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Novel oligonucleotide-based sorbent for the selective extraction of cadmium from serum samples

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Abstract

The objective was to develop a sorbent functionalized with aptamers for the selective extraction of cadmium from biological samples. Two oligonucleotide sequences reported in literature as specific to cadmium were covalently grafted on activated Sepharose, with grafting yields of 45%. Once the supports packed in cartridges, a thorough study of the percolation conditions favoring Cd(II) retention was performed, demonstrating the importance of the nature of this medium. A high selectivity was reached when applying the optimal conditions as a recovery of 85% was obtained using the sorbent functionalized with one of the specific aptamers and only 1% on the control sorbent grafted with a scramble sequence. A high specificity was also obtained as recoveries for most of other ions were lower than 15%. The capacity of this oligosorbent estimated to 180 ng of Cd(II) for 30 mg of support was perfectly adapted to the trace analysis of Cd(II). The extraction procedure was then applied to a serum sample which was first subjected to acid precipitation. The initial concentration of cadmium in the serum was estimated to 1.83 µg/L using standard addition method and an extraction yield of 75 ± 1.6% was measured. Comparison of these results with those obtained without oligoextraction (recovery of 57%) showed a significant reduction of matrix effects in ICP-MS thanks to the use of the oligosorbent, underlining its interest for a more reliable quantification of Cd(II). This result was confirmed by performing the oligoextraction protocol on a certified serum.

Keywords: Solid-phase extraction; Aptamers; Oligoextraction; Cadmium; ICP-MS

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1. Introduction

Cadmium is a naturally occurring metal, which belongs to the class of transition metals. It is now considered as one of the most toxic metals to humans. It is recognized as carcinogen [1] and can cause tissue damage by creating oxidative stress [2,3]. It can also compete with other ions, such as Fe(II) and Zn(II) [4], and disrupt their mechanisms of physiological action on the human body. Cadmium is commonly used for anthropogenic activities such as Nickel-Cadmium batteries or paint pigments [5]. The main source of population exposure to cadmium is the ingestion of cigarette smoke [6]. In order to monitor accidental exposure of the population, some countries have set levels of cadmium concentration to be found in the blood [7], which is 4 µg/L in France, for example [8].

Cadmium trace analysis is typically performed by inductively coupled plasma mass spectrometry (ICP-MS) [9]. However, matrix effects can compromise the accuracy of the results obtained with this instrument especially when dealing with complex samples such as biological fluids. In order to overcome these problems, a sample treatment technique is mandatory before the ICP-MS measurement. Solid phase extraction (SPE) is by far the most used technique to purify and preconcentrate ions in order to quantify them at the trace level [10]. Conventional sorbents such as cation exchange resins [11] have been largely used but their specificity is limited as some other cations are co-extracted. In order to overcome this lack of specificity, new highly specific and selective supports have been developed such as ion-imprinted polymers (IIPs) [12,13]. They are synthetic supports with cavities allowing a selective trapping of the target ion. The potential of IIPs has been largely demonstrated [14] but their synthesis remains a complex step in analytical development.

The objective of this study is to investigate the potential of aptamers to specifically and selectively extract cadmium from complex samples. Aptamers are single-stranded DNA or RNA (typically 20 to 110 bases) that have a specific and high affinity for molecules or ions. They are identified by a complex process called SELEX for Systematic Evolution of Ligands by EXponential enrichment [15]. Aptamers immobilized on solid supports have been widely reported for the extraction of molecules, from small drugs to proteins [16–18], but little for the extraction of ions. Concerning cadmium, cadmium-specific oligonucleotide sequences have been identified and applied to develop specific sensors [19–32].

A first cadmium-specific sequence, characterized by a dissociation constant of 34 nM and composed of a so-called specific sequence placed between two non-specific sequences at the 5' and 3' ends, was described for the development of a sensor [28]. This sequence was further modified at the 3' and/or 5' ends by deleting the non-specific part or by adding nucleotides or chemical groups to modify the sensor detection mode [19,21–24]. Some of the authors [23,32] deleted the non-specific part in 5', but kept it partially in 3' (the first 6 nucleotides) to improve the sensor sensitivity. Once the sensors developed, they were applied to the determination of cadmium in tap and river waters. A second cadmium-specific sequence, characterized by a dissociation constant of 220 nM [26], was reported [27]. During SELEX, after having identified several sequences showing some affinity for cadmium, a negative selection round was performed to eliminate the sequences with an affinity for zinc, chosen because of its similar chemical behavior to cadmium in solution. The sequences identified during the SELEX process were then compared in terms of affinity using circular dichroism and one of them was selected for the development of a light-up biosensor.

These two sequences were thus considered here to develop for the first time oligosorbents (OSs) to be used as cadmium-selective extraction sorbent. After grafting the reported sequences on CNBr-activated Sepharose and measuring the grafting yields, the performances of the two resulting OSs were evaluated and compared in terms of affinity, selectivity and specificity. The optimization of the percolation, washing and elution media allowed to demonstrate their strong influence on the affinity of the OS for Cd(II), i.e. on its retention. To evaluate the selectivity of the retention process, the behavior of Cd(II) on a given specific oligosorbent was compared to that of a support grafted with the same sequence but scrambled acting as a control OS. Based on selectivity toward the control OS and specificity towards other ions, one of the two specific OSs was selected. Once the extraction protocol was optimized, the performance of this specific OS was determined in terms of capacity and specificity towards several other cations. Finally, the protocol was applied to extract Cd(II) from spiked and certified serum samples before its analysis in ICP-MS and the results were compared with the ones corresponding to the analysis of the samples not submitted to oligoextraction. Once the oligoextraction support has been developed, initially for performing measurements using ICP-MS, future experiments using less expensive but less specific instruments can be considered. Furthermore, the use of this type of sample treatment allows for simplified utilization of ICP-MS without the addition of internal standards, for example.

2. Experimental

2.1. Reagents and samples

Metal standards (in 2% nitric acid), Trizma hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid tetrasodium salt (EDTA), sodium phosphate dibasic, sodium dihydrogen phosphate dihydrate, sodium acetate, sodium nitrate, sodium perchlorate, sodium chloride, potassium chloride, magnesium chloride, HEPES buffer, MES buffer,, 65% nitric acid, triethylamine (TEA), glacial acetic acid, human serum (a pool of male serum from USA with blood group AB), and CNBr-activated Sepharose (4B, 90 µm) were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Acetonitrile (ACN) was obtained from Carlo Erba (Val-de-Reuil, France). Hydrochloric acid 32% was obtained from Merck (Darmstadt, Germany). Serum certified for trace elements was obtained from UTAK (Santa Clarita, California, USA). The serum come from lot C9377, which has a certified cadmium value of 8 µg/L with an uncertainty of ± 1.2 µg/L. High-purity water was obtained using a Milli-Q purification system (Millipore, Saint-Quentin-en-Yvelines, France). The ICP-MS was daily tuned using an Agilent tuning solution (Ce, Co, Li, Mg, Tl, and Y at 1 µg/L in 2% HNO_3 (Les Ulis, France)). 5'-amino-modified with C_6 spacer arm DNA oligonucleotides (sequences 5'-GGACTGTTGTGGTATTATTTTTGGTTGTGCAGTATG-3' for AC1, 5'-ATGATTGATGTTAGGTGTTAGTTGCTCGTTGTGTGT-3' for scramble AC1 (named c1), 5'-CTCAGGACGACGGGTTCACAGTCCGTTGTC-3' for AC2, and 5'-GATGAAGTGCAGTGCTTGCTCACCGCCCTG-3' for scramble AC2 (c2)) were synthetized and purified by Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) from Eurogentec (Angers, France). The sequence modeling was done thanks to Mfold web server provided by M. Zuker.

2.2. ICP-MS

The determination of ion concentrations in solutions was performed with an ICP-MS 7850 from Agilent Technologies (Les Ulis, France) equipped with a glass MicroMist nebulizer and a quartz Scott spray chamber with an ASX-500 autosampler. The parameters applied during the measurement were as follows: RF power, 1550 W; sampling depth, 8 mm; plasma gas flow rate, 15 L/min; auxiliary gas flow rate, 0.9 L/min; Helium flow rate, 4.3 mL/min. During the daily performance check, the monitoring of ^{7}Li, ^{89}Y, ^{205}Tl, CeO/Ce ratio and Ce^{2+}/Ce ratio was carried

out in order to ensure constant performances on all measurements. The limit set for ratios was CeO/Ce ≤ 1.2% and Ce^{2+}/Ce ≤ 2%. The measurements were carried out with five replicates with 100 sweeps per replicate with an integration time of 1 s. The isotopes measured for the tested ions is: ^{24}Mg, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{111}Cd, ^{208}Pb, ^{207}Pb, and ^{208}Pb. All the samples were diluted to 1/10 in 2% HNO_3 before ICP-MS measurement. To quantify ions in the different solutions, calibration curves were daily studied between 5 ng/L and 5 µg/L. The limit of quantification (LOQ) was determined by measuring 10 blank solutions (HNO_3 2%). The equation described in Equation S1 (see Supplementary data) was then applied and the calculated LOQ value for Cd(II) was 4.2 ng/L. The LOQ values for the interfering ions were determined using the same method and are given in Table S1 (see Supplementary data). Another method was also used for determining the Cd(II) LOQ. It consisted of studying the calibration curve and determining the relative standard deviation (RSD) values obtained using 3 independent solutions for each concentration level and of considering a concentration level as quantifiable when the RSD values are lower than 5 %. Results are reported in Table S2. The minimum quantifiable concentration for Cd(II) was 5 ng/L, that is in good agreement with the previously calculated LOQ of 4.2 ng/L.

