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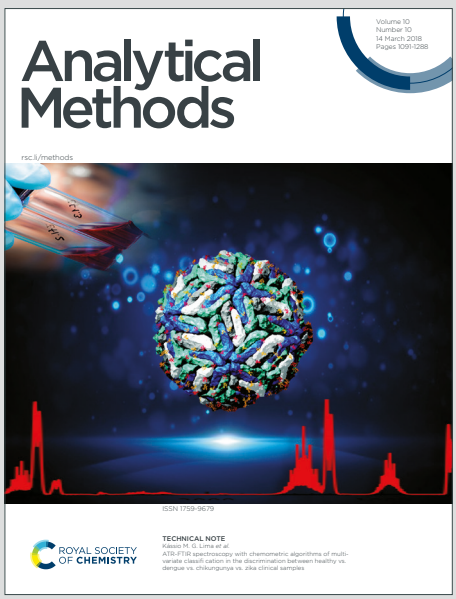

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Nanostructured paper-based device for phenylalanine neonatal screening by LED-induced fluorescence

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ABSTRACT

In this work, a novel paper-based analytical device (PAD) coupled to LED-induced fluorescence (LIF) detection (fPAD) for the rapid, selective, and sensitive quantification of phenylalanine (Phe) in neonatal samples was developed. Enzymes Phenylalanine dehydrogenase (PheDH) and diaphorase were immobilized on a paper microzone previously modified with zinc oxide nanoparticles (ZnONPs) coated with chitosan (CH-ZnONPs). Phe was extracted from the blood spots collected samples on filter paper and was mixed with nicotinamide adenine dinucleotide (NAD^+) and resazurin. Then the mixture was deposited on the reaction microzone of the fPAD where PheDH converts the Phe and NAD^+ to phenylpyruvate and NADH, respectively. Finally, NADH was oxidized by diaphorase with the consequent reduction from resazurin to resorufin. This latter was detected by LIF using an excitation wavelength of 535 nm and an emission of 580 nm in a synchronized video microscope.

We compare the responses of the PADs with and without nanomaterials to demonstrate the improved analytical performance of the developed devices. For this, the PADs were modified with the same concentration of horseradish peroxidase (HRP). The fluorescent signal obtained from the PADs with nanomaterials was higher than that of the unmodified PADs.

Our method exhibited within- and between-assay variation coefficients below 5.23% and 6.67%, respectively. The detection limit obtained by the developed device was 0.125 μM. The proposed fPAD allowed the simple, rapid, low-cost, and sensitive detection of Phe in neonatal blood samples.

Keywords: Fluorometric detection, Paper-based analytical device, zinc oxide nanoparticles, Enzymatic method, Phenylalanine.

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1. Introduction

In recent years, paper surfaces have been widely used as a support for the design and construction of paper-based analytical devices (PADs). The use of this material involves several advantages such as easy application, portability and low cost [1].

PADs can be designed by different procedures, such as photolithography, polydimethylsiloxane tracing, inkjet printing, plasma treatment, paper cutting, wax immersion and wax printing [2,3]. Among them, wax printing is the most commonly used for the fabrication of paper devices, due to its easy processing and the possibility of mass production [4]. Besides, PADs coupled to different detection systems have been reported. Fluorescent and colorimetric techniques allow obtaining analytical results by direct visual read-out. Nevertheless, fluorescence sensors exhibit higher sensitivity and lower detection limit than the colorimetric sensors [5].

PADs surface could be modified with different polymers and nanomaterials as a strategy for improving their properties. An interesting polymer to be incorporated on paper surface is chitosan, a cationic polysaccharide ecologically friendly [6,7]. Furthermore, chitosan easily forms covalent bonds with the cellulose fibers treated with oxygen plasma, allowing the subsequent incorporation of nanomaterials on the surface. This modification procedure improves the overall physical/chemical properties of the paper [8].

In this context, zinc oxide nanoparticles (ZnONPs) represent an attractive for the development of biosensors. These NPs are non-toxic materials which exhibit good biocompatibility, chemical stability and enhanced surface area for the immobilization of biological recognition agents [9–11].

One interesting application of PADs is the phenylketonuria (PKU) new-born screening, measuring phenylalanine (Phe) in neonatal samples. PKU is a phenylalanine

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metabolism error caused by a congenital deficiency of phenylalanine hydroxylase activity. In the absence of treatment, PKU induces damage to the central nervous system with irreversible mental retardation [12–14]. The Phe determination in the blood is essential for the early dietary therapy implementation to avoid the clinical manifestations of the disease.

