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STUDY AND OPTIMIZATION OF A MICROFLUIDIC PAPER BASED ANALYTICAL DEVICE FOR THE QUANTIFICATION OF SALIVARY COPPER

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Thesis presented to Escola Superior de Biotecnologia of the Universidadde Católica Portuguesa to fulfil the requirements of Master of Science level in Biomedical Engineering

by

Adriana Raquel Gomes Tavares Almeida

Supervision: Dr. Raquel B. R. Mesquita

Co-Supervision: Dr. António O. S. S. Rangel

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Resumo

O diagnóstico precoce de uma patologia permite promover um melhor tratamento e recuperação. Assim, o desenvolvimento de dispositivos de avaliação da condição de saúde de forma rápida, contribuem para este processo. Neste sentido, nos últimos anos tem havido um crescimento no desenvolvimento de dispositivos de baixo custo e que sejam fáceis de ser utilizados.

Neste trabalho foi desenvolvido um dispositivo analítico baseado na análise por microfluxo em papel (µPAD) para a determinação de cobre em saliva humana, sendo uma forma de teste rápido. O dispositivo é adequado para a detenção de excesso de cobre como consequência de alteração na condição de saúde. O desenvolvimento do dispositivo envolveu vários estudos de modo a conseguir uma determinação rápida, precisa, de baixo custo e de fácil utilização.

Foi escolhida a reação colorimétrica entre o cobre e a difenilcarbazida formando um complexo de cor vermelha. Após decorrer o tempo de reação, utiliza-se um digitalizador para obter uma imagem digital da zona de deteção do µPAD, de forma a calcular a absorvância com base na intensidade de cor de cada local de análise, obtida pela utilização do software Image J.

O dispositivo desenvolvido, permite a determinação de cobre num intervalo de 80,0 – 600 µg/L, com uma curva de calibração A= (0.0701±0.0008) $[Cu^{2+}]$ – (0.0043 ± 0.0004), um limite de deteção de 17 µg/L e o limite de quantificação de 56 µg/L.

Após a aplicação das amostras no µPAD, a leitura da cor pode ser feita entre 20-40 min. A exatidão dos resultados foi aferida por calculo de percentagens de recuperação utilizando salivas de voluntários.

Palavras-chave: Cobre; µPAD; saliva; reação colorimétrica

Abstract

Early diagnosis of pathology can promote a better recovery and treatment. Thus, the development the devices the rapid evaluation of the health condition, contribute to this process. In this context, in the recent years there has been growth in the development of low cost, easy to use devices.

In this work, a microfluidic paper-based analytical device (µPAD) was development for the determination of copper in human saliva, being a form of rapid test. The device is suitable for the detection of excess copper, as consequently to change the healthy status. The development of the device involved several studies in order to get the rapid determination, accurate, cheaper and easy to use.

Colorimetric reaction was chosen between copper and diphenylcarbazide create the complex of red colour. After the reaction time has elapsed, a scanner is used to obtain the digital image of µPAD detection zone, in order to calculate the absorbance based on the intensity of colour of each analytical local, obtained by Image J software.

This device provides a copper determination in the range of 80.0 – 600 µg/L, a calibration curve of $A= (0.0701\pm0.0008) [Cu^{2+}] – (0.0043 \pm 0.0004)$, the limit of detection of 17 µg/L and the limit of quantification of 56 µg/L.

After inserting the sample in the µPAD, it can be digitized, within a time period of 20 - 40 min. The accuracy of the results was assessed by calculating recovery percentages using saliva from volunteers.

Keywords: Copper; µPAD; saliva; colorimetric reaction

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Contents

1- Introduction

Nowadays, society follows several types of habits that, for a long period of time, are reflected in the health of the human body. Usually, when we think about unhealthy habits, tobacco, high body mass index, alcohol, low portion of fruits and vegetables, among others, are the ones that we think more about.

For the year 2018, the World Health Organization estimated that cancer was the second cause of death, statistically corresponding to 9.6 million deaths. Furthermore, about one-third of deaths from cancer are due to the unhealthy habits referenced before. However, Figure 1 shows a prediction for the next twenty years, for that disease to grow significantly, principally in poor countries (Word Health Organization *et al*., 2021).

Cancer is the general word for the diseases that can start in any organ or tissue of the body, where abnormal cells grow uncontrollably. Sometimes the cells boundaries are breached and the disease spreads to other organs. (Word Health Organization *et al*., 2021).

One of many cancers is head and neck cancer, which is the seventh most common malignancy worldwide. The demographic variation of incidence of this cancer is related to the habits of tobacco use and alcohol consumption, which increase almost 80% of all head and neck cancer diagnosed globally. The countries that have higher risk are India, Sri Lanka, Bangladesh and Pakistan (Vigneswaran *et al*., 2014; Schutte *et al*., 2020).

Moreover, in these regions the population is vulnerable due to various features like, age – children or older, geographical isolation, poor sanitary condition and low economic status. Thus, the access to hospitals and emergency centre where diagnosis is done, is essential, but most of the time impossible. So, the diagnosis must be in close proximity to the populations and adapted to real-life conditions (Vigneswaran *et al*., 2014; Augusto-Oliveira *et al.,* 2021).

In this context, the search to identify new, non-invasive and low-cost methods for detection of this disease grows. Saliva is the ideal non-invasive biological fluid for these new methods of detection given that the process to obtain it is better accepted by patients of different cultural and educational backgrounds (Augusto-Oliveira *et al.,* 2021).

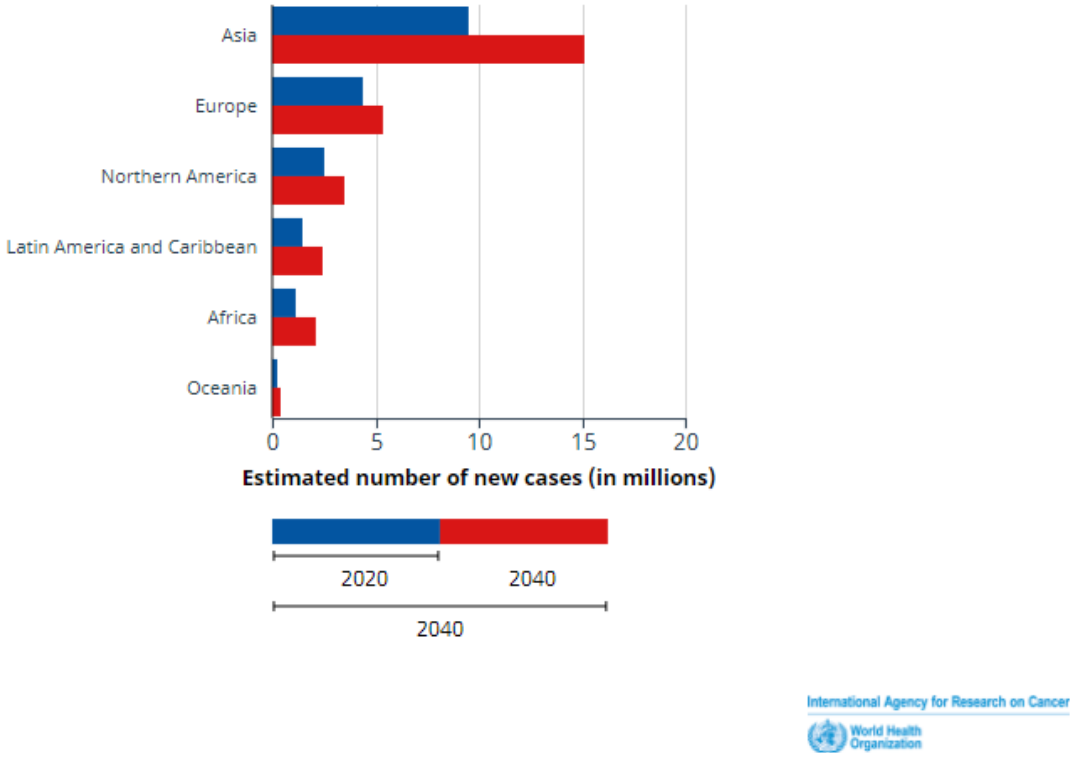

Figure 1 Estimated number of new cases of cancer from 2020 to 2040 (International Agency for Research of Cancer *et al.*, 2020).

1.1 Copper in human body

Copper is an essential microelement present in the body. Healthy people's copper content is between 50-150 mg. It's located mainly in the skeleton and muscles (50%-70%) but there's also a high concentration in the liver (20%), followed by the brain, kidney and heart (Wang *et al.,* 2021).

Copper is also fundamental for important processes in the body like, respiration, free radical eradication, energy production, metabolism of oxygen and iron, maturation of extracellular matrix and neuroendocrine signalling. But its essential role is preliminarily catalytic, and it acts as cofactor for many enzymes such as, cytochrome oxidase, tyrosine, ceruloplasmin, among others (Ingle *et al*., 2018).

In addition, it is the third most prevalent mineral, so it's absorbed in the stomach and small intestine, particularly by the duodenum, which absorbs about 40% of its total. Then copper is transported into the liver, where it binds with protein albumin.

