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Determination of amino acids in sugarcane vinasse by ion chromatographic using nickel nanoparticles on reduced graphene oxide modified electrode☆

José Luiz da Silva *, Maísa Azevedo Beluomini, Graziela Cristina Sedenho, Nelson Ramos Stradiotto

São Paulo State University (Unesp), Institute of Chemistry, Araraquara Campus, Department of Analytical Chemistry, Street Prof. Francisco Degni, 55, JardimQuitandinha, 14800-060 Araraquara, São Paulo State, Brazil

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ABSTRACT

The present work is aimed at investigating nickel nanoparticles on reduced graphene oxide modified electrode as an amperometric detector along with anion-exchange chromatography. The modified electrode was found to exhibit electrocatalytic oxidation efficiently in relation to amino acids with high stability. The effects of hydroxide and acetate as mobile phase on the retention time, retention factor, separation factor, and peak resolution were subjected to thorough evaluation. The proposed method presented linear ranges from 2.5×10^{-6} to 1.0×10^{-3} mol L^{-1} with excellent correlation coefficient of at least 0.9997 in optimized anion-exchange chromatographic conditions. The limits of detection for all the compounds analyzed ranged from 8.7×10^{-7} to 2.0×10^{-5} mol L^{-1}. The amperometric sensitivity was found to be within the range of 3.3×10^5 and 5.3×10^6 nA mol^{-1} L. The recovery study showed that the method has good accuracy and repeatability, with recoveries between 95 ± 4.7 and 100 ± 1.7%. Experiment results obtained demonstrated that the sensor has relatively good long-term stability, accuracy, and sensitivity, besides being devoid of interference. The proposed analytical method was applied towards the determination of amino acids in sugarcane vinasse sample obtained from a sugar and ethanol plant.

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1. Introduction

Sugarcane vinasse (also known as stillage, dunder or restyle) is a co-product generated from ethanol production through yeast fermentation of carbohydrates in sugar and ethanol plants. Stillage is a highly pollutant matter with a degree of pollution of about a hundred times that of domestic sewage, and requires high chemical oxygen demand (COD) within the range of 50 to 150 g L^{-1} [1,2].A wide array of studies have reported the application of sugarcane vinasse into the soil via irrigation and its use as organic fertilizer (fertilization) on agricultural fields, which is considered a traditional practice in agriculture as a result of its high nutrient and organic matter content [1,3,4]. Nonetheless, it is an indisputable fact that vinasse once discarded into the environment causes serious environmental problems given its high concentration of organic matter. Furthermore, this co-product contains in its composition a mixture of organic and inorganic compounds, such as organic acids, polyols, carbohydrates, alcohols, phenols, amino acids, mineral salts, and heavy metals [1,3,5–7].

Amino acids can be easily separated by high-performance liquid chromatography (HPLC), high performance anion-exchange chromatography (HPAEC) and capillary electrophoresis [8–23]. The detection of these analytes via traditional methods such as spectrophotometry UV–vis absorption or fluorescence is not always simple to achieve given the absence of natural strong chromophores and fluorophores, respectively. As a result, the most advanced methods essentially require some type of pre or post-column derivatization [8–10]. Derivatization is, however, often seen to be affected by the nature of complexity of the matrix, which tends to render the process incomplete. Underivatized detection methods are known to use conductivity, refractive index, evaporative light scattering, chemiluminescent nitrogen, nuclear magnetic resonance, and mass spectrometry [8]. Conductivity detection is found to be unselective, as such any species can generate a signal including the eluent, though some cases require the use of chemical suppression [24]. Refractive index and evaporative light scattering are universal detectors with lower detection sensitivity, which thus explains their limited use [8, 25].Chemiluminescent nitrogen detector possesses high sensitivity and specificity in addition to low selectivity [8]. Nuclear magnetic resonance and mass spectrometry require a more complex sample preparation besides being relatively expensive to perform. Electrochemical detection (ED), usually operated in amperometric mode, is combined with HPAEC for amino acids determination without derivatization [9–18]. It is noteworthy that the amino groups in amino acids can be deprotonated in

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* Corresponding author.
E-mail address: silva_jl@iq.unesp.br (J.L. da Silva).

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basic medium. Hence, the application of HPAEC with the direct use of strong alkaline solution as mobile phase can simultaneously meet the requirements of ED and chromatographic separation.

Bulk electrodes such as platinum and gold are generally employed in electrochemical detection [13–18].However, these electrodes may be leached by the mobile phase and lose catalytic activity owing to the adsorption species on the surface. Alternatively, chemically modified electrodes (CMEs) have emerged in order to overcome these shortcomings. Generally, CMEs have greater surface area, sensitivity, selectivity and most importantly stability compared to conventional bulk metal electrodes when it comes to HPAEC analysis of amino acids in alkaline medium [9–11,26]. Non-noble transition metals and their oxides, such as cobalt [27,28], iron [29], copper [10,30] and nickel [9,11,26] have been attracting considerable interest as modifying materials as a result of their electrocatalytic property in alkaline media in relation to amino acids oxidation. Among the various materials used in the modification of electrodes, graphene has spawned a wider attention due to its optical, chemical and electronic properties, in addition to its wide range of applications in nanomaterials, nanotechnology and electrochemical sensors [31, 32]. Graphene is essentially a two-dimensional monolayer of carbon atoms in a closely packed honeycomb. It is endowed with many advantages including exceptional thermal and mechanical properties, excellent electrical conductivity, fast electron transportation ability and a high surface area that allows the immobilization of large amounts of substances and nanoparticles [25,32–34]. Moreover, metallic nanoparticles and their oxides have been extensively studied given their chemical and physical properties, such as better mass transport, catalysis, high surface area and effective control of the microenvironment of the electrode compared to bulk metal electrodes [35–38]. By virtue of their unique properties, graphene and non-noble transition metal nanoparticles have, in effect, been considered attractive new materials for the development of electrochemical sensors aiming at amino acid detection. To date, several kinds of functional materials such as cobalt nanoparticles (CoNPs) [27], CoNPs in graphene oxide (GO) [28] and iron nanoparticles on GO [29] have been applied towards the determination of amino acids. Nickel nanoparticles combined with reduced graphene oxide-modified electrodes may provide a means of enhancing the detection of amino acids while contributing towards improving our knowledge on this subject matter in the field of sensors and detectors.