2.3. LC-UV analysis of aptamer

This method was adapted from previous studies realized by our group [33]. Grafting yields were determined by analyzing aptamer content in the solutions recovered after the grafting procedure by ion pair RP-HPLC-UV (Reverse Phase High Performance Liquid Chromatography) using an Agilent 1200 series HPLC system equipped with an 1100 series autosampler and a 1200 series UV detector. The aptamers were monitored at 260 nm. The separation was achieved on a Kinetex core shell C18 column (150 x 3 mm i.d., 5 µm, 100 Å) from Phenomenex (Le Pecq, France) at 50°C. The column was connected to a precolumn filter (0.5 µm frit, 2.39 x 1.65 mm, Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase was composed of water with 0.1 M triethylammonium acetate (TEAA, prepared with TEA and acetic acid) pH 7 and acetonitrile. The gradient applied is as follows: 7% ACN for 5 min then a ramp of 15% ACN is performed in 15 min, 15% ACN is then maintained for 5 min before re-equilibrating the column to 7% ACN during 10 min. The flow rate was set at 200 µL/min and the injected volume was 20 µL. Before sample injection, a calibration range for each aptamer sequence was performed from 0.1 to 10 µg/mL in 200 mM Na_2HPO_4 and 5 mM $MgCl_2$.

2.4. Oligosorbent synthesis

The procedure used to immobilize aptamers on CNBr-activated Sepharose has been previously described by our group to develop OS for organic molecules [33]. Briefly, prior to immobilization, the dried aptamers were dissolved at 1 g/L in a buffer (200 mM Na_2HPO_4 and 5 mM $MgCl_2$, pH 8) and denatured by heating at 75°C for 5 min. The solution was then left at room temperature for 30 min. CNBr-activated Sepharose (30 mg) was swollen and washed 6 times with 1 mM HCl and then rinsed with 1 mL of ultrapure water. The aptamer solution (150 µL) was then mixed with CNBr-activated Sepharose gel overnight at room temperature. The oligosorbent was then placed in a 1 mL SPE cartridge between two frits and washed 3 times with 1 mL of 200 mM Na_2HPO_4 pH 8. These washing solutions were recovered and diluted in 200 mM Na_2HPO_4 and 5 mM $MgCl_2$ in order to measure their content in non-grafted aptamers and then to calculate the grafting yield for each support. A 0.1 M Trizma solution was percolated through the cartridge during 2 h to block the remaining active groups of Sepharose. Finally, the OS was washed 3 times alternately with 2 mL of an acetate buffer (0.1 M acetate + 0.5 M NaCl, pH 4) and 2 mL of a Trizma buffer (0.1 M + 0.5 M NaCl, pH 8). The sorbents were conditioned with 5 mL of ultrapure water. The different oligosorbents synthetized in this study are called OS1 (oligosorbent grafted with the sequence AC1), OS2 (oligosorbent grafted with the sequence AC2) and OSc1 and OSc2 for the non-specific OS grafted with the respective c1 and c2 scramble sequences.

2.4. Extraction procedure

2.4.1. Pure media

Before each extraction, the OS was conditioned with 5 mL of the tested buffer. The ions are eluted from the cartridge at a flow rate estimated at 1.5 mL/min.

The percolation solution consists of 1 mL of the tested buffer spiked at 30 µg/L with Cd(II) and eventually other ions (Mg, Co, Ni, Cu, Zn or Pb, each at 30 µg/L) to study the specificity. The sorbent was further washed with 1mL of the same tested buffer. Finally, elution is performed with 2 x 500 µL of 1 mM EDTA. All these steps were performed with solutions maintained at 4°C. Each SPE fraction (percolation, washing and elution) was then diluted 1/10 in 2% HNO_3 for ICP-MS analyses. After determining the optimal percolation medium (25 mM Na_2HPO_4 + 25 mM NaH_2PO_4 + 150 mM NaCl), the capacity of the most promising OS was determined using the same protocol described above but by percolating 1 mL containing increasing amounts of Cd(II) from 30 to 600

ng on the OS. The elution fractions were then diluted in 2% HNO_3 with a dilution factor adapted to the introduced amount of Cd(II) to be in the calibration curve range.

2.4.2. Serum samples

A first step of acid precipitation of the serum samples was performed to eliminate proteins that may complex cadmium. For this, 500 µL of serum (spiked or non-spiked) were introduced in an Eppendorf tube and mixed with 30 µL of 65% HNO_3. The mixture was centrifuged (10 min, 12 000 rpm) and the supernatant was then recovered and submitted again to centrifugation (10 min, 12000 rpm). The resulting supernatant was placed in a 5 mL polypropylene tube to be diluted with 4.5 mL of buffer (25 mM Na_2HPO_4 + 25 mM NaH_2PO_4 + 150 mM NaCl, pH 6.6). The pH was adjusted to 6.6 with 2 M NaOH. The optimized oligoextraction protocol was applied on 1 mL of treated serum sample. After oligoextraction, the elution fractions were diluted 1/10 in 2% HNO_3 and analyzed by ICP-MS. The determination of cadmium concentrations was done thanks to a calibration curve performed before each sequence (an example is provided in Figure S1). To demonstrate the potential of oligoextraction, the non-certified serum was spiked at concentrations of 2, 4 and 30 µg/L of Cd(II) and the content of serum sample determined after oligoextraction was compared to the Cd(II) content determined after the analysis of a serum sample only submitted to an acid precipitation and a dilution step (dilution rate of 50 in order to obtain the same dilution factor as after the oligoextraction). Two replicates were performed for each spiking level. The same protocol was also applied to the certified serum.

3. Results and discussion

3.1. Oligosorbent synthesis and grafting yields

As previously mentioned, the Cd-specific sequence first described [28] has been modified by many authors [19–25] in order to adapt it to the immobilization procedure (addition of thiols or amino functions) or detection mode (fluorescence, electrochemistry, ...) for the development of aptasensors. Indeed, only a part of the sequence allows the recognition of the Cd(II) ion. As done by some authors, [23,32] it was chosen to delete the non-specific part identified by the first team [28] while keeping the first 6 nucleotides in the 3' part, this sequence being named AC1 in this work. A second sequence, named AC2, also described in the literature although less widely used, [27] was also selected. Two control sequences were also synthesized, respectively c1 and c2.

These control sequences have the same composition as AC1 and AC2 but with a different nucleotide base order randomly established through a base shuffling tool provided by the Genscript website. While this kind of control sequences were not used in the development of the aptasensors, they allow the selectivity of the Cd(II) retention process to be assessed. This ensures that any observed retention is attributed to the conformation of the sequence rather than nonspecific interactions that could occur between Cd(II) ions and the nucleotide base and their anionic phosphate groups.

The sequences were then grafted on CNBr-activated Sepharose and the grafting yields were estimated by determining the residual amount of the non-grafted aptamers in the recovered solution of grafting and in the solutions used for washing after the immobilization step by ion pair RP-HPLC-UV analysis. The values of grafting yields are reported in Table 1. They are between 35% and 63%, similar to those obtained when grafting aptamers specific of organic molecules on CNBr-activated Sepharose [18]. Two independant syntheses were carried out for each OS, demonstrating a good repeatability of the grafting for each sequence (a maximum of 11% of difference between each synthesis for a given aptamer). Once the grafting yield is known, it is possible to determine the theoretical capacity of each OS functionalized with a Cd(II) specific aptamer by assuming that one mole of aptamer can retain one mole of cadmium ion. The theoretical capacity values for SPE cartridges containing 30 mg of grafted Sepharose are also reported in Table 1. They are between 680 and 840 ng (i.e. between 23 and 28 ng/mg of grafted Sepharose), which is sufficient to extract trace amounts of cadmium from serum.

Table 1. Grafting yields for each OS and the corresponding theoretical capacities of the SPE cartridge containing 30 mg of grafted Sepharose. The two values correspond, for each sorbent, to two independent immobilization procedures.

Oligosorbent	Grafting yield (%)	Theoretical capacity (ng)
OS1	46 ; 57	680 ; 840
OS2	45 ; 42	810 ; 750
OSc1	63 ; 63	-
OSc2	35 ; 41	-

3.2. Selection of the percolation media

The first step of an SPE procedure consists in the percolation of the sample, during which retention of the target analyte is expected. It is therefore necessary to select a percolation buffer promoting the Cd(II) retention on OS1 and OS2. Although no medium promoting the retention of

Cd(II) on an OS has been described in the literature, it is commonly admitted that choosing a buffer as percolation medium with a composition close to the aptamer sequence selection buffer should favor retention during the percolation step since an optimal affinity is then expected. Furthermore, it is reasonable to assume that a buffer used for Cd(II) measurement with an aptasensor should also be a buffer favorable to the Cd(II) retention on the OS functionalized with the same aptamer. However, for the two selected sequences, the nature of the measuring buffer was not clearly described and of very variable composition (buffers, salt solutions...). Therefore, several percolation media including those described in the works related to aptasensors, were studied with the objective to retain Cd(II) on OS1 and OS2. Two ions were also introduced with Cd(II) in the percolated buffer for these first experiments in order to compare the behavior of the target ion Cd(II) and some other ions, to evaluate the specificity of the retention process. Co(II) and Pb(II) were selected for their double charge and for their easy determination in ICP-MS. Selectivity was also studied by comparing the ability of the support functionalized with the specific sequence to retain Cd(II) with that of the support functionalized with the control sequence. Once percolation was done, a washing step of the supports was carried out to eliminate the interfering ions potentially retained during percolation. Initially, the washing medium used was similar to that used during percolation. Subsequently, an optimization of the washing step was performed by changing the volumes and/or the nature of the washing medium. Then, the elution of the ions retained on the support was carried out with 1 mM EDTA selected for its ion chelating properties. After dilution of all the recovered fractions with 2% HNO_3, ions were quantified by ICP-MS.

