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Staple-based paper electrochemical platform for celiac disease diagnosis

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Abstract: *A staple-based electrochemical platform is proposed for the first time as a simple and low-cost detection system for paper-based devices. The system, that incorporates small and disposable stainless-steel staples as electrodes, is combined with a paper strip. The staple acting as working electrode is modified with carbon ink. The platform was carefully optimized with ferrocene carboxylic acid. As a proof-of-concept, it was employed for the enzymatic (HRP-based) immunoelectroanalytical detection of human tissue anti-transglutaminase (anti-tTG), biomarker for celiac disease diagnosis. The intensity of the current due to the electrochemical reduction of TMB (HRP substrate) was recorded chronoamperometrically at -0.2 V in different paper areas. A linear relationship between the current measured at 30 s and the logarithm of the concentration of anti-tTG in the range comprised between 3 and 100 U.mL^{-1} was obtained. Negative and positive controls produced expected values. Results demonstrated that the paper/staple-combined platform is very convenient for the detection of electroactive analytes and other compounds that can be determined indirectly in bioassays.*

Celiac disease (CD) is the immune-mediated intolerance to dietary gluten, a protein contained in wheat, rye and barley in genetically susceptible persons. A correct diagnosis is extremely important [1] due to: i) the permanent nature of the celiac condition, ii) the treatable character of this autoimmune disease, requiring implementation of a lifelong gluten-free diet, iii) the wide spectrum of clinical symptoms [2], including cases with either intestinal or extraintestinal features as well as silent forms, occasionally discovered after serological screening, iv) the number of organs or tissues that can be potentially affected by this disease and, v) the predisposition to reduced life quality and the incremental use of health services and medicines in delayed diagnosis [3]. Therefore, the number of analysis performed to diagnose celiac disease is continuously increasing. In this context, the immunochemical determination of serum antibodies against tissue transglutaminase (tTG) is among the main tests. Examples of commercial tests for anti-tTG determination are the Biocard™ celiac test [4] and the EliA™ Celikey® kit [5]. They are based on a lateral flow qualitative immunoassay and an enzyme-linked immunosorbent assay (ELISA), respectively. In this work we have combined both strategies in: i) a low-cost paper-based detection, but with quantitative results, and ii) an ELISA test, but using portable and simple instrumentation allowing the decentralization. It has been possible by integrating an electrochemical detection, which fits perfectly with the concept of point-of-care tests. This is mainly due to its simplicity and low cost while maintaining sensitivity and selectivity [6]. Actually, enzymatic immunosensors have been developed on nanostructured carbon electrodes [7] or gold electrodes modified with transglutaminase [8], even with complicated architectures [9]. However, simplicity and low cost remain as unmet needs in the diagnosis of the celiac disease.

Whitesides' group has opened a new field with the generation of microfluidic paper-based analytical devices [10]. Later on, Henry's group has integrated the electrochemical detection, based on carbon ink electrodes [11]. Since then, the combination of paper with electrochemistry has attracted significant interest. As an example, electrodes were printed on foldable hydrophobic paper with a pen filled with graphite ink for immunochemical determination of a malarial antigen [12]. Wax printing [13] and heating allows generating hydrophilic regions delimitated by hydrophobic barriers that can be converted into electronic and electrofluidic paths by the addition of conductive elements [14]. In the design of a three-electrode amperometric cell, electrodes have to be separated from each other. This could be made by: i) using multiple layers [14], ii) direct printing (e.g., pencil-drawn [15], pen-on-paper [12,16] or laser scribing [17]), iii) stencil- or screen-printing electrodes [11, 18-20] or iv) using external wire electrodes [21-24]. Moreover, thin- or thick-film working electrodes (WEs) do not require the use of stencils if reference and counter electrodes (RE, CE) are located on the opposite side of the film [23-].

A very different low-cost strategy for ink electrodes is based on the use of pins as a support for the ink. Whitesides et al. proposed the use of three stainless-steel pins, the one used as working electrode modified with carbon ink, for paper- and thread-based devices [26]. Later on, these conductive elements of reduced size and cost have been employed for enzymatic glucose biosensing, in static [27], or flow systems [28], and

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epinephrine determination by batch injection analysis [29]. Stainless steel seems to be a suitable material for electrochemical purposes. It has been proposed as a substitute of conventional Ni electrodes for diagnosis of the sudomotor dysfunction [30].

We report in this article the use of staples, for the first time, as low-cost, disposable and mass-produced conductive elements for electroanalysis. We have combined common stainless-steel staples with paper for developing a portable and user-friendly electroanalytical device of compact design. We have demonstrated its utility in the determination of anti-tTG, (IgA class) antibodies that appear at high titers in CD.

