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Use of electrodes modified with carbon nanotubes for the analysis of thiols in samples of biological interest

Utilització d'elèctrodes modificats amb nanotubs de carboni per a l'anàlisi de tiols en mostres d'interès biològic

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Education is the most powerful weapon which you can use to change the world.

Nelson Mandela

REPORT

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SUMMARY

Thiol groups play a crucial role in maintaining biological systems. This groups can be oxidized producing disulfur bridges. Cysteine (aminoacid) and glutathione (peptide) are examples of biomolecules that have thiol groups. Different methods have been reported for the determination of biomolecules with thiol groups, among them, electrochemical methods. The electrochemical oxidation of these molecules using conventional electrodes presents low kinetics and this leads to low sensitivities.

Screen-printed electrodes (SPE) are a kind of electrodes that present low cost and simplicity of operation, but they also present a low sensitivity for the determination of thiols. In order to increase this sensitivity, a modification of the screen-printed carbon electrodes with carbon nanotubes has been studied, due to due to the fact that the electrocatalytic activity of these nanoparticles is able to accelerate the oxidation of thiol groups.

In the present work, a method for thiol determination in samples of biological interest has been described. For that purpose, an optimization of the electrode modification with carbon nanotubes has been studied by voltammetric methods using cysteine solutions to do the experiments. Then, an optimization of the applied potential for the amperometric determination of thiol groups has been done. In order to have an idea of the optimal potential, cyclic and differential pulse voltammetry have been performed and after that, the optimal potential has been found by flow injection analysis (FIA).

Under optimized conditions, the linear detection range and the limits of detection and quantification that provide the electrodes modified with carbon nanotubes have been determined.

Finally, the concentration of thiols in a human plasma sample has been determined by flow injection analysis. In the analysis, it have been able to check that the described method acceptable for the screening of thiols in biological samples and to establish a starting point for the development of a method for the thiol analysis in this kind of samples by liquid chromatography.

RESUM

Els grups tiol juguen un paper fonamental en el manteniment de sistemes biològics. Aquets grups es poden oxidar donant ponts disulfur. Un exemple de biomolècules que contenen grups tiol són la cisteïna (aminoàcid) i el glutatió (pèptid). Diferents mètodes per a la determinació de biomolècules amb grups tiols han estat estudiats, entre ells, mètodes electroquímics. L'oxidació electroquímica d'aquestes molècules presenta una cinètica lenta en elèctrodes convencionals i això produeix una baixa sensibilitat.

Un tipus d'elèctrodes que presenten un baix cost i facilitat de operació son els elèctrodes serigrafiats (SPE), però aquests elèctrodes també presenten una baixa sensibilitat a l'hora de determinar tiols. Per tal d'augmentar aquesta sensibilitat s'ha estudiat la modificació dels elèctrodes serigrafiats de carboni amb nanotubs de carboni, ja que aquestes nanopartícules presenten una activitat electrocatalítica que pot accelerar l'oxidació dels grups tiol.

En aquest treball s'ha descrit un mètode per a la determinació de tiols en mostres d'interès biològic. Per això, s'ha estudiat l'optimització de la modificació dels elèctrodes amb nanotubs de carboni mitjançant mètodes voltamperomètrics utilitzant solucions de cisteïna a l'hora de fer els experiments. A continuació, s'ha procedit a optimitzar el potencial a aplicar per a la determinació amperomètrica de grups tiols. Per tenir una idea del potencial òptim, s'han realitzat experiments amb voltamperometria cíclica i diferencial d'impulsos i després, s'ha trobat el potencial òptim mitjançant l'anàlisi per injecció en flux (FIA).

Un cop optimitzat els paràmetres per a l'anàlisi amperomètrica de tiols, s'ha determinat l'interval de linealitat i els límits de detecció i quantificació que presenten els elèctrodes modificats amb nanotubs de carboni.

Finalment, s'ha analitzat la concentració de tiols en una mostra de plasma humà per injecció en flux (FIA). En l'anàlisi s'ha pogut comprovar que el mètode descrit es vàlid per a l'estimació de tiols en mostres biològiques i establir un punt de partida per al desenvolupament d'un mètode per a l'anàlisi de tiols en aquest tipus de mostres per cromatografia líquida.

1. INTRODUCTION

Sulfur, as a biogenetic element, acts in multiple functions, principally in the reduced form. Thiols are similar to alcohols but with a sulfur in the place of the oxygen and can be oxidized generating disulfide bonds. There are thiol groups present in some amino acids, such as cysteine (Figure 1A), or in peptides as glutathione (Figure 1B) [1].

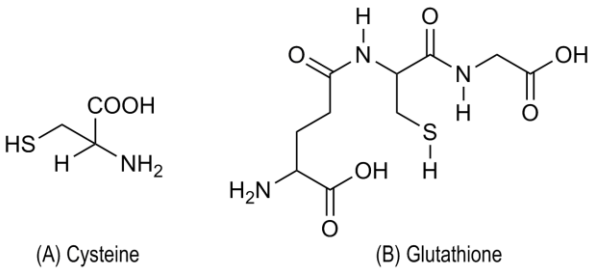
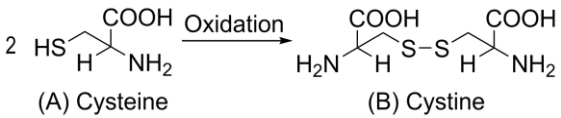


Figure 1. Structures of principal thiols.

Sulfur containing amino acids contribute substantially to the maintenance and integrity of cellular systems by influencing cellular redox state and cellular capacity to detoxify toxic compounds, free radicals and reactive oxygen species.

Cysteine is a primary sulfur-containing amino acid in mammals. It is non-essential, as it is synthesized in the metabolism of the methionine. It contributes significantly to the cellular pool of organic sulfur and generally to sulfur homeostasis [2]. Dimerization of cysteine by oxidation produces cystine, composed by two molecules of cysteine linked by a disulfide bond (Scheme 1). These disulfide bonds have a great importance in the structure of proteins, due to the folds that they generate between different parts of the protein molecule or between two different polypeptidic chains [3].



Scheme 1. Oxidation of cysteine to cystine.

Glutathione is a thiol-containing tripeptide composed of the amino acids glutamine, cysteine and glycine. It plays a role in detoxification and in other cellular reactions, including the reduction of ribonucleotides to deoxyribonucleotides and regulation of protein and gene expression via thiol:disulfide exchange reaction. This tripeptide can exist intracellularly in either an oxidized (GSSG) or reduced (GSH), although in a healthy cell, 95% is in the reduced form. Reduced glutathione protect against oxidative stress by chelating copper ions, regenerating vitamin C and preventing the oxidation of thiol groups. It may also be able to scavenge several radical species by reacting with them to remove the lone electron and become a glutathione thiyl radical (GS•) [4, 5].

Thiol imbalance has been associated with multiple disorders, including vascular disease, Alzheimer's, HIV and cancer [2]. For instance, decreased concentration of reduced glutathione has been observed in patients with Parkinson's disease. In fact, treatment with glutathione has been investigated as a method of slowing progression [5].

Various methods have been reported for the determination of thiols. Among them, the leading positions are held by techniques as high performance liquid chromatography (HPLC) and capillary electrophoresis separations coupled with different detection methods. Among the detection methods, the most prevalent ones are the optical detections, especially the fluorescence due to its simplicity and low detection limit. Nevertheless, these detection techniques involve specific reactions with the thiol groups in order to introduce chromophore or fluorophore groups. Mass spectrometry techniques have also been reported as detection methods, which provide specificity and decreased time for sample clean up and derivatization [6, 7]. Even so, these techniques are expensive, destructive and need experienced laboratory personnel. Electrochemical detection methods offer an attractive alternative due to the relatively low cost and simplicity of the analysis [8].

A variety of electrode materials has been employed for thiol detection in different biological and environmental samples, and their use is well reviewed and documented [8, 9]. However, there have been few studies using screen-printed electrodes (SPEs) for thiol detection to date. For example, it has been reported the use of SPE modified with a polymer film [10] or carbon SPE coupled with MnO_2 reactor [11]. The screen-printing technology relies on printing patterns of conductor and insulators onto surface of planar (plastic o ceramic) substrates. To that end, various conducting and insulating ink materials are avilable for this task. The process of screen-printing yields mass-producible (uniform and disposable) electrodes of different shapes or sizes

and their electrochemical reactivity and overall performance are depenent on the composition of the ink employed and the printing and curing conditions [12]. In general, the main advantages of this kind of electrodes are associated to their low cost, simplicity of operation and the compact detector arrangement containing the working, auxiliary and reference electrodes. Their low cost makes it possible to use a new electrode for each measurement avoiding possible poisoning of the surface by products of the electrochemical processes if necessary.

Even though, electrochemical methods based on the direct oxidation of thiols at solid electrodes are slow and usually large overpotentials are required [6]. The aim of the present work is to develop an easy to go procedure for the electrochemical determination of thiols, specially cysteine and glutathione, with carbon screen-printed electrodes modified with multiwalled carbon nanotubes (MWCNTs) in order to solve the problem of the slowness and large overpotencial requirements. This type of modification with carbon nanotubes (CNTs) have attracted increasing research in recent years and it has been also exploited for thiol electrochemical detection [13-16]. Carbon nanotubes represent an increasingly important group of nanomaterials with unique geometric, mechanical, electronic, and chemical properties [17]. CNTs can be divided into single-wall carbon-nanotubes (SWCNTs) and multiwall carbon nanotubes (MWCNTs). The former are made from a single grafite sheet rolled flawlessly producing a tube diameter of 1-2nm, while the latter are made of concentric and closed grafite tubules having diameters ranging from 2 to 50 nm (Figure 2). The electrocatalytic activity of CNTs has been attributed to the presence of edge plane defects at their end caps [18].

