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PORTO

# A MICROFLUIDIC PAPER-BASED SENSOR FOR IODINE MONITORING AS A PUBLIC HEALTH INDICATOR

by

Mafalda Gomes Pereira

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# A MICROFLUIDIC PAPER-BASED SENSOR FOR IODINE MONITORING AS A PUBLIC HEALTH INDICATOR

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfil the requirements of Master of Science degree in Biomedical Engineering.

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# RESUMO

Neste trabalho foi desenvolvido um novo dispositivo microfluídico baseado em papel (μPAD) para a quantificação de iodeto. Como o iodo tem um papel fundamental para o funcionamento da tiroide, torna-se um micronutriente essencial para os seres humanos, revelando-se um parâmetro fundamental para avaliar o estado de saúde. A deficiência de iodo (ID) tem contribuído para o subdesenvolvimento contínuo de milhões de pessoas, tornando-se a causa mais incidente de danos cerebrais. Se os níveis de iodo necessários para o corpo humano não forem satisfeitos, diversas doenças por deficiência de iodo podem surgir. Posto isto, a ingestão de iodo deve ser monitorizada para prevenir a ID.

O dispositivo em papel foi baseado na reação de 3,3',5,5'-tetrametilbenzidina (TMB) com $H_2O_2$, na qual o iodeto é catalisador. A reação resulta na oxidação do TMB, que passa a estar na sua forma oxidada azul.

O μPAD desenvolvido consistiu em duas camadas de discos de papel de filtro, em que a camada superior (Whatman 4) foi embebida numa mistura de ácido acético e peróxido de hidrogénio e a camada inferior (Whatman 1), impregnado com TMB. O dispositivo foi digitalizado aos 25 minutos e analisado no software ImageJ, onde a intensidade da cor formada foi convertida em valor de absorvância. Após otimização, um limite de deteção de 0,89 mg/L (7.0 μM) e um limite de quantificação de 2,8 mg/L (22 μM) foram obtidos.

Para monitorizar a ingestão/excreção de iodeto, várias amostras foram testadas, como urina, sal e suplementos alimentares. No entanto, as amostras de sal apresentaram interferências devido ao cloreto, e as amostras de urina apresentaram níveis de iodeto abaixo do limite de deteção do método desenvolvido.

Por último, este método foi validado através da comparação dos resultados obtidos para suplementos alimentares com um método e verificou-se que não havia diferenças significativas. O μPAD foi desenvolvido com sucesso, visto que permitiu uma resposta rápida para a análise de iodeto em diferentes amostras, apresentando um baixo custo, uma vez que os consumíveis de cada dispositivo custam aproximadamente 0,12 €. Assim, é possível uma determinação económica, simples e rápida de iodeto, que até agora exigia métodos complicados e morosos, e sem a necessidade de pré-tratamentos de amostras.



**Palavras-chave:** Dispositivo microfluídico em papel (μPAD); determinação de iodeto; iodo; doenças por deficiência de iodo; análises *on site*; suplementos alimentares.

## ABSTRACT

In this work, a new microfluidic paper-based analytical device (μPAD) was developed for on-site iodide quantification. As iodine is an essential micronutrient for humans, due to its fundamental role in producing the thyroid hormones, it is a key parameter to assess health condition. Iodine deficiency (ID) has contributed to continued underdevelopment for millions of people, making it the single most important and preventable cause of brain damage. In case of not fulfilling the iodine requirements for the human body, the synthesis of thyroid hormones is impaired, causing several iodine deficiency disorders. Consequently, iodine intake needs to be monitored to prevent ID.

The paper-based sensor was based on the reaction of 3,3',5,5'-tetramethylbenzidine (TMB) with $H_2O_2$, in which iodide is a catalyst. This reaction results in the oxidation of TMB, which goes from colourless to its oxidized blue colour.

The design of the μPAD consisted of two layer of filter paper discs, the top layer (Whatman 4 paper) embedded in a mixture of acetic acid and hydrogen peroxide and the bottom later (Whatman 1 paper) impregnated with TMB. The μPAD was scanned at 25 minutes and analysed in ImageJ software, where the intensity of the formed colour product was converted to absorbance values. After optimization, a limit of detection of 0.89 mg/L (7.0 μM) and a limit of quantification of 2.8 mg/L (22 μM) were attained. Different types of samples were targeted, such as urine, salt and dietary supplements, corresponding to the intake and outtake of iodide. However, the salt samples presented some interferences from chloride, and the urine samples proved to have a significantly lower iodide content than the current limits of detection of the developed method.

Lastly, this method was validated by comparing the obtained results for dietary supplements with potentiometric method and no significant differences between both methods were found. The μPAD was successfully developed, as it allowed for a rapid response for analysing iodide in different samples and still being a low-cost approach, since the consumables for each device cost approximately 0.12 €. The developed device allows for an economic, simpler and faster determination of iodide, which until now required complicated and time-consuming methods, adding the advantage of not needing to pre-treatment the samples to be used.



**Keywords:** Microfluidic paper-based analytical device (μPAD); iodide determination; iodine; iodine deficiency disorders; on-site analysis; dietary supplements.

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# CONTENTS

# 1. INTRODUCTION

## 1.1. Iodine, iodide metabolism and Iodine Deficiency Disorders

Iodine is an essential nutrient to human life, and it is naturally occurring or added to food [1]. Due to its crucial role in producing thyroid hormones, it is clear that iodine is a key parameter in assessing the public health condition [2]. However, little to no attention was paid to iodine deficiency until 50 years ago, when some studies started to be performed, regarding the supplementation of some foods with iodine and also salt iodization. The typical diseases caused by impaired function of the thyroid, prompt by insufficient iodine, are classified as Iodine Deficiency Disorders (IDD) [3]. Different kinds of these disorders can affect several age groups, with goitre and hypothyroidism being the most prevalent ones in adults. Despite the severity of the referred disorders for adults, in children the effects can be worse. The lack of iodine in this age group can contribute to impaired mental function, delayed physical development and cretinism. As for pregnant women, low levels of this analyte can cause spontaneous abortion, stillbirth and congenital anomalies [4]. Iodine deficiency is one of the most preventable causes of brain damage worldwide, being that low levels when in utero and in early childhood can lead to the loss of IQ points [5]. The potential of a whole community can be disrupted in an environment where there is not sufficient iodine, where everybody may seem slow and sleepy, with poor quality of life and even the domestic animals and livestock are affected [4]. Children from 0 to 5 years of age are recommended to have about 90 μg of daily iodine intake and from 6 to 9 years old the daily intake should be around 120 μg. As for adolescents and adults, the recommended intake is 150 μg and pregnant or lactating women should have a daily intake of 250 μg of iodine [4].

This analyte can be found essentially in the ocean, being the world's main reservoir [6]. Typically, coastal regions are much richer in iodine than inland regions. Although the soils also contain iodine, the levels are significantly lower, which means the locally raised and grown food will also have low iodine content. The problematic of iodine deficiency can be aggravating as time goes by, due to different climate scenarios, such as strong winds, floods, snow and glaciation. Iodine is usually found in the soils and the oceans as iodide [7].

Table 1.1 – Different sources of iodine, retrieved from [6].

| | |
|---|---|
| **Soil** | Sodium Iodine |
| | Sodium Periodate |
| **Seaweed** | Potassium Iodide |
| | Sodium Iodide |
| | Iodine |
| | Iodide |
| **Seawater** | Iodide |

A healthy person contains about 15 mg of iodine, the majority in the thyroid gland. The daily required levels of iodine intake are 100 to 150 µg per day, but in pregnancy, this requirement is increased to 200 µg per day. Iodine can be ingested in several forms, but it is absorbed in the stomach and the duodenum as iodide and circulates in the plasma as inorganic iodine [3], [7]. Since iodine is used in the production of thyroid hormones, it is important to understand its metabolism and how it circulates in the human body.

The iodide cycle starts with its ingestion, becoming then trapped in the thyroid. In the thyroid, it binds to tyrosine in order to form thyroglobulin, which residues will form T4 and T3 hormones, allowing the human body to regulate its energy and metabolism [8]. After this process, the hormone has done its job and is deiodinated, and the remaining iodide is reused or excreted in the kidney, which later is excreted in urine [7].

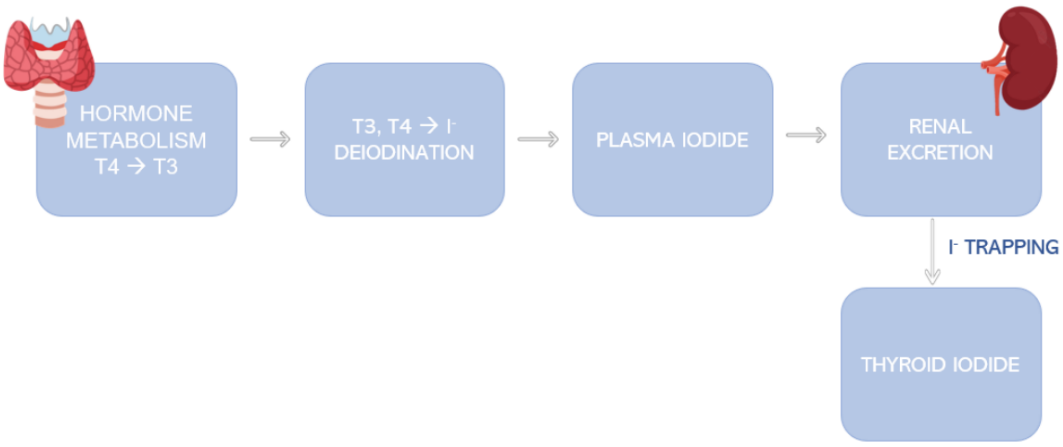


Figure 1.1 – Iodine metabolism in the human body, adapted from [7].

The iodine content in most foods is low. Foods of marine origin, seaweed, bread and milk are the major sources of iodine in a typical human diet [3]. Iodine supplementation is the most cost-effective solution to IDD. As so, in 1993, the World Health Organization (WHO), the United Nations Children's Fund (UNICEF) and the International Council for the Control of Iodine Deficiency Disorders Global Network (ICCIDD) recommended salt iodization, as an attempt to reduce and prevent the IDD. However, this tactic is dependent on policies in each country and an elevated salt intake can result in high blood pressure. It can also be associated with several cardiovascular diseases and since most of the populations already consume exceeding levels of salt, this approach might not always be viable. Despite this fortification, in pregnant women and children under two years of age, it might be necessary to introduce other types of dietary supplements, to achieve the required iodine levels [2]. Additionally, in some countries in which the IDD had been eliminated, the fortification through salt iodization programs fell apart and the IDD recurred [5]. As so, IDD is still a current problem, with about two billion individuals not having adequate iodine intake. Although a global effort is being made, a high number of European countries, about 44%, still report iodine deficiency and there is still missing up-to-date data regarding all these problems [9].

