Manuscript Number: EA19-4180R1

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Article Type: Research Paper

Keywords: electrochemical grafting; peptide-modified sensors; screen-printed electrodes; stripping voltammetry; metal determination

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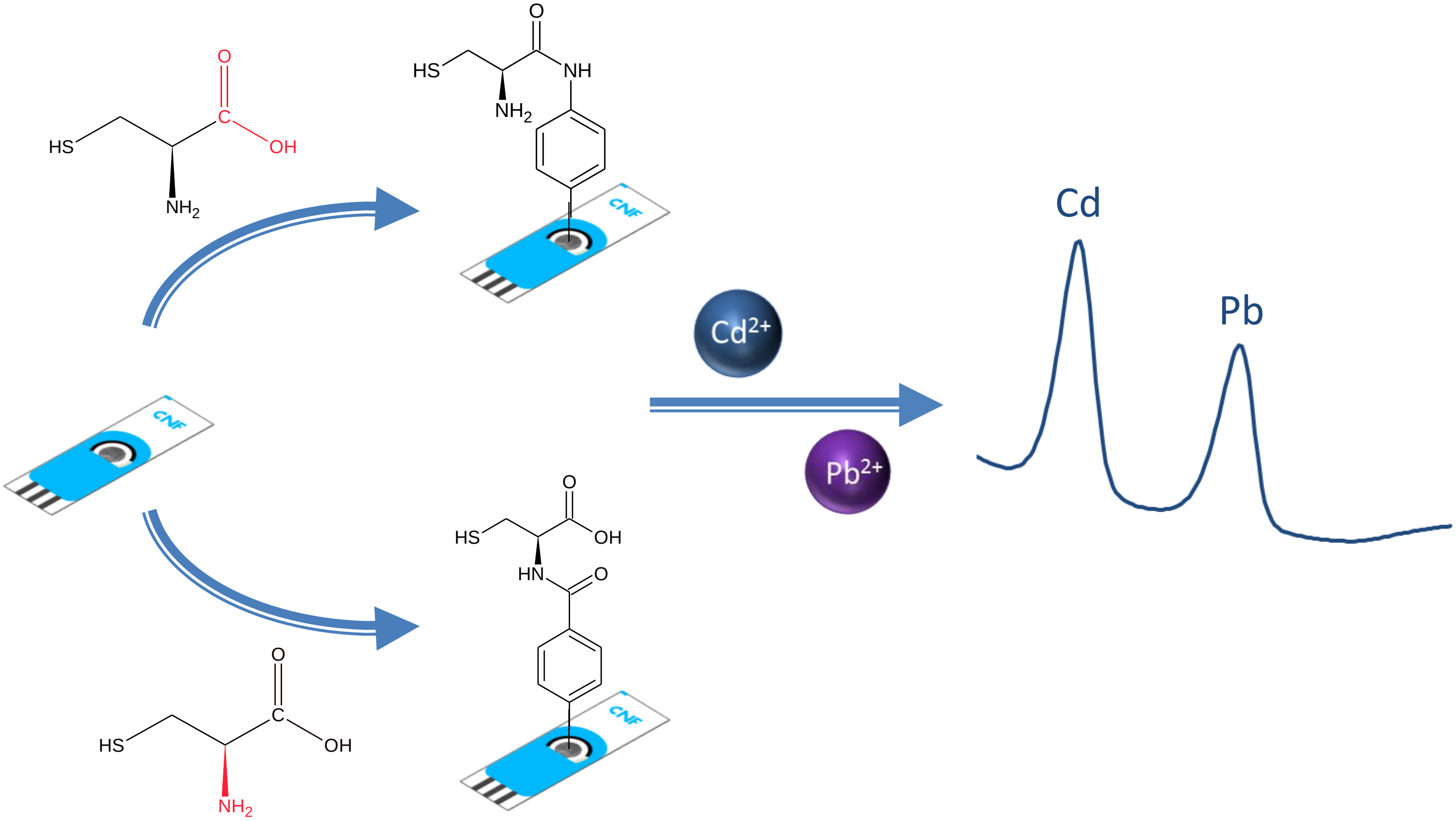
Graphical Abstract (for review)



Expanding the posssibilities of electrografting modification of voltammetric sensors through two complementary strategies

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ABSTRACT

Two different modification strategies by means of aryl diazonium salt electrografting were compared for the development of voltammetric sensors. In this sense, L-cysteine was immobilized onto a screen-printed carbon-based electrode surface through either its $–NH_2$ or its –COOH group and the performance of the resulting modified sensors was tested for the simultaneous determination of Pb(II) and Cd(II) by anodic stripping voltammetry. The results obtained indicate that attachment through the –COOH group of cysteine, despite being a much less frequent electrografting strategy, improves the analytical performance of the resulting sensor achieving lower LODs, at low µg L^{-1} levels, for Cd(II) and Pb(II). Furthermore, this strategy allows the quantification of both metal ions below the legal limits established by the European Water Framework Directive, which represents a great improvement with respect to similar sensors reported in the literature.

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1. INTRODUCTION

The problems associated with metal ions contamination have been known for years. During the last decades, metal emissions have significantly decreased, mainly due to their banning in some specific products (e.g. lead in gasoline or mercury regulations) or the decrease of their use and/or smelting [1,2]. However, the amount of metals emitted to the environment is still preoccupying and this, coupled to their non-biodegradable character, makes it still important to develop new analytical methods for the determinations of *on-site* trace metal ions.

