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Electroanalytical paper-based device for reliable detection and quantification of sugars in milk

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ABSTRACT

Knowing the level of sugars in food and beverages is essential because they are related to diseases such as diabetes, and obesity, among others. In this regard, an approach has been designed, characterized, and applied to develop the first all-in-one paper-based microfluidic analytical device for the quantification of sugars in milk samples. In particular, the powerful combination of the paper-based platform, the facile manufacturing, and the on-demand customization, allowed to obtain a stand-alone platform that was capable to store all the reagents necessary for the assay, to treating the sample by removing major interferences due to lipids and proteins, and to visualize the results in minutes. A wax-patterned chromatographic paper was finely designed to provide a reagent-free platform for the use of non-specialists applied towards the analysis of sugars' content (lactose as the main sugar). Sugars were electrochemically quantified down to 0.16 g L^{-1} with a repeatability of 5%. To strengthen the efficacy of the novel platform, the electrochemical paper-based analytical device has been applied to commercial milk, including skimmed, semi-skimmed, whole, and lactose-free, confirming satisfactory performance with analytical reliability. This paper-based architecture opens novel avenues for portable (bio)sensing of other relevant species, not only in the agri-food field.

1. Introduction

To ensure that products comply with established standards, food analysis in conjunction with quality control provide information about their characteristics from reception of raw ingredients to the final product; and determine whether the product is satisfactory or not [1,2]. Thus, toxic substances are removed from food products and the quality of life of people with allergies, diabetics and those who cannot consume some species/products is improved [1]. Among these categories, lactose represents a sugar of high interest.

Lactose is the main carbohydrate in milk and dairy products and is a unique disaccharide because it is only produced in the mammary gland of mammals [3,4]. The most important role of lactose on milk yield is to control the osmotic pressure of milk, since more than 50% of the osmotic pressure of milk is defined by the lactose that is secreted by the mammary epithelial cells [3]. In addition, many benefits of lactose consumption via milk have been identified, such as the main energy source, shaping of the intestinal microbiota, and facilitating some mineral absorption, among others [5–9]. For all these reasons, milk and dairy products are widely consumed by humans, but, unfortunately, many people have lactose intolerance (more than 70% of the world population) [5,6]. This disorder causes several symptoms, such as diarrhea, nausea, bloating, abdominal pains, loss of appetite, weakness, and joint or pain in the muscle [5–7,10].

Abbreviations: **ePAD**, electrochemical paper-based analytical device; **LOD**, limit of detection; **LOQ**, limit of quantification; **μPAD**, microfluidic paper-based analytical device; **POCT**, *point-of-care* testing; **PONT**, *point-of-need* testing; **SPE**, screen-printed electrode; **SWV**, square wave voltammetry; **TS_{MILK}–OsVI**, total sugars from milk labeled with the electrochemical tag Os(VI); **TEMED**, N,N,N′,N′-tetramethylenediamine; **WHO**, World Health Organization.

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Traditionally, many methods have been used to determine lactose concentration in milk, but the official methods are HPLC (according to ISO 26462:2010 and AOAC Official Method^{SM} 2020.08) [11], enzymatic assays (according to ISO 22662:2007) [12,13], and mid-infrared spectroscopy (according to AOAC Official Method^{SM} 972.16) [14]. However, these methods may not be feasible for laboratories and small companies due to the complexity and the high cost of equipment and analysis. Furthermore, these devices are not portable and require long analysis times, which together hinder their applicability for *point-of-care* testing (POCT) applications.

In this sense, POCT is a top priority for World Health Organization (WHO), especially, in resource limited settings [15]. WHO has established some criteria that POCT must fulfill: Affordable, Sensitivity, Specific, User friendly, Rapid and Robust, Equipment-free, and Deliverable to end users (ASSURED) [15]. Recently, new criteria have been added: Real-time connectivity and Ease of sample collection (REASSURED) [16]. These characteristics are highly demanded by developing countries [17]. These well-established features for the *point-of-care* approach can be adapted and extended to other domains of decentralized analysis and *point-of-need* testing (PONT). The rapid identification of frauds or allergens, such as the case of lactose in milk, could be a relevant case.

