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# A paper-based device for glyphosate electrochemical detection in human urine: A case study to demonstrate how the properties of the paper can solve analytical issues

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ABSTRACT

In the ever-growing demand for agricultural production, the use of pesticides and the consequential health risks is an issue that remains in the spotlight. The biomonitoring of pesticides in biological matrices is a mandatory task to point out the adverse effects on those people that are particularly exposed (i.e., occupational exposure) and to customize the use of pesticides for safer and more aware agricultural practices (i.e., precision agriculture). To overcome the bottleneck of costs and long sample treatments, we conceived a paper-based analytical device for the fast and smart detection of glyphosate in human urines, which is still the most widespread pesticide. Importantly, we demonstrate how to face the analytical interference given by uric acid to develop an electrochemical sensor for glyphosate detection using paper as a multifunctional material. To this purpose, a sample treatment was pointed out and integrated into a paper strip to decrease the level of uric acid in urines, finally delivering a ready-to-use device that combines lateral and vertical flow. The effective decrease of uric acid after the paper-integrated treatment is verified by direct oxidation in differential pulse voltammetry, whereas glyphosate detection can be carried out by enzyme inhibition assay in chronoamperometry. The system showed a limit of detection for glyphosate of 75 μg/L and a linear range of 100 - 700 μg/L. Additionally, the sustainability of the paper device was assessed and compared with reference chromatographic methods. Overall, this work provides an example of how to design green sensing solutions for addressing analytical challenges in line with the White Analytical Chemistry principles.

## Introduction

Since the spread of pesticides in agricultural practices after the second half of the twentieth century, several studies have been carried out to understand their effects on the environment and living beings. Continuous exposure to contaminated agricultural products leads to the bioaccumulation of these chemicals in living organisms with harmful effects on human and animal health [1,2]. Among pesticides, glyphosate is the most ubiquitous herbicide and crop desiccant in the world [3], representing a main concern due to its toxicity, including its carcinogenic potential [4]. The U.S. National Nutrition Examination Survey revealed that glyphosate is still the most used pesticide in the U.S.A., finding its presence in representative urine samples of the population [5]. In European countries, glyphosate has been found widespread in urine samples, particularly for people who are exposed to pesticides for occupational reasons [3]. Faniband et al. [6] studied the excretion of glyphosate in human urine upon oral administration to volunteers, observing that the concentration reaches the maximum levels (higher

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than 200 μg/L and 1000 μg/L for women and men, respectively) within 2 h after the intake. These findings highlight that the analysis of glyphosate in human urine can promptly reveal that severe exposure has occurred, as in the case of occupational exposure. Early biomonitoring of glyphosate intake in urines is important to control health risks and for the management of glyphosate usage in agricultural practices, which could be customized using the approach of precision agriculture [7] to minimize adverse effects.

In this framework, the use of conventional analytical methods, such as chromatography and mass spectrometry [8,9], allows for reliable but limited monitoring, due to the need for a well-equipped laboratory, skilled personnel, and time/reagent-consuming as well as expensive/time-consuming analytical procedures (e.g., urine pre-treatment). As demonstrated in many fields, electrochemical (bio) sensors represent a valid option to obtain more sustainable analytical strategies, based on fast and cost-effective screening responses that can be carried out before performing confirmatory techniques [10–12].

In the sensor field, paper-based analytical devices have been exploited for a variety of applications resulting in an innovative approach to face the complexity of real matrices and answer the call for sustainability [13–15]. Over the last 10 years, the principle of sustainability has been translated into the sector of analytical chemistry defining new criteria for assessing sustainable analytical approaches [16]. For instance, *Green Analytical Chemistry* [17] focuses on the green aspects, listing 12 principles including the simplification of sample pre-treatment, the amount of sample needed for the analysis, the choice of miniaturized sensing devices, the decrease of waste after the analysis and the use of toxic reagents. A few years later, *White Analytical Chemistry* [18] introduced additional aspects, namely the analytical efficiency (e.g., maximizing the number of detectable analytes, the range of applicability, and compatibility with various types of samples) as well as practical/economic aspects (e.g., cost-efficiency, time-efficiency, simplicity of the overall method as well as the minimal need for advanced equipment, infrastructure, and personnel qualifications in favor of portability and easiness of usage). It is challenging to design and develop new sensing strategies able to fulfill all these criteria, but paper can provide breakthrough solutions.

In the view of sustainability and screening biomonitoring, the paper combines multiple properties capable of matching many requirements needed for developing green analytical tools [19]. Using paper typically provides devices with competitive analytical results and the advantage of being cost-affordable and disposable sensing platforms (e.g., by incineration) for screening monitoring [20]. Cellulose structure has intrinsic microfluidic properties useful to store the reagents, manage sample solutions by capillarity, and promote reactions in a confined space [19]. Such versatility has been presented by the group of C. S. Henry in a pioneering study in 2009, in which the paper microfluidics was employed for the first time to screen-printing electrochemical cells for the multiplex detection of glucose, lactate, and uric acid in human serum [21]. This device was conceived as an enzymatic biosensor, exploiting glucose oxidase, lactate oxidase, and uricase to catalyze the oxidation of the corresponding substrates and measure the enzymatic by-product $H_2O_2$ in chronoamperometry, using Prussian Blue as electrochemical mediator. For the measurement, a 5-μL drop of sample solution was dropped on the device, allowing capillarity forces to drive the sample toward the reaction areas, where the enzymes were previously loaded. This smart approach showed the suitability of the paper in designing ready-to-use microfluidics devices with promising analytical performances also in a complex biological matrix such as human serum.