Among all the available analytical techniques for metal ions determination, electrochemical techniques and, more specifically anodic stripping voltammetry (ASV), are particularly suitable. ASV measurements fulfil all the analytical requirements (high reproducibility and repeatability, low detection limits, high sensitivity, multielement determination…) and at the same time provide fast measurements with relatively low cost and portable equipment [3]. These last features are further enhanced by the coupling of ASV measurements with screen-printed electrodes (SPE), which are miniaturized, versatile, disposable and economical devices that can be mass-produced in a reproducible manner [4,5].

ASV measurements are strongly influenced by the chosen working electrode. Several types of working electrodes have been successfully reported for metal ion determination including metal films, sputtered electrodes, chemically modified electrodes, biosensors and metal nanoparticles or carbon nanostructured electrodes [6–12].

Among all of these, chemically modified electrodes present the advantage that sensitivity and selectivity can be regulated by the incorporation of a convenient functional group onto the electrode surface. In this sense, several types of complexing agents such as EDTA [13–15], dimethylglyoxime [16], crown ethers [17,18] or thiolic compounds [19–22] have been used as electrode modifiers for metal ion determination. In particular, peptides are effective ligands for a great variety of metal ions because they contain a large number of potentially donor atoms. In any peptide, a metal ion can interact through the amino groups, the amide nitrogen and/or the carboxylic group present on its backbone and, additionally, this peptide-metal interaction can be further enhanced by other donor groups present in the side chains. This would be the case, for example, of thiol-rich peptides [23,24].

A good strategy reported in the literature for the immobilization of peptides and ligands in general onto carbon surfaces is based on the aryl diazonium salt electrografting [25]. This strategy allows the covalent linking of the modifier to the surface providing structural flexibility, chemical stability and control on the immobilization orientation and resulting in sensors with great reproducibility and durability [26]. Although carbon surfaces can be functionalized with different aryl diazonium salts, the most usual electrografting approach found in the literature is based on the *in-situ* generation of 4-carboxybenzenediazonium, which results on the functionalization of the electrode surface with carboxylic groups that allows the immobilization of modifiers containing an amino group through a peptide bond facilitated by cross-linking agents. A less common electrografting approach is based on the *in-situ* generation of 4-nitrobenzenediazonium. In this case, the electrode surface is functionalized with amino groups allowing the immobilization of modifiers through different functional groups such as carboxylic acids.

In order to compare these two electrografting based- strategies for sensor modification, in this work L-cysteine (L-Cys) was immobilized onto a screen-printed carbon-based electrode surface through either the generation of 4-carboxybenzenediazonium or 4-nitrobenzenediazonium and the performance of the resulting electrodes was studied for the simultaneous determination of Cd(II) and Pb(II).

2. EXPERIMENTAL

2.1 Chemicals

Hydrochloric acid, acetic acid, sodium acetate, 2-(N-morpholino)-ethanesulfonic acid (MES) and potassium ferrocyanide were supplied by Merck (Darmstadt, Germany). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (sulfo-NHS), 4-nitroaniline (PNA), 4-aminobenzoic acid (ABA), sodium nitrite, potassium dihydrogen phosphate, sodium monophosphate and L-cysteine (L-Cys) with a purity greater than 99% were obtained from Sigma-Aldrich (St. Louis, MO, USA). Potassium ferricyanide was purchased from Panreac (Barcelona, Spain). Cd(II) and Pb(II) stock solutions 10^{-2} mol L^{-1} were prepared from $Cd(NO_3)_2 \cdot 4H_2O$ and $Pb(NO_3)_2 \cdot 4H_2O$ respectively and standardized complexometrically [27]. In all cases analytical grade reagents were used and solutions were prepared with ultrapure water (Milli-Q plus 185 system, Millipore).

2.2 Apparatus

Stripping voltammetric measurements were carried out on an Autolab System PGSTAT12 (EcoChemie, The Netherlands) attached to a Metrohm 663 VA Stand

(Metrohm, Switzerland). GPES software version 4.9 (EcoChemie) was used for data acquisition.

Ag|AgCl|KCl (3 mol L^{-1}) reference electrode and Pt wire counter electrode were acquired from Metrohm (Switzerland). A carbon nanofiber screen-printed electrode (SPCNFE, 4 mm diameter), supplied by Dropsens (Spain), was modified with Cys and used as working electrode. A flexible cable (ref. CAC, DropSens) was employed for the connection of screen-printed electrodes.

pH measurements were carried out on a Crison micro pH 2000 pH-meter and all measurements were performed in a glass cell at room temperature (20 ºC) without oxygen removal.

2.3 Preparation of modified SPEs

Cys modified SPEs were prepared by two different methods according to the schemes shown in Figure 1. Both methods are based on the electrografting of an aryl diazonium salt followed by the covalent immobilization of Cys.

2.3.1 Preparation of (ABA)Cys-SPCNFE

The preparation of (ABA)Cys-SPCNFE is described elsewhere [28]. Briefly, 2 mmol L^{-1} $NaNO_2$ was added drop by drop to a cooled solution of 73 mmol L^{-1} of ABA in 1 mol L^{-1} aqueous HCl and left to stir in an ice bath for 30 min to generate the aryl diazonium salt. Then, the electrografting process was conducted in this solution by CV; applying 15 cycles form 0 to -1 V at 200 mV s^{-1}.

After rinsing the electrodes with Mili-Q water and ethanol, the carboxyl groups generated on the electrode surface were activated by dropping 10 µL of a 5 mg mL^{-1} EDC and 7.5 mg mL^{-1} sulfo-NHS solution in 0.1 mol L^{-1} MES buffer (pH 4.5) onto the functionalized electrodes and leaving it to incubate for 1 h. Finally, 10 µL of a 2.9

mg/100 µL Cys solution in 0.1 mol L^{-1} MES buffer (pH 4.5) were dropped onto the electrode and left to react overnight at 4 °C.