Recently, portability has become an increasingly important goal for developing sensing platforms for many applications. On the one hand, colorimetric sensors offer several advantages. They are fast, adaptable, cheap, sensitive, do not require cleanroom facilities, and can be detected with the naked eye [18–21].

On the other hand, the portable nature and low power demands of electrochemical sensors satisfy many of the requirements for *in situ* measurements. These electrochemical sensors offer high sensitivity, selectivity, a wide linear range, miniaturization, independence of sample turbidity, and high compatibility with smart devices, such as wearables and smartphones, enabling automatic data transfer to "the cloud" (that can make diagnostic decisions in real-time). They are fast, easy-to-use, simple, low-cost, small, require low sample volumes (typically in the microliter range), and they will have major impact on PONT [22–26].

In contrast to colorimetric assays, electrochemical methods provide a unique advantage: they are not affected by the color/turbidity of the matrix of the sample, which is often the cause of interference.

Several interesting approaches have been reported for lactose determination using electrochemical-based portable devices [27–29]. Pazzona's group developed and tested a portable device to monitor the health status of Sarda breed sheep by the measurement of conductivity on-site of this milk [27]. The results for lactose (4.75 ± 0.50%) were within the range reported for Sarda sheep. The device showed a sensitivity of 73.1%, and a good accuracy of 73.5%. A total of 68 Sarda sheep milk samples were analyzed, but they needed 50 mL of milk to obtain the correct electrical conductivity reading [27]. In another paper, Pérez-González *et al.* validated a portable potentiometric electronic tongue to evaluate the quality of milk with different fat and nutritional contents [28]. The device showed an accuracy of 90.8% and lower relative error was achieved for lactose contents with respect to values obtained by traditional methods. In contrast, a volume of 100 mL for each sample was necessary for analysis and 65 milk samples were analyzed [28]. In the last example, Mathews *et al.* developed a portable digital handheld hydrogen breath monitor (GIMate) in diagnosing lactose malabsorption compared to a US Food and Drug Administration (FDA) cleared device (H_2 Check) [29]. The main result was the 100% positive and negative percent agreement between GIMate and H_2 Check. A total of 31 patients with lactose malabsorption or lactose intolerance were analyzed [29].

Within portable devices, paper-based analytical devices (PADs) have garnered significant interest because they offer several advantages such as light, customization, biocompatibility, easy-to-use, cost-effectiveness, minimal reagent consumption, low-cost (especially useful in developing countries with limited resources), liquid flow via capillary forces without external driving forces, and simple and safe disposal by incineration. Furthermore, the porous structure of the paper allows for storage of all reagents necessary for the assay before the measurement (delivering the "reagent-free" devices), to filter the sample, to block gross impurities present in the real matrices (avoiding electrode fouling), and it is a support for chemical and electrochemical reactions [30–36]. Microfluidic paper-based analytical devices (μPADs) offer spatial control of fluids forming hydrophobic patterns by microchannels and chambers. The combination of the advantages of electrochemistry and microfluidics and the use of paper as a support for the reactions, led to the development of electrochemical paper-based analytical devices (ePADs). ePADs have attracted widespread attention in biosensing due to their benefits in biomedical analysis [30–36].