Several methods have been developed for detection of Phe such as fluorometric [15], spectrophotometric [16], chromatographic [17,18] and enzymatic [19–22]. In this regard, enzymatic assays have great potential to be implemented in routine analysis. However, it is essential to emphasize that the native NADH fluorescence generated in this method is low and, therefore, the detection limit obtained is deficient [23].

A strategy to enhance the sensitivity of the enzyme assay is the diaphorase-resazurin coupling, which generates the rapid amplification of the signal [24]. In this amplification system, diaphorase enzyme catalyzes the reduction from weakly fluorescent resazurin to highly fluorescent resorufin in the presence of NADH.

Here, a novel PAD coupled with LED-induced fluorescence (LIF) detection (fPAD) applied to the Phe quantification in neonatal blood samples was developed. For the construction of the fPAD, the paper microzone was modified with zinc oxide nanoparticles coated with chitosan (CH-ZnONPs). The enzyme mixture was immobilized in the modified paper microzone which acts as reaction support. The fluorescent product generated by the enzyme reaction was detected by LIF detection using an emission wavelength of 580 nm. The proposed fPAD exhibited good selectivity, stability, and reproducibility for the neonatal screening of PKU.

2. Materials

2.1. Reagents and solutions

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All the reagents used were of analytical grade. Phenylalanine (Phe), phenylalanine dehydrogenase (PheDH), diaphorase from *Clostridium kluyveri*, β-nicotinamide adenine dinucleotide (NAD) sodium salt, resazurin sodium salt, chitosan (CH) (high purity, M_v 140,000–220,000), nitrate zinc hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$), Peroxidase from horseradish (HRP) and 10-Acetyl-3,7-dihydroxyphenoxazine (ADHP) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium hydroxide (NaOH), sodium chloride (NaCl) and glutaraldehyde (GLA) (25% aqueous solution) were obtained from Merck (Darmstadt, Germany). Paper devices were made of Whatman N° 1 chromatographic paper. The aqueous solutions were prepared using purified water from a Milli-Q system.

High- and low-level of Phe samples and controls were supplied by Blood Spot PHENYLALANINE NEONATAL-MW (enzyme-linked colorimetric assay) Kit (MP Biomedicals, USA).

2.2. Instrumentation

Fluorescent measurements were performed using a synchronized video microscope SVM340TM (LabSmith, Livermore, California, USA). All pH measurements were made with an Orion Research Inc. (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.). The fluorescent emission spectrum of zinc oxide nanoparticles and resorufin were studied using a QE65000-FL scientific-grade spectrometer (Ocean Optics, USA). The morphology and elemental composition of the nanoparticles on the paper surface were studied by scanning electron microscopy (SEM) LEO 1450VP (UK) and energy dispersive spectroscopy (EDS) EDAX Genesis 2000 energy dispersive spectrometer (UK), respectively. A Xerox Phaser printer from XEROX (Xerox

ColorQube 8870, ARG) was used to print the paper device. The treatment by oxygen plasma was carried out with a PDC-32G device (Micro Technology Co. Ltd., USA).

2.3. Synthesis of CH-ZnONPs

The synthesis of the ZnONPs was achieved by a wet chemical process under high-speed agitation. An aqueous solution of 0.90 M NaOH was added drop by drop to an aqueous ethanol solution of zinc 0.50 M ($Zn(NO_3)_2 \cdot 6H_2O$) and allowed to react for 2 h. Then, the supernatant was carefully separated, and the remaining solution was centrifuged for 10 min. Subsequently, the precipitate was cleaned and dried in one atmosphere of air at 60 °C to generate ZnO from $Zn(OH)_2$. Finally, 0.25 mg of the obtained ZnONPs was dispersed in 5 mL of CH solution (0.05 M in an acetate buffer, pH 4.50) and stirred for 8 h at room temperature until obtaining a dispersion.

2.4. Paper-based devices (PADs) fabrication

The fabricated PADs have a hydrophilic reaction microzone confined by hydrophobic wax barriers. Paper microzones with a diameter of 6 mm were designed on Whatman # 1 filter paper using graphic software (Corel Draw 9). Once the design was printed, to melt the wax through the paper and create a hydrophobic barrier, the platforms were placed at 90 °C for 5 min. Finally, with the aim to produce aldehyde groups over the reaction microzone, the wax-penetrated paper was treated for 4 min by oxygen plasma using an excitation frequency of 13.56 MHz and a power of 100 W.

