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Development of an electronic tongue based on screen-printed electrodes for the analysis of aminothiols
Desarrollo de una lengua electronica basada en electrodos serigrafiados para el analisis de aminotioles

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If you want to have good ideas you must have many ideas. Most of them will be wrong, and what you have to learn is which ones to throw away.

Linus Carl Pauling

Quiero dedicar esta memoria a mis padres Ricardo Augusto y Maria Ivoneide por toda paciencia, dedicación y esfuerzos para proporcionarme la mejor educación posible

Quiero agradecer primeramente a Dios por me haber concedido fuerza, salud y determinación para superar todos los imprevistos y obstáculos.

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REPORT

CONTENTS

1. SUMMARY

In this work it was studied the voltammetric behavior of different types of commercial screen-printed electrodes (SPE) in the oxidation of peptides of biological interest that containing thiol groups, such as: cysteine, glutathione, and homocysteine. Gold and carbon screen-printed electrodes were used as working electrode and some modifications with gold nanoparticles and carbon nanotubes were tried in the carbon electrode. The electrodes with higher signal intensities and more different voltammograms have been used to form an electronic tongue to do simultaneous voltammetric measurements by linear sweep voltammetry (LSV).

Chemometric methods such as principal component analysis (PCA) and partial least squares (PLS) were applied along with methods of variable selection as interval PLS (iPLS) and genetic algorithm (GA) to extract useful information from the obtained voltammetric data to differentiate and to determine the concentration of different proportions of peptides in the samples.

Using an electronic tongue provided with SPE of gold with high temperature of curing, gold with low temperature of curing and carbon modified with carbon nanotubes produced satisfactory results. In the case of cysteine a RMSECV of 0.3951×10^{-5} mol.L^{-1} and R^2_{cv}=0.9761; were obtained; for glutathione a RMSECV of 0.3264×10^{-5} mol.L^{-1} and R^2_{cv}=0.9816 were attained; and for homocysteine a RMSECV of 0.8429×10^{-5} mol.L^{-1} and R^2_{cv}=0.8937 were achieved.

Keywords: Electronic tongue, Screen-printed Electrodes (SPE), Aminothiols, Cysteine, Glutathione, Homocysteine, PCA, PLS, Voltammetry, GA, iPLS, Multivariate Calibration.

2. RESUMEN

En ese trabajo se ha estudiado el comportamiento voltamperométrico de diferentes tipos de electrodos serigrafiados (SPE) en la oxidación de péptidos que contienen grupos tioles, tales como cisteína, glutatión y homocisteína. Se han utilizado electrodos serigrafiados como electrodos de trabajo, y se han realizado algunas pruebas modificando con nanopartículas de oro y nanotubos de carbono el electrodo de carbono. Se han seleccionado los electrodos con señales de intensidad más elevada y voltamperogramas más diferentes para constituir una lengua electrónica y hacer medidas de voltamperometria de barrido linear (LSV).

Se han utilizado métodos quimiométricos como análisis de las componentes principales (PCA) y mínimos cuadrados parciales (PLS) junto con técnicas de selección de variables como mínimos cuadrados parciales en intervalos (iPLS) y algoritmo genético (GA) para extraer informaciones relevantes de los datos voltamperometricos obtenidos y así diferenciarlos y poder determinar la concentración de los péptidos en diferentes proporciones en las muestras.

Utilizando una lengua electrónica equipada con SPE de oro con alta temperatura de curación, oro con baja temperatura de curación y un electrodo de carbono modificado con nanotubos de carbono, se obtuvieron resultados muy satisfactorios; concretamente, para la cisteína un RMSECV de 0.3951×10^{-5} mol.L^{-1} y R^2_{cv}=0.9761; para glutatión un RMSECV de 0.3264×10^{-5} mol.L^{-1} y R^2_{cv}=0.9816; y para la homocisteína, un RMSECV de 0.8429×10^{-5} mol.L^{-1} y R^2_{cv}=0.8937.

Palabras clave: Lengua Electrónica, Electrodos Serigrafiados (SPE), Aminotioles, Cisteína, Glutatión, Homocisteína, PCA, PLS, Voltamperometría, GA, iPLS, Calibración Multivariante

3. INTRODUCTION

3.1. THIOL GROUPS

Aminothiols are amino acids with low molecular weight containing thiols. They are compounds chemically and biologically actives, present in human tissues and fluids, performing functions diverse as: homeostasis, cell signaling, detoxification of the free radicals and peroxides, protein synthesis, and as extracellular reducing agents; but, their main characteristic is that their concentration levels in the organism are related with the pathological system, serving as a tool to: prevent, diagnostic and monitor the presence of diseases, such as: cardiovascular diseases, arteriosclerosis, leukemia, diabetes, acquired immunodeficiency syndrome (AIDS). Being, cysteine (Cys), glutathione (GSH), and homocysteine (Hcys) are the principals compounds to observe this pathological factors[1,2,3,4,5,6,7].

Cysteine (Cys, figure 1a) is an amino acid produced in the liver, responsible for many functions in the organism, such as protein synthesis acting as critical substrate, detoxification, active site in catalytic function of enzymes and other peptides, and precursor in the GSH and taurine synthesis. The level of cysteine in the human fluids is an indicator of some disorders as cystinosis, cancer[1,2,3,4,5].

Glutathione (GSH, figure 1b) is a tripeptide that occurs in animals, vegetables and bacteria cells, being the major non-protein thiol. It can be obtained by the diet or synthetized in the liver; acts in the protein and DNA synthesis, transports, catabolism, metabolism, helping in the regeneration of the other antioxidants, protecting against free radicals, and maintaining the thiol-protein status. Alterations in the level of GSH in the body can indicate some pathogenesis and diseases, such as Alzheimer's disease, AIDS, alcoholic liver disease, asthma, pulmonary diseases, arteriosclerosis, occlusive vascular, leukemia, and diabete[2,3,4,5].

Homocysteine (Hcys, figure 1c) consists in an endogenous thiol generated by the demethylation of methionine, an essential amino acid derived from dietary proteins. It acts as a precursor for cystathionine, cysteine and further metabolites, as a mean of the conservation of the methionine, and as substrate in the recycling of tissue folates. Altered level of Hcys in the

organism indicates of pathological conditions, such as Alzheimer's and Parkinson's disease, autoimmune deficiency syndrome, arteriosclerosis, cardiovascular diseases[2,3,4,5,8].

Several methods have been reported in the literature for the analysis of thiols, among them, liquid chromatography with ultraviolet detection[4], liquid chromatography with mass spectrometry[5,7], liquid chromatography with fluorescence[6] detection and capillary eletrophoresis[9]. However, such chromatography methods usually require derivatization to introduce chromophore or fluorofore groups[10].

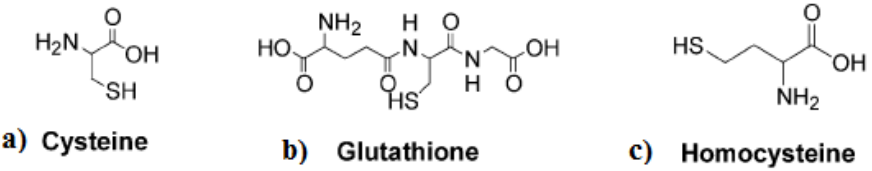

Figure 1.