In this work, we developed a new approach to quantify lactose and total sugar content in milk samples, which is based on an integrated paper-based screen-printed electrochemical sensor. Since sugars are electrochemically inactive on carbon electrodes, their electrochemical quantification needs the use of a label-based approach. In this sense, an osmium (VI) complex was used as electrochemical probe, which specifically link to the carbohydrates [37–40], allowing a response in the range of 10–16 h. However, with regards to this reported method, herein the adoption of Os(VI) complex with sugars has been designed and adapted onto a chromatographic strip, decreasing the assay time to 15 min. The herein-reported device is a fully printed, namely an all-in-one, paper-based electrochemical device for application to complex samples, e.g., milk. Its unicity is highlighted by several features, as follows: i) mostly all the processes are exploited onto the structure of chromatographic paper, dramatically simplifying the tasks for final users, ii) the merging of screen-printing, wax printing and drop casting approaches, allows to obtain a customized platform for specific application, iii) the separation of the protein fraction is autonomously performed on the paper-based strip, thus avoiding extra-tasks for treatment, and iv) due to the low-cost procedures adopted, the final device appears affordable for all the contexts of use, especially conceived for limited-resource countries.

2. Material and methods

2.1. Reagents and materials

Lactose, potassium phosphate monobasic, potassium phosphate dibasic, hydrochloric acid, acetic acid glacial, potassium osmate (VI) dihydrate, and N,N,N′,N′-tetramethylenediamine (TEMED) were purchased from Sigma–Aldrich (Darmstadt, Germany). Conductive inks (Ag/AgCl and carbon) were acquired from Acheson (Italy). Skimmed milk, semi-skimmed milk, whole milk, and lactose-free milk (all of them cow milks) were purchased in the local market.

All reagents and solvents were of analytical grade. All solutions were prepared in Milli-Q water.

Whatman Chromatography Paper 1 CHR was purchased from Merck (Darmstadt, Germany). Scotch adhesive tape was purchased in the local market.

2.2. Instrumentation

Adobe Illustrator was used to design the wax pattern of the ePAD, and they were printed on a sheet of filter paper (Whatman 1 CHR) using a solid ink printer, namely, ColorQube 8580 from Xerox (USA, CT, Norwalk).

All the electrochemical measurements were carried out with the use of a portable potentiostat, EmStat3 (PalmSens, The Netherlands), connected to a laptop. Currents were recorded and displayed on a laptop by using the software PSTrace 5.9.

2.3. Procedures

2.3.1. ePAD design and fabrication

Fig. 1 shows the schematic procedure of the sensor fabrication. The manufacture of the ePADs is composed by wax printing and screen-printing, as follows [41,42]:

To draw the pattern of the ePAD, Adobe Illustrator software was used (Fig. 1**A**). The pattern was printed onto a filter paper (Whatman 1 CHR) using an office wax printer (Fig. 1**B**). Then, waxed paper was cured in an oven at 100 °C for 1 min (Fig. 1**C**), allowing the wax to diffuse through the paper, producing a hydrophobic confinement (wax, green) around the hydrophilic testing area (paper, white). This step is very important to define the testing area and confine the solution in the delimitated electrochemical cell area, avoiding its diffusion toward the electrical contacts and affecting the readout [41,42]. After the hydrophilic area was surrounded by a hydrophobic wall, the three-electrode system was manually screen-printed onto the hydrophilic zone (Fig. 1**D**) using a squeegee and two masks, first one for the Ag/AgCl ink (Electrodag 477 SS), that was used to print the connections and the reference electrode, and the other one for the carbon ink (Electrodag 421), that was used to print the working and counter electrodes. After each printing step, the screen-printed electrodes (SPEs) were cured at 60 °C for 30 min. Thermally curing was necessary to make the printed ink stable for electrochemical measurements [41,42]. Next, the backside of the printing surface was covered with adhesive tape (without covering electrical connections) to prevent the solution from leaking out underneath the ePAD.

To produce a reagent-free platform in the analysis of milk samples, the microfluidic channel was impregnated with a hydrochloric acid solution (Fig. 1**E**), which was used as an agent for the acidic precipitation of milk proteins [43–45]. 20 µL of 10 M HCl were added to the hydrophilic area of test of the microfluidic channel of ePAD (at the beginning of the channel), and it was dried completely in an oven at 60 °C for 2 min prior the milk sample was added and analyzed. Then, the ePAD was ready to use (Fig. 1**E**).