All the tested percolation media are listed in Table 2, which presents also the results in a simplified way. The detailed ones are presented in Figures S2-S13 in supplementary data. Due to the large number of media tested and the need to assay ions all the fractions (percolation, washing and elution fractions), some extraction procedures were carried out only once. Nevertheless, this approach enabled us to obtain a trend in the behavior of the various ions as a function of the percolation media. In the first column (Cd retention), the positive sign means that Cd(II) is retained on the specific OS during the percolation. In the second column (Cd vs Pb and Co), the positive sign indicates that only cadmium is retained until elution, the ± sign indicating that cadmium but also one of the two interfering ions tested is retained until elution. The minus sign indicates that Cd(II), Co(II) and Pb(II) are retained until elution, thus indicating a poor specificity of the retention process. In the third column, the positive sign indicates that Cd(II) is retained until the elution only on the specific OS and not on its control OS thus meaning that selectivity is obtained using this

percolation and washing media. For this column, the minus sign indicates that cadmium is retained until the elution on the specific OS but also on its control OS (meaning that no selectivity was observed).

Table 2. Evaluation of cadmium retention during percolation, specificity (retention of Cd(II) vs. Co(II) and Pb(II) until elution on the specific OS) and selectivity (retention of Cd(II) until elution on the specific OS vs the control OS) for all the percolation media tested. The results are presented in detail in Figures S2-S13.

Percolation media	Cd retention		Cd vs Pb & Co		Cd behavior	
	OS1	OS2	OS1	OS2	OS1 vs OSc1	OS2 vs OSc2
ultra-pure H_2O	+	+	-	-	-	-
EDTA (10^{-5} M to 10^{-3} M)	-	-	-	-	-	-
NaCl (10 mM pH 6.0)	+	+	-	-	-	-
$MgCl_2$ (5 mM pH 6.0)	-	+	-	±	-	+
$NaNO_3$ (10 mM pH 6.0)	+	+	±	-	-	-
Na_2HPO_4 (100 mM pH 7.0)	-	+	-	±	-	+
HEPES (30 mM pH 6.5)	+	+	-	-	-	-
MES (50 mM pH 6.0)	+	+	-	-	-	-
Tris-HCl + KCl + NaCl + $MgCl_2$*	-	+	-	±	-	+
Acetate + perchlorate**	+	+	-	+	-	+
PBS1	-	+	-	±	-	+
PBS2	**-**	**+**	**-**	**+**	**-**	**+**

*Tris-HCl (20 mM), KCl (25 mM), NaCl (50 mM), MgCl2 (5 mM), pH 7.4. **Sodium acetate (10 mM), sodium perchlorate (10 mM), pH 6.0. PBS1: Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (137 mM), KCl (2.7 mM), pH 6.6. PBS2: NaH_2PO_4 (25 mM) + Na_2HPO_4 (25 mM) + NaCl (150 mM), pH 6.6.

When percolating spiked ultra-pure water (Figure S2), both specific OSs (OS1 and OS2) retained cadmium during percolation, however neither selectivity or specificity were observed, but EDTA 1 mM induced the ion elution. Using increasing amount of EDTA from the percolation to washing and next elution solutions (Figure S3) to potentially observe a difference in the behavior of Cd(II) vs. Pb(II) and Co(II) (by expecting that the interfering ions would elute with a lower EDTA concentration), none of the ions were retained on the OSs, even at a very low EDTA concentration (10^{-5} M). As it is well known that some ions favor the conformation change of aptamers, different solutions containing NaCl (Figure S4), $MgCl_2$ (Figure S5), $NaNO_3$ (Figure S6) or Na_2HPO_4 (Figure 2A and S7) were then evaluated as percolation media. None of its salts allowed to obtain a selective and specific retention of cadmium on OS1. In return, some selectivity and specificity for

Cd(II) vs. Co(II) was observed on OS2 using $MgCl_2$ and Na_2HPO_4, even if Pb(II) was retained as was Cd(II). Then, buffers such as HEPES (Figure S8) or MES (Figure S9) were investigated as they were reported as measurement media of some aptasensors developed for Cd(II). In these buffers, only Co(II) appears to be partially lost in the washing fraction but neither specificity of Cd(II) vs. Pb(II) or selectivity were observed on the OSs. A Tris buffer (Figure S10) containing salts was also tested on the OSs, as it was used as measuring medium of aptasensors. In this medium, only OS2 led to the retention of Cd(II) until elution and selectivity and specificity towards Co(II) were observed. However, the lack of specificity towards Pb(II) persisted. Based on literature, an acetate + perchlorate medium was next tested (Figure S11). Cd(II) was retained on OS1 during percolation but not until elution. On OS2, for the first time, a specific and selective retention was observed. Finally, the use of PBS (phosphate buffered saline) was envisaged as it was also reported in literature, even if its composition has not been precisely detailed. Two PBS buffers of two different compositions and ionic strengths were then tested (Figure 2B, S12 and S13). On OS1 none of the two PBS buffers allowed the retention of Cd(II) during the percolation step. On OS2 when percolating PBS1 containing Na^+ and K^+ cations (Figure S12), Cd(II) was retained along with Pb(II) until elution whereas with the PBS2 containing only Na^+ as cation, only Cd(II) was retained until elution, leading to both selectivity and specificity (Figure S13). This illustrates that ions present in the percolation and washing solutions strongly impact the OS behavior, affecting the conformation of the aptamer and competing with target ions to potentially interact with the aptamer.

To conclude with OS1, none of the tested media allowed to obtain the expected SPE performances in terms of specificity and selectivity. As illustrated by Figure 1, which presents the best SPE profile obtained with OS1, some specificity was observed as Co(II) was only very weakly retained by OS1, but selectivity was low, since Cd(II) retention was the same on OS1 and OSc1. Therefore, this sorbent has not been characterized further in the following.

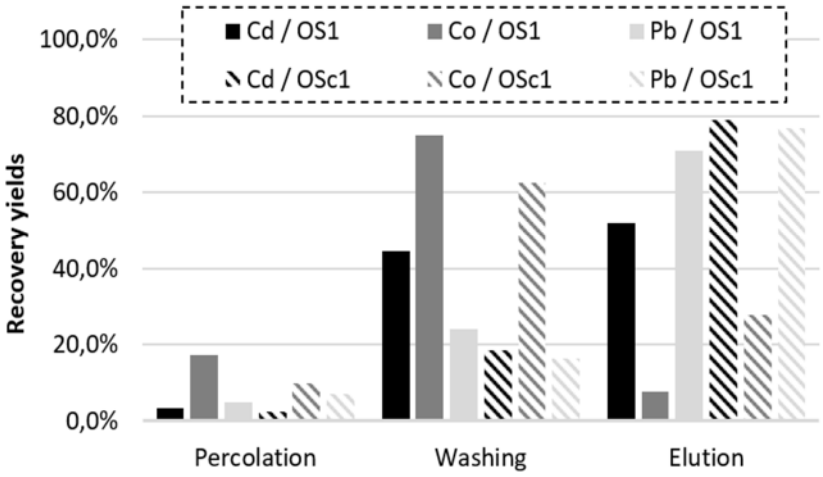

Fig. 1. Extraction profiles of Cd(II), Co(II) and Pb(II) ions in optimized media for OS1 and its control. Extraction procedure: P: 1 mL of 30 mM MES + 10 mM $NaNO_3$ spiked with 30 ng of Cd(II), Co(II) and Pb(II) each; W: 4 mL of 10 mM $NaNO_3$; E: 2 mL of 1 mM EDTA.

With OS2, only two of the media tested (NaH_2PO_4 + Na_2HPO_4 + NaCl (PBS2) or CH_3COONa + $NaClO_4$) generated specificity since the recovery of Pb(II) in the elution fraction is less than 20%. However, when using perchlorate + acetate, specificity was only observed during the washing steps, whereas when using PBS2, the interfering ions were mostly eliminated during the percolation step. Furthermore, as the objective was to selectively extract Cd(II) from serum samples, the percolation medium chosen for further study was the one that most closely resembles the composition of serum, i.e. a phosphate buffer. This is why, PBS2 was selected.

Then, the extraction procedure was optimized by varying the volume of the washing and elution fractions. The resulting extraction profiles of Cd(II) and interfering ions on OS2 and its control are reported on Figure 2B. An extraction yield of 85% ± 2.6% (n=3) was obtained for Cd(II) in the elution fraction of OS2, whereas it was only 1% ± 0.3% with the control, demonstrating that the retention on OS2 was due to interactions with the specific AC2 aptamer. Regarding Co(II) and Pb(II), they were mostly removed from OS2 and its control during the percolation of the sample.