## 1.2. Iodine/Iodide Analysis

To analyse the iodine content from food, plants, soil or biological samples, different methods are used. All the methods established to this day, are relatively expensive, and not easily acquired by many laboratories [8]. One of the most known methods is Sandell-Kholtoff. However, this method requires a digestion step, where the possible intereferences from urine are eliminated, and, after that, there is still a very two-step slow reaction [10]. On top of that, the arsenic reagent used is carcinogenic and the chloridric acid can be toxic [11].

Over the years, new methods were developed, with new types of equipment and techniques that can efficiently determine iodine or iodide in different kinds of samples. In Table 1.2 there is a summary of the currently developed and used methods for iodine/iodide determination.

Table 1.2 – Some iodine and iodide analysis methods from ISI search ("iodide determination", "iodine determination", accessed in October 2023).

| Method | Iodine form | Sample | Analysis time per assay | LOD | Reference |
|---|---|---|---|---|---|
| Sandell-Kolthoff (spectrophotometry) | Iodine | Urine | 1h30 | < 25 μg/L | [12] |
| ICP-MS | Iodine | Urine | Faster than S-K | 0.95 μg/L | [13] |
| Voltammetry | Iodide | Salt | ≈ 1 min | 0.3 mg/L | [14] |
| Potentiometry | Iodide, Iodate | Urine, Salt | 2 min | $1.39\times10^{-6}$ M, $1.77\times10^{-6}$ M | [15] |
| ICP-OES | Iodine | Food | 5 min | 0.049 mg/L | [16] |
| "Fast B" method (spectrophotometry) | Iodine | Urine | Rapid test | n.a.[1] | [17] |
| Microplate method (spectrophotometry) | Iodine | Urine | ≈ 1h30 | ≈ ICP-MS | [17] |
| Chip-based spectrofluorimetry | Iodine | Salt, pharmaceuticals and algae | — | 0.028 μmol/L | [18] |
| Total reflection X-ray fluorescence | Iodine | Water, dietary supplements | < 15 min | 180 μg/L | [19] |
| HPLC (spectrophotometry) | Iodide | Urine | 13 min | — | [20] |
| μPAD (spectrophotometry) | Iodide, Iodate | Seaweed | 1 min | 9.8 μM, 0.6 μM | [21] |

[1] n.a. – non applicable due to being a semi-quantitative method

As it is possible to observe, there are several methods to determine iodine or iodide in different kinds of samples, from food to biological samples. There are differences regarding the limits of detection, being that the Sandell-Kolthoff classical method is the most time-consuming and laborious. It is also possible to conclude that there is only one method similar to the one developed in this work, emphasising the importance of this kind of test.

It is important to understand that iodate analysis was not deeply analysed due to the fact that iodate is reduced to iodide, when in the human body, becoming only present in that form. As so, contact between iodate and the body is really reduced, so it will not be detected when testing biological samples [22].

## 1.3. Microfluidic paper-based analytical device

Paper devices have been used since the 1700s, when litmus paper was used as a pH indicator [23]. It was not until 1930 that an advance was made regarding paper tests, with

spot tests being created for metal ions, establishing a pioneer of on-site analysis. The following revolution in this field was the lateral flow assays, for molecular detection [24]. However, the research field regarding paper testing became more popular in 2007, when Whitesides reported the first μPAD (microfluidic paper-based analytical device) for detecting protein and glucose in only 5 μL of urine, using photolithography as the fabrication technique [25].

Nowadays, people are becoming more self-aware and paying more attention to health in general, so it is urgent to create new diagnosis tools that are simple and effective, allowing everybody to do rapid tests, either in health, food or environmental samples [26][27]. According to the ASSURED criteria, established by WHO, diagnosis tests must be "affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and derivable to end-users" [28]. With these conditions reunited, is possible to ensure a paper test that is inexpensive, portable and can be applied to different kinds of samples. Some advantages of this type of paper device are the ability it allows for testing in remote locations, the easy usage that it permits to untrained personnel and the free-equipment factor that it provides, without needing any kind of exterior power source or reagents [25].

A μPAD is a recently developed platform, where channels are defined with millimetric dimensions, and two different zones are established, a hydrophobic and a hydrophilic zone [29]. These devices can be two or three-dimensional, already a big change from the first devices developed for lateral flow, which were one-dimensional. The 2D μPAD was presented by Whitesides, and it allowed a significantly different dynamic from the previous devices, due to its multi-direction flow. However, it was not until the 3D μPAD that the devices started to become more complex and personalised for each reaction, with different ways of being prepared, such as folding, bending, twisting, or stacking papers, in order to create microfluidic channels in between layers [30]. The paper used to create a μPAD presents itself as an advantage due to being low-cost, environmental-friendly, flexible, and biocompatible and also its white colour allows for the clear distinction of the developed colour, which is of great importance in a colorimetric reaction [27], [31]. In Figure 1.2 is possible to see more advantages of using paper for fabricating μPADs [32].


Figure 1.2 – Advantages of paper being used for fabricating µPAD, adapted from [33].

Additionally, the microfluidic paper device, as opposed to the traditional methods, allows for the incorporation of the required pretreatment or digestion of all kinds of samples, usually associated with environmental contaminants, medical diagnosis, food safety and bacteria detection [30].

Regarding the typical flow in the paper devices, the solution goes through the hydrophilic area, that is established by the paper itself, allowing for the solution to contact with a large contact area. The flow is crucial for the traditionally assembled µPAD, as it is generated by gravity, or by hydrodynamic or electrical forces, but in this way requires external equipment [30].

There are several ways for a µPAD to be fabricated. There are different techniques, that can be divided into two general categories, such as shaping or cutting the paper and patterning the different areas of the device directly on paper [32]. The different fabrication techniques are presented in Table 1.3.

Table 1.3 – Different techniques for fabricating μPADs, information retrieved from [24].

| Technique | Advantage | Disadvantage |
|---|---|---|
| Photolithography | High precision, which allows well-defined areas on the paper | Expensive and time-consuming |
| Plasma Treatment | Fewer steps that photolithography | Expensive plasma oxidizers and required mask, not compatible with organic solvents |
| Wax Printing | Low-cost, simple fabrication, rapid and robust | Not compatible with most organic solvents, poor resolution |
| Pen Plotting | One-step technique, cheaper than photolithography, compatible with different substrates | Cannot form channels with high reproducibility and resolution, not suitable for some organic solvents |
| Wax Dipping | Not complex and expensive and does not require chemical compounds | Only coats the surface so it requires a wax printer |
| Inkjet Printing | Compatible with organic compounds, reproducible | Ink cannot be replaced and some solutions cannot be kept for a long time |
| Flexographic Printing | Polystyrene used as inks does not need heat treatment and is biocompatible | Low resolution, still need further studies |
| Laser Treatment | Highly efficient and solvent-free, not necessary as many steps as inkjet printing | Expensive and limited shelf life, fabrication process is complicated |
| Stamping | Low-cost mass production, able to retain and mix the samples in the channels | Low resolution, needs a new stamp for each pattern |

Regarding the μPADs with colorimetric detection, different approaches can be used, such as pH indicators, redox indicators, formation of complexes or nanoparticles [33]. On a paper-based device, the detection process can be qualitative, providing only a yes or no response, semi-quantitative, by observing the colour gradient, or quantitative, by analysing the colour with a computer software [27]. The quantitative analysis requires the pre-established calibration curve, attained from the colorimetric signal obtained from a set of standard solutions [30].

For the image analysis, it is necessary to scan the device after the established time of reaction. This is usually done with a scanner, and the scanned side corresponds with the side of the μPAD that contains the colour reagent [34]. The JPEG image is then treated in

a software, where the RGB data is used to set different intensity values and, therefore, calibration curves [35]. To obtain the highest intensity for each colour measurement, the RGB filter chosen corresponds to the complementary colour of the developed from the reaction. Each absorbance measurement is obtained through the values obtained with the following equation, where $I_0$ is the intensity of the signal of the blank, $I_s$ is the intensity of the sample signal, and A is the absorbance obtained [36].

$$A = \log\left(\frac{I_0}{I_S}\right)$$

### 1.3.1. Colorimetric determination of iodide

Regarding the determination of iodide, only one paper [21] using a µPAD was found in the literature. However, this paper has some problematics associated, such as using a waxprinting method technology and the 3D assembly (origami), which demands the usage of the personalized holder for the smartphone-assisted determination. As so, it could be interesting to devise a µPAD with an alternative technology developed in our laboratory [29], [37]–[39] and base this method on a different reaction described by Lin *et al.* [40], in order to potentially increase the sensitivity of the determination. This reaction involved the reaction between 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide. The TMB is a colourless reagent which turned blue in the presence of hydrogen peroxide, since this reagent oxidizes TMB. Since this reaction was very slow, iodide was used as a catalyser (Figure 1.2).

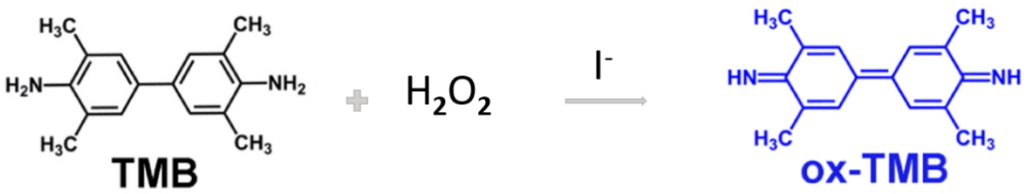


Figure 1.3 – Schematic representation of the reaction.

In this scenario, one can take advantage of iodide being a catalyst to use this reaction for the determination of iodide. This was the case in this work, in which this catalytic method was used for the production of a paper-based sensor.