After this first example, numerous studies have been carried out for biological samples [15,22] as well as for environmental monitoring [14,23] also exploiting origami-like architectures [24]. Particularly, one of the most recent examples of paper-based devices was reported by Arduini et al. [25] for the detection of three classes of pesticides namely organophosphorus insecticides, phenoxy-acid herbicides, and triazine herbicides. In detail, paraoxon, 2,4-dichlorophenoxyacetic acid, and atrazine were determined in aqueous solutions obtaining linear ranges within 2–20 μg/L, 100–600 μg/L, and 10–100 μg/L, respectively. The detection principle was based on enzyme inhibition assays, by exploiting the inhibitory activity of these pesticides on specific enzymes, namely butyrylcholinesterase, alkaline phosphatase, and tyrosinase, respectively. Later, the same group reported a paper-based flower-like biosensor for the detection of pesticides from the aerosol phase [26]. In this case, butyrylcholinesterase, alkaline phosphatase, and hydrogen peroxidase (HRP) enzymes were used for inhibition assays to detect paraoxon, 2,4-dichlorophenoxyacetic acid, and glyphosate, with linear ranges within 2–20 μg/L, 50–400 μg/L, and 50–150 μg/L, respectively.

In the present work, we exploited the paper properties to develop a paper-based origami biosensing strategy for the analysis of glyphosate in human urine. Taking inspiration from the previous works, the electroanalytical sensing of glyphosate is here based on the inhibition of HRP enzyme activity using 3,3′,5,5′-tetramethylbenzidine (TMB) as the substrate [26]. Considering the analytical interference due to the presence of uric acid in human urine, we designed an easy sample treatment to reduce the amount of this species in the sample by precipitation under acidic conditions. Notably, this sample treatment was integrated into a paper strip, configured to combine lateral flow and vertical flow microfluidics in a single architecture (Fig. 1). By exploiting the porosity of the paper, all the reagents were pre-loaded in specific areas of a paper platform, allowing for the sample treatment as well as for the enzymatic inhibition assay without the use of any additional reagents. To carry out glyphosate detection and to check the effective decrease of the uric acid level after treatment, two separate paper-based printed sensors have been integrated into the same platform. They were included to perform, respectively i) evaluation of residual uric acid in the sample by differential pulse voltammetry (DPV) and ii) glyphosate detection through enzymatic inhibition assay by chronoamperometry. Our work demonstrates the possibility of integrating a sample treatment into a ready-to-use paper-based analytical device to finally obtain a smart and sustainable analytical approach for glyphosate detection to overcome uric acid interference. Being characterized by low-impact, sustainable materials (i.e., paper), nontoxic reagents, and minimal waste production, this integrated device is also respectful of the principles of the White Analytical Chemistry, as demonstrated by the application of the sustainability assessment. This paper-based sensing platform can promote an early screening of glyphosate in human urine allowing for the biomonitoring of excess exposure and the prompt activation of adequate interventions.

## Materials and methods

### *Reagents and equipment*

HRP, TMB, glyphosate (N-phosphonomethyl glycine, 99.2% w/w), uric acid, ammonium chloride, sodium chloride, disodium sulfate, urea, creatinine, and Nafion™ 117 containing solution was purchased from Sigma Aldrich. All solutions were prepared with MilliQ water (18 MΩ×cm resistivity). Britton-Robinson buffer (BRB) at a concentration of 0.2 M was prepared using $H_3PO_4$, $H_3BO_3$, and $CH_3COOH$; to adjust its pH to 5, adequate amounts of NaOH (0.2 M) were added. Phosphate buffer saline (PBS) at a concentration of 0.1 M and a pH of 7.0 was prepared with a concentration of 0.1 M $Na_2HPO_4$ and $NaH_2PO_4$, adding 0.05 M of KCl. Syringe filters (PTFE/∅13 mm/0.22 μm/Organic) were purchased from Biocomma. The office paper-based screen-printed electrodes (SPE) were supplied from SENSE4MED Company (Rome, Italy) and consisted of graphite working and counter electrodes and Ag/AgCl pseudo-reference electrode (S4M-OP02), with geometric working electrode surface area equal to 12.6 $mm^2$. Further commercial carbon SPEs (DR.−110) were purchased from Dropsens-Metrohm (geometric working electrode surface area equal to 12.6 $mm^2$). Paper pads and paper strips were wax-printed on filter paper (67 $g/m^2$, Cordenons, Italy), as previously described [14]. Commercial carbon black (CB,

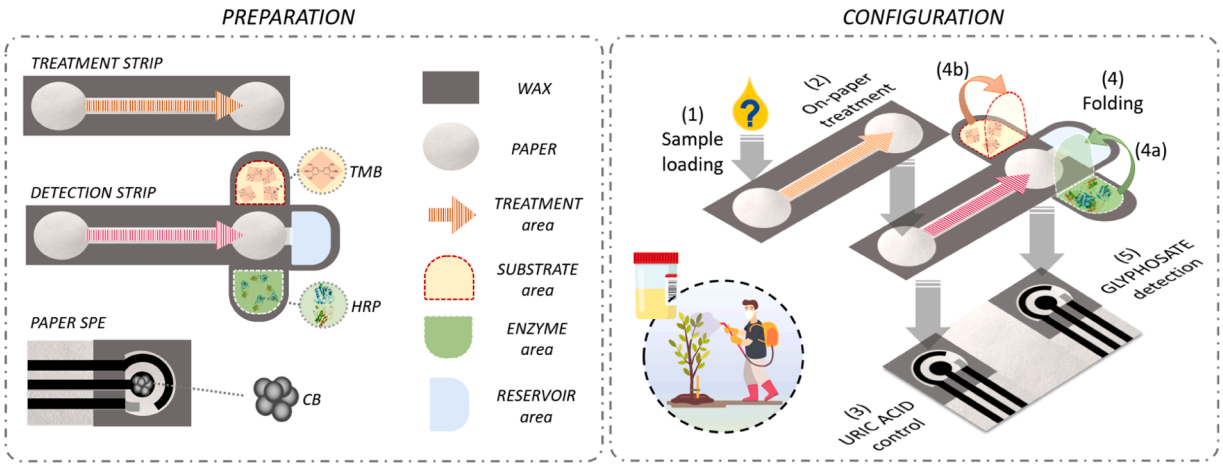


**Fig. 1.** Illustration of our paper-based electrochemical biosensing platform for glyphosate detection in urine samples. Left side: description and preparation of the paper strips. Right side: origami-like configuration of the paper platform, composed of three paper layers overlapped: the treatment strip, the detection strip, and the layer with the screen-printed sensors. The preparation and application procedures are described in detail in paragraphs 2.5 and 2.6.