After plasma treatment, 5 µL of CH-ZnONPs previously obtained was dropped onto the reaction microzone, and dry at room temperature. The aldehyde groups of the cellulose were used to crosslinking amino groups of CH [25], allowing the covalent binding of the CH-ZnONPs in the reaction microzone (CH-ZnONPs-PADs).

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To carry out the enzyme incorporation, the CH-ZnONPs-PADs surface was exposed to 5 µL of GLA 5% w/w solution (acetone medium, pH 5.00) for 1 h at room temperature and washed three times (GLA-CH-ZnONPs-PADs). Finally, 5 µL of a solution containing 6 U mL^{-1} of PheDH and 7 U mL^{-1} of diaphorase was incubated with GLA-CH-ZnONPs-PADs overnight at 5 °C. This treatment allowed to form covalent bindings between the amino groups of the chitosan and enzymes by crosslinking with GLA. Finally, the device was washed three times with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C.

2.5. Fluorometric measurements

The Phe determination in neonatal blood samples consisted in placing into an eppendorf tube 9 µL of the eluted samples (see samples extraction in the electronic supplementary information), followed by the addition of 3 µL of an assay cocktail containing 50 µM resazurin and 2.5 mM NAD^+ solution in 0.2 M glycine buffer pH 8.00. Then, 5 µL of this mixture was added to the device. PheDH catalyzed the NAD-dependent deamination of Phe to phenylpyruvate and NADH. This cofactor was oxidized by the diaphorase with the consequent reduction of resazurin to fluorescent resorufin, which was detected by LIF in the synchronized video microscope using an excitation wavelength of 535 nm and emission of 580 nm. Figure 1 shows the schematic representation of the device construction and Phe determination.

3. Results and discussion

3.1. PADs Characterization

The reaction surface was characterized by SEM. Figure 2(a) shows the typical scanning electron micrograph image of the unmodified paper surface, Figure 2(b)

reveals the aggregation of CH-ZnONPs uniformly spread on the paper surface. Besides, the elemental composition was disclosed by EDS analysis in which strong signals of Zn were observed at 1 keV, while signals from C and O were also recorded confirming the presence of CH-ZnONPs (Figure 2c).

Figure 2(d) can be observed the emission spectra of CH-ZnONPs and resorufin excited at 340 and 535 nm, respectively. The CH-ZnONPs exhibits emission bands at 390, 420, 485, 530 nm, while the resorufin shows an emission band around 580 nm. Therefore, the fluorescent signal obtained during the assay is only due to the enzymatic product.

In order to demonstrate the enhanced fluorometric performance of the devices , we compared the responses of PADs with and without NPs. For this, both PADs with the same reaction microzone dimensions (6 mm of diameter) were modified with the same HRP concentration. The experiment consisted of adding 5 µL of different H_2O_2 concentration with ADHP, to perform the fluorescence measurement after 2 min of incubation time. As shown in Figure 2(e), the fluorescent response of the CH-ZnONPs-PADs was higher than the non-modified PADs. Therefore, the use of the CH-ZnONPs in the PADs development improved the analytical performance.

3.2. Optimization of the experimental variables

In this section, relevant studies of experimental parameters that affect the performance of Phe determination were analyzed. A Phe standard of 900 µM was employed for the assays.

One of the parameters evaluated was the optimal PheDH concentration to be immobilized on the paper microzone surface. For this assay, the concentration of PheDH enzyme ranged from 1 to 12 U mL^{-1}. The highest fluorescence intensity was

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observed with a PheDH concentration of 6.0 U mL^{-1}. Hence, 6.0 U mL^{-1} PheDH in the enzymatic mixture was used for all experiments (Figure 3a, black line).

The diaphorase concentration was another significant parameter evaluated in the enzymatic study. The measured fluorescence intensity is weakly dependent on the diaphorase concentration above 7.00 U mL^{-1}. This enzyme concentration generated an acceptable signal/noise ratio due to allowing an optimal reduction from resazurin to resorufin. The high concentrations of diaphorase did not produce an improvement in detection capability. Therefore, a diaphorase concentration of 7.00 U mL^{-1} was used for the immobilization on the paper microzone (Figure 3a, blue line).

