Wearable wristband-based electrochemical sensor for the detection of phenylalanine in biofluids

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Abstract

Wearable electrochemical sensors are driven by the user-friendly capability of on-site detection of key biomarkers for health management. Despite the advances in biomolecule monitoring such as glucose, still, several unmet clinical challenges need to be addressed. For example, patients suffering from phenylketonuria (PKU) should be able to monitor their phenylalanine (PHE) level in a rapid, decentralized, and affordable manner to avoid high levels of PHE in the body which can lead to a profound and irreversible mental disability. Herein, we report a wearable wristband electrochemical sensor for the monitoring of PHE tackling the necessity of controlling PHE levels in PHE hydroxylase deficiency patients. The proposed electrochemical sensor is based on a screen-printed electrode (SPE) modified with a membrane consisting of Nafion, to avoid interferences in biofluids. The membrane also consists of sodium 1,2-naphthoquinone-4-sulphonate for the in situ derivatization of PHE into an electroactive product, allowing its electrochemical oxidation at the surface of the SPE in alkaline conditions. Importantly, the electrochemical sensor is integrated into a wristband configuration to enhance user interaction and engage the patient with PHE self-monitoring. Besides, a paper-based sampling strategy is designed to alkalinize the real sample without the need for sample pretreatment, and thus simplify the analytical process. Finally, the wearable device is tested for the determination of PHE in saliva and blood serum. The proposed wristband-based sensor is expected to impact the PKU self-monitoring, facilitating the daily lives of PKU patients toward optimal therapy and disease management.

Keywords
Wearable electrochemical sensor, phenylalanine detection, phenylketonuria, biofluids analysis, screen-printed electrode, point-of-care testing

1. Introduction

Wearable health monitoring is driven by new advances in materials, sensors, artificial intelligence, and engineering (Haick and Tang, 2021; Zohar et al., 2021). Wearable sensors have demonstrated the ability to detect (bio)chemical molecules (Yang and Gao, 2019) enabling personalized therapies (Xu et al., 2020) or nutrition (Sempionatto et al., 2021). Particularly, electrochemical sensors have transitioned from bench bulky devices to miniaturized systems (Mahato and Wang, 2021) with even self-powered capabilities (Parrilla and Wael, 2021) that allow wearing the lab on the body for the detection of many different targets such as ions (Parrilla et al., 2019), biomolecules (Wiorek et al., 2020), or drugs (Teymourian et al., 2020).

Today, there still exist several unmet clinical needs in terms of monitoring and detection of targets for proper diagnostic or therapeutic purposes (Li et al., 2020). From one side, the detection of (bio)molecules that can induce diseases is paramount to keep on healthy levels. A successful example is glucose monitoring for diabetes management. In this case, the glucose level is controlled by the proper insulin administration. Interestingly, closed-loop therapies have been recently designed to automized the treatment (Li et al., 2021). On the other side, the administration of therapeutic drugs and the continuous monitoring of the exogenous drug level on the body represent an essential step toward personalized medicine, and potentially an optimization of the healthcare expenditure. Maintaining the drug level at an optimal therapeutic range enables fast recovery of the patient while minimizing side effects or toxic consequences. Besides, pharmacokinetics and pharmacodynamics are dependent on each patient, thus the coupling of closed-loop systems with artificial intelligence will play an important role in digital medicine (Zhang et al., 2021).

Phenylketonuria (PKU) is the most prevalent disorder caused by an inborn error in amino acid metabolism (i.e. phenylalanine hydroxylase deficiency –PAH–) with a prevalence of about one case per 10 000 livebirths (Blau et al., 2010). PKU disease results in the lack of ability to metabolize L-phenylalanine (PHE) which in turn can cause an accumulation of PHE in the brain leading to a profound and irreversible mental disability (Mitchell et al., 2011). The combination of current therapies, PHE-

restricted diet, and blood PHE monitoring would enable a safe lifestyle among PKU patients. Hence, tools for the fast detection of PHE in biofluids would allow a daily control of PHE consumption within physiological limits.

PAH deficiency is categorized according to the PHE levels in the blood (120 – 1200 µM) (**Table S1**). However, the use of blood can be cumbersome due to painful extractions. Hence, a point-of-care test (POCT) through noninvasive biofluid is desirable for PAH subjects. In that sense, salivary PHE levels have been correlated with PHE blood levels, opening an opportunity for noninvasive PKU monitoring (Hall et al., 2000) (**Table S1**). Besides, salivary PHE has been linked with the diagnosis of oral squamous cell carcinoma (OSCC) (Hema Shree et al., 2019; Reddy et al., 2012), nasopharyngeal carcinoma (Qiu et al., 2016) as well as related to periodontal diseases (Aimetti et al., 2012). Therefore, salivary PHE detection in fasting subjects with a wearable POCT device might be clinically relevant for monitoring several diseases (i.e. PHE levels of 16 – 111 µM for PKU, and 105 – 368 µM for OSCC, **Table S1**).

In the last decade, electrochemical sensors have been designed for the detection of PHE. As PHE is not electroactive using regular electrodes' materials (e.g. carbon, gold, platinum), enzymes (e.g. phenylalanine dehydrogenase) (Arslan et al., 2020), antibodies (Tachibana et al., 2006), and aptamers (Hasanzadeh et al., 2018) have been used as the recognition element coupled with electrochemical transducers. Interestingly, screen-printed electrodes (SPEs) have been used as an affordable solution for PHE biosensing (Moreira et al., 2018). The use of SPEs poses a significant advantage for the development of POCT due to its low-cost and scalable manufacturing. However, the employment of (bio)recognition elements increases the cost per test and might hinder the shelf-life of the sensors. As a result, other strategies such as chemically modified electrodes (Xu et al., 2019) or the use of molecularly imprinted polymers (Dashtian et al., 2020) can contribute to the development of affordable POCT for PHE determination.

Herein, we present a chemically modified SPE integrated into a wearable configuration (i.e. 3D-printed wristband, **Fig. 1A**) for the user-friendly detection of PHE in biofluids. The wearable device combines a miniaturized potentiostat under a lid which is connected with a single-use modified SPE for the electrochemical analysis (**Fig. 1B**). The wearable device can be worn on the wrist of the subject for easy handling and portability (**Fig. 1C**). The SPE is functionalized with a mixture of sodium 1,2-naphthoquinone-4-sulphonate (NQS) and Nafion layer for an *in situ* derivatization of

PHE with NQS toward an electroactive product, which is thereafter oxidized at the surface of a carbon electrode (**Fig. 1D**). The current output obtained by square-wave voltammetry (SWV) is used as an analytical readout for PHE quantification in biofluids (i.e. saliva and blood serum) (**Fig. 1E**). First, optimization of the compounds in the membrane is performed. Subsequently, a deep analytical characterization including a complete study of interferents is obtained showing excellent analytical features for the PHE detection in biofluids. Finally, the wearable electrochemical device is investigated for the detection of PHE in spiked saliva and blood serum samples. This wearable concept is expected to open a new way for PKU monitoring in biofluids following a similar therapeutic use case as glucose monitoring, thus facilitating the lifestyle of patients with PHE-associated disorders.