This metabolism process is highly complex and intervenes with many proteins; however, copper arrives at the bloodstream via ceruloplasmin (plasma protein), in this part the metabolism of copper is controlled and it is excreted by the bile. This metabolism needs

equilibrium with zinc and manganese, because zinc interferes with the absorption of copper in the small intestine (Ingle *et al.,* 2018; Wang *et al.,* 2021).

The uptake of copper needs two oxidation states, by action of reductase enzyme, the first stage is Cu (I)/Cu^{+} (cuprous ion) and then Cu (II)/Cu^{2+} (cupric ion), which allows it to behave either as a recipient or as a donor of electrons (Chen *et al.,* 2020).

This complex process is important to maintain the balance of copper by intestinal absorption and biliary excretion.

Hence, some national and international organizations, such as, World Health Organization (WHO), U.S. Institute of Medicine (IOM), The European Safety Authority (EFSA), recommend standards concentration of copper for healthy human beings.

WHO proposes 1.3 mg of copper per day in adults. However, this quantity of copper is different between organization and countries (Ingle *et al*., 2018).

1.1.1 Pathologies relation with copper

The copper is an essential element in cells when it has the adequate concentration, as it is responsible for the regulation of the complex mechanisms of healthy people.

Despite that, a high copper concentration is worrying, due to the onset of undesirable diseases. Hence, a deficit or excess of copper occurs, it leads to an imbalance in the different mechanisms.

In Table 1 the relation between some diseases and copper disfunction is shown.

On one hand, when there's an excess of copper circulating in the human body, it is due to the failing of the copper homeostasis mechanism, leading to the development of diseases. These are classified as Wilson disease and non-Wilson disease, both harmful and fatal harmful and fatal the same, cancer being an example of them. (Ingle *et al*., 2018)

On the other hand, when the decrease of copper quantity circulating inside the body occurs, several deficiency syndromes happen. Ingle and Paralikar (2018), report that a diet that contains less than 1mg of copper per day, can lead to unfavourable effects, for the good function of human body. These can lead to some diseases such as Menkes disease.

Table 1 Diseases related to the biological concentration of copper. (Adaption: Ingle *et al*., 2018)

Deficiency	Menke's Disease	
	Cardiovascular Disease	
	Occipital Horn Syndrome	
	Zinc-Induced Myeloneuropathy	
	ATP7A – Related Isolated Distal Motor Neuropathy	
	Temporary Deficiency of Copper	
	Aceruloplasminemia	
	…	
Excess	Wilson's Disease	
	Non-Wilson's Disease	Indian Childhood Cirrhosis
		Idiopathic Copper Toxicosis
		Endemic Tyrolean Infantile Cirrhosis
		Alzheimer's Disease
		Diabetes
		Cancer
		…

The diagnosis of pathologies associated with cancer is long, but this is a disease that it is constantly progressing, hence late treatment will not have the same effectiveness. However, the more accessible and rapidly the diagnosis process is, the bigger the impact will be on slowing down the growth of disease.

Furthermore, tracing a chemical element like copper has a crucial role in numerous physiological and metabolic processes in humans, given that, around 25% of the enzymes in human body need to be activated by them (Okade *et al*., 2015).

As to what cell functioning is concerned, this metal is important for the initiation and progression of cancer. Additionally, copper has procardiogenic effects. Thus, the quantification of this element could be of prognostic significance (George *et al*., 2017).

In this sense, the concentration of salivary copper is different in healthy people and in people that have a pathologic relation with copper. In Table 2, the expected concentration values

of salivary copper for head and neck cancer are indicated, showing that copper levels of patients are higher than in healthy people, who are the control group.

Table 2 Reported expected concentration of copper in saliva.

Article	Group	Concentration
Okade *et al*., 2015	Control Group	52 µg/mL
	Oral submucous fibrosis	87 µg/mL
George *et al*., 2017	Control Group	97.4 µg/mL
	Head and neck cancer	197 µg/mL

For quantification of salivary copper, different methods can be used, although each assays allow obtaining better precision and higher processing speed leading to a minimum error of measurement. Thus, some methodologies are used, such as spectrophotometric assay, immunoassays and "omics" (Tvarijonaviciute *et al*., 2020).

In spite of these methodologies having higher sensitivity, they need professionals with knowledge on manipulating each method. Moreover, vulnerable populations, as well as people that live in remote locations, do not always react well to blood sample harvest. Since the blood needs specific conditions to be stored and kept for conservation, it might not be the best biologic fluid. In this situation, a fast, less invasive test, such as saliva gathering brings benefits for patient. Furthermore, these tests require less manipulation, the price is relatively cheaper, when compared with alternative methods and it is safe.

1.2 Saliva

The biological fluid, saliva, is the most abundant one in the oral cavity; a healthy adult produces between 500 – 1500 mL per day of serous and mucinous saliva, this is approximately 0.5 millilitres per minute as basal flow rate (Bellagambi *et al*., 2019).

This oral fluid is synthesized and secreted by two glands: the major glands, which include the parotid submandibular and sublingual. The others are the minor glands, the labial, buccal

lingual and palatal. Usually, the major glands yield 90% and the others only 10% of the total saliva (Roblegg *et al*., 2019; Bellagambi *et al*., 2019).

Hence, the principal component is water (94-95%), but it contains other components like, enzymes, electrolytes, proteins, nucleic acids, hormones, cytokines, carbohydrates, salts and non-protein nitrogen (urea, uric acid, amino acids and creatinine). Besides that, saliva contains antibacterial, antifungal and antiviral properties, by action of salivary immunoglobulins (Khan *et al.,* 2017; Chojnowska *et al*., 2018).

Due to the complex composition, this fluid has a high buffer capacity, where the pH levels between 6 and 7. This behaviour of saliva happens due to the presence of three buffers: bicarbonate, phosphate and proteins (Bellagambi *et al.,* 2019).

Saliva is a key element of the oral cavity, given that, it conserves oral health and homeostasis, but also, contributes for an efficient digestion, lubrification, moisture of the tissues that support swallowing, chewing, talking and tasting (Bellagambi *et al*., 2019). According to Chojnowska and Baran (2018), these characteristics create a secure environment for different adverse stimuli, such as, mechanical (e.g., friction), biological and chemical.

Salivary diagnostics changed the way that traditional diagnostics – which used blood and urine – were regarded. It's necessary to create a point-of-care (PoC) technology, with saliva, given that, salivary analysis decreases the risk of infection, and due to being non-invasive, painless, customizable and inexpensive, makes it a worthwhile option. Moreover, this type of diagnostic brought many advantages like, automation, integration, multiplexed detection ability, quick analysis, small sample size and minimal training (Khan *et al.,* 2017).

Thus, saliva is a potential source of clinical information, and according to Khan (2017) and Bellagambi (2019), the property of saliva is a "mirror of body health" because this fluid is an ultra-filtrate of blood - due to high vascularized salivary glands -and some molecules, present in the blood stream, have the capacity to pass through the wall of the blood vessel by transcellular and para-cellular means and thus being part of the composition of saliva. Thereby the variation of components of blood also affected the biochemical components of saliva.

Nowadays, these types of analysis are practiced in different areas like pharmacotherapy, epidemiology and bioterrorism, but in medicine many diseases are detected, according to the literature, are summarized in Appendix 1 -Table 1 **(**Roblegg *et al*., 2019).

Otherwise, the saliva sampling, is the first step to started this diagnostic. Hence, saliva can be collected from specific salivary glands or the whole liquid produced by all glands. Although, the method of salivary collection influences the precision and determination of biomarkers (Chojnowska *et al*., 2018).

There are different methods, however for the process of sampling for whole saliva, the most used is, passive drooling and draining, (Figure 2) which consist of letting the patient accumulate saliva in their mouth, while having their head tilted forward, then transferring the liquid to a sterilized plastic tube. Also, can be useful to use some material that helps transfer the liquid to plastic tube such as, gauze, cottons swabs or rolls. With this method there is no stimulation of saliva production, which is beneficial given that in biological studies the effects of the flux in saliva composition are disregarded (Tvarijonaviciute *et al*., 2020).

This method contains advantages like high sample volume, less interference of substances or materials used for sampling or promoting the salivary flow, and, as referred before, this a better method for gathering basal unstimulated secretions. Also, as all methods it has disadvantages, like a extend time for sampling, and when the patients are very young children, sleepers and frail elderly, it's difficult for them to expel the fluid, so in this case there are other appropriate techniques like absorption (Bellagambi *et al*., 2019).