The optimal concentration of the cofactor NAD^+ in the reaction mixture was studied using different NAD^+ concentrations (1-5 mM). The optimal response was observed with a NAD^+ concentration of 2.5 mM, and it was used in the subsequent analysis (Figure 3b, black line). Following this line, the optimal resazurin concentration was evaluated. The highest fluorescent response was achieved at a resazurin concentration of 50 μM. When higher concentrations were analyzed, no significant differences were observed (Figure 3b, red line).

The effect of pH on the fluorescence signal was analyzed. Taking the optimal pH ranges of the PheDH and diaphorase enzymes, the influence of pH was studied at 25 ºC in a range from 6.00 to 12.00. The pH variation of the 0.2 M glycine buffer was no significant effect on the fluorescence intensity in a range of 7.00 and 9.00. Therefore, the selected pH range for resorufin detection was 8.00 (Figure 3c).

Finally, the study of optimal incubation time was performed. As illustrated in the Figure 3(d), the fluorescence generated for different Phe concentration (240, 420 and 900 μM) increased by increasing the incubation time up to 1.30 min and remained constant at higher times. Therefore, an incubation time of 2 min at 25 °C was used in all

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assays to ensure the complete enzymatic reaction. Table 1 shows the optimal experimental conditions for Phe determination using the fPAD.

3.3. Phe determination by fPADs

The developed method was applied to the Phe determination in neonatal blood samples under the optimized conditions described above. The relative fluorescence response (RFU) of the enzymatic product is proportional to the concentration of Phe present in the samples.

A linearity was achieved in the range from 5 to1620 μM for the proposed method by analyzing seven controls (5, 20, 60, 240, 420, 900 and 1620 μM) in 0.2 M glycine pH 8.00 (n = 6). The linear regression equation was RFU = 39.33 + 11.44× C_{Phe}, with a linear regression coefficient of 0.998 (Figure 4). The linearity range achieved is in accordance with the clinical relevance, owing to that the cut-off for the Phe levels in neonatal samples is of 240 μM [26,27]. Furthermore, the limit of detection (LOD) for fPADs was 0.125 μM calculated by LOD = (3.3 σ_o)/A, where σ_o and A, are the controls deviation of the blank and the slope of the calibration curve, respectively.

To evaluate the selectivity of the proposed method, (see Figure 5a) fortified blood samples (252 μM Phe) were analyzed in the presence of 1200 μM bilirubin (Bl), 6000 μM hemoglobin (Hb), 1164 μM tryptophan (Trp), 1074 μM tyrosine (Tyr) and 660 μM ascorbic acid (AA). It was found that AA can interfere with the Phe analytical signal at levels higher than the normal physiological range. However, no interference was observed when AA was tested at normal physiologic levels (12 - 120 μM) (Figure S1).

The fPAD was compared with a Phenylalanine neonatal MW enzyme assay kit (commercial colorimetric method) for the Phe quantification in eight neonatal samples (three high-level and five low-level) and seven controls (three high-level and four low-

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level). The fluorescence response obtained for our fPAD was proportional to the concentration of Phe in the blood samples. The slope obtained was reasonably close to 1, indicating a good correspondence between the two methods (Figure 5b). Besides, compared with the colorimetric enzyme assay (LOD = 18 μM, linear range of 60 to 1620 μM), our method showed an enhancement in the detection limit, which allowed to determine very low levels of Phe in neonatal samples.

The within- and between-assay variation coefficients were determined. The within-assay precision was tested with six measurements for three levels of controls (60, 420, and 900 μM, n = 6). These series of analyses were repeated for three consecutive days to estimate the between-assay precision. The values obtained within- and between-assay were 5.23% and 6.67%, respectively (see electronic supplementary information).

The developed fPAD was applied for the Phe quantification in spiked blood samples (0.5, 10, 40, 100, 500 and 1000 μM). The designed device accuracy was evaluated. Obtained recovery values ranged from 98.60% to 102.40%. Therefore, the accuracy of the proposed method is suitable for the quantitative detection of Phe (Table 2).

Finally, our method was compared with other methods reported in the literature for the determination of Phe. During the last years, several articles based on different methodologies for the Phe determination in neonatal blood samples were reported [17,28–31]. Our system was designed on a paper support, a disposable and practical material to the generation of portable devices. This support was modified with zinc nanoparticles, allowing us to obtain one of the lowest LODs according to published articles. Moreover, the proposed system requires an analysis time of 2 min, less than the compared methods (Table 3).

