

Technical Evaluation of a Paper-Based Electrochemical Strip to Measure Nitrite Ions in the Forensic Field

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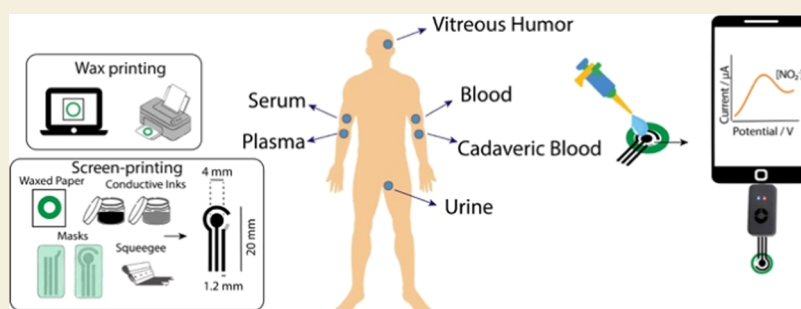
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ABSTRACT: Nitrite is a compound used as a food additive for its preservative action and coloring capability, as well as an industrial agent for its antifreezing action and for preventing corrosion, and it is also used as a pharmaceutical in cyanide detoxification therapy. However, even recently, because of its high toxicity, it has been used as a murder and suicidal agent due to its affordability and ready availability. In this technical report, we describe an electrochemical paper-based device for selectively determining nitrite in complex biofluids, such as blood, cadaveric blood, vitreous humor, serum, plasma, and urine. The approach was validated in terms of the linearity of response, selectivity, and sensitivity, and the accuracy of the determination was verified by comparing the results with a chromatographic instrumental method. A linear response was observed in the micromolar range; the sensitivity of the method expressed as the limit of detection was $0.4 \mu\text{M}$ in buffer measurements. The simplicity of use, the portability of the device, and the performance shown make the approach suitable for detecting nitrite in complex biofluids, including contexts of forensic interest, such as murders or suicides in which nitrite is used as a toxic agent. Limits of detection of ca. 1, 2, 4, 5, 3, and $4 \mu\text{M}$ were obtained in vitreous humor, urine, serum and plasma, blood, and cadaveric blood, also highlighting a satisfactory accuracy comprised between 91 and 112%.

KEYWORDS: screen-printed, paper-based, nitrite, forensic, electroanalysis

INTRODUCTION

Nitrite (NO_2^-) is a very toxic species of nitrogen that plays an important role in the nitrogen cycle and has serious consequences for human health and the environment.¹ Nitrite is commonly used for food preservation and meat curing, despite the fact that its toxicity may be due to over-consumption of NO_2 .² The World Health Organization (WHO) recommends a daily nitrite level in drinking water of less than 3 mg L^{-1} because of the risk of nitrite poisoning to human health.³ Maximum nitrite concentrations in drinking water are regulated by both the United States Environmental Protection Agency (EPA) and the European Union (EU). The maximum level of EPA contamination is 1 ppm ($1.45 \times 10^{-5} \text{ M}$). The EU has set the maximum allowable concentration an order of magnitude lower at 0.1 ppm ($1.45 \times 10^{-6} \text{ M}$).⁴ It is important to keep in mind, however, that the allowable levels

of nitrites can undoubtedly be helpful as preservatives,⁵ blood pressure medications,⁶ in stainless steel industries,^{7,8} and as fertilizers.^{9,10} However, more than $0.3\text{--}0.5 \text{ g}$ can cause severe poisoning in humans, and 3 g of nitrite can be fatal.¹¹

Despite the strict restrictions imposed by the government, poisoning accidents caused by nitrite occur frequently.