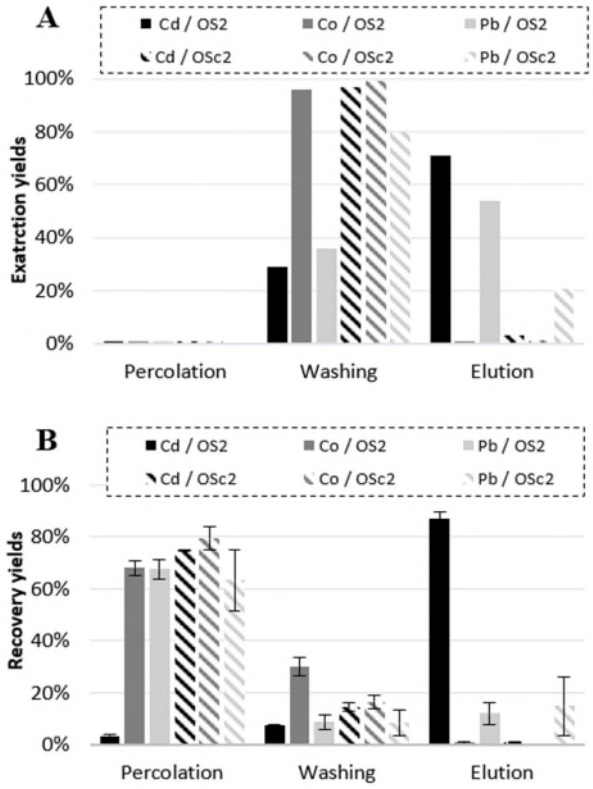

Fig.2. Extraction profiles of Cd(II), Co(II) and Pb(II) ions percolated on OS1 and its control in (A) Na_2HPO_4 100 mM or (B) PBS2 after optimization of the whole extraction procedure (n=3). SPE procedure: (A): Percolation: 1 mL of Na_2HPO_4 100 mM spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 2 mL of the percolation solution; Elution: 2 mL of EDTA 1 mM + NaCl 50 mM + Tris-HCl 10 mM, pH 7.0; (B): Percolation: 1 mL of Na_2HPO_4 25 mM + NaH_2PO_4 25 mM + NaCl 150 mM (PBS2) spiked with 30 ng of Cd(II), Co (II) and Pb(II); Washing: 1 mL of PBS2; Elution: 500 µL of EDTA 1 mM.

3.3. Simulation of aptamer conformations

In order to try to understand the impact of the cation nature on the aptamer conformation, and thus the consequent OS2 behavior, the AC2 sequence was modelled. Unfortunately, in the software typically used, only the concentrations of Mg^{2+} and Na^{+} can be fixed whereas the presence of other ions cannot be simulated. However, a simulation was done with a Na^{+} concentration of 225 mM (corresponding to the sodium concentration in the optimal percolation medium PBS2), 10 mM Na^{+}, a concentration which did not allow to obtain selectivity or specificity on the OS2 support (as shown in Figure S4) and also 5 mM $Mg2^{+}$, a medium which provides selectivity and specificity for Co(II) but not for Pb(II) No difference in the secondary structure of AC2 was observed between the three tested media(the modelization obtained is shown in Figure S14It seems therefore that, although it could be of great help in limiting the number of conditions to be evaluated in this type of study, this type of modelling is still not sufficient to understand the mechanisms involved in the retention of cadmium on the OS2 support grafted with the AC2 sequence.

3.4. Experimental capacity of the oligosorbent

Once the optimal extraction conditions fixed, the capacity of OS2 was determined. It corresponds to the maximum amount of analyte that can be retained on a given amount of OS by specific interactions. In order to measure it, the optimized procedure described in Figure 2B was applied using PBS2 as percolation medium spiked with increasing amounts of Cd(II) (from 30 to 300 ng). The amounts of Cd(II) retained on OS2 and its control, corresponding to the amount found in the elution fractions, are reported in Figure 3. For OS2, the curve is composed of two parts. In the first part, the quantity retained increases proportionally to the quantity percolated, corresponding to a constant extraction yield. It is in Figure 2B (85% ± 2.6%, n=3)). The curve then reaches a plateau indicating the saturation of OS2 by Cd(II) ions. For OSc2, regardless the Cd(II) amount introduced, the slope of the curve remains constant and close to zero, illustrating once again the selectivity of the SPE procedure previously optimized where no non-specific interactions occur with the optimized SPE protocol. According to Figure 3, the OS2 capacity (which corresponds to the break in the slope) is about 180 ng of Cd(II) per 30 mg of sorbent (i.e. a capacity of 6 µg/g). This experimental capacity is about four times smaller than the calculated theoretical value (cf. Table 1, 2nd synthesis). One hypothesis for this difference is steric hindrance between grafted aptamers, which prevents them from assuming a conformation suitable for Cd(II) trapping. However, even if the experimental capacity value seems low, it is perfectly adapted to the percolation of samples contaminated at the trace level such as blood, since the threshold value of Cd(II) in this fluid is 4 µg/L.

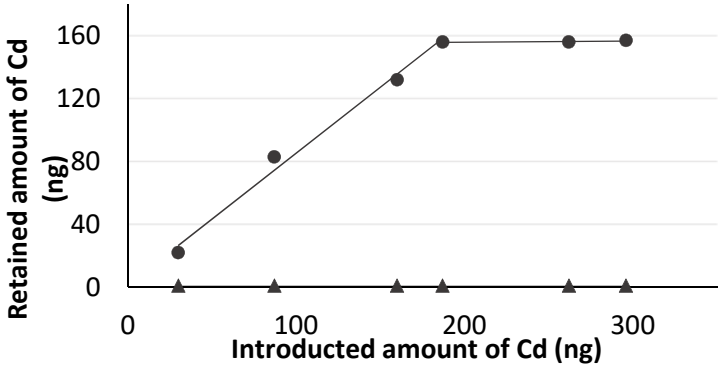

Fig. 3. Capacity curves obtained for Cd(II) after the percolation of an increasing amount of Cd(II) on OS2 (circle) and OSc2 (triangle). The retained amount of Cd(II) corresponds to the amount found in the elution fraction after applying the optimized extraction procedure described in Figure 2B.

3.5. Specificity of the oligosorbent

In order to carry out a more detailed study of the specificity of OS2, a mixture containing the ions Mg(II), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Pb(II) was percolated on OS2 and OSc2, the sum of their percolated amount being lower than the capacity. The results are reported in Figure 4. Except for Cu(II), all interfering ions were not retained on OS2 since they are mostly eliminated during the percolation step and their extraction yields in the elution fraction are below 20%. Regarding Cu(II), it is retained on OS2 until elution. However, a similar behavior is observed on the control, which means that it is retained by non-specific interactions on OS2 unlike Cd(II) which is retained in a specific way on OS2 (because not retained on OSc2). It can also be noticed that some authors that had used the AC2 sequence for the development of Cd-aptasensors also mentioned the existence of interference with Cu(II) [26,27] (Table S3). However, in return to aptasensors, the use of ICP-MS to analyze the extract after the oligoextraction step allows to distinguish Cu from Cd thus removing the risk of false positive in Cd determination. Moreover, if necessary, the total removal of Cu(II) from the extract can be achieved by percolating the sample first on OSc2 which allowed Cu(II) to be trapped without trapping Cd(II) which can be then selectively retained on OS2. Nevertheless, to complete this study related to the selective potential of the OS, it was further applied to serum samples.

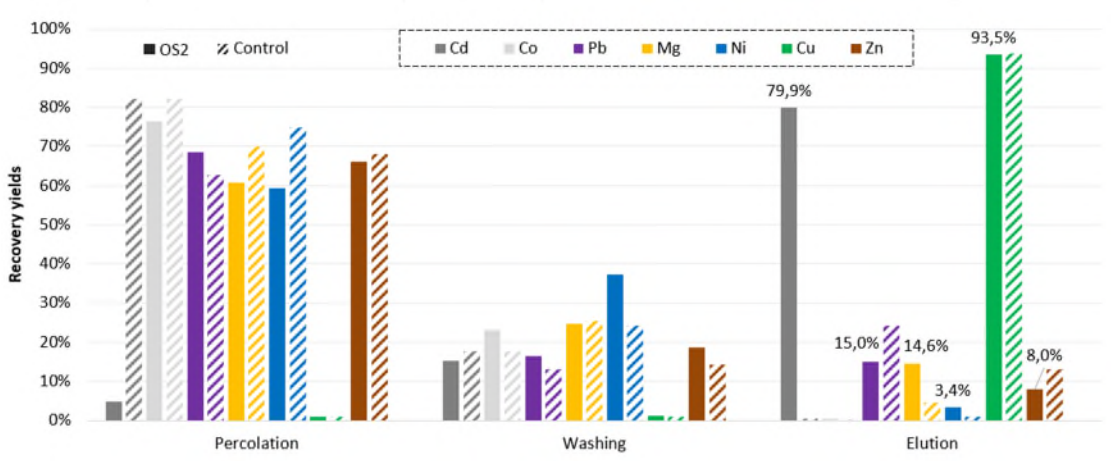

Fig. 4. Extraction profiles of Cd(II) and interfering ions (Co(II), Pb(II), Mg(II), Ni(II), Cu(II), and Zn(II)) on OS2 (full histograms) and OSc2 (hatched histograms). SPE procedure: percolation: 1 mL of Na_2HPO_4 25 mM + NaH_2PO_4 25 mM + NaCl 150 mM spiked with 15 ng of Cd(II), Co(II), Pb(II), Mg(II), Ni(II), Cu(II), and Zn(II); Washing: 1 mL of the percolation buffer; Elution: 500 µL of EDTA 1 mM.