N220) of the industrial standard grade was obtained from Cabot Corporation. The solutions of synthetic urine with a well-defined chemical composition that can be changed to test the effect of different components on enzymatic inhibition were prepared according to the procedure reported in our previous works [27]. A portable potentiostat, Sensit Smart (PalmSens, Netherlands) connected to a laptop was used to carry out the electrochemical measurements. All data were recorded with PSTrace5 software and elaborated using OriginPro 8.5.

*Electrochemical enzymatic assay preliminary tests*

The inhibition of HRP enzymatic activity was estimated via chronoamperometric analyses using SPE previously modified via drop-casting with 2 μL of 1 mg/mL CB dispersion. A drop of 20 μL of synthetic urine, was put in contact with the working electrode surface of the SPE-CB. A drop of 2 μL of 25 U/mL HRP in PBS solution was added followed by an incubation time of 5 min to activate possible enzyme inhibition. Then, 20 μL of TMB solution diluted 1:5 v/v with synthetic urine was added to the drop, followed by a second incubation time of 2 min, necessary to activate the enzymatic reaction. Finally, the product of the enzymatic reaction was detected via chronoamperometric analysis by polarizing the working electrode a −0.2 V vs. pseudo-Ag/AgCl for 100 s.

In each case, the inhibition percentage (I%) was calculated with Eq. (1):

$$I\% = \frac{I_0 - I_i}{I_0} * 100 \quad (1)$$

where $I_0$ and $I_i$ are the currents recorded from the HRP enzymatic assay using TMB as substrate in the absence and in the presence of glyphosate, respectively.

*Urine sample pre-treatment*

Real urine samples were provided from the Microbiology Laboratory of the "Casa di Cura Giovanni XXIII of Monastier" in Treviso(Italy). The samples (volume of 5 mL), stored at −20 °C just after the sampling, were defrosted at 4 °C just before the analysis and brought to room temperature (20 °C). In the preliminary tests with real urine, the initial pH of each sample was measured using a pH electrode (HI1083B, Hanna Instruments™) and, depending on the value recorded, small aliquots of 1.0 M solution of either HCl or NaOH were added to reach pH 5.0 and pH 8.0, to induce UA precipitation. A minimum volume of either HCl or NaOH was added to avoid significant dilution. For samples brought to pH 8.0, NaCl was added to a final concentration of 0.5 M to further promote UA precipitation. After 5 min, all samples (pH 5.0 and 8.0) were filtered using a syringe filter (polytetrafluorethylene, pore size 0.45 μm). The filters were previously activated with methanol and washed with MilliQ $H_2O$ before treating urine samples. The pre-treated samples were stored at 4 °C until testing. It is worth noting that the same pre-treatment steps were applied also to synthetic urine samples, as described in Section 3.2. For all spiked samples, the addition of glyphosate or uric acid was performed before the pre-treatment.

*Voltammetric detection of uric acid level*

The level of uric acid in the samples after the treatment was monitored by direct oxidation using the DPV technique, in the potential region between −0.1 to +0.6 V vs. pseudo-Ag/AgCl, applying a potential step of 7 mV and a potential pulse of 40 mV with a time pulse of 100 ms and a scan rate of 0.015 $Vs^{-1}$. For tests in these solutions, 40 μL of the sample were directly drop-cast on the SPE-CB to perform DPV analysis. For tests on the paper strip, 60 μL of samples were drop-cast on the sampling area, then the sample flowed through the treatment area, reaching the uric acid detection area where DPV analysis was carried out at SPE-CB.

*Paper-based platform design*

The origami-like paper platform, composed of three paper layers overlapped: the treatment strip, the detection strip, and the layer with the screen-printed sensors (Fig. 1). The treatment strip (about 6 cm-length) presents a microfluidic pathway (about 0.7 x 8.0 cm) defined by wax, where 20 μL of BRB at pH 5.0 are pre-loaded for providing the on-paper treatment area. The wax pattern was obtained by using a ColorQube 8580 Xerox printer. The treatment strip is overlapped on the detection strip to allow the sample to flow through the layers by a vertical flow, reaching the underlying layer where two SPE-CB sensors are placed. At the end of the detection strip, which presents another lateral microfluidic pathway (about 0.7 x 8.0 cm), two pads are provided, confined by the wax barrier to pre-load separately the HRP enzyme (2 μL of a 25 U/mL HRP in PBS solution) and the TMB substrate (20 μL of TMB solution diluted 1:5 v/v in PBS), and left to dry at room temperature before use. Finally, a reservoir area allows the dropping of 0.1 M PBS buffer when the enzyme inhibition assay takes place, to ensure the dissolution of the reagents from the cellulose matrix and their reactions. The sample undergoes a lateral flow, first along the treatment strip and then the detection strip, in addition to the vertical flow through

the paper layers.

*Analytical protocol*

When 60 μL of the sample is drop-cast at the beginning of the treatment strip, it flows through the lateral microfluidic pathway along which the treatment occurs. In the treatment area, the pre-loaded BRB (pH 5.0) creates the acid conditions that induce uric acid precipitation, as described in Section 2.4. After the treatment area, the sample flows vertically through the layers, reaching the first SPE-CB to detect the residual uric acid amount by DPV. The treatment can be repeated with other treatment strips until the signal due to uric acid oxidation is decreased sufficiently (below 20 μA).