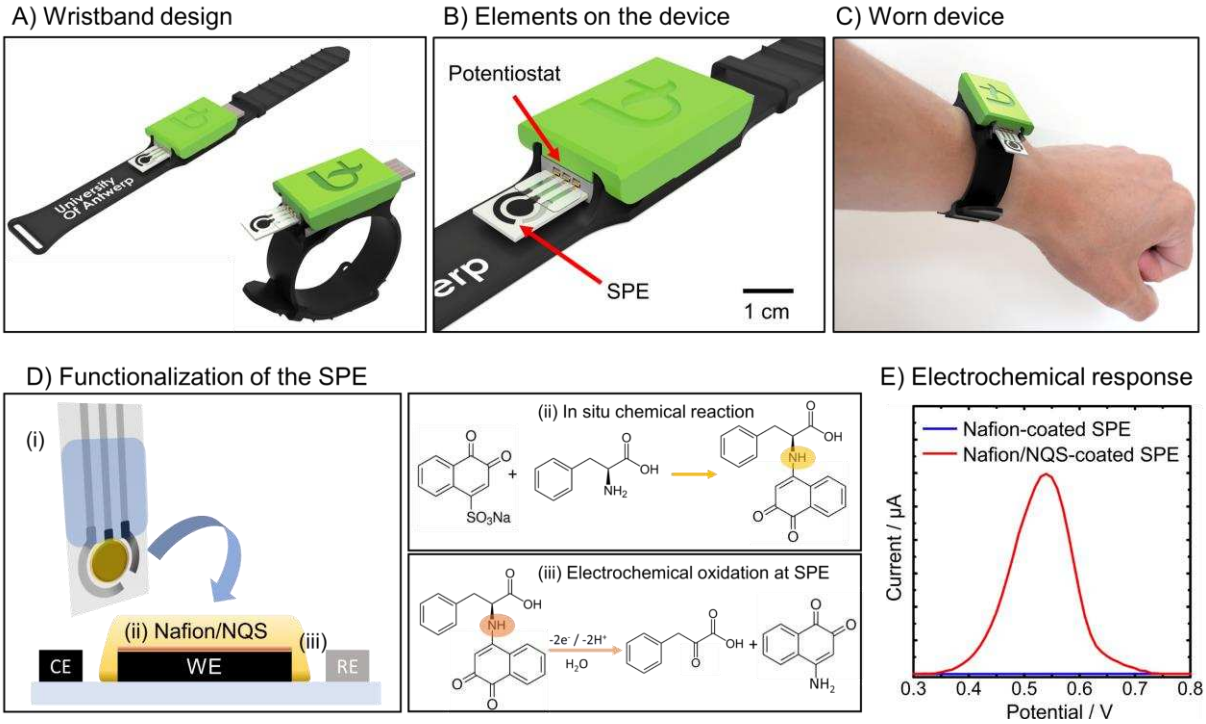

Fig. 1. *The wearable electrochemical device for the detection of PHE: A) Rendering of the design of the 3D-printed wristband. B) Rendering displaying the elements of the wearable sensor including a 3D-printed wristband that embeds, a miniaturized potentiostat capable of performing electrochemical analysis which is connected to a disposable functionalized SPE for PHE determination. C) Image of the wearable device worn on the wrist of a subject. D) Schematic representation of the electrochemical process on (i) the functionalized SPE indicated by the deposition of an NQS layer mixed with Nafion; (ii) chemical reaction occurring when PHE molecules interact with the immobilized NQS, and subsequent (iii) electrooxidation of the product triggered by the SWV analysis. E) SWV output of the PHE detection.*

PHE=phenylalanine, NQS=sodium 1,2-naphthoquinone-4-sulphonate, SPE=screen-printed electrode, SWV=square-wave voltammetry.

2. Materials and methods

2.1. Materials

Analytical grade salts of potassium chloride, potassium phosphate, sodium borate, sodium bicarbonate, sodium acetate, and potassium hydroxide were purchased from Sigma-Aldrich (Overijse, Belgium). 1,2-naphthoquinone-4-sulphonic acid sodium salt (NQS) (>98%) was purchased from Tokyo Chemical Industry Co., LTD., Japan. Phenylalanine (PHE), Nafion™ 117 containing solution (~5% in a mixture of lower aliphatic alcohols and water), filter paper Whatman No 1, and human serum from human males were purchased from Sigma-Aldrich (Overijse, Belgium). All solutions were prepared in 18.2 MΩ cm^{-1} doubly deionized water (Milli-Q water systems, Merck Millipore, Germany). The pH was measured using a pH-meter (914 pH/Conductometer, 2.914.0020, Metrohm, Switzerland).

2.2. Electrochemical Methods

Electrochemical methods were recorded using a MultiPalmSens4 and Emstat Pico potentiostats (PalmSens, The Netherlands) with PSTrace/MultiTrace. Disposable ItalSens screen-printed electrodes (SPE) (PalmSens, the Netherlands), containing a graphite working electrode (Ø = 3 mm), a carbon counter electrode, and a (pseudo) silver reference electrode were used for all measurements. Cyclic voltammetry (CV) parameters that were used: scan rate of 0.1 V s^{-1} with a step potential of 0.010 V s^{-1}. The square-wave voltammetry (SWV) parameters that were used: potential range from 0.0 to 1.2 V, frequency 25 Hz, 90 mV amplitude, and 10 mV step potential. Voltammograms are background corrected using the internal “moving average correction” (peak width = 1) tool in the PSTrace software in which the capacitive current generated during the voltammetric procedure is removed. This data treatment enables an improved identification of the oxidation processes occurring at the electrode. Electrochemical tests were performed in 20 mM hydrogen carbonate buffer (HCB) solutions with 100 mM KCl at a suitable pH by applying 50 µL of the solution onto the SPE. The derivatization of phenylalanine in solution into an electroactive compound is performed by mixing HCB with an optimal concentration of NQS (see

below), and subsequently, drop cast 50 µL of the solution on the SPE. Each modified SPE is designed for single use and it is therefore discarded after one measurement.

2.3. Design of the wearable interface

The wearable wristband was designed using Solidworks 2020 (Dassault Systèmes, France) (**Fig. 1A**). First, the potentiostat was measured with Mitutoyo's Absolute Digimatic Caliper Series 500 (Mitutoyo, Japan). These measurements were inputted in Solidworks to generate the design around these dimensions. The prototype consists of two parts, a black flexible strap and a green rigid cover (**Fig. 1B**). The design is optimized for production with a fused deposition modeling 3D printer. Both parts were printed on an Original Prusa Mini FDM 3D-printer (Prusa Research, Czech Republic). The strap was printed in Realflex flexible filament 1.75 mm (Real Filament, Netherlands) because the filament exhibits excellent self-adhesion, resulting in strong, semi-flexible 3D-prints. The rigid cover is printed in Prusament PLA 1.75 mm (Prusa Research, Czech Republic) which offers excellent printability and high strength. It can be printed without the need for supporting scaffolds due to the lack of large overhangs. The model is built completely parametrically to allow for easy scaling according to the users' needs and for adjusting tolerances to achieve an optimal fit. The renderings of the design were generated using Keyshot 9 (AESC B.V., Netherlands).

