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# Paper-based enzymatic platform coupled to screen printed graphene-modified electrode for the fast neonatal screening of phenylketonuria

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ABSTRACT

*Introduction:* The PKU is an inborn error of amino acid metabolism, in which phenylalanine (Phe) accumulated in the blood causing alterations at the central nervous system. We report a novel paper-based enzymatic platform coupled to screen printed graphene-modified electrode for the neonatal screening of phenylketonuria (PKU0.
*Methods:* The paper-based analytical device coupled to electrochemical detection (EPAD) is based on the use of paper microzones modified with phenylalanine dehydrogenase enzyme (PheDH). The modified PADs were placed on the surface of an electrode modified with electrochemically reduced graphene (ERGO). PheDH in the presence of $NAD^+$ catalyzes the reversible deamination of Phe to form phenylpyruvate, ammonia, and NADH. The electrochemical oxidation of NADH was monitored by differential pulse amperometry (DPA) at 0.6 V. The method was linear in the concentration range from 1 to 600 µmol/L of Phe with a LOQ of 1 µmol/L and LOD of 0.2 µmol/L. Within day precision was 5.7% across 3 levels of control samples. Between-day precision was 8.3%. The comparison with the standard Phe enzyme assay kit showed good agreement. The time required for the overall assay was < 5 min. The non-sophisticated equipment required, the short assay time and the appropriate LOQ and LOD achieved by our EPAD make it an attractive and easy to use alternative compared to existing methods applied to the screening of PKU in neonatal samples.

## 1. Introduction

Phenylketonuria (PKU) is an inborn error of amino acid metabolism generated by the decrease or deficiency of the activity of phenylalanine hydroxylase hepatic enzyme complex. The activity alteration of this enzyme which catalyzes the conversion of phenylalanine (Phe) to tyrosine causes the accumulation of Phe in blood, used as a biomarker for the PKU screening [1].

Phe increased level causes severe mental retardation, developmental delay, epilepsy and behavioral alterations [2,3]. To prevent these clinical anomalies a rapid diagnosis and the introduction of a reduced diet in phenylalanine is required before 3 or 4 weeks of life [4].

Routine diagnosis of PKU relies mainly on the use of enzymatic assays to detect increased levels of Phe [5–7]. Other methods include chromatography [8,9] and mass spectrometry [10–13]. These techniques allow the development of sensitive methods, but they have some disadvantage such as time-consuming, large volume of sample and expensive equipment. Consequently, the development of a precise, disposable and straightforward device represents a challenge in the clinical diagnosis of PKU.

In recent years, the paper has become a new platform for the construction of paper-based analytical devices (PAD). The use of paper in the development of biosensors has significant advantages such as large-scale production, low cost, versatility and ease of handling (printing, coating, and impregnation). Moreover, the paper allows the physical adsorption of proteins (enzymes), without denaturation [14].

The PADs can be combined with different detection methods, such as colorimetric [15], absorbance [16], electrochemistry [17,18], chemiluminescence [19], Raman spectroscopy [20] and mass spectrometry [21]. Among them, the electrochemical detection represents a suitable technique for the development of compact, portable and economic devices [22,23].

In this context, the use of carbon-based nanomaterials for the modification of working electrodes improves the electrochemical signal in analytical devices [24]. Graphene has emerged as an interesting nanomaterial for the development of electrochemical biosensors. Graphene oxide (GO) is usually obtained via Hummers' method [25], followed by chemical reduction of GO with hydrazine [25]. The electrochemical reduction (ER) of GO enhance the electrochemical conductance and increase the specific surface area of the working electrode, improving the analytical performance [26].

The enzymatic determination of Phe is based on the detection of β-

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nicotinamide adenine dinucleotide (NADH) which acts as a cofactor of the enzyme phenylalanine dehydrogenase (PheDH). NADH generated in the enzymatic reaction can be oxidized over the working carbon-based electrode generating an anodic signal which requires an over potential of about one volt [27]. The use of graphene for the modification of carbon-based electrodes decreases the overpotential of NADH, due to the high density of edge-plane-like defective sites in the graphene which provide many active sites for the transfer of electrons to NADH [28–31].