In the meanwhile, the sample flows through the lateral microfluidic pathway along the detection strip, reaching the area for the enzyme inhibition assay. The assay is carried out by folding the pads previously loaded with the enzyme and the substrate. Before the assay, 10 μL of PBS (pH 7.4) is dropped in the reservoir area to facilitate the diffusion of the sample throughout the microfluidic pathway of the detection strip, to ensure the dissolution of the reagents, and the correction of the solution pH for the enzyme inhibition assay. Thus, the HRP-loaded pad is folded on the strip to expose the enzyme for 5 min to the sample solution. Then, the TMB-loaded pad is folded to overlap the HRP-loaded for a reaction time of 2 min. Finally, the chronoamperometric signal is recorded by applying a potential of - 0.2 V vs pseudo-Ag/AgCl for 100 s. The chronoamperometric response in the presence of glyphosate is used to calculate the inhibition percentage (I%) with respect to the $I_0$ (current of the enzymatic activity in the absence of inhibitors), according to Eq. (1).

## Results and discussion

*HRP-based assays in urine environment*

The inhibition capability of glyphosate towards HRP was exploited by Caratelli et al. [26] to develop a paper origami device for detecting glyphosate among multiple pesticides in the aerosol phase. They pre-loaded HRP and its substrate (TMB) on separated paper areas of a paper-based device to carry out an enzyme inhibition chronoamperometric assay by just exposing the HRP-loaded paper pad to an aerosol containing glyphosate as the inhibitor. In the present work, we translated this detection principle to urine samples, addressing the issue in a biological matrix. Chronoamperometry was chosen as the readout method for the HRP-TMB assay instead of colorimetric ones to ensure high sensitivity and selectivity for a complex matrix, such as urines. The electroanalytical performance was boosted by using CB, which allows for carrying out the HRP-TMB assay at a potential of - 0.2 V vs pseudo-Ag/AgCl [26].

To test eventual interfering compounds in urines, we evaluated the response of HRP-based assays in the presence of some main components in urines, namely uric acid, urea, or creatinine, all tested in standard solutions at physiological urine concentrations. In addition, synthetic urine with a complete composition, including uric acid at a physiological concentration, was tested as a medium for the HRP-based assay. These experiments were performed by dropping 60 μL of solution on an SPE-CB and collecting chronoamperometric responses (see Section 2.2). By comparing the current values recorded in these conditions (Fig. 2a), we can observe that urea and creatine are not responsible for any significant changes in the assay response, while the presence of uric acid decreases the current by about 60%. A similar result was observed when the enzymatic assay was carried out in the synthetic urine medium. These findings suggested that the HRP-based assay is significantly affected by the presence of uric acid in the solution. Indeed, a decrease in the HRP enzymatic activity was observed also in samples of real urine. These observations are consistent with previous studies, which demonstrated that uric acid can act as an enzymatic substrate for HRP [28,29].

To better understand how this species affects the HRP-based assay, synthetic urine samples containing various amounts of uric acid within the physiological range (between 1.2 and 4.4 mM [30]) were mixed with HRP and left in contact for 5 min. Then, TMB was added to the sample solution and the chronoamperometric analysis was recorded after an additional reaction time of 2 min. A current decrease of about 85–100% was obtained in the chronoamperometric signal for uric acid concentration higher than 1 mM (Fig. 2b). Knowing that the physiological concentration of uric acid in human urine is between 1.2 and 4.4 mM [30], it represents an issue that limits the application of the HRP-based enzyme inhibition assay for glyphosate detection in urine.

*Treating physiological levels of uric acid in urine samples*

To tackle the issue of uric acid analytical interference in HRP-based assays, we used the properties of the paper as the key functional material to decrease the concentration of this species in the sample, exploiting the precipitation phenomena occurring in both acid and alkaline environments. Uric acid, in fact, is a weak diprotic acid with $pK_{a1} = 10.3$ and $pK_{a2} = 5.6$, whose precipitation equilibria are strongly dependent on the pH, $Na^+$concentration, and ionic strength of the solution. The solubility

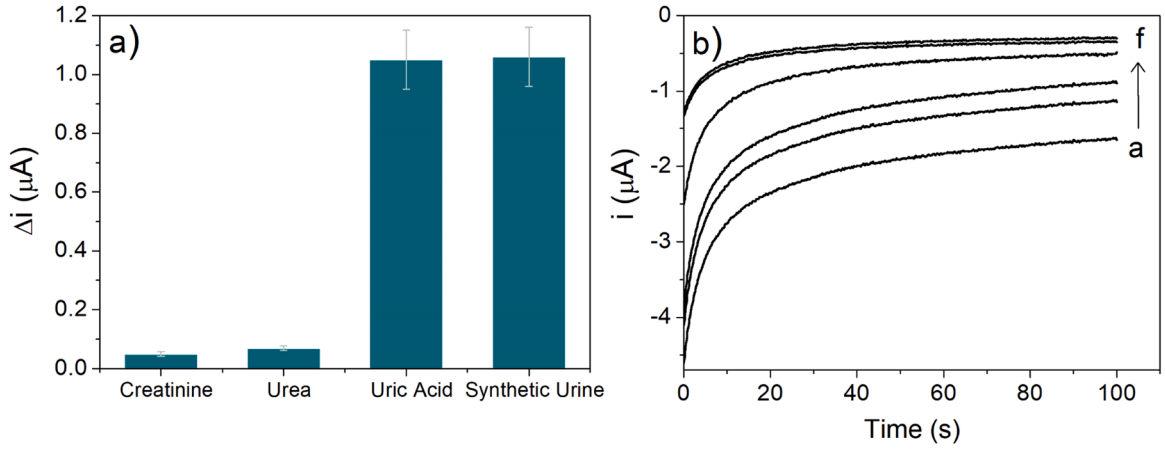


**Fig. 2.** a) Current decrease obtained from chronoamperometric responses of HRP-based assay obtained after the addition of TMB substrate in a drop of solution (60 μL) containing creatinine (17.7 mM), or urea (412.5 mM), or uric acid (2.1 mM), or synthetic urine composition [27]. The current intensity is expressed as the difference (Δi) between the $i_0$ of the HRP/TMB system in buffer solution and $i_f$ recorded in the presence of the different compounds listed above. b) Chronoamperometric responses of HRP-based assay for 60 μL-drop in the presence of TMB substrate and uric acid at various concentrations: 0, 0.50, 0.75, 1.5, 2.5, 4.0 mM. Measurements were carried out in triplicate at SPE-CB, $E = -0.2$ V vs Ag/AgCl pseudo reference electrode.

curves of uric acid and the relevant monosodium urate form follow a specific trend with pH [31]. At pH values close to 8, a high $Na^+$ concentration can increase uric acid supersaturation with respect to the monosodium urate form, promoting its precipitation. On the contrary, for pH values equal to or lower than 5, the uric acid concentration is limited by the low solubility, inducing precipitation from the supersaturated urate solutions [32].