## 1.4. Objectives

The goal of this work was to develop a microfluidic paper-based analytical device for quantifying iodide in different kinds of samples, based on the reaction between TMB and hydrogen peroxide. To assess both intake and outtake of iodide, the goal was to help the end-user to analyse different samples, such as urine, salt and dietary supplements, without any pretreatment, that is almost mandatory in most traditional methods.

# 2. MATERIALS AND METHODS

## 2.1. Reagents and Solutions

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

The 0.1 M iodide stock solution (Hanna HI4011-01) was used to weekly prepare intermediated iodide standard solutions of 5 mM and 0.250 mM. From the 250 μM iodide solution, the working standards within a range of 25 to 150 μM were obtained.

The 0.8 M acetic acid solution was prepared by diluting 4.6 mL of acetic acid (glacial) 100% (Merck) in 100 mL of water. Then, the pH was adjusted to 3.6, with NaOH 2.5 M, as reported by Lin *et al.* [40].

The hydrogen peroxide solution was prepared by diluting 13 mL of 8M hydrogen peroxide (Merck) and completed to 20.00 mL.

A daily mixture of acetic acid and hydrogen peroxide was prepared, combining 240 μL of acetic acid and 10 μL of hydrogen peroxide.

The 3,3',5,5'-tetramethylbenzidine (TMB) solution was prepared by dissolving 24 mg of TMB (Sigma-Aldrich) in 50 mL of 96% ethanol (Labchem) and then adding 50 mL of water, resulting in a 1 mM concentration, as described by Palladino *et al.* [41]. This solution was stored in a dark bottle and kept in the fridge.

A silica suspension was prepared for every 2 μPADs, by mixing 15 mg of the powder in 5 mL of water.

The synthetic urine used was prepared with the following concentrations: 10 g/L urea, 0.07 g/L uric acid, 0.8 g/L creatinine, 5.2 g/L sodium chloride, 0.1 g/L lactic acid, 0.4 g/L citric acid, 0.37 g/L calcium chloride dihydrate, 0.49 g/L magnesium sulfate heptahydrate, 1.41 g/L sodium sulfate, 0.95 g/L potassium dihydrogen phosphate, 1.2 g/L potassium hydrogen phosphate and 0.49 g/L glucose, as reported by Ferreira *et al.* [38].

A $KNO_3$ solution worked as an ionic strength adjustor (ISA), prepared by dissolving 40 g of the solid in 200 mL of water, resulting in a concentration of 2M, which was then diluted to a final concentration of 0.4M (ISA WS).

## 2.2. Design and assembly of the μPAD

The assembled μPAD consists of establishing a hydrophilic detection area, for the reaction to occur and a hydrophobic area to separate the independent detection areas. To set the hydrophobic area, a 75 x 110 x 0.125 mm plastic laminating pouch (Q-Connect) was perforated with twenty-four 3 mm holes for sample insertion, establishing 6 columns and 4 rows arrangement. The hydrophilic area consists of twenty-four colour reading units established with the overlapping of two filter paper discs aligned with the holes of the plastic laminating pouches.

For the described iodide determination, two different types of Whatman filter paper were used and filter paper discs of 9.5 mm of diameter were obtained using a paper puncher (3/8" EK tools).

The top layer (TL) consists of Whatman Grade 4 (W4) filter paper discs, which were embedded in a silica suspension solution for 30s with manual agitation, and then placed in the oven to dry for 30 minutes at 50 ºC. After that, these discs were loaded with 10 μL of the mixture of acetic acid and hydrogen peroxide, and were set to dry in the oven, at 50ºC for 10 minutes.

The colour reagent layer, the bottom layer (BL), was prepared by loading 10 μL of TMB and was left to dry at room temperature for 20 minutes.

Following these procedures, the two-disc units were aligned with the sample hole into the plastic pouch and passed through the laminator (A3-330C High Quality Laminator), to seal the pouch and properly establish the twenty-four reading units with the two distinctive hydrophobic and hydrophilic areas.

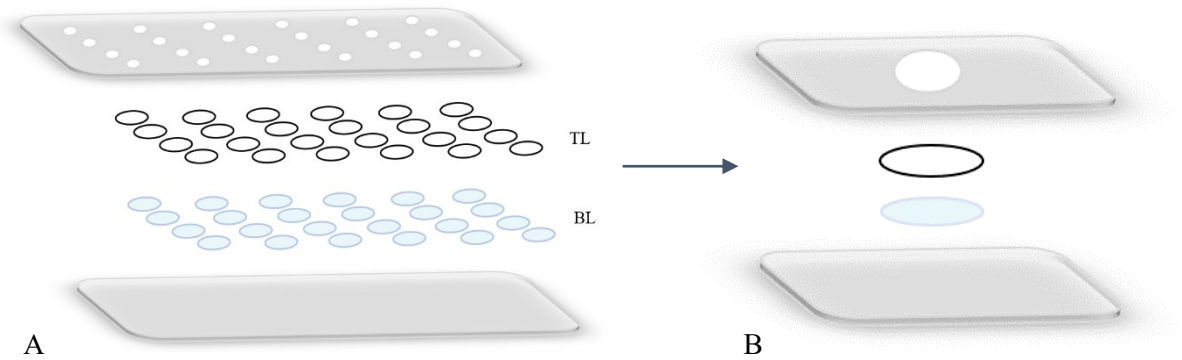


Figure 2.1 - μPAD alignment (A). BL: Bottom Layer; TL: Top Layer. Reading unit (B).

## 2.3. Iodide Determination and Data Processing

To determine the iodide concentration, 20 µL of the standard solutions, or the samples, was inserted through the previously cut-out 3 mm holes of the top sheet of the laminating pouch. Then, after waiting 25 minutes for the colour to develop, the µPAD was scanned (EPSON Stylus SX100) on the colour reagent side, bottom sheet of the laminating pouch, to analyse the colour intensity of each reading unit. In fact, according to Figure 2.1, the sample is inserted on the top layer (TL) and the scan is made on the opposite side, corresponding to the bottom layer (BL), the layer with the TMB reagent.

To measure the colour intensity, a free image processing software was used (ImageJ). This software allows to obtain the intensity value by using RGB filters (red, green and blue). In this specific case, given that the developed colour was blue, the red filter was used as the complementary colour of the formed product.

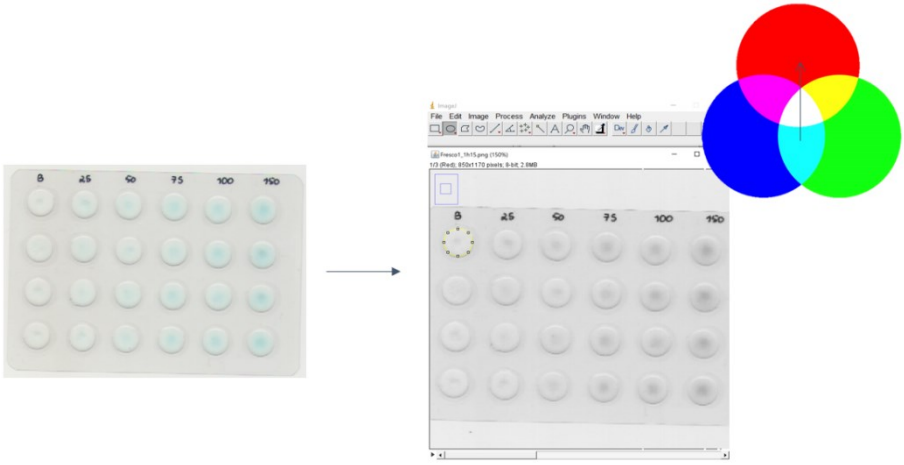


Figure 2.2 – ImageJ analysis of the scanned µPAD, with the selected RGB filter.

After obtaining the intensity value for each disc in the device, the data was imported into Microsoft Excel and the absorbance calculated, using the formula:

$$A = \log\left(\frac{I_0}{I}\right)$$

where A is the absorbance value, I is the mean measured intensity of the standard or sample (#4 replicas), and $I_0$ is the mean measured intensity of the blank (#4 replicas).

Then, a calibration curve set the correlation between the calculated absorbance values and the standard solution concentration. This allows the quantification of iodide present in samples, by analysing the given colour intensity and interpolating the calculated absorbance in the established calibration curve equation.

## 2.4. Sample Preparation

To prepare the salt samples, 1 g of each salt was dissolved in 15 mL of water.

The dietary supplements, including vitamin pills or powders, which implied pre-treatment for each one, as described in Table 2.1.

Table 2.1 - Samples tested (mass, dilution and filter process).

| Sample Type | m (g) | Volume (mL) | Sample ID | Spiked [$I^-$] (μM) | Filtering process & filter type |
|---|---|---|---|---|---|
| Pharmaceutical Y200 | 0.0608 | 5 | #Pharm 1 | - | Vacuum Nylon 0.45 μm |
| | | | #Pharm 2 | 50 | |
| | | | #Pharm 3 | 100 | |
| Pharmaceutical Y300 | 0.0923 | 5 | #Pharm 4 | - | Vacuum Nylon 0.45 μm |
| | | | #Pharm 5 | 50 | |
| | | | #Pharm 6 | 100 | |
| Pharmaceutical | 0.1105 | 5 | #Pharm 7 | 100 | Vacuum Filter 595 (7μm) |
| | 0.1677 | 5 | #Pharm 8 | 100 | Vacuum Filter 595 (7μm) |
| Vitamins KELP | 0.1914 | 5 | #Supplement 1 | - | Vacuum (2x) Nylon 0.45 μm |
| | | | #Supplement 2 | 50 | |
| Dried Seaweed | 1.0970 | 50 | #Algae 1 | 50 | Syringe Nylon 0.45 μm |
| | | | #Algae 2 | 100 | |
| Certified Sea Lettuce | 1.003 | 25 | #RM 1 | 50 | Syringe (3x) Nylon 0.45 μm |
| | | | #RM 2 | 100 | |
| Certified Hay Powder | 1.003 | 25 | #RM 3 | 50 | Syringe Nylon 0.45 μm |

## 2.5. Comparison Method

To validate the developed method, a comparison was made between the paper device and the Ion Selective Electrode method. For performing the potentiometric method, standard solutions (in the range of $1\times10^{-5}$ to $5\times10^{-3}$ M) were prepared and the analyte concentrations were calculated by interpolation in the corresponding calibration curve: $E= f (\log [I^-])$. For each reading, 5 mL of standard or sample were mixed with 5 mL of the ionic strength adjustor (ISA WS).