The objective of this study was the development of a novel EPAD for the PKU screening in neonatal samples. For the construction of our EPAD, the enzyme PheDH was immobilized over the paper microzones by physical adsorption. Then, the modified PADs were placed on the surface of a screen printed carbon electrodes modified with ERGO. The electrochemical oxidation of the enzymatically generated NADH was monitored by differential pulse amperometry (DPA) applying a constant potential of 600 mV. The oxidation current of NADH was directly proportional to the levels of Phe in neonatal blood samples. The proposed EPAD represents an attractive and easy to use alternative in comparison to the existing methods applied in the screening of PKU in neonatal samples.

## 2. Experimental

### 2.1. Materials and reagents

All reagents were of analytical grade. Phenylalanine (Phe), phenylalanine dehydrogenase (PheDH), β-nicotinamide adenine dinucleotide (NAD) sodium salt and graphite powder were from Sigma Chemical Co. Glycine buffer pH 10.50 composed of glycine (Gly), sodium hydroxide, sodium chloride, sodium nitrate, sulfuric acid, potassium permanganate and hydrogen peroxide were from Merck. The Whatman® qualitative filter paper, grade 1 was chosen as a working paper. The graphene oxide was synthesized from graphite according to Hummers's method [26]. Double distilled water for preparing all solutions was used.

### 2.2. Instruments

Electrochemical measurements were performed using a BAS 100 B/W (Bioanalytical Analyzer Electrochemical Systems). Cyclic wave voltammograms and amperograms were obtained using a screen-printed carbon electrode (SPCE, ItalSens IS-C–PalmSens), which is composed of a circular graphite working electrode (∅ = 3 mm). Silver and graphite electrodes were used as the pseudo-reference and auxiliary electrode, respectively All pH measurements were made with an Orion Research Inc. (Orion Research Inc.) Model EA 940 equipped with a glass combination electrode (Orion).

In the production of oxidized graphene by the exfoliation procedure was used an ultrasonicator (Testlab) model TB02 of 50/60 Hz.

The morphology of graphene nanofilms on the surface of the working electrode was studied by scanning electron microscopy (SEM) LEO 1450VP.

### 2.3. Synthesis of GO

GO dispersion was prepared from graphite powder according to the modified Hummers' method [25]. In brief, 48 mL of a concentrated $H_2SO_4$ solution containing 1 g $NaNO_3$ was added to 1 g of graphite powder, and the mixture was cooled down to 0 °C in an ice bath. Then, 6 g of $KMnO_4$ were gradually added, and the mixture was allowed to react for 3 h under mechanical stirring. Next, 200 mL of $H_2O_2$ 3% were added to the mixture, and it was stirred for 30 min. For the purification step, the mixture was washed with bidistilled water until reaching neutral pH. The obtained solid was resuspended in water until getting a brown dispersion. Then, the mixture was kept under ultrasound (50–60 Hz) during 8 h for the exfoliation of GO. Finally, the non-exfoliated GO was removed by centrifugation (3500 rpm, 30 min) and the solid was discarded.

### 2.4. Modification of the SPCE with graphene oxide and electrochemical reduction

To modify the working electrode surface of the SPCE, 5 μL of GO dispersion (30 μg/mL in $H_2O$) was added to the working electrode surface. The GO thickness was around 10 nm, accepting that the density of the solution was near to unity. Therefore, the concentration and volume of the GO solution regulate the thickness of the produced GO film used to cover the surface of the electrode. The ER of the oxygen-containing functional groups of GO was made applying a constant potential value of −1200 mV (vs. Ag-SPCE) in 0.5 mol/L pH 4.00 $NaNO_3$ for 800 s. Then, the modified electrode (ERGO-SPCE) was rinsed with ultra-pure water by mechanical stirring for 30 s, and finally, it was dried thoroughly with pure nitrogen gas.

### 2.5. Immobilization of the PheDH on the paper microzones

Paper microzones with a diameter of 6 mm were designed on Whatman # 1 filter paper using Corel Draw 9 software. The wax patterns were printed using a Xerox ColorQube 8870 printer. Once printed, the paper microzones were placed at 90 °C on a hot plate for 5 min to melt the wax through the paper, creating a 3-dimensional hydrophobic barrier. The PheDH enzyme was immobilized on the paper microzones surface by physical adsorption. Subsequently, the dried microzones were impregnated with 5 μL of a PheDH solution 12.50 U/L in glycine buffer 0.2 mol/L pH 10.50, incubated at 25 °C for 10 min, washed and dried at room temperature. Finally, the modified microzone was placed on the surface of an ERGO/SPCE.