For the evaluation of new processes involving the application and the production or extraction of compounds from sugarcane vinasse, it is fundamentally crucial for us to accurately characterize the chemical composition of this industrial co-product if we are to add greater value to this biomass and to enhance its applications in bioenergy. In this present study, the analytical performance of glassy carbon electrode modified with reduced graphene oxide decorated with nickel nanoparticles (NiNPs-RGO/GCE) for the detection of underivatized amino acids in HPAEC was investigated. The application of the established new method using direct electrooxidation of amino acids without derivatization in sugarcane vinasse was performed using NiNPs-RGO/GCE.

2. Experimental

2.1. Reagents and solution

Solutions were prepared from analytical reagent grade chemicals without further purification and with ultrapure water (MILLI-Q). Graphene oxide (GO, 4 mg mL^{-1}), nickel(II) sulfate hexahydrate ($NiSO_4.6H_2O$, 99%), amino acids (glycine ≥99%, L-alanine ≥98%, L-asparticacid ≥ 98%, L-glutamicacid ≥99%, L-leucine ≥ 98%), sodiumhydroxidesolution (NaOH, 50–52% in H_2O)andsodium sulfate (Na_2SO_4, ≥ 99%) were obtained from Sigma-Aldrich and nitric acid (HNO_3, ≥ 65%) from Merck.

2.2. Apparatus

An Autolab PGSTAT-30 (Eco Chemie B.V.) potentiostat/galvanostat installed with NOVA 1.11 software was used for electrochemical measurements while electrochemical impedance spectroscopy (EIS) was carried out using the FRA module. Cyclic voltammetry (CV) and EIS experiments were performed in a conventional three-electrode cell using the glassy carbon (GCE, geometric area: 0.070 cm^2) modified with nickel nanoparticles decorated with reduced graphene oxide (NiNPs-RGO/GCE). Ag/AgCl (KCl 3.0 mol L^{-1}) electrode and platinum wire were used as working, reference and auxiliary electrodes, respectively. All experiments were carried out at room temperature (25 °C).

The spectroscopic characterizations of the modified electrodes were conducted on glassy carbon plates (GC) with a covered area of 0.070 cm^2. Scanning Electron Microscopy (SEM) was used for the morphological characterization of the modified glassy carbon plates (GC). A scanning electron microscope with Jeol field emission gun (SEM-FEG), model JSM 7500F and a coupled module of energy dispersive X-ray spectroscopy (EDX) were used for conducting the measurements.

Pulsed chronoamperometric measurements in flowing streams were performed using a Model IC Amperometric detector (Metrohm) and an electrochemical Wall-Jet cell (Metrohm) consisting of NiNPs-RGO/GCE as working electrode (3.0 mm diameter), while palladium and platinum were employed as reference and counter electrodes respectively. A microcomputer equipped with a MagicNet, version 3.1 software was used for the acquisition and processing of the chromatograms. Chromatographic experiments were carried out by an ion chromatograph 850 Professional IC Cation-HP-Gradient (Metrohm) with extender module (pump C, post-column), loop of 20 μL and 863 compact autosampler (Metrohm).

Chromatographic separations were performed using a CarboPacPA1 (Dionex, 250 × 4 mm I.D., 10 μm) anion-exchange column coupled to a guard CarboPac PA1 column (50 × 4 I.D., 10 μm). The temperatures employed in the column oven and detector were 32 and 36 °C, respectively.

2.3. Electrode modification with reduced graphene oxide and nickel nanoparticles

Prior to electrodeposition, the GCE (3.0 mm diameter) was immersed in nitric acid (20% *w*/w) for five minutes so as to remove metal impurities off the electrode surface. The surface of the GCE was polished with α-alumina (0.3 μm) powder on a polishing microcloth in order to improve the uniformity as well as the adhesion of the RGO and NiNPs, and was subsequently washed with ultra-pure water.

The working electrode surface was modified with RGO based on previously published reports [39,40] and NiNPs by chronoamperometry. All electrode modification experiments were performed in triplicate ($n = 3$). First, the RGO was electrodeposited on the GCE surface using 0.50 mg mL^{-1} graphene oxide suspension in 0.10 mol L^{-1} Na_2SO_4 at a potential of − 1.4 V for 500 s. Parameters including the concentration of Na_2SO_4 and GO suspension, electrodeposition potential and time were all optimized. The obtained modified electrode was washed and dried at room temperature for 10 min. After that, the electrode was subsequently immersed in 0.10 mol L^{-1} Na_2SO_4 containing 2.0×10^{-2} mol L^{-1} $NiSO_4$ and electrochemically reduced at a constant potential of − 1.1 for 90 s based on previous optimization conditions. The resulting modified electrode was washed and immersed in 0.10 mol L^{-1} of NaOH prior to being subjected to 30 cyclic voltammograms between 0.10 and 0.75 V at a scan rate of 100 mV s^{-1} for the oxidation of nanoparticles.

2.4. Chromatographic conditions

The mobile phase was prepared by mixing 0.20 mol L^{-1} of NaOH solution with ultrapure water.The isocratic elution strength of the NaOH/

H_2O solvent and the flow rate were all investigated. Stock solutions of amine acids (1.3×10^{-1} mol L^{-1}) were prepared in mobile phase and standard curves were constructed. The limit of detection (LOD) was evaluated as a signal to noise ratio equal to 3 (S/N = 3) from the lowest injected concentration. The linear range, amperometric sensitivity, correlation coefficient and repeatability of the analytes were also assessed. The method validation was conducted via the standard addition method as described in the next section.

2.5. Sample preparation

Sugarcane vinasse sample was obtained from a sugar and ethanol plant. The sample was initially centrifuged at 4000 rpm for 20 min aiming at removing the solid particles present therein. Afterwards, the supernatant was collected and filtered (filters 5.0, 0.47 and 0.22 μm from Millipore).Prior to being injected into the column, the sample was diluted with ultrapure water while relevant solutions were diluted at 1:100 for the determination of alanine, leucine and glycine, and at 1:10 for the quantification of aspartic and glutamic acids. The standard addition method was used to determine the concentration of amino acids present in the samples. Three different concentrations of alanine, glycine, leucine (2.5×10^{-4}, 5.0×10^{-4} and 7.5×10^{-4} mol L^{-1}), aspartic acid and glutamic acid (2.5×10^{-5}, 5.0×10^{-5} and 7.5×10^{-5} mol L^{-1}) were added into the samples.

3. Results and discussion

3.1. Morphological and electrochemical characterizations of NiNPs-RGO/GCE

The modified GCE with RGO and NiNPS were confirmed by SEM images as shown in Fig. 1. Fig. 1(A) shows unmodified GCE while Fig. 1(B) and Fig. 1(C) depict RGO and NiNPs electrodeposited on the surface of the GCE at optimized conditions (Fig. S1 and S2). The wrinkled nature of the RGO sheets is found to be highly favorable to modification with NiNPs, besides contributing towards increasing the surface area of the electrode. From the SEM image, it is possible to observe that the NiNPs on the RGO/GCE surface display sizes ranging from 11 to 121 nm (300 nanoparticles were counted), and mean diameter of 46 ± 20 nm (Fig. 1D). Furthermore, the nanoparticles are seen densely occupying the RGO sheets.