3.6. Oligoextraction of Cd(II) from serum samples

Once the performance of OS2 evaluated with spiked buffers, the extraction procedure was applied to real serum samples. An acid precipitation step was performed before oligoextraction to remove proteins that could complex Cd(II) and plug the SPE cartridge [34,35]. The precipitated serum was further centrifuged and the supernatant was either diluted with the phosphate buffer PBS2, to be passed through OS2 before the ICP-MS analysis of the elution fraction, or diluted with HNO_3 2% and directly analyzed by ICP-MS. This procedure was applied on a human serum not spiked or spiked at levels ranging from 2 to 30 µg/L (n=2 for each concentration level). Results are reported in Figure 5. The calculated concentrations were plotted as a function of the spiking levels. The concentration in the raw serum is then determined by extrapolating the line to y = 0. Hyperbolas of 95% confidence are plotted to determine the uncertainty on either side of the raw serum value. The Cd(II) concentration in raw serum is 1.83 µg/L and is between 1.02 and 2.69 µg/L with a 95% confidence interval. The slope of the curve allows the determination of the extraction yield and a value of 75% with a standard deviation of 1.6% (n=6) was obtained. This extraction yield is slightly lower than the one obtained previously with the phosphate buffer and the difference can be explained by some Cd(II) loss during the acid precipitation step and the associated centrifugation steps. To confirm this hypothesis, the serum extract was spiked with Cd(II) at 30 µg/L after performing these steps. In this case, a Cd(II) extraction yield of 81% was obtained, confirming a partial loss of Cd(II) during the serum pretreatment. By analyzing Cd(II) in the diluted supernatant (without an oligoextraction step) by ICP-MS, the recovery was estimated at 57% revealing a matrix effect in comparison with the results obtained with the oligoextraction step (75%).

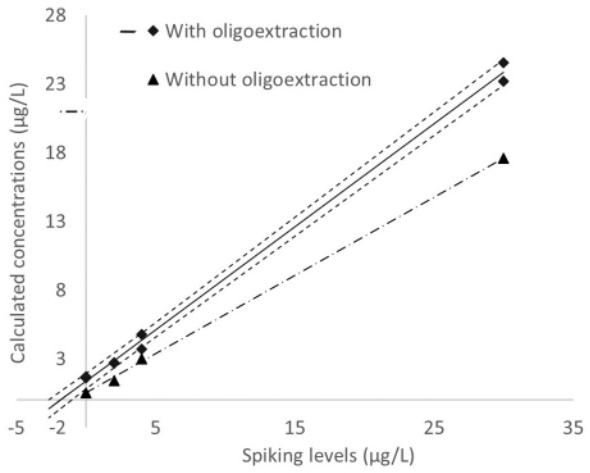

Fig. 5. Calibration curves for the analysis of Cd(II) in a human serum submitted to an acid precipitation step and with (solid lines and square markers) or without (dotted lines and triangle markers) oligoextraction. Spiking levels are between 0 to 30 µg/L. The 95% confidence hyperbolas for the results with oligoextraction are shown as dotted lines.

In order to pre-validate the whole method, the protocol was applied to a certified serum (certified concentration value in Cd(II) of 8 ± 1.2 µg/L). The concentration level determined in the certified serum sample after applying a purification step on OS2 was 8.84 ± 0.21 µg/L (n=3), which corresponds to the expected value. The calculated concentrations take into account the applied dilution as well as the recovery determined by the standard additions in the serum. In return, a value of 5.24 µg/L was obtained when determining Cd(II) after the acid precipitation but without the oligoextraction step, which is far from the certified value (a difference of more than 30%).This confirms the presence of matrix effects in ICP-MS when analyzing the digested extract of serum after a dilution but without an oligoextraction step, thus preventing a reliable quantification of Cd(II). All these results confirm the high potential of oligoextraction as an extraction method of cadmium from a complex biological sample, allowing its reliable quantification by reducing the matrix effects in ICP-MS.

It is also important to notice that, over 40 extraction procedures in pure medium over a period of 18 months were carried out and no loss of retention was observed. The reusability of the support in real samples was also investigated and no loss of retention was observed after more than 20 oligoextraction procedures applied to plasma samples, demonstrating the excellent stability of the sorbent.

Conclusions

The objective of this study was to develop for the first time a selective and specific extraction sorbent based on aptamers to extract Cd(II) from a serum sample. After identifying two promising sequences in literature, they were grafted on CNBr-activated Sepharose. If these two sequences had previously led to good results when used in aptasensors, this study revealed, by studying in parallel other interfering cations but also the retention of ions on a control support prepared from a scrambled aptamer, that the achievement of selectivity and specificity results from the careful optimization of the extraction procedure. In this respect, the nature of the percolation medium appears to be a key factor in promoting affinity between the aptamer and the target ion and therefore retention. This study finally showed that once the procedure was optimized, its application to a real sample led to performances similar to those obtained in pure medium (75 % vs 85 % in pure media) and guaranteed an equally reliable analysis of cadmium in pure medium

by suppressing matrix effects. Even if aptamers are highly sensitive to the percolation medium, it seems that a dilution of serum in percolation buffer is sufficient to ensure not only cadmium retention but also the specificity and selectivity observed in pure medium. On a fundamental level, other studies could then be carried out, in particular by more detailed modelling taking into account the presence of all the ions present in the media in order to better understand the mechanisms involved in the retention process. On a more applicative level, although rarely reported in literature, the use of control sequences is essential to demonstrate the selectivity of the retention process. In the future, miniaturization of the OS could also be considered in order to couple it on line with the ICP-MS, which would limit the handling steps by an operator. Another future prospect is the use of less specific but less costly instruments than ICP-MS, such as ICP optical emission spectrometry or graphite furnace atomic absorption spectrometry, which further justifies the use of highly specific support such as OS2.

Author Contributions

Conceptualization: V. Pichon, N. Delaunay. Formal analysis and investigation: F. Gignac. Writing-original draft preparation: F. Gignac. Writing-review and editing: V. Pichon, N. Delaunay. Funding acquisition: V. Pichon. The manuscript was written through contributions of all authors.

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Supplementary data

Novel oligonucleotide-based sorbent for the selective extraction of cadmium from serum samples

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Supplementary material

$$Limit\ of\ quantification = \frac{10\ \sigma}{k}$$

Equation S1. equation for calculating the limit of quantification. σ is the standard deviation in counts per second (cps) of 10 blank solutions (in 2% HNO_3) and k the sensitivity of the instrument (cps/µg/L)

Table S1. LOQ of ^{59}Co, ^{208}Pb, ^{63}Cu, ^{24}Mg, ^{60}Ni and ^{66}Zn in 2% HNO_3 determined by ICP-MS using Equation S1.

Element	LOQ (ng/L)
Cobalt	1
Lead	45
Copper	51
Magnesium	508
Nickel	44
Zinc	224

Table S2. Cd(II) concentration average, standard deviation and RSD values calculated for each calibration solution analyzed in replicate (n= 3) in 2% HNO_3 in ICP-MS.

Concentration of calibration solutions (ng/L)	Average values and standard deviation (ng/L)	RSD (%)
0.5	0.53 ± 0.06	10.7
1	1.13 ± 0.14	12.1
3	3.02 ± 0.33	11.0
5	5.20 ± 0.25	4.8
10	9.86 ± 0.46	4.7
50	49.03 ± 0.77	1.6
100	96.51 ± 0.99	1.0
500	491.74 ± 2.15	0.4
1000	984.32 ± 14.71	1.5
3000	2923.39 ± 12.75	0.4

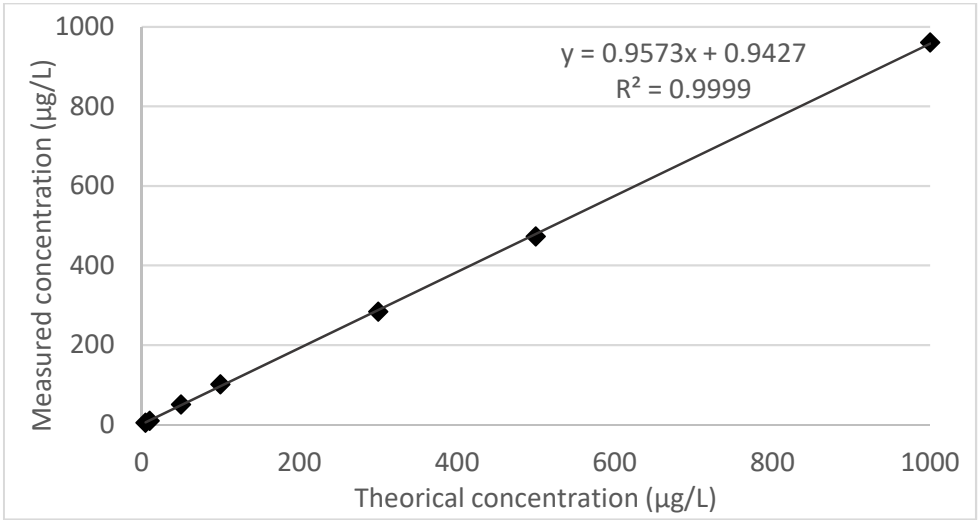

Figure S1. typical calibration curve for ^{111}Cd obtained by analyzing in ICP-MS solutions of Cd(II) in HNO_3 2% with concentrations between 5 and 1000 µg/L.

