

Crown ether-modified electrodes for the simultaneous stripping voltammetric determination of Cd(II), Pb(II) and Cu(II)

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ABSTRACT

This work describes the immobilization of 4-carboxybenzo-18-crown-6 (CB-18-crown-6) and 4-carboxybenzo-15-crown-5 (CB-15-crown-5) assisted by lysine on aryl diazonium salt monolayers anchored to the surface of graphite-epoxy composite electrodes (GEC), and their use for the simultaneous determination of Cd(II), Pb(II) and Cu(II) by differential pulse anodic stripping voltammetry (DPASV). These modified electrodes display a good repetitivity and reproducibility with detection and quantification limits at levels of $\mu\text{g L}^{-1}$ (ppb), confirming their suitability for the determination of Cd(II), Pb(II) and Cu(II) ions in environmental samples. The overlapped nature of the multimetal stripping measurements was resolved by employing the two-sensor array CB-15-crown-5-GEC and CB-18-crown-6-GEC, since the metal complex selectivity exhibited by the considered ligands could add

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some discrimination power. For the processing of the voltammograms, Discrete Wavelet Transform and Causal Index were selected as preprocessing tools for data compression coupled with an artificial neural network for the modelling of the obtained responses, allowing the resolution of mixtures of these metals with good prediction of their concentrations.

Keywords: crown ether-modified sensors; electrochemical grafting; metal determination; stripping voltammetry; artificial neural network

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4 **1. INTRODUCTION**
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6 Determination of trace heavy metal ions in environmental samples is nowadays an area of
7 major concern, since heavy metals from pollution sources can affect people's health when
8 absorbed or inhaled interfering with organ system function. Heavy metals are especially
9 hazardous since they tend to bioaccumulate in animal and human organisms, this means that
10 they become toxic when they are not metabolized by the body and accumulate in the soft
11 tissues [1].
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21 Stripping voltammetry methods are the most efficient electrochemical techniques for trace
22 and species analysis due to their high sensitivity and selectivity, being particularly suitable
23 for the determination of trace heavy metal ions in environmental samples [2]. The
24 performance of voltammetry is strongly influenced by the working electrode material. For
25 many years, electrochemical stripping methods were associated with the use of working
26 mercury electrodes for the determination of trace metal ions due to the extensive cathodic
27 potential range [3]. However, mercury is toxic and poses a significant health and
28 environmental hazard. Thus, the development and use of chemically modified electrodes as
29 working electrodes for the detection and quantification of metal ions in natural samples is
30 also a subject of high interest.
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45 Macrocyclic compounds, as crown ethers, can be employed as modifiers for metal
46 determination, where crown compound acts as host, complexing its metal. Compounds of
47 this type may be introduced into the electrode to enhance its selectivity and to lower the
48 detection limit of analyte. Moreover, crown ethers have a high degree of selectivity being
49 able to strongly bind certain metals, since their structural properties allow the complex
50 formation by means of ion-dipole interaction with these metal ions [4]. So, the complexing
51 ability and crown cavity size which is suitable for a particular analyte have to be considered
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4 for the selective detection of metal ions. Particularly, crown ethers are well-known to form
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6 complexes with alkaline ions, alkaline earth metals, lanthanides and transition metal ions.
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8 Surprisingly, studies devoted to complexation with transition metal ions are scarce and only
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10 few works using crown ether-modified electrodes are published for the determination of
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12 lead [5-7], mercury [8, 9], silver [10], thallium [11], palladium [12] and copper [13].
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16 An important aspect in the design of these electrodes is the chemical modification
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18 procedure. In this sense, few approaches were described in the literature to present day
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20 including the crown ethers coating with graphite powders and the modification of a gold
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22 electrode with a crown ether yielding a self-assembled monolayer (SAM) [14].
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24 Nevertheless, as reported for peptides, an alternative strategy would be the crown ether
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26 immobilization on aryl diazonium salt monolayers anchored on the electrode surface
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28 resulting in a simple, flexible and valuable alternative for forming stable complexing
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30 monolayers [15, 16].
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34 Crown ether-modified electrodes can be used for metal determination as a single-electrode
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36 sensor or in combination with others forming a multi-sensor array, in which each electrode
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38 in the array is modified with a recognition crown ether that is largely selective for one of
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40 the metal ions to provide a multivariate response [17].
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46 The aim of this work is to report the modification and analytical characterization of
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48 graphite-epoxy composite electrodes (GEC) with 4-carboxybenzo-18-crown-6 (CB-18-
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50 crown-6) and 4-carboxybenzo-15-crown-5 (CB-15-crown-5), which were immobilized on
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52 aryl diazonium salt monolayers anchored to the electrode surface, for the simultaneous
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54 determination of Cd(II), Pb(II) and Cu(II) using differential pulse anodic stripping
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56 voltammetry (DPASV). An artificial neural network model was proposed as a tool to
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4 maximize the information obtained from the voltammetric data sets using CB-18-crown-6-
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6 GEC and CB-15-crown-5-GEC sensors that a priori are difficult to understand.
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10 11 **2. EXPERIMENTAL**

12 13 14 **2.1 Chemicals**

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16 Potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, 2-(N-
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18 morpholino)-ethanesulfonic acid (MES), potassium dihydrogen phosphate, sodium
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20 monophosphate, methanol, perchloric acid, hydrochloride acid, *N*-hydroxysulfosuccinimide
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22 (sulfo-NHS), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and
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24 sodium nitrite were purchased from Sigma (St. Louis, MO, USA). 4-aminobenzoic acid
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26 (ABA) and DL-lysine monohydrochloride were provided by Acros (Geel, Belgium). 4-
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28 carboxybenzo-18-crown-6 with a purity of 99% and 4-carboxybenzo-15-crown-5 with
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30 purity greater than 98% were provided by Acros and Sigma respectively. All other reagents
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32 used were from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). All reagents
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34 were of analytical grade. Pb(II), Cd(II) and Cu(II) stock solutions 10^{-2} mol L⁻¹ were
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36 prepared from $Pb(NO_3)_2 \cdot 4H_2O$, $Cd(NO_3)_2 \cdot 4H_2O$ and $Cu(NO_3)_2 \cdot 3H_2O$ respectively and
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38 standardized complexometrically [18]. 0.1 mol L⁻¹ acetic acid / acetate buffer solution (pH
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40 4.5) was used for pH control. Ultrapure water from MilliQ System (Millipore, Billerica,
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42 MA, USA) was used in all experiments.
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51 52 53 **2.2 Apparatus**