# 3. RESULTS AND DISCUSSION

## 3.1. Preliminary studies

### 3.1.1. Conventional batch measurements

For the development of the microfluidic paper device, it was necessary to assess the dynamic concentration range obtained with the chosen reaction as well as the reaction parameters, so a study was carried out in a batch-wise approach. The initially chosen protocol was based on the one developed by Lin *et al.* [40], where 880 µL of $CH_3COOH$, 10 µL of $H_2O_2$, 10 µL of $I^-$ and 100 µL of TMB, were mixed respectively, and then placed at 37ºC for 5 minutes (Figure 3.1). This procedure was done with a range of standard solutions, with concentrations between 50 µM and 1 mM.

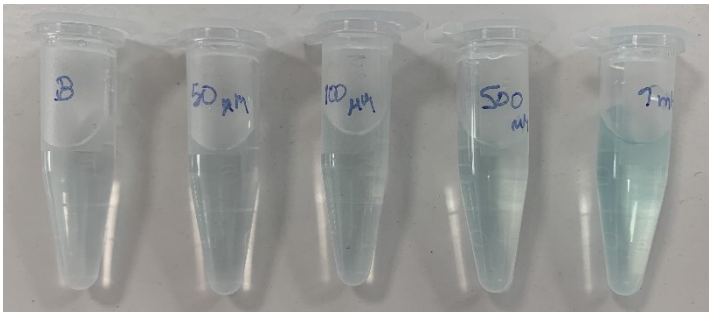


Figure 3.1 – Developed colour of the reaction, after 5 minutes of incubation.

Mainly because of the prolonged reaction time necessary for the colour development, a waiting time of 30 minutes was chosen before the absorbance readings for each standard solution, resulting in the absorbance spectrum corresponding to Figure 3.2.

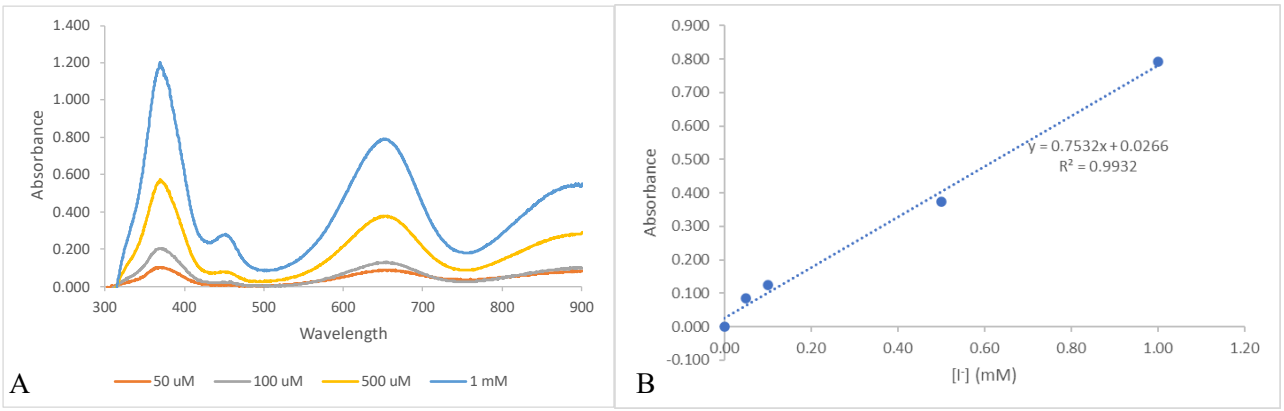


Figure 3.2 - Absorbance spectrum after 30 minutes of reaction time (A) and calibration curve (B), obtained with the absorbance values at 615 nm.

In this study, other reaction times were also studied, but 30 minutes showed a higher sensitivity if compared to other waiting periods (such as 20 minutes, for example).

When transferring this process to a paper-based platform, it was necessary to understand that each layer usually corresponds to a different reagent, which in this case

would result in a three-layer arrangement. This could lead to a problem of inefficient mixture and an increase of analysis time. So, in this case, because several reagents are necessary for the colour reaction, a mixture of acetic acid and hydrogen peroxide was previously prepared to be used in the same layer, therefore resulting in a two-layer device. An additional challenge of using the chosen reaction on paper was the incubation step, which was not feasible for the paper device. Another issue to account for was the different order of the reagents that was required due to the µPAD structure. In batch conditions, the order of reagents being added was acetic acid, hydrogen peroxide, iodide and TMB. However, in the paper platform, iodide would be the last reagent being added to the reaction, since the determination of iodide in different samples was the focus of the work. In this context, studies were conducted, by establishing calibration curves in different conditions, to study the influence the mixture of the two referred reagents, the elimination of the incubation step and iodide being the final reagent, as shown in Figure 3.3.

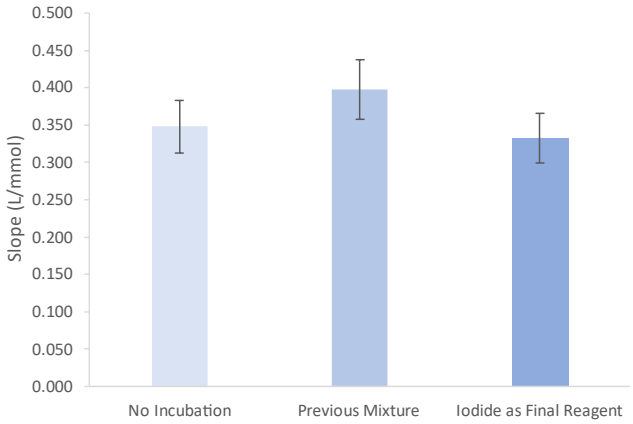


Figure 3.3 – Sensitivities (slope of the calibration curves) obtained under different conditions of the batch studies. The error bars represent 5% deviation.

As it was possible to observe, none of the different conditions showed a significant difference in sensitivity, so the chosen process was: no need for incubation, the acetic acid and hydrogen peroxide mixed previously and iodide placed as the final reagent.

### 3.1.2. Measurements in the µPAD platform

The following step consisted of testing the previously settings in a paper-based approach.

As mentioned, although the iodide quantification with the chosen reaction involved three reagents, two could be previously mixed, so it was necessary to have two paper layers in the device. The mixture was prepared with the acetic acid and hydrogen peroxide

and would correspond to the top layer of the μPAD. The bottom layer consisted of TMB reagent. This way, the reaction order was kept as similar as possible to the batch studies, since a vertical flow was promoted after the insertion of the sample, as pictured in Figure 3.4.

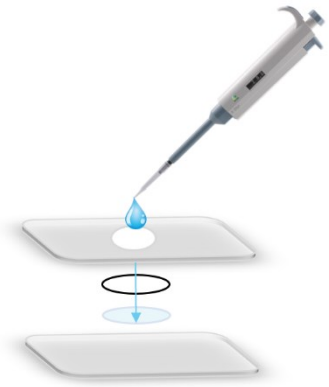

Figure 3.4 – Vertical flow promoted by sample insertion, in a reading unit.

Some adaptations of the batch-wise reaction studies had to be made, namely the reduction of TMB volume, due to the limited capacity of the paper disc, which cannot hold 100 μL of reagent, so 10 μL was used.

Because in the μPAD assembly the filter paper discs must be dry, so that they can absorb the sample/standards, the drying process of the discs after loading the reagents was an essential parameter to be assessed. So, the oven temperature, where the discs were put to dry, was also tested, between 37ºC for 15 minutes, and 50ºC for 10 minutes. In the end, the highest temperature showed improvement regarding the slope of the calibration curve, thus this option proved to be the best, and also time-saving.

Before moving along to deeper studies regarding the reaction and the paper itself, it was crucial to understand if the device could also measure iodate instead of only iodide. For that, iodate standards were prepared and tested on the μPAD. The results regarding this study showed that the device showed no sensitivity for iodate. Therefore, it could be concluded that this μPAD would not measure iodate.

In the development of a μPAD, studies usually consist of assessing the influence of the number of layers, sample volume and the amount of reagent.

### 3.1.3. Standards solutions stability

A fundamental component in an analytical procedure is the standard solutions, which play a crucial role as they are used to establish the calibration curve, and the concentration in the sample is calculated by interpolation in that curve. Due to this, a study was conducted to understand the stability of these solutions and how frequently it was necessary to prepare new standards.

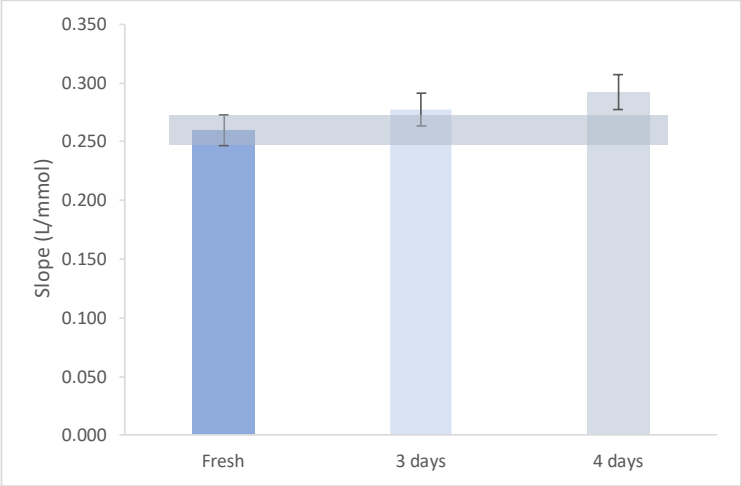


Figure 3.5 – Comparison of the slope of the calibration curves (sensitivity), for the different day standard solutions. The shadow area represents 5% deviation for the fresh standard solution.

Observing Figure 3.5, it was possible to conclude that no sensitivity was lost during a week. No further studies were performed regarding the longer duration of the solutions. For that reason, new standard solutions were prepared every week.

## 3.2. Type of paper

As a result of the preliminary studies, a primary design of a two-layer μPAD was established. Initially, the filter paper used was the same for both layers, consisting of 9.5 mm diameter discs of Whatman 1 qualitive filter paper. The top layer disc was loaded with 10 μL of the mixture of $CH_3COOH$ and $H_2O_2$, and the bottom layer disc with 10 μL of TMB.