Due to the large number of media tested and the need to assay ions all the fractions (percolation, washing and elution fractions), some extraction procedures were carried out only once. Nevertheless, this approach enabled us to obtain a trend in the behavior of the various ions as a function of the percolation media.

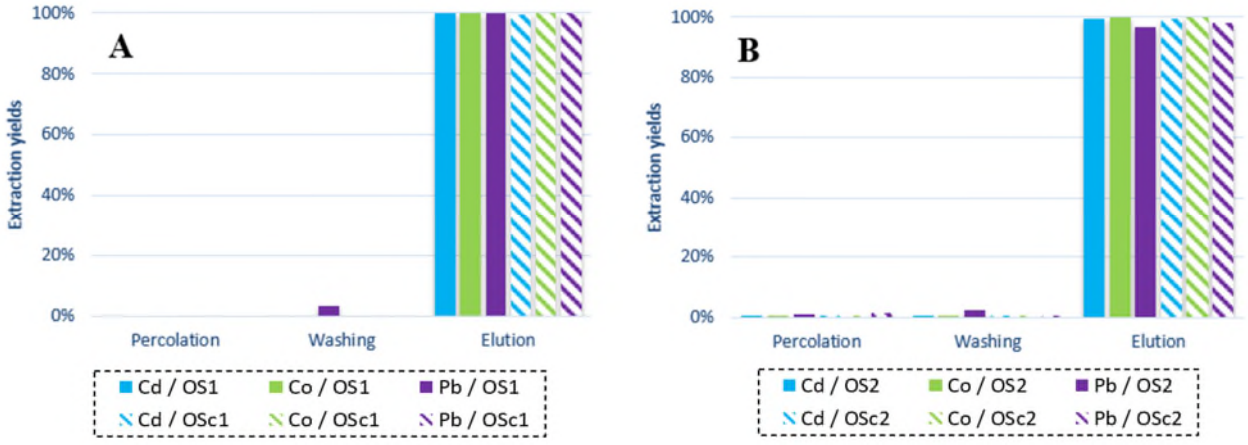

Figure S2. Extraction profiles obtained when percolating ultra-pure water spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL ultra-pure water spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of ultra-pure water; Elution: 1 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

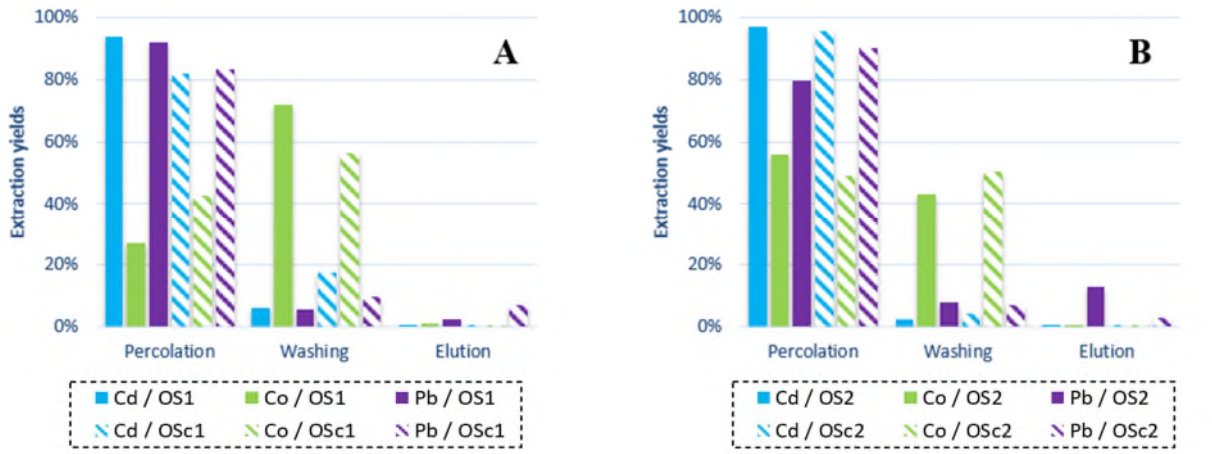

Figure S3. Extraction profiles obtained when percolating EDTA 10^{-5} M spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of EDTA 10^{-5} M spiked with 30 ng of Cd(II), Co (II) and Pb(II); Washing: 1 mL of EDTA 10^{-4} M; Elution: 500 µL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

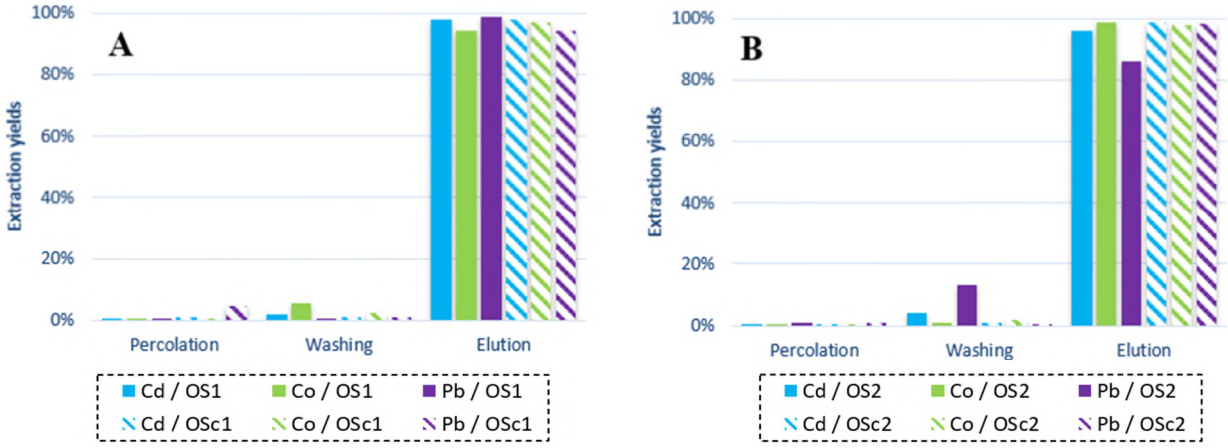

Figure S2. Extraction profiles obtained when percolating NaCl (10 mM, pH 6.0) spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of NaCl (10 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation solution; Elution: 3 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

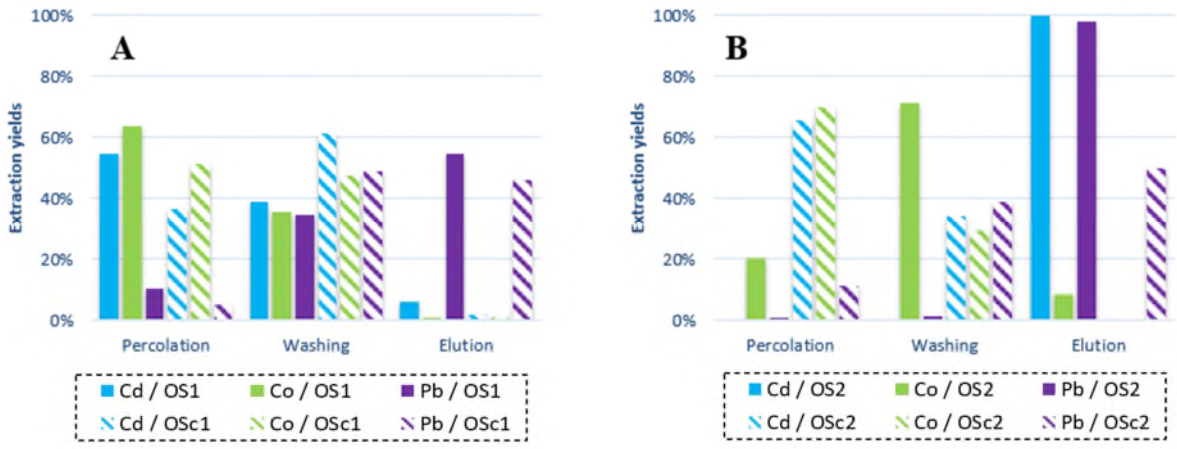

Figure S3. Extraction profiles obtained when percolating $MgCl_2$ (5 mM, pH 6.0) spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of $MgCl_2$ (5 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation solution; Elution: 1 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