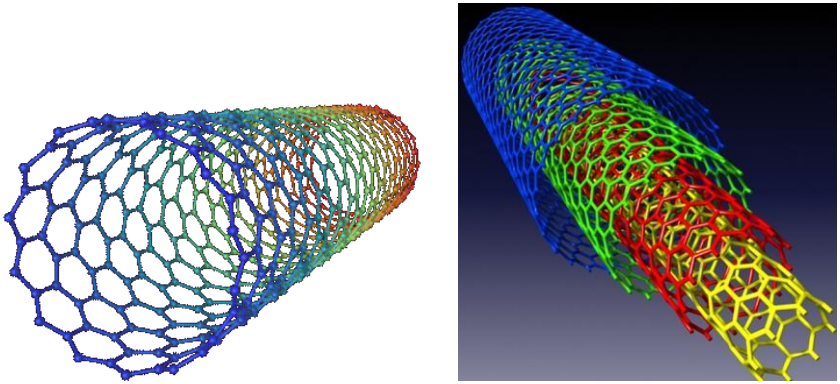


Figure 2. A model of SWCNT (left) and a MWCNT (right) [19, 20].

In order to carry out this work, voltammetric methods, such as cyclic voltammetry (CV) and differential pulse voltammetry (DPV), will be performed to develop static experiments. The

voltammetry is based on the measurement of the current produced in an electrochemical cell at higher (or lower) potentials than the oxidation (or reduction) potential of the compound to be measured. In the measurement, a slightest quantity of the analyte is consumed. CV and DPV differ in the way that the potentials are applied (Figure 3) [21].

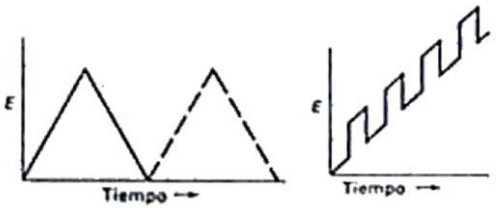


Figure 3. Application of potential in CV (left) and DPV (right) [21].

Cyclic voltammetry is the most widely used technique for acquiring qualitative information about electrochemical reactions. It offers a rapid location of redox potentials of the electroactive species and convenient evaluation of the effect of media on the redox process. It consists of scanning linearly the potential of a stationary electrode in an unstirred solution using a triangular potential waveform (Figure 3 (left)). During the potential sweep, the potentiostat measures the current resulting from the applied potential [12]. On the upper side of the voltammogram, the oxidation of the specie is produced and on the lower side the reduction is done. If the voltammogram presents a peak on each side (Figure 4 (left)), a reversible reaction has occurred because it has produced the oxidation as well as the reduction of the compound. If the voltammogram presents a peak on only one side (Figure 4 (right)), the reaction is irreversible.

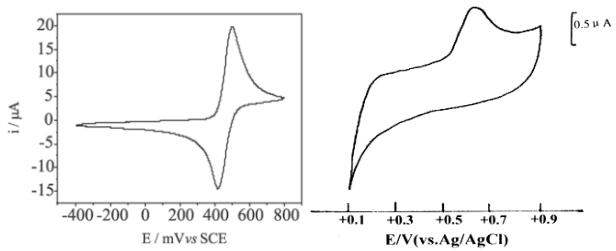


Figure 4. Cyclic voltammogram of a reversible reaction (left) [22] and an irreversible reaction (right) [23].

Differential pulse voltammetry is an extremely useful technique for measuring trace levels of organic and inorganic species. In differential pulse voltammetry, fixed magnitude pulses superimposed on a linear potential ramp are applied to the working electrode (Figure 3 (right)). The current is sampled twice, just before the pulse application and again late in the pulse life.

The first current is instrumentally subtracted from the second, and this current difference is plotted against the applied potential. The resulting differential-pulse voltammogram consists of current peaks, the height of which is directly proportional to the concentration of the corresponding analytes. The peak potential on DPV occurs near the peak potential in CV but it is usually not the same [12].

After the static voltammetric experiments, dynamic experiments will be performed with flow injection analysis (FIA) system. The FIA system simulates a chromatographic method but without the need of a column, due to the fact that the interest of the assay is to try the electrochemical detection of thiols with the modified SPE. Chromatographic methods need higher pressures than FIA because the sample must pass through the column, but both types of methods need flow cells to detect the analytes. Due to the simplicity of the FIA system, it brings the opportunity to study the behavior of the electrodes for a possible later application in chromatography. The main advantage of FIA system is that it is the simplest way of mechanizing practically all operations that need to be made with the sample in the whole practical procedure. Then, instead of measuring the sample volume, using several pieces of glassware, transferring a sample between them (which is the main source of incidental contamination), waiting for a reaction to occur and waiting for a steady detector response, in a flow system, the only operations to be done for each individual sample are its delivery to the flow analyzer and reading or recording of transient or steady signals. So, the basic advantage of flow-analytical measurements compared to manual procedures are better reproducibility (precision) of determinations, a larger throughput and reduction of the sources of contamination [24].

2. OBJECTIVES

The main objective of this work is to improve the amperometric detection of thiols using screen printed electrodes modified with carbon nanotubes. This main goal has a double purpose:

- To establish a screening method with FIA system useful to determine an approximate thiol concentration in samples of biological interest, in this case, human plasma, without separating the different compounds present in the sample.
- To establish a starting point for a development of an amperometric detection of different thiol compounds separated by liquid chromatography. This study requires a longer investigation and cannot be done using commercial flow cells.

3. MATERIAL AND METHODS

3.1. CHEMICALS

All reagents were analytical grade. L-Cysteine (Cys) and sodium hydrogenphosphate anhydrous (Na_2HPO_4) were provided by Sigma-Aldrich. Reduced glutathione (GSH) and N,N.dimethylformamide (DMF) were provided by Merck. Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) was provided by Panreac.

Carboxyl functionalized Multi-Walled Carbon Nanotubes (MWCNT-COOH) were purchased from DropSens, S.L. (Oviedo, Spain) (ref. DRP-MWCNTCOOH).

Phosphate buffer solution 0.1 mol L^{-1} (PBS) of pH 7.0 was used for all voltammetric measurements as well as the carrier solution in FIA. The solution was prepared by dissolving 0.6 g of $NaH_2PO_4 \cdot H_2O$ and 0.8 g of Na_2HPO_4 in 1L of ultrapure water (Millipore MilliQPlus 185 system). PBS was deoxygenated with nitrogen before every use to prevent the oxidation of thiol compounds. Thiol solutions were prepared using deoxygenated PBS as a dissolvent and generally used within 2 h.

For the treatment of human plasma, plasma and perchloric acid ($HClO_4$) (70%) were purchased from Sigma-Aldrich. The human plasma was stored at -80˚C until analysis.

3.2. INSTRUMENTATION

Screen-printed carbon electrodes (SPE) and a cable connector were purchased from DropSens, S.L. (ref. DRP-110 and DRP-CAC, respectively). These sensors have been already described elsewhere [25]. They include a traditional three-electrode configuration: a carbon

working (4 mm diameter), carbon auxiliary and silver pseudoreference electrodes printed on an alumina substrate (Figure 5). An insulating layer serves to delimit the working area and electric contacts.

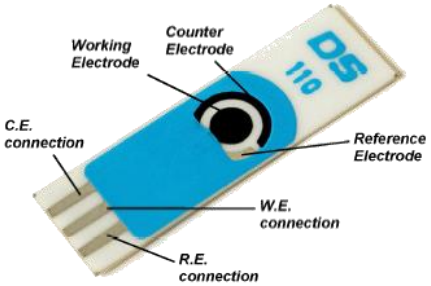


Figure 5. The screen-printed carbon electrode [26].

Electrochemical measurements were performed in a VA Stand 663 (Metrohm, Herisau, Switzerland) connected via an IME-663 module to a computer-controlled potentiostat – μAutolab Type III with GPES software (Eco Chemie, Utrecht, The Netherlands) (Figure 6). All measurements were carried out using a glass cell at thermostated room temperature (20˚C).

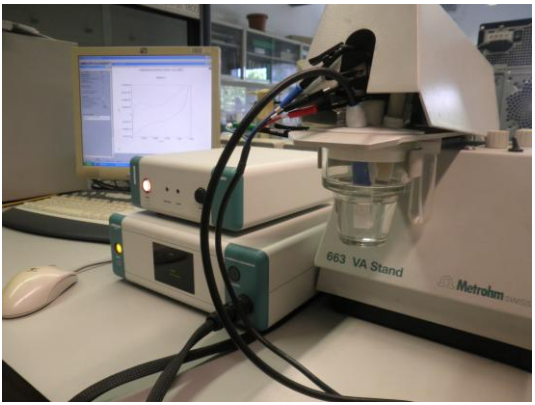


Figure 6. System for voltammetric measurements.

The flow-injection analysis equipment (Figure 8 (right)) included a peristaltic pump Gilson (Middleton, USA) equipped with fluidic Tygon tubing with an inner diameter of 0.08 mm and a six-way injection valve (Supelco, Rheodyne, Bellafonte, PA) model 5020 for low pressure volume injection. This injection valve has 2 positions, load position (Figure 7 A) and injection position (Figure 7 B).

