Development of CPE/ssDNA-Based Electrochemical Sensor for the Detection of Leucine to Assess Soil Health

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Article

Development of CPE/ssDNA-Based Electrochemical Sensor for the Detection of Leucine to Assess Soil Health

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Abstract

For the first time, the interaction between the amino acid leucine (Leu) and thermally denatured single stranded (ss) DNA has been demonstrated by applying voltammetry. As a result of interaction, the characteristic peak of ssDNA, due to the oxidation of guanine residues. decreased upon interaction time. The interaction behaviour between leucine and ssDNA was studied with UV-vis spectrophotometry, the obtained results are in good agreement with voltammetric ones. The results of the interaction study were exploited in order to develop a SWV method for the determination of leucine at the ssDNA modified carbon paste electrode (CPE). Different parameters were tested to optimize the conditions of the determination. The peak of guanine was at around +0.86 V. Linearity was observed in the range of 0,213 - 4,761 μg/L(r=0,9990) while LOD equals 0,071μg/L. The method was applied to a spiked soil sample and gave satisfactory results.

Keywords: leucine; square wave voltammetry; ssDNA; carbon paste electrode; electrochemical biosensor; soil

1. Introduction

Amino acids are essential as can support plants under extreme conditions including high concentrations of potentially toxic elements as well as extremely high or low temperatures [1,2], or high concentrations of salts and severe water shortages which may occur in the soil environment [3–5] Leucine has also the potential to reduce oxidative damage by regulating plant's antioxidant system, according to a study on *Panax notoginseng* [6] In the investigation of Bayranvand et al. [7] it was found out that leucine resulted in an increase in the activity of appropriate enzymes activating the nitrogen cycle in soils. Also, it can enhance the nitrogen metabolism [8] and subsequently it can lead to an increase in plant resistance to stress from high Cu concentrations ([5].

Leucine is also used as an indicator of the stress that a plant organism undergoes when stressed by extreme toxicity conditions, as in the case of emerging pollutants and environmental hazards or where an amendment has been used in the soil tested [9].

Variations in the amount of soil organic matter can provide valuable information on possible scenarios of land use, fertility levels, the percentage of the land area under cultivation, as well as the cultivation method [10]. Agricultural soils usually contain higher amounts of organic matter, as they either contain manure or other organic soil amendments [11]. In urban soils, the organic matter is usually oxidized, and the amounts are low [12].

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Soil plays an important role in reducing the carbon footprint by storing and sequestering carbon dioxide from the atmosphere. A healthy and fertile soil can increase its carbon storage capacity, helping to reduce emissions [13]. Therefore, the existence of leucine could also lead to conclusions regarding soil health.

Clunes and Pinochet [14] in their research concluded that leucine presence in soils and its variation level provides a satisfactory assessment of the soil's ability to retain soluble carbon compounds easily degradable. Furthermore, it provides an indirect estimation regarding the effects of the dynamics of land use changes on carbon sequestration capacity and the way such changes are relevant to the biodiversity of soil micro-organisms [15] In other words, it can be an indicator about the changes that climate crisis may induce in soils, affecting the microbiota and the rate of carbon sequestration [16].

Due to these important properties of leucine, research on its determination in both soil and plants is valuable. The extractant used depends on the soil fraction and the type of plant in which the concentration of leucine should be determined.

Leucine, like many other amino acids, can be isolated in the exchangeable soil fraction and in clay minerals, as it can be bound to them [14]. In such applications, leucine is determined in the aqueous extract of CH_3COONH_4 0.1M (pH = 7). More often, experiments involve the addition of quantities of the amino acid, followed by incubation for a specific period of time and under specific conditions, and then determination of the remaining amount of amino acid.

The method commonly used is based on the colorimetric determination after ninhydrin reaction at pH of 5.0 [17]. However, this method's main problem is that the soil extracts must be adjusted to pH=5 and this may modulate significant parameters in the remaining components of the soil extract, resulting in fluctuations in the amount of amino acid that is actually free (rather than bound) in order to be quantified [14].

Therefore, a method that could be carried out at pH values ranged between 5-8 would be more preferable. For the quantitative determination of leucine in soil samples, an HPLC method in combination with mass spectrometry was developed by the researchers [18] using 6-aminoquinolyl-N-hydroxysuccinyl imidyl carbamate (AQC).Linearity was observed in the range 50-800 mol L^{-1} . Detection limits were 0.20-0.60 pmol μL^{-1} in the column and 0.07-0.24 $\mu g\ g^{-1}$ soil.