2.3.2 Preparation of (PNA)Cys-SPCNFE

10 mmol L^{-1} $NaNO_2$ was added drop by drop to a cooled solution of 12 mmol L^{-1} of PNA in 1 mol L^{-1} aqueous HCl and left to stir in an ice bath for 30 min to generate the aryl diazonium salt. Then, the electrografting process was conducted in this solution by CV; applying 15 cycles form 0 to -1 V at 200 mV s^{-1}.

After rinsing the electrodes with Mili-Q water and ethanol, the nitro groups were reduced with 5 cycles of CV from -0.4 to -1.2 V at 200 mV s^{-1} in 10 mmol L^{-1} HCl. After CV, the electrodes were rinsed again with Mili-Q water and ethanol.

Finally, the amino groups reacted overnight at 4 °C with 10 µL of a 2.9 mg/100 µL Cys solution in 0.1 mol L^{-1} MES buffer (pH 4.5) previously activated with 8.25 mg/100 µL EDC and 5.25 mg/100 µL sulfo-NHS for 1 h.

2.4 Voltammetric measurements

Differential pulse anodic stripping voltammetric (DPASV) measurements of Cd(II) and Pb(II) using (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE were performed in acetate buffer 0.1 mol L^{-1} pH 4.5 or 5, depending on the considered sensor, applying a deposition potential (E_d) of -1.0 V for a deposition time of 120 s with stirring. Deposition was followed by a rest period (t_r) of 5 s and a scan from -1.0 to -0.3 V with pulse times of 50 ms, pulse amplitudes of 100 mV and step potentials of 5 mV was recorded. In order to remove any remaining bound metals, an electrochemical cleaning stage consisting on the application of a conditioning potential (E_{cond}) of 0 V for 60 s was introduced between measurements.

Electrode characterizations at different modification steps and pHs was performed by CV using 2 mmol L^{-1} ferrocyanide/ferricyanide in 100 mmol L^{-1} phosphate buffer as a redox probe. The potential was scanned from -0.5 to 0.7 V at 100 mV s^{-1}.

3. RESULTS AND DISCUSSION

3.1 Electrodes modification

As a first step, aryl diazonium salts were generated *in situ* from ABA and PNA in 1 mol L^{-1} HCl and grafted onto the SPCNFE surface. CV measurements observed during the reduction of the diazonium salts are presented in figure 2a and 2b for 4-carboxybenzenediazonium and 4-nitrobenzenediazonium respectively. In both cases, a broad irreversible peak that decreases with every scan can be observed. This decrease in the peak intensity is due to the formation of an organic layer on the surface of the electrode, which prevents the access of the diazonium cations [25].

Additionally, for the preparation of (PNA)Cys-SPCNFE, the electrografting process was followed by the reduction of the nitro groups generated onto the electrode surface. This reduction was performed by CV in 0.01 mol L^{-1} HCl and, as it is shown in figure 2c, a broad irreversible cathodic peak can be observed at -0.775 V, which is characteristic of the six electron reduction reaction of the nitro groups to the amino groups [29]. The disappearance of this peak in the following cycles shows that the reduction of the nitro groups was completed.

These steps allowed the generation of either carboxylic or amino groups onto the electrode surface, which were later used to immobilize Cys through the formation of a peptide bond assisted by EDC/sulfo-NHS crosslinking agents. This way, (ABA)Cys-SPCNFE has a free thiol and a free carboxylic group whereas in (PNA)Cys-SPCNFE the thiol and amino groups are available, which will be protonated o deprotonated depending on the working pH value (Figure 1).

3.2 Effect of pH on the electrode surface

The immobilization of Cys through the amino group in (ABA)Cys-SPCNFE and through the carboxylic group in (PNA)Cys-SPCNFE, both assisted by EDC/sulfo-NHS crosslinking agents, leads to the availability of different pH-dependent groups for each electrode. Thus, the electrode response was studied at different pHs.

For this purpose, CV measurements were carried out in a 2 mmol L^{-1} ferrocyanide/ferricyanide at several pHs. Since ferrocyanide/ferricyanide is a non-pH dependent redox probe, the difference in the peak intensities can be explained by the electrostatic interactions of the electrode surface and the redox probe. Figure 3 shows the evolution of anodic current peaks *vs.* pH for (ABA)Cys-SPCNFE, (PNA)Cys-SPCNFE and bare SPCNFE. As it can be seen, at pH 3 the highest response corresponds to (PNA)Cys-SPCNFE, which could be associated to the positive charge of the amino group in **D** that creates a favourable electrostatic interactions with $[Fe(CN)_6]^{3-/4-}$. In both (ABA)Cys-SPCNFE and bare SPCNFE the surface charge should be neutral and the lower response for (ABA)Cys-SPCNFE could be attributed to the presence of the cysteine-based layer that hinders the access of $[Fe(CN)_6]^{3-/4-}$. As the pH increases from 3 to 6, a current decrease is observed for both (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE. Although this behaviour cannot be associated to the theoretical acid-base equilibriums, a possible explanation could be related to the first steps of the modification procedure. As it can be seen in Figure 1, in the case of (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE, the electrode surface is functionalized with benzoic acid and aniline units, respectively, prior to cysteine attachment. Although most of these units are later linked to cysteine through the EDC/sulfo-NHS crosslinking agents, it is possible that some of them remain unmodified. These unmodified units would have theoretical pK_a values of 4.2 (benzoic acid) and 4.9 (aniline), which would explain the above-mentioned current decrease. In the case of bare SPCNFE, no surface functionalization was performed and, therefore, this current decrease is not observed.