The ePAD consisted of one hydrophilic rectangular zone (microfluidic channel, 50 mm (length) x 5 mm (width)), and one hydrophilic semicircle zone (electrochemical cell, 10 mm diameter). For Ag/AgCl ink, three 19 mm × 1.2 mm rectangles were used for electrode traces, and the reference electrode was an arc of 4 mm × 1.2 mm (length x width). For carbon ink, the working electrode was a circle of 4 mm diameter, and counter electrode was an arc of 13 mm × 1.2 mm (length x width). The entire design of the ePAD was reported in the Supplementary Information file (Fig. S1).

In a single sheet, 32 devices were screen-printed. Consequently, each ePAD was cut to obtain disposable devices after use.

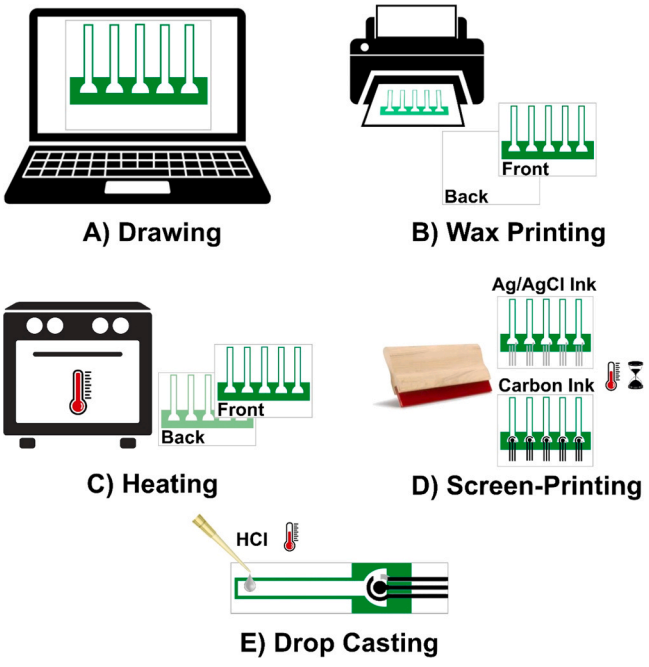

Fig. 1. Schematic representation of the fabrication of the electrochemical paper-based analytical device (wax printing, screen-printing, and reagent-free platform).

2.3.2. Preparation of electrochemical tag ($Os(VI)O_2(OH)_2$TEMED complex)

The preparation of $Os(VI)O_2(OH)_2$TEMED complex was carried out according to the literature [37,46]. First, 18.4 mg of potassium osmate (VI) dihydrate (50 µmols) were suspended in 1.22 mL of water. Then, 5.8 mg of TEMED (50 µmols) and 0.41 mL of a 0.2 M potassium phosphate buffer pH = 7.0 solution were poured into the previous solution. Finally, 10 µL of 10 M HCl solution were added. The solution was shaken for 1 h to allow the formation of $Os(VI)O_2(OH)_2$TEMED complex and finally, the solution was filtered using a syringe Nylon filter 0.45 µm (Tecno-Air). The final concentration of Os(VI) complex (electrochemical tag) obtained was 30.3 mM.

2.3.3. Labeling protocol of lactose with $Os(VI)O_2(OH)_2$TEMED complex

Lactose labeling with $Os(VI)O_2(OH)_2$TEMED complex (Lac–Os(VI) adduct) was prepared as follow, with slight modifications [46]: 1.0 mg of lactose was dissolved into 234 µL of 50 mM potassium phosphate buffer pH = 7.0 using a microtube. Then, 16.5 µL of Os(VI) $O_2(OH)_2$TEMED solution were added in the previous solution and it was kept 15 min at 90 °C using an oven. Final concentration was 4000 mg L^{-1} for Lactose. Finally, working solutions were prepared by dilution of stock solution in 50 mM potassium phosphate buffer pH = 7.0.