Aminoacids, in free or polymeric form, contribute significantly to most ecosystems' nitrogen levels and so play an important role in the soil nitrogen cycle. Some plants' ability to absorb amino acids straight from the soil may provide a competitive advantage, particularly in nitrogen-limited situations. While inorganic N concentrations (NH_4 and NO_3^-) are regularly measured in either soil solutions or soil water/KCl extracts, a supplementary technique is required to estimate free amino acids and all plant-available pools.

The purpose of this research [14] is to create and evaluate a procedure for rapidly and sensitively determining total free amino acids in soil solutions and soil extracts (water or 2 M KCl). The spectrofluorometric technique is based on the reaction of free amino acids with o-phthaldialdehyde and b-mercaptoethanol. The fluorometric method is substantially more sensitive (working range 0.1-50 mM) than standard spectrophotometric analysis procedures for free amino acids, which use the ninhydrin reagent (10–500 mM). Furthermore, the method requires only tiny sample quantities, is quick, easy to use, and linearity in the range 0.1-100 mM. Free amino acid concentrations were determined in a variety of ecosystems (upland and lowland grasses, forests, heathlands, and coastal saltmarsh). Overall, the concentration of free amino acids in soil solution was low and generally unaffected by soil type. Free amino acids typically account for 10-40% of total soluble N in soil solutions, making up a large soluble N and plant accessible pool in soil.

In the last decades, extensive research in electrochemistry has advanced the detection of biological molecules. Among these, amino acids (AAs) have garnered significant interest from scientists and researchers. Electrochemical sensors and biosensors are simple to use, while present high selectivity, sensitivity, and timesaving.

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Regarding analytical methods for the determination of leucine an important comparison of analytical methodologies is being discussed in [19].

A comparison of the analytical characteristics of the proposed method with the already established ones so far in the literature, mainly in biological substrates and absence of applications in soil samples is underlined, is presented in Table 1.

Table 1. Comparison of the analytical characteristics of the already established methods, with the proposed work.

Ref.	Application	LOD mol/L	Linearity mol/L	Electrode
[20]	Blood	3×10^{-12}	10^{-11} - 10^{-8}	α-CD/ZnO/nanoC
[21]		8×10^{-16}	25-700x10^{-6}	v-NiNWs
[22]	spiked urine, milk and serum	$37.5 \pm 0.2\ 10^{-12}$	0,1-100x10^{-9}	SrO NR
[23]	-			Diamond paste
[24]	-	2 x10^{-6}	10 - 600 x10^{-6}	Amperometric bienzyme ScPE
[25]	blood, urine samples	3.0×10^{-6}	$9.0 \times 10^{-6} - 5 \times 10^{-3}$	MWNTs
This work	spiked soil	4,9.10^{-10}	1,4.10^{-9} - 3,5.10^{-8}	CPE/ssDNA

The immobilization of DNA at electrode surfaces has been recognized as an excellent strategy for forming a conductive thin layer with nanostructures, hence increasing the electrode's surface area for the future construction of efficient biosensors [26]. Furthermore, this method can produce thin coatings with negative charges on the electrode surface, enabling for the adsorption of positive-charged target molecules while limiting undesired adsorption on the substrate. Typically, DNA is deposited at the surface of conductive materials to form a bilayer modified electrode. The application of conductive materials to the electrode can increase its surface area and interfacial conductivity. These bi-layer modified electrodes are capable of sensitively detecting tiny compounds, including medicines, carcinogens, and pollutants, that interact with DNA.

A rare metabolic disease caused by large amounts of branched-chain amino acids (b AAs) i.e., leucine, isoleucine, and valine, and reported MSUD and b AAs was studied as an assay based on electrochemical (bio)sensing [26].

The interaction study between leucine and DNA can be the main trend in the detection of amino acids with electrochemical (bio)-sensors is the use of biomolecules. In general, all electrochemical approaches in both simple electrodes and advanced biosensors may be suitable for the electrochemical detection of amino acids, due to the low detection limit required.

However, simple electrodes are probably not the most suitable solution in the analysis of complicated samples, since biomolecules improve the selectivity, sensitivity and reproducibility of the analysis. In this sense, the damage resistance of biomolecule modified electrodes is particularly important, since they can perform various analyzes without altering their analytical characteristics.