2.4. Functionalization of the SPE

To obtain an in situ derivatization of PHE, NQS is immobilized onto the SPE surface. First, NQS is dissolved in doubly deionized water at a 0.1 M. Subsequently, the membrane cocktail is prepared by mixing Nafion solution and NQS solution at a concentration of 0.5% and 40 mM, respectively, in a 1:1 ethanol/water solution. After thoroughly mixing in a vortex, 0.5 µL of the cocktail is deposited by drop-casting on the working electrode of the SPE. These parameters were selected after a careful optimization (see below). The cocktail is freshly prepared every time a batch needs to be manufactured. After depositing, the functionalized SPE can be stored at room temperature.

2.5. Phenylalanine detection in biofluids

Saliva samples used for analytical characterization (from fasting subjects or at least 2 hours after consuming food or taking any medication) were collected immediately

before analysis by spitting or drooling into a 3 mL tube. Aliquots of the saliva were taken in 1.5 mL tubes and were spiked with the corresponding amount of PHE. Besides, saliva samples were collected during different times of the day for the analysis of PHE. Aliquots of the human serum were taken in 1.5 mL tubes and were spiked with the corresponding amount of PHE before analysis.

A preliminary test was performed with diluted saliva samples by diluting 2-fold in 150 mM HCB to maintain the alkaline pH of the solution for optimal detection. The diluted saliva was deposited on the SPE for the electrochemical analysis.

To avoid the above sample treatment (i.e. the dilution step), a paper-based sampling strategy was carried out. Conventional filter paper grade 1 was cut in 1 cm^2 area, subsequently, drop cast with 50 µL of 150 mM HCB pH 11, and finally dried in an oven for 30 min at 80°C. Once the paper is dried, it is deposited on the surface of the Nafion/NQS-coated SPE. The paper is thus impregnated with the number of salts able to maintain an alkaline pH, which is essential for the in situ chemical reaction. Importantly, the biofluid is deposited and mixed on the paper without yielding a dilution of the target sample. The paper-based sampling approach was tested on whole saliva and whole human serum for the detection of PHE by depositing the sample on the paper.

3. Results and discussion

3.1. Electrochemical exploration of the derivatization of phenylalanine into an electroactive product.

The first set of experiments was performed to demonstrate the use of NQS as a fast derivatization reagent for PHE. It is suggested that after mixing both reagents, the primary amine of PHE reacts with the sulfonate group of NQS potentially leading to a secondary amine that is redox-active (**Fig. 1D**) (Hartke and Lohmann, 1983). Hence, a similar approach was previously followed for amphetamine detection (Parrilla et al., 2021). First, NQS and PHE were mixed at a concentration of 10 mM and 0.5 mM, respectively, in HCB solution. Immediately after mixing, 50 µL of the mixture was deposited on the SPE for the electrochemical interrogation. Upon mixing of NQS and PHE, the chemical reaction leads to an electroactive compound which is unraveled as a characteristic electrochemical profile (**Fig. S1A** and **Fig. S1B,** raw and background corrected profiles, respectively). **Fig. S1C** displays the SWV of the derivatized product from PHE at different pH (i.e. pH 9 to 12). Corresponding **Fig. S1D** exhibits the signal

upon data treatment (i.e. background removal) showing a pH-dependency. Besides, blank experiments were performed at the same conditions by only employing NQS solution without PHE. **Fig. S1E** and **Fig. S1F** show the corresponding results with raw and background corrected profiles, respectively. Importantly, in none of the conditions, the peak of the oxidation of the derivatized PHE was shown, thus proving the realization of the chemical reaction in **Fig. S1A** and **Fig. S1B**. Besides, a preliminary test with other amino acids containing primary amines (i.e. glycine –GLY– and tyrosine –TYR–) was carried out to show the potential of the derivatization step with other targets (**Fig. S1G** and **Fig. S1H**). Interestingly, the electrochemical profiles were different for each amino acid which would allow identifying each target by its peak potential (E_p) and voltammetric pattern.

However, this method needs a preliminary step of mixing a NQS solution with a PHE sample to trigger the chemical derivatization of PHE which makes it difficult to be applied as a POC test. For this reason, the immobilization of NQS onto the surface of the SPE was addressed to improve the usability of the test.

3.2. Functionalization of the SPE toward in situ derivatization of phenylalanine

The functionalization of the SPE was performed by mixing NQS with a membrane and subsequently drop cast on the SPE. The membrane selected for this approach was Nafion due to its outstanding properties in biosensing (Parrilla et al., 2017; Wisniewski and Reichert, 2000). Nafion confers the polymeric structure with nanosized hydrophilic pores for NQS-PHE in situ reaction as well as the ability to reject charged interferents (e.g. ascorbic and uric acid) encountered in saliva (Ngamchuea et al., 2018) due to its negatively charged sulfonate groups. Despite Nafion being a proton-exchange membrane, the thin layer deposited on the working electrode is quickly equilibrated with the alkaline buffer, avoiding any change in the pH at the surface of the SPE, and therefore, allowing for the suitable environment for the in situ derivatization. Therefore, a mixture of NQS with Nafion to a final concentration of 20 mM and 0.5% in ethanol, respectively, is subsequently drop cast (0.5 µL). After a drying step of 10 min at room temperature, the modified SPE can be used or stored.

After the modification of the SPE, an evaluation of the electrochemical sensor was performed by CV. Hence, bare SPE, Nafion-coated SPE, and Nafion/NQS-coated SPE were interrogated with 0.5 mM PHE and without target by CV (**Fig. 2A**). An oxidation peak at 0.59 V was only displayed when Nafion/NQS was deposited on the

SPE in presence of PHE proving the in situ derivatization step and corresponding electrooxidation of the product (**Fig. 1D** and **1E**). Besides, the NQS oxidation (-0.13 V) and reduction (-0.23 V) phenomena were displayed in both conditions when Nafion/NQS membrane is present, showing the redox behavior of the quinone group of NQS. **Fig. 2B** displays five consecutive scans of the Nafion/NQS-coated SPE with 0.5 mM PHE in HCB, exhibiting the irreversibility of the oxidation of the derivatized PHE. Besides, the redox peaks from the NQS decreased upon increasing scans due to the potential fouling by the products of the electrooxidation of the NQS-PHE (**Fig. 1D**). As the sensor is designed to be disposable, this phenomenon does not hinder its use for a POC determination. To attain a faster and sensitive electrochemical technique, SWV was employed using the same previous conditions (**Fig. 2C**). Following the same pattern, an oxidation peak corresponding to the derivatized PHE was only observed in the Nafion/NQS-coated SPE at 0.56 V. For easy interpretation, background-corrected data were plotted in **Fig. 2D**. Note that this data treatment was also employed in the rest of the experiments.