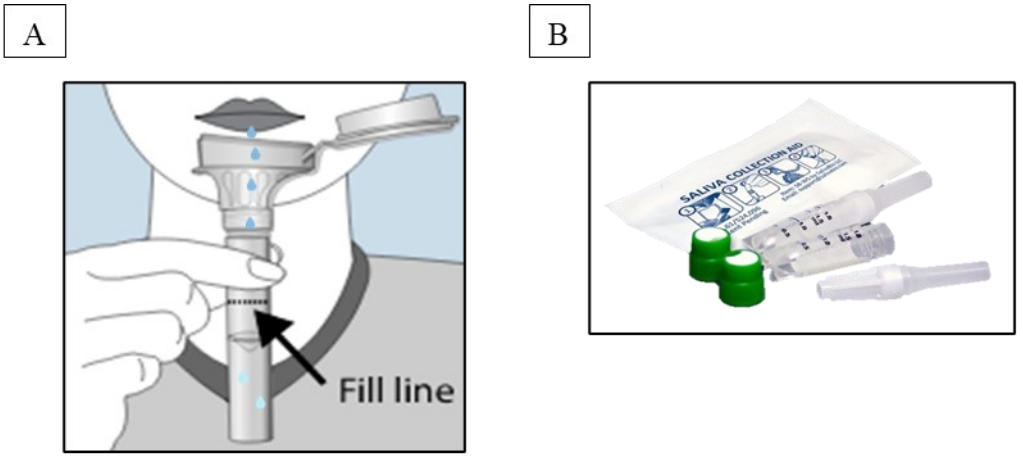

Figure 2 Methods for sampling saliva. (A) Passive drooling and draining method. B) Passive drool using the saliva collection aid (Salimetrics - SalivaBio Collection Aid) (Adaptation: Molecular Vision.; Salimetrics).

1.3 Microfluidic Paper – Device

The year 2007 was the first time that Whitesides Group of Harvard University described this device; although since 19th century paper patterning with hydrophobic materials were used, where the reaction zones were traced with paraffin **(**Lim *et al.,* 2019).

Microfluidic paper based analytical devices (µPADs or micro-PADs) incorporate the science and technology of devices based on paper or other porous membranes, that manipulate small volumes (10^{-3} to 10^{-6} mL) of fluids by capillary action (Nishat *et al.,* 2021).

Thus, in recent years, there have been different techniques for µPAD production, including two groups; the chemical patterning and the physical patterning. Each technique has different resolution of the patterns, fabrication time, chemical compatibility and requirements for equipment and reagents. Although most laboratories will have to apply the technique without a significant investment in new equipment (Nishat, A., *et al* 2021).

The main role of these technique applications was to create two distinct zones: the first are the hydrophobic barriers, usually made of silicon, glass or polymers; the other zone is the hydrophilic usually composed of cellulose that creates the millimetre sized walls of the capillary channels. **(**Nishat *et al.,* 2021; Lisowski *et al.,* 2013).

As it was referred before, the hydrophilic zone is made from cellulose, but normally there were two types of porous membranes used for assays: cellulose-based paper and nitrocellulose membranes (Figure 3).

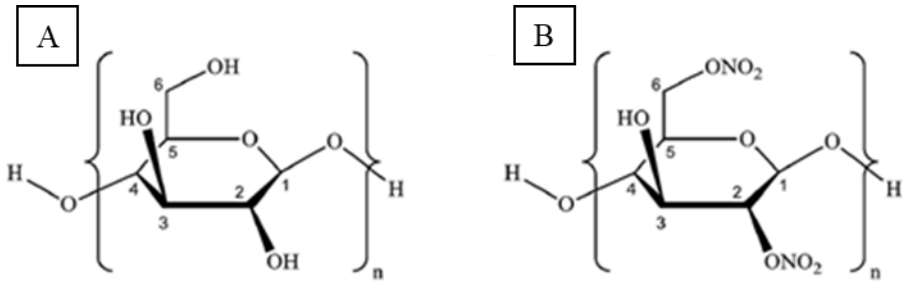

Figure 3 Chemical structure of cellulose [A] and nitrocellulose [B] (Nishat *et al*., 2021)

The first one, cellulose-based paper is made of cellulose fibers with a high density of hydroxyl functional groups (-OH) and few carboxylic acid groups (-COOH). Commonly, the

development of µPADs used two different categories of cellulose-based paper: filter paper and chromatography paper (Nishat *et al.,* 2021).

The nitrocellulose membranes were composed of nitrating cellulose, in this case the polymer allows to control the porosity of the membrane. Furthermore, it has huge protein-binding affinity when used in lateral -flow assays or with the western blots technique (Nishat *et al.,* 2021).

Paper has several advantages because it is inexpensive, suitable for processing techniques and a wide range of printing. Moreover, the reagents and samples are easily placed on paper either manually or using high-speed printing techniques. Besides the white colour of the paper, it an ideal background to analyse and visualize the results of colorimetric tests (Nishat *et al.,* 2021).

Otherwise, the flow of the paper occurs by capillary action, hence in this device the fluids move without any pumps or external sources of power. Thus, it was important to know the flow behaviour of µPADs, to create them in a highly accurate, predictable and programmable way. Paper wet-out is the condition in which the fluid comes into contact with the pores for the first time. However, the wicking in paper is of a level lower than Reynolds' number, thus the flow is classified as being a laminar flow regime **(**Lim *et al.,* 2019).

The detection methods for µPADs are essential, as they allow a simple, versatile, miniaturized, stable, and low-cost detection of analytes. There were different detection methods, although the most common technique in studies is colorimetric.

In this method, the intensity of colour changes during the reaction, in this situation, the intensity was proportional to the concentration of analyte in the sample. Then, with help of the naked eye or a visual aid, the qualitative or quantitative results can be observed (Lim *et al.,* 2019).

For the quantitative results, it is necessary to use a digital image colorimetry (DIC), that can be gathered in a variety of ways, such as with mobile phone, digital camera or scanner. However, the sensitivity and detectability are influenced by type of the modes of detection and which detector is used in the measurement. Detection is based on the reflection and transmission of radiation, which is influenced by the relationship between the visible radiation and the colourful product in a solvent or a solid substrate. In the case of µPADs the light is reflected only on the surface of the analysis zone (Fernandes *et al.,* 2020).

Thus, to measure pixel colour intensity a specific application for colorimetric assays, such as Image J, MATLAB or ColorScan, is necessary (Nishat *et al.,* 2021).

Then, to obtain the pixel intensity, the value of absorbance was converted through the Beer-Lamber law (Equation 1), as will be described later.

Even though there are some disadvantages for the colorimetric technique, as a lower sensitivity to readout in the visible range, and the background paper colour or lighting damage in the automated readout. Otherwise, the association of μPAD and DIC produces results with error below 10% for assays performed (Lim *et al.,* 2019; Nishat *et al.,* 2021).

The characteristics of the μPADs are accessible to researchers and an excellent support for the development of point-of-care (POC) diagnostic tests, for infectious diseases such as, COVID-19, tuberculosis, malaria and non-communicable diseases, such as diabetes, cancer and cardiovascular disorders (Nishat *et al.,* 2021).

Hence, the microfluidics paper exponential growth, whose interest is evidenced by the number of publications in this field. A search done in recent literature from PubMed, with the key-words "paper-based microfluidics" or "microfluidic paper based analytical device", presented 2979 papers published between 2007 and 2020 **(**Nishat *et al.,* 2021).

In addition, several papers are focused on this area, with notable work on biomedical applications, electrochemical analysis, flow control and biosensors. With this research being developed it's possible to reach developing countries where this analysis technique can be applied. In said countries there's a scarcity of diagnosis resources where 75-80% of all deaths are due to a reduce number of diagnosis that would allow a timely treatment of pathologies (Nishat *et al.,* 2021).

Accordingly, the World Health Organization, applied the abbreviation "ASSURED" criteria to μPADs, which means Affordable, Sensitive, Specific, User friendly, Rapid and Robust, Equipment free and Deliverable to end-users (Nishat *et al.,* 2021).

These characteristics make μPADs accessible, easy to produce without clean room facilities or computer-controlled pumps to operate, with quickly available materials; they are portable, biodegradable analytical, inexpensive devices. Furthermore, the potential for μPADs is not only for use in diseases diagnostics, it can also be applied to expand to others fields, like drug.

1.4 Colorimetric reaction of copper

The formation of colour is an important and exploited aspect in chemistry, because the change of colour in a solution indicates that a chemical reaction happened and that colour intensity could be related to the concentration of the products included in that reaction (Fernandes *et al.,* 2020).

Futhermore, the choice of the colorimetric reagent, is the essencial point for identification of copper in the samples, given that the sensibility and sensitivity are some of the parameters to make the best choice of reagent.

According to the literature, there were diferent methods to quantify copper however, some of these methods are more specific than others. Moreover, in the case of the μPAD's, it was necessary to use the method that included the colorimetric reagent and also, this reagent must be able to determine micromolar copper concentration. Hence, the colorimetric reagents most recognized are the 1,5-Diphenylcarbazide (DPC), zincon and azo reagents like, (1Z)-1-[2-(pyridin-2-yl) hydrazin-1-ylidene]-1,2-dihydronaphthalen-2-one (PAN) and 4-(2-Pyridylazo) resorcinol (PAR) (Marczenko *et al*., 2000).

DPC has benefits when compared to other reagents because it displays a high sensitivity to copper, as well as a large molar absorptivity (Turkington *et al.,* 1958) (Crespo *et al*., 2005).