Firstly, the top layer paper type influence in the iodide calibration curve was studied, maintaining the bottom layer with the same conditions as before. Calibration curves in the range of 25-150 μM of iodide were established using different filter papers and the one producing the highest calibration curve slope was chosen, as corresponding to the highest sensitivity. Filter papers with different pore sizes were tested: 20-25 μm

(Whatman 4, W4), 10-20 µm (Whatman 1, W1) and 2.5-5 µm (Whatman 5, 42, 50 and 542, W5, W42, W50, W542). Additionally, filter paper with different paper treatment was also studied, where there was qualitative (Whatman 1, 4 and 5), ashless (Whatman 42), hardened (Whatman 50) and hardened-ashless (Whatman 542). The tests were performed using different sample volumes, namely 10 and 15 µL (Figure 3.6).

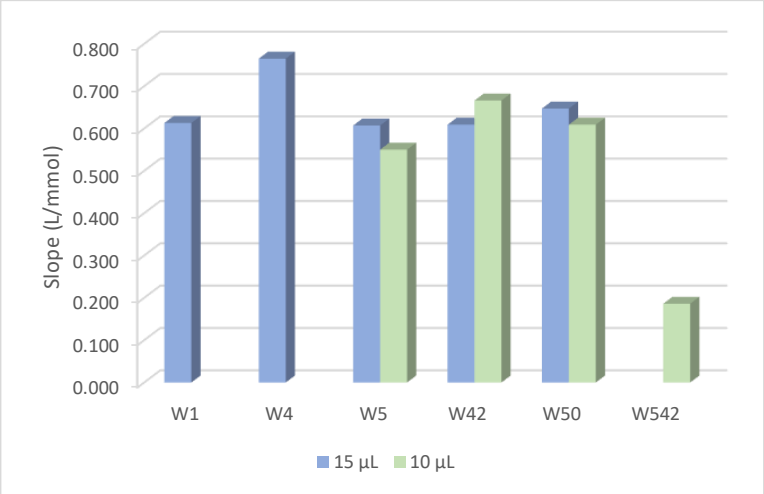


Figure 3.6 – Comparison of the slope of the calibration curve (sensitivity) using different filter papers for the top layer.

It was possible to conclude that the Whatman 4 filter paper resulted in a higher sensitivity, possibly due to the bigger pore size and the possibility it allowed for the sample to flow to the bottom layer.

Because the Whatman 4 was the chosen paper for the top layer, the bigger pore size allowed to use a larger sample volume. Therefore, the bottom layer studies were performed using 15 µL of sample volume.

As before, different pore sizes and paper treatments were tested for the bottom layer (Figure 3.7). Because this was the layer that was scanned and analysed, two different scanning times were used, to guarantee that the reaction was given enough time to develop a sufficient colour intensity. The paper that provided the highest slope of the calibration curve was Whatman 1 paper, for both scanning times, so this filter paper was chosen.

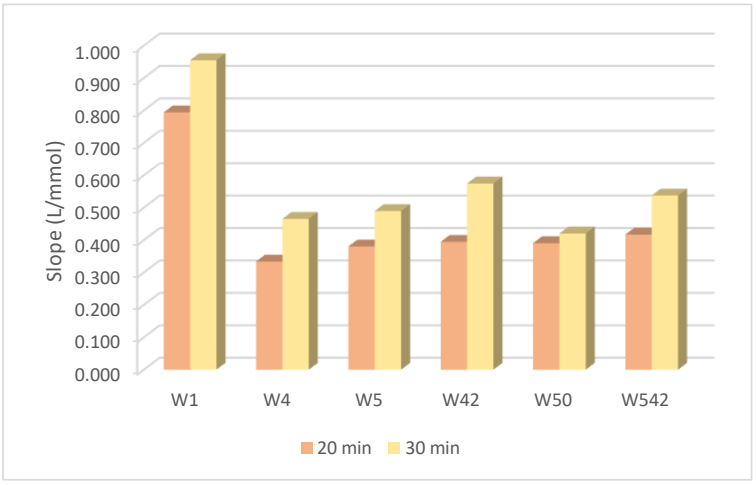


Figure 3.7 - Comparison of the slope of the calibration curve (sensitivity), using different filter papers for the bottom layer.

## 3.3. Colorimetric reaction

### 3.3.1. Influence of TMB concentration

All the studies previously carried out in the μPAD were based on the concentrations and reagents preparation described in the method developed by Lin *et al.* [40]. However, as the reaction on a paper platform may show a different behaviour when compared with the reaction in-vitro, the concentration of the TMB in the reaction sensitivity was analysed (Figure 3.8). To do so, three concentrations were tested, maintaining the rest of the reagents the same as before.

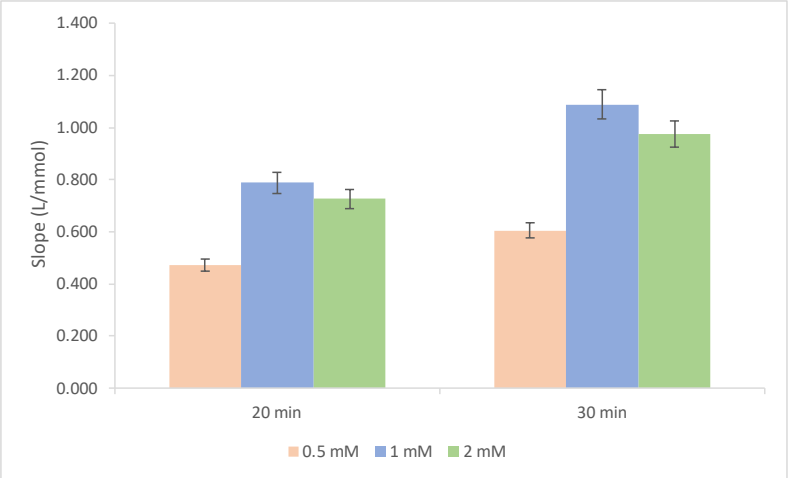


Figure 3.8 – Comparison of the slope of the calibration curves (sensitivity), for the different TMB concentrations. The shadow area represents 5% deviation of the 1 mM concentration.

As can be observed in Figure 3.8, the 1 mM TMB concentration produced a higher slope, with no significant differences between 1 mM and 2 mM at 20 minutes. Consequently, the chosen concentration was 1 mM because it showed greater sensitivity, for both times analysed.

### 3.3.2. Influence of peroxide concentration

To study the influence of the other reagents involved in the reaction, namely the mixture of hydrogen peroxide in acetic acid, the amount of peroxide added (5, 10 and 20 µL) to the acetic acid (final volume 250 µL) was varied, resulting in different final concentrations (Figure 3.9). Considering that the volume of acetic acid was quite similar, its concentration was considered to maintain 0.8 M.

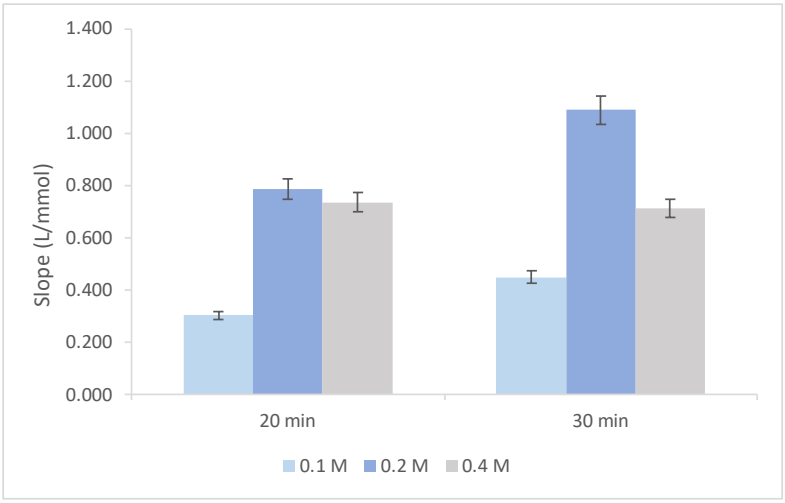


Figure 3.9 – Comparison of the slope of the calibration curves (sensitivity), for different concentrations of hydrogen peroxide. The error bars represent 5% deviation.

The proportion originally used was 10 µL of hydrogen peroxide and 240 µL of acetic acid corresponding to a peroxide concentration of 0.2 M. As shown in Figure 3.9, it can be concluded that this concentration showed the greater slope, so, it was chosen for the final mixture to be used in the µPAD.

### 3.3.3. Detection conditions

The developed colour of the reaction could range from blue to purple when using different concentrations of iodide, so it became necessary to evaluate what RGB filter was the best to be used in the image processing software. The filter should be chosen according to the complementary colour of the formed product (Figure 3.10). The

intensities of the different standards were calculated using all RGB filters, namely red, green and blue, and the resulted calibration curve compared (Figure 3.11).

Figure 3.10 – Wheel of complementary colours, used to choose the appropriate filter.

The obtained colour for the lower standards was light blue, and its complementary colour was red, so the red filter would be the appropriate choice.

However, when the iodide concentration was higher than 200 mM, the colour started to be more purple, so the green filter should be chosen. Despite that fact, as illustrated in Figure 3.11, the standards with concentration below 0.150 mM, could not be distinguished when using this filter.

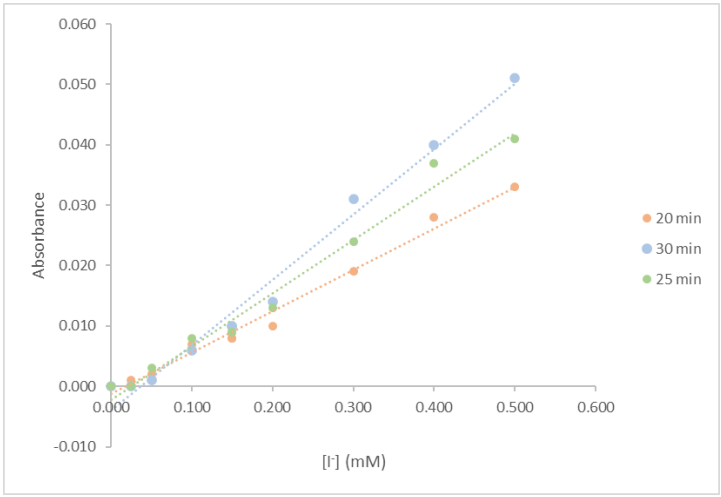


Figure 3.11 – Comparison of the calibration curves, using the green filter, with different waiting times.