Studies have also shown that NO_2^- in the blood can react with the iron(III) in hemoglobin to form methemoglobin, causing the hemoglobin to carry less oxygen and causing health

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problems.¹² Due to the generation of carcinogenic N-nitroso compounds, high NO_2^- concentrations in humans are linked to a range of health problems including methemoglobinemia, stomach cancer, and hypertension.¹³ From a toxicological point of view, the detrimental action of nitrite ions in the bloodstream causes the oxidation of iron(II) contained in the hemoglobin structure to iron(III). The resulting macromolecule, identified as methemoglobin, has a limited capability to bind oxygen. As a function of the methemoglobin blood content, different effects are caused, in particular, dyspnea, nausea, and tachycardia if the level is below 30%. Values up to 50% cause lethargy, stupor, and deteriorating consciousness. Death may occur when methemoglobin levels exceed 70%.¹⁴ The half-life of nitrite is on the order of 30–50 min, causing an almost complete conversion to nitrate in one h in whole blood. On this basis, only 30% of this toxic compound is excreted through urine, and the remaining part is eliminated as nitrate.¹⁵ Recently, several fatal nitrite-related intoxications due to contaminated foods¹⁵ and accidental¹⁶ or voluntary¹⁷ intake were reported in the literature. In addition, this trend is further exasperated by the simplicity of obtaining the toxic compound also using the Internet.¹⁸ Concerns about the exponential increase in the accumulation of pollutants in the environment due to human activities have increased, in line with the growing public awareness of the dangers these substances pose to human health.

It has led to important advances in food safety, water quality management, disease detection, and forensic investigation, and more recently, fundamental biological studies on the physiology and pathology of nitric oxide.¹⁹

Therefore, a practical test for the quantification of nitrite is not only an indispensable tool in traditional disciplines but also occupies a prominent position in basic scientific research related to nitric oxide. This is because a practical test can directly measure the nitrite concentration. The deleterious effects of nitrite on physiological systems and human health emphasize the need for nitrite detection. There is a need for a real-time detection and analysis method that is highly specific, sensitive, cost-effective, and quantitative.²⁰

On the other hand, most of these publications focus on laborious methods used in the laboratory rather than on-site or portable tests, such as spectrophotometric assays^{21,22} and chromatographic,^{23,24} chemiluminescent,^{25–27} capillary electrophoresis,^{28,29} and electrochemical detection methods.^{30–32} The aforementioned approach has benefits and drawbacks of its own. Most of these methods have high specificity and sensitivity, but their advantages are accompanied by tedious sample preparation, long run times, complicated equipment, high prices, and poor reproducibility.²⁰ To overcome these limitations, electrochemical (bio)sensors might represent a solid option to realize diagnostics in home healthcare or remote situations. Electrochemical portable devices are based on simple methods to measure markers in complex clinical samples with high selectivity, simplicity, ease of fabrication, and low cost; moreover, it has the potential to be miniaturized for on-site detection.^{33–35} As a result, several researchers have made great efforts to develop electrochemical sensors for nitrite. To detect nitrite, Yang and colleagues developed an electrochemical sensor based on rod-shaped Co@carbon. With a detection limit of $0.17 \mu\text{M}$ for nitrite in sausage samples and tap water, the proposed sensor exhibits excellent selectivity, stability, repeatability, and reasonable applicability.³⁶ Ding et al. synthesized and characterized a copper-based metal–

organic gel (MOG-Cu) containing multiwalled carbon nanotubes (MWCNTs) (MOG-Cu-MWCNTs) for the electrochemical detection of nitrite. The sensor has reasonable anti-interference capability, excellent sensitivity, and a low detection limit of $0.086 \mu\text{M}$.³⁵ A photoelectrochemical biosensor for nitrite based on graphene and TiO_2 nanomaterials was reported by Muthuchamy et al. The developed biosensor showed a detection limit of 0.225 mM and a wide linear range up to 9 M.³⁷