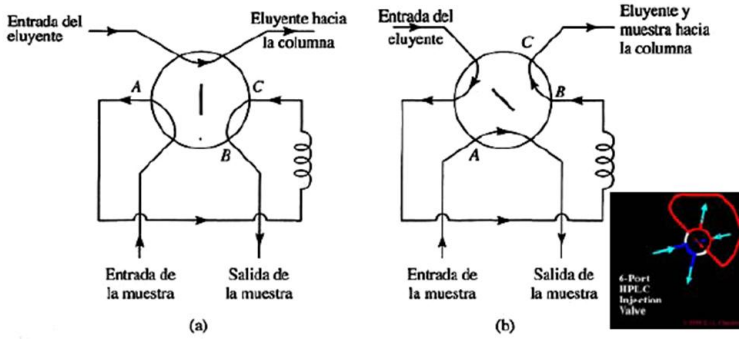


Figure 7. Six-way injection valve in load position (a) and in injection position (b) [21].

The injection loop was 120 µL. A transparent methacrylate wall-jet flow cell where the SPEs were placed was purchased from DropSens, S.L. (ref. DRP-FLWCL) (Figure 8 (left)). The cell includes two pieces of transparent methacrylate with an easy open-closing system based on magnetic bars. The upper methacrylate block has inlet and outlet flow channels forming an angle of 30˚ [25].

Figure 8. FIA system (left) and the wall-jet flow cell (right).

3.3. MODIFICATION OF SCREEN-PRINTED ELECTRODES WITH CARBON NANOTUBES

The modification of the SPE with carboxyl modified multiwalled carbon nanotubes was previously described elsewhere [25].

3.3.1. Preparation of carbon nanotube solutions

Two solutions of different carbon nanotube concentrations (0.1 and 0.5 mg mL^{-1}) were prepared, used for different SPE modifications.

Carbon nanotubes have a tendency to coagulate in water or in polar solvents. Thus, dispersing of tubes is usually performed in non-polar organic solvents. In this case, 1 and 5 mg of MWCNT-COOH were mixed with 1 mL of DMF at 2400 rpm for 12 h using EppendorfMixmate and followed by 1 h of ultrasonification. At this time, stable homogeneous and stable nanotube solutions were achieved. After that, the solutions were diluted by adding 4 ml of DMF:H_2O (1:1) and 5 ml of water obtaining 0.1 and 0.5 mg mL^{-1} of carbon nanotubes solutions respectively. The presence of water in the solution allows its use with all kinds of SPEs based on any substrate material or ink composition as pure DMF solutions are not suitable to be combined with the majority of the plastic substrates and conductive inks of SPEs [27].

3.3.2. Modification of screen-printed electrodes

Two different modifications of SPE with different amounts of carbon nanotubes were performed in order to find one which gives the most sensitive measurements. The modifications were carried out by sequential depositing on the working electrode surface of 4 and 4 µL of 0.1 mg mL^{-1} solution (modification 1, M1) and sequential depositing of 4 and 4 µL of 0.5 mg L^{-1} solution (modification 2, M2). After each deposition, the solution was left to dry at room temperature (20°C) until its absolute evaporation. Afterwards, the electrodes were carefully washed with 1:1 DMF:H_2O mixture and water, and dried under nitrogen stream.

3.4. TREATMENT OF HUMAN PLASMA

The treatment of human plasma for electrochemical detection of thiols has been described elsewhere [28, 29]. 100 µL of plasma were treated with 50 µL of perchloric acid (3 mol L^{-1}) in order to precipitate the large molecules, such as proteins, which could be present in the plasma. After precipitating the macromolecules, the sample was centrifuged at 4500 rpm for 6 minutes and the supernatant was collected.

As the collected sample's pH was acid, it was diluted in PBS in order to achieve pH 7. The dilutions depended on the measured concentration of thiols in the pretreated sample and the linearity of the obtained patron line. For that purpose, some trials were done to decide the optimal dilution of the plasma sample in the phosphate buffer solution. It was found that the

appropriate dilution of the sample was to mix 10 µL of pretreated plasma with 290 µL of PBS. Consequently, 300 µL of sample solution at pH 7.0 were obtained.

3.5. ANALYTICAL PROCEDURES

3.5.1. Optimization of thiol determination

Voltammetric methods were used in order to optimize the modification of SPEs and study de variation of the signal with some parameters. Afterwards, flow-injection analysis was performed to optimize the measuring potential and study the linearity and sensitivity of the method.

3.5.1.1. Voltammetric methods

Cyclic Voltammetry was performed between 0 and 1.2 V so as to study the behavior of the different electrodes in the presence of thiols (L-cysteine or glutathione). The measurements were made with each SPE (non-modified, M1 and M2). First, 25mL of PBS were placed into the cell and purged with nitrogen for 20 min, and the blank voltammogram was recorded in order to check the absence of any contamination. Afterwards, an addition of 1 mL of 2.6 mol L^{-1} of L-cysteine or glutathione solution was done, in each case, in order to obtain a resultant solution with the concentration of $1 \cdot 10^{-4}$ mol L^{-1} of thiol species, and the corresponding voltammograms were recorded. After each addition, the resultant solution was purged with nitrogen for 5 min so as to avoid the presence of oxygen in the measurements. In each measurement, 10 scans were recorded at 0.1 V/s scan rate. In these experiments, it was seen that glutathione did not give significant signals with any of the three different SPEs, so it was decided to do the next experiments only using cysteine as a thiol compound.

After the election of the optimal modification of the carbon SPE, an analysis of the signal variation with parameters, such as the scan rate and the concentration of Cys, was performed with the non-modified and modified SPEs. To that end, Cyclic Voltammetry (CV) was performed with standard solutions with Cys concentrations between $5 \cdot 10^{-5}$ and $5 \cdot 10^{-4}$ mol L^{-1} at 0.1 V/s scan rate in order to study the variation caused by different concentrations and with a $2 \cdot 10^{-4}$ mol L^{-1} solution at scan rates between 0.025 and 0.15 V/s so as to observe the variation with different rates. To do these measurements, first, 25 ml of PBS were placed into the cell and purged with nitrogen for 20 min, and a blank voltammogram was recorded so as to check the absence of any contamination. Afterwards, the subsequent additions of Cys solution were done, purging the resultant solutions after each addition with nitrogen for 5 min. The additions came

from a stock solution that was $2.7 \cdot 10^{-3}$ mol L^{-1} so as to obtain resultant solutions in the range previously mentioned. The volume of each addition was 0.5 mL. Consequently, the following solutions were achieved after the additions (Table 1).

Addition	Cys concentration (mol L^{-1})
1	$5.24 \cdot 10^{-5}$
2	$1.03 \cdot 10^{-4}$
3	$1.51 \cdot 10^{-4}$
4	$1.98 \cdot 10^{-4}$
5	$2.43 \cdot 10^{-4}$
6	$2.86 \cdot 10^{-4}$
7	$3.28 \cdot 10^{-4}$
8	$3.68 \cdot 10^{-4}$
9	$4.07 \cdot 10^{-4}$

Table 1. Concentrations measured in voltammetry after each addition.

In the case of the different concentration solutions, Differential Pulse Voltammetry (DPV) was also performed for each concentration in order to observe the variation of the signal with the increase of the Cys concentration and to have an idea of the optimal measuring potential.

3.5.1.2. Flow-Injection Analysis (FIA)

In order to evaluate the possibility of using the described electrodes for the detection of thiols in flow systems, a Flow-Injection Analysis (FIA) system coupled to a DropSens wall-jet flow cell especially designed for the screen-printed electrodes has been employed. So as to optimize the potential applied for electrochemical detection in flow analysis, a standard solution of $3 \cdot 10^{-4}$ mol L^{-1} Cys was repetitively injected in the FIA system with M1 SPE-NT increasing the applied potential from 0.7 V to 1.1 V in 0.1 V increments. All these measurements were made pumping PBS as a carrier solution at 1.0 mL min^{-1} flow rate and checking the absence of air bubbles. The transparent methacrylate allowed controlling the presence of air bubbles in the carrier flow and inside the cell. When a constant baseline was reached at a fixed potential, the standard solution of the thiols were injected into the carrier flow by means of the six-port valve,

and the fiagram was recorded. In all experiments, the flow rate of eluent solution was 1.0 mL min^{-1}.

After fixing the optimal applied potential for electrochemical detection of thiols with the M1 SPE-NT, fiagrams of different Cys concentration solutions have been recorded with non-modified and M1 SPE in order to study the linearity and sensitivity that each electrode provides. The measured Cys solutions concentrations were, first, in the range of $1 \cdot 10^{-5} – 1 \cdot 10^{-3}$ mol L^{-1}. For the purpose of obtaining the solutions with those concentrations, first, 100 mL of a stock solution of 0.01 mol L^{-1} of Cys was prepared and 50ml of standard solutions in the previously mentioned range of concentrations were obtained by the following dilutions of the stock solution (Table 2).

Standard solution	Cys concentration (mol L^{-1})	Added volume of stock solution (ml)
1	$1.00 \cdot 10^{-5}$	0.050
2	$1.35 \cdot 10^{-4}$	0.675
3	$2.60 \cdot 10^{-4}$	1.300
4	$3.85 \cdot 10^{-4}$	1.925
5	$5.10 \cdot 10^{-4}$	2.550
6	$6.35 \cdot 10^{-4}$	3.175
7	$7.60 \cdot 10^{-4}$	3.800
8	$8.85 \cdot 10^{-4}$	4.425
9	$1.01 \cdot 10^{-3}$	5.050

Table 2. Preparation of standard solutions by dilutions of the 0.01 mol L^{-1} stock solution.

After seen that different linear intervals could be observed for the M1 SPE-NT, another linearity study was done for a range of $5 \cdot 10^{-7} – 3 \cdot 10^{-5}$ mol L^{-1}. In order to obtain the solutions between that range of concentrations of Cys, 100 mL of a stock solution of $1 \cdot 10^{-3}$ mol L^{-1} were prepared and 50 mL of standard solutions were obtained doing the following dilutions of the stock solution (Table 3).