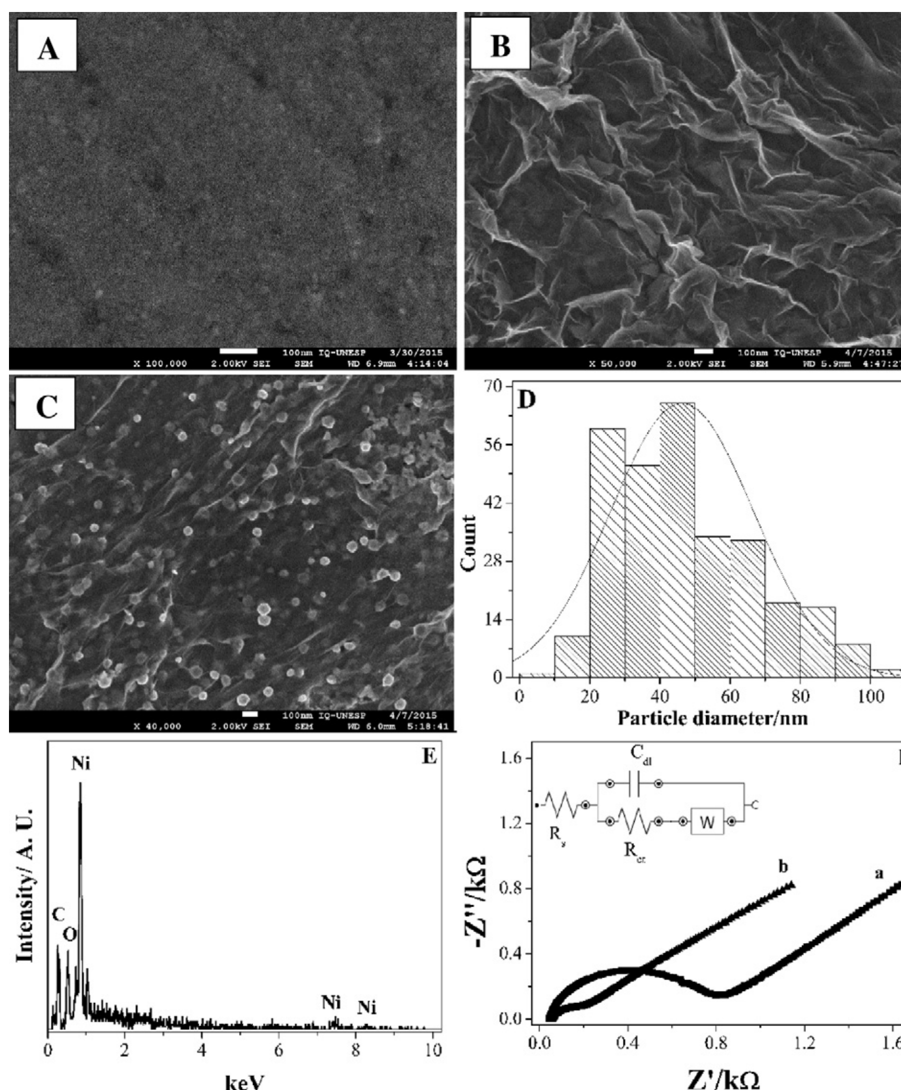


Fig. 1. SEM images of the (A) unmodified GCE, (B) GCE modified with reduced graphene oxide (RGO/GCE) and (C) RGO/GCE modified with nickel nanoparticles (NiNPs-RGO/GCE). (D) Histogram showing nanoparticle diameter distribution. (E) EDX analysis of NiNPs-RGO/GCE surface. (F) EIS spectra of (a) unmodified GCE and (b) NiNPs-RGO/GCE in 10×10^{-3} mol L^{-1} $Fe(CN)_6]^{4-/3-}$ in 0.10 mol L^{-1} KCl, and equivalent circuit inset.

EDX analysis was performed with the aim of verifying the composition of the NiNPs-RGO/GCE. The obtained EDX spectrum is shown in Fig. 1(E).The elemental composition obtained for the modified GCE indicated the presence of carbon, oxygen and nickel on the electrode surface. This result confirms the modification of the electrode with NiNPs.

EIS is widely used in investigating the electrochemical process regarding the transfer of electrons between the electrode/electrolyte interface. Fig. 1(F, curves a and b) presents the EIS diagram which typically includes a semi-circular part of the Nyquist plot and a linear part in the frequency range of 0.10 Hz to 100 kHz. The Randles circuit was used as an equivalent circuit for EIS data. The circuit was consisted of solution resistance (R_s), charge transfer resistance (R_{ct}), double layer capacitance (C_{dl}) and Warburg impedance (W) in parallel (inset in Fig. 1(F)). The characterization by EIS was performed with a solution of 1.0×10^{-2} mol L^{-1} $K_3[Fe(CN)_6]$ in 0.10 mol L^{-1} KCl which showed that the electron transfer resistance on the NiNPs-RGO/GCE (Rct, 153 Ω) surface decreased approximately 4.8 times compared to the unmodified GCE (Rct 738 Ω). The results obtained thereby confirm the success of the electrode modification with RGO and NiNPs in addition to the observed improvement in electron transfer.

3.2. Electrochemical behavior of the amine acids in alkaline medium

A comparative study on the electrochemical behavior of different electrodes was conducted in 0.10 mol L^{-1} of NaOH aqueous solution in the range of 0.10 to 0.80 V with a scan rate of 100 mV s^{-1} using CV. No redox peaks were observed on the unmodified GCE (Fig. 2, curve a) and RGO/GCE (Fig. 2, curve c). Interestingly, the RGO/GCE showed higher background current, indicating an increase in the active surface area of the RGO. When the GCE (Fig. 2, curve b) and RGO/GCE (Fig. 2, curve d) were modified with nickel nanoparticles, a pair of well-defined redox peaks was obtained in the cyclic voltammograms of the modified electrodes. The anodic peak potential (E_{pa}) observed at about 0.49 V along with the cathodic peak potential (E_{pc}) at about 0.38 V are attributed to the Ni(II)/Ni(III) redox couple on the NiNPs/GCE in alkaline medium. The redox peaks with a peak to peak separation (ΔE_p) of approximately 0.11 V can be observed on the NiNPs/GCE as shown in Fig. 2 (Curve b).The NiNPs-RGO/GCE exhibited a pair of redox peaks with E_{pa} at 0.57 V, E_{pc} at 0.32 V, ΔE_p of about 0.25 V while the peaks were all found to be higher and wider than those of NiNPs/GCE (Fig. 2, Curve d).The anodic peak current of NiNPs-RGO/GCE was found to be 3.2 times higher than that of NiNPs/GCE; this can be attributed to the larger surface area, excellent conductivity, the enhanced loading amount of the NiNPs electrocatalyst on the RGO surface and better electrocatalytic properties of the NiNPs-RGO/GCE. The electrochemical