In the pH region from 6 to 11 the slight current decrease observed can be explained by the acid-base equilibriums of cysteine forms. Particularly, in the case of (ABA)Cys-SPCNFE, this decrease can be associated to the deprotonation of the thiol group (**B** – **C**), with an experimental pK_a value, calculated from the dI/dpH *vs.* pH, close to 9. On the other hand, for (PNA)Cys-SPCNFE only one experimental pK_a value close to 8 was observed, which could be attributed to the loss of both amino (**D** – **E**) and thiol (**E** – **F**) protons.

3.3 Application to the determination of metal ions

Both (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE were tested for the determination of Pb(II) and Cd(II) as a model metal ion system by DPASV. First of all, some experimental parameters including pH, E_d and t_d were optimized. Figure 4 shows the response evolution *vs* pH of both considered sensors for a solution containing 50 µg L^{-1} of Pb(II) and Cd(II). pH is a critical parameter in this type of determination since it affects most of the processes involved such as protonation/deprotonation of functional groups with affinity for metal ions (-SH, $-NH_2$, -COOH), electrostatic interactions between the metal ion and the electrode surface and metal hydrolysis. For (ABA)Cys-SPCNFE the optimal pH was established at 5, which can be justified by presence of COO^- groups on the electrode surface that allow a favourable electrostatic interaction with the metal ions. At lower pHs this COO^- groups are protonated and this electrostatic interaction is not present whereas at higher pHs metal ion hydrolysis can be observed. A similar behaviour is shown by (PNA)Cys-SPCNFE, although in this case the lower response at pHs lower than 4 could be explained by the non-favourable electrostatic interaction with metal ions caused by the positively charged protonated aniline units present on the surface.

Regarding E_d, different voltammetric measurements were carried out from -1.4 V to -1.0 V in a solution containing 50 µg L^{-1} of Pb(II) and Cd(II) using both (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE. In both cases, the highest current response for both Cd(II) and Pb(II) was found at an E_d of -1.0 V and, therefore, this value was selected for further experiments. Finally, t_d was also studied from 30 to 300 s and a t_d of 120 s was selected as a compromise between peak area and analysis time.

Once the experimental conditions were optimized the analytical performance of both sensors was studied considering repeatability, reproducibility, sensibility, linear range and limits of detection (LOD) and quantification (LOQ). All these values are summarized in Table 1.

Repeatability was computed from 5 repetitive measurements carried out with the same sensor on a solution containing 50 µg L^{-1} of Pb(II) and Cd(II) in acetate buffer achieving similar RSD(%) values for both metal ions and both sensors, lower than 1.6 % in all cases. Reproducibility was estimated from the slope of three independent calibration curves carried out from 1 to 150 µg L^{-1} of Pb(II) and Cd(II) using three different sensors. The obtained values were lower than 1.3 % in all cases, which proves that both modification strategies produce very reproducible sensors, being slightly better in the case of (PNA)Cys-SPCNFE.

Simultaneous Pb(II) and Cd(II) calibration curves were performed at the optimal experimental conditions from 1 to 150 µg L^{-1} using both (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE. As it can be seen in Figure 5 two separate and well-defined peaks were obtained for both considered metal ions. Similar shapes and intensities were obtained for Cd(II) using both sensors whereas in the case of Pb(II) a more gaussian-shaped peak with higher intensities was observed using (PNA)Cys-SPCNFE.

Regarding sensitivity, computed as the slope of the calibration curve, both sensors present a higher sensitivity for Cd(II) than for Pb(II) which is in agreement with the hard-soft classification scheme, where softer ligands such as thiols present more affinity to softer metals like Cd(II) [23]. This higher preference for Cd(II) is accentuated in the case of (ABA)Cys-SPCNFE. On the other hand, LODs and LOQs, calculated as three and ten times the standard deviation of the intercept over the slope of the calibration curve respectively, were in all cases at µg L^{-1} levels. It should be pointed out that both sensors are able to quantify Pb(II) at lower concentrations than the established legal limit (10 µg L^{-1} according to the European Water Framework Directive 2000/60/EC) whereas only (PNA)Cys-SPCNFE can quantify Cd(II) at the more demanding limit established by the same directive for this metal (5 µg L^{-1}). This fact could be justified by the presence of amino groups in (PNA)Cys-SPCNFE that also have a strong binding preference for soft metals like Cd(II) [23].

Therefore, considering both peak shapes and analytical performance, we can conclude that attaching cysteine through its carboxylic group, i.e. (PNA)Cys-SPCNFE, is a better option than attaching it through the amino group since it provides a better sensor for the determination of metal ions at trace concentration levels, being able to simultaneously quantify both Cd(II) and Pb(II) at the legal limits established by the European Water Framework Directive. Moreover, it should be highlighted that the developed (PNA)Cys-SPCNFE sensor clearly improves the results previously reported for peptide-modified sensors [19–21,30], none of which provides a LOQ below the legal limit, without increasing the analysis time (t_d is the same or even lower than that used in the cited works).

In general terms, after studying the behaviour of both electrografting approaches, it can be said that the approach based on 4-nitrobenzenediazonium, although being less common, is not only an alternative strategy to the most usual electrografting procedure based on 4-carboxybenzenediazonium but can also give rise to improved sensors in some cases. It should also be pointed out that, although requiring an additional modification step to reduce the nitro groups to amino groups (Figure 1), the total sensor development time is similar or even slightly shorter than the procedure based on 4-carboxybenzenediazonium, with the additional advantage of allowing the attachment of modifiers with carboxylic groups that otherwise would require the use of a lysine spacer to assist their immobilization with the more usual electrografting based on 4-carboxybenzenediazonium.