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55 Cyclic voltammetric (CV) measurements were carried out using a μ STAT 200 from
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57 Dropsens (Oviedo, Spain) using Dropview (Dropsens) software for data acquisition and
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4 control of the experiments. A three electrode configuration was used to perform the CV
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6 measurements for modification and characterization of the electrodes: a commercial
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8 platinum counter electrode (Model 52-67, Crison Instruments, Barcelona, Spain), a
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10 reference double junction Ag/AgCl electrode (Thermo Orion 900200, Beverly, MA, USA)
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12 and the modified GEC as the working electrode.
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16 Differential pulse anodic stripping voltammetric (DPASV) measurements were performed
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18 in an Autolab System PGSTAT 30 (EcoChemie, The Netherlands), in a multichannel
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20 configuration, using GPES Multichannel 4.7 software package (EcoChemie). The
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22 voltammetric cell was formed by the two working graphite epoxy electrodes (GECs)
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24 modified with 4-carboxybenzo-18-crown-6 (CB-18-crown-6) and 4-carboxybenzo-15-
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26 crown-5 (CB-15-crown-5) respectively, a commercial platinum counter electrode and a
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28 double junction Ag/AgCl reference electrode.
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33 A pH meter GLP 22 (Crison Instruments, Barcelona, Spain) was used for pH
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35 measurements.
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38 All measurements were carried out at room temperature (20 °C).
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42 43 **2.3 Procedures**

44 45 ***2.3.1 Preparation of graphite epoxy electrodes***

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47 Graphite epoxy composite electrodes (GECs) were fabricated by using a PVC tube body (6
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49 mm i. d.) and a small copper disk soldered at the end of an electrical connector. The
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51 working surface is an epoxy-graphite conductive composite, formed by a mixture of 20%
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53 graphite powder (Merck, Darmstadt, Germany) and 80% of epoxy resin, Epotek H77, and
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55 its corresponding hardener (both from Epoxy Technology, Billerica, MA, USA), deposited
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57 on the cavity of the plastic body [19]. The composite material was cured at 80 °C for 3
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4 days. Prior to their functionalization, the electrode surface was moistened with MilliQ
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6 water and then polished on abrasive sandpaper (400, 600, 800, 1000 and 1200 grit) and
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8 finally on alumina polishing strips (301044-001, Orion) in order to obtain a reproducible
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10 electrochemical surface.
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13 14 15 16 **2.3.2 Preparation of modified GECs** 17

18 The principle of the modification of the GEC is illustrated in Figure 1, with specific steps
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20 described below [20].
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23 24 *2.3.2.1 Diazonium salt electrografting* 25

26 The *in situ* generation of the aryl diazonium was performed by adding $5 \cdot 10^{-3}$ equivalents of
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28 sodium nitrite to an acidic solution (1 M aqueous HCl) of ABA. These solutions were
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30 mixed for about 30 min in an ice bath, prior to the electrochemical grafting process [21]
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32 conducted by scanning the potential at 0.2 V s^{-1} from 0 V to -1 V for 100 cycles. The
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34 functionalized electrodes were thoroughly rinsed with Milli-Q water and methanol to
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36 remove any physisorbed compounds.
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40 41 *2.3.2.2 Covalent immobilization of crown ethers via carbodiimide coupling* 42

43 The carboxyl groups of the electrografted diazonium salt were activated by incubating the
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45 functionalized electrodes in a 26 mM EDC and 35 mM sulfo-NHS solution in 100 mM
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47 MES buffer (pH 4.5) for 1 h. In order to conjugate the carboxy-functionalized electrode
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49 with the carboxy-modified ligands, a lysine spacer was intercalated in between, by using its
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51 two amino functionalities to form amido bonds [22-25]. The surface activated groups
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53 reacted overnight with the α -amine group of the lysine at 4°C. Prior to cross linking with
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55 EDC/sulfo-NHS, 2.9 mg of 4-carboxybenzo-18-crown-6 or 4-carboxybenzo-15-crown-5
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57 were incubated with 100 μL 5 mM lysine in 0.1 M MES buffer for 3 h.
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7 **2.3.3 DPASV measurements**
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9 Before each set of measurements, the electrodes were scanned in acetate buffer solution in
10 order to get stable voltammetric responses.
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13 DPASV determinations using CB-18-crown-6-GEC and CB-15-crown-5-GEC of Cd(II),
14 Pb(II) and Cu(II), were done at a deposition potential (E_d) of -1.4 V, applied with stirring
15 during a deposition time (t_d) of 300 s and followed for a rest period (t_r) of 10 s.
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17 Determinations were done by scanning potential from -1.4 to +0.2 V using a step potential
18 of 4 mV and pulse amplitude of 50 mV. Calibration plots were obtained by increasing
19 metal concentrations in pH 4.5 acetic acid / acetate buffer media.
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23 In order to eliminate any remaining bound metals from the electrode, an electrochemical
24 cleaning stage was considered between measurements. This stage was performed by
25 applying a conditioning potential (E_{cond}) of 0.5 V for 30 s after each measurement, in a cell
26 containing fresh buffer solution.
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30 To allow the multimetal simultaneous determination, a response model was built using
31 artificial neural networks (ANN). For this aim, DPASV scans of a total set of 35 samples in
32 the concentration range 1.5 - 200 ppb, manually prepared by appropriate dilution from the
33 prepared metal stock solutions in pH 4.5 acetic acid / acetate buffer, were recorded at the
34 same experimental conditions as calibration plots. The set of samples was divided into two
35 data subsets: a training subset formed by 27 samples (79.4%), which were distributed in a
36 cubic design [26] and used to establish the response model; plus 7 additional samples
37 (20.6%) for the testing subset, randomly distributed along the experimental domain, and
38 used to evaluate the model predictive response (Figure 2).
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60 All experiments were carried out without any oxygen removal.
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7 **2.3.4 Data processing**
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9 In order to reduce the large amount of information generated for each sample (2 sensors x
10 390 current values at different potential) a preprocessing stage was necessary to compress
11 the original data. The objective of this step is to reduce the complexity of the input data
12 while preserving the relevant information; also the compression of the data allows to reduce
13 the training time, avoid redundancy in input data and to obtain a model with better
14 generalization ability.
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17 The chosen method was the Discrete Wavelet Transform (DWT) [27], each voltammogram
18 was normalized and then compressed using *Daubechies 4* wavelet mother function and a
19 fourth decomposition level. In this manner, the original data was reduced to 60 coefficients
20 without any loss of relevant information; additionally Causal Index [28] was employed to
21 further refine the model by eliminating the inputs that make relatively small contributions
22 to the model. With this double compression-pruning approach, the 780 inputs per sample
23 were reduced down to 21 coefficients, achieving a compression ratio up to 97.31%.
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26 Chemometric processing of data was performed by specific routines written by the authors
27 using MATLAB 7.1 (MathWorks, Natick, MA) and its Neural Network Toolbox (v.4.0.6).
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48 **3. RESULTS AND DISCUSSION**
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51 **3.1 GEC characterization**
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53 The electrochemical response using 2 mM ferrocyanide/ferricyanide as redox probe in 100
54 mM phosphate buffer (pH 7.4) was investigated at each functionalization step using cyclic
55 voltammetry (CV). CV measurements were performed in unstirred conditions scanning the
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4 potential at 0.1 V s^{-1} from -0.7 to 1 V . Electrografting resulted in decreasing current as
5 expected (Figure 3). Covalent binding of complexing agents through the α -amine group of
6 the lysine also resulted in lower current peaks compared to bare electrode, as shown in
7 Figure 3 for CB-18-crown-6-GEC. These observed changes in the voltammograms
8 confirmed the modifications occurring on the electrode surface.
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19 **3.2 Repetitively and reproducibility**