The proposed fPAD is a novel methodology with an appropriate LOD for the Phe quantification. The non-sophisticated equipment required, the accuracy and the achieved LOD represent relevant parameters, particularly when the routine diagnostic of phenylketonuria is needed.

4. Conclusions

In this article, a novel and disposable paper-based analytical device combined with a fluorescent detection system (fPAD) for neonatal screening of phenylketonuria (PKU) was developed. The PAD fabrication process included the wax printing technique and plasma oxidation treatment which allowed to obtain a delimited reaction area in the paper surface. The incorporation of zinc nanoparticles on the paper microzone increased the enzymes immobilization capacity of the sensing surface generating a strong affinity towards Phe, conferring thus high sensitivity and selectivity to the system. Furthermore, the developed method exhibited suitable stability and excellent accuracy. According to the previously described features, the proposed fPADs represents an alternative option for the simple, rapid and low-cost neonatal screening of PKU.

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Neonatal samples and control samples were provided by the Blood Spot PHENYLALANINE NEONATAL-MW Kit. The manufacturer's instructions specifies how these samples were obtained: blood samples were spotted in filter paper number 903 in the center of a 1 cm circle.

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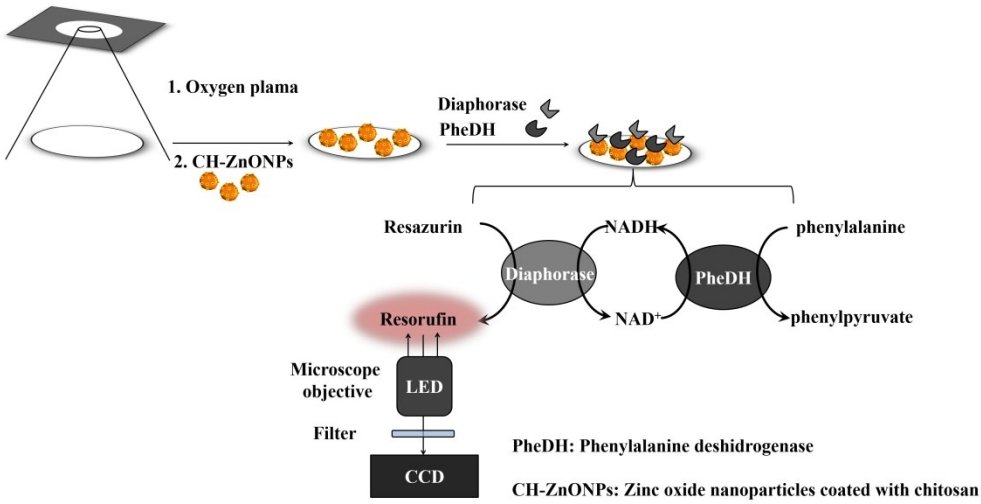

Analytical procedure for Phe determination in neonatal samples.

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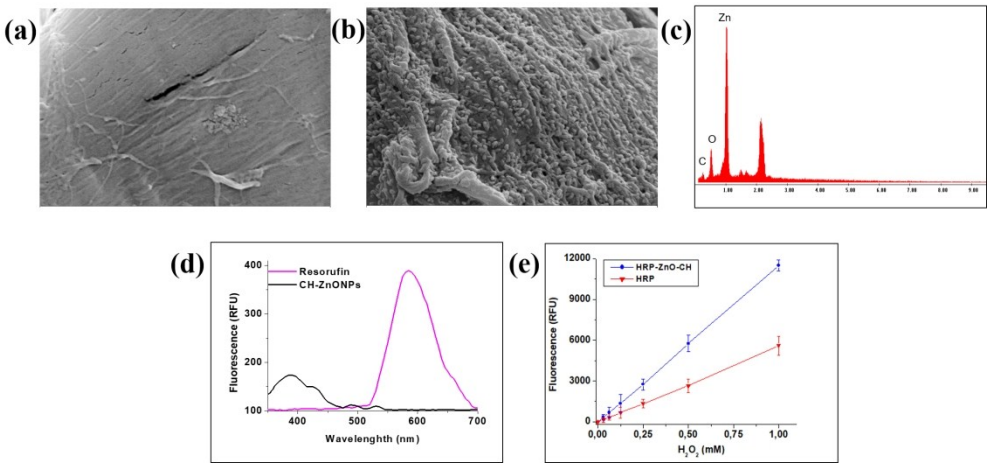