In the literature DNA interaction studies along with leucine were realized in the following studies where is being concluded that aliphatic amino acids alanine, isoleucine, leucine, and valine show low propensity in both binding specificity groups [27–29] Whi le leucine interaction with DNA is due to the shortness of its side chain [27].

Another review discusses the structure and function of single-stranded DNA (ssDNA) binding proteins (SSBs), as well as the structural characteristics that determine SSB binding selectivity. Machine learning-based approaches to predicting SSBs from double-stranded DNA (dsDNA) binding proteins (DSBs) are extensively studied [28].

All forms of amino acid side chains interact more favorably with ssDNA, with the exception of positively charged side chains, with aromatic and aliphatic side chains intercalating particularly well. While positively charged side chains and sodium ions preferentially bind to cytosine in ssDNA and

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negatively charged side chains and chloride ions preferentially bind to guanine. In the study is shown the intercalation of a leucine side chain between bases [29].

Aliphatic amino acids alanine (A), isoleucine (I), leucine (L), and valine (V), the negatively charged glutamate (E) and cysteine (C) show low tendency in both specific binding (SP) and non specific binding (NS) groups with ssDNA [30].

The conclusions of the above mentioned studies showed that exist important findings that in favour ssDNA-leucine interactions.

Considering the foregoing, the presence of high leucine levels may be an indicator of urban or rural origin of the soil samples. High concentrations of leucine indicate agricultural soil and low concentrations indicate urban soil and less fertile soil. Therefore, the analytical method we propose could help a traceability tool of the soil samples analyzed being a measure of fertility and or urban or agricultural origin.

In this context the objective of the proposed study is the development of an electrochemical DNA biosensor for the detection of leucine. In particular, in this study, the methodology of preparation, their voltammetric behavior, the conditions of DNA immobilization and the analytical characteristics of the sensor were studied.

2. Materials and Methods

2.1. Chemicals and Reagents for the Development of DNA Biosensor

Double-stranded deoxyribonucleic acid (dsDNA) from fish sperm was supplied from Sigma-Aldrich as a lyophilized powder. Single stranded deoxyribonucleic acid (ssDNA) was prepared by thermal denaturation. dsDNA (1000mgL^{-1}) was heated in 10mM Tris-HCl (8) at 100 °C for 15 min and was immediately placed in iced bath for cooling for 20 min. The stock solution of DNA gave a ratio of UV absorbance at 260/280 nm of ~1.90 absorbance, indicating that the DNA was sufficiently free of protein contamination. In this work, ultra-pure water (18 Ω) was used for the preparation of all solutions (Elgastat, England), and the chemicals used were of analytical grade. Experiments were performed at room temperature (22.0–25.0 °C)

2.2. Solutions

Solvents, acids, bases, and standard solutions were all analytical grade. They were used as received unless otherwise noted. Sodium hydroxide (NaOH), acetic acid (CH_3COOH) and potassium dihydrogen phosphate (KH_2PO_4) tris hydroxymethyl amino-methane (Tris 99.8%, ACS) and hydrogen chloride (HCl) were supplied from Merck (Darmstadt, Germany). Potassium chloride (KCl), potassium iodide (KI), potassium fluoride (KF), sodium chloride (NaCl) and sodium fluoride (NaF). Amino acids L-leucine (Leu), L-isoleucine (Ile), L-valine (Val), and L-methionine (Met) CELLPURE® ≥99% were purchased from Carl ROTH GmbH + Co. KG (Karlsruhe, Germany), while L-phenylalanine (Phe) ≥98 % was from Tokyo Chemical Industry Co., Ltd. (TCI, Japan). All aqueous solutions were prepared with deionized water. A magnetic stirring bar of 8 × 3 mm, PTFE (HEINZ HERENZ HAMBURG, Hamburg, Germany), was also used

2.3. Apparatus

The voltammetric analysis was conducted using a PalmSens potentiostat/galvanostat from Echo Chemie, based in the Netherlands . The electrochemical cell employed in the experiment consisted of CPE with a 3 mm inner and 9 mm outer diameter for the PTFE sleeve which was used as a "working electrode" ,an Ag/AgCl reference electrode (RE) saturated with 3 mol·L^{-1} KCl, and a platinum wire counter electrode (CE) (Metrohm, Switzerland). All weighings were performed using Sartorius-type scales (Kernew 220-30014 and Denver Instrument XE-310), with procedures conducted at ambient temperature and solution pH measured using a Consort C830 pH meter. The electrochemical cells (with a 25 mm diameter) were washed and rinsed with deionized water and cleaned with dilute nitric acid.