Knowing this, a pre-treatment procedure based on pH-driven precipitation [33] was developed to reduce the uric acid physiological level in urine samples. The samples (both synthetic urine and real urine) were treated to reach either pH 5.0 or 8.0 to promote uric acid precipitation and then were filtered to remove the precipitate. Note that 0.5 M NaCl was added to samples at pH 8.0, as indicated in Section 2.3. The changes in uric acid levels of both synthetic and real urine samples were evaluated considering the current intensity of the oxidation peak ($I_{pa}$) of uric acid [34] before and after the pre-treatment step, studied via DPV (Fig. 3). The responses were consistent with a meaningful reduction of uric acid content (30–40%) when the sample pH was adjusted to either pH 5.0 or 8.0 and then filtered (Fig. 3a, b, d, e, green curves). Although both the acid and alkaline conditions were suitable for reducing uric acid content in solution, the pre-treatment at pH 5.0 resulted in a more effective decrease in real urine (45–50% with respect to the original signal), even more than in synthetic urine (30–40%), as observed from Fig. 3c and f. Thus, the acidic treatment followed by filtration was considered the optimal pre-treatment protocol to be applied in this study. The effective decrease of the uric acid level in both synthetic and real urine samples enables the decrease of the interference on the HRP enzymatic reaction, allowing for carrying out the enzymatic inhibition assay based on glyphosate detection, as shown in the next paragraph.

*Paper-based origami platform for glyphosate detection in real urine*

Having verified the role of uric acid in HRP-based assays and pointed out a protocol for the pre-treatment for pH-filtration to decrease the level of interference in urine samples, we adapted this analytical protocol to the customized paper-based origami platform. This latter was specifically designed to integrate the pre-treatment of the samples as well as the detection step within a disposable, easy-to-use, sustainable, and cost-affordable paper strip, thanks to the paper microfluidic. To achieve this purpose, the platform was loaded with all the reagents simply via drop-casting, including BRB for pH treatment, and HRP and TMB for the enzymatic assay, resulting in a fast preparation protocol (Fig. 1).

The first step in designing this platform has been focused on integrating the treatment pointed out in Paragraph 3.2 within the cellulose matrix. Herein, the pH-filtration step was achieved by pre-loading a solution at pH 5.0 in the treatment area (Fig. 1, treatment area), exploiting the intrinsic adsorption/filtering properties of the paper to carry out the filtration in the selected acidic conditions. In detail, 20 μL of 0.5 M of BRB at pH 5.0 were simply drop-cast in the treatment area and left to dry. Thus, 60 μL of the real urine sample was dropped in the sampling area to flow through the treatment area, where it got into contact with the pre-loaded BRB. By dissolving the BRB salts, the acid conditions are restored in the cellulose matrix and the pH treatment occurs together with the filtration process, thanks to the intrinsic properties of the filter paper in which the treatment takes place. Thus, the $I_{pa}$ resulted in a decrease of about 50%, in agreement with the decreased percentage obtained for the pH-filtering treatment carried out in the liquid phase. No significant changes in the precipitation efficiency were observed by loading different volumes of 0.5 M BRB (data not shown).

If the uric acid level still results too high after the on-paper treatment, a second treatment strip can be added to the platform before running the detection strip. In this way, the on-paper treatment can be repeated 1–2 times to further reduce uric acid levels. We observed that when the response of uric acid is decreased to a current lower than 20 μA, the glyphosate quantification can be carried out without significant uric acid interference. For instance, the use of a second treatment strip was found to provide an additional decrease of uric acid of 25% (data not shown).

The final platform was completed with a paper layer composed of two office paper screen-printed sensors, with the working electrodes