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23 The selected E_d , t_d and t_r were firstly optimized to ensure the detection of each metal at each
24 ligand-modified electrode in the selected concentration range; the compromise conditions
25 were for all cases -1.4 V as the applied accumulation voltage with stirring during a t_d of 300
26 s and followed by a rest stage of 10 s at the same applied potential.
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33 Stripping measurements were carried out using both ligand-modified electrodes in order to
34 test their repetitively and reproducibility. The response obtained from three different
35 modified GEC units for each complexing agent was reproducible within a series of five
36 repetitive measurements of a $125 \mu\text{g L}^{-1}$ of Cd(II), Pb(II) and Cu(II) solution (mixed
37 solution) by applying the optimized voltammetric conditions yielding a RSD of 3.1 %, 2.1
38 % and 2.2 % for Cd(II), Pb(II) and Cu(II) respectively for the CB-18-crown-6-GEC and 6.4
39 %, 2.7 % and 3.0 % for Cd(II), Pb(II) and Cu(II) respectively for the CB-15-crown-5-GEC.
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50 The repetitively produced a RSD of 3.1 %, 4.3 % and 2.2 % for Cd(II), Pb(II) and Cu(II)
51 respectively for the CB-18-crown-6-GEC and 5.2 %, 3.3 % and 1.6 % for Cd(II), Pb(II) and
52 Cu(II) respectively for the CB-15-crown-5-GEC.
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3.3 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

First of all, separate calibration of Cd(II), Pb(II) and Cu(II) ions by stripping voltammetry (DPASV) was carried out on each CB-18-crown-6-GEC and CB-15-crown-5-GEC respectively. The LOD was calculated as 3 times the standard deviation of the intercept over the slope of the calibration curve of the target ions. LOQ was evaluated by considering 10 times the previous ratio. The lowest value of the linear concentration range was established from the corresponding limit of quantification (LOQ). For LOD and LOQ determinations, ten different standards of the considered ions were used to build the calibration lines. Figure 4a, 4b and 4c show, as an example, the evolution of DPASV signals of each metal using the CB-15-crown-5-GEC sensor when the concentration of Cd(II), Pb(II) and Cu(II) respectively increases (CB18-crown-6-GEC with equivalent behavior). In all cases, well defined stripping peaks without any evidence of signal splitting were observed over the considered concentration range. Linear calibration curves were obtained for Cd(II), Pb(II) and Cu(II) up to a maximum concentration level of 191.1, 186.5 and 177.3 $\mu\text{g L}^{-1}$ respectively. The corresponding regression equations and the correlation coefficient for both sensors are shown in Table 1. It must be pointed out that similar voltammetric responses for Pb(II), Cd(II) and Cu(II) were also observed using CB-18-crown-6-GEC sensor at the same experimental conditions. Nevertheless, regarding the sensitivities considered as the value obtained from the slope of the calibration curves, it can be mentioned that: i) using CB-18-crown-6-GEC, Pb(II) was the metal ion showing better sensitivity; and ii) using CB-15-crown-5-GEC, Cu(II) was the most sensitive metal. The LOD of the assay for the three metal ions in both modified electrodes varied from 1.5 to 4.7 $\mu\text{g L}^{-1}$ depending on the metal ion (Table 1) and the LOQ ranged from 5.0 to 15.7 $\mu\text{g L}^{-1}$ depending on the metal ion (Table 1). For Pb(II) and Cu(II) the obtained results are similar

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4 than the values reported in earlier studies [6, 7, 13]. In the case of Cd(II) no previous LOD
5 and LOQ data is available in the literature. Therefore the reported calibration data suggest
6 that both CB-18-crown-6-GEC and CB-15-crown-5-GEC sensors could be fully suitable
7 for the determination of Cd(II), Pb(II) and Cu(II) at the ultra-trace level in environmental
8 samples. The second observation is that the use of the two electrodes as an array could add
9 some discrimination power to resolve a multimetal mixture.
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21 **3.4 Metal complex selectivity**

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23 Taking into account that the immobilized crown ethers on the GEC surface are used as
24 molecular collector with ability to selectively coordinate with the metal ions, both the ionic
25 diameter of metal ions and the cavity size of the crown ethers play a crucial role for the
26 complex formation by means of ion-dipole interaction with metal ions. The ability of each
27 considered metal ions for forming the complex with both CB-18-crown-6 and CB-15-
28 crown-5 is displayed in Figure 5a. Voltammetric peak current responses for equal
29 concentrations of metal ion solution ($175 \mu\text{g L}^{-1}$ of Cd(II), Pb(II) and Cu(II)) show that
30 CB-18-crown-6 exhibits the highest selective complex forming ability with Pb(II) followed
31 by Cu(II); on the contrary, CB-15-crown-5 offers the highest interaction with Cu(II)
32 followed by Pb(II). In both considered crown ethers, Cd(II) shows the smallest ion-dipole
33 interaction being CB-15-crown-5 a little bit more selective than CB-18-crown-6.
34 Comparing the ionic diameter of the considered metal ions (1.5 \AA for Cu(II), 1.9 \AA for
35 Cd(II) and 2.4 \AA for Pb(II)) with the cavity size of both crown ethers (cavity diameter, 1.7 -
36 2.2 \AA for CB-15-crown-5 and 2.6 - 3.2 \AA for CB-18-crown-6), the described behavior is
37 consistent with studies by Christensen *et al.* in 1971 who suggested that cation diameter to
38 host cavity size ratios of 0.75 - 0.90 are favorable for direct ion-crown ether binding [4, 29].
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4 Thus, for Pb(II) a ratio of 0.75-0.92 were achieved for CB-18-crown-6 reflecting the size
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6 match for that ion. Likewise, ratios of 0.86-1.11 and 0.68-0.88 for CB-15-crown-5 were
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8 obtained for Cd(II) and Cu(II), respectively, consistent with the observed selectivities. The
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10 hypothesis is, therefore, that the use of the two electrodes as an array will provide higher
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12 information to resolve a multimetal mixture than that obtained from a single electrode. Two
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14 were the electrodes considered here, given that these are the ligands commercially
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16 available. With synthetic approaches, the number of differently modified sensors might be
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18 increased at will.
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26 **3.4 Multimetal Stripping Voltammetric Measurements**