Thus, the reaction between copper (II) ion and DPC has many intermediate species and reactions. However, it depends on the concentration levels of the copper and the pH medium. If the medium contains high pH above 7, its conducive to the oxidation of DPC (Figure 4 - A) to diphenylcarbadiazone (DPDO) (Figure 4 - C), given that the redox potential of oxygen decreases. Simultaneous with this process, there is formation of an intermediate anion of diphenylcarbazone (DPCO) (Figure 4 - B) that exhibits high molar absorptivity. However, it's difficult to identify only DPCO, because the oxidation of DPC to either DPCO or DPDO were very close. Otherwise, if the medium has the lower pH below 7, it contributes to the development of neutral copper (II) complex. (Crespo *et al*., 2005)

Hence, copper (II) must intervene as a selective catalyst in this reaction, where the mole ratio of the cationic complex is 2 moles of DPC to 1 mole of copper (II); copper (II) bonds to the oxygen (Figure 4 – B_1) in the form of enol and resulting in a chelate complex. (Figure 4 – B_2) During this process, the formation of the proton occurs, which contributes for movement of the reaction towards alkaline solutions and its restriction in acidic conditions. Thus, the final

product contains an orange - red colour with the maximum of absorbance at 495 nm (Feng *et al.,* 2011; Crespo *et al.,* 2005).

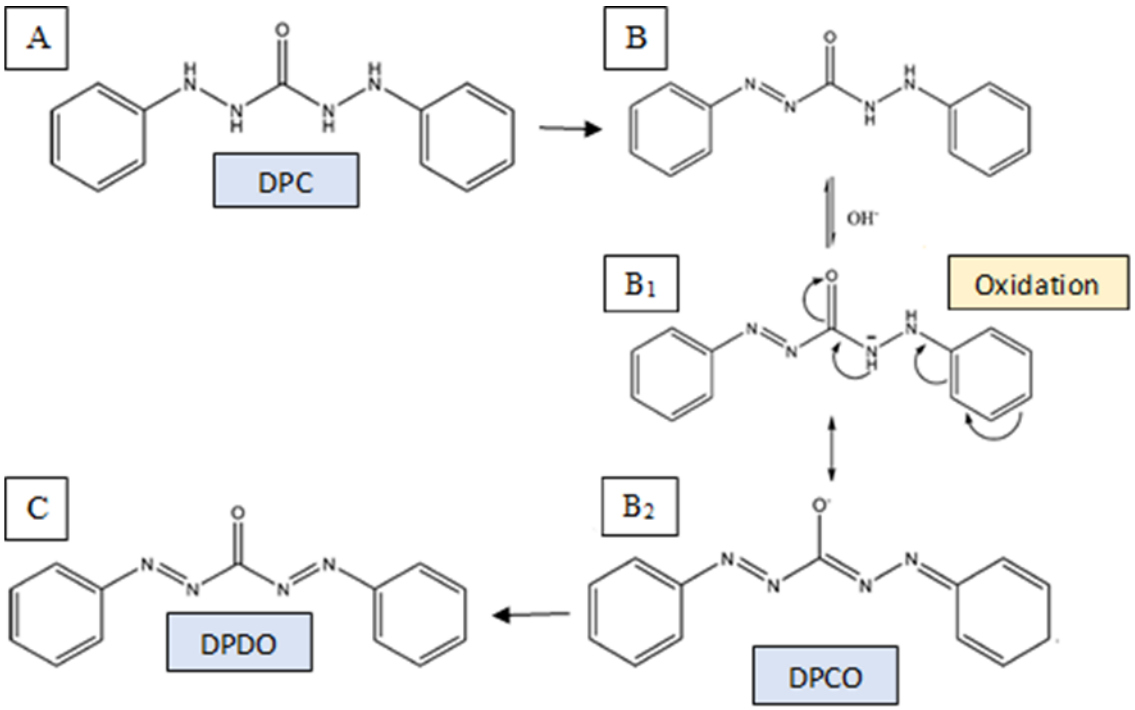

Figure 4 Oxidation reaction from DPC to DPCO (Adaptation: Crespo *et al*., 2005).

1.5 Objectives

In this work, an improved microfluidic paper – based analytical device (µPAD) for copper quantification from in saliva samples was developed based on the structure of a previously described µPAD (Silva, M. *et al* 2019).

The aim was to adapt the design, that was developed, for other features as a fast, effective method and inexpensive. This way, it was proposed to study new reagents, to obtain a device that is more sensitive to copper determination, as well the reagent consumption was the minimum volume.

Additionally, it was expected to achieve a lower value of limit of detection (LOD) in order to reach the better application of the sample on the device. Also, this diagnostic tool was developed to give results for copper determination, in hand and in moment.

2- Materials and Methods

2.1 Reagents and solutions

All solutions were prepared with analytical grade chemicals and Milli-Q water were used (resistivity > 18 MΩ/cm, Millipore, USA).

The diphenylcarbazide reagent solution (DPC) was prepared dissolving 50 mg of 1,5-Diphenylcarbazide (Sigma-Aldrich, USA) in 25 mL of ethyl alcohol (95%) to a final concentration of 2 g/L.

A stock solution of sodium hydroxide (Panreac chemical, Barcelona Spain) was prepared dissolving 16 g of NaOH in 200 mL of water to a final concentration of 2 M.

The 0.01 M NaOH buffer solution was obtained from dilution of the stock solution of 2 M and the pH adjusted to pH = 11.

Synthetic saliva was prepared according with Batista *et al* 2016, dissolving 447.3 mg of potassium chloride (KCl – Merk, Darmstadt Germany), 108.87 mg of potassium phosphate monobasic (KH_2PO_4 – Panreac chemical, Barcelona Spain), 953.2 mg of 4-(2-hydroxyethly) -1-piperazine ethanesulfonic acid (HEPES – Sigma-Aldrich, USA), 15.5 mg of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$ - Merk, Darmstadt Germany), 3.8 mg of magnesium chloride ($MgCl_2$ - Merk, Darmstadt Germany and 540.2 mg of bovine serum albumin (BSA - Sigma-Aldrich, USA) to a final volume the 200 mL of water.

A copper standard stock solution of 50 mg/L, was prepared by diluting a commercial solution with 1000 mg/L (Sigma-Aldrich, Germany) and used to prepared an intermediate solution with 1 mg/L. The copper working standards in the range 80-600 µg/L, were weekly prepared from proper dilution of the intermediate solution in synthetic saliva.

2.2 Design of the µPAD

The basic structure of the µPAD was based in the work of Silva, M. *et al* 2019. It consisted of a hydrophilic area, composed of two layers of filter paper discs, delimited by a hydrophobic area composed of laminated plastic pouch.

For the µPAD assembly (Figure 6A) 24 analysis units were aligned in a 6 columns x 4 lines arrangement samples insertion holes (3 mm) in the plastic pouch (Figure 6B -A).

Each analysis unit is composed of a layer of reagent filter paper disc (Figure 6B - B) aligned over a layer of empty filter paper disc (Figure 6B - C). To prepare the reagent layer (Figure 6B - B), 10 µL of mix reagent was loaded in the 9.50 mm diameter filter paper discs (Whatman Grade 42) and then set to dry in the oven for 10 min at 50ºC. The empty layer (Figure 6B - C) consisted of 12.7 mm filter paper discs (Whatman Grade 3).

The sample insertion holes (Figure 6B - A) on the laminating pouch(Q-Connect Glossy - 75 x 110 mm) were made using laser cutting (Laser Machine - Model 3040).

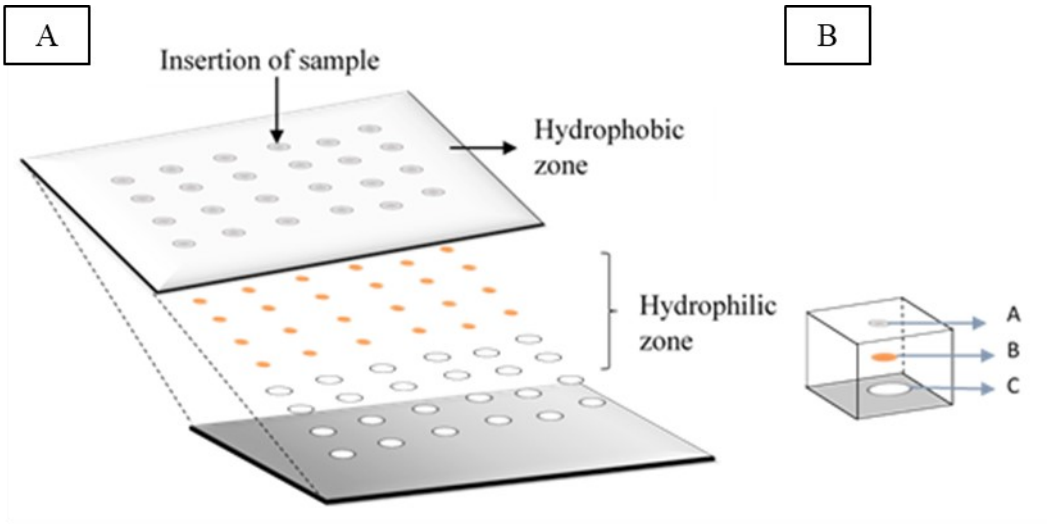

Figure 6 Schematic representation of construction of µPAD for copper determination (A) Shows the different zones. (B) Represent the different layers.

2.3 Copper determination

Once the µPAD was ready to use, 25 µL of the sample/standard was inserted inside the sample holes (Figure 7 - A). Then, after 20 min at room temperature, the µPAD was scanned for capturing the colour product formation. The 20 min between sample insertion and image acquisition intend to ensure the complete absorption of the sample/standard volume and colour product formation (Figure 7 – B).