As reported in the literature, some methods have been reported to detect nitrite in various matrices, biological and environmental, with the adoption of various (bio)recognition elements. However, the development of novel and more sustainable portable devices for the use of nonspecialists represents a leitmotiv in the field of measurement science. In particular, the field of electrochemical-based sensors and biosensors is being characterized by the implementation of novel methods that are based on the use of paper-based substrates.^{38,39} Paper is becoming increasingly common among materials for the production of POC devices due to its many advantages including sustainability, portability, low production cost, ease of use, and the possibility of customization and modification.⁴⁰ However, the different paper-based substrates are characterized by diverse properties, such as thickness and pore size. A wide variety of substrates such as chromatography paper, filter paper, absorbent paper, and office paper are available to produce paper-based analytical devices (PADs) from which one can choose, based on the characteristics to improve the sensitivity of the analysis. For example, Miglione et al.⁴¹ designed an analytical device for copper detection by combining the characteristics of filter paper, which was used to preconcentrate the analyte and store the reagents; it was combined with office paper that was chosen as the substrate where the electrode was screen-printed because of its wide availability and to reduce production costs.

The main advantage of using office paper is related to the reduction of the cost, avoiding the use of polyester-based electrodes. In fact, as pointed out,⁴² the production cost of an electrochemical strip can go from ca. 0.033, 0.025, and 0.018 Euro, respectively, using polyester, Whatman #1 filter paper, and office paper. In this work, office paper was used as the substrate where the electrodes were screen-printed, and the work area was delimited by a layer of wax,⁴³ which acts as a hydrophobic area and allows the working solution to remain in the working area. It should be noted that the use of a wax printer and wax-based inks allows the reduction of the experimental tasks needed to obtain hydrophobic/hydrophilic areas; for instance, even if photolithography was convincingly used to pattern a photoresist embedded into paper, as reported by Whitesides et al. in 2007,⁴⁰ the use of a wax printer is characterized by a higher simplicity and lower costs.⁴⁴ Herein, an office paper-based electrochemical sensor has been designed, characterized, and applied toward the analysis of several biofluids, highlighting the suitability of carrying out measurements in complex matrices without the adoption of time-consuming and complex architectures. In addition, the satisfactory accuracy was evaluated with complementary analysis carried out through HPLC.

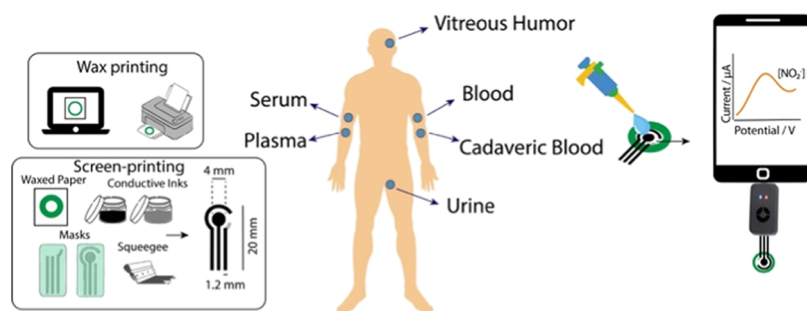


Figure 1. Schematic representation of the manufacture of the electrochemical strip based onto office paper (wax printing and screen printing) for the determination of nitrites in various biological matrices.

MATERIALS AND METHODS

Reagents and Instruments

All of the reagents have been purchased from Merck (Germany) and the real samples have been obtained from the Unit of Forensic Medicine at the Department of Diagnostics and Public Health, University of Verona. All of the electrochemical measurements have been carried out using a portable potentiostat (PalmSens, The Netherlands) connected to a smartphone and/or laptop. The chromatographic measurements have been obtained using an LC-MS/MS equipped with an IonKey coupled to an Acquity system (Waters, Milford, MA).