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28 The behavior of the stripping signals of different mixtures of Cd(II), Pb(II) and Cu(II) was
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30 studied inside the concentration range 1.5 - 200 $\mu\text{g L}^{-1}$ using both CB-18-crown-6-GEC
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32 and CB-15-crown-5-GEC sensors in order to detect possible interactions between metal
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34 ions. As an example, a sample of five stripping voltammograms obtained using CB-15-
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36 crown-5-GEC (arbitrary concentrations) is displayed in Figure 5b. As it can be seen, in
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38 contrast to the individual signals of metals (Figure 4), an overlapping effect and the
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40 formation of some intermetallic compounds hinder the direct determination of the mixtures.
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42 Comparing both individual and multimetal stripping measurements the potential of the
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44 oxidation peak of each considered metal in the complex voltammograms could be assigned
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46 at *ca.* -0.75 V, -0.55 V and 0.03 V for Cd(II), Pb(II) and Cu(II) respectively. A comparison
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48 between the voltammograms provided by both modified sensors displayed no significant
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50 differences in metal peak shapes and peak potentials, however different levels of metal
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52 interactions were observed in agreement with metal complex selectivity. Thus, for example,
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54 in the calibration mixture of 100.7 $\mu\text{g L}^{-1}$ of Cd(II), Pb(II) and Cu(II) the greatest
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4 voltammetric peak currents were obtained for Cu(II) and Cd(II) using the CB-15-crown-5-
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6 GEC, and for Pb(II) using CB-18-crown-6-GEC (inset in Figure 5b). In this way, the
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8 stripping voltammetric response will be different depending on the metal ion concentration
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10 in each calibration mixture, the used modified sensor and the metal complex selectivity. For
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12 example, in the calibration mixture of 146.5, 116.0 and 24.4 $\mu\text{g L}^{-1}$ of Cd(II), Pb(II) and
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14 Zn(II) using the CB-15-crown-5-GEC (Figure 5b, (D) thin line), the peak current of Cd(II)
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16 and Pb(II) increases substantially with respect to the mixture of equal concentration of
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18 metal ions (Figure 5b, (A) thick line), whereas the peak current of Cu(II) decreases
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20 considerably.
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26 Apart from the initially observed complexity, as the next step, the sets of voltammograms
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28 of heavy metal mixtures obtained from the two-sensor array were postulated to be used to
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30 calibrate Cd(II), Pb(II) and Cu(II) using an appropriate ANN model that may consider any
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32 non-linearity or overlapping in the determination of the considered metal ions.
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38 **3.5 Quantification of the metal mixtures**

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40 Once the data were compressed by use of wavelet transform and causal index in this study
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42 case, the first step in building the appropriate ANN model is choosing the topology of the
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44 neural network used. Normally, given the difficulties to predict the optimal settings in
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46 advance this is a trial-and-error process, where several parameters (training algorithms,
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48 number of hidden layers, transfer functions, etc.) are fine-tuned in order to find the best
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50 configuration that optimizes the performance of the model [30].
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55 In consequence, the samples from the training subset were used for building the ANN
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57 model, and its accuracy was then evaluated towards samples of the external test subset by
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59 employing the built model to predict the concentrations of the metals of those samples
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4 (external validation). Taking into account that the external test subset data is not used at all
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6 for the modelling, its goodness of fit is a measure of the completed modelling performance.
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9 With the aim of facilitating the verification of the prediction ability of the obtained ANN
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11 model, comparison graphs of predicted *vs.* expected concentrations for the considered
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13 compounds were plotted, both for training subset and testing subsets. Once calculated the
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15 root mean square error (RMSE) [31], the best model will be the one that has the lowest
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17 RMSE values and additionally, regression parameters from the comparison graphs close to
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19 the ideal values (i.e. slope and correlation coefficient equal 1, and intercept equal 0).
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24 In our case, the resolution of the Cd(II), Pb(II) and Cu(II) mixtures was attempted using the
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26 data from the two voltammetric sensors. To this aim, the set of samples was measured with
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28 the two electrodes (CB-15-crown-5-GEC and CB-18-crown-6-GEC) and the obtained
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30 voltammetric responses were compressed employing DWT+CI and the different ANN
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32 models were optimized. After a systematic study optimizing the different parameters, the
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34 final architecture of the ANN model had 21 neurons in the input layer, 4 neurons and *logsig*
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36 transfer function in the hidden layer and three neurons and *tansig* transfer function in the
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38 output layer, providing the concentrations of the three species considered. Afterwards,
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40 comparison graphs of predicted *vs.* expected concentrations for the considered compounds
41
42 were built (Figure 6). As it can be observed, a satisfactory trend was obtained for all three
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44 metal ions with regression lines close to the theoretical ones. Additionally, the obtained
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46 linear comparison parameters were calculated (Table 2) resulting close to the ideal values,
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48 with intercepts close to 0 and slopes and correlation coefficients close to 1, meaning that
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50 there are no significant differences between the values predicted by the ANN model and
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52 those expected and provided by the reference method.
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4. Conclusions

In this work, it has been demonstrated that CB-15-crown-5 and CB-18-crown-6 were successfully immobilized through the assistance of lysine on aryl diazonium salt monolayers anchored on a graphite epoxy composite electrode surface, constituting an alternative to the more widespread ether crown modified electrodes. These proposed modified electrodes exhibit a good repetitivity and reproducibility and can be used for a large set of measurements without signs of degradation or loss of sensitivity, allowing the simultaneous determination of Cd(II), Pb(II) and Cu(II). In agreement with their good performance, these modified electrodes could be a promising implement for the determination of heavy metals in real samples as a healthier alternative to mercury electrodes. Related to their analytical performances, normal-shaped stripping signals were obtained for individual stripping measurements of Cd(II), Pb(II) and Cu(II) achieving LODs and LOQs at levels of $\mu\text{g L}^{-1}$, whereas an overlapping effect and the formation of some intermetallic compounds were observed in the stripping measurements of Cd(II), Pb(II) and Cu(II) mixtures in both CB-15-crown-5-GEC and CB-18-crown-6-GEC.

These considered crown ethers exhibit a high metal complex selectivity. In agreement with propitious cation diameter to host cavity size ratios, Pb(II) shows a more favorable complex forming ability with CB-18-crown-6 whereas Cu(II) fits snugly into CB-15-crown-5. For Cd(II), CB-15-crown-5 is a little bit more selective than CB-18-crown-6.

Taking advantage of the crown ethers highest selective complex forming ability with metal ions, the use of the two-sensor array CB-15-crown-5-GEC and CB-18-crown-6-GEC can add some discrimination power to resolve the metal ion mixture. In this sense, the results provided in this work prove that the combination of the set of voltammetric measurements

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4 with chemometric tools helps us to determine the considered metal ions despite the intricate
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6 origin of the signals. Thus, voltammetric data preprocessed by DWT+CI and coupled with
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8 an artificial neural network permitted to obtain satisfactory results for the quantification of
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10 Cd(II), Pb(II) and Cu(II). The experiments exhibited similar performance in all training and
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12 testing correlation coefficients, obtained from the predicted *vs.* expected concentrations
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14 comparison graphs, which were in all cases higher than 0.942. The multivariate models
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16 created with ANN properly describe the complexity in the voltammograms caused by the
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18 overlapped peaks. At the view of the satisfactory results the proposed methodology is
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20 perfectly suitable for the determination of heavy metals ions in environmental and
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22 biological samples at the ultra-trace level.
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Table 1. Calibration data for the determination of Cd(II), Pb(II) and Cu(II) on CB-18-crown-6-GEC and CB-15-crown-5-GEC at E_d of -1.4 V using a t_d of 120 s at pH 4.5.

