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Design and development of a Microfluific Paper-based Analytical Device (μPAD) for magnesium determination in saliva

by

Juliana Isabel Soares Aguiar

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Design and development of a Microfluific Paper-based Analytical Device (μPAD) for magnesium determination in saliva

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science level in Biomedical Engineering

by

Juliana Isabel Soares Aguiar

Place: CBQF – Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Supervision: Raquel B. R. Mesquita

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

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RESUMO

De todos os catiões presentes no corpo, o magnésio é o segundo mais importante catião e o quarto mais prevalente. Doenças que envolvem o magnésio são classificadas em dois grupos: hipomagnesemia (défice de magnésio) e hipermagnesemia. Desta forma, a determinação de magnésio despertou grande interesse, porque auxilia no contexto clínico e em pesquisas epidemiológicas.

Portanto, o objetivo deste trabalho foi desenvolver um dispositivo microfluídico analítico em papel (μPAD) para quantificar magnésio em amostras salivares.

Neste caso em concreto, o μPAD baseia-se numa reação colorimétrica entre o magnésio e o eriocrómio de cianina, formando uma cor laranja/avermelhada intensa. Após a reação, é necessário utilizar um scanner de mesa para obter uma imagem de alta resolução da zona de deteção do μPAD para determinar a intensidade de cor laranja/ avermelhada de cada unidade de teste, medida através do software Image J.

Sob condições ótimas, o método para o μPAD proposto foi caracterizado por um intervalo de calibração para a concentração de magnésio entre 0.082 – 0.247 mmol/L. Os limites de deteção e quantificação foram 0.062 mM e 0.081 mM, respetivamente. O gasto dos reagentes, eriocrómio cianina, NH_4Cl e NH_4OH foram 0.043 mg, 1.62 mg e 13.0 mg por curva de calibração. O gasto da solução padrão/amostra foi 120 μl por cada determinação.

Palavras-chave: Saliva, Magnésio, Dispositivo Microfluídico Analítico baseado em Papel (μPAD)

ABSTRACT

Of all the cations in the body, magnesium is the second most important intracellular cation and the fourth most prevalent. Disorders involving magnesium are categorized into two groups: hypomagnesemia (magnesium deficiency) and hypermagnesemia. In this way, the determination of magnesium has aroused great interest, because it helps in the clinical context and epidemiological research.

Therefore, the objective of this work was to develop a microfluidic paper-based analytical device (μPAD) for the quantification of magnesium in saliva samples.

In this case, the μPAD is based on the colorimetric reaction between magnesium and eriochrome cyanine to form an intense orange/reddish dye. After the reaction, it is necessary to use a flatbed scanner to obtain a high-resolution image of the detection zone for determination the intensity of the orange/reddish colour within each detection zone measured with Image J software.

Under the optimum conditions, the proposed μPAD method was characterized by a linear calibration range for magnesium concentration 0.082 – 0.247 mmol/L. The detection and quantification limits were 0.062 mM and 0.081 mM, respectively. The reagents, eriochrome cyanine, NH_4Cl and NH_4OH consumption were 0.043 mg, 1.62 mg and 13.0 mg per calibration curve and the sample consumption was 120 μl per each determination.

Key Words: Saliva, Magnesium, Microfluidic Paper-based Analytical Device (μPAD)

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1. INTRODUCTION

1.1. Saliva

In most laboratory diagnostic procedures, blood is the most commonly used sample type because of its cellular and chemical constituents, and because it reflects the real concentration of the various analytes [1]. However, in recent years, there has been an increasing need to study alternative blood plasma samples, as this type of sampling is done invasively and has associated risk potentials such as infections and bruises. Some of these alternatives can be another type of biological fluid, such as sweat, urine and saliva [2]. When compared to the other alternatives, saliva is easily accessible, can be collected non-invasively and without violating patient privacy [3]. Saliva, due to its various constituents (e.g., biological material, proteins and microorganisms), has become a type of clinical interest sampling and has been implemented in multiple diagnostic tests for monitoring health conditions and certain diseases [4].