Standard solution	Cys concentration (µmol L^{-1})	Added volume of stock solution (ml)
1	0.5	0.025
2	1	0.050
3	2	0.100
4	4	0.200
5	8	0.400
6	10	0.500
7	13	0.650
8	16	0.800
9	20	1.000
10	25	1.250
11	30	1.500

Table 3. Preparation of standard solutions by dilutions of the 0.001 mol L^{-1} stock solution.

3.5.2. Determination of total thiols in human plasma

After finding the linear intervals for the modified electrode, a determination of thiols in human plasma was done by flow-injection analysis. As only 300 µL of sample was available for each injection, it was injected just at the moment of the measurement. Meanwhile, the PBS solution was flowing through the system all the time. The injections of the sample solutions were made when the linear base was stable.

For the purpose of quantifying the concentration of thiols (in Cys equivalents) in the human plasma, a linear calibration curve was calculated. In order to fix the range of Cys concentration values needed for the calibration line, some trials were done to guess the approximate concentration of thiols (supposing that each different thiol present in the sample has the same electrochemical response). After the trials, it was found that standard solutions should be in the range of 2-20 µmol L^{-1}. In order to obtain these solutions, 100 ml of a 0.001 mol L^{-1} Cys stock solution were prepared and 50 ml of standard solutions were obtained by the following dilutions of the stock solution (Table 4).

Standard solution	Cys concentration ($\mu mol\ L^{-1}$)	Added volume of stock solution (µL)
1	2	100
2	5	250
3	10	500
4	20	1000

Table 4. Preparation of standard solutions for the quantification of thiols in human plasma.

After calculating the linear calibration curve, 300 µL of human plasma sample was injected repeatedly in order to quantify the concentration of total reduced thiol. This quantification was done not considering that the different thiols present in the sample may have different electrochemical responses. It was calculated as if all the thiols had the same behavior as cysteine.

4. RESULTS AND DISCUSSION

4.1. OPTIMIZATION OF THE ELECTROCHEMICAL DETERMINATION OF THIOLS

4.1.1. Optimization of SPE modification

The electrochemical behavior of the three different screen-printed electrodes (non-modified, M1 and M2) in the presence of L-cysteine and glutathione was studied in order to find the optimal modification of the electrode.

The following voltammograms were recorded with the bare carbon SPE (Figure 9).

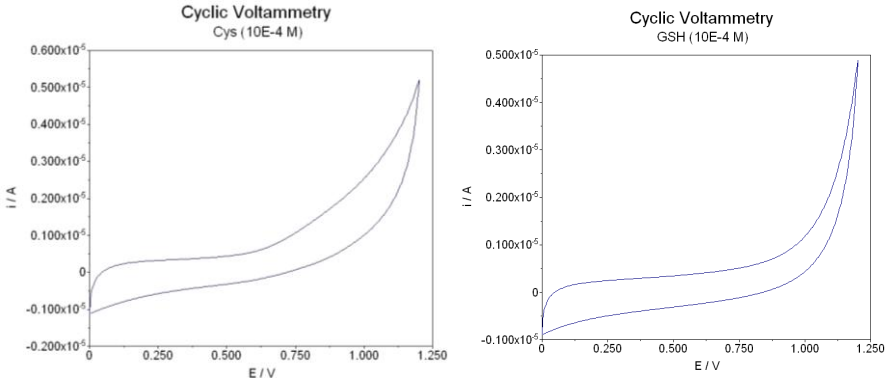


Figure 9. Voltammograms of Cys and GSH solutions with bare carbon SPE.

It can be seen that the bare carbon SPEs can detect cysteine much better than glutathione. In the voltammogram of Cys solution, an increase of intensity is observed, at potentials between 0.7 V and 1.1 V. For the voltammogram of GSH solution, there is no significant difference with the blank voltammogram.

Using M1 modified SPEs, instead of non-modified ones, the following voltammograms were recorded (Figure 10).

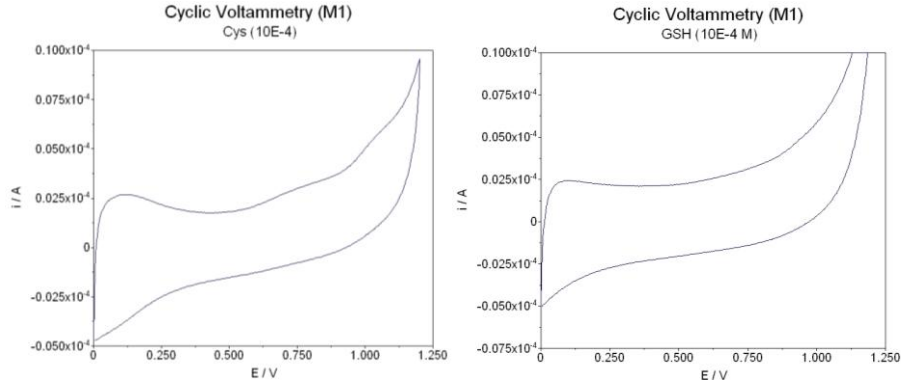


Figure 10. Voltammograms of Cys and GSH solutions with M1 SPE.

In the presence of cysteine, two peaks can be observed between 0.6 and 1.2 V. Otherwise, in the presence of glutathione, no significant difference is observed comparing with the blank voltammogram.

When M2 modified SPE was used, the following voltammogram was recorded in the presence of only PBS. (Figure 11).

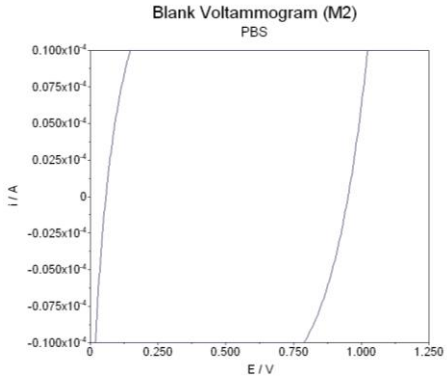


Figure 11. Blank Voltammogram with M2 modified SPE.

It can be deduced that there are too many carbon nanotubes in the electrode and, in consequence, too high intensities are obtained in the voltammetry. Due to that fact, these modified SPEs cannot be used to detect thiols.

Looking at all the voltammograms, it can be deduced that the presence of cysteine can be detected much clearer than the presence of glutathione. Both types of SPE which provide the detection of thiols can detect cysteine, but cannot detect glutathione clearly. M2 modified SPEs cannot be used to detect thiols, because they produce too high intensities. Comparing non-modified and M1 modified SPEs, it can be deduced that the modified electrodes provide a higher response. Due to that fact, it was concluded that the next experiment would be done only with cysteine solutions, and using the bare carbon and the M1 modified SPEs.

On the other hand, it can be deduced that the dimerization of cysteine is irreversible because the peak of oxidation is present on the voltammograms but no reduction peak is present.

4.1.2. Analysis of signals with variation of parameters

After seen that M1 modified SPEs provide the best detection of cysteine, the variation of the signal was studied for different concentrations and different scan rates.

First, cyclic voltammetry was done for the additions **1 - 5** of Cys (Table 1) in order to study the variation of the signal at different Cys concentrations. At the same time, differential pulse

voltammetry was also done for the additions **1 – 4**. The following voltammograms were recorded (Figure 12).

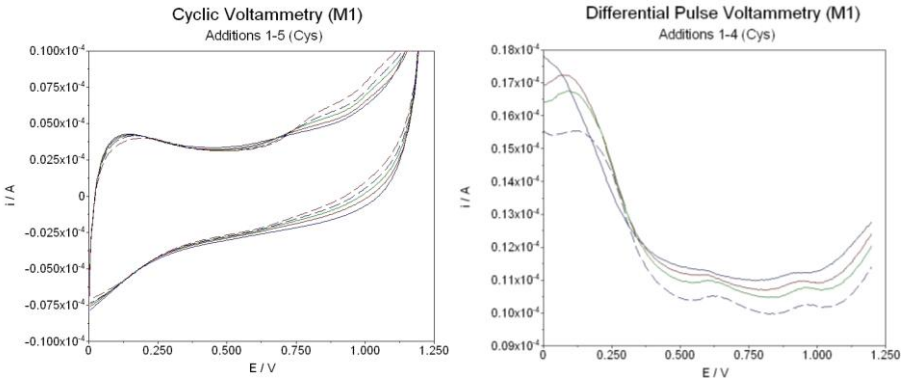


Figure 12. Voltammograms of Cys at different concentrations (M1 SPE).

Addition	Line color (Figure 12)
1	Continuous blue
2	Continuous red
3	Continuous green
4	Discontinuous blue
5	Discontinuous red

Table 5. Colors of the lines for each addition in figure 12.

In the cyclic voltammetry, an increase of the intensity between 0.7 and 1.2 V can be observed with the increase of Cys concentration. In the differential pulse voltammetry, despite the intensities for higher concentrations are lower, the peaks are sharper. This fact is due to a lower base line for higher concentrations. These peaks are present for potentials around 0.6 V and 1.0 V. It is usual due to the fact that in DPV, the peaks use to appear at lower potentials than in CV.

The variation of the signal with different scan rates was also studied. The following scan rates were fixed to do cyclic voltammetry of a $2 \cdot 10^{-4}$ mol L^{-1} Cys solution with the M1 modified SPE (Table 6).

Experiment	Scan rate (V s^{-1})	Line color (Figure 13)
1	0.025	Continuous blue
2	0.05	Continuous red
3	0.10	Continuous green
4	0.15	Discontinuous blue

Table 6. Studied scan rates with M1 modified SPE.

The following voltammograms were recorded for each scan rates (Figure 13).