	Cd (II)		Pb (II)		Cu (II)	
	CB-18-crown-6-GEC	CB-15-crown-5-GEC	CB-18-crown-6-GEC	CB-15-crown-5-GEC	CB-18-crown-6-GEC	CB-15-crown-5-GEC
Regression	$y = 0.016x - 0.13$	$y = 0.011x - 0.11$	$y = 0.021x + 0.31$	$y = 0.014x - 0.013$	$y = 0.015x - 0.052$	$y = 0.020x - 0.39$
R²	0.999	0.998	0.999	0.999	0.999	0.999
Linear range ($\mu\text{g L}^{-1}$)^a	7.9 – 191.1	15.7 – 191.1	5.0 – 186.5	10.9 – 186.5	5.1 – 177.3	7.7 – 177.3
LOD ($\mu\text{g L}^{-1}$)	2.4	4.7	1.5	3.3	1.5	2.3

^aThe lowest value of the linear range was considered from the LOQ.

Table 2

Table 2. Results of the fitted regression lines for the comparison between obtained vs. expected values, both for the training and testing subsets of samples and the different metal species (intervals calculated at the 95% confidence level).

Metal	Correlation	Slope	Intercept ($\mu\text{g L}^{-1}$)	RMSE ($\mu\text{g L}^{-1}$)	NRMSE ($\mu\text{g L}^{-1}$)	Total RMSE ($\mu\text{g L}^{-1}$)	Total NRMSE ($\mu\text{g L}^{-1}$)
Pb(II)	0.999	0.98±0.04	1.9±4.3	2.91	0.015		
tr Cd(II)	0.999	0.98±0.04	1.9±4.3	3.02	0.015	3.17	0.016
Cu(II)	0.996	0.98±0.05	1.5±5.5	3.66	0.018		
Pb(II)	0.942	0.77±0.64	14.9±52.0	14.2	0.071		
ts Cd(II)	0.957	1.04±0.72	-29.9±49.3	34.1	0.170	22.4	0.12
Cu(II)	0.989	1.31±0.45	-31.2±52.3	17.3	0.087		

tr: training subset; ts: testing subset; RMSE: root mean square error; NRMSE: normalized root mean square error

CAPTIONS FOR FIGURES

Figure 1. Scheme of the preparation of the two modified electrodes, CB-15-crown-5-GEC and CB-18-crown-6-GEC, by electrochemical grafting.

Figure 2. Experimental design using for training (●, solid line) and testing (○, dotted line) data subsets.

Figure 3. CVs plots recorded at each electrode functionalization step. Measurements were performed in a 2 mM ferrocyanide/ferricyanide solution in phosphate buffer.

Figure 4. DPASV measurements of (a) Cd(II), (b) Pb(II), and (c) Cu(II) recorded on a CB-15-crown-5-GEC sensor at pH 4.5 using a E_d of -1.40 V during 300 s and t_r of 10 s.

Figure 5. (a) Differential Pulse anodic stripping voltammetric peak current of $175 \mu\text{g L}^{-1}$ of Cd(II), Pb(II) and Cu(II) using both CB-15-crown-5-GEC and CB-18-crown-6-GEC sensors. (b) Some voltammograms generated during the build of the response model recorded at the same conditions as Figure 4. Sample composition: (A) $100.7 \mu\text{g L}^{-1}$ of Cd(II), Pb(II) and Cu(II); (B) $54.9 \mu\text{g L}^{-1}$ of Cd(II), $85.5 \mu\text{g L}^{-1}$ of Pb(II) and $177.1 \mu\text{g L}^{-1}$ of Cu(II); (C) $123.6 \mu\text{g L}^{-1}$ of Cd(II), $108.4 \mu\text{g L}^{-1}$ of Pb(II) and $169.4 \mu\text{g L}^{-1}$ of Cu(II); (D) $146.5 \mu\text{g L}^{-1}$ of Cd(II), $116.0 \mu\text{g L}^{-1}$ of Pb(II) and $24.4 \mu\text{g L}^{-1}$ of Cu(II); (E) $169.4 \mu\text{g L}^{-1}$ of Cd(II), $123.6 \mu\text{g L}^{-1}$ of Pb(II) and $93.1 \mu\text{g L}^{-1}$ of Cu(II). Inset in (b): comparison between the response of both sensors for sample composition (A).

Figure 6. Modelling ability of the optimized ANN for the two-sensor array. Comparison graphs of obtained vs. expected concentrations for (a) Cd(II), (b) Pb(II) and (c) Cu(II) metals, both for training (●, solid line) and testing subsets (○, dotted line). Dashed line corresponds to theoretical diagonal line ($Y=X$).