For that reason, the red filter was the chosen filter for the image processing step, since it allowed for a better sensitivity and linearity of the calibration curves, as well as the possibility of determining lower concentrations of iodide.

At this point, scanning the top or the bottom layer of the μPAD was also tested. This variation involved changing the order the layers were stacked, as well as the scanning side of the device and establishing calibration curves for each tested option. The schematic representation of this testing is detailed in Figure 3.12, together with the obtained results.

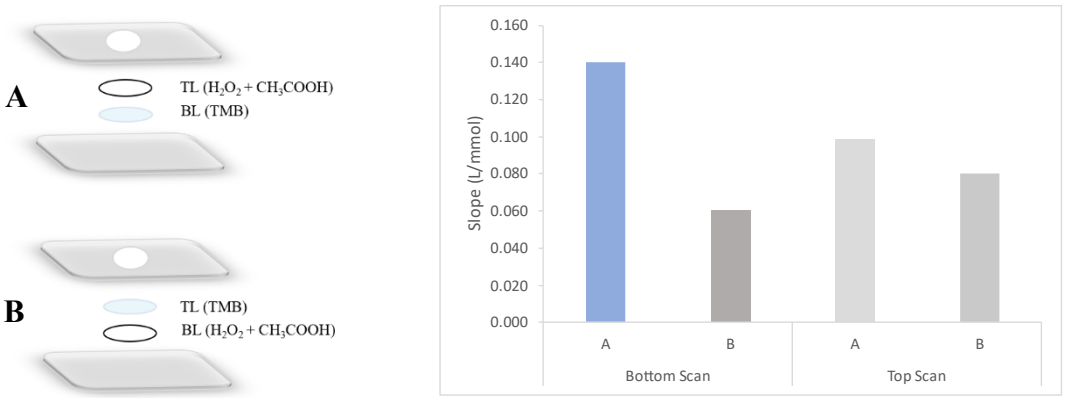


Figure 3.12 – Comparison of the slope of the calibration curves (sensitivity), when changing the order of layers and scanning side.

As can be observed, the original layout of the device (A) with the colour reagent in the bottom layer and scanning at the bottom, was the combination that allowed for a higher sensitivity, so no changes were made.

## 3.4. Sample volume

The influence of the standard or sample volume was studied to assess which volume would result in a higher sensitivity. To do so, volumes of 15, 20 and 25 μL were used in the preset design to establish calibration curves. For each volume, a scan was made at 20, 25 and 30 minutes, so it would also be possible to understand if higher volumes needed more time (Figure 3.13).

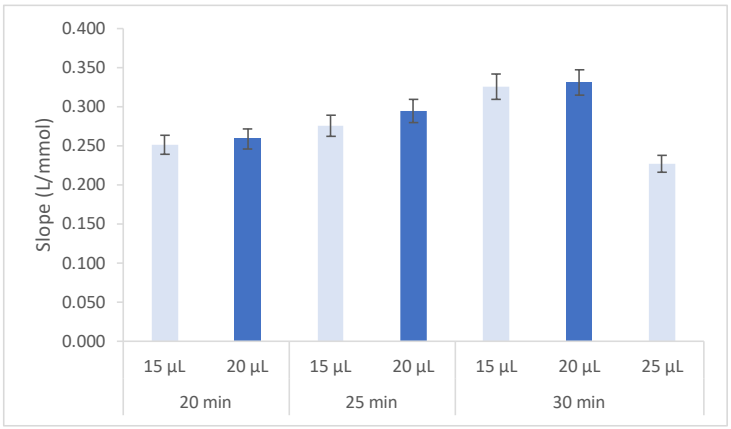


Figure 3.13 – Comparison of the slope of the calibration curves (sensitivity), for different volumes of sample/standard. The error bars represent 5% deviation for 20 µL, after 25 minutes.

From Figure 3.13, it was possible to conclude that 20 µL was the volume that allowed the device to be more sensitive. Although there was a difference between 25 and 30 minutes (about 12%), in both scanning times the 20 µL resulted in the highest slope.

## 3.5. Time to scan

The previous study showed some differences in the calibration curve slope when using different scanning times, so it was crucial to assess the time interval necessary before scanning the device (time to scan). From the in-vitro studies, it was possible to conclude that 30 minutes was the best time to wait before doing the reading of the absorbance. However, because the dynamic of the reaction in the paper is different, several waiting times were studied, going from 10 minutes to 30 minutes.

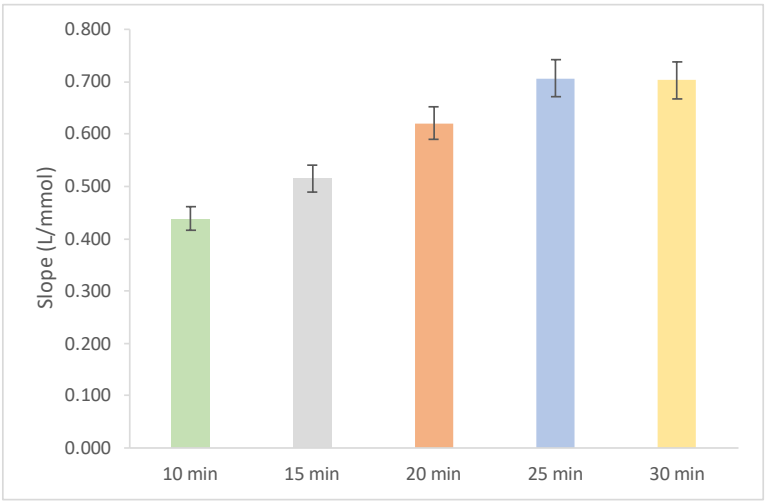


Figure 3.14 – Comparison of the slope of the calibration curves (sensitivity) for the different waiting times before scanning the device. The error bars represent 5% deviation.

Observing Figure 3.14, it was possible to conclude that the best sensitivity was obtained at 25 and 30 minutes. Since there were no significant differences, 25 minutes was to be chosen and, because one of the main features of a μPAD is the rapid response it allows, the 25-minute mark was chosen.

## 3.6. Use of silica to improve measurement repeatability

Since the coloured product formed lacked some colour uniformity, an additional study was performed, based on the method described by Evans *et al.* [42] that reported the use of silica nanoparticles in a microfluidic device to improve the colour intensity and uniformity. To test this hypothesis, calibration curves were established using devices without silica particles and devices with silica particles placed on the top layer. In this way, silica particles proved to increase not only the absorbance signal, but also the calibration curve slope (Figure 3.15). As seen in Figure 3.15, the slope of the calibration curve increased by about 30%, which means the sensitivity of the μPAD improved significantly.

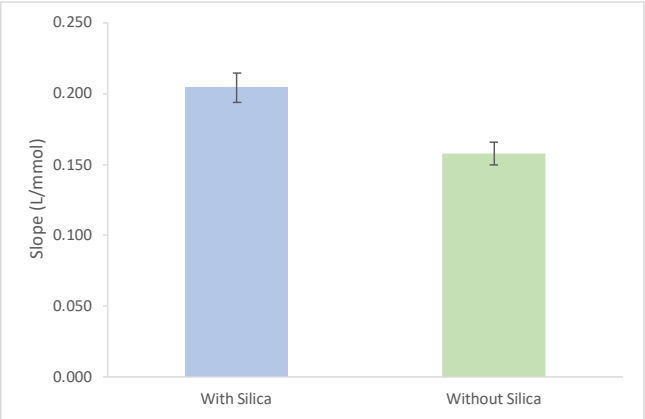


Figure 3.15 – Comparison of the slope of the calibration curves (sensitivity), with silica and without silica. The error bars represent 5% deviation.

With that being said, the chosen μPAD was the one prepared with silica powder in the top layer.

## 3.7. Influence of pH

Although the optimum pH reported for this reaction is 3.6 [40], a pH study was performed, where four different pH values for the acetic acid solution were studied: 1.13, 2.3, 3.6 and 4.75 (Figure 3.16). It was possible to conclude that the pH 3.6 was, in fact, the best pH providing the highest slope of the calibration curve, and, consequently, was the one that allowed a greater sensitivity for the device, so this was the set pH.

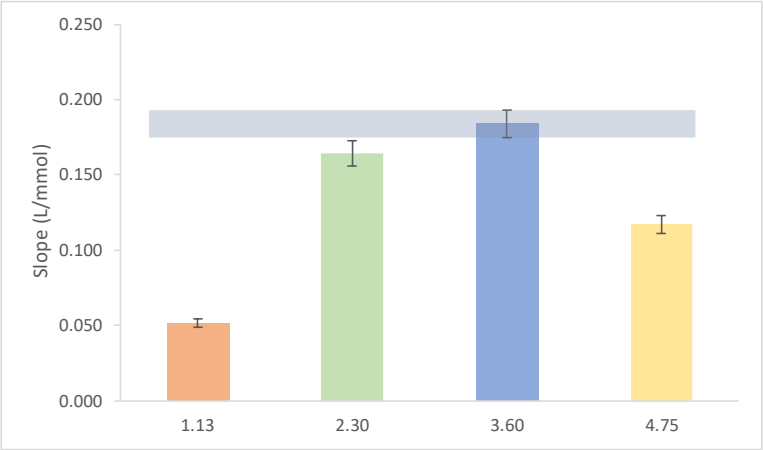


Figure 3.16 – Comparison of the slope of the calibration curves (sensitivity), for the different pH values. The shadow area represents 5% deviation for the 3.6 pH.

## 3.8. Features of the method

Having established the operation conditions for the determination of iodide in a microfluidic device, the features of the developed µPAD were summarized in the Table 3.1.

Table 3.1 - Features of the develop µPAD for the colorimetric determination of iodide.

| | |
|---|---|
| **Dynamic Range (µM)** | 25 – 150 |
| **Typical Calibration Curve [a]**<br>**(A = slope ± SD × [Iodide] mM + intercept ± SD)** | 0.188 ± 0.008 × [Iodide] + 0.070 ± 0.004 |
| **LOD (µM)** | 7.0 |
| **LOQ (µM)** | 22 |
| **Repeatability Intraday [a]** | 29% |
| **Repeatability Interday [a]** | 13% |
| **Time to scan** | 25 minutes |
| **Reagent Consumption / µPAD** | • Silica Powder: 15 mg<br>• $H_2O_2$: 1.7 mg<br>• $CH_3COOH$: 11 mg<br>• TMB: 58 mg |

[a] calibration curve slope: n= 5

The LOD and LOQ were calculated as the concentration corresponding to three and ten times, respectively, the standard deviation of the calibration curve intercept (n=5) following IUPAC recommendations [43].