3.2. VOLTAMMETRY

Voltammetric methods configure a group of the electroanalytical methods which obtain information about the analyte from the current intensity obtained under an applied potential. Voltammetry is based on the measurement of the current as a function of the applied potential. This technique uses basically three electrodes being a working electrode, reference electrode, and an auxiliary electrode. The measurements of the current intensities are obtained between auxiliary and working electrodes; and the applied potential is measured between the reference and working electrodes. Sometimes the use of supporting electrolyte is necessary, it consists in a non-reactive alkaline metal salt, which reduce the migration effects and decreases the resistance of the solution[11,12].

Several methods of excitation signals can be applied to the electrode to obtain the voltammograms, as linear sweep voltammetry (LSV), differential pulse, triangle, and square wave. Linear Sweep Voltammetry (LSV) is a type of voltammetry where the potential between the working electrode and the reference electrode varies linearly with the time to generate a

voltammogram, that consists of a graphic of the current intensity as a function of the applied potential[11,12].

The electroanalytical methods in contrast with the chromatographic methods generally used to analyze the aminothiols, can take advantage of the electroactive character of thiol groups (susceptible to be oxidized to disulfide forms) to develop simple, fast, low cost, and easily automated screening assays without need of any derivatization; however, due the poor voltammetric behavior the direct oxidation of thiols is generally dificulted[13]. To overcome this, has been seen in the literature the use of electronic tongue to improve the selectivity, sensitivity, signal-to-noise ratio[14]. Electronic tongue consists of an array of electrodes to interpret the complex electrochemical signals.

In this work disposable screen-printed electrodes (SPE) were used. They are commercially available in a large variety of materials and designs, with possibilities of modification, and their low cost as main advantage[15,16].

3.3. CHEMOMETRICS

Chemometrics is a chemical discipline that uses mathematics, statistics, and formal logic: to design or select optimal measurement procedures and experiments to provide maximum chemical information by analyzing chemical data; and obtain knowledge about chemical systems[17,18,19].

3.3.1 Data pretreatment

Voltammetric curves usually present potential shifts, scatter of the signals, differences in the baseline and noise; and these factors can affect the performance of multivariate calibration, so the use of the data pretreatment tools is necessary in order to avoid these problems. Several pretreatments methods are described in the literature, being multivariate scatter correction (MSC), Savitzky Golay derivative (DER) and smoothing (SMOTH), Baseline (BAS), Variance (std) scalling (VARSTD), and mean center (MC) the more used techniques. MSC is applied to correct the scatter of signals and shifting of the peaks. DER and BAS are used to eliminate

baseline differences. To prevent noise SMOTH and VARSTD are used. MC is applied to ensure that all results will be interpretable in terms of variation around the mean[20].

3.3.2 Principal Components Analysis

Principal component analysis (PCA) is an exploratory method that reduces the amount of data obtained from instrumental techniques but keeping most of the information. This is made by using linear combination variables of original variables containing information about each sample. Such combinations are called principals components (PC's), and are created in order to maximize the variation in the original observations. PCA is calculated with the factorization of the matrix $\boldsymbol{X}$ in a product between two orthogonal matrices, Scores $\boldsymbol{Q}$ and loadings $\boldsymbol{P}$. The factorization is shownd in eq.(1), where $\boldsymbol{P}^t$ represents the transpose of matrix $\boldsymbol{P}$.

$$\boldsymbol{X} = \boldsymbol{Q}.\boldsymbol{P}^t \quad (1).$$

Loadings represent a vector where it is possible to see the contribution of the original variables in the PC selected and scores represent the values of the experimental data using PC as a new set of variables. Scores show the relationship between observations and groups of observations can be used to classify types of samples[20,21].

3.3.3 Multivariate Calibration

Multivariate calibration consists of techniques used to establish a relationship between an *n X 1* vector $\boldsymbol{y}$ containing known reference values of a parameter of interest (such as: concentration, viscosity, pH, acidity, among others) and the *n X N* voltammetric matrix $\boldsymbol{X}$, where, n represents the number of curves, and N is the amount of variables, in this study represents the number of potential values in which the measurement was performed[19]. Sometimes an augmented matrix can be used, which consists of joining two or more $\boldsymbol{X}$ matrices obtained in different ways, thus increasing the number of variables.

3.3.3.1. Partial Least Squares

Partial Least Squares (PLS) represents one of the methods of multivariate calibration most commonly used in electroanalytical chemistry[22]. It relates two matrices ***X*** (voltammograms*)* and ***Y*** (concentrations), by linear combinations of the originals variables, called latent variables; to build a model of calibration. This model can predict concentration from new sets of measured voltammograms. Leave one out cross validation was used to determine the amount of latent variables to be selected. To evaluate the models, root mean square error of calibration (RMSEC), root mean square of cross validation (RMSECV), and the correlation coefficient to calibration (R^2_c) and to cross validation (R^2_{cv}) were used, according with the following equations.

$$\mathrm{RMSEC/RMSECV} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}, \quad (2)$$

being n the number of the samples, $\hat{y}$ the value predicted for the model of calibration or cross validation, and y the reference value.

$$R^2 = \frac{\sum(x_t - \hat{x})(y_t - \hat{y})}{\sqrt{(\sum(x_t - \hat{x})^2 \sum(y_t - \hat{y})^2)}}, \quad (3)$$

where, x_t is the independent variable, $\hat{x}$ is the average value of X, y_t is the measured parameter, and $\hat{y}$ is the average value of measured parameter.

3.3.3.2. Variable Selection

Variable selection methods are used in voltammetric data to select variables (i.e., potential) with the maximum of useful information according to the analyte of interest, discarding variables which have unusual and irrelevant information that interfere in the search of a good model. In the literature several types of variable selection exist, being interval partial least squares (iPLS) and genetic algorithm (GA) two of the most commonly used. In the IPLS method, the data are divided in intervals of the same length, and a PLS model is constructed for each. The GA is a popular heuristic optimization that uses a probabilistic, non-local search process inspired by Darwin's theory of natural selection[23,24,25].

4. OBJECTIVES

The objective of this study is to develop an electronic tongue based on linear sweep voltammetry using different types of screen-printed electrodes with the help of chemometric techniques such as preprocess, multivariate calibration, and variables selection. The electronic tongue is intended to be used as a fast and relatively cheap method to simultaneously determine different types of aminothiols, that represent a fundamental role in the biological systems, as cysteine, glutathione, and homocysteine.

5. EXPERIMENTAL

5.1. Chemical and Reagents

All reagents were analytical grade. L-Cysteine (Cys 97%), DL-Homocysteine (Hcys ≥ 95%), and trifluoroacetic acid (TFA 99%) were provided by Sigma-Aldrich (Sta. Louis, USA). Reduced Glutathione (GSH >98%) and N,N-dimethylformalmide (DMF) were obtained by Merck (Darmstadt, Germany). Multi-walled carbon nanotubes solution was purchased (MWCNT) by DropSens (Asturias, Spain). Potassium chloride and gold nanoparticles solution ($HAuCl4.4H_2O$) were provided by Panreac (Barcelona, Spain). All samples were prepared using ultrapure filtered water obtained by Milli-Q plus 185 purication system (Merck Millipore corporation, Germany).