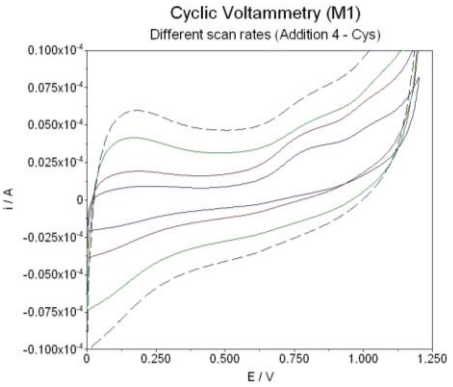


Figure 13. Voltammograms of Cys at different scan rates (M1 SPE).

It can be observed that at a higher scan rate, the recorded voltammogram is wider. It is due to the fact that there is less time to oxidize or reduce the species in the surface of the electrode and consequently, the process becomes more irreversible and the intensities are higher (in absolute value). The scan rate is not important in FIA system because in this system, the amperometric response is measured at a fixed potential. Nevertheless, it is useful to understand the electrochemical process.

In order to compare the M1 modified SPE with the bare carbon SPE, cyclic voltammetry of a 2.5 mol L^{-1} Cys solution was performed with both electrodes. In the Figure 14, the voltammogram recorded with the M1 modified SPE is in blue and the one recorded with the bare carbon SPE is in red.

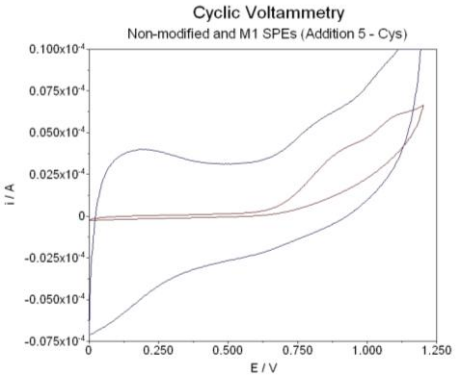
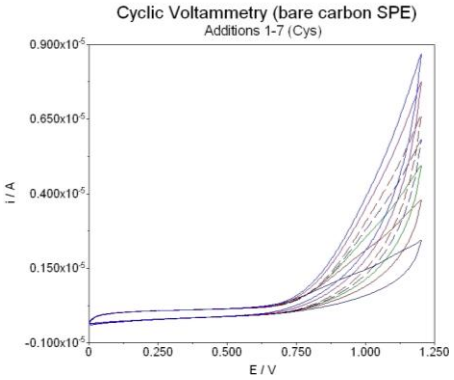


Figure 14. Voltammograms of a Cys solution with non-modified and M1 modified SPEs.

Higher intensities are obtained with the modified SPE, but in the same way, the base line is also higher. It can be observed that the peaks appear at the same potentials for both SPEs.

For a further study of the behavior of both SPEs with the concentration of Cys, cyclic voltammetry was performed with a bare carbon SPE for the additions **1 – 7** of Cys (Table 1) (Figure 15).



Addition	Line color
1	Continuous blue
2	Continuous red
3	Continuous green
4	Discontinuous blue
5	Discontinuous red
6	Continuous magenta
7	Continuous light blue

Figure 15. Voltammograms of Cys at different concentrations with bare carbon SPE.

It can be seen that higher intensities are obtained at higher thiol concentrations but there are not significant peaks observed as in the voltammograms with the modified SPEs. In order to compare the obtained responses with both types of electrodes, differential pulse voltammetry was performed with the bare carbon SPE for the additions **1 – 7** (Table 1) and with the M1

modified SPE for the additions **1 – 9** (Table 1). The following voltammograms were obtained (Figure 16).

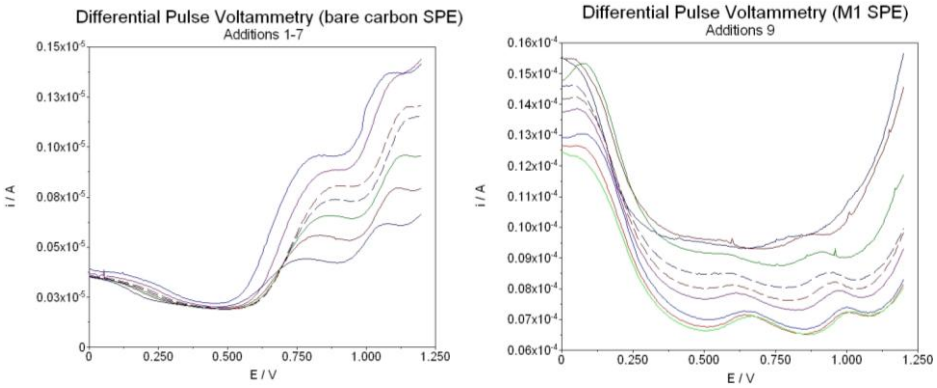


Figure 16. DPV voltammograms at different concentration of Cys with both SPEs.

Addition	Line color (Figure 16)
1	Continuous blue
2	Continuous red
3	Continuous green
4	Discontinuous blue
5	Discontinuous red
6	Continuous magenta
7	Continuous light blue
8	Continuous light red
9	Continuous light green

Table 16. Colors of the lines for each addition in figure 16.

With the non-modified SPE, higher intensities and sharper peaks are obtained at higher Cys concentrations. With the modified SPE, instead, lower intensities were obtained at higher concentrations, but the peaks are also sharper, as with the bare carbon SPE. As it has been explained before, the fact that the base line obtained at higher concentrations is lower affect to the intensities of the peaks, but the intensity difference between the base line and the peaks remain being higher for higher concentrations of Cys.

In both voltammograms, two peaks are obtained in the presence of cysteine. The first peak is obtained at potentials around 0.7 V with the bare carbon SPE and 0.6 with the modified SPE, and the second peak is obtained around 1.1 V with the bare carbon SPE and 0.9 – 1.0 V for the modified SPE. In order to compare both peaks and the response obtained with each type of SPE plots for peak height as a function of Cys concentration were made for each peaks' potential of each kind of SPE (Figure 17).

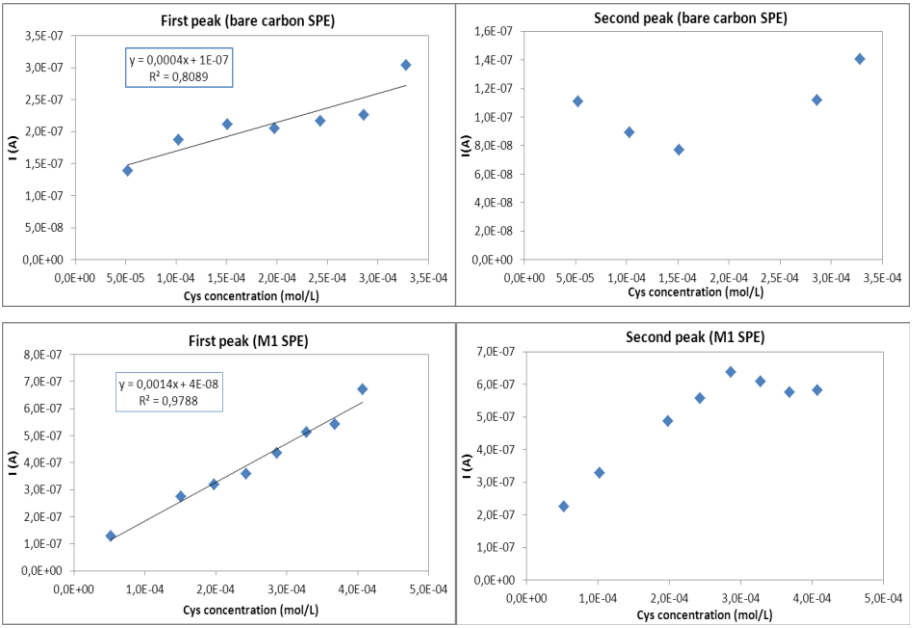


Figure 17. Relationship between peak height and Cys concentration with non-modified and M1 SPEs.

It can be seen that the second peaks with both SPEs provide worse information that the first ones. In the case of the bare carbon SPE, it can be observed that the recorded second peak heights do not depend on the concentration of thiols. The second peak heights recorded with the modified SPE depend on the concentration of Cys but it is observed that at high thiol concentrations, this relation between Cys concentration and peak height is lost.

Observing the recorded first peaks with both kind of SPEs, it can be observed that the peak height do depend on cysteine concentration. Linear regressions were made in order to measure the dependence of the peak height on thiol concentration. Higher slope is observed with the modified SPE and consequently a higher sensitivity is obtained with the M1 modified SPE than with the bare carbon SPE.

With the static experiments performed by voltammetric methods, it could be deduced that M1 modified SPEs provide best sensitivity than non-modified ones and that the optimal potential for the determination of thiols is around 0.7 – 1.0 V.

4.1.3. Effect of air bubbles in FIA measurements

Some trials were made with the FIA system to get acquainted with the technique. It those trials a lack of reproducibility was observed in the obtained signals as it can be observed in the figure 18.

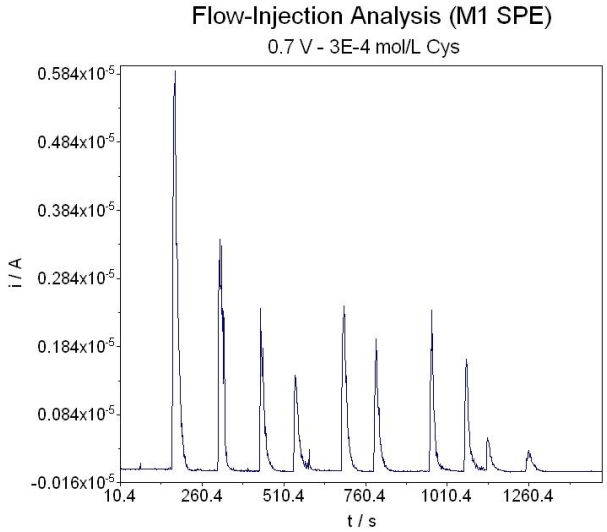


Figure 18. Fiagram of $3 \cdot 10^{-4}$ mol L^{-1} Cys obtained with M1 SPE at 0.7 V.