The interday and intraday repeatability was assessed by calculating the relative standard deviation (RSD) of the standard deviation of the calibration curve slope (n=5).

The reagent consumption per device was calculated accounting for 24 reading units and the reagent volume and concentration necessary for preparing that number of units.

### 3.8.1. Colour product stability

After inserting the standard or the sample in the µPAD, it was possible to obtain a colour reaction product and it was important to assess how long it was stable for. So, in order to study its stability, different scans were performed up to 90 minutes and the sensitivities for each scanning time were compared, as observed in Figure 3.17.

The sensitivity increased over time and, although the chosen reading time for the developed µPAD of 25 minutes, represented in blue, was less sensitive, it still enabled good results. Because one of the main focus of a microfluidic paper device is the rapid response it allows, this reaction time proved itself quite effective as a compromise choice. Nevertheless, if it would not be possible to scan the device in those 25 minutes, these results show that the device will not lose sensitivity, at least until 90 minutes after inserting the sample.

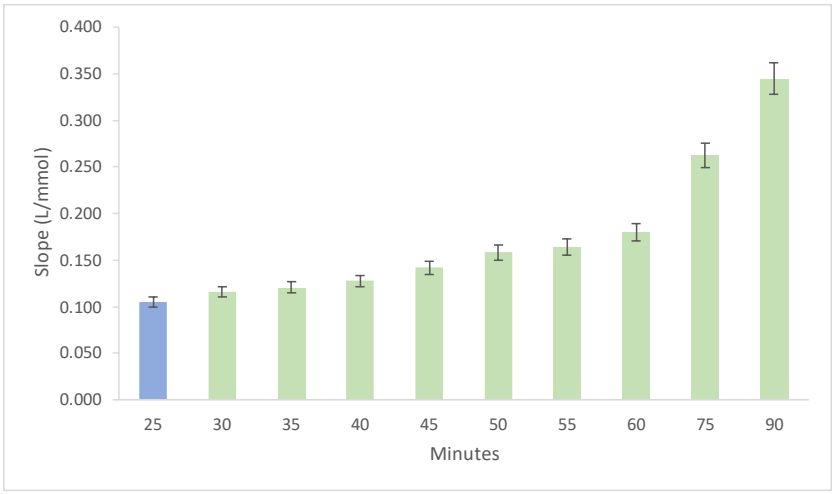


Figure 3.17 – Comparison of the slope of the calibration curves (sensitivity), along 90 minutes of reaction time. The error bars represent 5% deviation.

### 3.8.2. µPAD stability

In addition to knowing the reaction product stability, it was also crucial to understand the stability of the device itself, before introducing the standard/sample. So, it was studied for how long it could be stored prior to its use and still obtaining a good sensitivity. Different conditions were tested, such as temperature, which varied between room temperature and refrigerator temperature (approximately 4ºC), and vacuum or the presence of air. In all these conditions, the µPAD was protected from light, since they were all wrapped in aluminium foil. The periods of time studied were 1 day, 2 days and 1 week. After that time, the standards were inserted, as normally would be, and the obtained results were compared with a µPAD freshly prepared on that day, to see if there were significant differences in sensitivity.

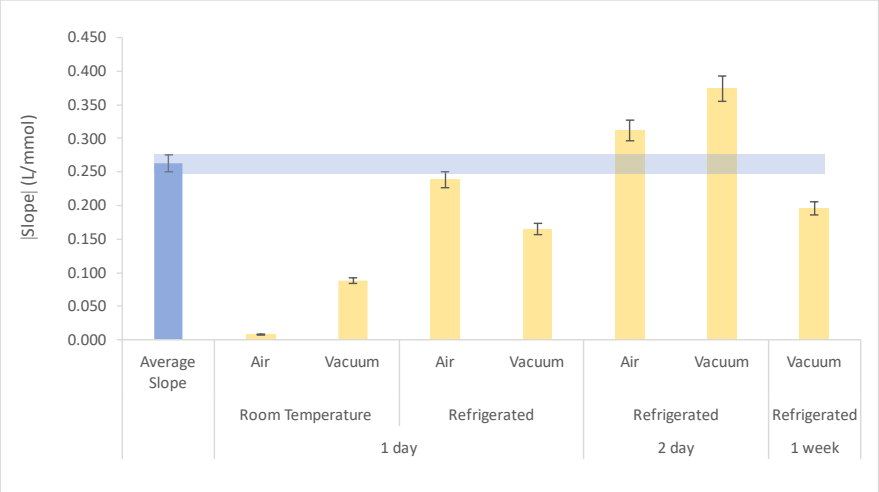


Figure 3.18 – Comparison of the slope of the calibration curves (sensitivity) for the µPAD stability. The shadow area represents 5% deviation from the average slope.

When stored at room temperature, even just for one day, it was no longer possible to obtain a calibration curve. Although when storing the µPAD in the refrigerator, it was possible to establish calibration curves up to one week, the results did not seem consistent. In fact, some of the calibration curve slopes were significantly lower than the one freshly prepared µPAD, while others were significantly higher.

## 3.9. Application to samples – matrix interferences

After the optimization studies, the following step consisted of applying the µPAD to the initially target samples: urine, salt and dietary supplements. However, before

introducing the sample directly into the device, it was necessary to evaluate the potential interferences from the different targeted matrixes display.

### 3.9.1. Urine samples

To see if the urine matrix would affect the sensitivity, a study was conducted, comparing calibration curves when using standards prepared in water and standards prepared in synthetic urine. The results, as shown in Figure 3.19, prove that there were no significant differences, as the slope presents only a 9% decrease, so it could be concluded that the synthetic urine matrix had no interference in the reaction.

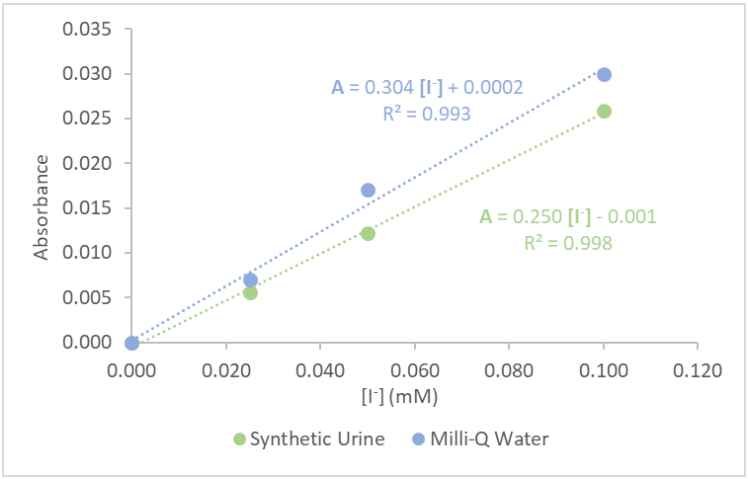


Figure 3.19 – Calibration curves for synthetic urine and water.

Since the iodide levels in urine are usually quite low and the lower standard of the calibration curve was 25 µM, the possibility of performing standard addition within the device was considered. To this aim, a study was performed to understand the difference it might have existed, if a third layer was added in the µPAD. This extra layer would be advantageous, since it would allow for the addition of an already known amount of iodide.

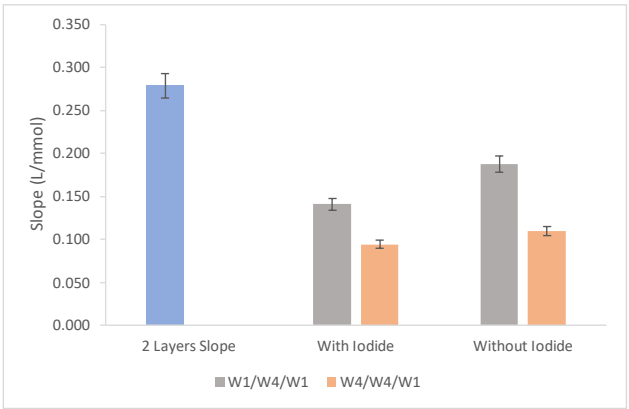


Figure 3.20 – Comparison of the slope of the calibration curves (sensitivity) adding a third layer, with and without iodide. The error bars represent 5% deviation.

As seen in Figure 3.20, it was possible to understand that adding a third layer decreased significantly the sensitivity of the μPAD. Additionally, when adding iodide in the third layer, the loss of sensitivity was even more significant, proving that this hypothesis was not advantageous for the quantification of iodide in urine. So, because the urinary iodide levels are very low, it was possible to conclude that, with this developed method, it would not be possible to determine iodide in urine.

### 3.9.2. Salt samples

One of the main sources of iodine intake for many people is iodized salt. As so, it was of great importance to understand if this matrix could interfere with the reaction.

The first salt samples introduced in the μPAD resulted in a different colour product than the usually observed with the iodide standards, as seen in the comparison in Figure 3.21.

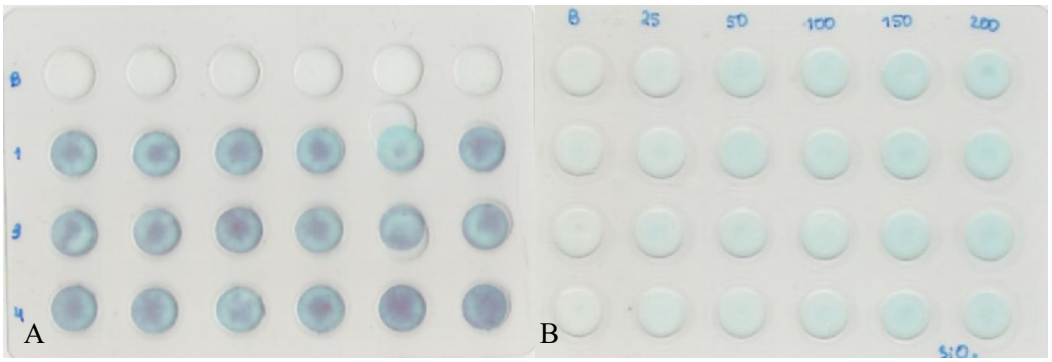


Figure 3.21 – Comparing the developed colour of a salt sample (A) and a standard solution (B) μPAD.