1.1.1. Salivary production, composition and functions

Saliva belongs to a large group of mucous fluids (e.g. tear fluid, nasal mucus, cervical mucus, etc.) and plays a critical role in the physiology of the organism [5]. Saliva is the fluid present in the oral cavity and is mainly produced by three salivary glands: parotid, submandibular and sublingual (Figure 1.1), and a large number of smaller salivary glands [6]. However, the whole saliva also comes from the non-glandular origin as it is composed of a complex mixture of fluids from other regions, such gingival fold fluids, oral mucosal transudate and mucus of the nasal cavity (bacteria, fungi, virus, upper airways secretions). Besides, oral fluid may also contain food debris, blood-derived compounds, traces of medicines or chemicals [7][8].

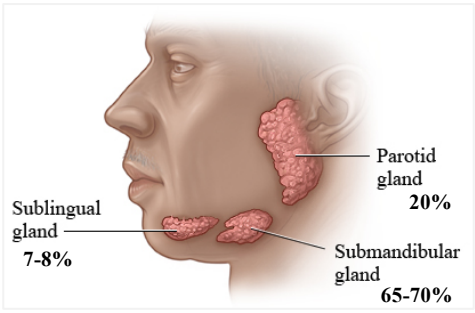

Figure 1.6 - *Salivary glands and respective % of saliva production for unstimulated salivary flow [8].*

The salivary liquid is mostly composed of water (about 99%). However, it is also comprised of inorganic compounds (e.g. ionic compounds), organic compounds (e.g. uric acid, glucose), protein/polypeptide compounds (e.g. amylase, albumin) and hormones (e.g. steroids) [9][10]. Saliva contains a variety of electrolytes (Table 1.1), the most abundant of which are sodium, potassium, chloride and bicarbonate. Calcium, magnesium and phosphate are present in lower concentrations [7][11].

Table 1.6 *- Electrolyte and total protein concentrations in whole human oral fluid and plasma [7].*

	Plasma	**Whole human *resting* oral fluid**	**Whole human *stimulated* oral fluid**
Na^+ (mmol/L)	145	5	20-80
K^+ (mmol/L)	4	22	20
Ca^{2+} (mmol/L)	2.2	1-4	1-4
Cl^- (mmol/L)	120	15	30-100
HCO_3^- (mmol/L)	25	5	15-80
Phosphate (mmol/L)	1.2	6	4
Mg^{2+} (mmol/L)	1.2	0.2	0.2
SCN^- (mmol/L)	<0.2	2.5	2
NH_3 (mmol/L)	0.05	6	3
$(NH_2)_2CO$ (mmol/L)	2-7	3.3	2-4
Protein (g/L)	70	3	3

Under healthy conditions, adults usually produce about 0.5 to 1.5 L of saliva per day, at a rate between 0 mL/min (during sleep) to 5 mL/min (during chewing or in the presence of stimuli). Total salivary flow can be classified as normal (1.0 - 4.0 mL/min), low (0.7 - 1.0 mL/min) and very low (< 0.7 mL/min) [12]. However, as salivary flow shows great biological variation, the composition (qualitatively) and the volume produced (quantitatively) are not only influenced by the time of day, but also due to various physiological and pathological conditions. Thus, salivary flow (SF) can be called stimulated and unstimulated SF [8]. Stimulated salivary flow represents saliva produced in the presence of mechanical, taste, olfactory or pharmacological stimuli, contributing about 80-90% of total salivary production. In addition to the presence of stimuli, saliva volume and composition may be influenced by factors such as hormonal changes (e.g. pregnancy), alcoholism and smoking, age, physical exercise, body weight, hereditary influences, and mouth hygiene. Unstimulated saliva represents a small

continuous salivary flow, called basal secretion, whose role is to moisturize and lubricate oral tissues continuously [13].