The labeling protocol of lactose/total sugar content was modified when milk samples were analyzed [43]. Briefly, 200 µL of milk sample was mixed with 50 µL of $Os(VI)O_2(OH)_2$TEMED solution in a microtube and it was kept 15 min at 90 °C using an oven.

2.3.4. Electrochemical measurements

All electrochemical measurements were carried out without pretreating the electrodes prior to the measurements. Electrochemical detection to measure lactose and milk samples was performed using square wave voltammetry (SWV). SWV was carried out in a range potential from − 1.5 to + 1.2 V, with a step potential of 0.005 V, amplitude of 0.05 V, and frequency of 50 Hz. Controls (Os(VI) complex, lactose, and milks) and blank (50 mM potassium phosphate buffer pH = 7.0) were also prepared and analyzed in the same way. All measurements were performed in triplicate.

2.3.5. Reagent-free approach for the determination of total sugar content in milk samples using ePADs

Milk is composed of water, proteins, carbohydrates, fats, vitamins, and minerals [47]. In the case of the reagent-free approach, 20 µL of 10 M HCl were loaded in the microfluidic channel (hydrophilic zone) and the sensor was ready to use after drying in an oven at 60 °C for 2 min prior to analysis of milk samples. Briefly, after the testing area of the microfluidic channel was dried, the sample solution can be added. 50 µL of milk labeled with the electrochemical tag Os(VI) (total sugars, TS_{MILK}–Os(VI)) were diluted in 200 µL of 50 mM potassium phosphate buffer pH = 7.0 in a microtube, the final volume being 250 µL. Then, a total of 100 µL of the previous solution were transferred at the beginning of the microfluidic channel, where the reaction of the HCl impregnated in the paper with the milk sample took place, and this solution reached the electrochemical cell (approx. 2 min). Finally, 100 µL of background electrolyte (50 mM potassium phosphate buffer pH = 7.0) were dropped on the SPEs of the ePAD homogeneously wet the three-electrode system.

All the SWV measurements were carried out by using a portable potentiostat interfaced to a laptop. Each ePAD was used for a single analysis since they were disposable devices.

3. Results and discussion

3.1. Electrochemical behavior of native and labeled lactose on ePAD

As was aforementioned, Os(VI) complexes react with hydroxyl groups from glycans to form stable adducts, which yield several peaks by SWV at carbon electrodes [37–40]. The oxidation potentials of these peaks depend on, among other factors, the pH and the kind of complex agent bound to the cation Os(VI) [37,46], as displayed in Fig. S2.

In our case, two peaks were obtained by SWV when analyzing the Lac–Os(VI) adduct. The first peak, at −0.75 V, is exclusively due to the electrochemistry of the Os(VI) [37–40], while the second peak, at + 0.40 V, corresponds to the electrochemistry of the adduct formed (Lac–Os(VI)) [48]. Sierra *et al.* [48] were the first to observe the appearance of a high and well-defined peak at + 0.40 V, when glucose was tagged with the Os(VI) complex. In our study, it has been possible to demonstrate that this peak (+0.40 V) also appears when lactose is tagged with the Os(VI) electrochemical probe, opening new directions for sugar electrochemical detection. These results demonstrated that this new methodology is valid for labeling and detecting lactose.

3.2. Optimization of the acidic medium required for protein precipitation in milk samples using ePAD

Due to the presence of proteins in the milk samples, a pretreatment in the microfluidic channel of the ePAD was needed. To this end, a selective acidic precipitation was used to separate total sugar content from proteins. All types of acid can be used as precipitating agents but the most used are hydrochloric and acetic acids [43–45,49]. As reported in Fig. S3, various levels of acetic acid, namely 0.17, 1.7, and 17.5 M, and hydrochloric acid solutions, namely 0.1, 1, and 10 M, were compared with phosphate buffer measurements. A solution of 10 M hydrochloric acid resulted the most suitable to conduct the protein precipitation, and subsequently volumes of 10, 20, and 50 µL were interrogated. 20 µL of 10 M HCl was chosen as the optimal volume (results not shown) because it was able to impregnate the entire microfluidic channel of the ePAD without reaching the electrochemical cell. 10 µL did not cover the entire microfluidic channel and 50 µL impregnated the entire ePAD.