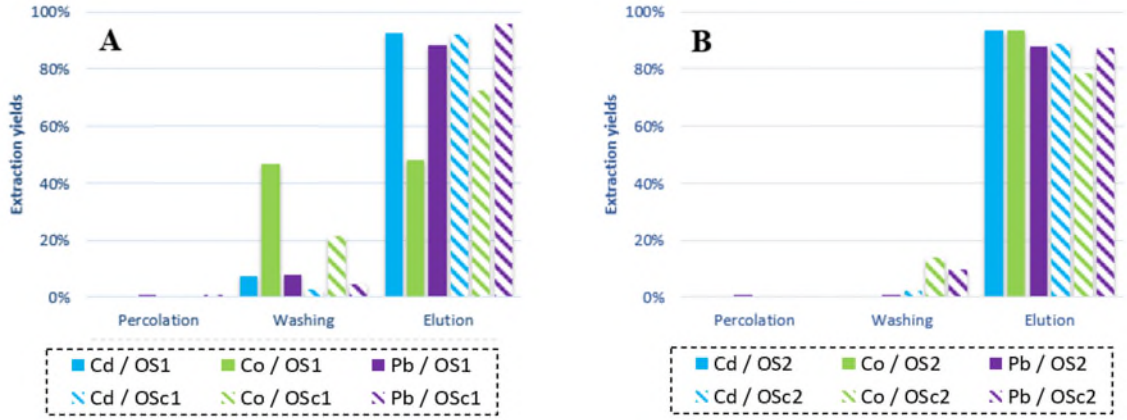

Figure S6. Extraction profiles obtained when percolating $NaNO_3$ (10 mM, pH 6.0) spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of $NaNO_3$ (10 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation solution; Elution: 1 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

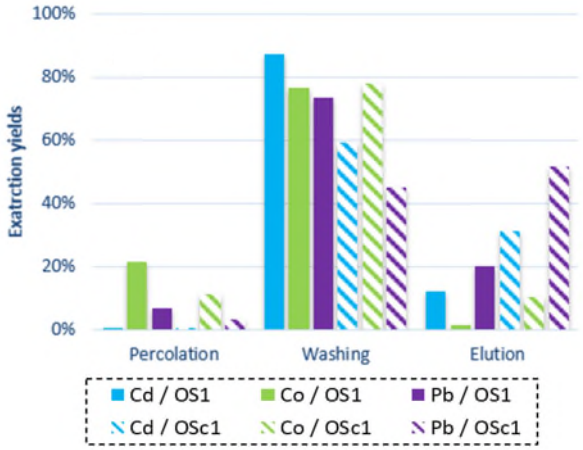

Figure S7. Extraction profiles obtained when percolating Na_2HPO_4 (100 mM, pH 7.0) spiked with Cd(II), Co(II) and Pb(II) on OS1 and on its corresponding control sorbent. SPE procedure: Percolation: 1 mL of Na_2HPO_4 100 mM mM spiked with 30 ng of Cd(II), Co (II) and Pb(II); Washing: 2 mL of the percolation buffer; Elution: 500 µL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

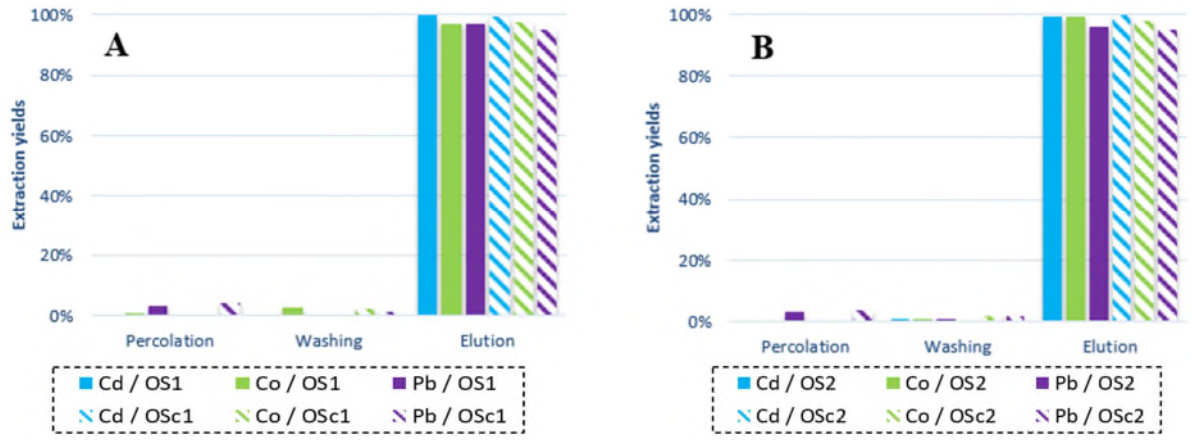

Figure S8. Extraction profiles obtained when percolating HEPES (30 mM, pH 6.5) spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of HEPES (30 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 2 mL of $NaNO_3$ (10 mM); Elution: 2 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

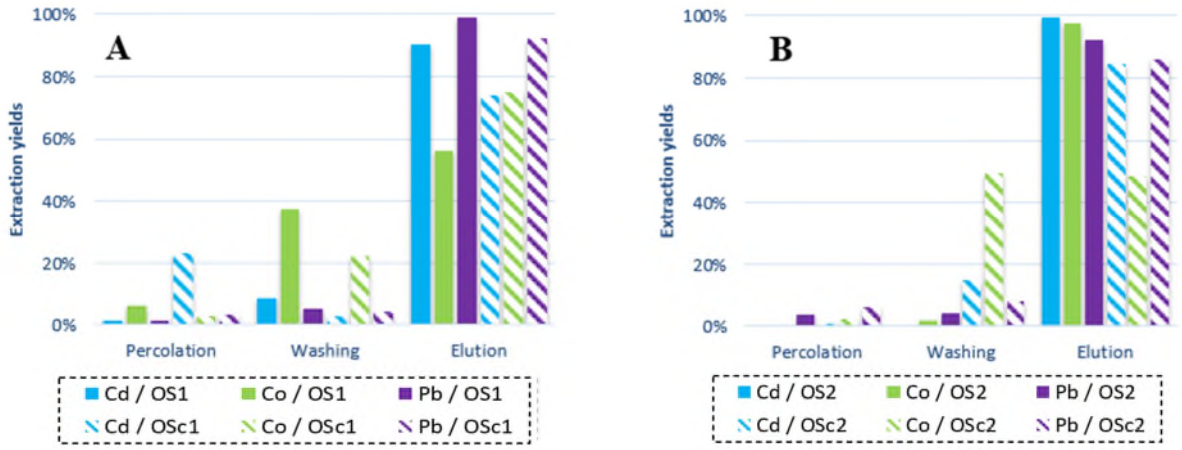

Figure S9. Extraction profiles obtained when percolating MES (50 mM, pH 6.0) spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of MES (50 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 2 mL of the percolation buffer; Elution: 2 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

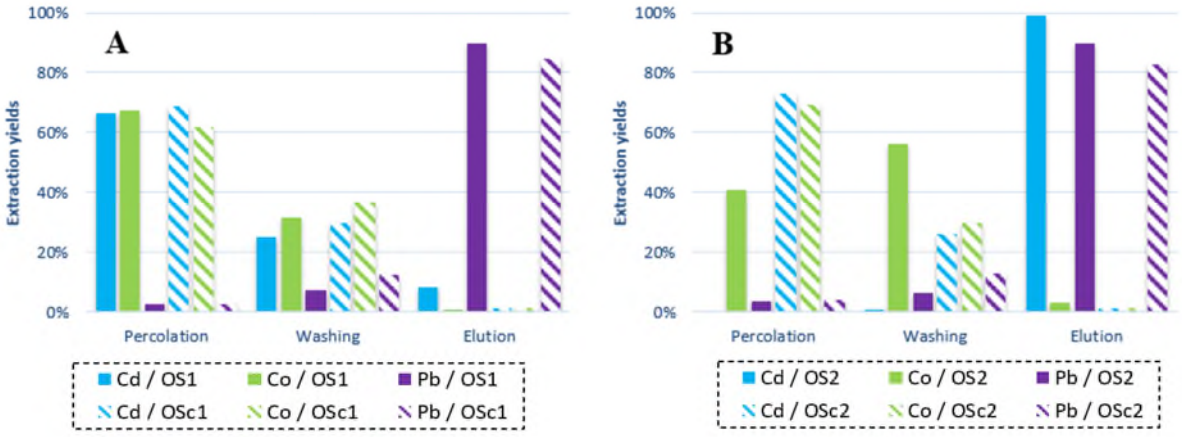

Figure S10. Extraction profiles obtained when percolating Tris-HCl (20 mM) + KCl (25 mM) + NaCl (50 mM) + $MgCl_2$ (5 mM), pH 7.4 spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of Tris-HCl (20 mM) + KCl (25 mM) + NaCl (50 mM) + $MgCl_2$(5 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation solution; Elution: 3 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

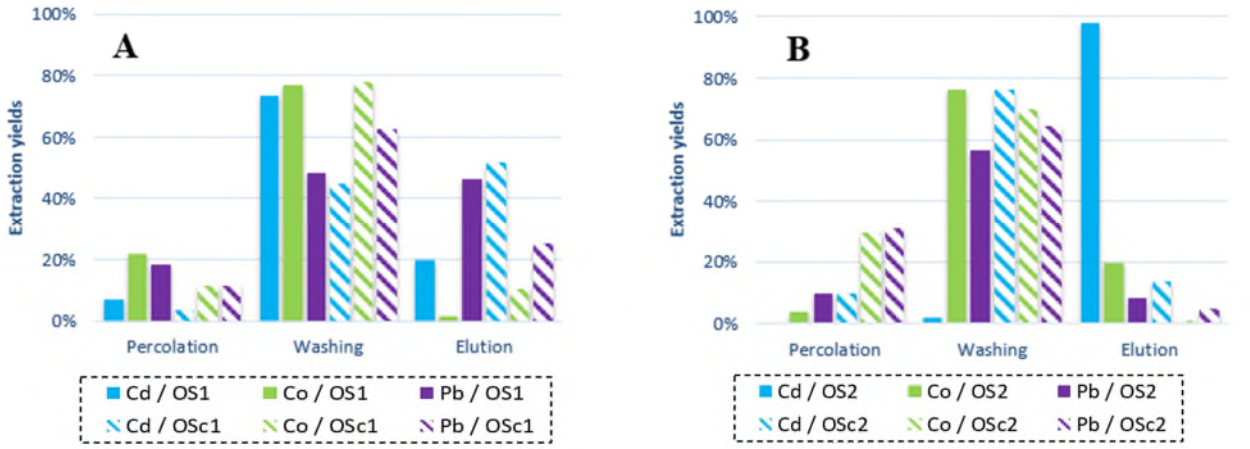

Figure S11. Extraction profiles obtained when percolating sodium acetate (10 mM) + sodium perchlorate (10 mM), pH 6.0 spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of sodium acetate (10 mM) + sodium perchlorate (10 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation solution; Elution: 2 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