4. Conclusions

At the view of the obtained results we can conclude that Cys was successfully linked to the SPCNFE surface through both its $-NH_2$ and $-COOH$ groups using aryl diazonium electrografting based strategies giving rise to two different sensors. The sensor performance was studied as a function of pH showing slightly different behaviour according to the free functional groups available in each case. Moreover, both sensors were analytically tested for metal ion determination using Pb(II) and Cd(II) as a model system. Firstly, the working pH was established, being 4.5 and 5.0 the optimal value for (PNA)Cys-SPCNFE and (ABA)Cys-SPCNFE respectively. At these pH conditions, metals are not affected by hydrolysis and the electrostatic interactions between metal ions and the electrode surface are favourable.

From an analytical point of view, although both developed sensors are very repetitive and reproducible and can be used for a large number of measurements without signs of degradation, (PNA)Cys-SPCNFE presents a better voltammetric behaviour conferred by the better shaped Pb(II) peak. Both (PNA)Cys-SPCNFE and (ABA)Cys-SPCNFE are sensible sensors that are able to detect Pb(II) and Cd(II) at trace levels with LODs around 1 and 2 µg L^{-1} for Pb(II) and Cd(II) respectively, resulting in suitable sensors for the detection of Pb(II) and Cd(II) at very low concentrations. However, (PNA)Cys-SPCNFE presents the additional feature of being capable of quantifying both metal ions below the legal limits established by the European Water Framework Directive, which represents a great improvement with respect to similar sensors reported in the literature. Thus, despite being a much less common electrografting strategy for the development of voltammetric sensors, the approach based on 4-nitrobenzenediazonium is also a valuable procedure that should be further taken into account.

Finally, it should be pointed out that the combination of both electrografting approaches clearly expands the versatility of the covalent modification strategies for the development of voltammetric sensors since it allows the attachment of modifiers with different functional groups, therefore broadening the list of modifiers that can be covalently linked to an electrode surface.

Acknowledgments

This work is supported by the Generalitat of Catalonia (Project 2017SGR311). Clara Pérez-Ràfols acknowledges the Spanish Ministry of Education, Culture and Sports for a Ph.D grant (reference FPU15/02140) and Clara Pérez-Ràfols and Miguel Rosal thank the Water Research Institute (IdRA) of the University of Barcelona for its support.

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Table 1. Calibration data for the simultaneous determination of Pb(II) and Cd(II) on (ABA)Cys-SPCNFE and (PNA)Cys-SPCNFE at acetate buffer pH 5 and 4.5 respectively applying an E_d of -1.0V and a t_d of 120 s. Standard deviations are denoted by parenthesis

	(ABA)Cys-SPCNFE		**(PNA)Cys-SPCNFE**	
	Cd(II)	**Pb(II)**	**Cd(II)**	**Pb(II)**
Sensitivity (a.u. µg^{-1} L)	5.39 (0.05)	2.75 (0.01)	4.63 (0.03)	3.44 (0.01)
R^2	0.998	0.999	0.999	1.000
Linear range (µg L^{-1})[a]	7.3 – 153.9	3.7 – 153.9	4.6 – 153.9	3.4 – 153.9
LOD (µg L^{-1})	2.2	1.1	1.4	1.0
Repeatability (%)	1.52	1.58	1.39	1.33
Reproducibility (%)	1.29	1.15	0.82	0.92

[a] LOQ was considered as the lowest value of the linear range.