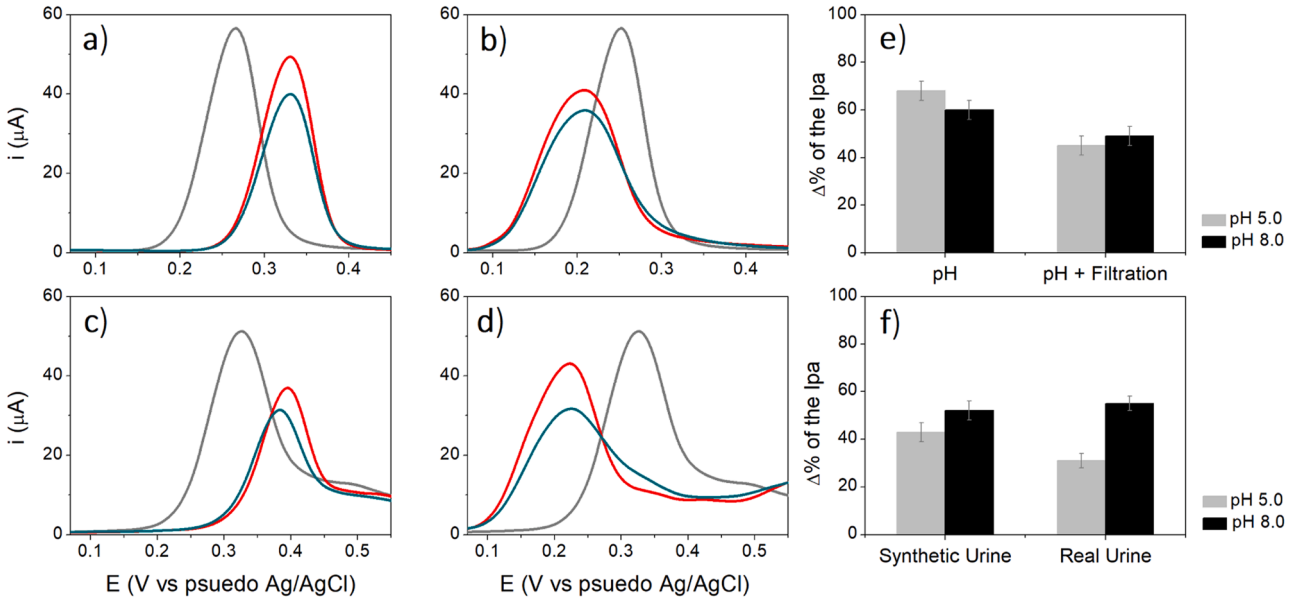


**Fig. 3.** (a, b, d, e) Comparison of the voltammograms recorded via DPV of urine samples before (*gray curve*), after pH treatment (*red curve*), and after pH treatment plus filtration step (*green curve*); results were obtained in synthetic urine samples with a pretreatment at pH 5.0 (a) and 8.0 (b), and in real urine samples with a pretreatment at pH 5.0 (d) and 8.0 (e). (c) Comparison of the decrease in the anodic peak current intensity ($I_{pa}$) of uric acid in synthetic urine, with a known concentration of uric acid (i.e., 2.2 mM), after the pH treatment and the pH treatment + filtration (e), at either pH 5.0 (*light gray*) and 8.0 (*black*). (f) Comparison of the efficacy of the whole pre-treatment (pH + *filtration*) in synthetic and real urine at either pH 5.0 (*light gray*) or 8.0 (*black*). "*Δ% of the* $I_{pa}$" is the percentage variation between the uric acid current peak registered before and after the pretreatment. Note that the peak potential changes are due to the pH variation influence on the uric acid oxidation process.

modified with CB (SPE-CB). The paper strips can be combined as shown in Fig. 1. The biosensing platform is assembled by overlapping the filter paper strips onto the SPE-CBs. When the sample is dropped at the beginning of the treatment filter paper strip, it runs along the strip thanks to horizontal microfluidic, undergoing the treatment directly inside the cellulose matrix. After the treatment, the sample reaches the first SPE-CB by vertical flow to control that the residual uric acid level with DPV analysis is under the 20-μA threshold. Thus, the sample flows along the detection strip until it gets in contact with the HRP-TMB system, which is allowed to react by folding the pads previously loaded with the HRP and TMB, separately. The folding sequence includes first the HRP pad, to get the enzyme in contact with the sample and eventually react with glyphosate, then the TMB pad, to enable the enzymatic reaction. The enzymatic reaction can be monitored in chronoamperometry using the second SPE-CB, which is wet by vertical flow, allowing for the detection of glyphosate through the enzyme inhibition assay. Conveniently, the total time for the analysis, from the sample addition to the final readout, is about 10 min, considering the 5-minute contact time between HRP and the sample solution, the 2-minute incubation in the presence of TMB, and the 1.7 min for running the amperometric scan.

The complete analytical protocol was tested on several real samples of human urine, spiked with known amounts of glyphosate up to 700 μg/L (4.14 μM). Fig. 4 shows the chronoamperometric responses corresponding to HRP-based detection and the calibration plot obtained considering the I% calculated for glyphosate inhibition according to Eq. (1). In this screening, the $I_0$ value corresponds to the chronoamperometric current recorded for real urine samples without any glyphosate contaminations. Increasing concentration of glyphosate resulted in a progressive decrease of chronoamperometric currents due to the inhibition of HRP (see inset of Fig. 4). A linear correlation between the I% and glyphosate concentration was observed in the range from 100 to 700 μg/L (i.e., 0.59 – 4.14 μM), corresponding to an inhibition percentage comprised between 10% and 50%, respectively. The LOD in real urine was calculated for I% = 10% resulting in a value of 75 μg/L, while for glyphosate concentrations ≥ 700 μg/L the signal reaches saturation, as shown in Fig. S1.

These outcomes suggest that the origami platform can be used to monitor glyphosate overcoming the interference from uric acid in real human urine samples and avoiding time-consuming sample pretreatment steps. The results are summarized in Table 1. This screening provided successful results since the real and experimentally deduced glyphosate concentrations showed a good agreement.

**Table 1**
Uric acid level and glyphosate (GLY) measured in real urine samples (A, B, C, D). The glyphosate concentrations calculated with our analytical method are compared with the known concentrations used to spike the real urine samples upstream of the analysis, resulting in good percentage recoveries.

| Sample | Glyphosate added (μg/L) | Glyphosate calculated (μg/L) | Recovery, % |
|---|---|---|---|
| **A** | 75.0 ± 0.1 | 74.3 ± 0.8 | 98.7 |
| **B** | 250 ± 1 | 247 ± 2 | 98.8 |
| **C** | 500 ± 1 | 490 ± 3 | 99.8 |
| **D** | 750 ± 1 | 745 ± 4 | 99.3 |

*Sustainability assessment*

The sustainability of the here proposed analytical approach was assessed by interrogating the method with the criteria of the White Analytical Chemistry, introduced by Nowak et al. [18] and based on previous sustainability assessment methods [17,35]. This assessment is an innovative tool that points out what are the most commonly recognizable key aspects to evaluate the sustainability of an analytical methodology. To apply this assessment, it is necessary to analyze a total of 12 principles, including the analytical performance (4 "red" principles), practical and economical aspects of the methodology (4 "blue" principles), and the environmental friendliness and safety aspects (4 "green" principles, in agreement with the Green Analytical Chemistry [17]). The red, blue, and green principles together provide a degree of "whiteness", analogously to the RGB color model, in which fully saturated red, blue, and green colors give a white color. The White Analytical Chemistry approach provides a simple algorithm to assign to each principle a score between 0 (not sustainable) and 100 (fully sustainable), and overall, quantify the degree of sustainability (the "whiteness"). Moreover, the algorithm, which is provided as an Excel file, enables the comparison among several analytical methods by their sustainability scores.