The design of the electrochemical cell is one of the key steps in the development of an electroanalytical platform. We selected (Figure 1A) a three-electrode system consisting of three stainless-steel (SS) staples, contacting the paper at their inner part. The one corresponding to the working electrode is modified with conductive carbon ink. A simple PDMS platform: i) embeds the connections to the potentiostat and ii) serves as a support for the paper platform.

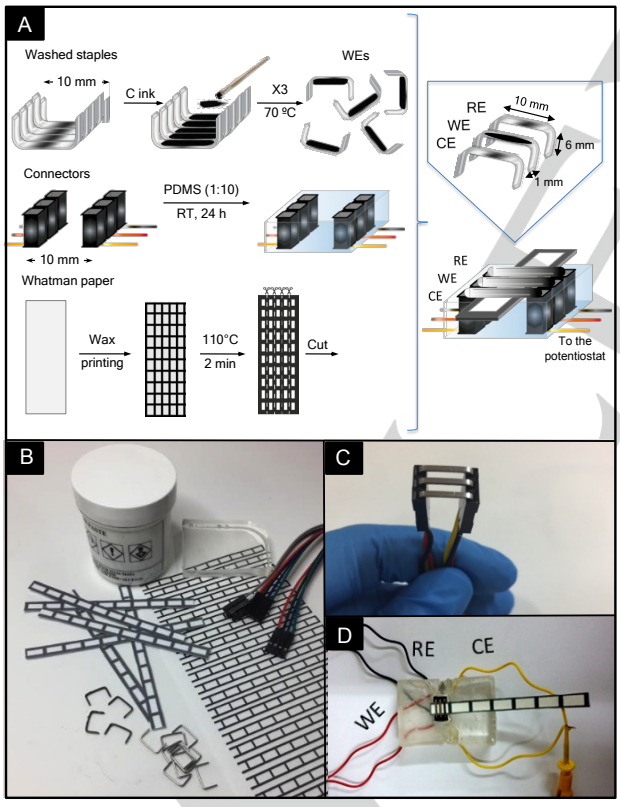

Figure 1. A) Schematic representation of the fabrication process of the stapled paper-based platform. Picture showing: **B)** the materials employed in the platform for quantitative electrochemical detection, **C)** staples inserted in two female pin connectors, and **D)** PDMS holder with connectors and three stainless-steel staples inserted.

We first evaluated the performance of the staples by testing them in combination with a conventional ceramic card with screen-printed electrodes (SPEs, DropSens, DRP-110, Spain) as seen in Figure S1. Working and counter electrodes are made of carbon ink, whereas the reference electrode is made of silver ink, and covers a smaller area. Stainless-steel staples (STANLEY Stainless Steel® 1/4" 6mm) were tested alternatively as WE, CE and RE. The other two electrodes, necessary to complete the cell, were those of the screen-printed card. Staples have a thin layer of a polymer covering them, so they were tested: i) directly as received, ii) washed with acetone to eliminate the polymeric layer and iii) painted with carbon ink after being washed. The carbon paste (Gwent Group, UK, Ref C10903P14) was diluted with N,N-dimethylformamide (DMF, Merck, Germany) in different ratios (26, 50 or 60% w/w of carbon paste in DMF) to generate the carbon ink for electrode modification. The mixtures were vortexed (REAX 2000, Heidolph) for a few seconds and then sonicated (Elmasonic P, Elma) for 1 h. Then, staples were all together adhered on a double-sided tape and the inner part of their crown was painted thrice with the carbon ink using a brush. Solvent evaporation took place for 15 min after each of the two first coatings. After the last one, staples were placed in an oven (Nabertherm®, D-2804 Lilienthal/Bremen) for 1 h at 70 ºC. The staples were painted 3 times to ensure full coverage of the stainless steel. A higher number of coatings produce thicker films that can be detached from the staple. On the other hand, a lower number could result insufficient.

The staple acting as reference, counter or working electrode substituted the corresponding electrode of the card. The staple was inserted in a PDMS block, with the SPE card laying on the PDMS below the staple (Figure S1 A). The card was inserted in a commercial interface that connects the electrodes to the potentiostat (either Autolab PGSTAT12 or μAutolab, Metrohm, Switzerland) controlled by GPES (General Purpose Electrochemical System) software. In our case, the corresponding alligator clip coming from the potentiostat is connected to the staple acting as WE, CE or RE by means of a hook clip (bypassing the interface). Ferrocene carboxylic acid (FCA, Sigma-Aldrich, Madrid), a well-known redox probe, was employed to evaluate the performance of the system by cyclic voltammetry. A volume of 40 μL of a 10^{-3} M solution (in 0.1 M phosphate buffer (PB) pH 7.0) was deposited on the screen-printed area, in such a way that the inner part of staple connected the drop (Figure S1 B).