Paper-Based Screen Printing

Briefly, the testing area is realized by printing a well-defined pattern, designed with Adobe Illustrator, onto an 80 g/m² office paper (Fabriano, Italy) using a wax printer machine (Xerox ColorQube 8580). Subsequently, the waxed paper is cured into an oven for 1 min at 100 °C in order to obtain a hydrophobic layer which defines the testing area. During this time at this temperature, the wax melts and diffuses through the office paper, defining a hydrophilic area which is surrounded by a hydrophobic area. After this step, the electrodes are manually screen-printed using an Ag/AgCl ink (C2130809D5, Sun Chemical) for the reference electrode and a graphite ink (C2030519P4, Sun Chemical) for both counter and working electrodes. Each printing step is performed by using ca. 2 g of each conductive ink and by using a squeegee to spread the inks through the masks. After each conductive ink is screen-printed onto the office paper, the electrodes are cured into an oven for 20 min at 80 °C. The procedure of screen printing has been previously reported in literature.⁴²

Application to Real Samples

The printed strips have been applied toward the analysis of spiked biofluids, using both cyclic voltammetry (CV) and differential pulse voltammetry (DPV), which have been responsible for the oxidation of nitrite ions. With regards to the use of SWV, the following parameters have been used for measurements: scan rate of 50 mV/s, E step of 20 mV, E pulse of 200 mV, and t pulse of 20 ms. All of the measurements have been carried through the use of only 100 µL of samples directly onto the testing area of the electrode, as reported in Figure 1.

In order to evaluate the accuracy of measurements, an ion chromatography system was used to perform measurements, and all of the conditions are reported in the Supporting information file.

RESULTS AND DISCUSSION

Office paper is a valuable substrate for producing electrochemical sensors and biosensors, as reported in the literature.^{36–39} As the starting point, the quality and the ability of our handmade printed strips to recognize nitrites were tested through the use of cyclic voltammetry (CV) in the presence of increasing concentrations of the target, using a phosphate buffer, pH = 7, as the working solution. As displayed in Figure 2A, a clear oxidation peak is visible at around 0.9 V

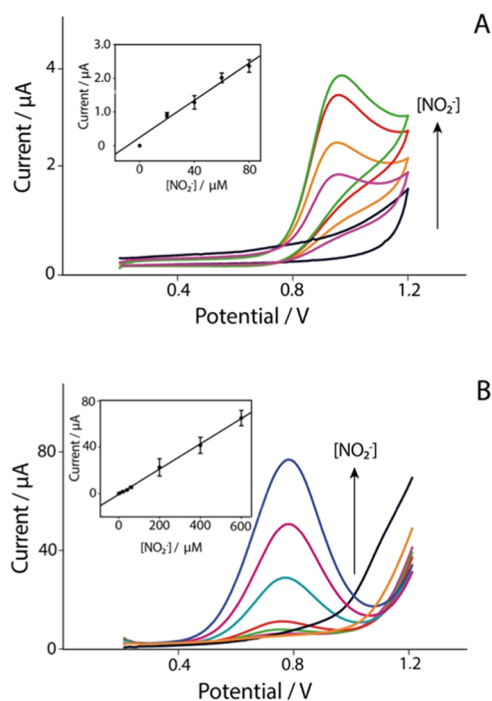


Figure 2. (A) Voltammetric curves obtained using CV and the corresponding calibration curve at increasing concentrations of nitrite ions up to 80 µM. (B) Voltammetric curves obtained using DPV and the corresponding calibration curve related to increasing concentrations of NO₂⁻ between 0 and 600 µM. In both the cases, the measurements were carried out in a phosphate buffer solution at pH = 7, using a scan rate of 50 mV/s. Each point is the result of three different paper-based devices.

that can be ascribed to the electro-oxidation of nitrite (NO₂⁻) to nitrate (NO₃⁻), as shown in following equation: NO₂⁻ + H₂O → NO₃⁻ + 2H⁺ + 2e⁻

A good linearity was obtained up to 80 µM, as described by the equation: $y = 0.03x + 0.24$, where y represents the signal current expressed in µA and x represents the concentration of nitrite ions. This means that the target is able to diffuse and reach the electrode surface without entrapment due to the paper-based structure.^{45,46} Considering this, the effectiveness of electro-oxidation was evaluated in phosphate buffer by using differential pulse voltammetry (DPV) as the detection technique, as already reported in literature.^{47,48} Figure 2B reports the voltammetric curves related to the increase of NO₂⁻, which displayed a satisfactory linearity up to 600 µM, with a sensitivity of 0.11 A/M and a detection limit of 0.4 µM. In addition, the response of the sensor has been compared with