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2.4. UV-Vis Study of ssDNA Along with Leucine

Absorption titrations were obtained on a Shimadzu 160A spectrophotometer-by using a fixed Leucine concentration (75 mM) to which increments of the ssDNA stock solution were added. Leu-DNA solutions were allowed to incubate for 15 min before the absorption spectra were recorded with the UV region from 200 to 450 nm. Absorption titrations were carried out by employing the Wolfe–Shimmer equation [31]: [DNA]/(|εA-εF|)=[DNA]/(|εB-εF|)+1/{Kb (|εB−εF|)}, where [DNA] is the concentration of DNA in base pairs; εA, εF, and εB correspond to Absd/[compound], the extinction coefficient of the free Leucine and the extinction coefficient of the compound in the fully bound form, respectively. In the plot of [DNA]/(|εA−εF|) versus [DNA], the intrinsic binding constant (Kb, in M^{-1}) is then given by the ratio of the slope to the intercept.

3. Procedures

3.1. Interaction of ssDNA with Leu at the CPE/ssDNA Based Biosensor with Leucine

CPE was pretreated by applying a potential at + 1.7 V for 1 min without stirring. The modification procedure involves:

a) ssDNA immobilization at the CPE electrode surface; CPE was modified with 190 mg L^{-1} ssDNA, in a stirred sample solution (0.2 M acetate buffer solution pH 5.0) for 60 s at + 0.5 V .

b) Interaction of Leu, with CPE/ssDNA based biosensor; ssDNA modified CPE allowed to interact with leucine for 60s in Tris-HCl 0,1M+0,008 M NaCl, pH=8 solution.

c)The transduction was performed in 0.1 M acetate buffer solution pH 5.0

Prior to each medium exchange, the electrode was rinsed carefully with water for 5 s.

In both procedures, the transduction was performed in a blank acetate buffer solution, and square wave voltammetry (SWV) was applied under the following conditions: Estep = 0.005 V, Epulse = 0.015 V, and frequency = 12 Hz (unless stated otherwise). The studied potential range varied between 0 to 1.3 V.

Native ssDNA yielded in this medium two positive peaks, the first within 0.82 and 0.86 V where guanine [G] residues are being oxidised and the second between 1.05 and 1.15 V was attributed to adenine [A] residues.

It must be noted that three experimental replicates were performed in all of the subsequent studies, unless stated otherwise

4. Results and Discussion

In order to favour the performance of the biosensor, various factors influencing the response of the biosensor, as ssDNA concentration, concentration of salts, applied potential and accumulation time, were investigated.

The current height of the oxidation peak of guanine and adenine residues increases with increasing ssDNA concentration up to 190 mg L^{-1}. Above this value, saturation of the CPE surface is observed, and the current stabilizes. Thus, the concentration of 190 mg L^{-1} is chosen as the most appropriate for the following studies, since the electrode coverage is complete.

The influence of different salts were studied, like NaCl, NaF, KI, KF, KCl. The effect of ionic strength was also studied as a parameter influencing the electrochemical behaviour of ssDNA, and a concentration of 0,005 M NaCl was found to be ideal.

Figure 1 shows the oxidation square wave voltammograms (SWVs) of bare CPE (black line), leucine on bare CPE (green line), ssDNA/CPE (blue line), ssDNA/CPE after its interaction with leucine (red line). The presence of leucine decreased the oxidation peak current on the CPE/ssDNA at +0.850 V (guanine) and +1.100 V (adenine) with a slight shift of the oxidation peak potential to more negative values by approximately +5 mV and +15 mV, respectively. In addition, Leucine's oxidation peaks were absent on the bare CPE. This phenomenon, in combination with the shift in the peak potential of ssDNA residues, suggests leucine's possible interaction with the prepared electrode ssDNA/CPE.