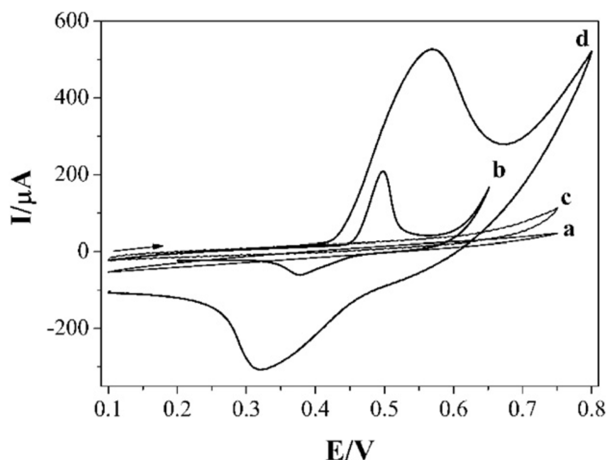
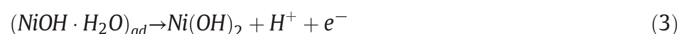


Fig. 2. Cyclic voltammograms obtained in 0.10 mol L^{-1} NaOH solution on (a) unmodified GCE, (b) NiNPs/GCE, (c) RGO/GCE and (d) NiNPs-RGO/GCE with a scan rate of 100 mV s^{-1}.

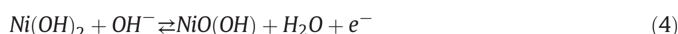
reactions of the coupled Ni(II)/Ni(III) in alkaline medium can be simply described as follows [41–43]:

$$Ni^0 + H_2O \rightarrow (NiOH)_{ad} + H^+ + e^- \quad (1)$$

$$(NiOH)_{ad} + H_2O \rightarrow NiOH \cdot H_2O_{ad} \quad (2)$$



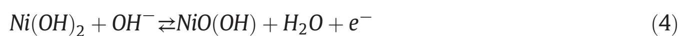
$$(NiOH \cdot H_2O)_{ad} \rightarrow Ni(OH)_2 + H^+ + e^- \quad (3)$$



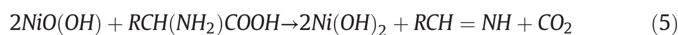
$$Ni(OH)_2 + OH^- \rightleftarrows NiO(OH) + H_2O + e^- \quad (4)$$

The electrooxidation of amino acids in 0.10 mol L^{-1} of NaOH was investigated by CV using unmodified GCE, RGO/GCE and NiNPs-RGO/GCE. Our observation was that 1.0×10^{-3} mol L^{-1} of amino acids in alkaline medium did not show significant current on the unmodified GCE and RGO/GCE at the potential range of − 1.5 and 1.5 V with scan rate of 100 mV s^{-1} (Fig. S3 and Fig. S4). When the RGO/GCE was modified with nickel nanoparticles, an oxidation peak of amino acids was observed. The NiNPs in alkaline medium electrochemically generate the NiOOH species which exhibit a powerful electrocatalytic activity during the oxidation of organic compounds [9,44]. Typical cyclic voltammograms for the electrooxidation of amino acids alanine, leucine, glycine, glutamic acidand aspartic acid in alkaline medium obtained at the potential range of 0.10 to 0.80 V are shown in Fig. 3.

Fig. 3(A) shows the cyclic voltammogram of the NiNPs-RGO/GCE in the absence and presence of alanine in alkaline solution. In the absence of alanine, a pair of well-defined redox peaks was obtained on the NiNPs-RGO/GCE. Following the standard addition of 5.0×10^{-3} mol L^{-1} of alanine, the anodic peak current of NiNPs-RGO/GCE was found to undergo a significant increase while the cathodic peak current declined, this behavior, in effect, evidences the catalytic oxidation of the amino acids by Ni(III) species. The anodic peak current of alanine tends to increase linearly with the increase in concentration of up to 1.0×10^{-2} mol L^{-1} in the region ranging from 0.44 and 0.73 V during the anodic sweep. Similar electrochemical behavior was observed for the amino acids - leucine, glycine and glutamic acid, as can be seen in Fig. 3(B), (C) and (D), respectively. In the presence of aspartic acid, the anodic peak current exhibited an oxidation peak ranging from 0.45 to 0.74 V while increasing linearly with the concentration of analytes of up to 1.0×10^{-2} mol L^{-1} (Fig. 3E).The Ni(II)/Ni(III) species on the electrode surface act as catalyst for the oxidation of amino acids. When amino acids reach the electrode surface, they are rapidly oxidized to aldehyde, carbon dioxide and ammonia by the Ni(III) species on the modified electrode. The electrocatalytic oxidation mechanism of amino acids on the NiNPs-RGO/GCE surface may be described by the following reactions [11,26,45,46]:



$$Ni(OH)_2 + OH^- \rightleftarrows NiO(OH) + H_2O + e^- \quad (4)$$



$$2NiO(OH) + RCH(NH_2)COOH \rightarrow 2Ni(OH)_2 + RCH = NH + CO_2 \quad (5)$$

$$2NiO(OH) + RCH = NH + H_2O \rightarrow 2Ni(OH)_2 + RCHO + NH_3 \quad (6)$$

Scan rate studies were conducted so as to aid our understanding of the electrochemical behavior of amino acids in 0.10 mol L^{-1} of NaOH solution. Scan rates were studied in the range of 10 to 500 mV s^{-1} and sixteen points were measured. Representative cyclic voltammograms of amino acids oxidation on the modified electrode surface with a scan rate of 100 mV s^{-1} are shown in Fig. 3. The anodic and cathodic peak currents were found to increase linearly with the square root of the scan rate in the range of 10 to 500 mV s^{-1}, indicating that the electron transfer reactions of the amino acids alanine, glycine, leucine, aspartic acid and glutamic acid are controlled by the diffusion process.

