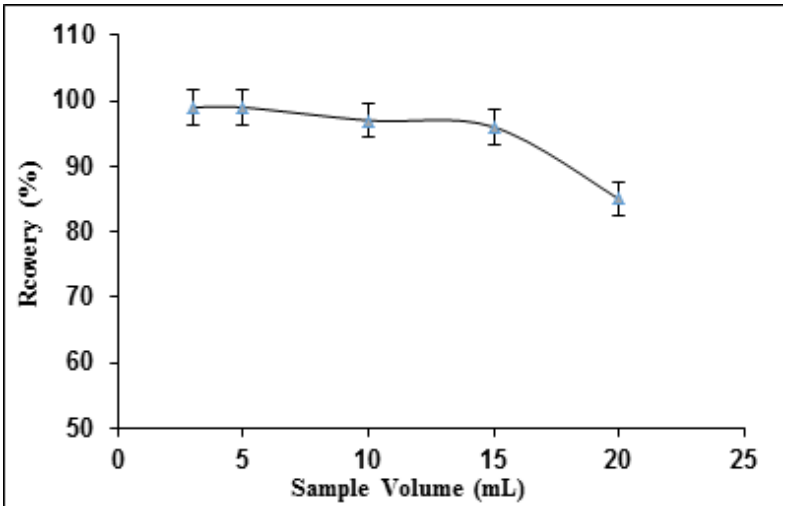

Fig. 6. The influence of sample volume on absorption of Ca (II) by USA-SPME

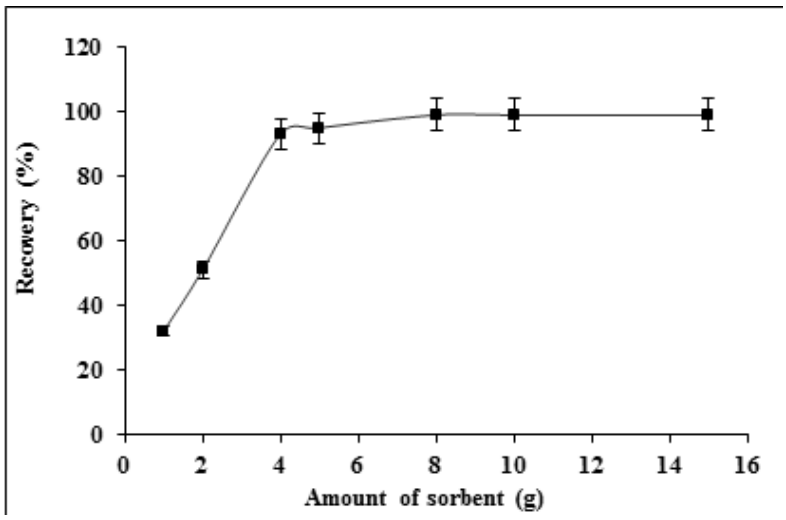

Fig. 7. The influence of amount of sorbent on absorption of Ca (II) by USA-SPME

4-4 Effect of matrix

FAAS is a very simple method with low interference for determination calcium in human body. By USA-SPME, the interference of some coexisting ions in blood and serum samples on the recovery of Ca (II) ions was evaluated for optimized parameters. The interference of coexisting ions effected on pre-concentration step by proposed method. The typical ions in blood and serum samples such as cofactors of Mg, Cu, Zn, Fe, Mn, Cr, Na, K, and Co which was interfered on calcium extraction were investigated. The proposed procedure was performed using a 10 mL sample containing 10-50 mg L^{-1} of analyte and 1─ 5 g L^{-1} of different concentration of matrix ions. The tolerate amounts of each ion were tested that caused less than 7% of the absorbance alteration. In optimized conditions, the ions such as, Zn^{2+}, Cu^{2+}, Cr^{3+}, Co^{2+}, Mn^{2+}, Mg^{2+}, Na^{+}, K^{+}, Fe^{2+} and Mg^{2+} do not interfere to lead extraction by USA-SPME procedure (less than 7%). On the other hand, tolerable concentration ratio of interfering ions versus Ca(II) ions for Ni^{2+}, HCO_3^{-}, SO_4^{2-} and CO_3^{2-}, NO_3^{-} , PO_4^{3-} , Br^{-}, Cl^{-} , F^{-} was less than 360 and 520, separately. The tolerable concentration ratio of interfering ions versus Ca(II) ions for Hg and Ag was obtained less than 45. The results showed us, the most of the probable concomitant cations and anions have no

Table 2. Effect interfering ions on the recovery of Ca (II) ions by USA-SPME procedure