Table 1. Comparison of Sensing Characteristics of Various Nitrite Sensors

materials	technique	LOD (μM)	linear range (μM)	ref
AuCu NCNs/GCE	DPV	0.2	10–4000	49
GC/AuMNNs	amperometry	0.4	0.4–800	50
AuNPs/Co-MOF/MWCNT	amperometry	0.4	1–1000	51
Ni(OH) ₂ /sr-GO	amperometry	0.07	0.1–663.6	52
PEDOT/AuNCs	amperometry	0.017	0.05–2600	53
Au/PANI/SnO ₂ /GCE	amperometry	0.08	0.25–2400	54
GO-Ag	LSV	0.24	1–1000	55
CB/NiCo ₂ S ₄ @CeO ₂ /GC	amperometry	0.003	0.2–7400	56
AuNPs@MoS ₂ /rGO	amperometry	0.038	0.2–2600	57
office paper carbon-based	DPV	0.5	1–600	this work

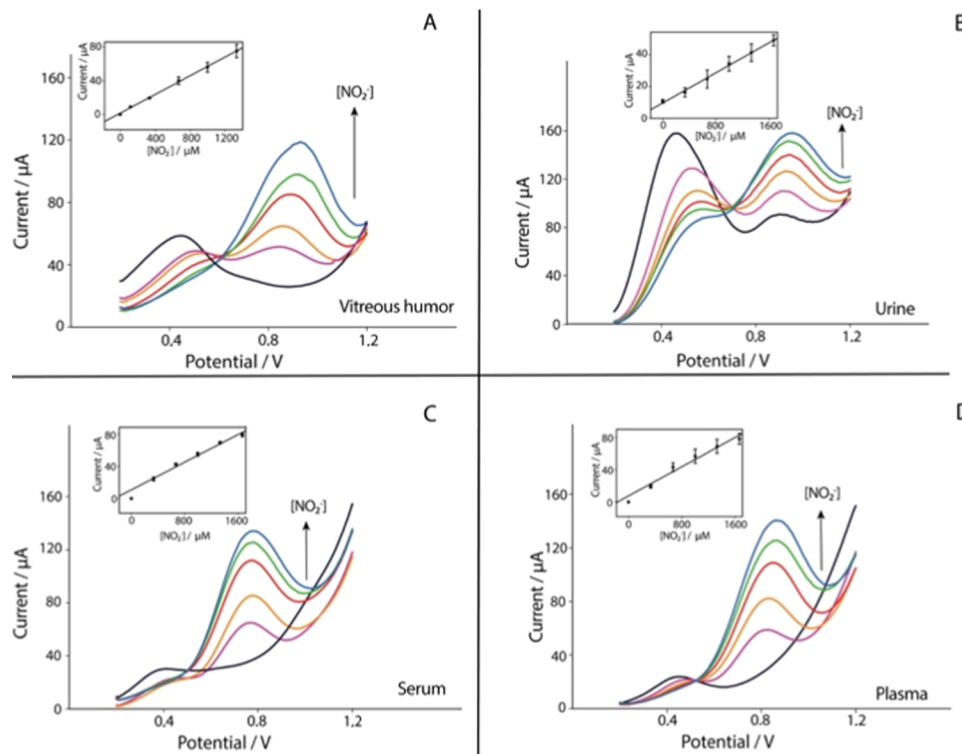


Figure 3. Voltammetric curves obtained using DPV and the related calibration curves at increasing concentrations of NO_2^- , between 0 and 1600 μM , tested in (A) vitreous humor, (B) urine, (C) serum, and (D) plasma, without dilution. Each point is the result of three different paper-based devices.