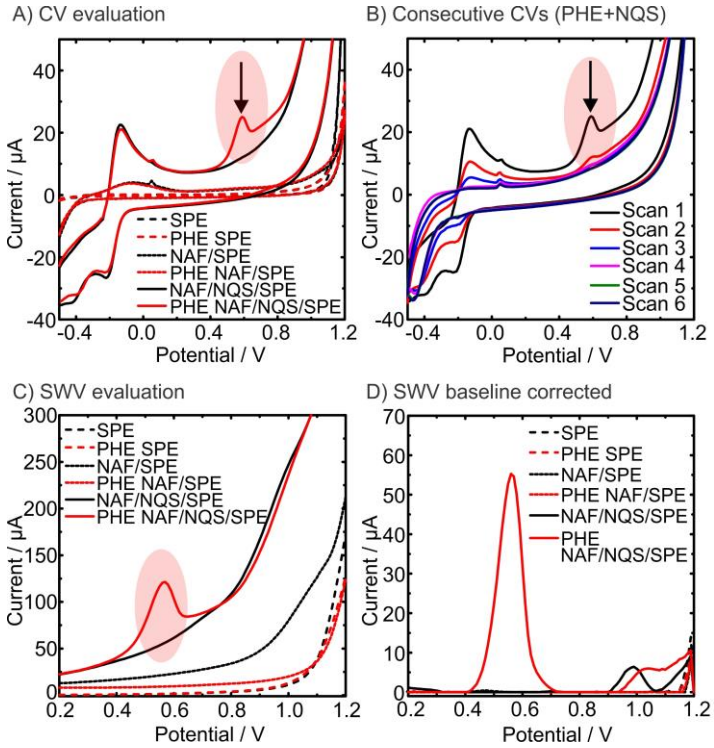

Fig. 2. *Electrochemical evaluation of the concept to detect PHE at modified SPE: A) CV of buffer (in black) and 500 µM PHE (in red) of unmodified SPE (dash line), Nafion-coated SPE (dotted line), and Nafion/NQS-coated SPE (straight line). B) Consecutive CV scans of the Nafion/NQS-coated SPE in 500 µM PHE. C) SWV of buffer (in black) and with 500 µM PHE*

(in red) of unmodified SPE (dash line), Nafion-coated SPE (dotted line), and Nafion/NQS-coated SPE (straight line). D) corrected baseline SWV in C). All measurements are performed in 20 mM hydrogen carbonate buffer pH 10. CV was performed at 0.1V s^{-1} with a start potential at -0.5V. SWV was launched after 60 s in situ reaction time.

3.3. Optimization of the in situ detection

Upon proving the feasibility of the in situ derivatization by employing a Nafion/NQS membrane at the SPE, several parameters were optimized to maximize the analytical performance of the sensor. Knowing that the NQS derivatization of a primary amine needs to be carried out in HCB (Parrilla et al., 2021), a preliminary pH screening using a Nafion/NQS membrane containing 20 mM NQS, 0.5% Nafion, and 0.5 µL deposition was performed between pH 9 and pH 12 (**Fig. S2**). As pH 10 and pH 11 exhibited the highest peak current (I_p), a narrower pH screening within these boundaries was executed (**Fig. 3A**). **Fig. 3B** indicates that pH 10.7 was optimal to obtain the highest I_p. Subsequently, the Nafion concentration was evaluated while keeping the 20 mM NQS and 0.5 µL deposition conditions (**Fig. 3C**). The condition with 0.5% Nafion exhibited the highest I_p (**Fig. 3D**). It is suggested that fewer amounts of Nafion would not retain the NQS at the surface of the SPE and a higher amount would create a thick polymeric layer that would hinder the diffusion of PHE toward NQS for the chemical reaction. The NQS concentration in the cocktail was also assessed from 0 to 50 mM (**Fig. 3E**). **Fig. 3F** displays an increment in I_p when introducing more concentrated NQS in the membrane reaching a plateau at 40 mM. Thereafter, the amount of membrane deposited on the SPE was evaluated. Similar SWV were obtained for volumes ranging from 0.5 µL to 2 µL **Fig. 3G**. However, the inset plot showed the best I_p when using 0.5 µL. Finally, the reaction time before launching the electrochemical procedure was assessed from 15 to 240 s. **Fig. 3H** and **Fig. 3I** exhibit the SWV and the I_p, respectively, upon increasing reaction times, reaching a plateau after 150 s. As expected, the increment of the I_p after increasing reaction time demonstrates a rapid chemical reaction occurring at the SPE interface. As minimizing reagent waste is paramount, 0.5 % Nafion and 40 mM NQS in the membrane, the deposition of 0.5 µL of cocktail, and a reaction time of 2 min were selected as optimal conditions for further experiments.