Platform characterization: (a) micrographs of the paper without CH-ZnONPs by SEM (ETH = 15.00 KV, WD = 12 mm, Zone Mag = 10.00 K X, Signal A = SE1), (b) micrographs of the paper with CH-ZnONPs by SEM (ETH = 15.00 KV WD = 11 mm Zone Mag = 10.00 K X Signal A = SE1), (c) spectra of CH-ZnONPs by EDS, (d) fluorescence intensity of CH-ZnONPs and resorufin excited at 340 and 535 nm, respectively, (e) effect of the presence of CH-ZnONPs on reaction platform.

622x286mm (96 x 96 DPI)

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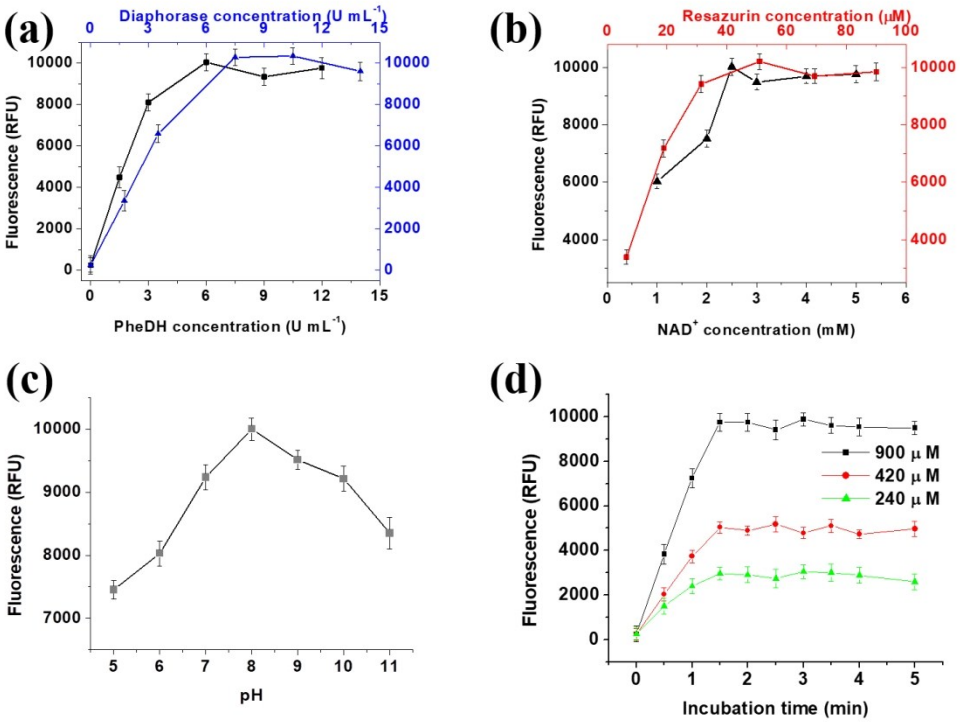

Optimization of experimental variables: (a) PheDH concentration (1-12 U mL-1) (black line) and Diaphorase concentration (1-14 U mL-1) (blue line), (b) NAD+ concentration (1-5 mM) (black line) and resazurin concentration (6-90 μM) (red line), (b) pH of the enzyme assay (5-11), (d) incubation time (0-5 min).

402x306mm (96 x 96 DPI)