### 2.6. Preparation/pretreatment of sample

For the PKU assay, the samples were collected and pretreated following the procedure proposed by Seia et al. [32] with the following modifications. In brief, the blood samples were spotted in the center of a 1 cm circle on filter paper number 903, trying to avoid tearing or disrupting the filter paper surface. Then, the samples were allowed to air dry overnight, avoiding heat, sunlight and absorbent surfaces. Finally, the samples were stored in a plastic envelope at 4 °C until assay. For the PKU assays, we used neonatal samples and control samples provided by the Blood Spot MW Phenylalanine enzyme assay kit..

In the Phe determination, a disk was punched from a blood spot collected filter paper, and it was placed into an Eppendorf tube with 200 μL in 0.2 mol/L pH 10.50 0.2 mol/L glycine buffer and exposed to a sonication procedure for 2 min. Finally, the content of all tubes was aspirated, and the eluted samples were stored at 4 °C until use. The total time for sample preparation was about 3 min.

### 2.7. Analytical performance studies

Analytical performance of the method was assessed according to the Clinical Laboratory Standards Institute (CLSI) guidelines [33]. Linearity, precision, quantification limit and method comparison were included in the parameters studied.

Linearity testing was performed adopting CLSI EP06-A guideline using mixtures of standard materials provided by the Blood Spot MW Phenylalanine enzyme assay kit. A series of concentrations were prepared by dilution of the standard material (600 μmol/L), which was mixed in different proportions with a blank standard material to cover the analytical measuring range, from 1 to 600 μmol/L of Phe. The mixtures of standard materials were then analyzed in duplicate, and the linearity of the obtained values was evaluated through linear regression analysis.

Precision was evaluated according to the CLSI EP05-A2 guideline

using control samples provided by the Blood Spot MW Phenylalanine enzyme assay kit. Briefly, between-analysis precision was assessed by measuring 3 levels of control samples in duplicate, using 2 separate analysis each day over 10 days ($n = 20$). Within-analysis precision was assessed by measuring 3 levels of control samples over the course of a single analysis (n = 20). The CV was calculated for each level of control samples.

Functional sensitivity (LOQ) was estimated following the CLSI EP17-A. Patient samples with Phe levels > 240 μmol/L were pooled. This pooled sample was then diluted at 1:2, 1:4, 1:16, and 1:32 using the blank serum and analyzed in triplicate. The concentration of Phe at which the imprecision exceeds a CV of 10% was determined.

In addition, a method comparison was carried out according to the CLSI EP9-A2 guideline. For this study, 20 blood samples were analyzed. Samples were measured using the proposed method, and the results were compared to samples measured the commercial Blood Spot MW Phenylalanine enzyme assay kit. Linear regression analysis was conducted along with Bland-Altman plots to assess the degree of correlation and bias between methods.

### 2.8. Phe determination

The enzymatic assay for the Phe determination in neonatal samples consisted in placing into an Eppendorf tube 9 μL of pretreated samples and 1 μL of 25 mmol/L $NAD^+$ solution in 0.2 mol/L glycine buffer pH 10.50. Then, 5 μL of this mixture was added over the modified PADs. PheDH in the presence of Phe from the blood samples catalyzes the NAD-dependent deamination of Phe to phenylpyruvate and NADH. The electrochemical oxidation of the enzymatically generated NADH was motorized by differential pulse amperometry (DPA) applying a constant potential of 600 mV (Fig. 1).

## 3. Results and discussion

### 3.1. Electrode characterization

For the electrochemical quantitation of Phe, the SPCEs were modified with electrochemically reduced GO. The electrochemical reduction of the oxygen-containing functional groups of GO was made applying a constant potential value of −1.20 V, which allows a slow reduction of the GO film avoiding defects in its structure, which usually appears in RGO generated by the repetitive reduction-oxidation of GO films using cyclic voltammetry [34]. Electrochemical reduction promotes the increase of the electrostatic attraction force between the electrode and the ERGO film by the elimination of the oxygen functional groups of GO sheets and the renovation of sp2 carbon. After the reduction process, we obtain a more compact, firm and stable ERGO film [34]. The Fig. 2a shows the electrochemical reduction profile of GO obtained using a constant voltage of −1.20 V. The reduction current grew almost linearly during the first seconds before leveling, indicating that the GO film became more conductive during the reduction process until the reaction completion.