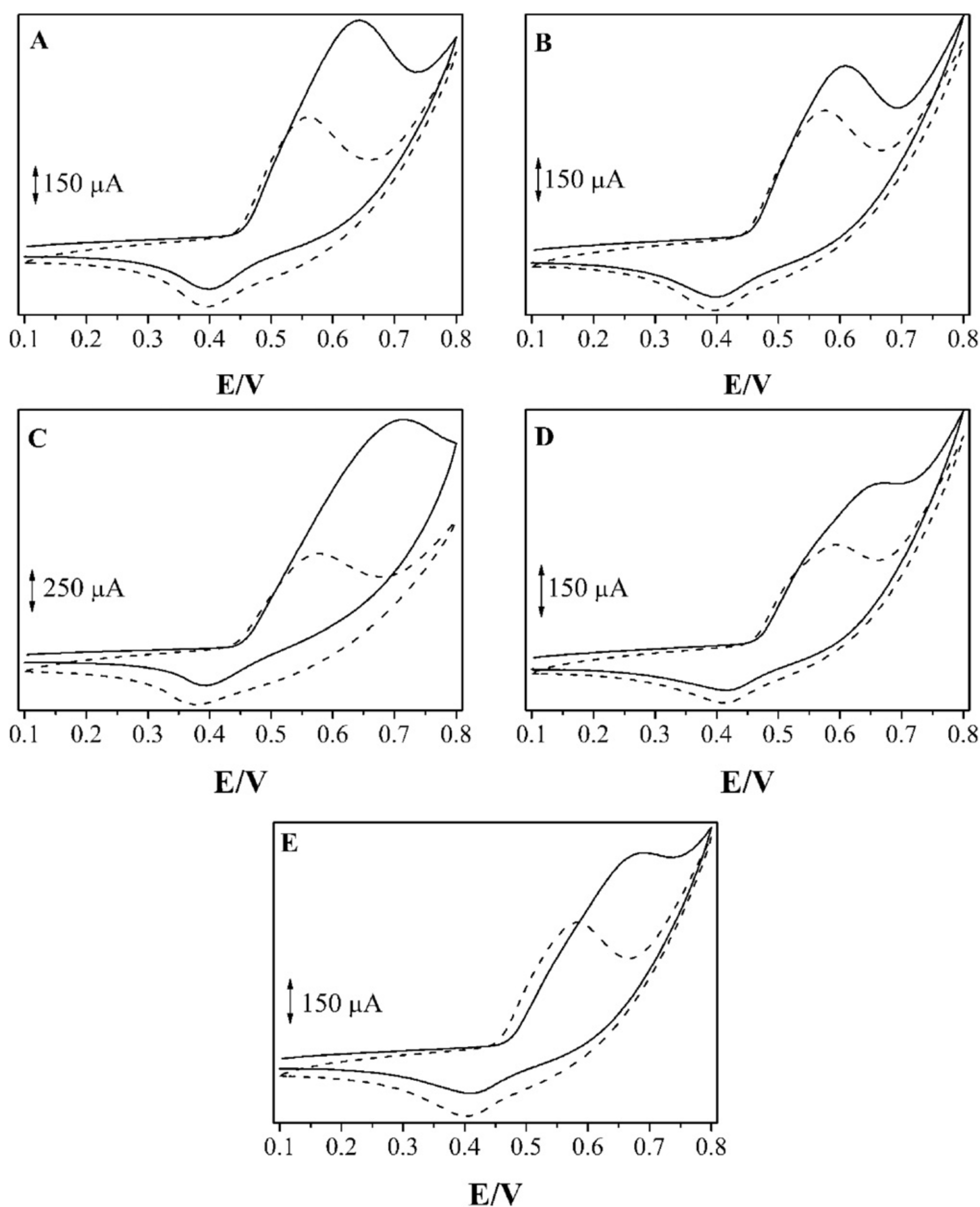


Fig. 3. Cyclic voltammograms on NiNPs-RGO/GCE in 0.10 mol L^{-1} NaOH solutions (dashed curves) and in the presence of Alanine (A), Leucine (B), Glycine (C), Glutamic acid (D) and Aspartic acid (E). Scan rate, 100 mV s^{-1} with concentration of 5.0×10^{-3} mol L^{-1}.

3.3. *Effect of applied potential and stability of the modified electrode*

An investigation was initially carried out on the potential of detection of the amino acids. The study of the applied potential using NiNPs-RGO/GCE as the amperometric sensor was undertaken using chronoamperometry. Chronoamperometric measurements were conducted at the potential range of 0.45 to 0.70 V in 0.10 mol L^{-1} of NaOH solution under agitation of 1000 rpm. These same conditions were used to simulate those under flow and stability of the modified electrode (Fig. 4). Fig. 4(A) shows the response of the NiNPs-RGO/GCE in amino acids solution. Following a period of 100 s to stabilize the current, an 80 μL aliquot of 1.3×10^{-1} mol L^{-1} of amine acid in 0.10 mol L^{-1} of NaOH stock solution was added to 10 mL of the supporting electrolyte (0.10 mol L^{-1} of NaOH solution), resulting in a 1.0×10^{-3} mol L^{-1} of amino acid solution. The current responses of analytes can be seen to increase as the potential increased from 0.45 to 0.50 V for the aspartic and glutamic acids. When the potential increases above 0.50 V, the analytical signs of these amino acids tend to decrease, negatively affecting the most sensitive detection of the analytes. The maximum current responses of the amino acids - alanine, glycine and leucine were observed at 0.55 V. Above this potential, the current responses of the amino acids are found to decrease with the increasing applied potential detection. Thus, an applied potential of 0.55 V was chosen as the optimal detection potential by virtue of it being more sensitive to a large number of analytes.