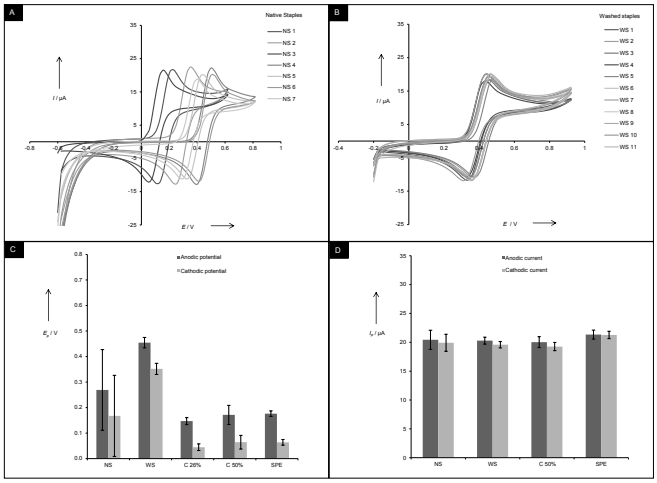

Figure 2. Successive CVs recorded on drops of 10^{-3} M FCA solution at 100 $mV.s^{-1}$ using: **A)** native and **B)** acetone-washed staples as REs. Dispersion of the: **C)** peak potentials (using different staples as REs) and **D)** peak currents (using different staples as CEs). Error bars correspond to the standard deviation of five measurements (NS = native staples; WS= washed staples; C 26%, C 50% = staples modified with different dilutions of the carbon ink, SPE = screen-printed electrode).

We have evaluated the use of staples as quasi-reference electrodes. Native staples presented CVs (Figure 2A) with similar peak current intensities, i_{pa} = 20 ± 1.0 µA; i_{pc} = 20 ± 1.1 µA (n= 7), but with a great deviation in peak potentials (E_{pa} = 370 ± 130 mV; E_{pc} = 270 ± 140 mV, n = 7). However, when the polymeric layer is removed, this dispersion decreases considerably (3.2 % (anodic) and 5.7 % (cathodic)), as can be seen in the 11 CVs presented in Figure 2B. Acceptable dispersion was obtained for those washed and painted with carbon ink (Figure 2C). However, for the sake of simplicity and lower cost, washed staples without carbon ink were used for the rest of the experiments. The values of the peak current intensities obtained are presented in Figure S2 A.

When the staples washed with acetone were used as CEs (Figure 2D) they provided acceptable dispersion in peak current intensity (RSD lower than 3.0% for 7 staples). It is recommended that the area of the counter electrode is substantially larger than that of the working electrode [31]. However, here, even when the geometric area of the SPE WE is 12.6 mm^2, and the one corresponding to the staple in contact with the drop is 6 mm^2, it seems to be enough for obtaining appropriate currents. Dispersion increases slightly when washing is not performed but even though, the current is maintained. The staple modified with 50% of C ink could also be employed but, as happened before, a just-washed staple was chosen. The values of the peak potentials obtained are presented in Figure S2 B. No corrosion was observed in any of the staples all along the work. It has been reported (for AISI 316L stainless steel) that with increasing pH the corrosion rate decreases [32].

Finally, staples were checked as WEs in combination with SPEs. In Figure 3A, CVs recorded on drops of FCA solution using a washed staple and a staple modified with 26 % of C ink are shown. It is evident the need of electrode modification. The redox pair of ferrocene is well defined, with i_{pa}/i_{pc} = 1.04 ± 0.02 and ΔE = 97.8 ± 0.5 mV (n = 5). When staples with different C ratio were evaluated, no significant difference was observed in i_p (Figure 3B). Then, a 50 % of C ink was chosen for further studies, to ensure better coverage of the staple.

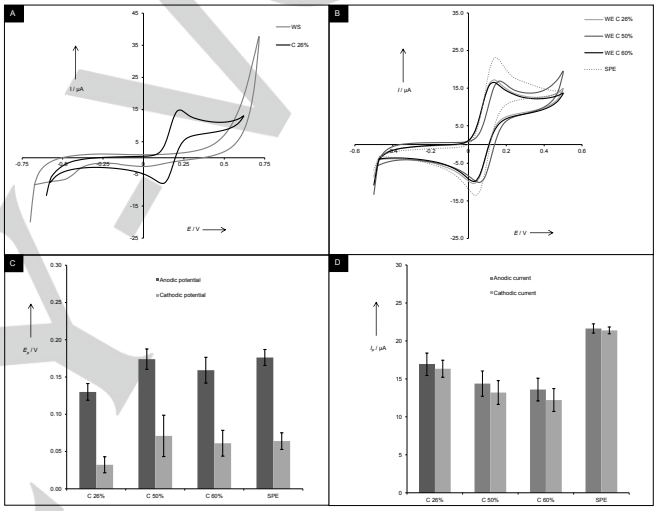

Figure 3. CVs recorded on a 10^{-3} M FCA solution at 100 $mV.s^{-1}$ using as WE: **A)** a native staple and a staple modified with C (26%) and **B)** staples modified with different ratios of C ink / DMF (w/w %) and SPE. Dispersion of: **C)** E_p and **D)** i_p using different WEs (WS= washed staple; C 26%, C 50%, C 60% = staples modified with different C ink / DMF ratios, SPE = screen-printed electrode).