The high-resolution SEM images allowed the observation and characterization of the graphene-modified electrode surface. The Fig. 2b shows that GO was uniformly spread over the surface of SPCEs due to good dispersion in aqueous solution by a large number of oxygen-containing groups, such as the epoxy/ether, carbonyl, and carbon carboxylate group. During electrochemical reduction stage, the aggregation of GO occurs due to the increase of π -π interaction between the layers of graphene (Fig. 2c). The Fig. 2d shows the EDS spectrum of GO and ERGO for the analysis of the elemental composition of the modified SPCE. The C/O atomic ratio of GO compared to ERGO increased 3.25 to 14.5, showing that most of the oxygen functional groups in the ERGO were eliminated.

The ERGO/SPCEs were electrochemically characterized by CV of 1 mmol/L $Fe(CN)_6^{4-/3-}$ solution in 0.1 mmol/L KCl (pH 6.50). The potential scan was ranged from −100 to 800 mV at a scan rate of 100 mV/s. The Fig. 3a shows a well-defined CV for an unmodified SPCE and ERGO/SPCE. The peak current obtained for ERGO/SPCE was larger than the one observed for bare SPCE, indicating that the RGO improved the conductivity and increased the active surface area of the electrode.

The optimum concentration of GO was studied between 1 and 100 μg/mL of GO in aqueous solution. After the electrochemical reduction of GO, each ERGO/SPCE was used for monitoring a $Fe(CN)_6^{4-/3-}$ solution. The effect of the scan rate on CVs obtained using the ERGO-SPCE was also studied (Fig. 3b). The oxidation and reduction current peaks exhibit a linear correlation with the square of scan rate (Fig. 3b inset) in the studied range (20–300 mV/s). These results indicate that this is a fast electrochemical and diffusion-controlled process. The Fig. 3c shows the evaluation of the ratio of the anodic and cathodic peak currents (Ipa/Ipc) as a function of the graphene concentration. The electrochemical reversibility decay when the graphene concentration is > 30 μg/mL. For this reason, the 30 μg/mL of GO solution was used for electrode modification.

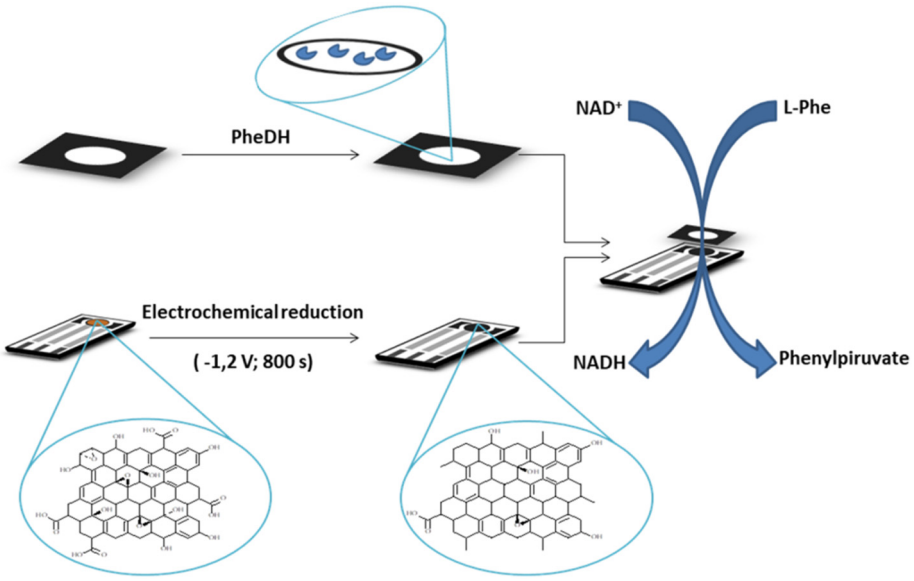


**Fig. 1.** Schematic representation of the electrochemical paper-based analytical device for the quantitative determination of Phe in neonatal samples.