It was possible to conclude that this reaction was also sensitive to chloride and not just iodide. Therefore, since salt is in its majority sodium chloride, it became necessary to evaluate the chloride concentration the salt solutions. So, a titration was made, and it was possible to conclude that the typically prepared salt solution had about 3.4 M of chloride.

That being said, standard solutions of sodium chloride were prepared, ranging from 0.0005 M to 3.4 M, in order to understand this interference in the reaction and in the μPAD. The results are in Figure 3.22.

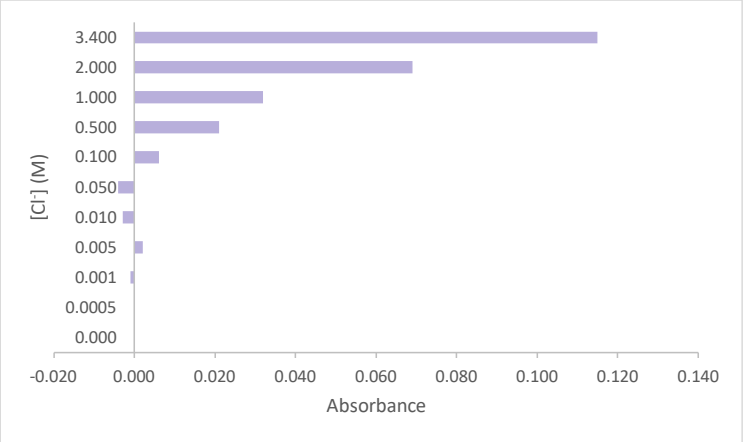


Figure 3.22 – Calibration curve for chloride standard solutions.

After observing the obtained results, it was clear that chloride concentrations below 0.100 M cannot be quantified by the μPAD. However, concentrations above that value were quantified and, because of that, it interfered with the iodide determination in salt samples, since each of them has chloride concentrations of about 3 M. One simple solution for this problem would be the dilution of the salt solutions. Nevertheless, because this would have to be, at least, a solution with ten times less concentration, the iodide concentration would also be very reduced and would not be possible to quantify in the μPAD.

In a similar way than with the urine samples, an hypothesis would be to add a masking agent (for example silver nitrate) and a third layer would possibly have some advantages. In this context, it would allow to add other solutions containing agents that could complex with chloride, and the reaction with iodide would still occur. However, when adding the third layer, there was linearity loss and the sensitivity of the μPADs significantly decreased. Still, an attempt of using $AgNO_3$ was made. The results of this study did not show any linearity or greater sensitivity than the originally developed μPAD, with two layers. Due to all these results not working the way it was hoped to work, the salt samples were dismissed.

### 3.9.3. Dietary supplements samples

Dietary supplements are also a source of iodide intake, so samples were prepared to insert in the μPAD. As they can be easily obtained, pharmaceutical pills and vitamins can supply the required levels of iodide for the human body. In addition to these kinds of supplements, there are also some edible algae that were tested for their iodide content.

## 3.10. Accuracy assessment – dietary supplement samples

Given that, at this point, all the optimization studies were conducted, and all the features were established, its accuracy assessment was performed.

The results validation was obtained by analysing several supplement samples in the μPAD and the potentiometric reference method using an ion selective electrode (ISE). The iodide content was determined in each sample, and the relative deviation between both methods was calculated ($[Iodide]_{\mu PAD}$ and $[Iodide]_{ISE}$). The obtained results are in Table 3.2.

Table 3.2 - Comparison of the results obtained with the developed μPAD and the reference method (ISE) by calculating the relative deviation percentage (%RD); SD, standard deviation.

| Sample ID | $[Iodide]_{ISE}$ ± SD (μM) | $[Iodide]_{\mu PAD}$ ± SD (μM) | % RD |
|---|---|---|---|
| #Pharm 1 | 24.4 ± 0.5 | 26.2 ± 5.7 | 7.4 |
| #Pharm 2 | 73.2 ± 3.3 | 70.6 ± 29.5 | -3.6 |
| #Pharm 3 | 118 ± 1 | 106.2 ± 36.5 | -9.9 |
| #Pharm 4 | 32.2 ± 0.9 | 35.1 ± 8.7 | 9.0 |
| #Pharm 5 | 75.1 ± 3.3 | 71.2 ± 7.7 | -5.2 |
| #Pharm 6 | 138 ± 1 | 158.9 ± 41.8 | 15.1 |
| #Pharm 7 | 182 ± 1 | 168 ± 56 | -7.3 |
| #Pharm 8 | 203 ± 1 | 206 ± 27 | 1.1 |
| #Supplement 1 | 23.5 ± 0.5 | 26.2 ± 7.8 | 11.5 |
| #Supplement 2 | 72.2 ± 1.6 | 68.6 ± 5.9 | -5.0 |
| #Algae 1 | 44.2 ± 1.0 | 42.1 ± 3.1 | -4.8 |
| #Algae 2 | 95.9 ± 2.1 | 84.6 ± 21.4 | -11.8 |
| #RM 1 | 42.1 ± 3.9 | 37.8 ± 10.6 | -10.2 |
| #RM 2 | 45.3 ± 1.0 | 41.7 ± 7.5 | -7.9 |
| #RM 3 | 93.5 ± 3.6 | 86.1 ± 8.7 | -7.9 |

From this comparison with the ISE method, it was possible to conclude that a range of iodide can be determined in these samples, going from 23.5 to 203 μM. A linear relationship was still established, and the following equation was obtained, represented in Figure 3.23.

$$[\text{Iodide}]_{\mu PAD} = 0.991 \pm 0.089 \times [\text{Iodide}]_{ISE} - 1.429 \pm 8.878$$

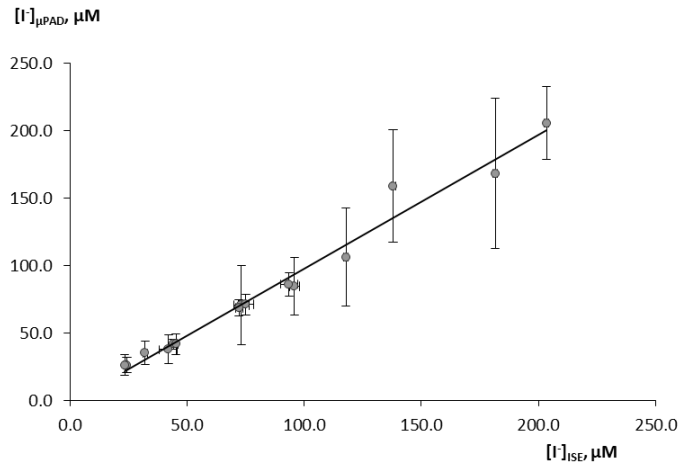


Figure 3.23 - Linear relationship between the iodide levels in the supplements analysed in the developed µPAD ([Iodide]µPAD) and the reference method ([Iodide]ISE)

These results made it possible to conclude that that there were no differences between the two methods, as the obtained slope was not statistically different from 1 and the intercept is close to 0, with a 95% confidence interval. Thus, there is no evidence for significant differences between the two set of results [44].

## 3.11. Cost analysis

In order to develop a rapid response device but also low-cost, a simple economic analysis was performed, to understand the evolving cost of assembling the device.

Each µPAD consisted of 24 detection units that worked as replicas, so there were 24 discs of Whatman 4 filter paper aligned with 24 discs of Whatman 1 paper. As already mentioned, in each layer there were different reagents, consisting of 240 µL of the mixture of acetic acid and hydrogen peroxide, and 240 µL of TMB, which were taken into account when analysing the final cost. Additionally, a plastic laminating pouch was used. The respective price of the µPAD is presented in Table 3.3.

Table 3.3 – Price of consumables used in the developed µPAD and total price per unit.

| Whatman 4 | Acetic Acid + Hydrogen Peroxide | Whatman 1 | TMB | Plastic Pouch | Total |
|---|---|---|---|---|---|
| 0.040 € | 0.008 € | 0.022 € | 0.005 € | 0.046 € | ≈ 0.12 € |

# 4. CONCLUSIONS AND FUTURE WORK

In conclusion, the developed µPAD proved to be an innovative analytical platform iodide determination for in-site analysis.

The chosen colorimetric reaction between 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide, with iodide as a catalyst, enabled a dynamic range from 25 µM to 150 µM and the limit of detection and quantification were 7.0 and 22 µM, respectively.

The designed µPAD consisted of 24 reading units, composed by two layers of filter paper (9.5 mm of diameter). Once the standard/sample is introduced, it flows through the top layer, containing a mixture of acetic acid and hydrogen peroxide, to the bottom layer containing the colour reagent TMB. This procedure enabled an accurate iodide determination, with a time to scan of 25 minutes, which was applied to various samples. The targeted samples were chosen based on the idea of monitoring iodide levels, through the intake and outtake, as a health indicator. A recognized iodine status parameter is the urinary iodine content, so it was the first sample to be analysed but showed levels below the limit of detection. As for the dietary intake of iodide, two samples were tested, namely salt and dietary supplements. Due to chloride interference, only the latter were in fact analysed. The method was validated by comparison with a potentiometric determination, with a iodide selective electrode, and no significant differences between methods.

This method improves on simplicity when compared to other iodine quantification procedures, together with a low-cost production in terms of consumables, rounding 0.12€ per device.

The µPAD, as a rapid test, follows the ASSURED guidelines provided by the World Health Organization, as it is inexpensive, user-friendly, rapid, robust, environmental friendly and easily disposable. In this way, this device could be used in developing countries, where the access to health is more difficult and not affordable, providing a good alternative to in-site diagnosis and analysis. In order to assess this possibility, the µPAD stability was evaluated under different storage and temperature conditions. Apparently, when refrigerated (4 ºC), the µPAD is stable up to one week. However, these studies were not conclusive.

As future work, it would be really interesting to improve the µPAD in order to apply to urine samples. The stability of the device also needs to be re-evaluated, to be able to be used in remote locations that cannot have access to other diagnosis tools. As for salt,

as a relevant tool for supplementing iodine worldwide, a different reaction would have to be implemented.

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