We have designed then a three-electrode platform to be coupled to a paper strip, with three washed SS staples, one of them (WE) modified with carbon ink (50 %). The use of staples is very advantageous since they are: i) low cost (1000 staples/pack for $4.49), ii) reduced in size (one staple is 1.0 cm x 0.1 cm x 0.6 cm), iii) easy to store (compact rectangular boxes of 1000 units), iv) made of stainless steel, conductive material, adequate for use in electroanalysis, v) able to be modified with conductive ink to act as WEs and generate adequate electrochemical processes, vi) appropriate REs (stable potential during many measurements, and very precise between different staples) and CEs without modification. Initially, staples were inserted directly in a PDMS block and connected to the potentiostat using hook clips. Later, they were set in 2 three-pin

Dupont female connectors (Figure 1B), with 1.5 mm between staples. This makes handling easier and increases the precision. Once connected, a potential was applied between WE and RE, with the current flowing between WE and CE (both of approximate area). We embedded the two reusable connectors in a PDMS matrix (Figure 1C) with a 10-mm distance in between in order to easily insert and remove the staples. Wires at the bottom are connected through hook clips in a highly efficient way with low electrical resistance. This block is also a solid support for placing adequately the paper strip.

We created hydrophobic areas on a Whatman® chromatographic grade 1 (100 x 300 mm^2 GE Healthcare Life Science, 180-μm thick) paper strip by wax printing with a pattern designed with Inkscape, Draw Freely 0.92.2. Well-defined separated working areas are created and then, liquid is not dispersed out of the strip. Printing was made with a solid ink printer (ColorQube 8570DN-42PS. Xerox®). Further heating at 110ºC for 5 min on a hot plate (IKA® RTC classic) melted the wax creating hydrophobic barriers that delimited 3 x 7 mm^2 working areas. To avoid liquid dispersion through the back with cross contamination between samples, and to handle and slide easily the strip on the holder, a rigid thin polymer backing (Laminated cards 60 x 301 mm^2, Millipore) was adhered to the opposite side to this printed area.

We placed the paper strip over the PDMS and below the staples (partially inserted in the connectors) in such a way that it can be easily slid until staples are over the hydrophilic portion of the working area (Figure 1C). The staples were pushed slightly until the crown reached the connector. In this way, errors due to human variation were avoided. The pressure of the staples on the paper is an important parameter. The rigid backing has an adequate thickness to approximate paper and staples but without contact. If the same backing, connectors and sample volume are employed, similar electrode area is obtained.

On paper-based electroanalysis the volume of solution that is added influences, considerably, the intensity of the analytical signal. We can distinguish between under and over saturation of the paper with the solution. In the first case, the staples have to be pressed against the paper in order to contact sufficiently the solution for measuring. In the second one, there is no need for physical contact. It has been reported that when a wire is in contact with the paper surface, a confined area is created between the electrode and the paper with thin layer-like behavior [22]. On the other hand, the highest the paper/electrode contact, the lowest the solution/electrode contact. In Figure 4A (top), CVs recorded at different times in a hydrophilic area of a paper strip after depositing a 1.5-μL drop of a 10^{-3} M FCA solution are shown. In this case, staples are inserted directly on the PDMS to contact the paper and reach the solution. Figure 4B corresponds to CVs recorded on paper areas where 8 μL of the same solution were added (platform with embedded connectors is employed). The difference in the intensity of the current is notorious. If a volume of 1.5 μL instead of 8 μL is deposited, a decrease in the current of *ca.* 85.3 % is observed. Part of the electrode area is contacted by paper fibers and consequently, i_p decreases considerably.

The effect of solvent evaporation can be seen in Figure 4A (bottom) where the anodic and cathodic peak currents are plotted *vs.* time (considered from the addition of 1.5 μL). Although the measurement platform is a semi-open area (1-mm wide staples, separated 1.5 mm one from each other, on a 7-mm long hydrophilic area) that would prevent evaporation to a certain extent, this occurs due to the small sample volume and large paper surface. The continuous evaporation can be followed by cyclic voltammetry. The anodic peak current decreases 44.2 % from the first to the second minute for a 1.5 μL-drop. However, as seen in Figure S3, the decrease is not appreciable when a drop of 8 μL is added. In the first case, the signal decreases until 11 min, where the contact between solution and staples is lost. Whole evaporation is indicated by the line at zero current (no connection between the electrodes).