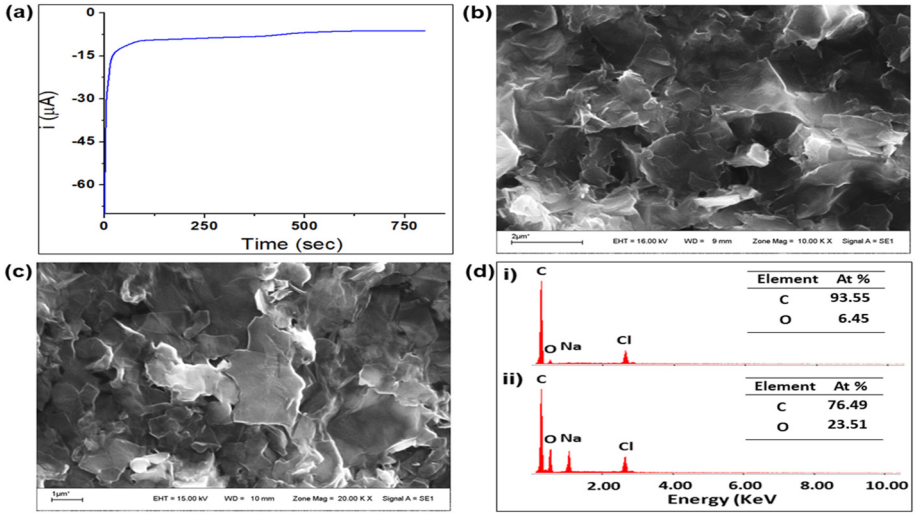


**Fig. 2.** Characterization: (a) i-t curve obtained from electrochemical reduction of GO at −1200 mV (vs. Ag/AgCl) in 0.5 mol/L $NaNO_3$ pH 4.00. (b) SEM image of GO/SPCE, (c) SEM image of ERGO/SPCE and (d) i) EDS spectra of ERGO, ii) EDS spectra of GO.

The electrochemical oxidation of NADH was evaluated by CV. The Fig. 3d shows the voltammograms of NADH 1 mmol/L in glycine buffer 0.2 mol/L pH 10.50 using an unmodified SPCE and ERGO/SPCE for the potential range 0–1000 mV (scan rate: 100 mV/s). The voltammograms exhibit a single irreversible anodic peak at 710 mV for the unmodified SPCE and 550 mV for ERGO/SPCE. Both voltammograms correspond to a one-electron transfer mechanism and expose the irreversible oxidation process of NADH in this reaction medium. The Fig. 3d reveals that the modification of the electrode surface with ERGO decreased the overpotential of NADH and increased the oxidation peak current and hence, the sensitivity of the method.

### 3.2. Parameters optimization

The Phe quantitation procedure required the optimization of several parameters: concentration of PheDH, the concentration of $NAD^+$ optimum pH value, the concentration of glycine buffer and time monitoring. These optimizations were done analyzing a Phe standard of 300 μmol/L.

The concentration of PheDH enzyme to be immobilized on the paper microzones surface was studied in the range of 5–15 U/mL to ensure the optimal measurement of Phe in blood spot samples. The optimal signal was observed with a PheDH concentration of 12.50 U/L, and it was used for all experiments. Also, the optimal concentration of the cofactor $NAD^+$ was studied in the range of 1 to 5 mmol/L. The optimal response was observed with an $NAD^+$ concentration of 2.5 mmol/L, and it was used in subsequent analyses.

The influence of the glycine buffer concentration was tested in the range of 0.01 to 0.5 mol/L to obtain the maximum signal for the system. The concentration of 0.2 mol/L was optimal, and therefore it was used in for all analyses. Also, the influence of pH over the enzymatic response of our biosensor was studied at 25 °C in the pH range of 6–12, exhibiting a maximum value of response at pH 10.50. The response rate increased from pH 6.0 to 10.5 and over these values the response dramatically decreased. Then, the pH used to evaluate the variables was 10.50 in 0.2 mol/L glycine buffer.

With the aim of optimizing the time for the electrochemical measurement, the electrochemical oxidation of the enzymatically generated NADH was monitored using our EPAD by DPA at 0.6 V for 5 min. The current response increased linearly until reaching a maximum at 1 min. Insignificant differences were observed above this time; thus, a monitoring time of 1 min was used. Table 1 shows the experimental conditions for Phe determination using the proposed methodology.