The stability of the NiNPs-RGO/GCE was investigated by recording the current response for 1.0×10^{-3} mol L^{-1} of glycine in 0.1 mol L^{-1} of NaOH. Following the current stabilization period of 300 s, the analyte was added to the alkaline solution. Fig. 4(B) presents the chronoamperometric response on the NiNPs-RGO/GCE for the addition of 1.0×10^{-3} mol L^{-1} of glycine in 0.10 mol L^{-1} of NaOH solution at an applied potential of 0.55 V, from which a rapid response to the addition of glycine (within 0.38 s) can be obtained. The response time for the amino acids - alanine, aspartic, glutamic and leucine were 0.92 s, 0.84 s, 0.61 s and 0.57 s, respectively. The electrochemical response of the modified electrode was only found to diminish at about 0.060%

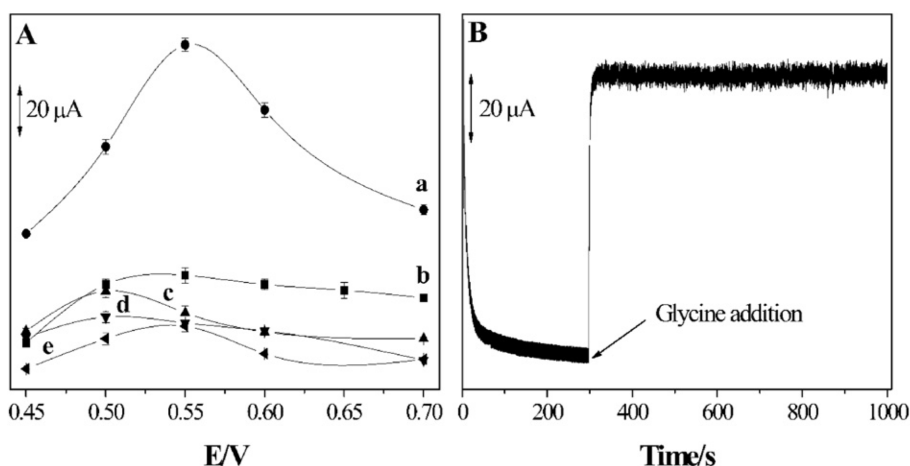


Fig. 4. (A) Chronoamperometric responses of the modified electrode to amino acids (a) glycine, (b) alanine, (c) aspartic, (d) glutamic and (e) leucine at various detection potential ($n = 3$). (B) Chronoamperometric response of glycine for a period of 1000 s at a potential of 0.55 V. Conditions: in a stirring solution of 0.10 mol L^{-1} NaOH (1000 rpm) and additions of 80 μL aliquot of 1.3×10^{-2} mol L^{-1} amino acids stock solutions.

after 700 s. In addition to that, the repeatability of the modification procedure ($n = 3$) of the GCE with RGO and NiNPs was also investigated. Chronoamperometric experiments were performed using the procedure described above for the potential detection study of amino acids. The repeatability of modifications, expressed as percentage of relative standard deviation (RSD, %) of glycine oxidation current (138 ± 2.6 μA with detection potential of 0.55 V) for a set of three consecutive modifications was approximately 2.0%. The results clearly demonstrate the excellent stability that can be achieved once this proposed modified electrode is employed - i.e. NiNPs-RGO/GCE.

3.4. Optimization of chromatographic separation

Through the use of NiNPs-RGO/GCE as detector and pulsed amperometric detection (PAD), we conducted an investigation regarding the best pulse sequence applicable in the determination of the amino acids. The PAD technique was selected for the analysis of the amino acids owing to the fact that it prevents the contamination of the modified electrode surface by adsorbed products that can be generated from the analyte oxidation reaction which may interfere with the electrochemical signal of interest. Furthermore, this contamination, if not hindered, is capable of affecting the charge transfer rate between the modified electrode and the analytes. This PAD technique enables the electrode response to present a degree of stability for a longer period of time due to the periodic electrochemical cleaning performed during chromatographic runs, thereby preventing or reducing the contamination of the NiNPs-RGO/GCE surface [47]. A sequence of four potentials employed in the PAD was determined through the cyclic voltammograms of the NiNPs on the RGO/GCE surface (Fig. 2, curve d). This analysis was conducted based on our work presented previously [48]. The first potential pulse (E_1) is applied aiming at conditioning the NiNPs-RGO/GCE where the amino acids are oxidized. The second potential (E_2) applied is set less positively so as to reduce organic species that have a potential lower than the amino acids oxidation. The third potential (E_3) is set more positively to enable the oxidization of all the species present on the modified electrode surface. This oxidation of all the nickel species on the modified electrode surface takes place during the fourth pulse (E_4), which is, in addition, used to stabilize the baseline of the chromatogram. The potentials and time periods for the pulsed amperometric detection were: E_1, 0.55 (alanine, glycine and leucine) or 0.50 V (glutamic and aspartic acids) for 300 ms; E_2, 0.05 V for 30 ms; E_3, 0.75 V for 30 ms and E_4, 0.50 V for 100 ms (Fig. S5).

The effects of the eluent concentrations on the retention times as well as the separation resolutions of the amino acids were also investigated. Amino acids were seen to have a high electrocatalytic response on the NiNPs-RGO/GCE surface in alkaline medium (not shown). This result indicates that a high concentration of alkaline solution is needed to ionize and oxidize the amino groups of the amino acids. Furthermore, alkaline eluents are required for the separation and detection of analytes by ion chromatography. NaOH solutions are able to meet these requirements for the amino acids - alanine, glycine and leucine. An investigation was, thus, carried out on NaOH concentration so as to evaluate the retention time behavior of the amino acids dependent on the concentration of this eluent. An increase in the NaOH concentration

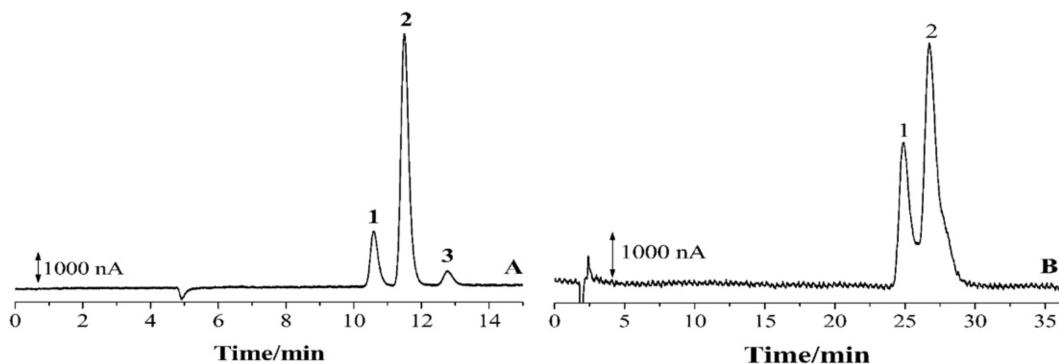


Fig. 5. (A) Chromatogram of a mixture of 5.0×10^{-4} mol L^{-1} alanine (1), glycine (2) and leucine (3).[a] (B) Chromatogram of a mixture of 2.5×10^{-4} mol L^{-1} glutamic (1) and aspartic acids (2).[b] [a]Conditions: Isocratic elution with mobile phase 0.18 mol L^{-1} NaOH, detection potential of 0.55 V vs. Pd and flow rate of 0.30 mL min^{-1}. [b]Conditions: Isocratic elution with mobile phase 0.10 mol L^{-1} CH_3COONa at 0.10 mol L^{-1} NaOH, detection potential of 0.50 V vs. Pd and flow rate of 0.8 mL min^{-1}.