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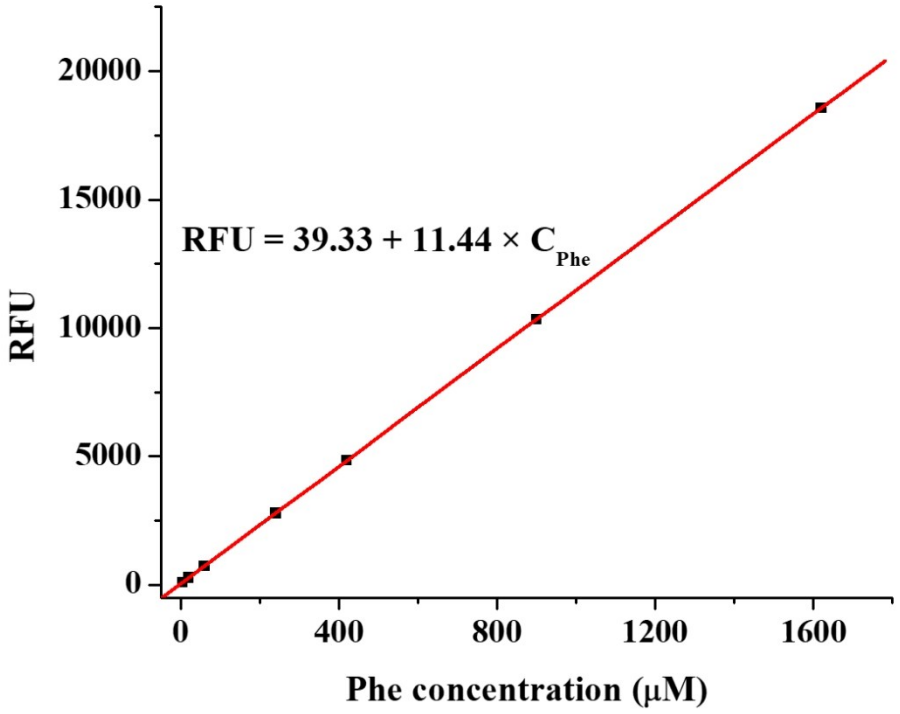

Linearity study for the proposed method by analyzing seven controls (5, 20, 60, 240, 420, 900 and 1620 μM) in 0.2 M glycine buffer pH 8.00 (n = 6).

307x252mm (96 x 96 DPI)

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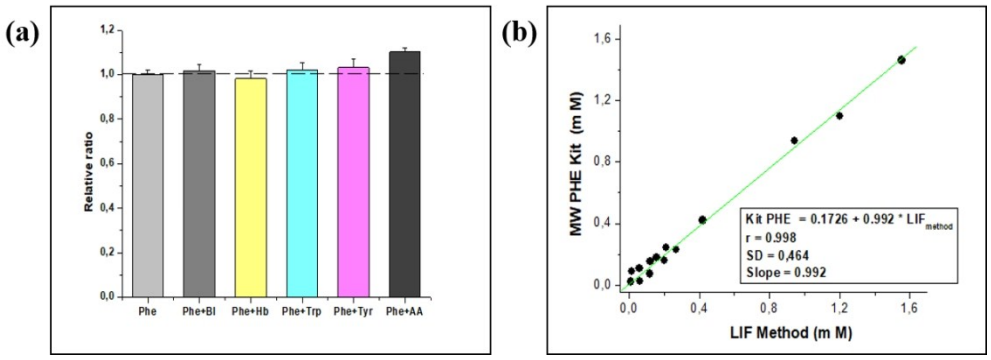

(a) Phe (252 µM) detection in presence of bilirubin (1200 µM) (Bl), hemoglobin (6000 µM) (Hb), tryptophan (1164 µM) (Trp), tyrosine (1074 µM) (Tyr) and ascorbic acid (660 µM) (AA). (b) Correlation between the colorimetric method and the proposed method analyzing eight neonatal samples (three high-level and five low-level) and seven controls (three high-level and four low-level).

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Optimized parameter	Evaluated range	Optimum value
PheDH concentration	1 – 12 U mL^{-1}	6.0 U mL^{-1}
Diaphorase concentration	1.75 – 14 U mL^{-1}	7.0 U mL^{-1}
pH of the enzyme assay	6 – 12	8.0
NAD^+ concentration	1 – 5 mM	2.5 mM
Resazurin concentration	6.5 – 90 µM	50 µM
Incubation time	0 – 5 min	2 min

Phe (μM)			Recovery (%)
Base	Added	Found	
120	0.0	119.85	-
120	0.5	118.81	98.60
120	10	132.08	101.60
120	40	163.84	102.40
120	100	218.02	99.10
120	500	613.18	98.90
120	1000	1144.64	102.20

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Methods	[a] LOD (μM)	[a] Linear Range (μM)	References
HPLC/fluorescence	10	10 - 1500	17
Biosensor/UV	10	10 - 10000	28
Fluorescence quenching	0.012	0.036 - 9.68	29
Sol–gel/spectrofluorimetric	5.2	50 - 2000	30
HPLC/fluorescence	0.3	1.25 - 200	31
PAPs/fluorescence	0.125	5 - 1620	This paper

[a] Phe concentration (μM)