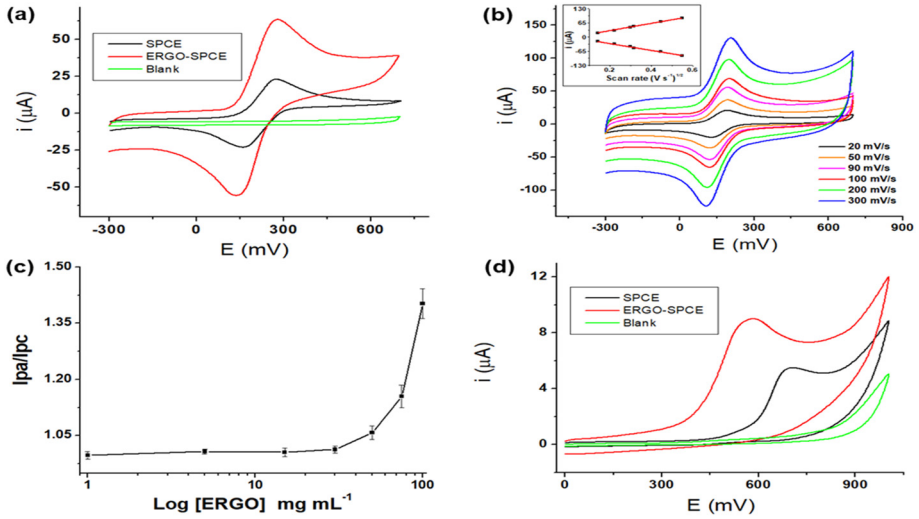


**Fig. 3.** (a) Cyclic voltammograms (scan rate: 100 mV/s) of unmodified SPCE and ERGO/SPCE in 0.1 mmol/L KCl (pH 6.50) containing 1 mM $Fe(CN)_6^{4-/3-}$. (b) CVs of modified electrode with ERGO in 5.0 mmol/L $Fe(CN)_6^{4-/3-}$ at different scan rates from 20 to 300 mV/s. Inset: plot of peak current (ip) vs. scan rate. (c) Effect of the variation of GO concentration for the SPCE modification process. (d) CV of unmodified SPCE, ERGO/SPCE in 1 mmol/L NADH solution in glycine buffer 0.2 mol/L pH 10.50. (scan rate: 100 mV/s and potential range: 0–1 V).

**Table 1**
Experimental conditions for Phe determination using the proposed methodology.

| Optimized parameter | Evaluated range | Optimum value |
|---|---|---|
| PheDH concentration | 5–15 U/mL | 12.50 U/mL |
| $NAD^+$ concentration | 1–5 mmol/L | 2.5 mmol/L |
| Glycine buffer concentration | 0.01–0.5 mol/L | 0.2 mol/L |
| pH of substrate solution | 6–12 | 10.5 |
| Monitoring time | 0.1–3 min | 1 min |

### 3.3. Phe quantitative determination

For the quantitative determination of Phe, the amperometric response of our system was evaluated at 0.6 V vs. Ag/AgCl at 25 ± 1 °C in 0.2 mol/L glycine buffer pH 10.50 containing 2.5 mmol/L $NAD^+$.

Under optimal conditions, the linearity was established. The Fig. 4a shows the linearity and demonstrates that the method is linear across the measuring range of standard materials, from 1 to 600 µmol/L, with an $r^2$ of 0.998. Linearity was not established below the lowest calibrator concentration because this range is related to the clinical relevance since the cut off for Phe levels in neonatal samples is 240 µM (provided by the Blood Spot MW Phenylalanine enzyme assay). The functional sensitivity, the concentration at which there is a 10% CV, was obtained at a concentration of 1 µM. Besides, the detection limit of the sensor was estimated at 0.2 µmol/L (signal-to-noise ratio = 3.3).

The precision of the EPAD was evaluated with a within-analysis and between-analysis using CLSI EP05-A2 protocol. Results from the precision studies (%CV) are shown in Table 2 and demonstrated an acceptable precision for the proposed method. Within-analysis precision was 5.7% across all 3 levels of control samples. Between-analysis precision was 8.2% across all 3 levels of control samples.

In this work, we compared our Phe detection procedure and the Phenylalanine MW enzyme assay kit (colorimetric method). For this experiment, 8 neonatal samples were analyzed by both methods (3 high-level and five low-level) and 12 control samples (6 high-level and 6 low-level). Results from the method comparison are shown in Figs. 4b and b. The Fig. 4b showed a good correlation across the measuring range (r2 = 0.996) and a slope of 0.976. The y-intercept of +8.52 µM indicates a proportional positive bias between the 2 methods. The overall relative mean bias (Fig. 4c) was +1.8% with 95% of the samples within 1.96 SD from the mean. As shown in Fig. 4b and c, we obtained similar Phe concentrations employing both procedures for all neonatal samples and control samples analyzed, indicating a good correspondence between them.