3.3. Analytical performance of ePADs towards lactose determination

The analytical performance of the ePAD for lactose determination was evaluated. Each point on the calibration plots corresponds with the use of three independent ePADs.

Calibration studies were carried out for Lac–Os(VI) adduct using the peak at −0.75 V, and at + 0.40 V (Fig. 2).

As shown in the insets of Fig. 2, a linear correlation has been obtained between the height of the current peaks and the concentration of lactose in the range of 0.5 – 11.1 mM, showing an excellent linear correlation coefficient for the signal at −0.75 V (r = 0.996) and good linear correlation coefficient for the signal at + 0.40 V (r = 0.990). The peak at −0.75 V was selected as the representative signal for Os(VI) complex for the following experiments because it provides higher sensitivity than the peak at + 0.40 V. The main characteristics of the methodological calibration are summarized in Table 1.

The reproducibility of ePADs in terms of calibration slopes was very good (RSD = 5%). The limit of detection (LOD) was 0.14 mmol L^{-1} (0.048 g L^{-1}) and limit of quantification (LOQ) was 0.5 mmol L^{-1} (0.16 g L^{-1}) (3 S/N and 10 S/N criteria, respectively, using the standard deviation of the point of lowest concentration). In this sense, LOQ was adequate for lactose determination in milk samples, because the normal range for lactose in cow milk samples is between 117 and 146 mmol L^{-1} (40 – 50 g L^{-1}) [5,7,10].

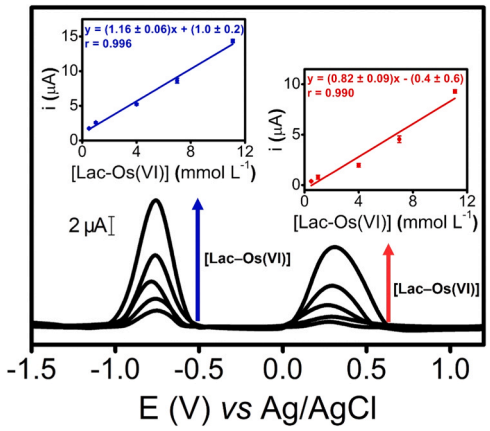

Fig. 2. Voltammograms corresponding to 50 µL of Lac–Os(VI) adduct from 0.5 to 11.1 mmol L^{-1} using the ePAD. Insets: Lac–Os(VI) adduct calibration plot for peak at −0.75 V (blue line), and peak at + 0.40 (red line). SWV parameters: start potential − 1.5 V, end potential + 1.2 V, frequency 50 Hz, amplitude 0.05 V, and step potential 0.005 V (the background signal was linearized) (n = 3).

Precision (in current height) was also evaluated using three independent paper-based electrochemical sensors: a Lac–Os (VI) solution (4 mmol L^{-1} lactose) prepared in 50 mM potassium phosphate buffer pH = 7.0 provided an RSD lower than 3% (n = 3) using the same batch, indicating a high reproducibility of the platform. In this way, the robustness coupled to the easy manufacturing process of the ePADs has been highlighted.

3.4. Reagent-free detection of total sugar content in milk samples using ePADs

Once the good analytical performance of the ePAD for lactose determination was demonstrated and, under the optimal conditions, the reagentless ePAD was evaluated for total sugar content determination in milk samples (lactose being practically the unique sugar present in milk) [3,5,6].