The summary of each principle is reported in Tables S1 and S2 while the criteria here used for score assignments are reported in Table S3, together with the Excel file, in the Supplementary material, in which a description of each principle is also reported. To critically evaluate the aspects of our method, a reference method for glyphosate detection was taken into consideration in this assessment. The chosen reference method is based on high-pressure anion exchange chromatography coupled with mass spectrometry (HPAEC-MS/MS) for glyphosate detection in several samples from a cattle farm [36], including urines. This method was selected among others not only for its relevance to our comparison but also because it reports enough information for the score

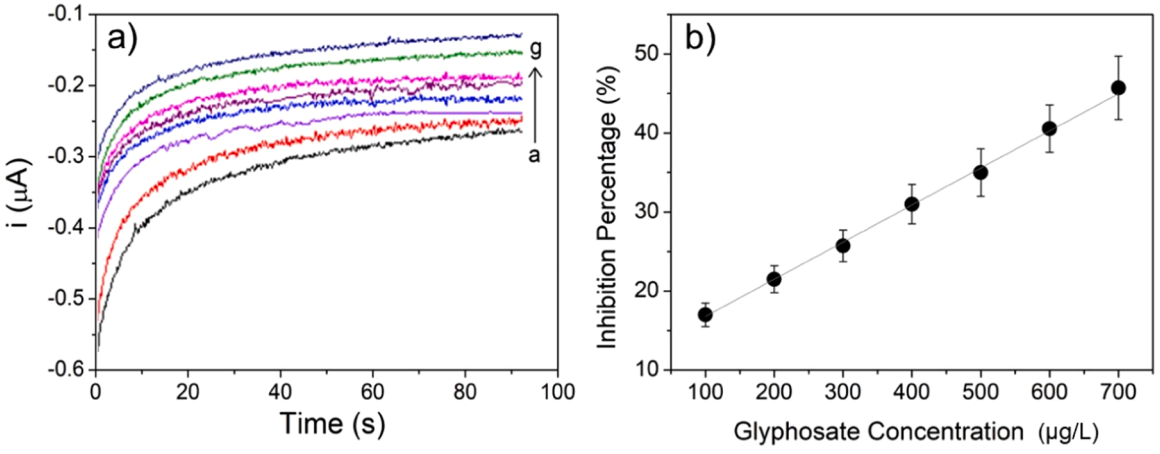


**Fig. 4.** a) Chronoamperometric measurements registered for real urine samples in the presence of glyphosate concentration ranging from 100 to 700 μg/L (0.59 μM to 4.14 μM) (from a to g), E = −0.2 V and t = 100 s. b) Calibration plot of glyphosate in spiked real urine samples, the error bars were calculated from triplicate measurements.

assignment of each sustainability principle.

The results of the assessment are illustrated in Fig. 5, with total scores (whiteness) equal to 87.2 and 58.3 for the paper origami platform and HPAEC-MS/MS, respectively. It can be observed that the whiteness of our proposed method is significantly higher than the reference method, thus demonstrating that our method more fully meets the sustainability criteria.

In detail, the analysis of the red principles results in medium-to-high scores equal to 66.3 and 91.3 for the paper origami platform and HPAEC-MS/MS, respectively. Among red principles, the resistance to the presence of potential interferences was assigned with the maximum score for both the paper origami platform and HPAEC-MS/MS method since both addressed this issue: the former is customized to overcome the possible interferences observed in urine samples (i.e., uric acid), while the latter allows for analyzing urine samples without any interfering effects. However, the linear range, the precision, and the LOD of our method are less performant than the ones of HPAEC-MS/MS, suggesting that the here proposed paper origami platform is recommendable as a sustainable tool for early screening applications.

The analysis of the green and blue principles further confirms the higher sustainability of our method. Indeed, the green aspects are clearly advantageous for the paper origami platform (scores equal to 97.5 and 62.9 for the paper origami platform and HPAEC-MS/MS, respectively), especially regarding the energy consumption, since our sensing platform, involving the need for only a portable potentiostat and a laptop, consumes much lower energy with respect to the chromatography/spectrometry-based instrumentation. Moreover, it is noteworthy that the very low volumes of reagents (< 50 μL) and sample (60 μL) used for our method, as well as the minimal waste production (e.g., the paper strips can be disposed of by incineration, reducing the waste and biological risk), strongly improve the sustainability of this method with respect to HPAEC-MS/MS.

Finally, the analysis of the blue principles resulted in scores equal to 97.9 and 20.8 for the paper origami platform and HPAEC-MS/MS, respectively. The aspects that rule this category, namely cost-efficiency, time efficiency, general requirements (including advanced instruments, skills, and facilities), and operational simplicity (including portability, automation, and miniaturization) point out a score markedly in favor of our paper origami platform, which encloses the advantages of very low total costs (< 5 euros), fast analysis (about 10 min), no need for skilled personnel or advanced instruments or skills, easy application with low consumption of sample (only 60 μL of sample needed for the analysis, directly dropped on the paper platform), in a handheld device that can be applied for at-line in situ analysis. Overall, this assessment shows that the combination of paper properties and the analytical strategy here conceived has produced a sensing device with competitive performance in terms of several aspects of sustainability, particularly suitable for screening analysis of glyphosate in human urine.