Figure 1

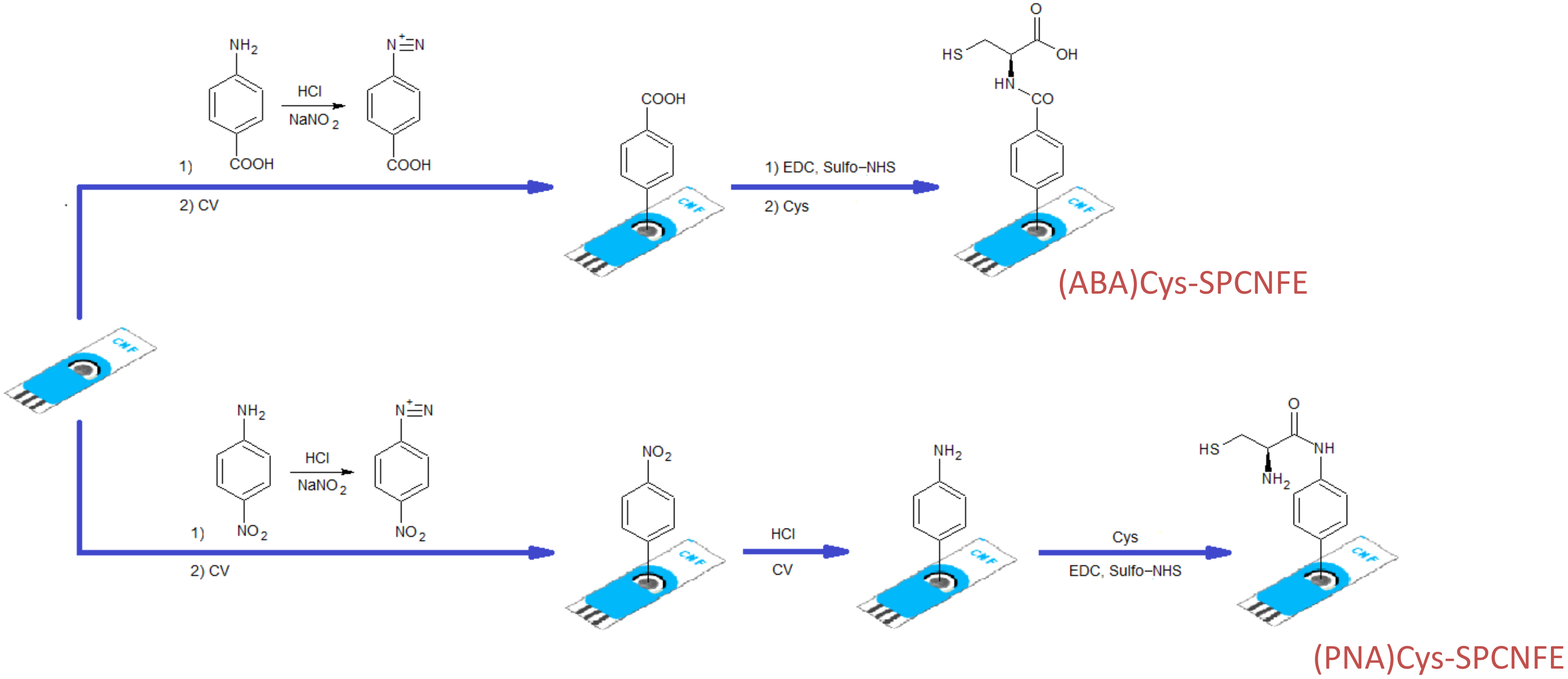

NH2
HCl
NaNO 2
N≡N
1)
COOH
COOH
2) CV
COOH
1) EDC, Sulfo–NHS
2) Cys
O
HS
OH
HN
CO
CNF
(ABA)Cys-SPCNFE
NH2
HCl
NaNO 2
N≡N
1)
NO 2
NO 2
2) CV
NO2
HCl
CV
NH2
Cys
EDC, Sulfo–NHS
O
HS
NH
NH2
(PNA)Cys-SPCNFE

Figure 2

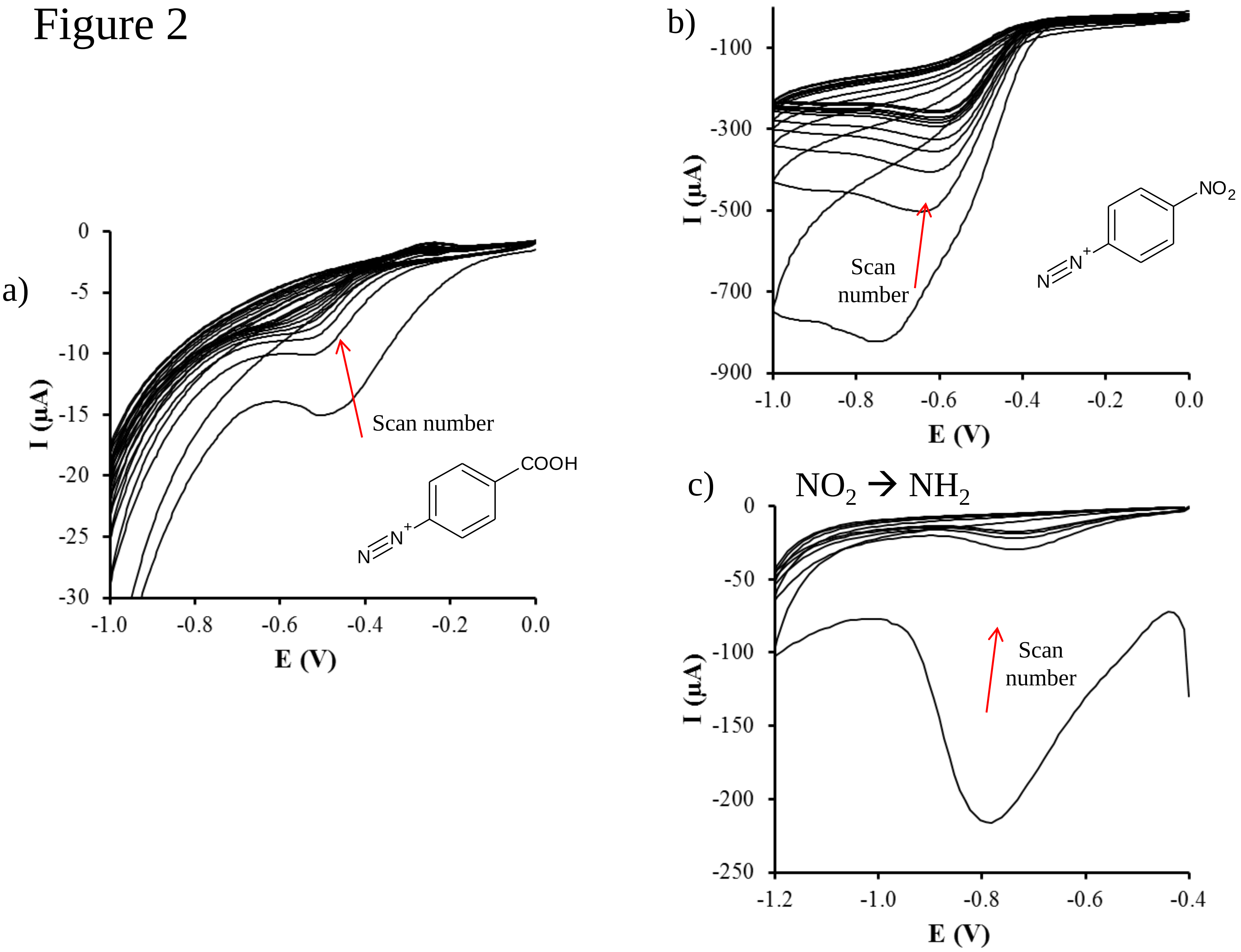


Figure 2
a)
0
-5
-10
-15
-20
-25
-30
I (µA)
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
E (V)
Scan number
COOH
N≡N+
b)
-100
-300
-500
-700
-900
I (µA)
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
E (V)
Scan number
NO2
N≡N+
c)
NO2 → NH2
0
-50
-100
-150
-200
-250
I (µA)
-1.2
-1.0
-0.8
-0.6
-0.4
E (V)
Scan number