Saliva is one of the most complex and vital body fluids for preserving oral tissue health and for a variety of physiological needs. This oral fluid plays a crucial role in preliminary digestion as it softens foods and assists in the preparation, chewing, digestion and swallowing of the bolus. Also, it protects tissues from mechanical damage by cleaning up the debris present in the oral cavity and also protects tissues from irritants agents such as bacteria, virus and fungi. Due to its composition, it also plays an essential role in maintaining mineralization of tooth enamel. Other important saliva functions include its role in speech, taste, ability to buffer acidic foods and antibacterial, breath odour control while maintaining the integrity of the oral and gastrointestinal mucosa [8][14].

1.1.2. Historic of salivary analysis and clinical applications

In recent years, several studies have been developed to assess and monitor health status to implement surveillance diagnoses of human health through saliva analysis. Surprisingly, however, saliva has been used in clinical diagnostics for over 2000 years. For ancient practitioners of traditional Chinese medicine, saliva and blood are “sister fluids” from the same origin, and changes in oral fluid are indicative of the wellness of the individual (e.g. viscosity, odour, taste, etc.). Theories such as excessive saliva secretion are related to heartburn or sweet-tasting saliva related to problems with spleen functioning were some of the historical hallmarks of early saliva applications for health monitoring [15].

The use of this type of sampling has been a bet in several studies and, in most of them, aimed to implement in the routine medical practice component. Demand to develop tests based on salivary specimens rather than using blood specimens has been quite high since specimen collection has no associated risk potential and can be achieved very affordably [16]. However, the biggest challenge associated with testing has been the discovery and hence, validation of salivary biomarkers for certain diseases [17].

In recent years, there have been several successful oral fluid-based diagnostic tests (Table 1.2), such as saliva-based tests for the detection of HIV antibodies, with high specificity and sensitivity similar to blood tests. (Roberts KJ) Saliva-based tests to

predict premature birth (estradiol analysis), evaluate female reproductive cycles (estrogen, testosterone and electrolyte analysis) and assess stress level (cortisol analysis) [16]. Saliva has also been widely used in dentistry for oral disease studies to avoid the risk of tooth decay by measuring saliva buffer capacity and assessing bacterial content [18].

There have been several study proposals for saliva research, namely, viral and bacterial infections (genomes and antibodies detection), cancer, pharmaceutical and abuse drugs, hormones, DNA tests (using oral cells), sialo chemistry analysis, etc. [6]. Besides, the importance of using saliva is not only useful in clinical practice for diagnosing disease but has also been increasingly used in law enforcement agencies to develop methods for detecting illegal poisoning [2].

***Table 1.7** - Current and potential use of saliva based diagnostics [18].*

Current existing assays with active development of new detection systems	**Potential use in near future**
Pharmacological monitoring	Autoimmune diseases
Therapeutic drugs	Allergic markers
Law enforcement applications	Cardiovascular diseases
Drug intoxication	Acute myocardial infarction
Illicit drugs	Cardiac risk
Forensics	Cancer screening and diagnosis
Smoking exposure cotinine and thiocyanate	Oral cancer
Steroid hormones	Breast cancer
Cortisol, estrogen, testosterone and progesterone	Cancer-specific markers
Infectious diseases	Periodontal diseases
Antibody testing: HIV, HCV and HBV	
Antigen detection: bacterial, viral, fungal DNA/RNA/Protein	
Microorganism recovery: bacterial, viral, fungal cultures	

1.1.3. Advantages and disadvantages of saliva samples

In recent years, there is a growing interest in studying other biological fluids, with the same constituents as blood plasma, to develop clinical trials. Blood is considered the best body fluid for developing systemic processes, as it reflects the actual concentration of analytes. However, the methodology used to collect such samples is quite invasive and involves some potential risk for the patient, such as bruises, discomfort, possible site infections and anaemia. Besides, such samples are also

less favoured when research involves children or cases of difficult venous access (severe diseases and the elderly) [13].