This lack of reproducibility was due to the air bubbles that could come into the wall-jet flow cell where the SPE was located. The bubbles caused a decrease of the contact area of the solution with the working electrode so the signal that arrived to the detector it was also lower. Thus, from this point forward, a special attention was paid not to have bubbles present in the cell.

4.1.4. Optimization of the potential for the amperometric determination of thiols

In order to find the optimal potential to be applied for the amperometric determination of thiols, fiagrams of a $3 \cdot 10^{-4}$ mol L^{-1} were recorded at potentials from 0.7 to 1.1 V doing 0.1 V increments (Figure 19).

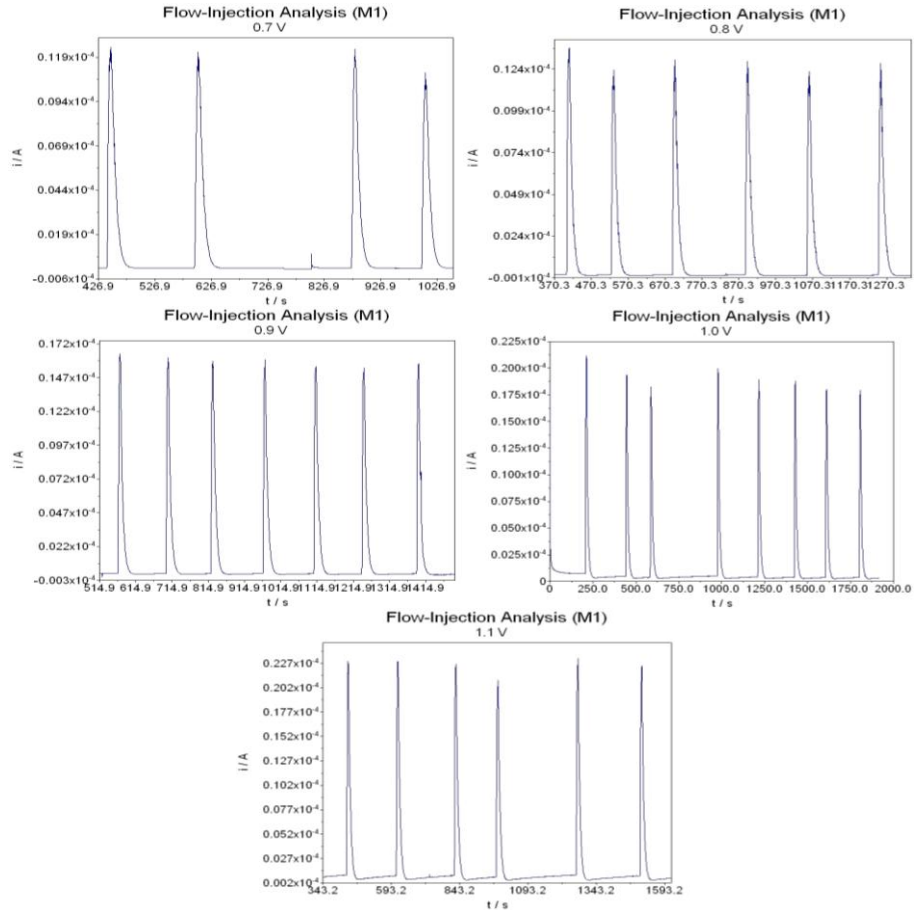


Figure 19. Fiagrams of $3 \cdot 10^{-4}$ mol L^{-1} Cys solution at different potentials (M1 SPE).

It can be observed that at higher potentials, higher peaks are obtained. The fiagrams obtained at 0.7, 0.8, and 0.9 V present a constant base line but at higher potentials, at 1.0 and 1.1 V, the base line is not constant. It can be observed that the base line intensity is increased while the carrier solution is going through the wall-jet flow. Thus, it is deduced that the best potential to apply to detect thiols in a solution is 0.9 V, due to the fact that this potential provides the higher peaks with a constant base line. Therefore, the following FIA experiments were done applying 0.9 V potential.

4.1.5. Linearity and limits of detection and quantification

First, the linearity provided by M1 modified SPE was studied in the range of $1 \cdot 10^{-5}$ – $1 \cdot 10^{-3}$ mol L^{-1}. It was analyzed by measuring the height of the peaks as well as the area. The following plots were obtained (Figures 20 and 21).

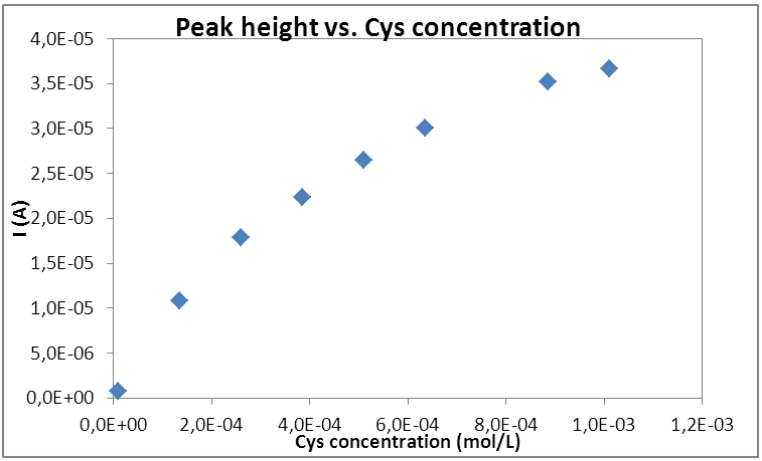


Figure 20. Variation of peak height with Cys concentration (M1 SPE).

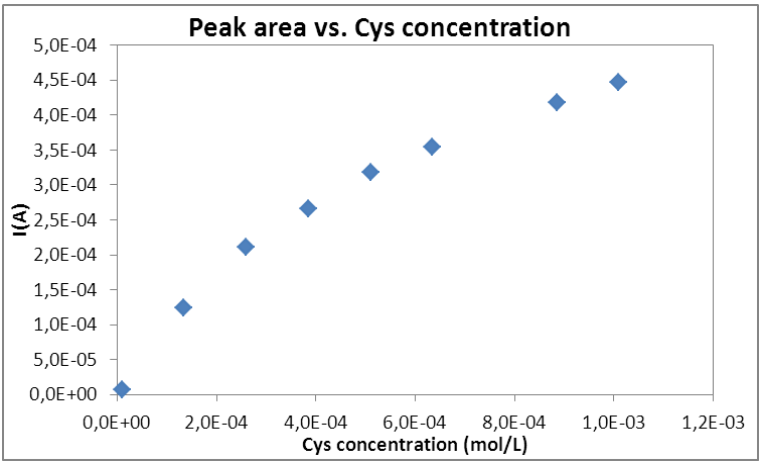


Figure 21. Variation of peak area with Cys concentration (M1 SPE).

It can be seen that the variation of peak height with Cys concentration is very similar to the variation of peak areas. It can also be observed that the relationship of the signal with the

concentration of thiols seems to be polynomial but some shorter ranges can be selected in order to have a lineal relationship (Figure 22).

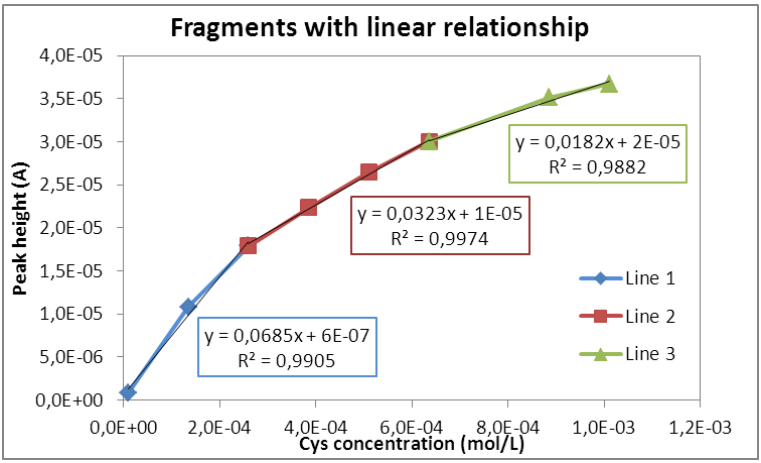


Figure 22. Possible fragments with linear relationship between signal and Cys concentration.

It can be deduced that depending on the range of cysteine concentration that is wanted measure, different ranges of concentration can be chosen and linear calibration can be made for the ranges. If this range is too large, a polynomial calibration can be made.

In order to check that the modified SPE provides an improvement in thiol determination, the same experiment was performed with a bare carbon SPE and the variation of the peak height and area were measures for different Cys concentration solutions. The following plots were obtained with the non-modified SPE (Figures 23 and 24).

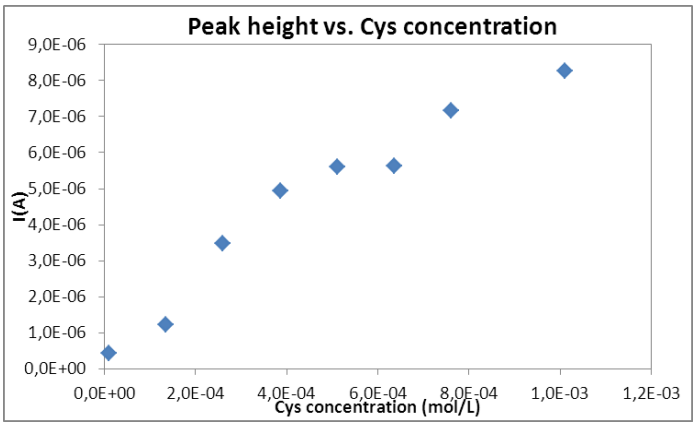


Figure 23. Variation of peak height with Cys concentration (bare carbon SPE).