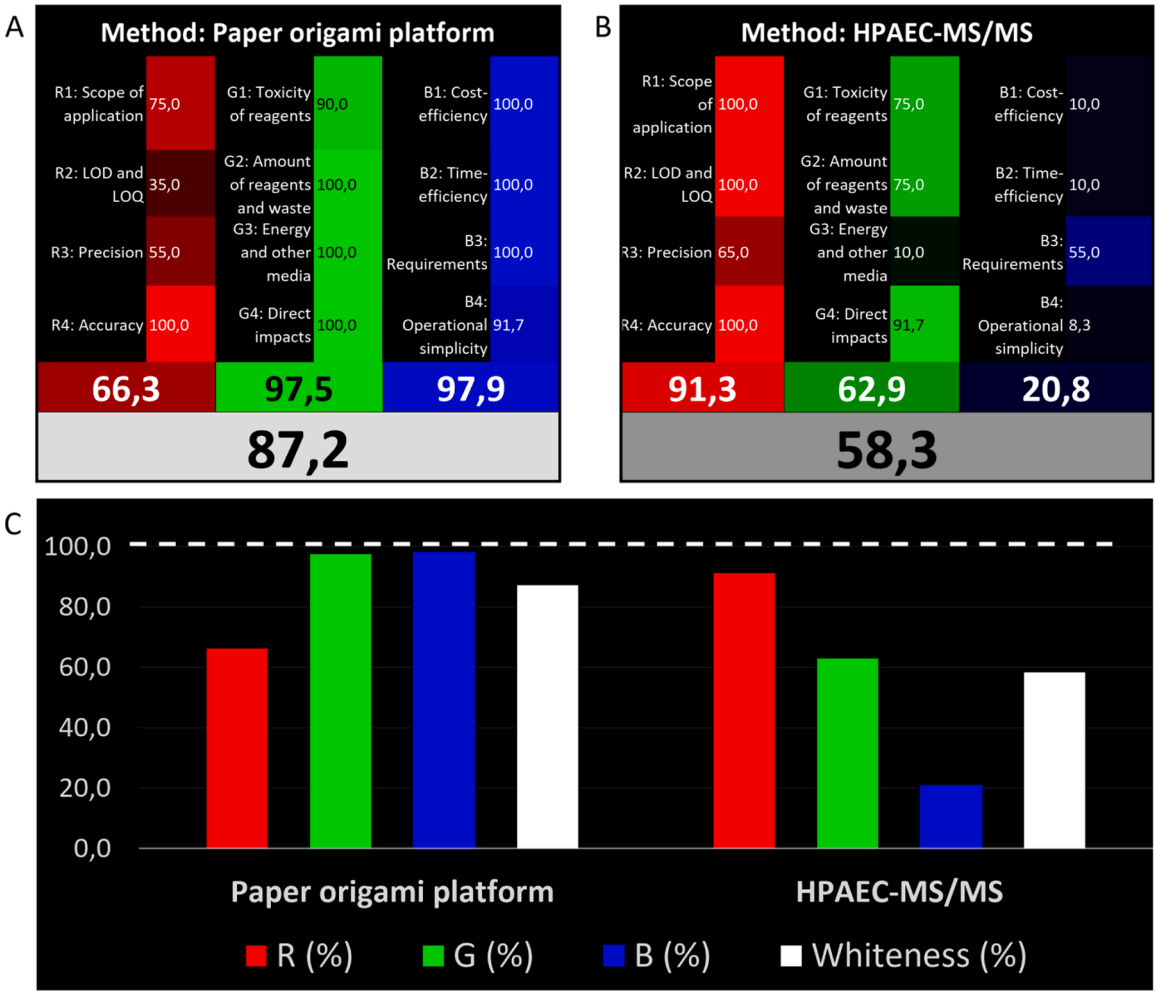


**Fig. 5.** Scores resulted from the application of the Whyte Analytical Chemistry assessment [18] for the method based on our paper origami platform (A) and a reference method for glyphosate detection, namely HPAEC-MS/MS (B). The scores for the *red*, *green*, and *blue* principles are illustrated in (C) with the resulting *whiteness*.

## Conclusions

In this study, we implemented the applicability of an HRP-based enzymatic inhibition strategy for glyphosate detection in a complex biological sample, namely human urine, as a key indicator for the presence of pesticide traces in the human body upon severe exposure to contaminated environments (e.g., occupational exposure). This strategy was combined with the decrease of uric acid level in human urine, which acts as the main interfering agent in our analytical methodology. To achieve this goal, we first developed an analytical pH-driven treatment capable of reducing the effect of this interfering species on HRP. Our studies highlight that uric acid in urine samples can be decreased by adjusting the pH of the solution either at acidic (i.e., pH 5.0) or alkaline (i.e., pH 8.0) values, coupled with a filtration step. Subsequently, we have integrated this treatment principle into a paper origami platform. By exploiting the properties of filter paper, we designed a device based on *i)* a treatment paper strip with a lateral flow microfluidic path, to drive the solution through the treatment area, where the required reagents are pre-loaded, and *ii)* a vertical microfluidic path to enable electrochemical analysis on CB-modified office paper SPEs, obtained by overlapping the treatment strip and the detection strip on the sensors.

Having pre-loaded all the needed reagents on the paper strip, we showed that our platform can perform multiple analytical steps in a single ready-to-use device, namely: i) to apply the pH-based treatment and decrease the uric acid level, ii) to assess the efficiency of the treatment by checking the level of the residue uric acid, and iii) to carry out the HRP-based inhibition enzyme assay for glyphosate detection. Overall, our proposed paper origami platform represents an advanced analytical solution that merges the problem-solving strategy of an analytical issue (i.e., interference in the real matrix), the use of green materials and reagents, minimized volumes and waste (e.g., the paper device can be easily incinerated), user-friendly design and operability, and reduced costs and time of analysis. These properties match efficiently the sustainability criteria, as demonstrated by applying the principles of White Analytical Chemistry, in comparison with chromatography/spectrometry-based methods for the detection of glyphosate in urine, resulting in a suitable analytical tool for inexpensive, easy-to-use, prompt, and in situ screening of severe exposure to glyphosate. The promising results achieved by our paper origami platform in terms of green, operational, and economical aspects are the outcome of an analytical strategy successfully driven by a sustainable view, to which the scientific community must drive its attention and efforts.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2023.100076.

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