5.2. Instrumentation and Software

The thiols oxidation signals were measured by Multi Potentiostat / Galvanostat model μStat 8000 DropSens (Asturias, Spain) attached to several screen-printed devices by means of a multichannel cable DRP-CABSTATMULTI (DropSens, Spain), one DRP-CAC cable for each screen-printed unit, and an USB cable to connect to computer. The multichannel cable was also connected to an external Ag/AgCl/3.0 mol L^{-1} KCl reference electrode Metrohm (Herisau, Switzerland), to which all potentials are referred, and to an external platinum auxiliary electrode, also by Metrohm. In this way, the potentiometer could simultaneously measure the signals of the working electrodes of all screen-printed units referred to a common reference electrode and a common auxiliary electrode. A glass cell and a cell support, both by Metrohm, were used to contain the cell solution and up to five electrodes (usually, the reference electrode, the auxiliary electrode and three screen-printed electrodes). Three types of the screen-printed electrodes (SPE) were used including a working electrode of 4 mm diameter, an auxiliary electrode (made of the same material as the working) and a silver pseudo-reference electrode printed on an alumina substrate. An insulating layer serves to delimit the working area and the electric contacts. The SPE's used were gold with high temperature curing (ref. 220-AT), gold with low temperature curing (ref. 220-BT), and carbon (ref. 110), all of them provided by DropSens (Asturias, Spain). In multichannel mode, the pseudo-reference and auxiliary electrodes of the screen-printed devices are not used during the measurements, being substituted by the common external reference and auxiliary electrodes. The scheme of the instrumentation is shown at Figure.2.

All linear sweep voltammetric (LSV) data were recorded using Drop View software (DropSens, Spain).The voltammetry curves were acquired at room temperature (20ºC), current intensities were obtained to each potential into the range of 0.1V–0.9V, being tested different potential steps (0.002V, 0.001V and 0.01V), and a scan rate of 0.05 V/s. A magnetic stirrer by IKA (Staufen, Germany) and purified nitrogen were also available for stirring and deaeration of the solutions, respectively.

To modify the carbon SPE with nanotubes a mixer Eppendorf MixMate PCB-08 (Hamburg, Germany), and an ultrasonic bath Bransonic model 2510EMTH provided by Branson Ultrasonics

(Danbury, USA) were used. A boxer connector for SPE DSC-BIDSC purchased by DropSens (Asturias, Spain) was used in the process to modify the carbon SPE with gold nanoparticles.

The steps of the loading, pre-processing, variable selection method, principal component analysis (PCA) and construction of the partial least squares (PLS) model regression were realized using Matlab® R2014a[26] (MathWorks, Natick, USA) with PLS-toolbox[27] version 7.8.2 (Eigenvector Research Inc., Wenatchee, USA). Different preprocessing techniques were evaluated, including mean center, baseline correction, multiplicative scattering correction (MSC), variance scaling, smoothing Savitzky-Golay , and 1st and 2nd derivative Savitzky-Golay.

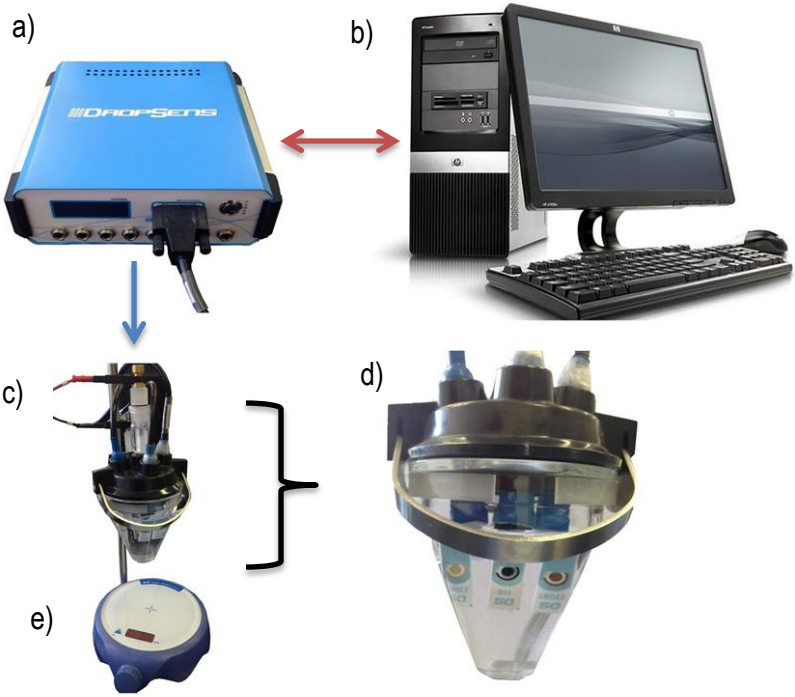

Figure 2. Instrumentation for the experiment. a) Multi Potentiosta / Galvanostat DropSens. b) PC equipped with Drop View software. c) Electrolytic cell. d) Electrolytic cell in perspective, showing the screen-printed electrodes. e) Magnetic stirrer.

5.3. Modification of the carbon electrode with carbon nanotubes

The modification of the carbon SPE was performed using a multiwalled carbon nanotubes (MWCNT) suspension. The suspension was prepared based on the described method at literature[28], where, 1.0 mg of MWCNT was mixed with 1.0 mL of DMF during 12h at 2000rpm using Eppendorf Mixmate, and after it was placed in an ultrasonic bath during 1h; at the end was made a dilution with DMF:H_2O (1:1) until a concentration of 0.5 mg/mL. Afterwards 4 µL of

the prepared solution was pipetted up the working electrode surface of the carbon SPE and placed to dry at room temperature.

5.4. Modification of the carbon electrode with gold nanoparticles

The method used to modify SPE with gold nanoparticles follows the used steps previously reported in the literature[29,30]. Nanoparticles solution to modify the SPE's was prepared with a concentration of 0.11 mmol L^{-1} $AuCl_4^-$ in acid solution 0.5 mol.L^{-1} H_2SO_4. Before the modification, the SPE was previously washed with ultrapure water and dried under nitrogen stream. 4 µL of nanoparticles solution was pipetted up to the working electrode surface of the carbon SPE, where the electrodeposition process was carried out with a sweep from 1.1V to 0.0V using the boxer connector for SPE connected with potentiostat / galvanostat during sixty seconds. Modified electrodes were again washed with ultrapure water and dried under a nitrogen flow to remove non electrodeposited nanoparticles.

6. RESULTS AND DISCUSSION

6.1. Determination of the instrument configuration

To determine the ideal instrument configuration parameters such as potential step and scan rate, two experiments were made using separately cysteine and glutathione solutions. The solutions were prepared with the concentration of the 1.0 x 10^{-5} mol.L^{-1} for each analyte in acid solution 0.05% TFA, a medium that provided good results in the amperometric detection of aminothiols separated by liquid cromatography[31]. The SPE's used were gold with high (AuAT) and low (AuBT) temperature curing, and carbon (CAR).

The measures have been carried out with three values of potential step (0.001V, 0.002V and 0.01V) using 0.05V/s for the scan rate, within a potential range of 0.3V-0.9V. For each solution and potential step, five curves were recorded. Figure 3 show the voltammograms obtained for cysteine and glutathione.

From the obtained curves it is possible to observe that all three potential steps represent a good signal, being hard to determine the best only visually. For this, a principal components analysis (PCA) was carried out. To construct the PCA models the data were previously preprocessed with MC. Table 1 shows the obtained results from the models, with the number of the principals components (PC's) and explained variance.