**Table 2**
Within-day and between-day precision for 3 levels of control samples.

| | [a] Mean (µmol/L) | (SD µmol/L) | CV (%) |
|---|---|---|---|
| Within-day | | | |
| Low | 53.2 | 1.8 | 3.4 |
| Medium | 304.8 | 17.3 | 5.7 |
| High | 589.4 | 28.3 | 4.8 |
| Between-day | | | |
| Low | 48.7 | 3.2 | 6.6 |
| Medium | 313.7 | 18.5 | 5.9 |
| High | 606.6 | 49.7 | 8.2 |

[a] Phe concentration (µmol/L).

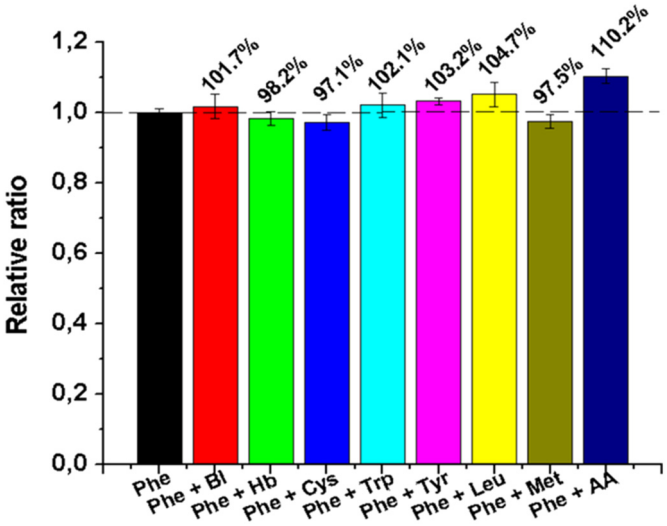


**Fig. 5.** Phe detection in the presence of bilirubin (Bl), hemoglobin (Hb), cysteine (Cys), tryptophan (Trp), tyrosine (Tyr), leucine (Leu), methionine (Met) and ascorbic acid (AA).

Furthermore, compared with the colorimetric method, the EPAD showed improved sensitivity and decreased assay time. Furthermore, the EPAD showed good stability during 2 months under storage conditions (4 °C), due to the device exhibited the same current signal response at this period.

The Fig. 5 shows the Phe analytical signal obtained in the presence

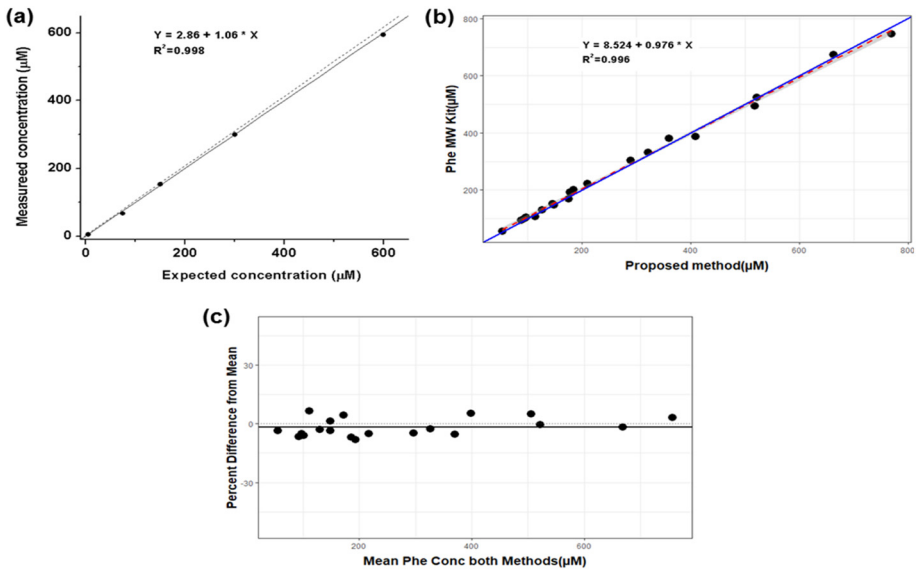


**Fig. 4.** Analytical performance of the method. (a) Graph showing linearity across the measuring range. The dashed line represents the perfect line of agreement. (b) Results from the regression analysis; 20 neonatal samples and control samples analyzed by the proposed method and the Phenylalanine MW enzyme assay kit which demonstrates acceptable agreement between both methods. The dashed line represents the perfect line of agreement. (c) Bland-Altman plot shows relative bias.