The µPAD was scanned (Epson Stylus Sx 100) at the top layer (Figure 7-C), and the images acquired in JPEG format; afterwards image analysis was conducted (Figure 7-D).

Moreover, for each sample/standard analysed in µPAD, there were 6 analytical measurements, to remove potential outliers, and still have enough measurements to calculate the average and standard deviation of each sample/standard.

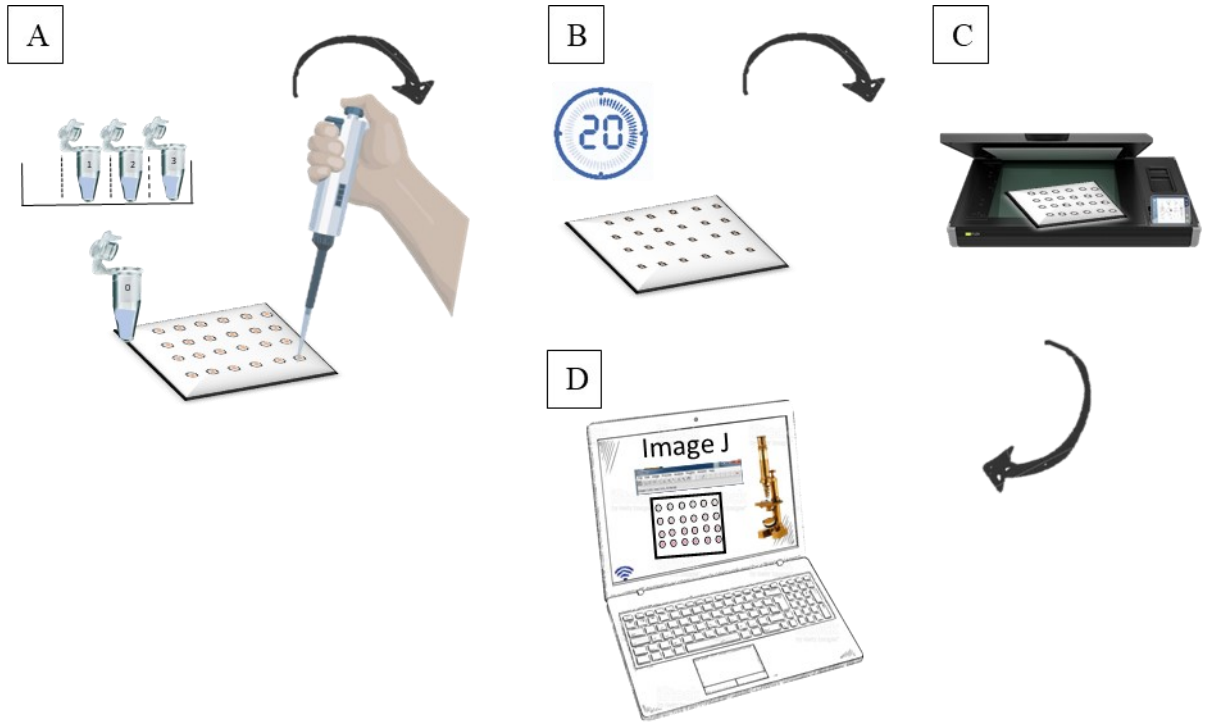

Figure 7 Schematic representation step by step of the copper determination in µPAD. (A) Insertion of sample/standard. (B) Waiting time. (C) Scanning. (D) Image analysis.

2.4 Image and data processing

The process of image analysis consists of measuring the intensity of the colour, where an intense colour corresponds to a higher concentration of copper in the samples/standard, as expected.

Therefore, the JPEG format images were analysis using Image J (National Institutes of Health, USA) by converting it into RGB images. The choice of the filter is based on the colour of the reaction product. In this case, it is the green filter was chosen because it corresponds to the complementary colour of red.

The complementary colour is the colours that have more contract between them and also, it obtained a high sensitivity, hence the chromatic circle, in the Figure 8, show the relation between different colours.

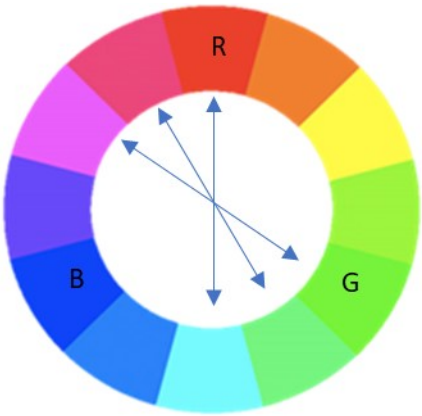

Figure 8 Chromatic circle

For each analytical measurement, a circular selection tool with 300x300 pixels was used to measure the intensity. Then the absorbance value was calculated as:

Equation 1 Beer -Lambert Law

$$A = \log_{10} \frac{I_0}{I_S}$$

Where A is the absorbance value, I_0 refers to the blank signal intensity and I_s refer to the sample signal intensity.

2.5 Saliva samples

The saliva samples used in this work were collected from healthy volunteers with their informed consent. The collection procedure consisted in placing a sterilize gauze (size 5x5 cm) in the mouth during 2 minutes. Then, the gauze was inserted in a 5 mL syringe, and squeezed with the help of the syringe plunger, to a 5 mL plastic tube.

The samples were diluted to half with synthetic saliva and then recovery assays were made adding an aliquot of copper from a 10 mg/L copper solution.

3- Results and Discussion

3.1 Selection of reagent

In a previous work (Silva, M. *et al* 2019), 1-(2-Pyridylaz)-2-naphthol (PAN), bathocuproine (BC) and dithizone (DTZ) were evaluated and BC proved to be the best sensitivity. However, aiming to further improve the sensitivity, three additional colorimetric reagents were tested: 4-(2-Pyridylazo) resorcinol (PAR), 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (Zincon) and 1,5 – Diphenylcarbazide (DPC). These reagents are not specific for copper determination and have been used for other metal ions determinations (Appendix 2 - Table1). Nevertheless, it is not supposed to be a problem because those metal ions are not expected to be in saliva. These additional reagents were compared to the previously set bathocuproina (BC) to compare the calibration curves sensitivities for copper determination in µPAD.

3.1.1 Preliminary studies – in vitro studies

Initially, studies were carried out in vitro using the spectrometer UVB- ISS-VIS (Ocean Optics) and the cuvette with 1.0 cm optical path and 3.0 mL the intern volume. Calibration curves were established for each reagent using copper standards in the range of 0.025 - 0.480 mg/L prepared in water. The conditions for the calibration curve for each reagent as well as the measurement wavelength are summarize in Table 3.

Table 3 Condition of calibration curve established for each tested reagent

Reagent	Wavelength (nm)	Condition
PAR	490	2.75 mL standard solution + 1.25 ml PAR solution + 100 µL H_3BO_3 (pH=11)
Zincon	660	1 mL standard solution + 3.5 mL Zincon solution
BC	479	2 mL standard solution + 0.5 mL BC solution + 50 µL HCO_3^- + 50 µL hydroxylamine
DPC	495	1 mL standard solution + 0.5 mL DFC solution + 0.5 mL H_3BO_3 (pH=9)

After comparing the calibration curve slope (Figure 9), PAR and DPC reagents were chosen for the further studies, since it presented a higher sensitivity for determination of copper (II).

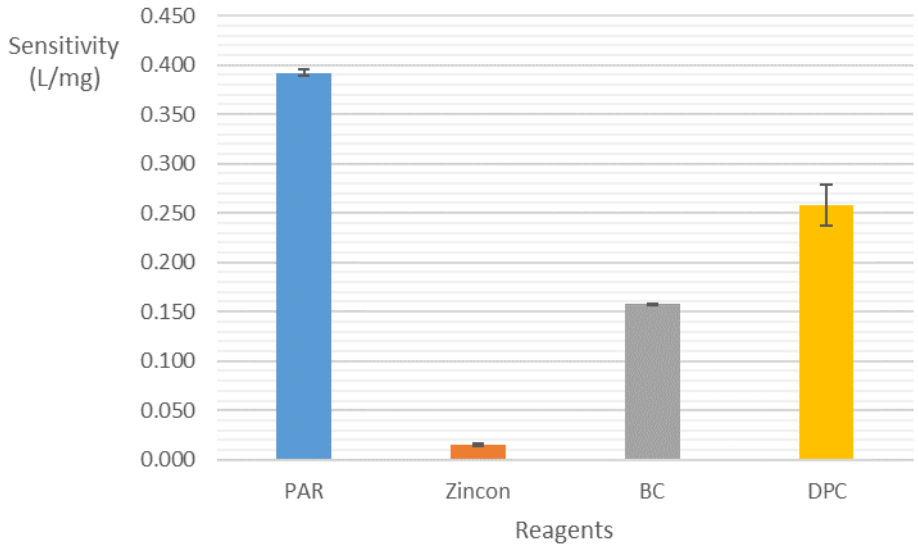

Figure 9 Comparison the sensitivity by assessing the calibration curve slope for determination copper with the different reagents studied.