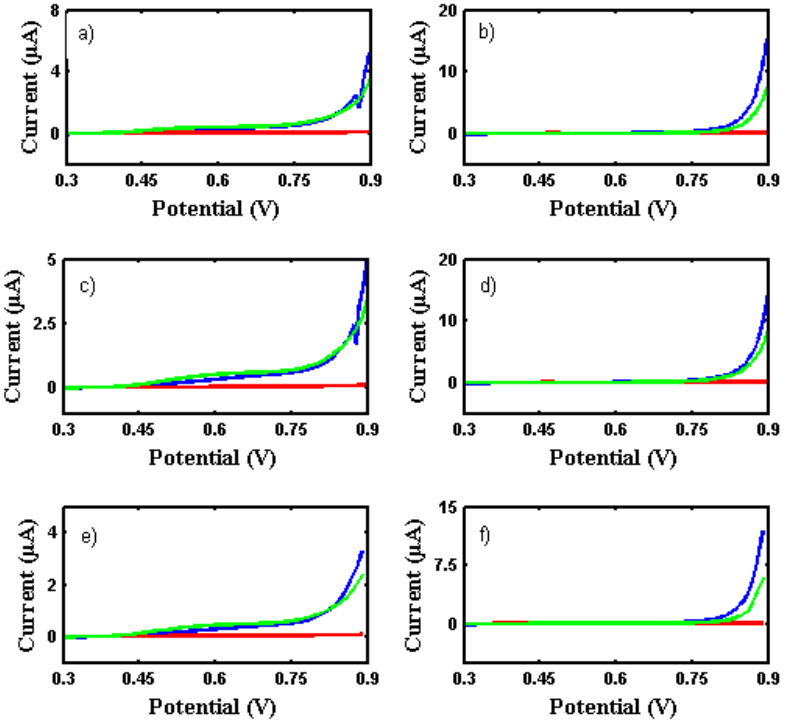

Figure 3. Voltammograms obtained.(AuAT — / CAR — / AuBT —).a)Cysteine with potential step of 0.001V. b) Glutathione with potential step of 0.001V. c) Cysteine with potential step of 0.002V. d) Glutathione with potential step of 0.002V. e) Cysteine with potential step of 0.01V. f) Glutathione with potential step of 0.01V

Model	PC's	Explained Variance (%)
Cysteine (step=0.001V)	2	99.74
Glutathione (step=0.001V)	2	99.98
Cysteine (step=0.002V)	2	99.54
Glutathione (step=0.002V)	2	99.76
Cysteine (step=0.01V)	2	99.78
Glutathione (step=0.01V)	2	99.99

Table 1. PCA models

Figure 4 contains plots of the PC1 scores *vs.* PC2 scores from PCA models. These plots show close points for similar samples and longer distances for samples with different properties. With PCA models it was possible a good separation of the replicates according to the electrode type for both analytes, but, mainly using 0.002V as potential step, which provides a better separation with respect to the type of electrode and a higher signal variation between replicates of the solution.

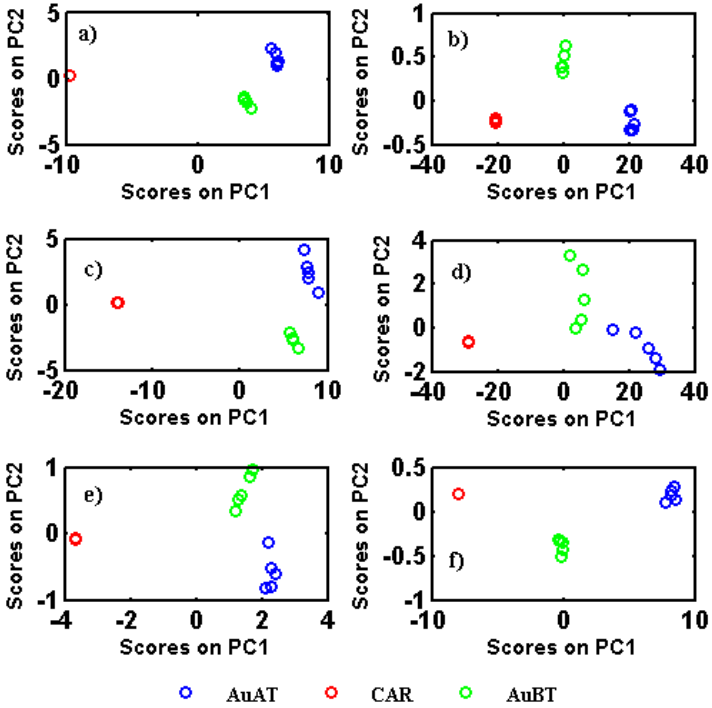

Figure 4. Scores PC1 *vs.* Scores PC2 from models:a) Cysteine (step=0.001V) b) Glutathione (step=0.001V) c) Cysteine (step=0.002V) d) Glutathione (step=0.002V) e) Cysteine (step=0.01V) f) Glutathione (step=0.01V)

The results demonstrate that the used SPE's have signals quite different with each other, with good intensity especially at 0.9V, except for the carbon electrode. In this case it was observed a very low signal intensity compared to the other two electrodes, and from the PCA models it is noticeable that the model practically does not detect variations, with the same signal and voltammograms to all replicates. It can be due the kinetic of oxidation reaction that occurs in the surface of this electrode, being very slow, and not providing good signal intensity. The change of the carbon electrode for a new electrode or its modification appeared to be necessary. Several methods are described for the modification of the carbon screen-printed electrodes in the literature, mainly using gold nanoparticles[30,31,32] or carbon nanotubes[28,29].

6.2. Modification of the carbon screen-printed electrode with gold nanoparticles

A modification of the carbon electrode with gold nanoparticles has been carried out. The modification procedure was made according with the method described previously in the experimental section. A test using a solution of cysteine with the concentration of 1.0×10^{-5} $mol.L^{-1}$ in acidic solution 0.05% TFA was done. Voltammograms were obtained in the range between 0.3V to 0.9V with 0.002V as potential step, and 0.05V/s as scan rate in five replicates using AuAT, AuBT and the carbon electrode modified with gold nanoparticles (CARnp). Figure 5 shows the voltammograms obtained; from this figure it is possible to see that this modification is not a significant improvement for the signals intensities. A PCA model was constructed using MC as preprocess, and two PC's with 99.44% of explained variance to analyze the data. Scores PC1 *vs* scores PC2 are represented in figure 6, where it can be seen that CARnp electrode provides slightly different signals if compared with the other electrodes, but this practically does not improve the behavior of bare carbon electrode.

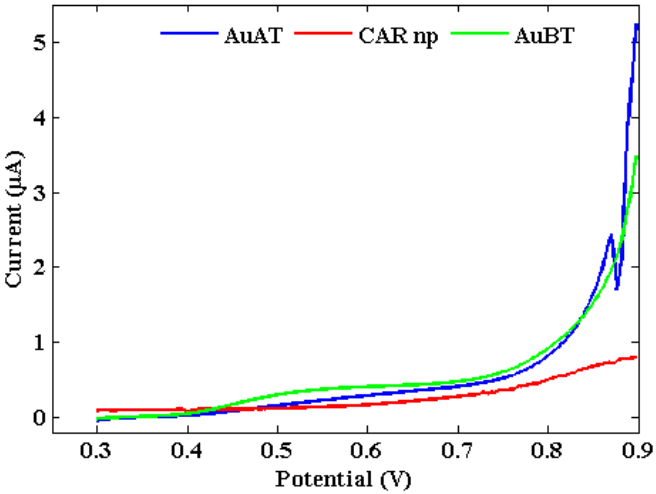

Figure 5 Voltammograms obtained for cysteine.