the presence of common interfering species like glucose, ascorbic acid, uric acid, and paracetamol, demonstrating no significant interferences within the physiological range (Supporting information file, Figure S1). The sensitivity of this system was also compared with commercial SPEs, namely, Metrohm DRP-110. Although the sensitivity appeared slightly better (a detection limit of 0.1 μM , Supporting information, Figure S2), we think our office paper-based SPEs represent the optimal choice, also considering the cost of both, namely, ca. 0.02 and ca. 3 Euro, respectively, for office paper-based and commercial SPEs. As reported in literature, the architecture reported herein represents the first paper-based electrochemical device to be applied toward the detection of nitrite in biofluids, especially considering all of the biofluids that are the objects of the current technical investigation. In particular, as reported in Table 1, although some of the reported electrochemical methods have displayed a better detection limit, it should be also noted how these are mainly characterized with the use of various (nano)modifiers, even

if sometimes the materials and procedure to synthesize some platforms might not be very sustainable in terms of manufacture. However, even if our office paper-based electrodes are not “nominally” modified with any additional materials, as declared by the producer of the conductive ink (Sun Chemical), it contains some amount of carbon black nanoparticles that can boost the electrochemical signal during detection.

Once the detection method in the buffer solution was established, the ability of our paper-based device to detect nitrite ions in biological matrices of different natures was evaluated, highlighting the suitability for potential use in crime scene investigations, i.e., the forensic field. In fact, nitrite ingestion is increasing in the recent years, as reported by the toxicology database and autopsy reports at Forensic Science SA (FSSA).⁵⁸ However, the concentration of nitrite ions that is ingested is not controlled, and the level found in postmortem whole blood samples can also reach 700–800 μM .⁵⁹ As a consequence, the application of the paper-based sensor toward

many biological matrices might represent a preliminary starting point toward a future fast screening on the crime scene. In the first instance, the behavior of the paper-based sensor toward nitrite ion detection was evaluated using a commercial serum, as reported in Figure S1 of the Supporting information file. Since good correlation and linearity were obtained, nitrite ions were detected into more complex matrices. The application toward complex biofluids is important toward a perspective of a device that is able to recognize nitrite ions on-site, with a small amount of sample and without a specific pretreatment of the sample. Consequently, the electrochemical response of nitrite ions was evaluated in four different matrices, namely, vitreous humor, urine, serum, and plasma, without dilution, where the presence of interferents that coexist, such as ascorbic acid and uric acid, might represent a limitation for the determination of the target ion. As reported in Figure 3, a good sensitivity was obtained for all of these nondiluted matrices, where the linearity of the response was evaluated in a wide range between 0 and 1600 μM .

As shown in Figure 3, the DPV measurements showed a satisfactory linearity, and the corresponding calibration curves were described by the following equations: $y = 0.09x + 9.6$, $y = 0.06x + 1.7$, $y = 0.04x + 10.7$, and $y = 0.04x + 7.4$, respectively, for vitreous humor, urine, serum, and plasma. The determination of nitrite ions in the reported matrices was characterized by the detection limit, calculated as the $S/N = 3$ ratio, equal to 1, 2, 4, and 5 μM . Moreover, considering the toxicological problems that nitrates can cause in the bloodstream, two other matrices were also tested, namely, blood and cadaveric blood. In these cases, a dilution was carried out because in addition to protein, the corpuscular part is also present and it can clutter the electrode surface, leading to a decrease in sensitivity. A 50% dilution was chosen for the whole blood, while a 90% dilution was chosen for the cadaveric blood, as reported in Figure 4.

As reported in Figure 4A,B, a good response was obtained in both matrices, where the calibration curves were described by the following two equations: $y = 0.04x + 0.75$ and $y = 0.05x + 10$ for blood and cadaveric blood, respectively, demonstrating the suitability of the developed method. The detection limits were calculated to be 3 and 4 μM , respectively, for blood and cadaveric blood. In addition, the accuracy of the reported paper-based architecture has been evaluated by measuring spiked samples through the use of ion chromatographic analysis, as reported in Table 2.