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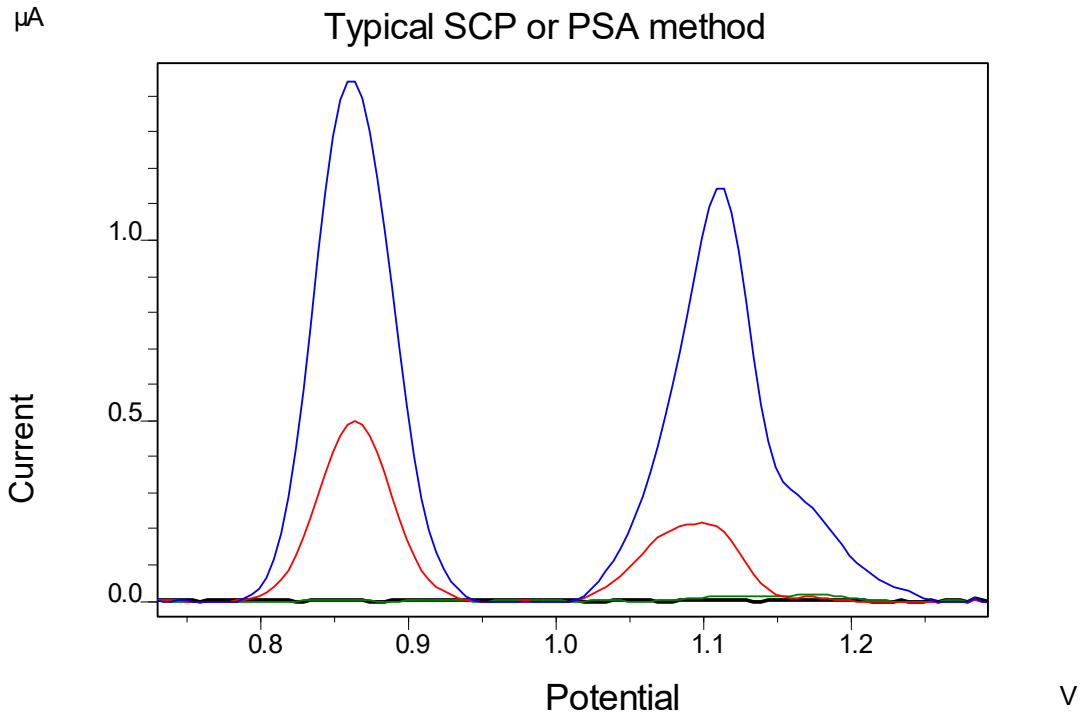

Figure 1. Bare CPE (black line), bare CPE +leucine (green line), CPE/ssDNA (blue line), CPE/ssDNA +leucine (red line). Conditions: 190 mg L^{-1} ssDNA, 11.50 μg L^{-1} Leu, interaction time 60 s and other experimental and instrumental conditions *described in & 3.*

The interaction time has a profound effect on the SWV response. The selection of the interaction time depended on changes in the characteristic oxidation peak of guanine in ssDNA. Increasing the interaction time of leucine with ssDNA, which is a relaxed and more accessible form of DNA, alters the current height of the characteristic oxidation peak of the guanine and adenine residues of DNA. These changes are directly related to the steric changes in the DNA backbone and its ability to adhere to the hard surface of the carbon paste. The dependence of the oxidation of guanine and adenine peak current upon interaction time with Leu is shown in Figure 2. It is shown that the current response was decreased as the interaction time was increased. The observed decrease in the peak current intensity of oxidation of guanine residues during the studied time period may be due either to some kind of equilibrium between the free and ssDNA-bound complex or to steric changes in the ssDNA structure which lead the electroactive groups of ssDNA to unfavorable positions for oxidation on the electrode surface a fact that means that the binding reaction of leucine with ssDNA reaches equilibrium. Consequently, optimum interaction time was found to be 60 s and was chosen for subsequent experiments. It should be noted that the adenine oxidation peak is not reproducible, and for this reason, only guanine residues were selected for study. Conclusively, this behavior cannot be attributed to electrostatic interactions phenomena as leucine is a neutral molecule; it is the result of the binding of Leu to the immobilized ssDNA which resulted in the decrease of both characteristic oxidation peaks of ssDNA [32–36].