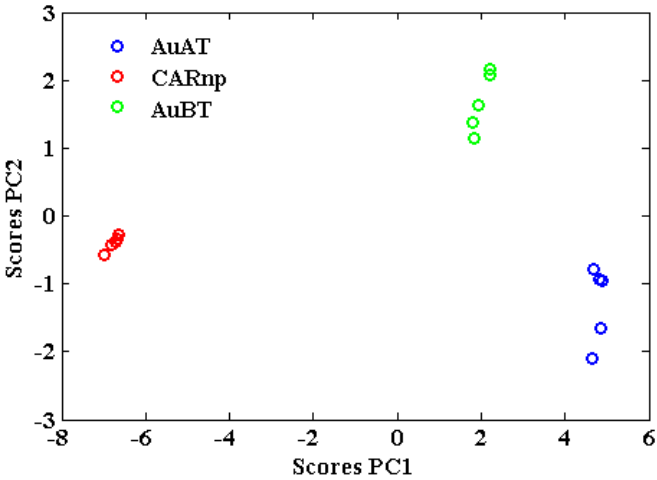

Figure 6. Scores PC1 *vs* Scores PC2 to cysteine.

6.3. Modification of the carbon screen-printed electrode with carbon nanotubes

To modify the carbon screen-printed electrode with carbon nanotubes it was used the method described previously in the experimental section.

In order to test the performance of the modified electrodes, two solutions were used; containing cysteine and glutathione, respectively, prepared similarly as in the previous experiment in acidic solution 0.05% TFA and concentration of 1.0 x 10^{-5} mol.$L^{-1}$for each analyte, but now using KCl 10^{-3} mol.L^{-1} as supporting electrolyte.

The voltammograms were obtained using potential step of 0.002V and 0.05V/s as scan rate in a potential range from 0.1V to 0.9V; being obtained five replicates to each solution. Figures 7 and 8 show the voltammograms obtained for cysteine and glutathione, respectively.

Carbon screen-printed electrode modified with carbon nanotubes (CNT) showed a significant improvement in the signal intensities in comparison with the carbon electrode, the higher of the intensities being obtained at 0.3V with a well-defined curve. Also, the voltammograms re quite different from these obtained with the other electrodes.

PCA models were constructed to evaluate the signals obtained and compare with the other electrodes. MC was used again to preprocess the dates before the analysis; the models have an explained variance of the 99.98%, 99.78%, and 99.52% for cysteine, glutathione and both, respectively.

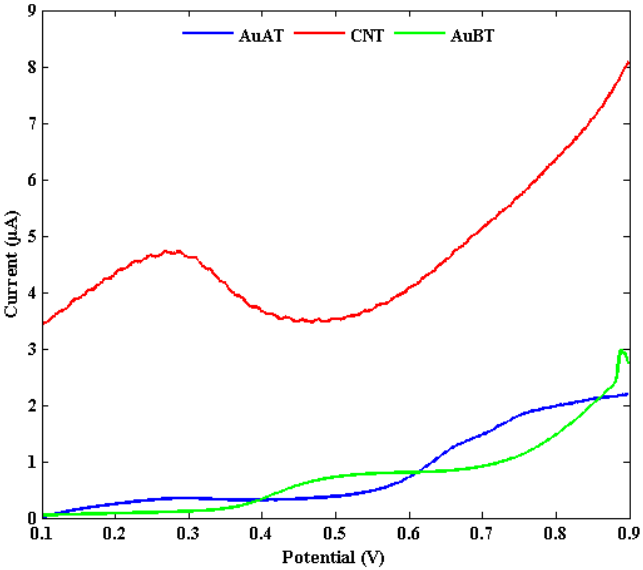

Figure 7. Voltammograms obtained for Cysteine.

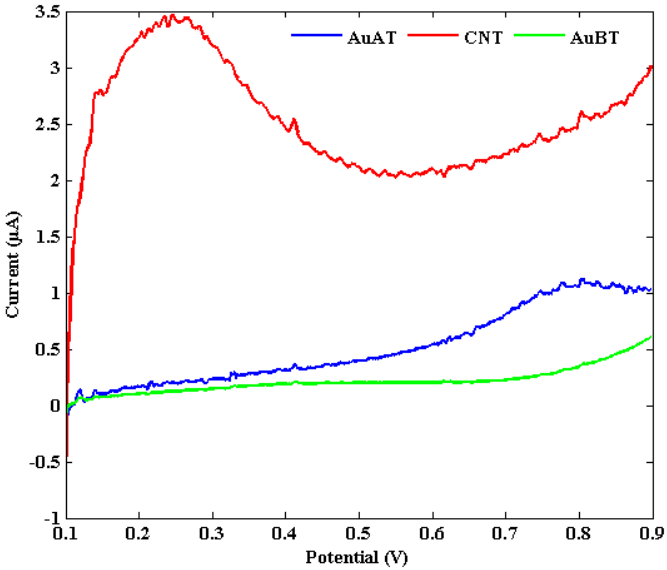

Figure 8. Voltammograms obtained for Glutathione.

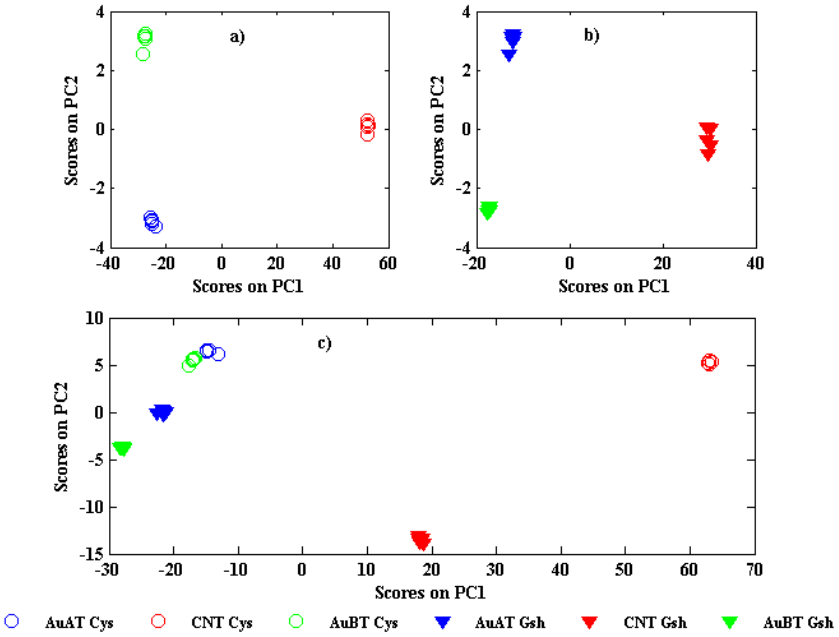

Figure 9. Scores on PC1 *vs.* Scores on PC2 to a) Cysteine b) Glutathione c) Cysteine and Glutathione using combined matrix.

The models showed good results, with a good separation of the replicates according to the type of electrode; as shown in the three plots of the scores in the figure 9, and it is possible to observe a variation between the replicate signals obtained from the same electrode type. This is good, because it indicates that the electrodes are sensitive, capable of detecting small variations in the composition of the solutions.

This section demonstrates that the carbon electrode modified with carbon nanotubes, shows an improvement in the responses and performs as a sensitive electrode to the analysis of the aminothiols. Thus, it can be used together with high and low temperature gold electrodes to obtain a sensor array for the analysis of aminothiols like cysteine or glutathione.