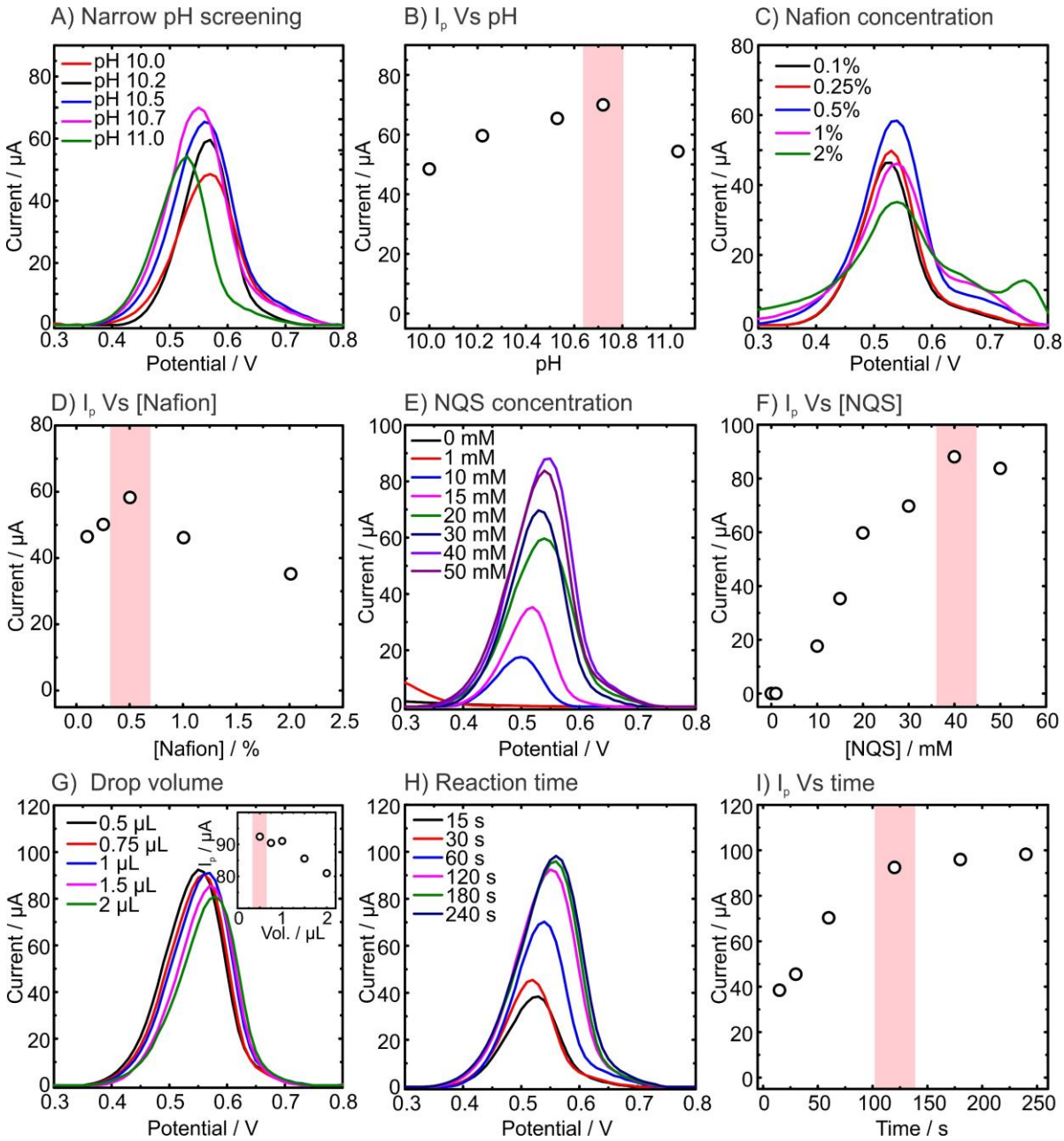

Fig. 3. *Optimization of the electrochemical detection of PHE at functionalized SPE: A) Influence of the pH in 20 mM hydrogen carbonate buffer, and B) corresponding peak current (I_p) from the SWV at each pH employing 0.5% Nafion and 20 mM NQS in the membrane. C) Evaluation of the Nafion concentration in the membrane on the electrochemical response, and D) corresponding I_p at each Nafion concentration pH using 20 mM NQS in the membrane and analysis at pH 10.8. E) Evaluation of the NQS concentration in the membrane on the electrochemical response, and F) Corresponding I_p at each NQS concentration using 0.5% Nafion at pH 10.7. G) Influence of the drop cast volume of the membrane's cocktail on the electrochemical response; inset shows the corresponding I_p at each deposited volume employing 0.5% Nafion, 40 mM NQS in the membrane at pH 10.7. H) Time of reaction dependence on the electrochemical signal, and I) corresponding I_p at each time using 0.5%*

Nafion, 40 mM NQS in the membrane at pH 10.7. PHE concentration was set at 500 µM PHE. All SWV were baseline-corrected. The reaction time was set at 120 s in all the experiments except for the reaction time assay.

3.4. Analytical performance of the modified SPE

Fig. 4 shows the analytical performance of the Nafion/NQS-coated sensor under optimal conditions (i.e. 0.5 % Nafion, 40 mM NQS, 0.5 µL of membrane, and 2 min reaction time in HCB pH 10.7). **Fig. 4A** displays the SWV upon increasing concentrations of PHE from 10 to 1250 µM (the range that allows the determination of PKU conditions in saliva/blood/plasma, **Table S1**). Accordingly, **Fig. 4B** indicates the corresponding calibration curve. Two linear relationships are determined: (i) from 20 to 100 µM, which exhibits a slope of 0.26 µA μM^{-1}, a limit of detection (LOD) of 3.0 µM and a limit of quantification (LOQ) of 9.1 µM based on the standard deviation of the response (Sy) and the slope of the calibration curve (S) according to the formula LOD = 3.3(Sy/S), and LOQ = 10(Sy/S), respectively; and (ii) from 100 to 1000 µM, which exhibits a slope of 0.11 µA μM^{-1}. **Fig. 4C** shows the SWV of a reproducibility test obtained at 500 µM PHE for intraday measurements with 10 different Nafion/NQS-coated SPEs. **Fig. 4D** exhibits the I_p of each SWV showing a relative standard deviation (RSD) of 3.6 % (N=10). Moreover, a shelf-life study was performed at room temperature within the same manufactured batch for more than 2 months. **Fig. 4E** displays the interday SWVs obtained by interrogating different modified sensors at 500 µM PHE. Each SPE was discarded after a single use. In accordance, **Fig. 4F** shows the I_p of each SWV obtained with the average and standard deviation of two sensors at each test exhibiting an outstanding RSD of 7.2% among all the measurements. Remarkably, the Nafion/NQS-coated sensor exhibited excellent stability showing promises for long-term storage at room temperature. Indeed. the employment of chemical compounds and the avoidance of biological elements on the modification of the sensor allows for highly stable sensors.

The complexity of biological matrices triggers the deep analysis of potential interferents to carefully evaluate the use of the sensor in real scenarios. One of the potential sources of error is the derivatization of other biomolecules containing primary amines. Therefore, a selectivity analysis was performed with primary amine-containing amino acids which can be present in biofluids (**Table S2**). **Fig. 4G** shows the SWV analysis with the Nafion/NQS-coated sensor of PHE, GLY, and TYR exhibiting a

higher signal for PHE (i.e. 4-fold higher for TYR and 10-fold higher for GLY). Thereafter, the effect of the targetted amino acids on the electrochemical signal of PHE was also evaluated (**Fig. 4H**). As the proposed sensor aims to determine high PHE levels in biofluids while keeping physiological levels of the other amino acids, the sensor was interrogated keeping GLY and TYR concentrations at 100 µM. Remarkably, the I_p from the SWV obtained with 500 µM PHE only exhibited an error of 1.1% showing the feasibility of the method to detect PHE in PKU conditions. However, a higher error of 15.2% was acquired for equimolar concentration.

Biological matrices also contain regular electroactive interferents that might alter the electrochemical signal of the analyte of interest. Hence, ascorbic acid (AA) and uric acid (UA), as well as potential molecules that might be ingested by the patient such as caffeine (CAF) and paracetamol (PAR). AA and UA were evaluated at physiological levels (i.e. 200 µM), and CAF and PAR were evaluated at low (100 µM) and high concentrations (500 µM) as their concentrations might vary depending on the ingested amount and pharmacokinetics of the patient. **Fig. 4I** shows the SWV of pure interferents at the aforementioned concentrations. UA and AA exhibited a small oxidation process at a similar potential window as in the oxidation of the derivatized PHE. This result suggests that AA and UA trigger some side reactions with the NQS leading to unspecific signals. In contrast, when the interferents were interrogated with 100 µM of PHE, the I_p presented was similar with only a 10.9 % of error in comparison to the pure PHE. Besides, when the interferents were analyzed with a high concentration of PHE (500 µM), the error decrease to 4.5 %, mainly caused by the UA SWV. Finally, the PHE analysis was determined in simulated saliva interferents at regular levels (i.e. 100 µM PHE in 5 µM AA, 200 µM UA, 10 µM CAF, and 100 µM PAR), and in simulated plasma interferents (i.e. 500 and 750 µM PHE in 40 µM AA, 300 µM UA, 10 µM CAF and 50 µM PAR) (**Table S2**). **Fig. 4H** displays a similar I_p exhibiting an error of 5.2 % among the conditions when compared with the standard PHE. Overall, the sensor shows a suitable linear range and selectivity to analyze PHE in real samples.

Overall, the analytical performance of the Nafion/NQS-coated sensor displays a similar linear range and LOD to recently published manuscripts (**Table S3**). Nevertheless, the advantages from the sensor presented in this work are: (i) easy and scalable manufacturing; (ii) long shelf-life at room temperature; (iii) employment of low-

cost materials; and importantly, (iv) the linearity fits with the physiological and PKU levels (**Table S1**).