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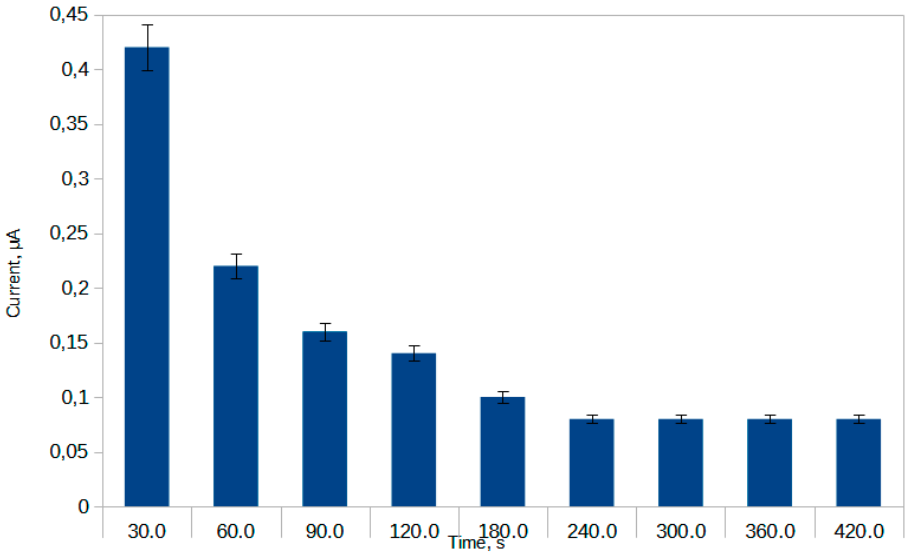

Figure 2. Dependence of the oxidation of guanine peak current upon interaction time with Leu. Conditions: 190 mg L^{-1} ssDNA, 11.50 µgL^{-1} Leu and *under the optimum experimental conditions,described in & 3* .

Figure 3 shows the dependence of the characteristic oxidation peak current of guanine on increasing concentrations of Leu, under the optimal experimental conditions at a given interaction time of 60 s. Up to the concentration 4.761 µg L^{-1}, a subsequent increase in the ssDNA peak was observed. above that value, it decreases up to the value of 7.000 µg L^{-1}, where it finally levels off, which means that the reaction of Leu with DNA reached the equilibrium. With the presence of Leu, new redox peaks do not appear, and there is no shift in the peak potential. This pattern of behavior suggests that the interaction between Leu and ssDNA depends on mass concentration.

In the case of the interaction of Leu with ssDNA immobilized at the CPE, the molecule binds preferentially to the sites of the biomolecule oriented towards the solution. We have to take into account that ssDNA is electrostatically adsorbed at the CPE surface through the negatively charged sugar-phosphate backbone and forms a flat layer on the electrode surface. Also, the side of ssDNA which is in intimate contact with the electrode surface cannot be easily accessible. At low concentrations of Leu the peak of ssDNA is increased because either the molecules of Leu are bound to the side of ssDNA opposite to its contact with the electrode surface. The decrease in guanine oxidation signal at high mass concentration of Leu could be due to the bending of the ssDNA molecule and its ability to adhere at the rough CPE surface. Moreover, it can be attributed either in terms of the equilibrium mixture of the free and ssDNA-bound complex to the electrode surface or of the conformational changes in ssDNA's structure that lead to a steric positioning of electroactive ssDNA residues at the electrode surface. Nevertheless, the data are not significant enough to lead to a safe conclusion.

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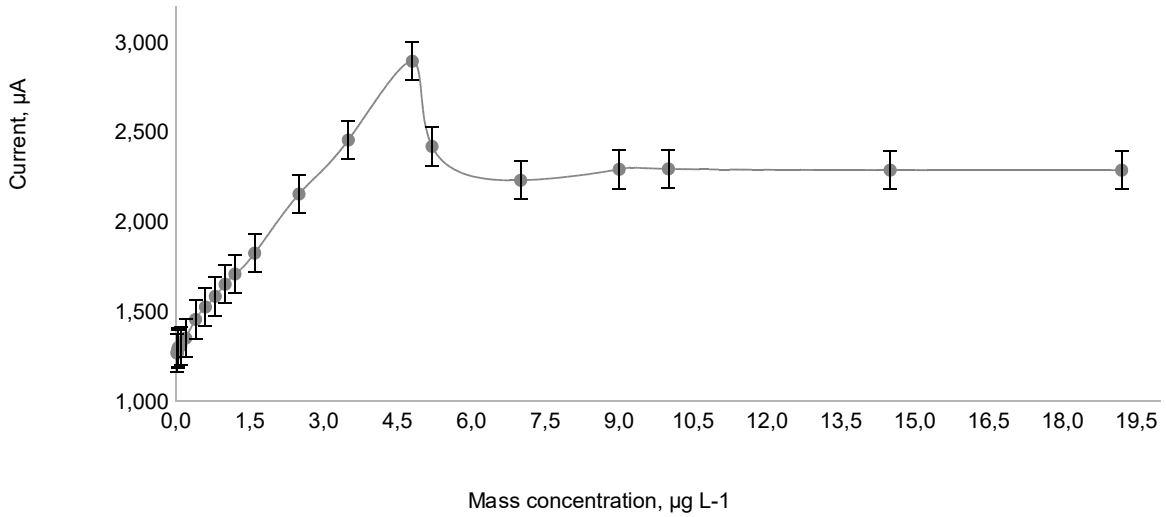