6.4. Simultaneous quantification of cysteine and glutathione

After the determination of the appropriate configuration of the instrument and the choice of suitable electrodes to analyze cysteine and glutathione separately, it has been carried out an experiment to quantify cysteine and glutathione into the same solution.

Sixteen synthetic samples were prepared, all of them in acidic solution 0.05% TFA and KCl 10^{-3} mol.L^{-1} with the concentration of analytes between 0.0 mol.L^{-1} and 6.0x10^{-5} mol.L^{-1} according to the table 2.

Sample	Cysteine Concentration mol.L^{-1}	Glutathione Concentration mol.L^{-1}
01	0.0	0.0
02	2.0 x10^{-5}	0.0
03	4.0 x10^{-5}	0.0
04	6.0 x10^{-5}	0.0
05	0.0	2.0 x10^{-5}
06	2.0 x10^{-5}	2.0 x10^{-5}
07	4.0 x10^{-5}	2.0 x10^{-5}
08	6.0 x10^{-5}	2.0 x10^{-5}
09	0.0	4.0 x10^{-5}
10	2.0 x10^{-5}	4.0 x10^{-5}
11	4.0 x10^{-5}	4.0 x10^{-5}
12	6.0 x10^{-5}	4.0 x10^{-5}
13	0.0	6.0 x10^{-5}
14	2.0 x10^{-5}	6.0 x10^{-5}
15	4.0 x10^{-5}	6.0 x10^{-5}
16	6.0 x10^{-5}	6.0 x10^{-5}

Table 2. Concentration of the cysteine and glutathione on the samples.

Voltammetric curves were acquired in five replicates of each sample with 0.05V/s as scan rate, and 0.002V as potential step from 0.1V until 0.9V. Figure 10 shows the mean of the voltammograms obtained by each of the 16 samples separated by electrode type. As can be seen, most curves have the higher intensity at 0.9V in all three electrodes but in CNT electrode a relative maximum is observed at 0.2V-03V. But all signals contain shifts, scattering and difference in the baselines; and this can be a problem to quantify the analytes. Therefore, the data need pretreatments to prevent this. Several methods of pretreatment such as MSC, BAS, VARSTD, DER, SMOTH and MC were applied in the data before the construction of the Partial Least Squares (PLS) models.

The PLS models were then constructed using the known value of the concentration of analytes as the reference value, and matrices with the intensity values. These could include data obtained for each separate electrode or be integrated into an augmented matrix with the data obtained by the three electrodes. Root Mean Square Error of the Calibration (RMSEC) and

Root Mean Square Error of the Cross Validation (RMSECV) were the factors used to determine the best models

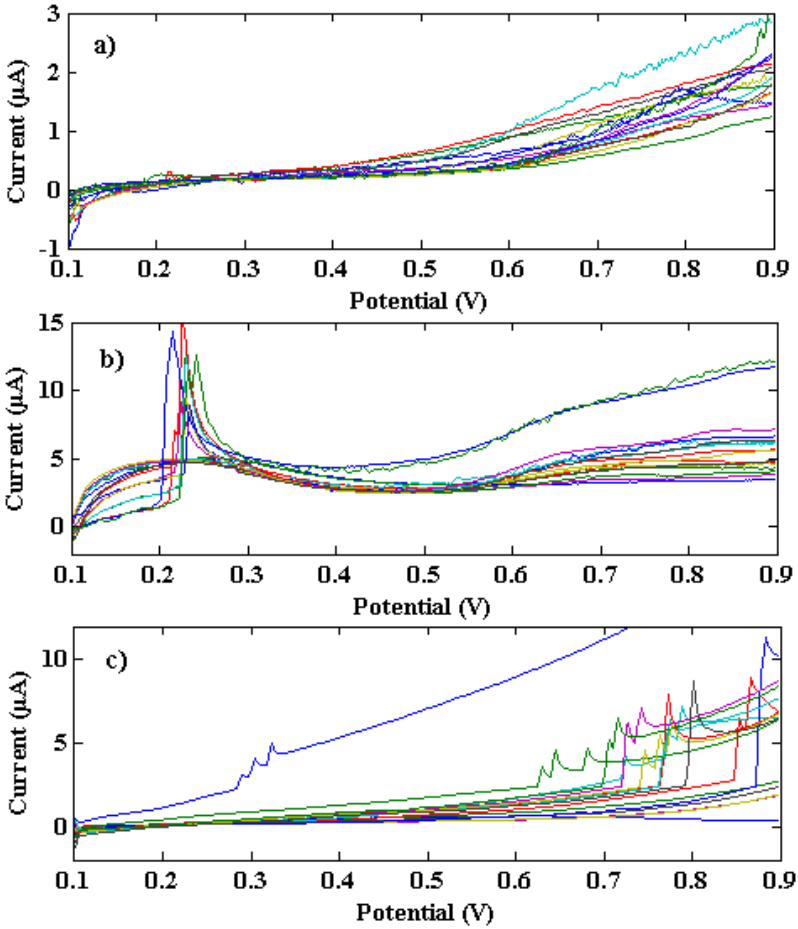

Figure 10. Voltammograms obtained by a) AuAT b) CNT c) AuBT

Due to the complexity of the data and the large number of variables obtained in this experiment, the use of the variable selection techniques was necessary. Two types of the variable selection algorithms (GA and iPLS) were applied in the data to reduce the number of variables and to select the variables with more information related with the analyte concentration. Table 3 presents the best models obtained to determination of the concentration of each analyte.

The best model for the quantification of cysteine was obtained using only AuAT electrode; with MC as preprocess method, and three latent variables. The model presents a RMSECV of 0.7787×10^{-5} mol.L^{-1} with an R^2_{CV} equal to 0.8797; to construct the model was realized firstly a variable selection by iPLS resulting in fifty selected variables, then this data were submitted to a

GA selection of variables being reduced to eleven selected variables. Figure 11a contains the mean of the curves to the samples obtained by AuAT electrode with the selected variables. The variables were selected in two principal regions of the curves, between 0.25V and 0.35V; and other in 0.85V.

To the quantification of glutathione the best model acquired was using the augmented matrix with the three electrodes; MC as preprocess method, and six latent variables were used. Selection of the variables has been carried out by iPLS algorithm; were selected ten intervals with five variables each. The ranges selected by iPLS to AuAT and AuBT are placed between 0.15V and 0.25V; and to CNT electrode between 0.1V and 0.15V, and among 0.7V and 0.85V. This selection can be seen in the figures 11b, 11c and 11d.

Model Pretreatment	Nº Latent Variables	RMSEC (x10^{-5} M))	RMSECV (x10^{-5}M)	R_c^2	R_{cv}^2	Electrode	Analyte
iPLS-MC	3	0.7810	1.0808	0.8502	0.7809	AuAT	Cysteine
iPLS-SMOTH(9pts)-MC	4	0.9887	1.3276	0.8044	0.6630	CNT	Cysteine
iPLS-BAS-VARSTD-MC	5	1.0395	1.4520	0.7838	0.5814	AuBT	Cysteine
PLS-MC	4	0.7768	1.1882	0.8793	0.7524	AuAT/CNT	Cysteine
iPLS+GA-MC	3	0.5760	0.7787	0.9336	0.8797	AuAT	Cysteine
iPLS-SMOTH(9pts)-MC	3	0.6390	0.7816	0.9183	0.8791	AuAT	Glutathione
iPLS-MSC-MC	5	0.4877	0.8250	0.9524	0.8642	CNT	Glutathione
iPLS-SMOTH(7pts)-MC	3	0.9624	1.1530	0.8147	0.7349	AuBT	Glutathione
GA-MC	4	0.3899	0.6133	0.9695	0.9252	CNT	Glutathione
iPLS-MC	6	0.3000	0.5658	0.9819	0.9366	AuAT/CNT/AuBT	Glutathione

Table 3. Best results obtained for the quantification of cysteine and glutathione.