3.1.2 Interferences assessment of matrix – in vitro studies

For assessment of potential interferences, standard solutions within the concentration range of 0.020 – 0.600 mg/L of copper were prepared in synthetic saliva described in materials and methods section. Additionally, different compositions of the synthetic saliva were used (Table 4).

Table 4 Different composition of the synthetic saliva used for preparing the standard solutions based on the synthetic saliva composition

Synthetic Saliva (SS)	BSA protein	Ca^{2+}	Mg^{2+}
Complete Synthetic Saliva (SS Comp)	X	X	X
Synthetic Saliva with Ca^{2+} (SS Ca)		X	
Synthetic Saliva with Mg^{2+} (SS Mg)			X
Synthetic Saliva with Ca^{2+} + Mg^{2+} (SS Ca+Mg)		X	X
Base Synthetic Saliva (SS Base)			

As both PAR and DPC were the chosen, as the reagents with higher sensibility with the standards prepared in water, they were both tested with the standards prepared in base synthetic saliva (Figure 10A).

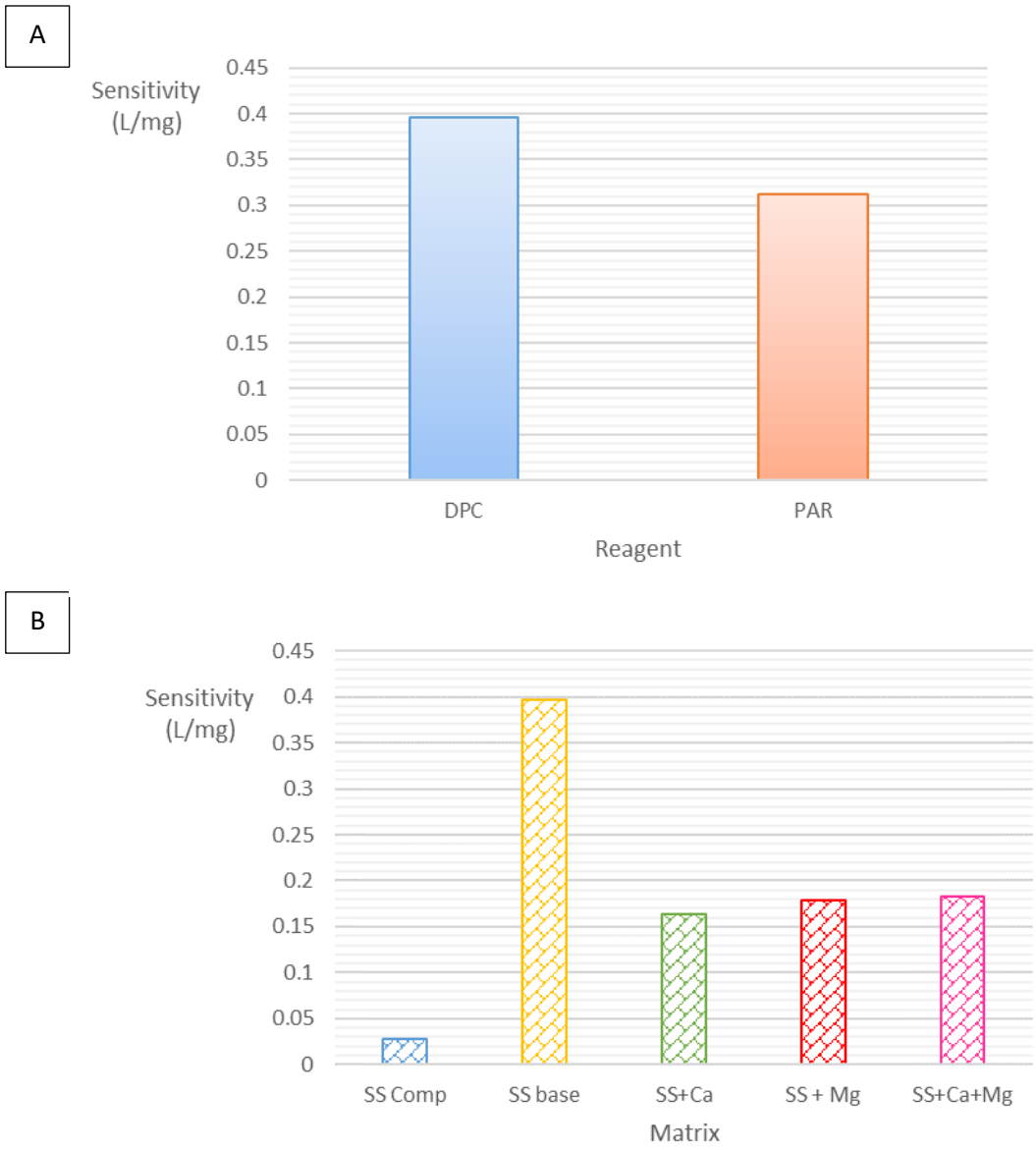

Figure 10 Comparison of the sensitivities of calibration curves for (A) different reagents; (B) different synthetic saliva matrix of standard for reagent DPC.

As DPC reagent provided a higher sensibility than PAR, it was the reagent used to test the different compositions of the synthetic saliva (Table 4) and the matrix composition proved to affect the calibration curve (Figure 10B). In order to maintain similar determination conditions for the calibration procedure and the target samples the composition chosen for the

standards was the synthetic saliva only without BSA protein (corresponding to the SS Ca+Mg in Figure 10B).

3.2 µPAD choice of RGB filter

As previously mentioned, the detection consisted in converting the colorimetric digital image to RGB images. The filter should be chosen in accordance with the colour of the reaction product, which the colour of the filter corresponds to a complementary colour (Figure 8). As the colour of reaction product is light red a study was carried out to evaluate which filter would be better the blue or the green filter (Figure 11).

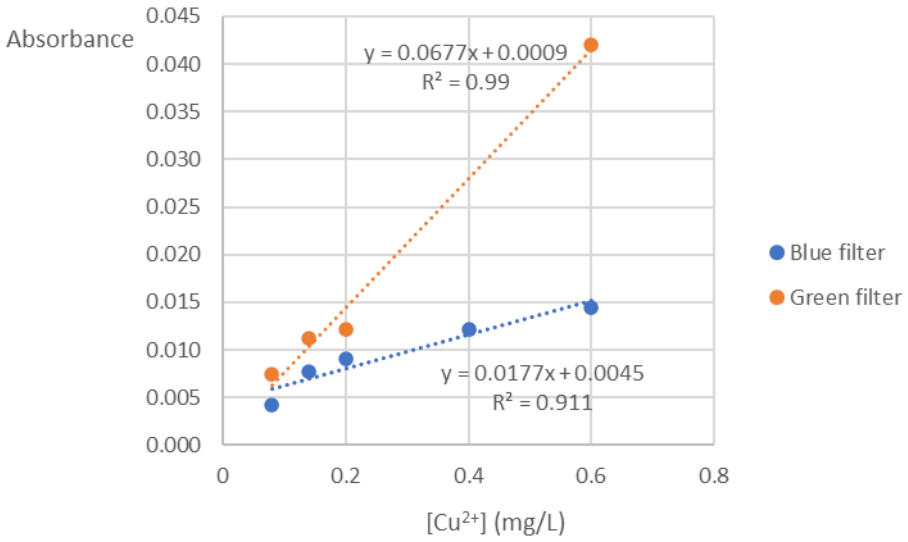

Figure 11 Study of the most appropriate RGB filter to be use in the image software in the copper determination with the develop µPAD.

The green resulted in a calibration curve with higher slope and better correlation factor so it was the chosen filter.

3.3 Study of the influence of the DPC reagent concentration

Different concentration of DPC were tested to establish the highest sensitivity with the minimal reagent consumption. Three solutions of DPC were prepared with concentrations of 0.5, 1 and 2 g/L (Figure 12).

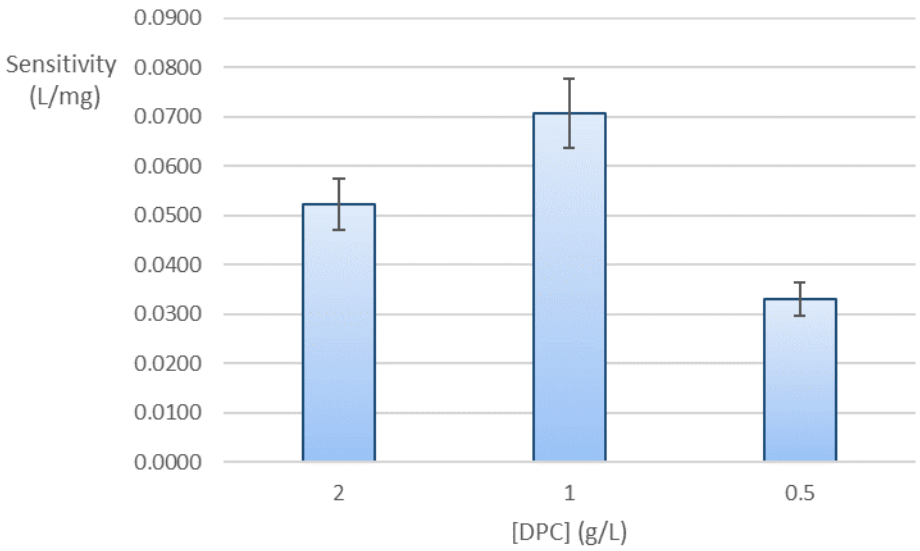

Figure 12 Influence of the concentration DPC in the calibration curve for copper determination; the error bars representation the variation range of error the ± 10%.