Figure 1

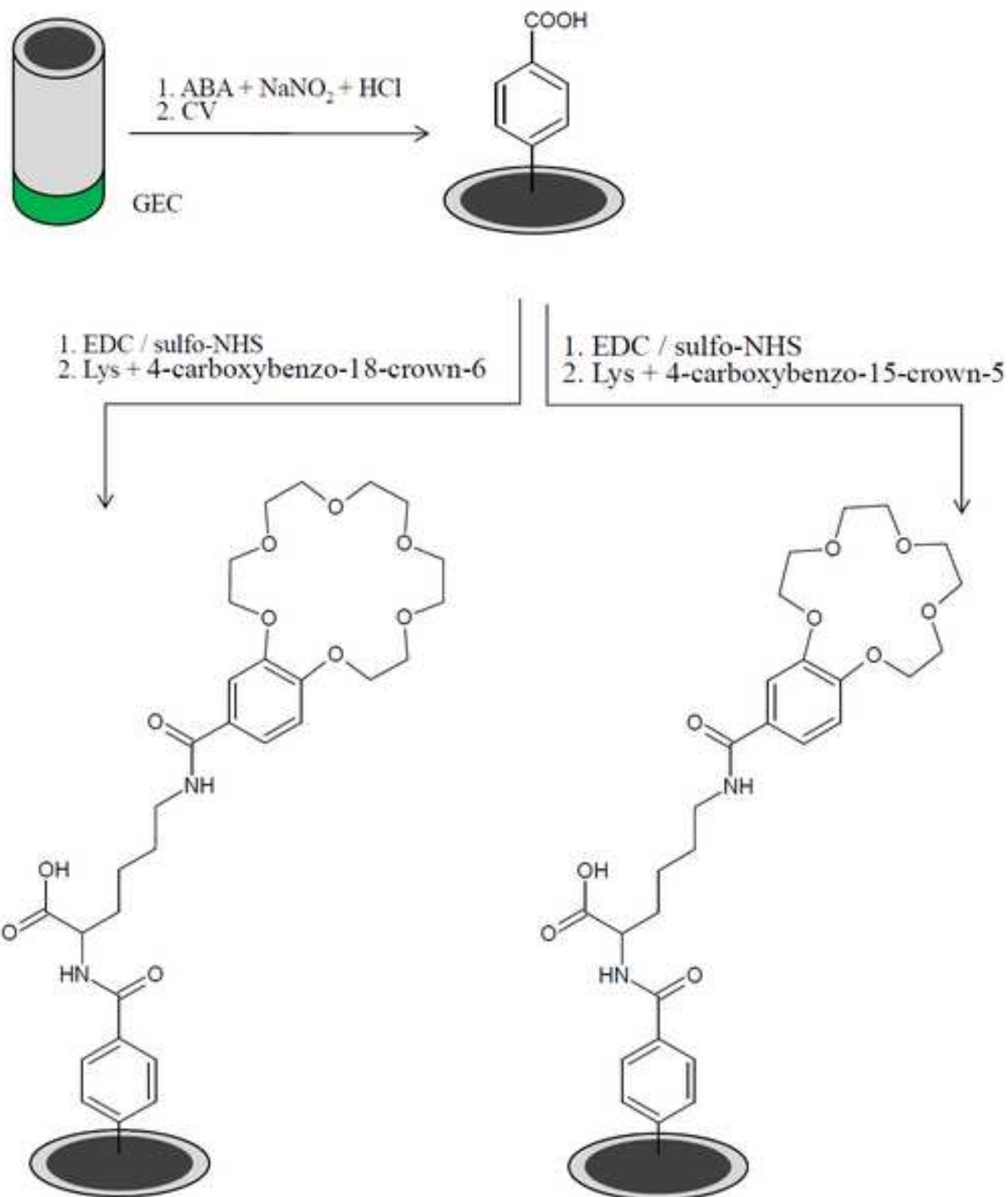


Figure 2

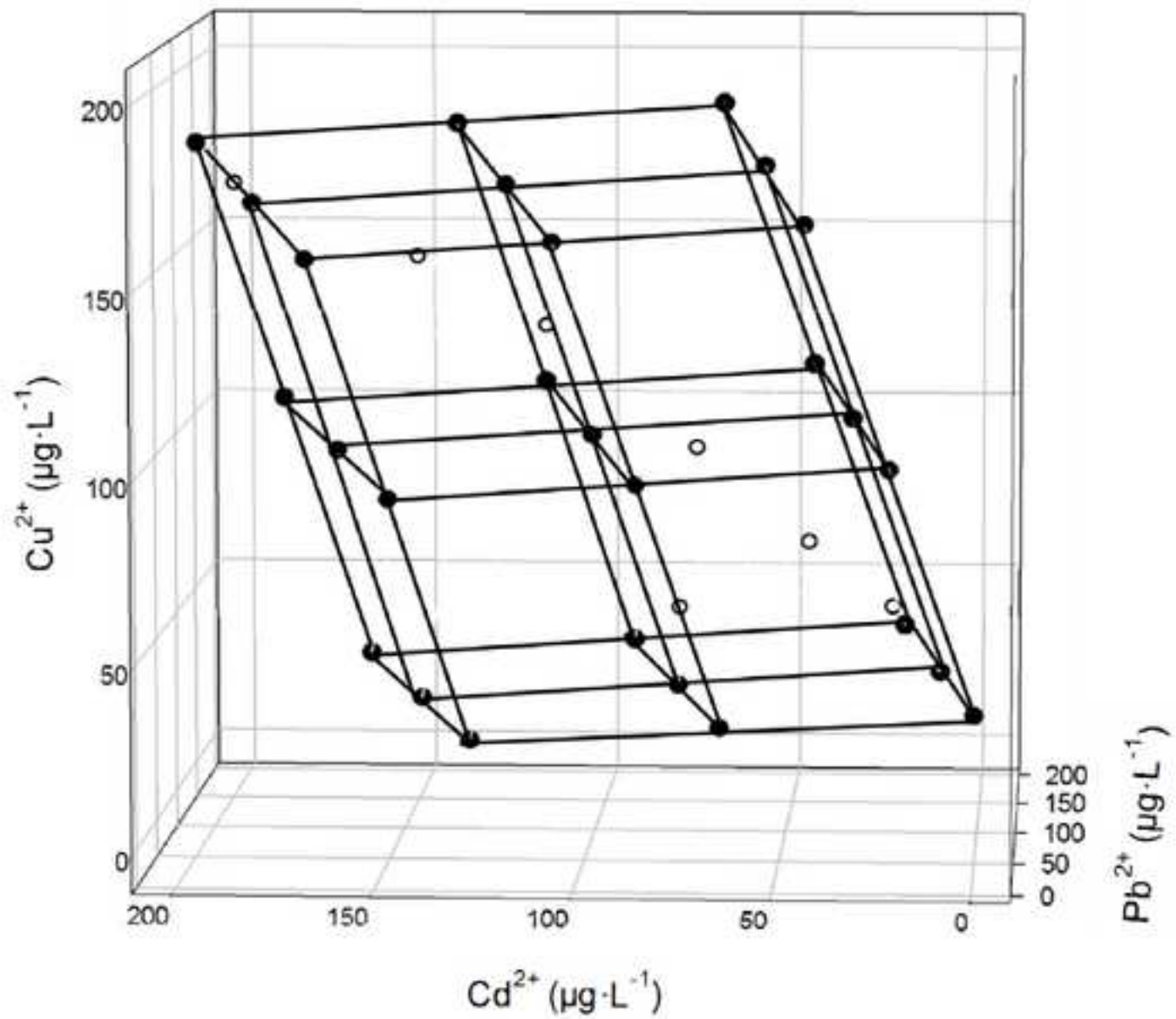