In Figure 4B, CVs recorded in 8 μL drops for precision studies, both intraelectrode (same three-staple group and different working areas, Figure 4B top) and interelectrode (different WEs, same RE and CE and different working areas, Figure 4B bottom) are shown. The precision in terms of standard deviation was adequate in both cases; not only for E_p but also i_p; with values always lower than 10 mV and 0.33 μA (n = 6) respectively. The precision was similar to that obtained with conventional screen-printed electrodes (12 mV and 0.58 μA for standard deviations of E_p and i_p respectively). CVs recorded on six different SPEs are shown in Figure S4. Although good results were obtained with 8 μL, in some cases noisy voltammograms were obtained and studies were conducted to find the optimum volume.

Considering a geometric paper area of 2.1 cm^2 and a saturation volume of 9 $\mu L.cm^{-2}$ [23], an 18-μL volume is required for saturation. We evaluated the performance of the platform recording CVs after adding two 9 μL-drops of 10^{-3} M FCA solution. The first drop wetted the hydrophilic area and the

second one generated a liquid layer that contacted the staples. Henares et al. [33] pre-wetted also a paper device with a drop, and later drops flowed freely on the lubricated surface. In closed microfluidic paper-based devices, hollow channels were also generated over cellulosic ones to allow free flow of solution with increased mass transfer [18]. In our case, the cellulose fibre network inside the hydrophilic area was first saturated with 9 µL via capillary action and then served as a surface where bulk liquid (other 9 µL) slips. This is more similar to voltammetry at open well electrodes, with higher currents as a result of maximal solution contact [22]. A saturation volume of 18 µL (added at once or in two drops of 9 µL) was chosen for the rest of the experiments to ensure staple/solution contact and to not depend on evaporation issues.

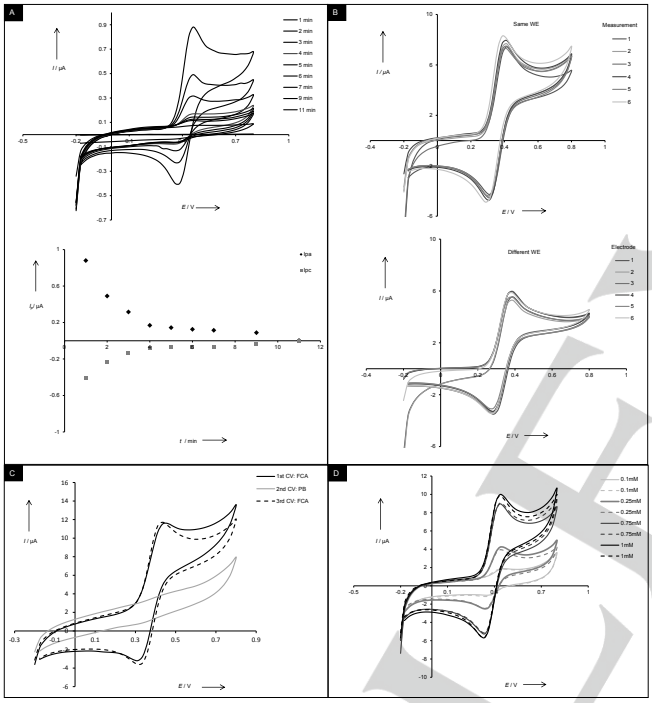

Figure 4. CVs recorded at 100 mV.s^{-1}, **A)** at different times after depositing 1.5 µL of 10^{-3} M FCA solution (top). At the bottom, anodic and cathodic peak current is represented *vs.* time, **B)** after depositing 8 µL of 10^{-3} M FCA solution in different working areas for intraelectrode (top) and interelectrode (bottom) precision studies and **C)** in three different working areas (with 10^{-3} M FCA, buffer and 10^{-3} M FCA solutions added) and **D)** depositing two 9-µL drops of FCA solutions of different concentration (0.1, 0.25 and 1.0 mM). Dashed lines correspond to CVs recorded for the same concentration with the same staples in a different area.

Figure 4C shows CVs obtained in 3 different working areas of the paper strip, with different solutions each one. After recording the voltammogram in a working area with 10^{-3} M FCA solution, a clean CV is obtained when buffer is added in a new area, indicating that staples could be reused. However, their low cost and the adequate precision make them also disposable, especially recommended when working with biological materials, susceptible to be adsorbed on the electrode.

Both i_{pa} and i_{pc} increased with the concentration of FCA solutions, as shown in Figure 4D. CVs of replicates measured in different working areas are represented with dashed lines and demonstrate the precision of the measurements.