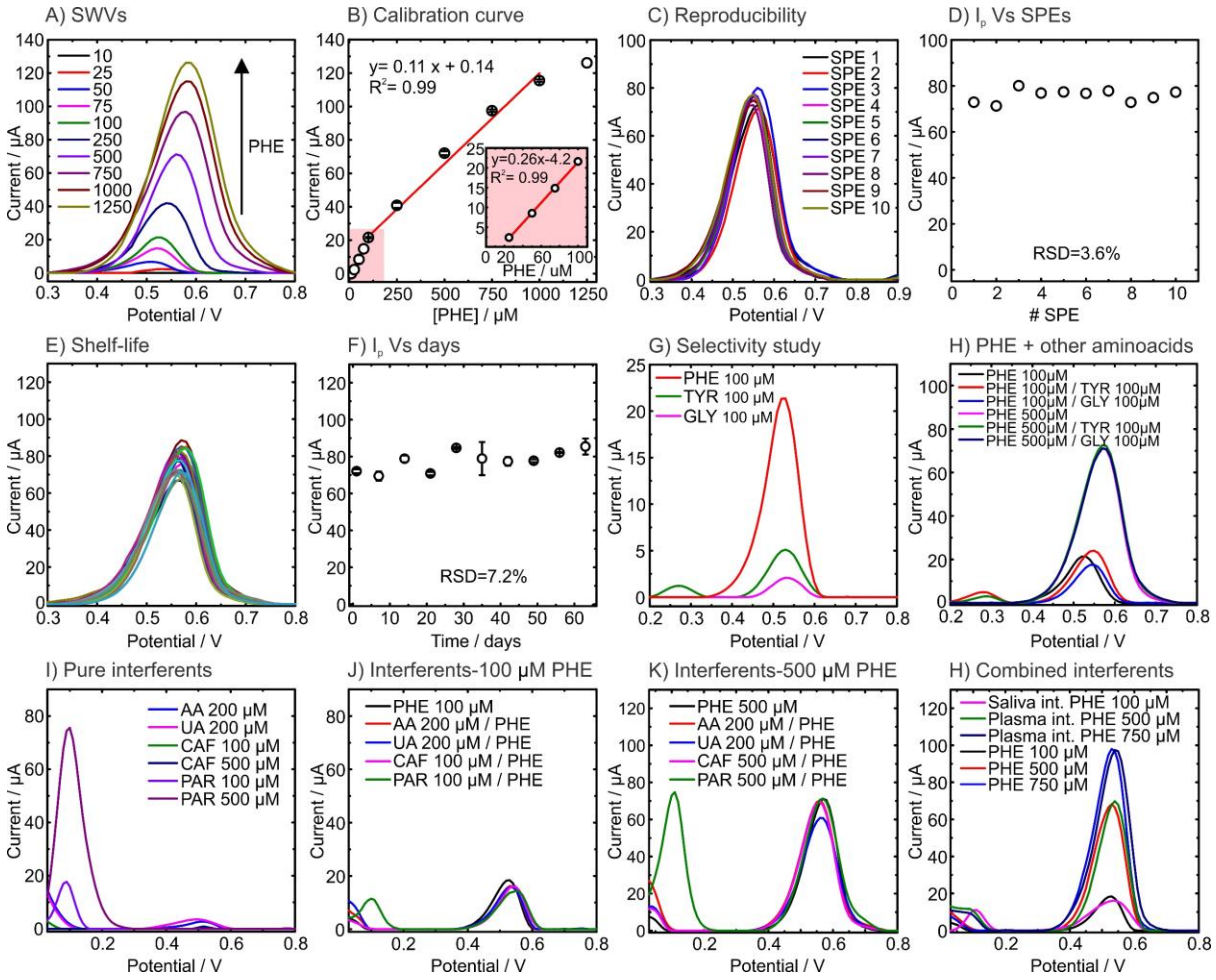

Fig. 4. *Analytical characterization of the Nafion/NQS-coated sensor for PHE detection: A) SWVs for the electrochemical oxidation of derivatized PHE in HCB in the range from 10 to 1250 µM. B) Corresponding calibration curve from the peak current values obtained from the SWVs. the inset shows the magnification of the PHE analysis at low concentration (i.e. 20 to 100 µM). Reproducibility test utilizing 500 µM PHE (N=10). C) SWVs obtained from the sensors, and D) respective peak potentials from the SWVs. Shelf-life study performed by a set of sensors stored at room temperature, E) overlapped SWVs from 20 sensors analyzed at 500 µM; and F) respective peak currents (N=2) at each time condition. Selectivity study with compounds regularly encountered in biofluids: G) pure amino acids containing primary amines (i.e. GLY and TYR); H) mixture of interferent aminoacids and PHE; I) pure interferents (i.e. AA, UA, CAF, and PAR), mixtures of interferents with J) 100 µM PHE and K) 500 µM PHE, and H) combination of several interferents found in saliva and plasma with different PHE levels. Simulated saliva interferents at regular levels (i.e. 100 µM PHE in 5 µM AA, 200 µM UA, 10 µM CAF and 100 µM PAR), and in simulated plasma interferents (i.e. 500 and 750 µM*

PHE in 40 µM AA, 300 µM UA, 10 µM CAF and 50 µM PAR). All experiments were set with 2 min reaction time after drop-casting the sample on the sensor. AA=ascorbic acid; CAF=caffeince; GLY=glycince; PAR=paracetamol; SWVs=square-wave voltammograms; TYR=tyrosine; UA=uric acid.

3.4. Phenylalanine detection in biofluids

An essential condition for the in situ derivatization of PHE for the electrochemical determination employing the Nafion/NQS-coated sensor is the analysis in alkaline pH with HCB (Parrilla et al., 2021). Thus, saliva samples were evaluated under 2-fold dilution using 100 mM HCB to bring saliva into alkaline conditions. Blank diluted saliva and spiked diluted saliva were assessed following the optimized protocol. **Fig. S3A** and **Fig. S3B** display the raw and baseline-corrected SWV of increasing concentrations of spiked PHE in diluted saliva samples. From the blank analysis, a small peak can be observed which is suggested to be from the remaining amino acids on saliva. The levels of amino acids in saliva are reported to be in the low micromolar to submicromolar range (Balci et al., 2021; Reddy et al., 2012). However, the levels might vary depending on the sampling method (e.g. time of sampling, mouth washing). In this work, the mouth was not washed before taking the sample and the subjects were not asked to be fasting as it would be in a potentially real scenario. Hence, higher levels of amino acids could be found in the analyzed saliva. Despite the variability of the amino acids, the test demonstrates the feasibility of PHE analysis in diluted saliva exhibiting an increase in the I_p (**Fig. S3B**). The calibration curve employing spiked saliva (from 25 to 400 µM) with subsequent 2-fold dilution exhibited excellent linearity with a slope of 0.047 µA μM^{-1}, a LOD of 20.9 µM (**Fig. S3C**). Besides, **Fig. S3D** indicates a reasonable reproducibility of the sensor when using diluted saliva (i.e. RSD=11.8%, N=4, 200 µM).

3.5. Wearable configuration for phenylalanine detection

User-friendliness is essential to ensure easy handling of the on-site analysis by the patient. Therefore, a wearable configuration through a wristband was developed to facilitate the PHE determination in decentralized settings. The wristband design allows for a quick test anywhere by an easy spitting process after approaching the sensor to the mouth. The wristband integrates a miniaturized potentiostat which can be

connected to a smartphone for an easy data display of graphical and numerical data and potential wireless transmission to a healthcare database.