Figure 3

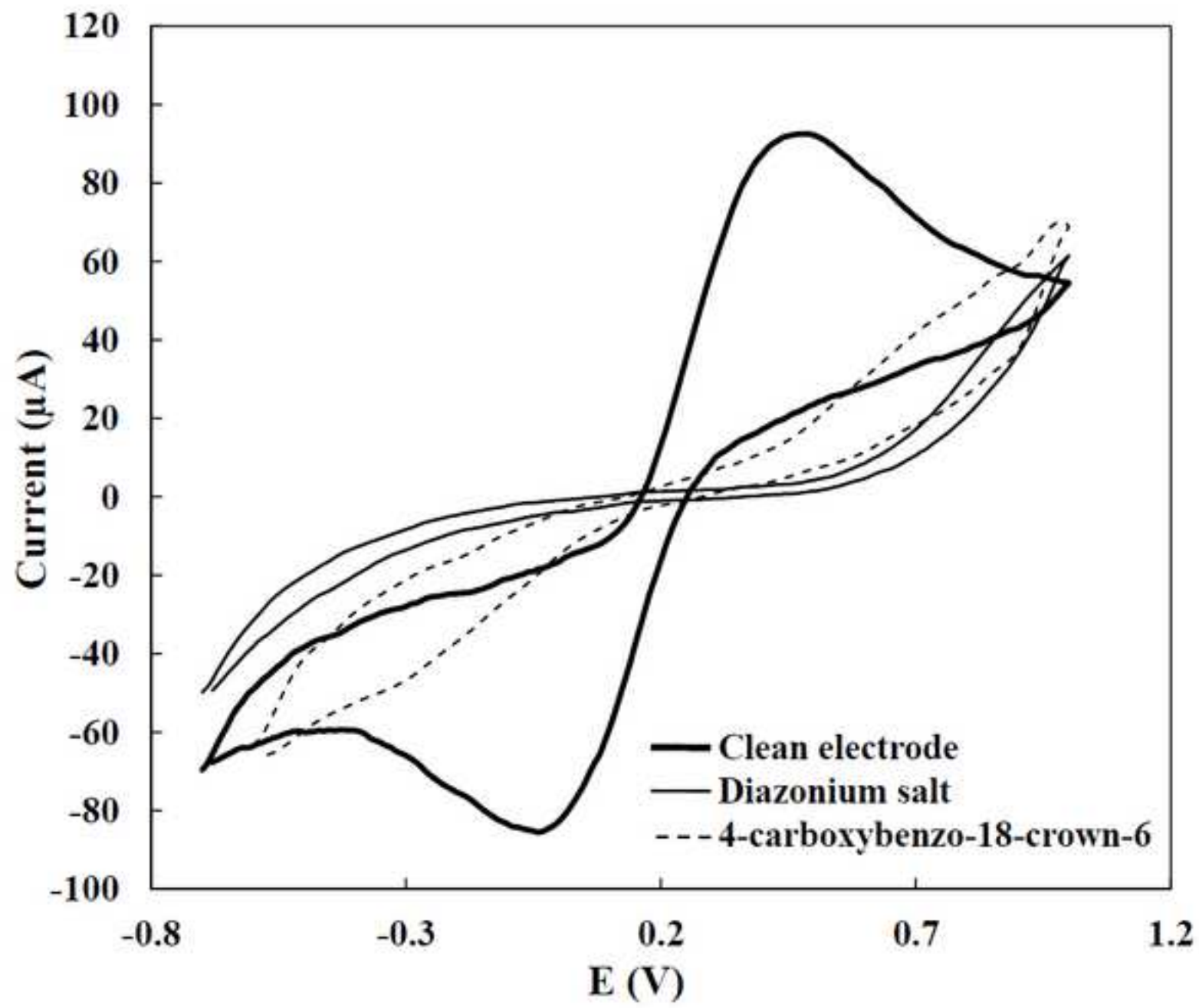


Figure 4

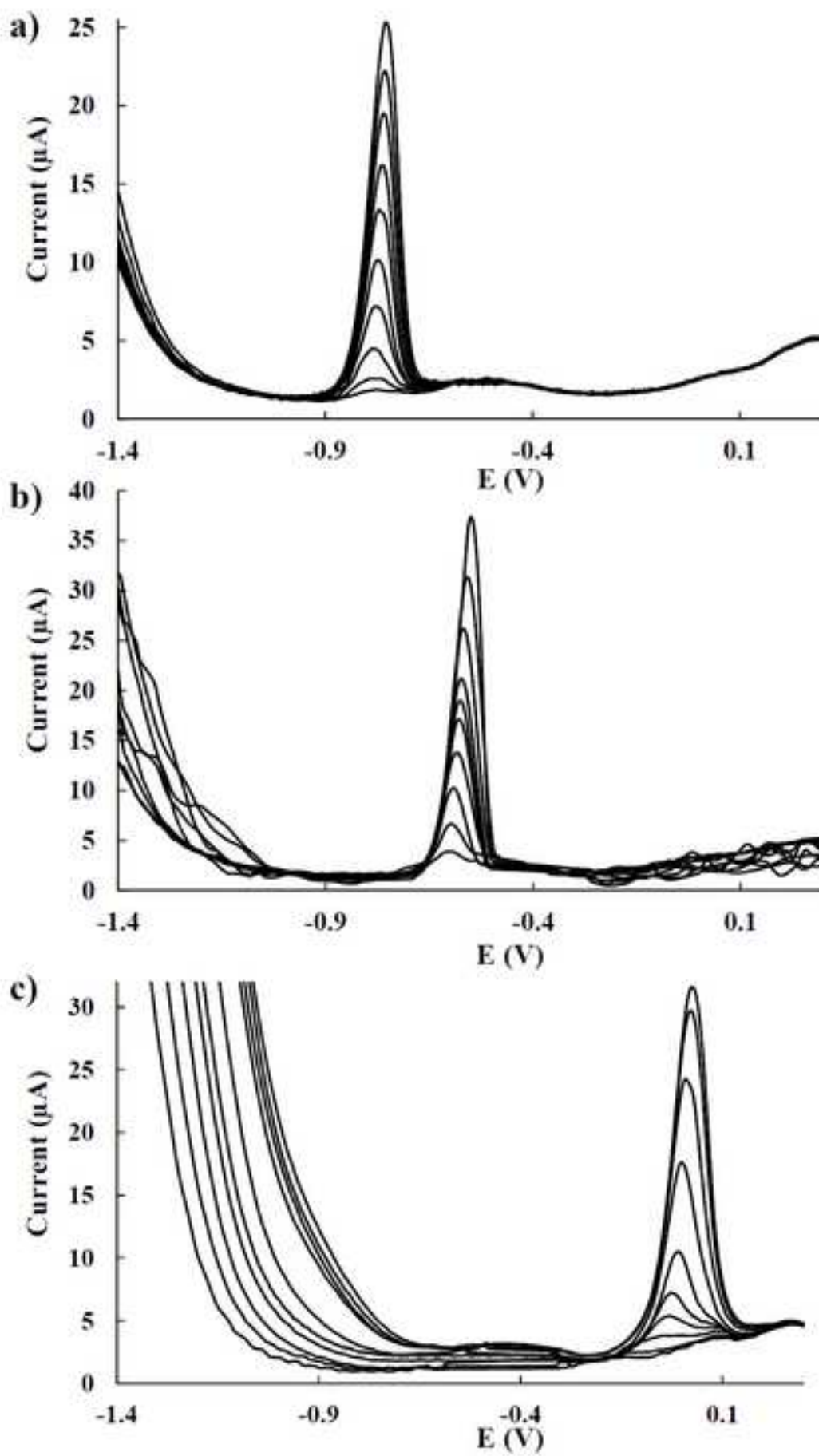