**Table 3**
Comparison of available methods for the determination of Phe.

| Method | Detection limit (μmol/L) | Linear Range (μmol/L) | Reference |
|---|---|---|---|
| Enzymatic electrode for amperometric detection. | 25 | 50–9100 | [7] |
| HPLC with fluorescence detection. | 10 | 10–1500 | [10] |
| Biosensor based on the optical detection with UV light emitting diode. | 10 | 10–10,000 | [36] |
| Cucurbit[7]uril sensitized fluorescence quenching method. | 0.012 | 0.036–9.68 | [37] |
| Biosensor Based on a Gold Electrode Modified with Graphene Oxide Nanosheets and Chitosan. | 0.416 | 0.5–15,000 | [38] |
| Nanocomposite of Silver Nanoparticles and Reduced Graphene for Biosensing. | 0.047 | 0.15–900 | [39] |
| Europium ion immobilized with sol-gel method for spectrofluorimetric determination. | 5.2 | 50–2000 | [40] |
| HPLC with fluorescence detection. | 0.3 | 1.25–200 | [41] |
| Molecularly imprinted polymer for enantioselective sensing by electrochemical | 13 | 13–100 | [42] |
| A paper-based analytical device with electrochemical detection | 0.2 | 5–600 | This paper |

of the following analytes: bilirubin (Bl), hemoglobin (Hb), cysteine (Cys), tryptophan (Trp), tyrosine (Tyr), leucine (Leu), methionine (Met) and ascorbic acid (AA). In this sense, only AA exhibited an increase (10.2%) in the analytical signal of Phe, but at a concentration higher than the physiological level. Also, other reagents were tested against Phe, and no significant differences were observed ($P < 0.01$, $n = 5$, *t*-test). For this reason, the results indicated the high selectivity of the biosensor.

In addition, for the proposed method the sample pre-treatment takes around 3 min, due to the extraction procedure is carried out under sonication conditions [32], while for the traditional method it takes 1 h [35]. The reagent preparations took < 1 min, due to the previously extracted sample and the enzymatic cofactor were added in the same step and were electrochemical measured at the same time, while the incubation time for the traditional method takes 45 min. Moreover, the modified electrode and functionalized paper platforms could be quickly prepared and stored according to the needed amount until use. For the proposed method the overall assay time, including the sample pre-treatment, reagents preparation, and electrochemical detection was < 5 min, which was smaller than the traditional method (around 2 h).

According to our search, there are only a few articles related to Phe biosensor with electrochemical detection. Table 3 summarizes and compares the detection limit, and the linear range concentration of Phe obtained in the present study with other methods described in the literature. It shows that our device obtained a better detection limit compared to previous studies due to the incorporation of the ERGO on the electrode surface, and exhibited a wide range of concentration. Moreover, our method is the only one based on paper support for enzymatic detection of Phe.

The proposed EPAD is a novel methodology with an appropriate LOD and LOQ for the quantification of Phe. The non-sophisticated equipment required, the accuracy and the achieved LOD and LOQ represent relevant parameters, particularly when the routine diagnostic of phenylketonuria is wanted. In addition, the developed device offers a fast, ultrasensitive, selective and inexpensive biosensing protocol for the determination of Phe in neonatal samples.

## 4. Conclusions

In this work, we described a novel EPAD for neonatal screening of Phenylketonuria (PKU). The results obtained showed that the paper matrix could provide an adequate microenvironment for the direct electrochemical measurement of PheDH. Moreover, the immobilization of the enzyme by physical adsorption did not affect its native structure, function, and electrocatalytic activity. The developed EPAD combines the high catalytic activity of PheDH and the inherent sensitivity of DPA with the advantage of the use of PADs, providing sensitivity, ease-of-use, low cost and short assay times. Furthermore, the modification of the SPCE with ERGO is simple, fast, reproducible and improved the sensitivity of the electrochemical measurements. The overall assay time employed (< 5 min) was shorter than the time reported for by the standard Phe enzyme assay kit frequently used in clinical diagnosis (around 2 h) without reduction of sensibility. The accurate determination of Phe in real samples demonstrates the great potential of our device for practical applications. Therefore, the proposed EPAD could be applied as an alternative method for the diagnosis of PKU.

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