Milk is a complex matrix because it contains proteins, carbohydrates, and fats, among others. A potential issue is given by the adsorption of proteins and fats contained in this matrix, which might produce an increase of the fouling processes reflected in lowered currents, and, therefore, a decrease in the sensitivity.

However, a possible strategy to limit these interferents is the porosity of the paper itself, since this porosity is very effective against the presence of gross impurities present in this complex matrix. For this reason, the reagent-free ePAD was loaded with 20 µL of 10 M HCl before measurement to form a yellowish precipitate (due to protein) when milk sample was added (see Fig. S4), as reported in **Section 2.3.5**.

Finally, four cow milk samples (skimmed, semi-skimmed, whole, and lactose-free milk) were analyzed following our methodology (see Fig. 3). Qualitatively, in contrast with the controls (buffer and sample without Os(VI), identical voltammogram profiles were obtained for the plethora of the milk samples studied without exception, identifying both peaks with excellent S/N features at −0.70 V and + 0.38 V in high agreement with the potential obtained for Lac–Os(V) adduct at –0.75 and + 0.40 V, respectively.

Quantitatively, Table 2 lists the amounts of total sugar contents in these samples, showing a high agreement between the results obtained using our ePAD-based approach and the declared values (E_r < 5%) with excellent precision as well (RSDs ≤ 2%).

The achieved results demonstrated the suitability of the developed ePAD as reliable microfluidic sensor for total sugar content detection in a complex matrix such as milk by an easy procedure and a cost-effective

Table 1
Analytical characteristics of calibration plots for Lac–Os(VI) using external standard method (n = 3).

Signal (V)	Linear range (mmol L^{-1})	r	$a \pm s_a$ (μA)	$b \pm s_b$ (μA L $mmol^{-1}$)	RSD_{Slope} (%)	LOD (mmol L^{-1})	LOQ (mmol L^{-1})
–0.75	0.5 – 11.1	0.996	1.0 ± 0.2	1.16 ± 0.06	5	0.14	0.5
+ 0.40	0.5 – 11.1	0.990	–0.4 ± 0.6	0.82 ± 0.09	11	0.15	0.5

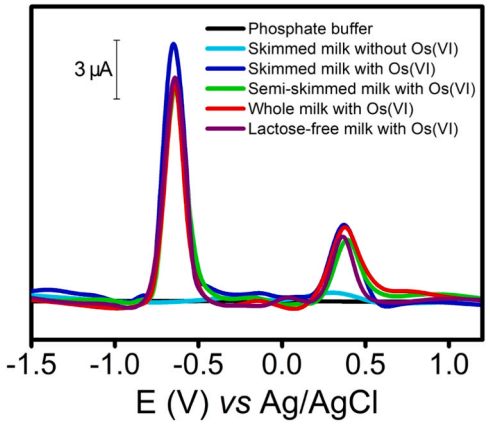

Fig. 3. Voltammograms corresponding to different commercial cow milks using the ePAD. SWV parameters: see Fig. 2. (The background signal was linearized) (n = 3).

Table 2
Analysis of total sugar content of different commercial cow milks by ePAD (n = 3).

Type of milk	Declared value (g/100 mL)	ePAD value		E_r (%)
		$\bar{x} \pm s$ (g/100 mL)	RSD (%)	
Skimmed	4.9	4.91 ± 0.09	2	0.2
Semi-skimmed	4.9	4.69 ± 0.08	< 2	4
Whole	4.9	4.67 ± 0.06	< 2	5
Lactose free	4.9	4.68 ± 0.09	2	4

device.

To our best knowledge, there is not any ePAD for total sugar content determination in milk samples reported in the literature, so our ePAD approach is the pioneer in this application and a potential device at the PONT. Several traditional methods have been reported in the literature for the analysis of lactose in milk [11–14], but most of these methods involve time consuming protocols and expensive benchtop equipment. For these reasons, there is a need for development rapid and *in situ* assays. Table 3 summarizes the main features of some portable devices for lactose determination along with our approach, where lactose is the main and majority sugar of the total sugar content in milk [3,5,6].