Figure 5

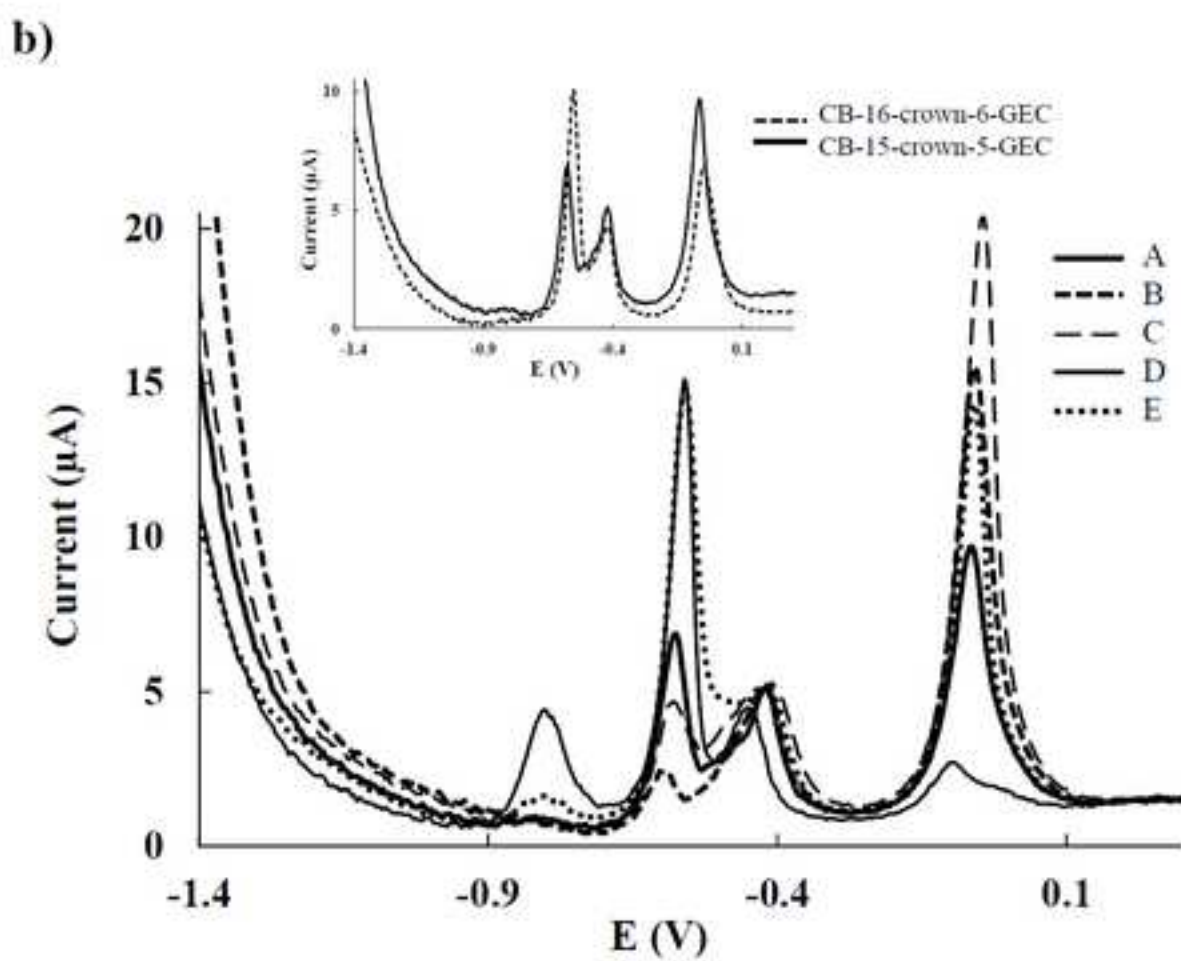
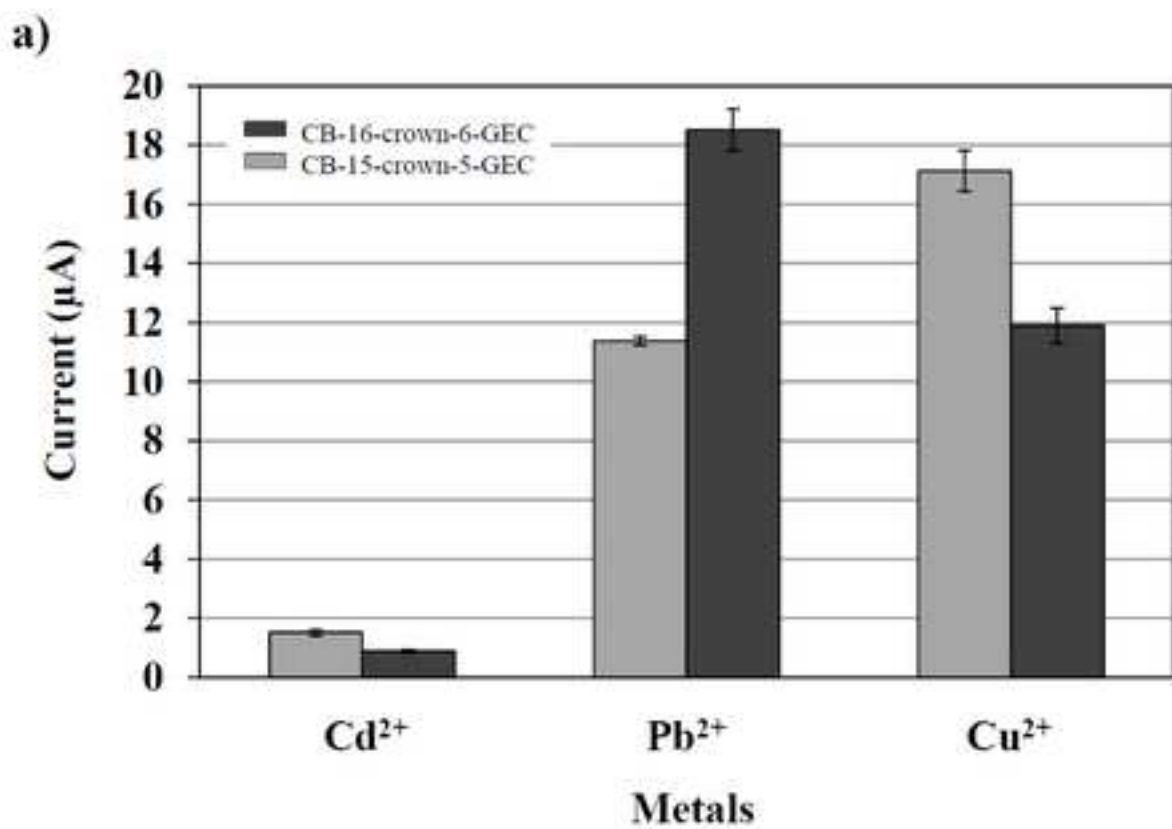


Figure 6