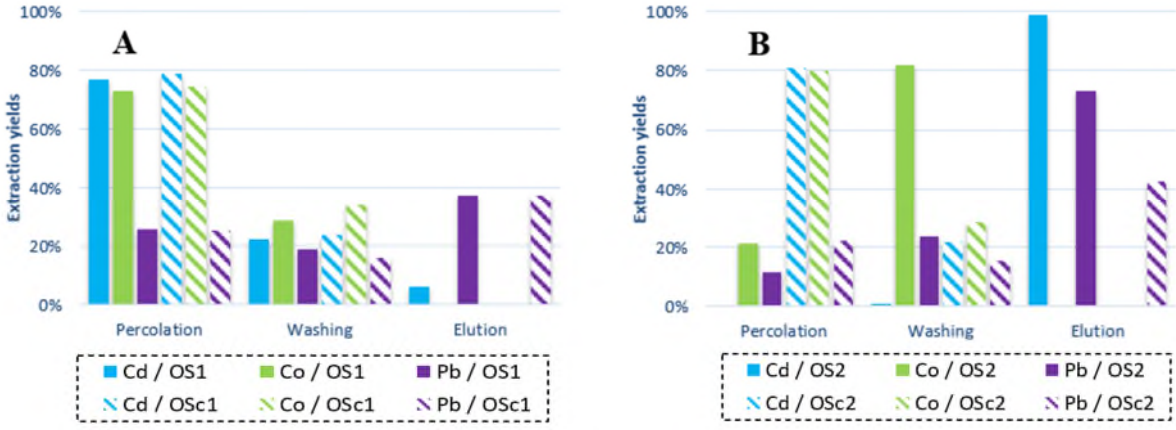

Figure S12. Extraction profiles obtained when percolating Na_2HPO_4 (10 mM) + KH_2PO_4 (1.8 mM) + NaCl (137 mM) + KCl (2.7 mM), pH 6.6 spiked with Cd(II), Co(II) and Pb(II) on (A) OS1 and (B) OS2 and on their corresponding control sorbents. SPE procedure: Percolation: 1 mL of Na_2HPO_4 (10 mM) + KH_2PO_4 (1.8 mM) + NaCl (137 mM) + KCl (2.7 mM)) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 4 mL of the percolation buffer; Elution: 2 mL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

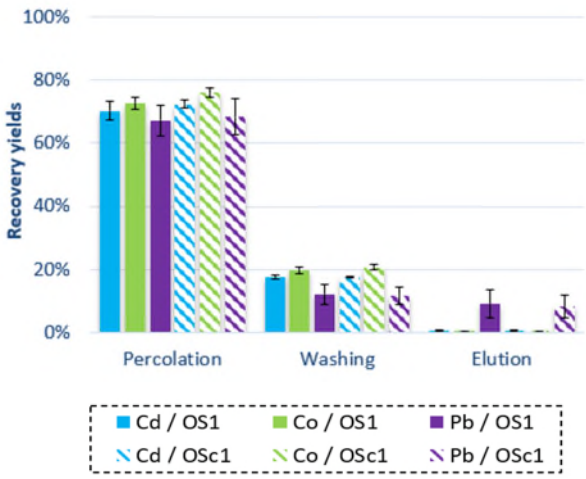

Figure S13. Extraction profiles obtained when percolating Na_2HPO_4 (25 mM) + NaH_2PO_4 (25 mM) + NaCl (150 mM), pH 6.6 spiked with Cd(II), Co(II) and Pb(II) on OS1 and on its corresponding control sorbent. SPE procedure: Percolation: 1 mL of Na_2HPO_4 (25 mM) + NaH_2PO_4 (25 mM) + NaCl (150 mM) spiked with 30 ng of Cd(II), Co(II) and Pb(II); Washing: 1 mL of the percolation buffer; Elution: 2 x 500 µL of EDTA 1 mM + NaCl 50 mM + Tris HCl 10 mM, pH 7.0.

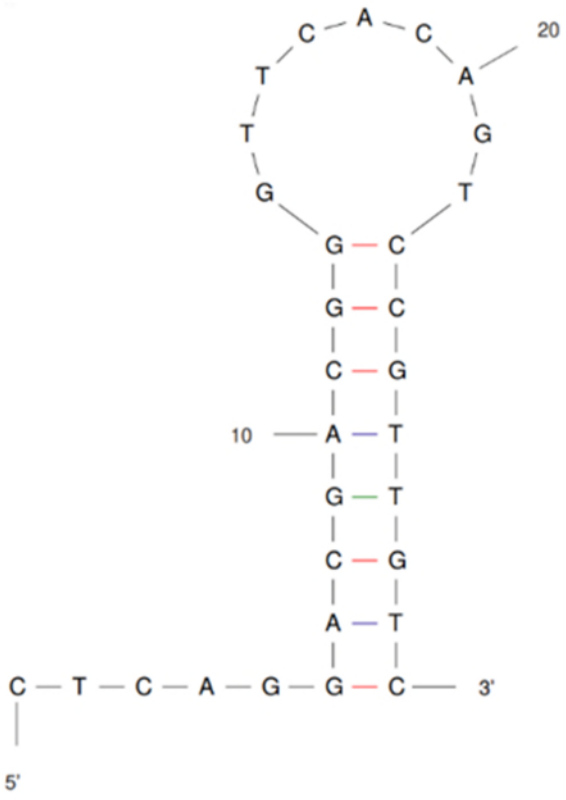

Figure S14. Secondary structure of AC2 obtained for [Mg2+] = 5 mM, [Na+] = 10 mM and [Na+] = 225 mM (concentration corresponding to the optimal percolation medium PBS2) at 4°C.

Table S1. Sequences identified in the literature, interfering ions tested and interferences observed. Two Cd(II)-specific sequences have been identified in the literature: the red sequence (which composed the AC1 sequence) and the blue sequence (which composed the AC2 sequence).

Sequences	Authors	Ref	Interferings ions tested	Interferings ions observed
ATG GGT CTC ACT ATG GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT ATG ACT A	D. Zhou	[1]	Hg, Pb, Cd, Ag, Zn, Mn, Ni, Cu	-
GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC	M. Park	[2]	Cu, Pb, Ag, Fe, Hg	Hg
GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT ATG	R. Y. Lai	[3]	K, Ca, Mg, Hg, Zn, Pb, Fe, Ag, Co, Cu, Mn, Cr, As, Se	-
	W. Sun	[4]	Ag, Hg, Cu, Fe, Pb, Ni, Se, Zn	Hg and Pb
GG GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT	Y. S. Wang	[5]	Na, Sr, Mn, K, Fe, Ca, I, Mg, Cr, Al, U, Zn, Hg, Pb, Ba, Tb, Cu, Ag	Ag
GGG GGG GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT	T.H. Kim	[6]	Zn, Pb, Mn, Co, Fe, Cu, Hg	Hg
A CCG ACC GTG CTG GAC TCT GGA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT ATG AGC GAG CGT TGC G	Z. Wang	[7]	Hg, Pb, Fe, Mn, Mg, K, Ag, Cu, Al, Zn, Ca	Hg
	S. Rabai	[8]	Hg, Pb, Zn	Hg
	P. Zhou	[9]	K, Ca, Mg, Hg, Zn, Pb, Fe, Ag, Co, Cu, Mn, Cr, Ba, Ni	Hg, Pb, Cu and Zn
	H. Wan	[10]	Pb, K, Cu, Ca, Mg, Na	Pb
GGG CTG GGA GGG TTG GGG TGT TGT TTT TGG TTG TGC CCT ATG	B. Zhou	[11]	K, Ag, Na, Ni, Cr, Hg, Zn, Mg, Ca, Ba	-
5'-GGG AGG GAA CTG TTG TGG TAT TAT TTT TGG TTG TGC AGT AGG GCG GG	B. Zhou	[12]	K, Na, Ba, Ca, Mn, Ni, Fe, Mg, Zn, Hg, Pb, Cu	-
AAA AAA AAA AAA AAA TTT TCG ACG GGT TCA CAG TCC GTT G	X. Yang	[13]	K, Na, Mn, Mg, Ni, Ca, Zn, Pb, Ba, Cu, Hg	Cu and Hg
CTC AGG ACG ACG GGT TCA CAG TCC GTT GTC	R. Pei	[14]	Ag, Hg, Ni, Ba, Ca, Co, Mn, Pb, Zn, Cu, Al.	Cu

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