Figure 3

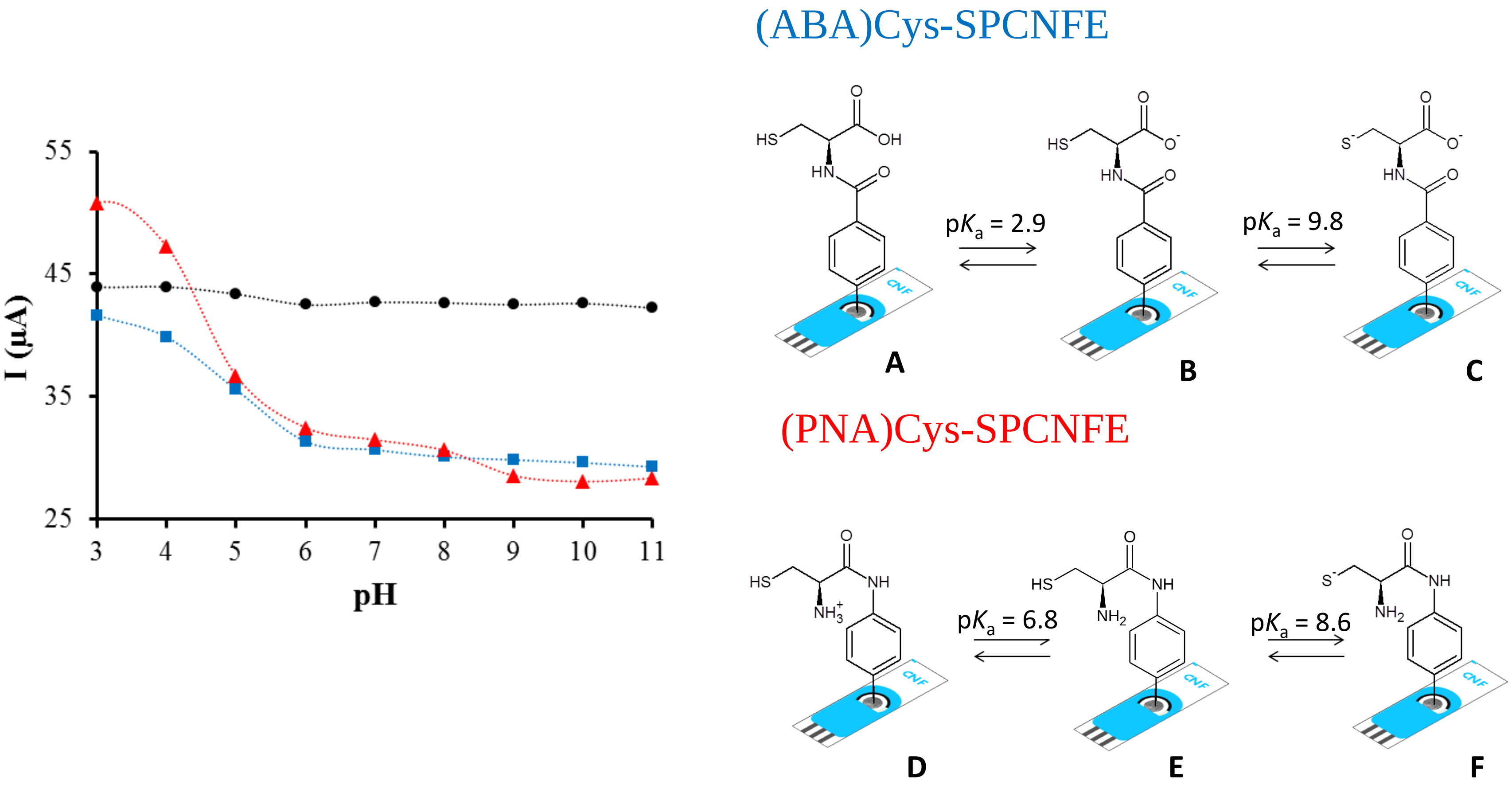

55
45
35
25
I (µA)
3
4
5
6
7
8
9
10
11
pH
(ABA)Cys-SPCNFE
HS
OH
O
HN
CNF
pK_a = 2.9
pK_a = 9.8
O^-
S^-
A
B
C
(PNA)Cys-SPCNFE
NH_3^+
NH
NH_2
pK_a = 6.8
pK_a = 8.6
D
E
F