Figure 3. Dependence of the characteristic oxidation peak of guanine with increasing concentrations of leucine, conditions: 190 mg L^{-1} ssDNA, 60 s interaction time and *under the optimum experimental conditions, described in & 3. Six experimental replicates were performed.*

Additionally, UV-vis absorption spectroscopy was used to investigate the *in vitro* interaction of leucine with ssDNA. The DNA-binding constant (K_b) was obtained by monitoring the changes in the absorbance at the corresponding λ_{max} with increasing concentrations of ssDNA and it is given by the ratio of slope to the y intercept in plots [DNA]/(ε_A-ε_f) versus [DNA], according to the Wolfe-Shimer equation. The K_b value was determined to be 0.0326 M^{-1}, indicating that leucine does not bind to DNA through intercalation, as expected since ssDNA was used. The obtained results of this study between ssDNA and leucine [37] are in good agreement with the performed voltammetric interaction study and the results presented so far in the literature [29,30,37]

After optimizing the biosensor's main parameters, a calibration curve was plotted (Figure 4). The results demonstrated that linearity was observed in the concentration range 0.213 – 4.761 (µg/L while LOD equals 0.073 µg/L (in standard solutions) and the regression equation: y=0.3371 (0.0042)x + 1.2913 (0.0075) (R^2=0.9980). It must be noted that the detection limit was determined by 3 s_b/slope where s_b is the standard deviation of the blank measurements and the slope of the calibration curve.

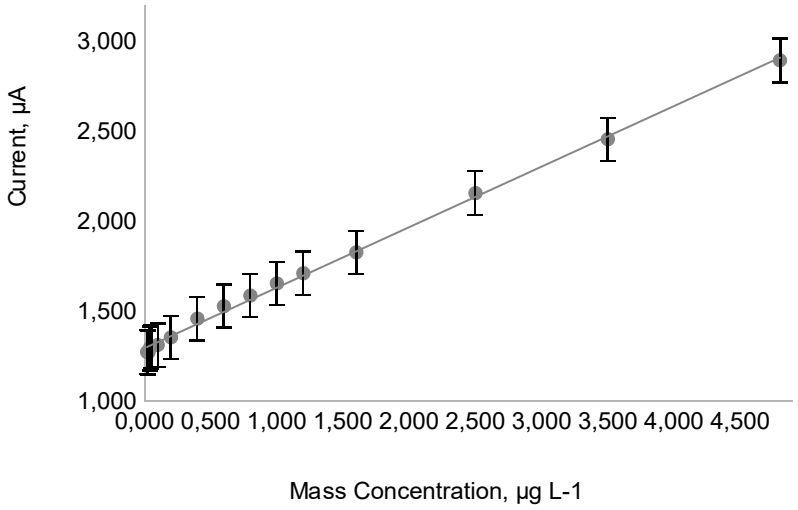

Figure 4. Calibration curve of Leu, under optimal experimental conditions.

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Interferences' Study

There is always the possibility of the interference of other amino acids to the detection of leucine. Aminoacids like Isoleucine, valine , phenylalanine, methionine were study as possible interferents. Therefore, the effect of foreign substances to the current response of the modified electrode was tested as shown in Table 2. The interfering effect of the studied amino acids was evaluated related to recovery, R%.

Table 2. Interference study mass ratio100:1.

Recovery, R% at 3 μg L⁻¹	*Interfering aminoacid*
100,00	*Leucine*
108,75	*Isoleucine*
100,95	*Valine*
102,4	*Phenylalanine*
97,63	*Methionine*

5. Application in Soil Sample

The analysed soil sample was a typical clayey loam soil of a rural area in the Greek countryside. For the extraction of leucine, shaking was performed for 1 hour with an aqueous solution of CH_3COONH_4 0.1M (pH=7), in a ratio of 1:10 (5g of air-dried soil with 50mL of solution). Subsequently, filtration was performed and the filtrate was subjected to 1:100 dilution with d.H_2O (sample solution)

CPE/ssDNA based biosensor was transferred to the diluted and stirred sample solution and allowed to interact with leucine for 60s in Tris-HCl 0,1M+0,008 M NaCl, pH=8 solution. The transduction was performed in 0.1 M acetate buffer solution pH 5.0. A recovery of 98,7% was obtained by the proposed method using the standard addition method.