On the other hand, Table 1 reports the precision of the device developed in the present work, and comparatively, details this of similar paper-based devices reported in the bibliography. Although different fabrication techniques or electrode materials were employed, all of them present adequate values of RSD (in most of the cases below 10%).

However, there are several advantages of the staple-based detection over other based on carbon electrodes, mainly: i) the use of staples as external electrodes. There are different degrees of integration between electrodes and paper and then, all the three electrodes can be included in the paper [11,12] or only some of them. In this case, it is usually the working electrode (RE and CE are external) [23,24] but in some platforms, reference and counter electrodes [18] are incorporated (WE is external). Alternatively, the three electrodes of the potentiostatic system can be external [22], as happens in this work. Staples are not integrated on paper and form part of the platform where the paper is inserted. Then, paper (bio)assays could be performed independently to the detection in case they are required. ii) Properties of paper are not changed by the incorporation of ink, because this conductive element only modifies the WE staple. iii) The ink modifies mass-produced elements with definite area, which adds precision to the device. iv) Modification does not require any expensive equipment. v) Since electrodes are external, washing can be easily performed in case different measurements want to be done. vi) Similarly, replacement of the electrodes (usually only WE) can be readily done. vii) Although disposable, staples are robust elements. They could be reused after eliminating the ink. viii) The staple system is is very versatile and can be used with different papers or other flat surfaces such as hydrophobic or thick paper, transparency film… and ix) paper or other surfaces can be easily slid under the staples to perform fast measurements.

Table 1.- Comparison of the precision of different paper-based devices with carbon working electrodes.

Paper device fabrication	Electrode material	Integration electrodes / paper	Redox probe / Electrolyte	Device precision (RSD of current intensity, n)	Reference
Wax printing Manual painting of the WE staple with ink and curing	Stainless steel staples and carbon ink	All external	FCA / PB pH 7.4	4.8%, 6	This work
Embossing and silanizing to render paper hydrophobic Manual painting of the WE pin and curing	Stainless steel pins and carbon ink	All external	FCA / PBS pH 7.6	6.3%, 7	Glavan *et al.* [26]
Embossing and silanizing to render paper hydrophobic Printing electrodes	Graphite ink with carbon nanotubes	All included on paper	FCA / PBS pH 7.6	2.9%, 7	Glavan *et al.* [12]
Single-step laser scribing Manual painting of RE	Carbon nanostructured electrodes	All included on paper	Picric acid / PBS pH 2.0	3.8%, 6	de Araujo *et al.* [17]
Wax printing and hollow channel cutting Screen printing electrodes	Carbon (WE, CE) and Ag/AgCl (RE) inks	All included on paper	FcMeOH / PBS pH 7.4	10%, 3	Renault *et al.* [18]
Wax printing Pencil-drawing electrodes	Graphite-based pencil leads	All included on paper	$Fe(CN)_6^{4-}$ / KCl	11%, 7	Dossi *et al.* [34]
Wax printing and cutting Stencil printing electrodes (Spray-coating method for rendering one side hydrophobic; self-powered)	Carbon (WE, CE) and Ag/AgCl (RE) inks	All included on paper	$Fe(CN)_6^{4-}$ / PBS pH 7.4	3.6%, 10	Pal *et al.* [35]
Photolithography Screen printing electrodes	Carbon (WE, CE) and Ag/AgCl (RE) inks	All included on paper	Au(III) / diluted aqua regia	5.1%, 10	Apiluz *et al.* [36]
Photolithography Screen printing electrodes (First work on ED for paper devices)	Carbon with Prussian blue (WE and CE), Ag/AgCl (RE) inks	All included on paper	Glucose, lactate, uric acid (enzymatic) / PB pH 6	<14%, 3	Dungchai *et al.* [11]
Wax printing Ink depositing (Stencil not required)	Carbon ink (WE), gold-plated RE and CE	WE included on paper; external RE and CE	Glucose (enzymatic) / Tris-HNO_3 buffer pH 7.6	4.8%, 9	Amor-Gutiérrez *et al.* [24]

FCA: ferrocene carboxylic acid; PBS: phosphate buffer saline solution; PB: phosphate buffer; ED: electrochemical detection; WE: working electrode; RE: reference electrode; CE: counter electrode

We have applied the paper platform to determine human tTG, a biomarker of celiac disease (CD). A sandwich-type immunoassay was performed using an ELISA kit, VARELISA (Celikey), with the capture agent (transglutaminase), immobilized on microplates. Figure 5D shows a scheme of the immunoassay. After incubation with sample (or standards), anti-tTG (IgA class, present in the serum of people with CD) is captured. Then, an anti-IgA conjugated with horseradish peroxidase (HRP) is added. After the incubation, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution are added and TMB is enzymatically oxidized. All the above-mentioned steps were followed by washing with the buffer solution provided in the kit. The reaction is stopped with 50 μL of 0.5 M H_2SO_4 solution. Since a paper platform is employed for detection, only a small volume is required for the measurement. Then, 18 μL of supernatant were transferred to the staple-based platform for obtaining the analytical signal. Even when the solution was acidified, staple corrosion was not observed.