Table 1
Effect of hydroxide and sodium acetate concentrations on the retention times (t_r) and retention factors (k) for aspartic and glutamic acids.[a]

Analyte		Sodium acetate/mol L^{-1}					Sodium acetate in sodium hydroxide/mol L^{-1} (1:1)				
		0.10	0.15	0.20	0.25	0.30	0.12	0.16	0.20	0.30	0.40
Aspartic acid	t_r (min)	65.5	39.8	26.5	19.1	15.2	51.0	29.9	20.3	11.3	8.64
	k	12.2	6.98	4.35	2.83	1.93	9.30	5.00	3.08	1.23	0.71
Glutamic acid	t_r (min)	70.6	42.2	27.7	19.5	15.2	48.0	28.5	19.9	11.3	8.64
	k	13.3	7.51	4.58	2.91	1.93	8.68	4.72	3.00	1.23	0.71

[a] Experimental conditions: Detection potential of 0.55 V, flow rate of 0.30 mL min^{-1} and 1.2×10^{-4} mol L^{-1} aspartic and glutamic acids.

was found to be accompanied by a decrease in the amino acid retention time while the current responses became more intense once the amino acids were being more easily oxidized to higher pH. Isocratic chromatographic separation of the analyte was completed within 15 min through the use of 0.18 mol L^{-1} of NaOH solution. A typical chromatogram of a mixture of amino acid standard solution detected on the NiNPs-RGO/GCE is shown in Fig. 5. A good chromatographic separation of the three amino acids obtained with symmetrical peaks in a short analysis time is presented in Fig. 5(A). For a faster eluting of the aspartic and glutamic acids, the addition of CH_3COONa in the mobile phase is required inasmuch as these amino acids interact stronger with the stationary phase column.

The effect of CH_3COONa concentration on the retention times of the amino acids (aspartic and glutamic acids) was investigated in the range of 0.10 to 0.30 mol L^{-1} in 0.10 mol L^{-1} of NaOH. The retention times of the analytes were found to decrease with increasing concentration of ion acetates owing to the fact that these ions preferentially interact with the stationary phase while the elution order remained the same in isocratic elution. Interestingly enough, however, we found that the addition of CH_3COONa in the mobile phase causes the analytes co-elution. The NaOH and CH_3COONa concentration at the ratio of 1:1 was evaluated with a standard solution mixture of 1.2×10^{-4} mol L^{-1} of aspartic and glutamic acids prepared with the mobile phase composition for isocratic elution in the range of 0.12 to 0.40 mol L^{-1}. It is worth pointing out that, increasing the concentration of the mobile phase is found to give rise to a decrease in the retention times and co-elution of the analytes, besides contributing towards an increase in the baseline noise. The results indicated that the best separation of aspartic and glutamic acids is promoted by the mobile phase composed of 0.10 mol L^{-1} of CH_3COONa and 0.10 mol L^{-1} of NaOH, as can be seen summarized in Table 1. The effect of flow rate on the retention times of the analytes was also an object of our assessment. The decrease in flow rate was found to lead to an increase in the retention times without inverting the order of elution. Fig. 5(B) shows the chromatogram of the separation of aspartic and glutamic acids obtained using 0.10 mol L^{-1} of CH_3COONa along with 0.10 mol L^{-1} of NaOH as mobile phase.

The chromatographic parameters such as dead time (t_m), retention time (t_r), adjusted retention time (t_r'), resolution (R_s), number of theoretical plates (N), separation factor (α) and retention factor (k), were all found to be within the expected results for a good chromatographic performance as can be seen in Table S1.

The analytical performance of NiNPs-RGO/GCE, used as amperometric detector, was evaluated according to the optimal chromatographic and PAD conditions described above. Standard solutions were prepared with concentrations within the range of 1.0×10^{-6} to 1.0×10^{-3} mol L^{-1}. Calibration curves were evaluated through the analysis of the standard solutions concentrations (x, mol L^{-1}) with respect to the peak areas (y, nA min). The linear range, detection limit (LOD), amperometric sensitivity, correlation coefficient and RSD% can all be found summarized in Table 2. The peak areas varied linearly with the concentration over a range of approximately two to three orders of magnitude for these five amino acids with minimum coefficient of correlation of 0.9997. LOD was determined for $S/N = 3$ from the lowest injected concentration. The LODs ranged from 8.7×10^{-7} mol L^{-1} for aspartic acid to 2.0×10^{-5} mol L^{-1} for leucine. The NiNPs-RGO/GCE used as amperometric detector presented high sensitivity for the analytes and ranged from 3.3×10^5 to 5.3×10^6 nA mol^{-1} L for leucine and glycine, respectively. Repeatability was expressed as a percentage of RSD% of twelve repetitive chromatographic injections at 1.0×10^{-4} mol L^{-1} concentration of each analyte. The RSD was found ranging from 1.1 (leucine) to 2.6% (aspartic acid) for the twelve successive measurements carried out, indicating excellent repeatability.

The stability of the NiNPs-RGO/GCE was investigated using developed method over a 24 h period employing a single modified electrode and 5.0×10^{-4} mol L^{-1} amino acids solutions. The results showed that the initial response could be maintained at $98 \pm 1.3\%$ (alanine, glycine and leucine) and $93 \pm 2.0\%$ (aspartic and glutamic acids) even after 24 h continuous detection and, thus, the NiNPs-RGO/GCE has good long-term stability.

3.5. Determination of amino acids in sugarcane vinasse sample

To demonstrate the applicability of the NiNPs-RGO/GCE as an amperometric detector in ion chromatography with pulsed amperometric detection, the determination of amino acids in sugarcane vinasse from sugar and ethanol plant was conducted. The method employed for the sample preparation has already been described in the *Sample preparation section*. The peaks identification was based on the retention time specific to the analytes and was confirmed by adding authentic standard solutions to the diluted samples. Representative chromatograms of sugarcane vinasse samples are shown in Fig.6. The amino acids were quantified in the sugarcane vinasse using the standard addition method. Three different concentrations of amino acids standard solutions were added to the sample. Analysis was performed in triplicate and the calibration curve was obtained by the plot of the peak area of the amino acid each versus the related added concentration. The quantitative results are shown in Table 3. As can be clearly observed, sugarcane vinasse sample contains in its composition alanine, glycine, leucine and aspartic acids. Glutamic acid was not detected while leucine presented the highest concentration in the analyzed sample. Good correlation coefficients (≥0.993) of the calibration plots apparently confirm the absence of adsorbed products capable of affecting the catalytic performance on the NiNPs-RGO/GCE surface. The average recoveries obtained by three chromatographic injections of each spiked sample ranged from 94.8%