As the highest sensitivity was obtained with the 1 g/L, it was the chosen concentration of DPC.

3.4 Study of the influence of the hydroxide reagent concentration

Aiming for a minimal consumption of hydroxide, a concentration of 0.01 M was chosen from the tested values of 0.005, 0.01 and 0.02 as it resulted in a highest sensitivity (Figure 13).

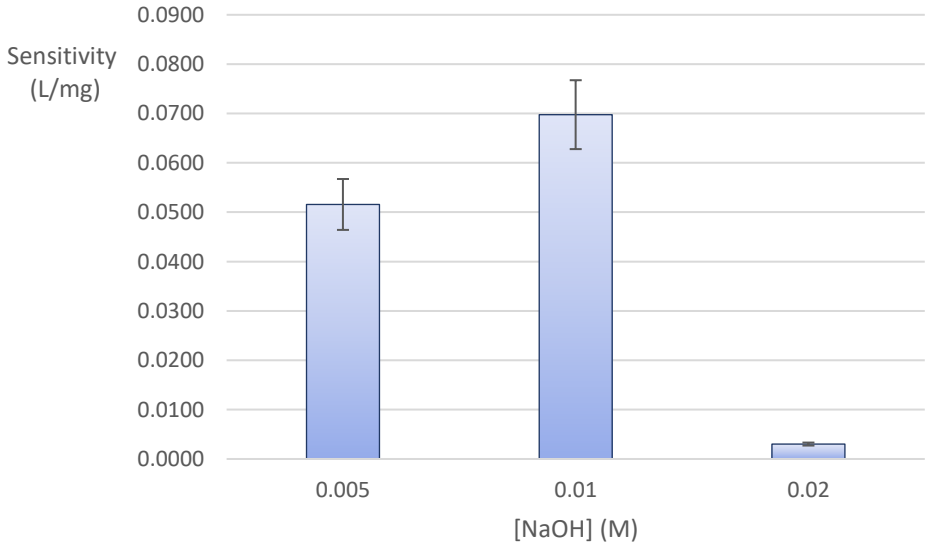

Figure 13 Influence of the concentration NaOH in determination copper; the error bars represent the ± 10% variation.

3.5 Analytical performance of the designed µPAD for copper determination

Several operational parameters were assessed to establish the main characteristics of the developed µPAD. The stability of the formed colored product indicates the time span for scanning the device after loading the sample. The stability of the device indicates how long, and in which conditions, the device can be storage prior to use. The repeatability and reproducibility were evaluated comparing the results obtained from different assemblies in the same day and in consecutive days, respectively and calculating the relative standard deviation.

3.5.1 Stability of the colour reaction product

To assess the colour product stability, the µPADs for establishing the calibration curve were prepared and scanned, for the signal detection, several times since 20 min up to 7 hours (Figure 14). The obtained slopes were compared and a 10% deviation considered to be significant difference.

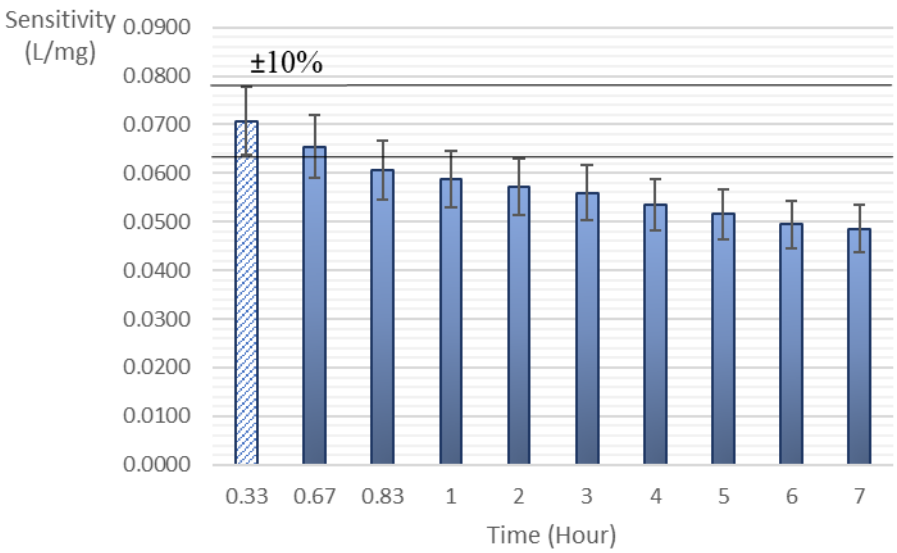

Figure 14 Stability of the colour reaction product of µPAD considering a variation range of error the ± 10%.

It was observed that the sensitivity of calibration curve decreased gradually along the 7 hours. Despite that, up to 1 hour there is an overlapping of the statistical intervals, but more reliable results are obtained when the signal registration is made until 0.67 hours (40 minutes).

This evaluation is fundamental to know, the maximum time that device can keep the results statistically significant.

3.5.2 Stability of µPAD

For this study, several µPADs were assembled and kept in two distinct atmospheric condition, air and vacuum, before sample insertion. In both conditions, the µPADs was introduced in transparent bags and for the vacuum condition the bags was sealed by pump vacuum, to remove all air inside the bags. The devices were stored protected from the light and at a dry place. The air storage condition was analysed for the next period of time, 24-hour, 48-hour, 1 week, 2 weeks, and 1 month (Figure 15). And at the same period of time that said, it was realized for vacuum condition, except the 24-hour. For each tested time period the device was used to set a calibration curve which was compared to a freshly prepared calibration curve. Therefore, an average of all the freshly prepared calibration curves was plotted (CF in the Figure 15) and a 10% variation considered.

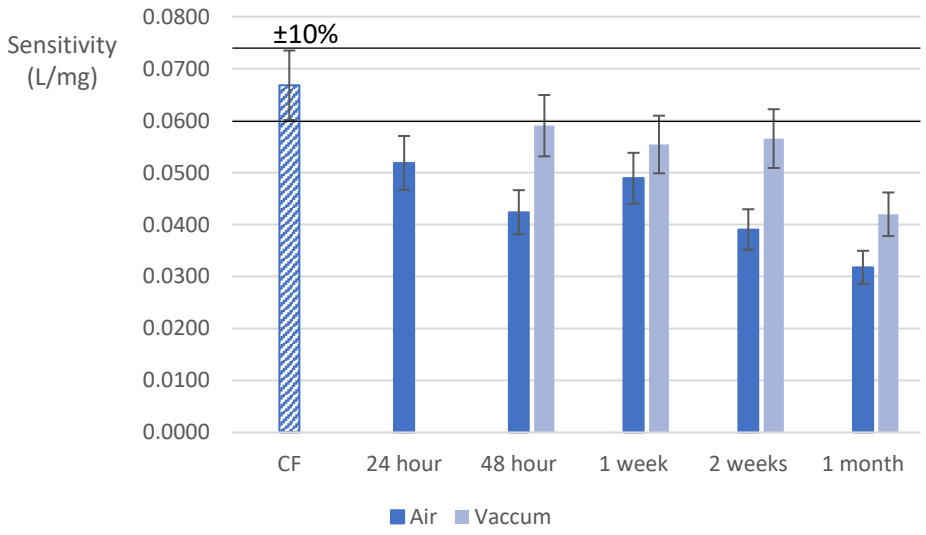

Figure 15 Stability of µPAD considering a variation range of error the ± 10%.

The storage condition of vacuum between the time 48 hours to 2 weeks, the variation of sensitivity was included within the ±10% variation of the freshly prepared average showing no statistical differences.

3.5.3 Analytical performance of copper determination in µPAD

The main characteristics of developed µPAD like, dynamic range, average calibration curve, limits of detection (LOD) and quantification (LOQ), relative standard deviation (RSD), reagent volume, sample volume, the range scanning time and the RGB image, are present in Table 5.

Table 5 Features of developed µPADs for copper determination; SD, standard deviation; R^2, Correlation coefficient linear; LOD, Limit of Detection; LOQ, Limit of Quantification

Dynamic range	0.0800-0.600 mg/L
Calibration curve* A=S ±SD x $[Cu^{2+}]$ +b ±SD R^2	A= (0.0701 ± 0.0008) $[Cu^{2+}]$ – (0.0043 ± 0.0004) 0.995 ± 0.001
RSD	0.0111
LOD*	16.9 µg/L
LOQ*	56.3 µg/L
Reagent consumption **	175 µg DFC 24 µg NaOH
Sample volume	25 µL
Scanning time	20-40 min
RGB image	Green

*number of calibration curves = 4

** per µPAD

The presented calibration curve resulted of the mean slope and intercepts of four calibration curves. The limit of detection and quantification were calculated as the concentration corresponding to three or ten times, respectively, the standard deviation of the intercept according to IUPAC (Currie *et al*., 1995).

The RSD was calculated by dividing the standard deviation of the typical calibration slope by the average slope of that calibration curve.

Reagent and buffer consumption was calculated by multiplication the final concentration of reagent or buffer by the final volume.