Although our LOD and LOQ obtained using ePAD are not the lowest in the literature, they meet the required levels of lactose in milk samples. Also, our approach is cheaper, easily portable, single-use (avoiding cross-contamination), reagent-free, and it requires very low sample volume (100 μL).

4. Conclusions

For the first time, a simple method to measure total sugar content in cow milk samples has been successfully developed by using a fully-integrated ePAD. Conceptually, the combination of filter paper as an active substrate, wax printing to define the testing area as well as to isolate the electrical contacts, and screen-printed electrodes on paper, offered a reagent-free customizable platform.

Table 3
Portable devices or enzyme assays for lactose determination in milk samples reported in the literature.

Platform	Technique	LOD (mmol L^{-1})	Experimental setup	Reference
3D prototype	Electrical conductivity	–	Conductivity probe, temperature probe, embedded electrical conductivity circuit, micro-controller, LCD screen, and container with 50 mL of milk	[27]
Potentiometric electronic tongue	Potentiometry	–	A five-hole acrylic tube filled with a conductive silver epoxy resin containing five sensors based on PVC membranes coupled to a data logger via electrical copper wires and immersed in a glass cell containing 100 mL of sample	[28]
High performance Raman spectrometer	Raman spectroscopy	19	The intensity of the Raman signal is affected by the instrument and the sample; therefore, crystal violet was selected as an internal standard	[50]
Gold disk electrode modified with an MPA-SAM	Enzyme-based amperometry	0.00046	β-Gal, GOD, HRP enzymes and TTF mediator are co-immobilized by a dialysis membrane on an MPA-SAM-modified gold disk electrode	[51]
ePAD	SWV	0.14	Reagent-free	This work

β-Gal: β-galactosidase; GOD: glucose oxidase; HRP: horseradish peroxidase; MPA: 3-mercaptopropionic acid; SAM: self-assembled monolayer; TTF: tetrathiafulvalene

It should be noted that the porosity of the paper allowed storing the necessary reagents as well as filtering the milk sample itself, removing the gross impurities of this complex matrix. In addition, the tasks required for the end-user are minimal and the possibility of combining these paper devices with a portable potentiostat, allow fast *in situ* monitoring.

Thanks to the selective acidic precipitation of milk proteins, the total sugar content was isolated from gross impurities, avoiding electrode fouling. Furthermore, these assays were carried out simultaneously on the same ePAD, achieving the integration of sample treatment and

detection on the device, which is one of the main challenges of microfluidic analytical devices.

Finally, this methodology was successfully applied to quantify total sugar content in commercial milk samples, showing an excellent and reliable agreement with the declared values in these samples.

The proposed strategy can be adapted and translated to other sensors and biosensor systems, not only in the field of food but in the clinical and environmental fields, where the complexity of samples often limits real-world application too. Since this is a sustainable approach due to the use of paper (environmentally friendly), ePADs can be safely disposed of after analysis, which is relevant in these fields.

One of the major highlights of this work is represented by the manufacturing procedures involved in the realization of the proposed device since everything has been obtained with the use of affordable and in-house procedures with a high throughput and excellent inter-devices fabrication reproducibility: screen-printing, wax printing, and drop casting of all the reagents needed. It might be very attractive for use in developing countries or remote areas, where access to clinical facilities is restricted or limited due to scarcity of resources. In addition, this ePAD meets several requirements of ASSURED criteria recommended by WHO guidelines.

CRediT authorship contribution statement

Silvia Dortez: Investigation, Conceptualization, Methodology, Writing - original draft, Writing - review & editing. **Agustín G. Crevillen**. Writing - original draft, Writing - review & editing. **Alberto Escarpa**: Funding acquisition, Writing - review & editing. **Stefano Cinti**: Conceptualization, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2023.134704.

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