Table 2
Analytical performance of amino acids using NiNPs-RGO/GCE as amperometric detector ($n = 3$).[a]

Analyte	Linear range (mol L^{-1})	Linear regression equation	LOD (mol L^{-1})	Sensitivity (nA mol^{-1} L)	Correlation coefficient	RSD (%)
Alanine	1.0×10^{-5} to 7.5×10^{-4}	$y = 1.1 \times 10^6x + 4.2$	4.3×10^{-6}	$1.1 \times 10^6 \pm 4.6 \times 10^3$	0.9999	1.8
Glycine	1.0×10^{-5} to 7.5×10^{-4}	$y = 5.3 \times 10^6x + 3.6$	1.9×10^{-6}	$5.3 \times 10^6 \pm 4.3 \times 10^4$	0.9998	1.4
Leucine	5.0×10^{-5} to 1.0×10^{-3}	$y = 3.3 \times 10^5x + 9.6$	2.0×10^{-5}	$3.3 \times 10^5 \pm 1.1 \times 10^4$	0.9997	1.1
Aspartic acid	2.5×10^{-6} to 7.5×10^{-4}	$y = 2.9 \times 10^6x + 2.6$	8.7×10^{-7}	$2.9 \times 10^6 \pm 9.2 \times 10^3$	0.9999	2.6
Glutamic acid	5.0×10^{-6} to 7.5×10^{-4}	$y = 1.3 \times 10^6x + 4.1$	3.0×10^{-6}	$1.3 \times 10^6 \pm 1.2 \times 10^4$	0.9997	1.5

[a] Experimental conditions as depicted in Fig. 5.

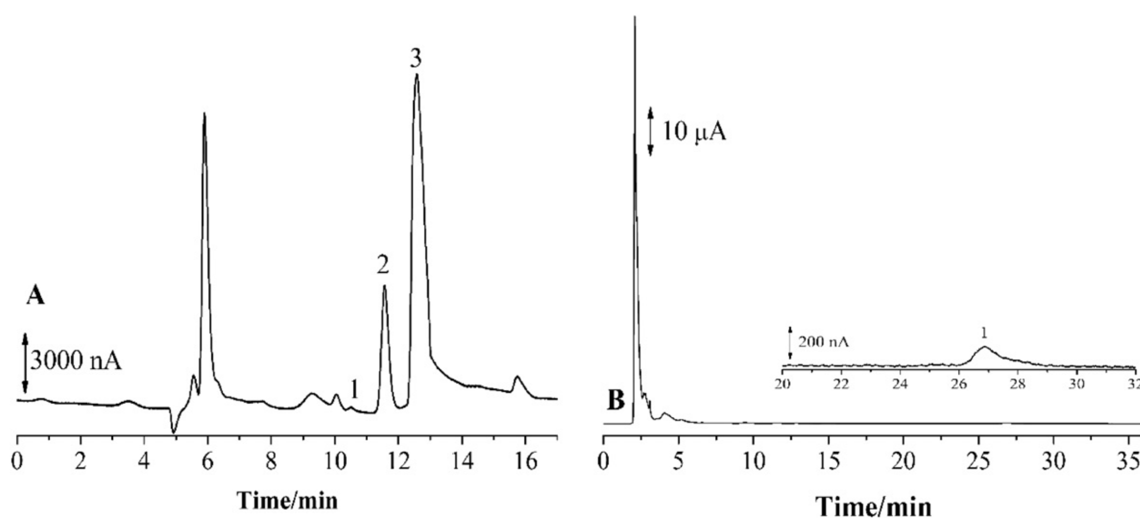


Fig. 6. (A) Chromatogram separation of a sugarcane vinasse sample diluted at 1:100 with ultrapure water. Peaks: alanine (1), glycine (2) and leucine (3). (B) Chromatogram separation of a sugarcane vinasse sample diluted 1:10 with ultrapure water. Peak: aspartic acid (1). Experimental conditions as in Fig. 5.

for alanine to 99.6% for glycine, indicating the good accuracy of the developed method. The precision was estimated through RSD% using chromatographic experiments in triplicate of the spiked samples with three levels of concentrations within the range of 1.7% to 4.9% for all the determined compounds. The found concentrations were statistically compared to nominal concentrations using unpaired Student *t*-test. The results at 95% confidence level presented t_{value} smaller than $t_{theoretical}$ for the amino acids analyzed, thereby indicating that the NiNPs-RGO/GCE can be used as amperometric detector for the determination of amino acids.

4. Conclusions

Reduced graphene oxide decorated with nickel nanoparticles on modified glassy carbon electrode presented high electrocatalytic activity with regard to amino acids oxidation characterized by repeatability, precision and stability. The use of mobile phase composed of 0.10 mol L^{-1} of CH_3COONa at 0.10 mol L^{-1} of NaOH in the ratio 1:1 as eluent solution enabled the isocratic separation of aspartic and glutamic acids in <30 min with good peak resolution and analytical sensitivity. The modified electrode was successfully applied as an amperometric detector for the determination of some amino acids by ion chromatography combined with pulsed amperometric detection having displayed essentially remarkable features including high sensitivity, good recoveries, interference free and accuracy in the sugarcane vinasse sample investigated in this work. The method appears to be useful and effective when it comes to the customary practical determination of common amino acids in complex real matrices without any derivatization procedure or extraction. Furthermore, the relatively good stability observed from our experiment results has proven the huge potential of this modified electrode as an amperometric sensor in analytical applications.

Table 3
Amino acids content in sugarcane vinasse.[a]

Analyte	Determined (mol L^{-1})	Recovery (%)	RSD ($n = 3$)	t_{value}[b]
Alanine	$1.5 \times 10^{-3} \pm 2.3 \times 10^{-5}$	95 ± 4.7	4.9	1.9
Glycine	$1.4 \times 10^{-2} \pm 4.0 \times 10^{-4}$	100 ± 1.7	1.7	1.1
Leucine	$2.4 \times 10^{-2} \pm 4.5 \times 10^{-4}$	98 ± 4.7	4.9	0.9
Aspartic acid	$3.2 \times 10^{-4} \pm 3.3 \times 10^{-6}$	99 ± 1.9	1.9	1.1
Glutamic acid	Not detected	99 ± 3.7	3.7	0.6

[a] Experimental conditions as depicted in Fig. 5.
[b] $t_{theoretical} = 4.3$ at 95% confidence level.

Notes

The authors have no any conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.microc.2017.07.007.

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