Figure 4

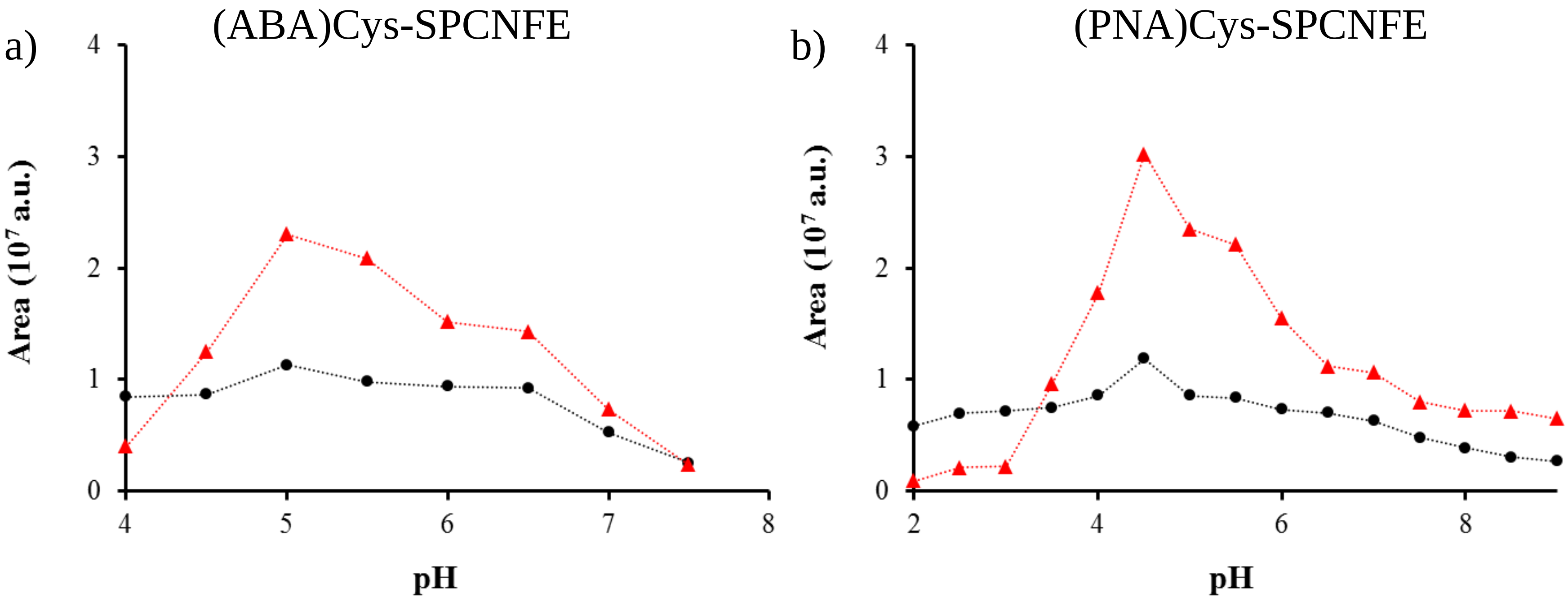
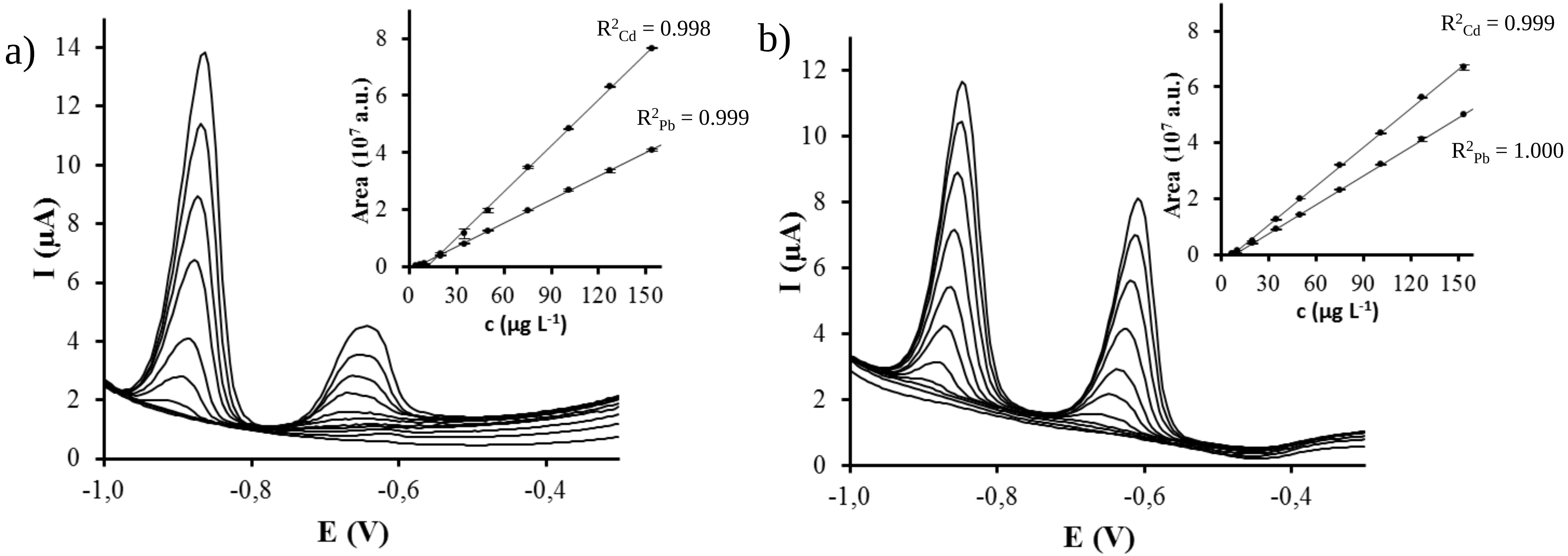

a)
(ABA)Cys-SPCNFE
Area (10^7 a.u.)
0
1
2
3
4
4
5
6
7
8
pH
b)
(PNA)Cys-SPCNFE
Area (10^7 a.u.)
0
1
2
3
4
2
4
6
8
pH

Figure 5


a)
14
12
10
8
6
4
2
0
I (µA)
-1,0
-0,8
-0,6
-0,4
E (V)
8
6
4
2
0
Area (10^7 a.u.)
0
30
60
90
120
150
c (µg L^{-1})
R^2_{Cd} = 0.998
R^2_{Pb} = 0.999
b)
12
10
8
6
4
2
0
I (µA)
-1,0
-0,8
-0,6
-0,4
E (V)
8
6
4
2
0
Area (10^7 a.u.)
0
30
60
90
120
150
c (µg L^{-1})
R^2_{Cd} = 0.999
R^2_{Pb} = 1.000