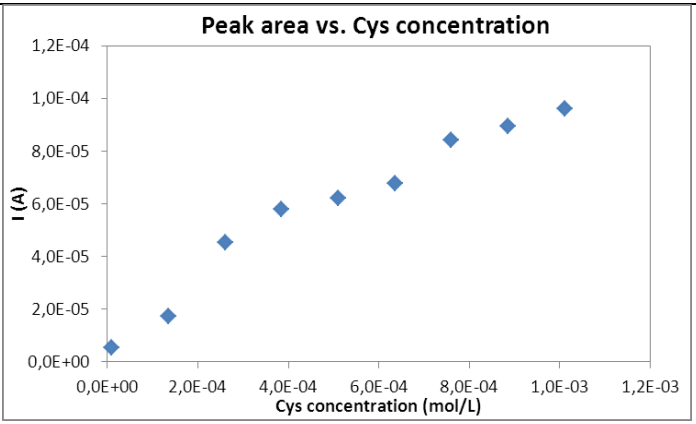


Figure 24. Variation of peak area with Cys concentration (bare carbon SPE).

The relationship between the signal and Cys concentration seems to be more irregular with bare carbon SPE than with the modified one. It can also be observed that the variation of the signal is lower with the non-modified SPE and consequently, the sensitivity is lower, as well.

As it has been shown before, different possible fragments can have a linear relationship between the amperometric signal and Cys concentration. Due to the fact that the aim of this work is to determine thiols in human plasma, the interesting range of Cys concentration for this work is the lower one. Thus, linearity of the method was studied for the range of 0.5 – 30 µmol L^{-1} of cysteine (Appendix 1). The following plot was obtained for the variation of the peak height (Figure 25).

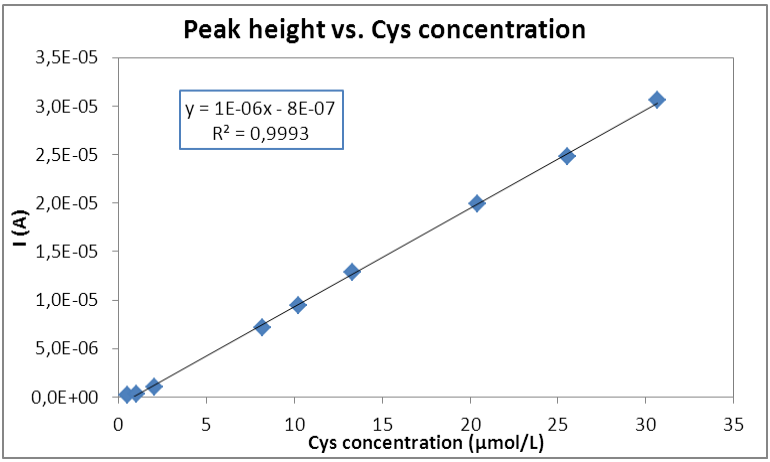


Figure 25. Variation of peak height with Cys concentration (M1 SPE). Linear range.

As it was expected, the relationship between amperometric signal and Cys concentration is linear. A linear regression was made in order to study its linearity, slope and limits of detection and quantification. In this case, the slope is $1.01 \cdot 10^{-6} \pm 1.00 \cdot 10^{-8}$ A·L·µmol^{-1} and it can be used as an indicator of sensitivity. Detection and quantification limits were calculated. It is assumed that the errors of the signal measured in the calibration procedure are negligible and computes $LOD = 3\ s_a / b$, where b is the slope of the calibration plot and s_a is the standard deviation of the intercept. The quantification limit computes $LOQ = 10\ s_a / b$ [30]. In this case, the standard deviation of the intercept is $1.63 \cdot 10^{-7}$. Consequently, the limit of detection (LOD) of this method is 0.48 µmol L^{-1} and the limit of quantification (LOQ) is 1.61 µmol L^{-1}.

The obtained detection limit is comparable with those reported in the literature for conventional and modified electrodes [10, 11, 13, 14, 16, 18] and with others reported based in fluorescence and colorimetric detection [6].

4.2. DETERMINATION OF TOTAL THIOLS IN HUMAN PLASMA

In order to calculate the approximate concentration of thiols in the sample and determine the best dilution of the pretreated sample for the analysis, a first FIA was performed injecting 100 µL of sample formed by 10 µL of pretreated plasma and 90 µL of PBS. The following fiagram was recorded (Figure 26).

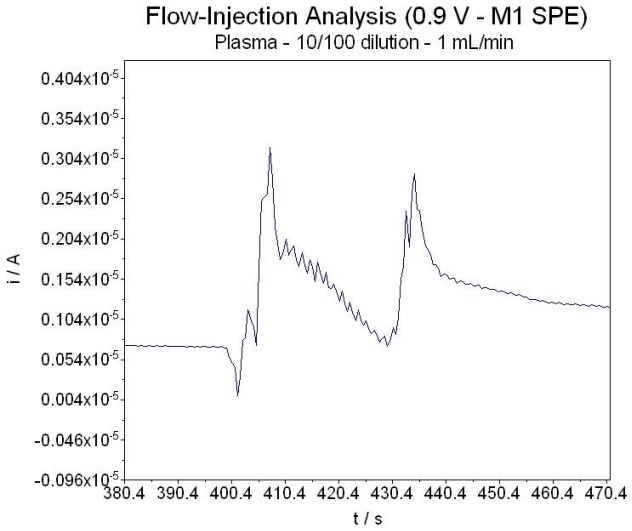


Figure 26. Fiagram of plasma sample at 10/100 dilution.

It can be observed that two peaks are obtained for each sample injection. The first peak is due to the sample but the second one seems to be caused by a change of matrix since it appears just at the moment that the whole sample has passed through the wall-jet flow cell and PBS starts to pass through it again. At first, it was thought that this second peak was due to a change of pH of the solution. As the first peak was too high to be in the described linearity range, a larger dilution of a sample was prepared in order to work in the range mentioned before and at the same time, to make sure that the pH of the sample solution was the same of the carrier solution. To that end, 300 µL of sample were prepared by diluting 10 µL of pretreated plasma in 290 µL of PBS. The next fiagram was recorded (Figure 27).

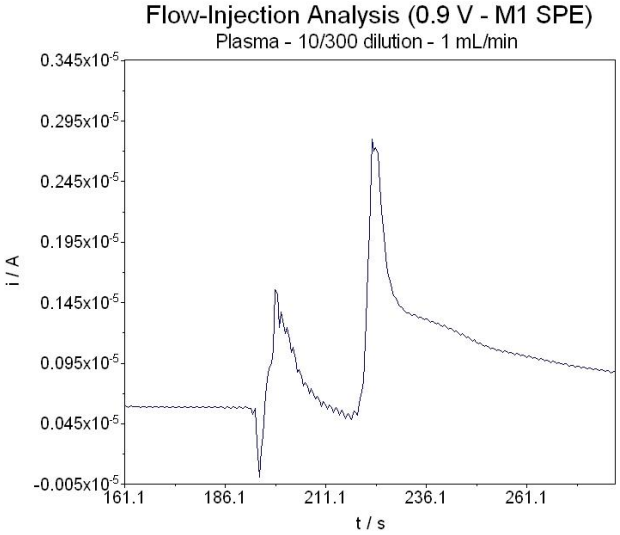


Figure 27. Fiagram of plasma sample at 10/300 dilution at 1 mL min^{-1}.

It can be seen that both peaks keep appearing for each sample injection, but in this case, the first peak is lower due to a higher dilution of the plasma. On the other hand, the second peak continues as high as in the fiagram of the less diluted sample. Therefore, it can be deduced that the second peak is not caused by a change of the pH of the solution. Another hypothesis is that a compound present in plasma could be retained by the tube's wall and its presence could cause a peak in the fiagram. In that case, the height and area of the second peak would depend on the flow rate, as well as the height and area of the first one. In order to check if the second peak was caused by a retained compound present in the plasma, the flow rate was increased to 2 mL min^{-1} and the following fiagram was recorded (Figure 28).

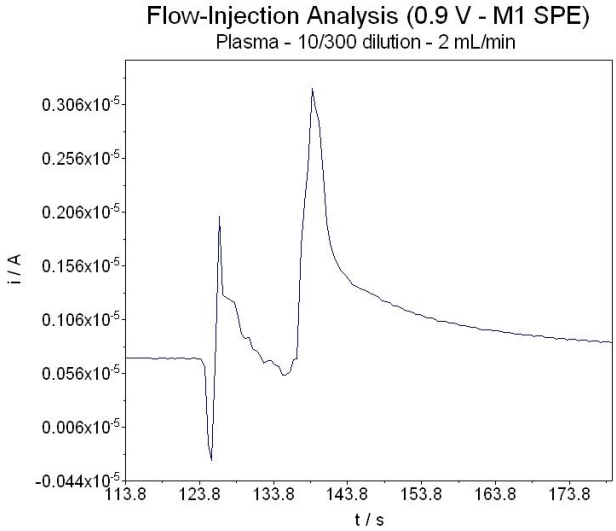


Figure 28. Fiagram of plasma sample at 10/300 dilution at 2 mL min^{-1}.