The corresponding voltammogram is presented in Figure 5. The regression equation for the soil sample was calculated to be y = 0.036 x + 0.721 (R^2= 0.9994).

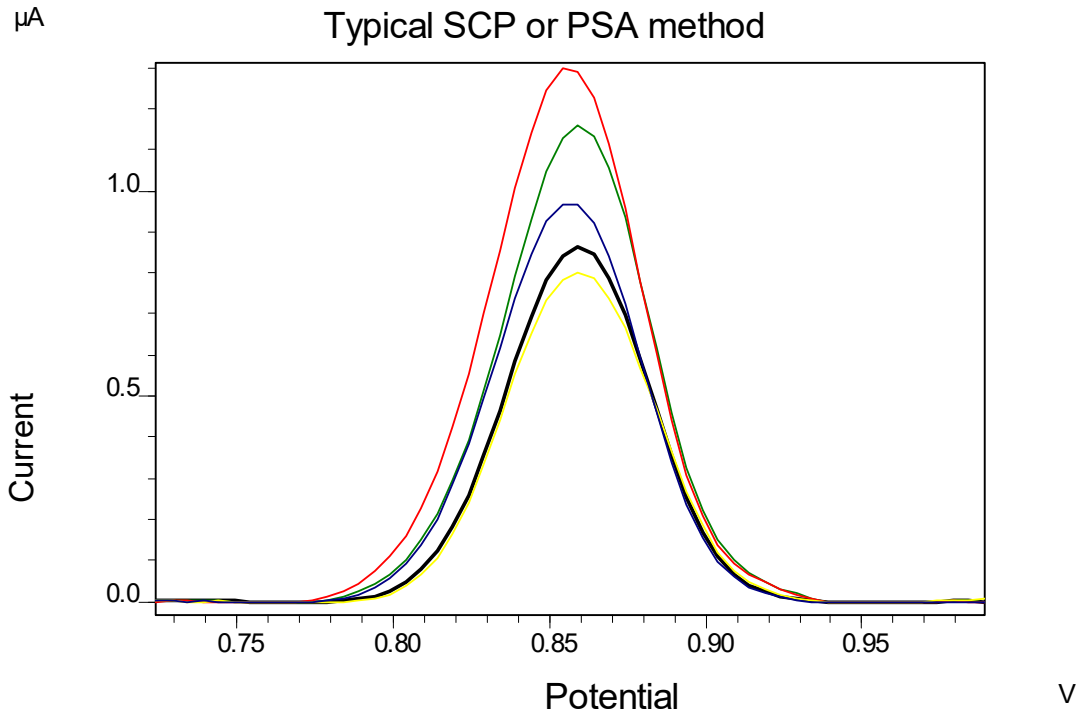

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Figure 5. (a) Overlay of standard additions in the soil sample extract under the optimum experimental conditions, described in & 3.[*yellow line; sample, dark blue line; 1st standard addition (0,5 μg L^{-1}), blue line; 2nd standard addition (1,0 μg L^{-1}), green line; 3rd standard addition(1,5 μg L^{-1}), red line; 4th standard addition (2,0 μg L^{-1})].*

6. Conclusions

The novelty of the present study relies in the fact that it is the first attempt to determine leucine using square wave voltammetry (SWV) applying a CPE/ssDNA biosensor. Furthermore, the proposed electrochemical sensor was shown to be very sensitive, with a lower detection limit than other techniques published in the literature, applied in samples of biological importance so far (serum, urine). The proposed biosensor is being developped based upon ssDNA interaction with leucine. Furthermore, the proposed approach was successfully applied to a spiked soil sample, demonstrating the sensor's potential relevance to real-world sample analysis. The proposed method can cover a range of concentrations, that can meet requirements for leucine determination in both organic and nitrogen-rich soils (e.g. organic and/or clayey), as well as sandy, acidic, or saline soils, where low levels of leucine are expected [38,39]. Additionally, the development of a simple, cost-effective, and environmentally friendly ssDNA biosensor is being demonstrated, successfully applied in the selective and sensitive detection of leucine in standard solutions and real samples.

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