We first investigated the electrochemical behavior of TMB on the staple system by CV, SWV (square wave voltammetry) and CA (chronoamperometry). The two first are important "diagnostic" techniques, being SWV a sensitive and fast technique. CA is commercially interesting due to its simplicity. We recorded SWVs in 18 μL of a 1:1.5 diluted TMB substrate solution (100 μL of TMB solution and 50 μL of 0.5 M H_2SO_4, employed as stop solution). Figure 5A shows 7 SWVs recorded in different test areas for the same staple system. A RSD of 4.8 % confirms the high precision of the methodology. Figure 5B shows the variation of the SWV signal for two concentrations (stock solution and 1:5 dilution) of the TMB solution, proving its potential for quantitative analysis. These experiments were made washing the staples between measurements by adding buffer on a new piece of paper. Contamination between solutions does not occur as

demonstrated in the CVs recorded in adjacent paper areas (Figure 5C). Therefore, the system can be accurately employed for quantitative measurements, even when the same staples are used.

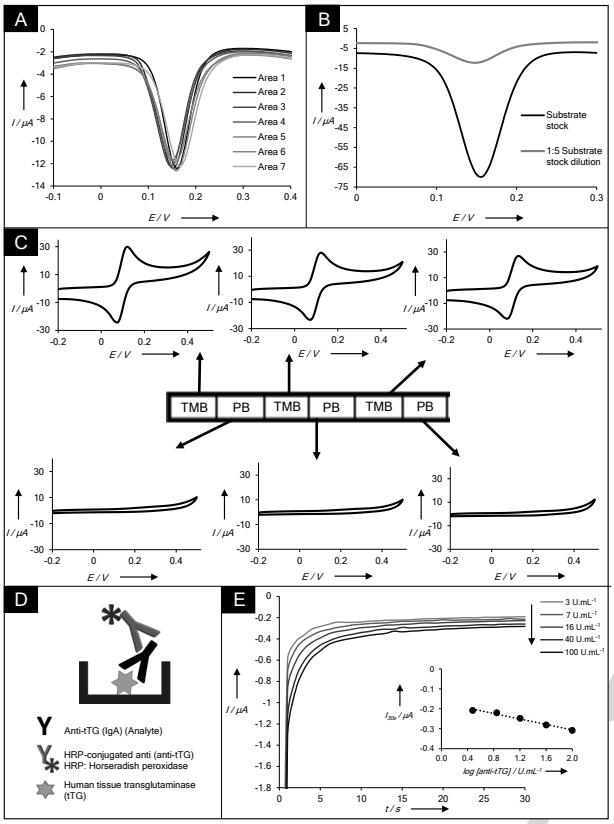

Figure 5. SWVs (f = 20 Hz, A = 20 mV, ΔE = 2 mV) recorded in the TMB solution with the same three-staple system in different paper working areas. **A)** Seven measurements of the same concentration (1:5 dilution in 0.1 M PB pH 7.0 of the commercial substrate system, stopped with 0.5 M H_2SO_4) **B)** Two measurements in substrate solutions, the stock and a 1:5 dilution with 0.1 M PB pH 7.0, both stopped with 0.5 M H_2SO_4 (100 μL of TMB solution with 50 μL of 0.5 M H_2SO_4). **C)** CVs (100 $mV.s^{-1}$) recorded on adjacent paper areas with the same three staples in 1:1.5 TMB solutions (100 μL of TMB solution and 50 μL of 0.5 M H_2SO_4) and in 0.1 M PB pH 7.0, alternatively. **D)** Schematic of the ELISA procedure. **E)** CAs recorded at -0.2 V after performing the ELISA in wells with immobilized tTG, for different concentrations of anti-tTG. The reaction is stopped and 18 μL of the final solution are added to the hydrophilic area. The inset shows the calibration curve.