It can be observed that the height and area of the first peak are lower at a higher scan rate because the time of contact of the sample with the SPE has been decreased. On the other hand, the height and area of the second peak remain constant. It can be deduced that the second peak do not depend on the flow rate of the FIA system. Therefore, the hypothesis of the presence of another compound that causes the second peak can be dismissed. It is deduced that the second peak is caused by the interface between the sample and the carrier solution. As the first peak is the only interesting peak for the determination of thiols in the sample, it is decided to ignore the second peak. An important fact to take into account is that the presence of the second peak can alter the area of the first one, so the height of the peak will be used to determine thiols in the sample instead of the area.

In order to determine thiols in human plasma, a calibration line was calculated. Four standard solutions of cysteine were prepared and fiagrams were recorded (Appendix 2). The following calibration line was obtained (Figure 29).

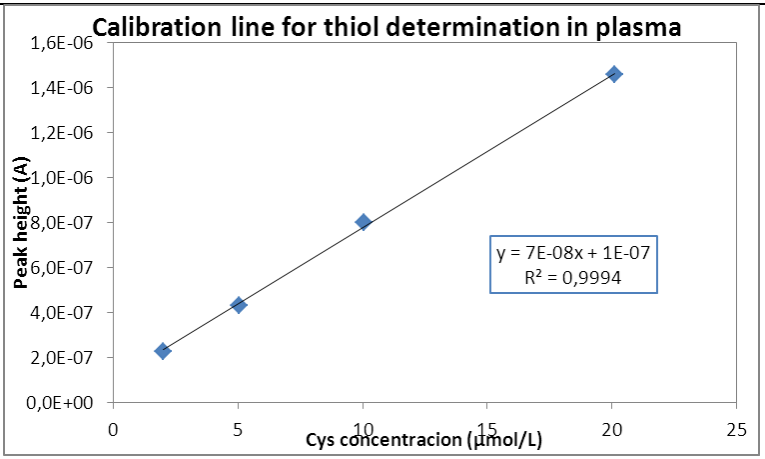


Figure 29. Calibration line for the determination of thiols in plasma sample (M1 SPE)

The calibration line that can be seen on the figure 29 ($y = 6.79 \cdot 10^{-8} x + 9.65 \cdot 10^{-8}$) is different to the line on the figure 25 due to the fact that each carbon SPE was modified manually so the distribution of the carbon nanotubes could not be controlled. Thus, it can be deduced that each modified SPE provides a different response for a same analyte and the calibration line obtained with each SPE can vary. On the figure 29 can be observed that the linear correlation is good enough for an analysis ($r^2 = 0.9994$).

After calculating the calibration line, three aliquots of plasma were injected in the FIA system so as to measure their response and calculate the concentration of thiols present in the human plasma. The next fiagram was recorded for the three aliquots (Figure 30).

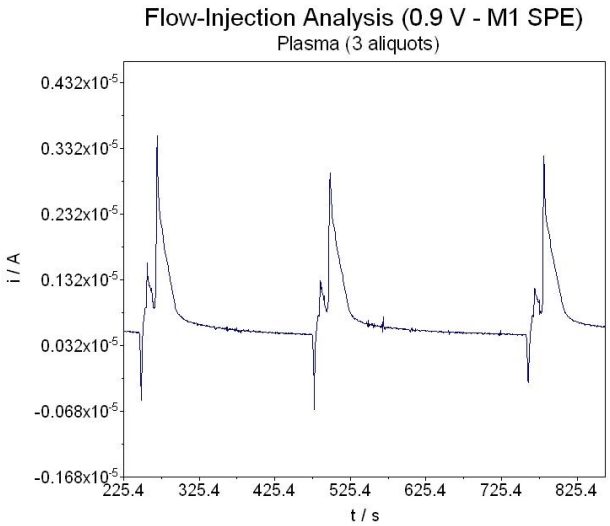


Figure 30. Fiagram of three plasma aliquots.

The height of the first peak for each aliquot was determined and using the calibration line described above, the thiol concentration was calculated. Then, thiol final concentration in human plasma was calculated for each aliquot taking into account the dilutions done in the treatment of the sample. In table X can be observed the obtained information.

Aliquot	Peak height (A)	Aliquot concentration (µmol L^{-1})	Final plasma concentration (µmol L^{-1})
1	7,56E-07	9,71	437,06
2	6,83E-07	8,63	388,44
3	7,07E-07	8,99	404,33

Table 7. Thiol concentration in the aliquots and final concentration in human plasma.

With the information described in table 7, the average concentration and standard deviation were calculated. The final thiol concentration in human plasma is 409 ± 25 µmol L^{-1}. The relative standard deviation (RSD) obtained in the analysis is 6 %.

The thiol concentration in plasma has been calculated with a calibration line formed by cysteine standard solutions. Due to that fact, the obtained thiol concentration is not exact because in the sample there can be present different thiol compounds, such as gluthatione,

cysteine and homocysteine. In addition, the recorded signal is not completely caused by thiols because there can be more compounds that can be oxidized at 0.9 V. This can be deduced comparing the results of this work with results in other articles [28, 29]. In these articles, thiol concentration in human plasma is much lower than the concentration calculated in this work due to the fact that the thiols are separated by chromatography before their determination. Thus, this analysis do not provide an exact concentration of thiols in human plasma at all but it is useful to check that the modified SPEs can be used to determine thiols in biological samples. Screen-printed carbon electrodes modified with carbon nanotubes can be used to determine thiols by a previous separation of the compounds by methods such as chromatography or electrophoresis. Additional investigations should be necessary to improve thiol determination by these separation systems. A wall-jet flow cell adapted for these screen-printed electrodes able to support the high pressures of these techniques should be also necessary.

5. CONCLUSIONS

In the present work screen-printed carbon electrodes modified with carbon nanotubes are shown as a useful tool for the detection of low molecular weight thiols, such as cysteine, in flow system. An optimal modification of the electrodes has been proved and an improvement on sensitivity of SPEs has been shown with the modification. The present setup using a flow cell and screen-printed electrodes offers a simple design and construction along with low cost and easy handle. The results obtained with modified electrodes are comparable to some previously reported results and cover a relevant analytical range for the analytes to be determined.

It has been shown that the performed method to determine an approximate concentration of thiols in human plasma by a FIA system coupled to a modified SPE as an amperometric detector can be used as a screening method to estimate the approximate thiol quantity in samples of biological interest. In the same way, it has been established a starting point for a development of an amperometric detection of different thiol compounds separated by liquid chromatography. Further investigations and a flow cell able to support higher pressures are required in order to develop such system.

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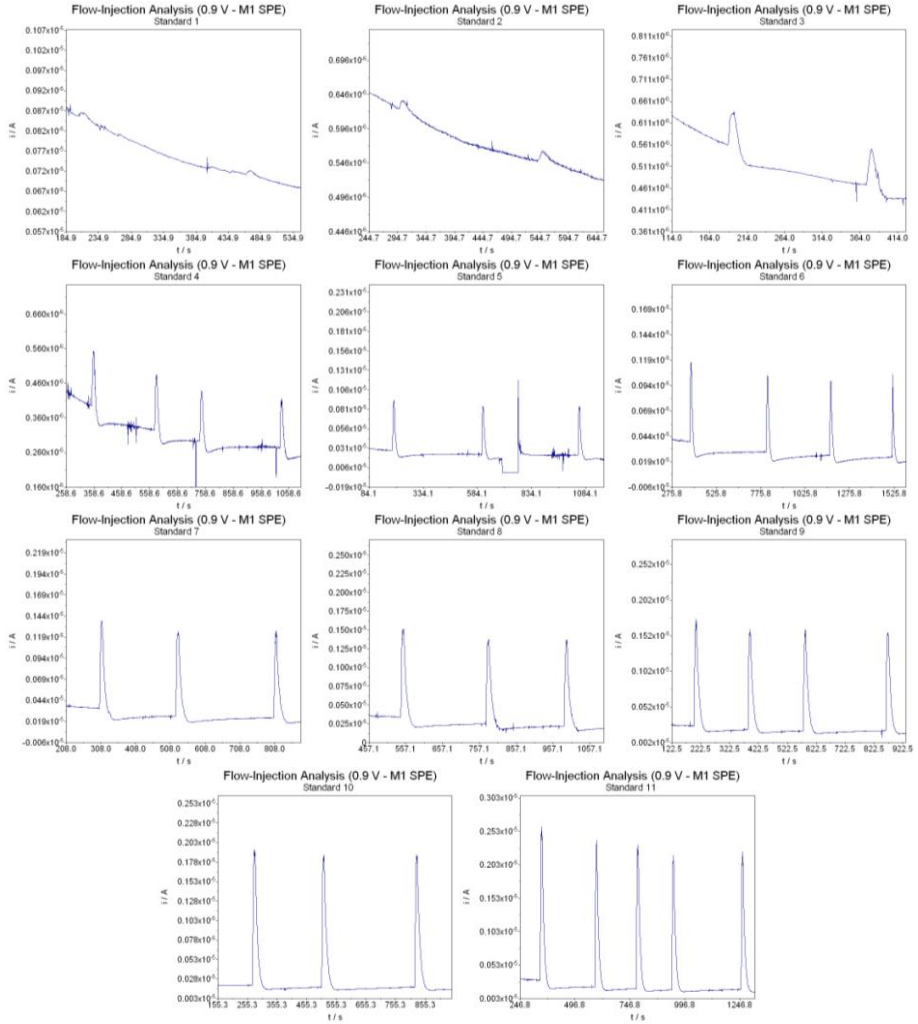
APPENDICES

APPENDIX 1. FIAGRAMS FOR LINEARITY ANALYSIS WITH M1 SPE (0.5-30 µMOL L^{-1})



APPENDIX 2. FIAGRAMS FOR THE CALIBRATION LINE TO DETERMINE THIOLS IN HUMAN PLASMA