	Concentration ratio ($C_{interferent\ ions}/C_{Ca^{2+}}$)			Mean of Recovery (%)		
Foreign Ions	**Standard**	**Blood**	**serum**	**Standard**	**Blood**	**Plasma**
Zn^{2+}, Cu^{2+}, Cr^{3+}, Co^{2+}, Mn^{2+}	1100	950	900	97.2	95.1	96.8
Mg^{2+}, Na^{+}, K^{+}, Fe^{2+}, Mg^{2+}	1200	1000	800	98.4	97.1	99.5
CO_3^{2-}, NO_3^{-}, PO_4^{3-}, Br^{-}, Cl^{-}, F^{-}	700	520	470	97.7	98.2	98.9
Ni^{2+}, HCO_3^{-}, SO_4^{2-}	450	360	320	96.2	95.0	97.3
Hg^{2+}, Ag^{+}	60	45	40	95.4	96.2	95.8

considerable effect on the recovery efficiencies of lead ions (Table 2).

4.5 Method Validation

The USA-SPME method based on NH_2-UVM_7, were applied to determine Ca (II) in water samples. The spiked samples were prepared to demonstrate the reliability of the method for determination of Ca (II). The remaining aliquots were spiked with increasing quantities of Ca (II) and then analyzed by the proposed method (Table 3). The recoveries of spiked samples are satisfactorily reasonable and were confirmed by using the additional method, which indicates the capability of the system in the determination of Ca (II) in standard and human blood samples (0.2 mL). Also, the results showed that the Ca (II) concentrations in blood samples ranged from 11.63- 15.17 mg L^{-1} and 8.58 ─ 10.76 µg L^{-1} in the renal failure subjects and control samples, respectively (Table 4). The intra mean concentration of Ca (II) in serum of hypercalcemia subjects (12.45 ± 0.59 µg L^{-1}) was significantly higher than healthy men controls (8.95 ± 0.44 µg L^{-1}) ($P< 0.001$). Also, total value of calcium in blood of hypercalcemia subjects is higher than the normal groups which were recommended by standard value of human biochemistry. The results showed that the Ca (II) concentrations in blood samples of hypercalcemia subjects (20N) were higher than in controls groups. There is no correlation between control and subject groups were achieved ($r \approx 0.1$).

5. Conclusions:

In this method, NH_2-UVM_7 nano-particles were

Table 3. Validation of calcium determination with FAAS by Ca (II) standard addition in human blood and water samples (mg L^{-1})

Sample	Added	Found *	Recovery (%)
[a]Blood	---	15.2± 0.6	---
	15.0	29.8 ± 0.7	98.0
[a]Blood	---	19.4 ± 0.8	---
	20.0	40.1 ± 1.7	103.3
[a]Blood	---	14.3 ± 0.6	---
	15.0	28.8 ± 0.8	96.6
wastewater	---	6.3 ± 0.3	---
	5.0	11.1 ± 0.5	96.0
Water	---	2.2 ± 0.1	---
	2.0	4.3 ± 0.3	105
Waste water	---	10.6 ± 0.1	---
	10.0	20.3 ± 0.1	97.0

*Mean of three determinations ± confidence interval (P = 0.95, n =5)
[a] 0.2 mL of blood samples diluted with DW up to 10 mL (DF:50)

Table 4. determination of calcium in serum, blood and urine by USA-SPME method (intra –day and inter day) (mg dL^{-1})

Sample	Hypercalcemia Men (n=20)		Healthy Men (n=20)		Hypercalcemia	
	Intra-day	Inter day	Intra-day	Inter day	r	*P value*
Serum	12.45 ± 0.59	12.62 ± 0.64	8.95 ± 0.44	9.08 ± 0.51	0.113	*<0.001*
Plasma	7.94 ± 0.46	8.02 ± 0.52	6.32 ± 0.32	6.53 ± 0.48	0.102	*<0.001*
Blood	13.04 ± 0.63	13. 27 ± 0.68	10.06 ± 0.48	9.87 ± 0.55	0.117	*<0.001*

*Correlations are based on Pearson coefficients (r). Statistical significance will be observed if $P < 0.001$
Mean of three determinations of samples ± confidence interval (P = 0.95, n =10)

used as a solid phase for extraction and separation of Ca (II) by USA-SPME. The developed method has the advantages of simplicity, relative selectivity, and high preconcentraion factor for Ca (II). A small amount of adsorbent, low volume of sample (0.2 mL) is employed in this procedure. The determination of Ca (II) in blood and environmental samples was successfully performed. The LOD, preconcentration factor, working range, and dilution factor for human samples was obtained 3.0 mg L^{-1}, 10.2, 9.8-75.9 mg L^{-1} and 50 respectively.

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