To facilitate the sampling method and avoid the dilution step for direct analysis on the wristband, a paper-based sampling strategy was engineered (**Fig. 5A**). A conventional filter paper was first impregnated with HCB and subsequently dried. Thereafter, the filter paper (1 cm^2) was deposited on top of the modified SPE for the analysis of whole saliva or serum. **Fig. S4** studies the effect of the paper-based sampling on the electrochemical response of the modified SPE. Hence, several conditions were evaluated showing a decrease in the I_p when using the paper on top of the SPE as well as when using the impregnated salts for the in situ alkalinization of the sample. The paper might hinder the diffusion of the analyte through the membrane, and the slow in situ alkalinization which can hamper the chemical reaction could explain the decrease in the electrochemical signal. However, the process still allows a fast and easy handling analysis of PHE in the whole saliva (**Fig. S4A**) and serum (**Fig. S4B**).

Fig. S5 and **Fig. 5B** display the raw SWV and baseline-corrected SWV of whole saliva samples spiked with PHE (i.e. from 25 to 400 µM). Accordingly, **Fig. 5C** shows the calibration curve with a slope of 0.043 µA $µM^{-1}$ and a LOD of 39.8 µM. As expected, a decrease in the sensitivity of the sensor was obtained due to the analysis in a complex matrix with high-protein loading that can hinder the electrochemical detection. Despite the matrix effect of saliva, **Fig. 5D** displays an excellent reproducibility of the sensor when using whole spiked saliva (i.e. RSD=4.7%, N=4, 400 µM). As a proof of concept for PHE detection, saliva samples from two subjects were analyzed at different times of the day during regular lifestyle (**Fig. 5E**, e.g. before and after coffee or food consumption). **Fig. 5F** exhibits the electrochemical signal of Nafion/NQS-coated SPE and Nafion-coated SPE (as a control condition) upon interrogation with saliva samples from a female and male subject during different times of the day. Importantly, the control sensors do not show any redox process, showing the necessity of NQS to determine PHE in biofluids. Accordingly, **Fig. 5G** shows the PHE levels during times of the day of the two subjects. At the first impression, the two subjects reveal different concentration profiles, subject 2 exhibiting considerably higher concentrations than subject 1. Interestingly, the PHE peak was reached after 2-3h lunchtime which could be explained by the metabolization of the lunch and diffusion of the amino acids to the peripheral biofluids. Similar levels occurred after 2h past breakfast. The lowest level of PHE was shown immediately after lunch because the

food was not still metabolized and the subject claimed to be hungry before lunch. Interestingly, subject 2 took 100 mL of black coffee at 10.30h which could explain the rise in the PHE level in the analysis at 12.30h, as coffee can contain free amino acids (Casal et al., 2005). From the PHE analysis, it seems that the PHE levels can vary depending on the condition and subject, thus further evaluation of the saliva matrix needs to be performed to be statistically and clinically relevant.

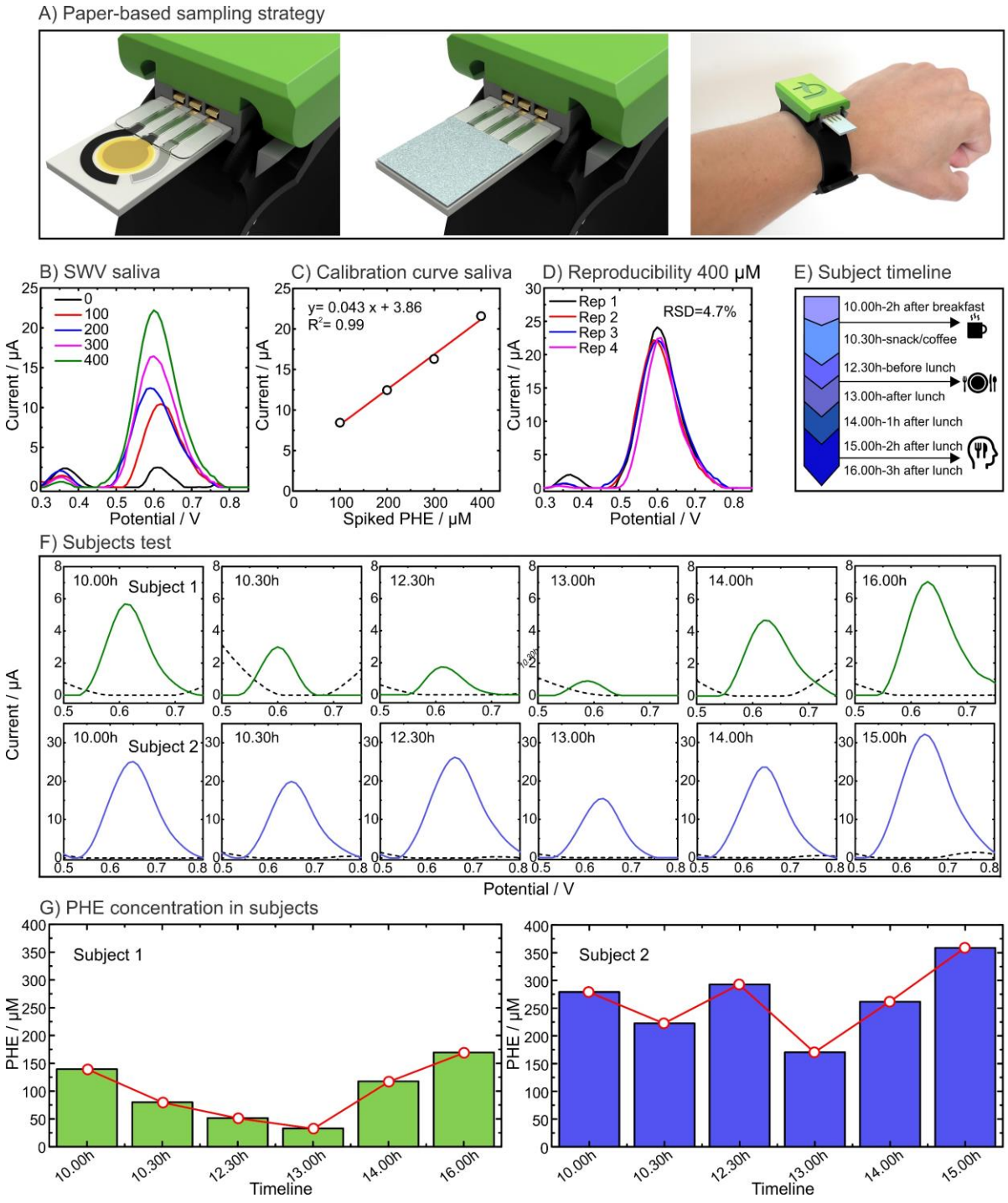

Fig. 5. *Wearable wristband-based sensor for the whole saliva detection of PHE: A) Schematic illustration of the paper-based sampling with the impregnated paper on the modified sensor*

strip. Electrochemical detection of PHE in spiked saliva from 100 to 400 µM, B) SWVs, C) calibration curve, and D) reproducibility study at 400 µM. E) Timeline of conditions when the saliva samples were collected. F) Electrochemical signal from saliva samples obtained during a regular day employing Nafion/NQS-coated SPE (straight line) and Nafion-coated SPE as a control (dashed line). G) Corresponding PHE levels in the saliva of the two subjects during daily activities. All experiments were set with 2 min reaction time after drop-casting the sample on the paper sampling element.