The accordance of the two methods has been calculated as the ratio among the level of nitrite ions quantified with the paper-based strip and the ion chromatographic analyses. In particular, it should be noted how a satisfactory accordance, comprised between 91 and 112%, has been obtained, demonstrating a good correlation between the two methods. To this regard, the performance of the office paper-based sensors has been satisfactorily compared with ion chromatography measurements, obtaining a correlation of 0.980.

CONCLUSIONS

In this work, a paper-based electrochemical printed strip has been applied toward the detection of nitrite ions in complex biological matrices. In particular, due to the importance of nitrite ions in the forensic field because of its toxicity and poisoning, the paper-based strip has been applied toward the detection of spiked nitrite ions in serum, saliva, plasma, vitreous humor, blood, and cadaveric blood. The main aim of

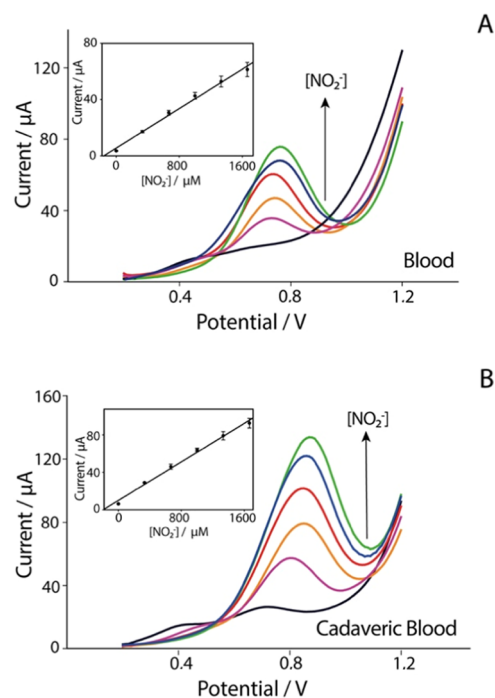


Figure 4. Voltammetric curves obtained using DPV and the related calibration curves at increasing concentrations of NO_2^- , between 0 and 1600 μM , tested in (A) blood diluted 1:2 and (B) cadaveric blood diluted 1:20. Each point is the result of three different paper-based devices.

Table 2. Comparison with Measurements Performed by Ion Chromatographic Analysis

matrix	paper-based strip (μM)	chromatography (μM)	accordance (%)
serum	45	42	107
plasma	50	49	102
urine	53	47	112
vitreous humor	44	46	96
blood	40	43	93
cadaveric blood	40	44	91

the work was to evaluate how a portable technology such as printed electrochemical sensors might be useful in potential forensic applications. As reported, the portable sensors displayed a satisfactory applicability to complex matrices, with a detection limit down to a low micromolar range and without the adoption of complex and time-consuming procedures. The accuracy was evaluated through the adoption of ion chromatographic measurements of spiked matrices, demonstrating satisfactory agreement within the range of 91 and 112%. Paper-based diagnostics offer valuable possibilities in all of the measurement science fields, and also the forensic practice might be positively affected by this in order to give the specialists portable and user-friendly tools for fast screening in decentralized settings.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmeasuresciau.3c00050>.

It contains measurements in commercial human serum (Merck, USA), HPLC parameters, and selectivity studies (PDF)

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Author Contributions

[†]A.R., A.M., and W.C. contributed equally to this work. The manuscript was written through the contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Ada Raucci** data curation, writing-original draft; **Antonella Miglione** data curation, writing-original draft; **Wanda Cimmino** data curation, writing-original draft; **Alessia Cioffi** formal analysis; **Sima Singh** data curation, writing-original draft; **Michele Spinelli** methodology, validation; **Angela Amoresano** validation, writing-original draft; **Giacomo Musile** conceptualization, writing-original draft; **Stefano Cinti** conceptualization, data curation, funding acquisition, methodology, writing-original draft.

Notes

The authors declare no competing financial interest.

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