We then performed ELISAs for different concentrations of human tTG (3, 7, 16, 40 and 100 $U.mL^{-1}$). In Figure S5 the results obtained for the assay with optical detection are presented. For the electrochemical detection, simple chrono amperometric measurements were employed. A potential scan is not required since only the current intensity obtained at a fixed is recorded. The intensity of the current obtained at a fixed time is correlated to the concentration. Then, after incubation with HRP, anti-IgA and TMB substrate solution, we deposited 18-μL drops of supernatant in the paper areas (one per well). We obtained the calibration curve from current intensities measured at 30 s in the chronoamperograms recorded applying a potential of -0.2 V (Figure 5E). This potential is negative enough to reduce the TMB enzymatically oxidized (as seen in the CVs shown in Figure 5C). A log-linear relationship was obtained: I_{30s} (μA) = -0.0688 log [anti-tTG] ($U.mL^{-1}$) – 0.169, with r = 0.992 for n = 5. The limit of detection, calculated as the concentration corresponding to a signal that is 3 times the standard deviation of the intercept, was 2 $U.mL^{-1}$. We determined the concentration of tTG in positive and negative controls, obtaining a concentration of 82.8 ± 0.1 and 3.8 ± 0.1 $U.mL^{-1}$, respectively. The provider establishes values below 5 and over 8 $U.mL^{-1}$, for negative and positive controls, respectively.

In conclusion, we have developed an electrochemical system for quantitative analysis on paper by using a simple, easy-handling and low-cost portable three-staple platform. This is the first report that describes the use of common staples for quantitative purposes. They are cheap supplies (1000 staples for less than $5.00) that we can found easily and use almost daily. While they were designed for specific applications, we proposed here an out-of-box application: quantitative low-cost analysis. In this approach, we used SS staples to avoid the presence of redox processes coming from components of the alloy. The staple-based electrochemical detection has several advantages: i) it is based on low-cost ($0.013 / 3 staples that can be combined with connectors that cost $1.98 / 2 three-pin connectors) and easy-to-obtain, transport and store materials of reduced size, ii) staples only require a washing with acetone and, in the case of the WE, modification with C ink, which can be done quickly with many staples at once, iii) staples can be easily disposed of. However, and depending on the analyte, they can also be reused, especially in the case of RE and CE with high precision, iv) staples can be coupled, through a PDMS block with embedded connectors, with disposable paper strips, using low volumes (20 μL or below) for detection purposes, and v) it employs a simple paper-sliding procedure for measuring different solutions. Washing with buffer in between is recommended for increasing precision and avoiding cross contamination when the same staples are employed. The distance between paper and staples is maintained even after changing paper strips and/or staples. This is ensured since connectors stop literally the staples and the thickness of the backing/paper remains constant. vi) It can

be combined with handheld potentiostats. This results very convenient not only for small laboratories but also, and especially, for resource-limited settings and decentralized analysis. vii) It can be employed with many different electrochemical techniques. We have recorded reproducible signals with CV, SWV and CA, main techniques included in modern small potentiostats [37, 38], but other are also possible.

We have successfully demonstrated, as a proof-of-concept, the great performance as detection system of an ELISA for CD diagnosis. We have proposed it for a specific application but it is very versatile since: i) it could be employed with several WEs for multiplexed measurements, ii) WEs could be easily modified with simple surface or bulk procedures, iii) the paper strip can be functionalised in different ways and various papers could be employed, iv) different flat surfaces can be combined with the platform (e.g., transparency films [39]), once located below the staples, v) more electrochemical techniques could be employed (e.g., anodic stripping voltammetry, differential pulse voltammetry, etc.) and vi) many different analytes could be determined. We really consider this platform could find multiple applications, using already mass-produced and low-cost easy-accessible elements.

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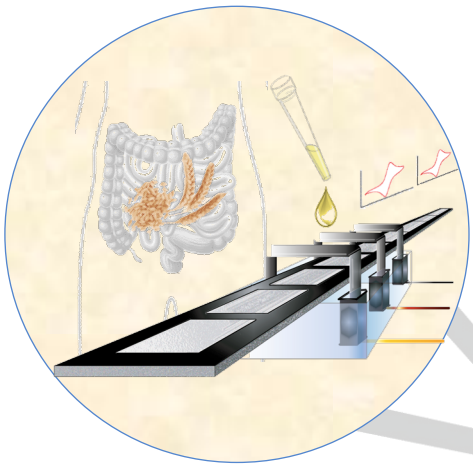
Keywords: Paper-based analysis • Electroanalysis • Staples • Decentralized analysis • Celiac disease

COMMUNICATION

***Staples diagnose celiac disease**: A staple-based electrochemical platform is proposed for the first time as a simple and low-cost detection system for paper-based devices. As a proof-of-concept, it was employed for the detection, through an enzymatic immunoassay, of human tissue anti-transglutaminase, an analyte related to the celiac disease. The strategy is based on the use of a three-electrode platform consisting of easy-accessible mass-produced stainless-steel staples. In the case of the working electrode, the inner surface was modified with carbon ink to favor electron transfer.*



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Page No. – Page No.

Staple-based paper electrochemical platform for celiac disease diagnosis

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