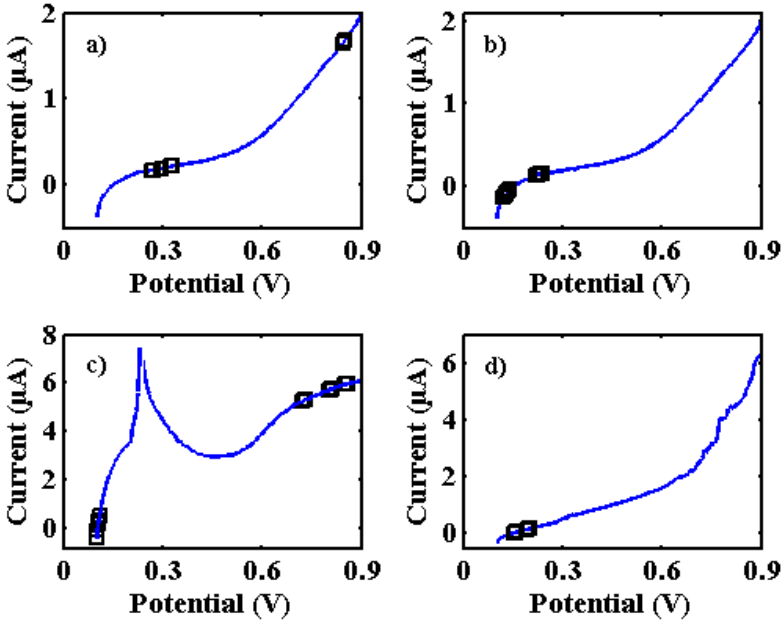

Figure 11. Variables selected to a) Cysteine determination using AuAT electrode. b) Glutathione determination using AuAT. c) Glutathione determination using CNT electrode. d) Glutathione determination using AuBT electrode.

6.5. Simultaneous Quantification of cysteine, glutathione and homocysteine

After obtaining satisfactory results in the simultaneous quantification of cysteine and glutathione from the data obtained in the previous experiment, a new experiment was made to quantify simultaneously cysteine, glutathione and homocysteine.

Fourteen synthetic samples were used in this experiment. All samples were prepared using ultrapure water, acid solution 0.05% TFA and a concentration of the KCl 10^{-3}M to act how as support electrolyte. The concentration for each analyte is inside a range between 0.0 mol.L^{-1} and 6.0 x 10^{-5} mol.L^{-1}. Table 4 show the composition of each sample according of thiol.

Voltammograms were recorded from 0.1V to 0.9V by applying a potential step of 0.002V and a scan rate of 0.05V/s; five measures were made to each sample. Figure 12 presents the mean curve of each sample. From figure 12 it is possible to see the presence of a peak to AuAT electrode at 0.8V; two peaks for CNT electrode at 0.2V and 0.7V; and a peak in the signal of AuBT electrode at 0.9V. It can be seen also the presence of the same problems that occurred in the previous experiment, such as displacement of the peaks, differences in baseline and scattering of the signals. Thus, various pretreatments were applied to the data to minimize these problems.

Sample	Cysteine Concentration mol.L^{-1}	Glutathione Concentration mol.L^{-1}	Homocysteine Concentration mol.L^{-1}
01	0.0	0.0	0.0
02	$6x10^{-5}$	0.0	0.0
03	$6x10^{-5}$	$6x10^{-5}$	0.0
04	0.0	$6x10^{-5}$	0.0
05	$3x10^{-5}$	$3x10^{-5}$	0.0
06	$3x10^{-5}$	0.0	$3x10^{-5}$
07	$3x10^{-5}$	$3x10^{-5}$	$3x10^{-5}$
08	$3x10^{-5}$	$6x10^{-5}$	$3x10^{-5}$
09	$6x10^{-5}$	$3x10^{-5}$	$3x10^{-5}$
10	0.0	$3x10^{-5}$	$3x10^{-5}$
11	$6x10^{-5}$	0.0	$6x10^{-5}$
12	0.0	0.0	$6x10^{-5}$
13	$6x10^{-5}$	$6x10^{-5}$	$6x10^{-5}$
14	0.0	$6x10^{-5}$	$6x10^{-5}$

Table 4. Composition of the samples.

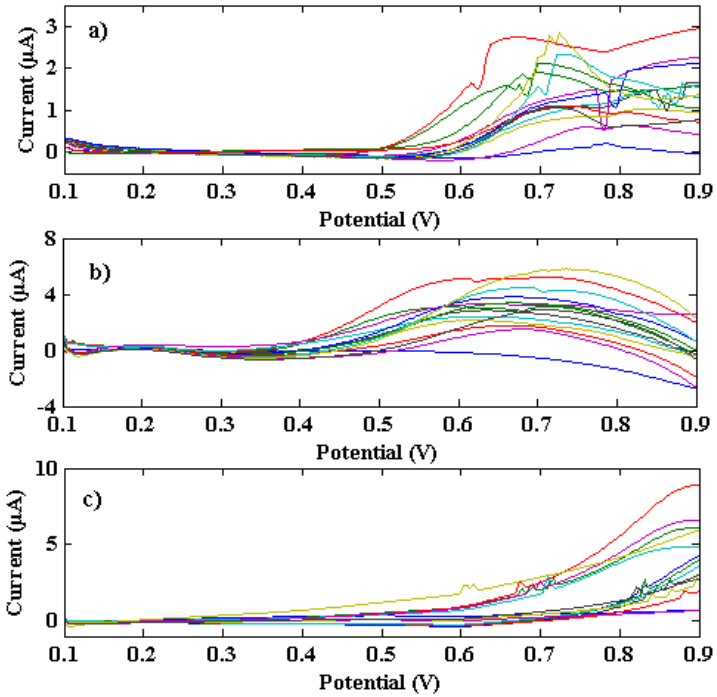

Figure 12. Voltammograms obtained by a) AuAT. b) CNT. c) AuBT.

PLS models were constructed separately according to the analyte and the electrode, and using an augmented matrix containing data from the three electrodes. Variable selection was again used to minimize the complexity of the data selecting variables with more correlated with the analyte concentration. To determine the best PLS models to each analyte, RMSEC and RMSECV were evaluated. Results of models are shown in table 5.