To further show the potential of the strategy to monitor PHE levels in biofluids, the wearable wristband-based sensor was used to analyze the whole serum by employing the paper-based sampling approach. Serum samples from male subjects were spiked with PHE to assess the analytical performance of the Nafion/NQS-coated SPE under this matrix (**Fig. 6**). Similar to saliva analysis, an oxidation peak was already encountered in serum. Indeed, PHE serum levels have been reported to be 95.86±11.45 µM (N=46) among healthy subjects (Corso et al., 2017). Despite the basal PHE level in the serum, a calibration curve from 250 to 1500 µM with spiked serum was performed. These values were selected according to the reported levels in PAH diseases to demonstrate the usability of the sensor for diagnostic and therapeutic purposes (**Table S1**). In a practical scenario, a fingerprick from the patient would be needed to extract the blood sample onto the wearable sensor.

Fig. S6A and **Fig. 6A** display the raw SWV and baseline-corrected SWV upon the increasing concentration of PHE, respectively. As the PHE levels in serum are at high concentration, the time of reaction was set at 1 min instead of 2 min to decrease the time of operation while maintaining sufficient sensitivity for proper PHE determination. **Fig. 6B** shows the corresponding calibration curve with a slope of 0.031 µA μM^{-1} and a LOD of 114.2 µM. Moreover, the reproducibility was evaluated in serum, spiked serum at 750 and 1000 µM (**Fig. S6B**, **Fig. 6C,** and **Fig. S6C**, respectively). Spiked serum samples displayed excellent reproducibility (i.e. RSD=5.9%, N=3, 750 µM, and RSD=4.5%, N=3, 1000 µM). Following the standard addition method, the predicted PHE concentration in serum was 261.4 µM. This PHE level is higher than the expected PHE level in the serum of healthy individuals (**Table S1**). It is suggested that the predicted high level can be a contribution from other amino acids in serum (e.g. glycine). Despite the obtained electrochemical signal, a much higher level of PHE should be expected from PKU patients with higher PHE in blood, thus an increase in

the oxidation peak should be envisaged. All in all, the Nafion/NQS-coated SPE integrated with a paper-based sampling approach on a wearable wristband configuration shows promises for the in situ monitoring of PHE levels in biofluids. Further electrochemical analysis of real samples from PKU patients needs to be assessed in comparison with standard methods to prove the reliability and accuracy of the wearable electrochemical device in therapeutic applications (Tu and Gao, 2021).

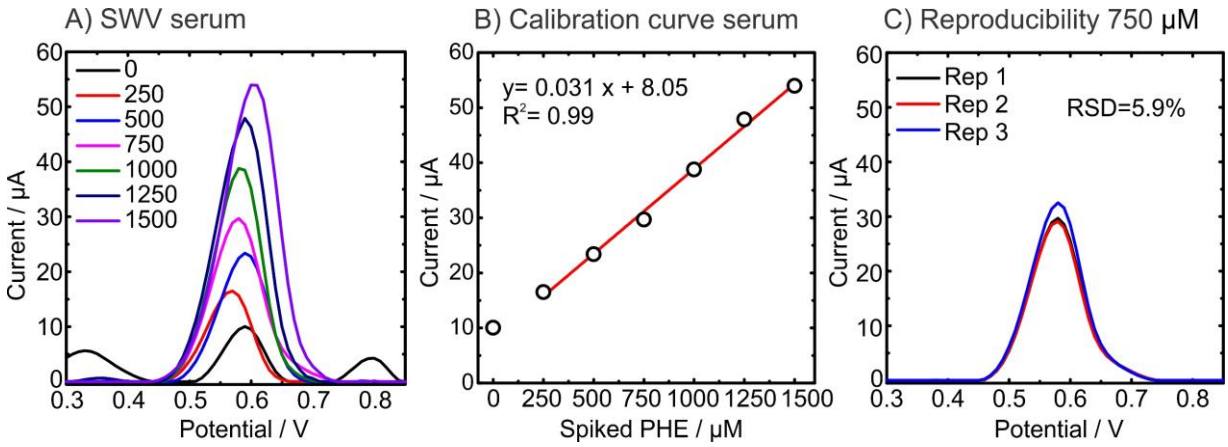

Fig. 6. *Whole serum detection of PHE with the wristband-based sensor: A) SWV of increasing concentrations of spiked serum from 250 to 1500 µM. B) Corresponding calibration curve of the spiked serum. C) Reproducibility study employing spiked serum at 750 µM. The serum sample was used without any treatment, directly deposited on top of the paper sampling element. All experiments were set with a 1 min reaction time after drop-casting the sample on the paper sampling element.*

4. Conclusions

We have demonstrated for the first time a fully integrated wristband-based electrochemical sensor platform for the monitoring of PHE levels in biofluids (i.e. saliva and serum). The embodiment consists of a miniaturized electronics module (i.e. potentiostat) coupled to a modified SPE for the PHE analysis in the biofluid. Importantly, the wristband platform allows for a rapid replacement of the disposable sensor strip thus avoiding contamination between tests. First, the electrochemical detection of PHE is presented by depositing a derivatization layer consisting of Nafion and NQS on top of the carbon SPE. Subsequently, the analytical performance of the sensor is carefully characterized exhibiting a dynamic range from 20 to 1000 µM in buffer allowing the potential monitoring of PKU levels from its healthy to alarming levels. Besides, the sensor exhibited high storage stability when considering the

lifetime of the derivatizing agent mixed with the Nafion layer. Finally, the proposed electrochemical sensor is embedded in a wristband configuration to develop a user-friendly interface able to monitor PHE levels in saliva and serum. The addition of a paper-based sampling intermediate previously soaked with the HCB buffer allows for the direct detection of PHE in undiluted saliva and undiluted serum without sample pretreatment, highlighting the relevance of the device for PKU patients.

The new wearable wristband concept can be readily expanded for the detection of other important biomarkers for health monitoring. Future steps involved the analysis of samples from PKU patients in comparison with results obtained with the standard methods, and the design of a mobile application to enhance the rapid decision-making process (e.g. take or adjust the medicine) by the physician or patient. Importantly, it is expected that the wearable electrochemical sensor can facilitate the lifestyle of PKU patients who can rapidly and easily control their PHE levels upon daily food consumption.

CRediT authorship contribution statement

Marc Parrilla: Conceptualization, methodology, investigation, data curation, writing - original draft, writing - review & editing. **Andres Vanhooydonck**: Conceptualization, methodology. **Regan Watts**: supervision, resources. **Karolien De Wael:** Investigation, resources, project administration, writing - review and editing.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version.

PDF file: Tables showing phenylalanine levels and interferents in biofluids as well as recently published PHE electrochemical sensors. Figures depicting the electrochemical detection of PHE in solution, pH screening using Nafion/NQS-coated

SPE, electrochemical evaluation of diluted saliva, the concept of paper-based sampling, raw SWV of spiked saliva, and complementary electrochemical evaluation of serum samples.

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Highlights

- Fabrication of a wristband-based electrochemical sensor for phenylalanine detection.
- Full analytical characterization of the Nafion/NQS-coated screen-printed electrode.
- The phenylalanine monitoring on saliva and serum has been demonstrated.

Graphical abstract