3.5.4 Repeatability

To assess the repeatability 4 calibration curves were established and the obtained slopes compared (Figure 16).

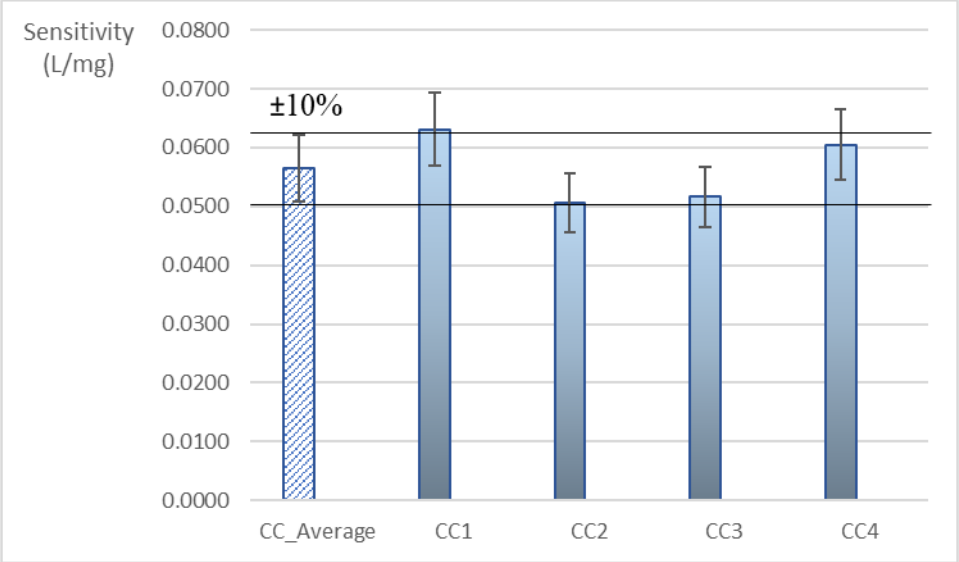

Figure 16 Sensitivity of each µPAD in same day considering a variation range of error the ± 10%.

The average of slope was calculated as reference curve (CC_average) and all the 4 calibration curves were comprised within the 10% confidence interval. The relative deviation was also calculated and a value of 8.7% obtained.

3.5.5 Reproducibility

To assess the reproducibility, four calibration curves were established in consecutive days (Figure 17). Also, the reference curve (CC_Average) was calculated, and with a standard deviation of 0.0038 and a relative standard deviation of 6% it is possible to concluded that there were not significant differences between the established calibration curves.

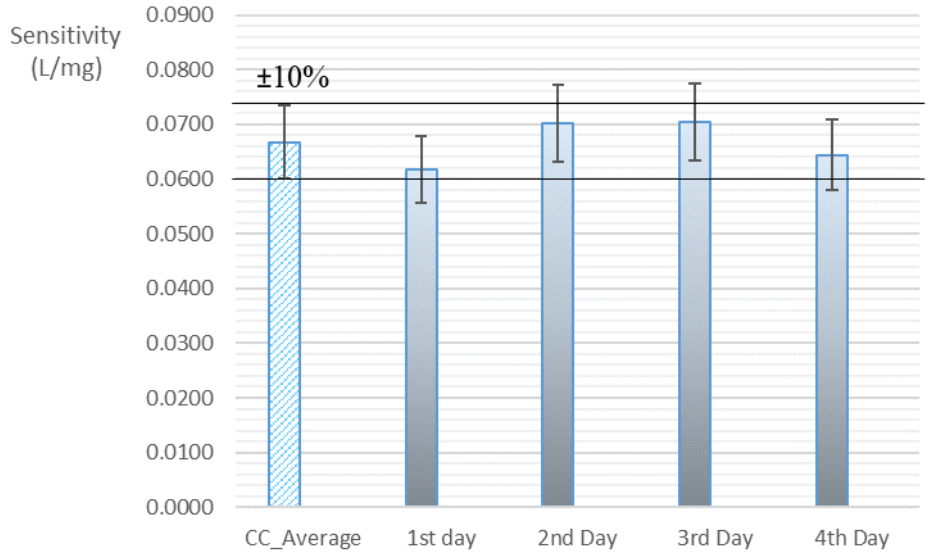

Figure 17 Sensitivity of µPAD in four days considering a variation range of error the ± 10%.

3.6 Method validation

To assess the accuracy of the developed µPAD, recovery studies were performed. The collected saliva samples were diluted to half and then 10 µL the copper standard of 10 mg/L was added to 500 µL of sample and the recovery percentages calculated, according to Burns, *et al.,* 2002 (Table 6).

Table 6 Recovery percentages calculated for copper determination in saliva samples using the µPAD; SD, Standard Deviation; RSD, Relative Standard Deviation; LOD, Limit of detection

Sample ID	Inicial			Added $[Cu^{2+}]$ [mg/L]	Found			Recovery percentage
	$[Cu^{2+}]$ [mg/L]	SD	RSD (%)		$[Cu^{2+}]$ [mg/L]	SD	RSD (%)	
S#1	<LOD			0.20	0.200	0.051	25%	102%
S#2	0.186	0.016	9%	0.20	0.340	0.067	20%	79%
S#3	0.171	0.137	38%	0.20	0.386	0.058	15%	110%

The average of recovery percentages was calculated, 99.5±4.0% and it was not significantly different from 100%.

4- Conclusion

The purpose of this work was to improve a microfluid paper-based analytical device (µPAD) for copper determination, that it was respected to designed and assembled, that it was development, and quantification in human saliva samples.

The performance of µPAD for copper determination reveals a potential tool to detect copper in saliva, that in the future can be used in remote locations or even in hospitals or emergencies center. The µPAD merge two areas, chemical and technology, that it is the colorimetric reaction and the Image J software, can provide the copper determination in hand and in moment for disease relation of copper toxicity.

Moreover, the final structure of the µPAD were result of several optimization studies. For it have well performance to used two layers of filter paper, where the top layer contains reagent. Also, the stability of colour reaction product is stable until 0.67 hour, that is 40 minutes.

In conclusion, the designed µPAD for determination of copper corresponds to the requirements proposed initially, which consisted in the determination of salivary copper. The first steps in validation, a couple of samples tested, anticipate that it is possible to make determination in saliva samples.

The development of these devices brings solution for diverse problems, such as to use small amounts of reagent and sample, easy-to-use, inexpensive and environmental-friendly because they are disposable by incineration. Besides that, the µPAD has sensitivity for measurement with few analytical material and equipment, and specialized technicians in the field.

However, the evolution in technology can transform these devices making the most efficient, in the data analysis phase, as well as improving device answer time. Given that, all analysis process can be performed by simple digital application that can be installed in mobile device, like smartphones.

Thereby, the population living in remote locations like rural or indigenous communities and with bad financial conditions don’t have access to good resources of health care. Hence, the development of devices based on saliva was more advantageous than blood protein markers due to need for conservation at low temperature and others specific condition, where the analytical infrastructure is limited.

4.1 Suggestion for future works

The μPAD of copper determination has advantageous properties to be used in the future. However, other complementary studies to these must be execute, in order to obtain a better applicability of the device.

As suggestion for future works, it would be interesting to certify the μPAD's applicability in on-location measurements. For this purpose, the stability studies should be revisited and other storing conditions tested, namely freeze/refrigerate, as it showed poor stability when stored (both air and vacuum).

Additionally, the μPAD was studied with standard in synthetic saliva, however the biologic fluid is far more complex and it would be interesting to study the influence of other ions present in saliva.

Also, for the validation process the determination of copper in saliva samples should be carried out with the developed μPAD and the results compared to an independent method namely atomic absorption spectrometry.

Likewise, to prove its applicability the developed μPAD should be employ with salivary samples from patients with pathologies associated with copper.

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Appendices

Appendix 1

Table 1 Diseases potentially detected from saliva (Chojnowska et al., 2018, Roblegg et al., 2019)

Oral squamous cell carcinoma	Ovaries cancer
	Lung cancer
	Breast cancer
	Pancreatic cancer
Autoimmunological diseases	Sjörgen's syndrome
	Celiac disease
	Hashimoto thyroiditis
Infection disease	Human immunodeficiency virus (HIV)
	Viral hepatitis
	Malaria
	Denga virus
Endocrinological diseases	Diabetes 1 and 2
	Cushing's syndrome
Diseases of gastrointestinal tract	Gastroesophageal reflux

Appendix 2

Table 1 Different application previously reported using the tested reagents.

Reagent	Analyte	Concentration of analyte	Reference
PAR	Zn^{2+}	10-40 µg/L	Ribas, T. et al 2020
	Cd^{2+}	20-2000 µg/L	Silva, E. et al 2009
	Ni^{2+}	50-2500 µg/L	
Zincon	Zn	100 – 1000 µg/L	Ribas, T. et al 2016
	Fe^{3+} Ni^{2+}	0.72 – 1.41 µg/L	Karatepe, A. et al 2011
DPC	Hg^{2+}	2 µg/mL	Zhai, Y. et al 2010
	Cr^{3+} Cr^{4+}	0.01-0.2 µg/mL	Tabani, H., et al 2020