Model Pretreatment	Nº Latent Variables	RMSEC (x10^{-5} M))	RMSECV (x10^{-5}M)	R_c^2	R_{cv}^2	Electrode	Analyte
GA-BAS-MC	4	0.7373	1.5714	0.9154	0.6433	AubT	Cysteine
iPLS-SMOTH(13pts)-BAS-VARSTD-MC	4	0.6048	1.0242	0.9430	0.8410	CNT	Cysteine
GA-SMOTH(11pts)-MC	6	0.4266	0.7350	0.9716	0.9183	AuAT	Cysteine
GA+iPLS-MC	6	0.2366	0.3951	0.9912	0.9761	AuAT/CNT/AuBT	Cysteine
GA+iPLS-MC	1	1.2832	1.4482	0.7131	0.6367	AuAT	Glutathione
GA+IPLS-SMOTH(13pts)-VARSTD-MC	5	0.7182	1.0509	0.9101	0.8091	AubT	Glutathione
PLS-VARSTD-MC	5	0.3733	0.7385	0.9757	0.9054	CNT	Glutathione
GA+iPLS-MSC-VARSTD-MC	5	0.0702	0.3264	0.9991	0.9816	AuAT/CNT	Glutathione
iPLS-SMOTH(7pts)-VARSTD-MC	4	1.5941	2.3608	0.6047	0.2561	AuBT	Homocysteine
PLS-MSC-BAS-VARSTD-MC	2	1.1185	2.4405	0.8053	0.3085	AuBT	Homocysteine
iPLS-BAS-MC	5	0.7247	2.2942	0.9182	0.3411	AuAT/CNT/AuBT	Homocysteine
GA+iPLS-SMOTH(7pts)-2ndDER-MC	6	0.4191	0.8429	0.9726	0.8937	AuAT	Homocysteine

Table 5. Best results obtained in the quantification of cysteine, glutathione and homocysteine.

In the analysis of cysteine good results were obtained using only the AuAT and CNT electrodes separately. It was possible to get RMSECV values similar or better than the best RMSECV obtained to quantify cysteine in the previous experiment; and using the augmented matrix with data from all electrodes it was obtained the best result, with a RMSEC of 0.2366 x10^{-5} M, RMSECV of 0.3951 x10^{-5} M, and R^2 of 0.9912 and 0.9761 to calibration and cross-validation respectively. The best model has been constructed using just MC as preprocess method, using six latent variables and being subjected to two variable selection, firstly by GA and after by iPLS, variables selected are shown at figures 13a, 13b and 13c. To AuAT electrode the variables were selected in the range of 0.3V and 0.7V; to CNT electrode in 0.6V and 0.85V; and to AuBT, between 0.2V and 0.5V.

To quantify glutathione, the best model was obtained using an augmented matrix preprocessed with MSC, VARSTD and MC; were realized two variable selection, being selected variables from AuAT and CNT electrodes resulting in a RMSEC of 0.0702 x10^{-5} M and RMSEP of 0.3264 x10^{-5} M, using five latent variables. In the figures 13d and 13e is possible to see the variables selected in the voltammograms of AuAT and CNT electrodes respectively; variables in the regions of 0.2V and 0.3V were selected for both electrodes, and in 0.85V to CNT electrode.

An electrode has been selected by the best model to quantify homocysteine. The best model presents a RMSEC of 0.4191 x10^{-5} M and a RMSECV of 0.8423 x10^{-5} M; was obtained using AuAT electrode preprocessed with second derivative (2ndDER), SMOTH with windows of 7 points, and MC. Six latent variables were used in the model; and GA and iPLS were applied in the data to select the variables within the ranges between 0.1V-0.5V, and 0.7V-0.9V, as it can be seen in figure 13f.

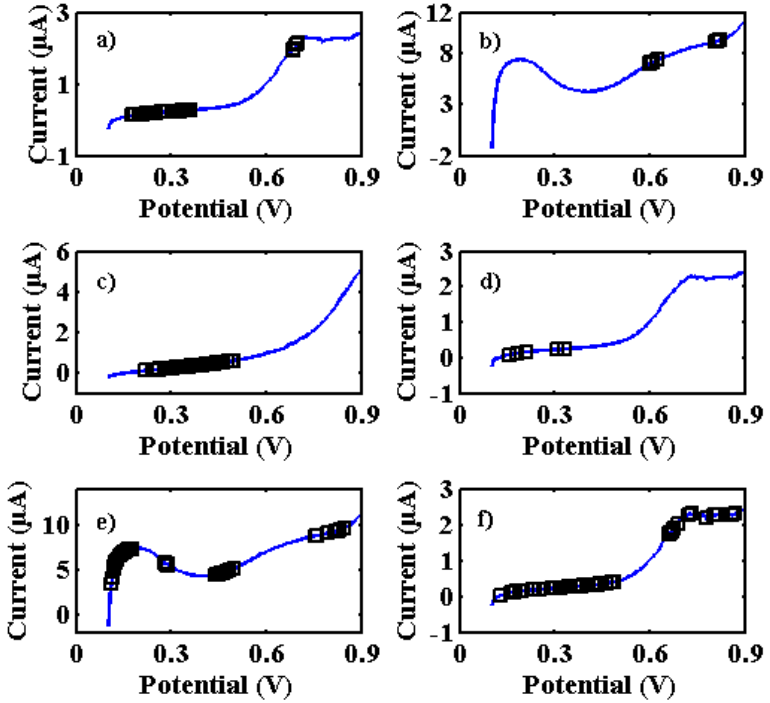

Figure 13. Variables selected for a) Cysteine determination using AuAT electrode. b) Cysteine determination using CNT electrode. c) Cysteine determination using AuBT electrode. d) Glutathione determination using AuAT electrode. e) Glutathione determination using CNT electrode. f) Homocysteine determination using AuAT electrode.

7. CONCLUSIONS

In this work, different experiments have been carried out to develop an electronic tongue for the determination of different thiols. The first experiment consisted of determining the best configuration of the instrument to do the measurements. PCA analysis has shown that configuration with 0.002V as potential step provides the best and the most different signals; and the need of changing the carbon SPE for another electrode type with higher current. In the second experiment the modification of carbon SPE with gold nanoparticles was tried, but, it was not successful because the modification does not present a significant improvement of the signal as compared with carbon electrode. A modification of the carbon SPE with carbon nanotubes was made with success in the third experiment, presenting a good improvement in the signal intensities. Thus, these first experiments could determine the best array configuration of the instrumentation to analyze thiols.

The following experiments, four and five, have been used to quantify simultaneously the thiols, being experiment four to quantify cysteine and glutathione, and experiment five to quantify cysteine, glutathione and homocysteine. Chemometrics techniques, mainly those of variable selection such as iPLS and GA, were applied to the data, and satisfactory results were obtained, showing that the electronic tongue represents a simple, fast, and low cost alternative method to analyze the thiols: cysteine, glutathione and homocysteine.

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9. ACRONYMS

SPE: Screen-printed electrode

LSV: Linear sweep voltammetry

PCA: Principal components analysis

PLS: Partial least squares

iPLS: Interval partial least squares

GA: Genetic alghorithm

RMSEC: Root mean square error of calibration

RMSECV: Root mean square error of cross-validation

R^2_c: Correlation coeffficient for calibration

R^2_{cv}: Correlation coefficient for cross-validation

Cys: Cysteine

GSH: Glutathione

Hcys: Homocysteine

MSC: Multiplicative scatter correction

DER: Derivative Savitzky Golay

SMOTH: Smoothing Savitzky Golay

BAS: Baseline correction

VARSTD: variance (std) scalling

MC: Mean center

PC: Principal components

MWCNT: Multi-walled carbon nanotubes

AuAT: Screen-printed electrode of gold with high temperature of curing

CAR: Carbon screen-printed electrode

AuBT: Screen-printed electrode of gold with low temperature of curing

CARnp: Carbon screen-printed electrode modified with gold nanoparticles

CNT: Carbon screen-printed electrod modified with carbon nanotubes