For this, biological fluids such as urine, sweat and saliva have been excellent alternatives to replace blood samples, since their collection is non-invasive. Urine allows the accumulated measurement of analytes. However, results are dependent on the patient's fluid intake, which may vary substantially. Urine specimen collection has contested as it aims to invade patient privacy in case of sampling supervision [7].

Unlike urine, saliva can be collected under supervision without any violation of privacy. In this case, the collection is simple, stress-free (procedure without needles), less discomfort, cheap and simple. Some advantages of using saliva as sampling are shown in Figure 1.2. These samples are relevant and useful if children and the elderly need to be evaluated or for large-scale screening, offering an economic approach [19]. Samples can be taken by patients themselves or by poorly trained people, and can even be applied to less-favoured areas or unconventional environments (e.g. developing countries) as it does not require very elaborate equipment [15].

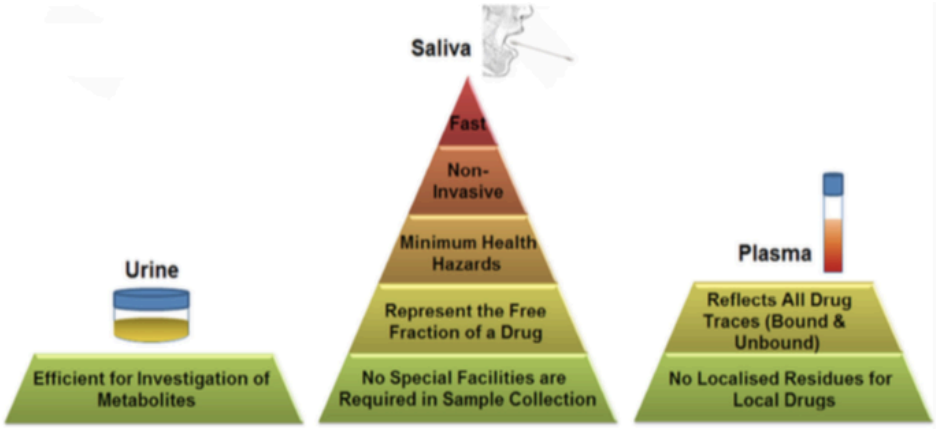

Figure 1.7 - *Schematic representation of the advantages of saliva specimen over other bio samples [2].*

However, saliva collection has some disadvantages, such as the fact that, compared to blood samples, their volume is limited and salivary biomarkers are still mostly unknown. The fact that salivary flow varies, both in volume and composition, in the presence of stimulating factors, it should be emphasized that the results depend on the cooperation of the individual, that is, depending on the psychological status and whether they consumed medicines [16]. Also, there is a need for more sensitive quantification methods, which may be related to the fact that the salivary matrix is quite complex, making interpretation of the results difficult [19].

1.1.4. Collection and sample storage

As mentioned above, the stimulated salivary flow is dependent on the presence of stimuli, and consequently, their composition may be altered. Thus, unstimulated saliva collection generally correlates with clinical conditions more accurately than stimulated saliva. An essential requirement for collecting salivary samples is to minimize all possible sources of variation in salivary composition. The most traditional way to obtain this fluid is to place the patient sitting with the head bent forward and allow the saliva to drip from the mouth into a collection container passively. However, this procedure is not very comfortable for the patient. Another method for collecting saliva is to ask the patient to spit gently into a vial. However, spit samples have been shown to contain up to 14 times more bacteria than passive drool [1][20].

When the above procedures are not possible, the only alternative is to use materials to collect saliva more efficiently. However, by introducing the elements into the patient's mouth, it stimulates the production of saliva and consequently may change its composition. The collection can be done through specific devices (larger sample volume), use of sterile swabs (faster), pipet aspiration under the tongue, chewing a piece of standard size paraffin, among others. The purpose of the devices facilitates sample collection in young children who have difficulty spitting. When using sterile swabs, the patient should remain with the gauze in their mouth for a few minutes until saturated, and then taken to the centrifuge to collect the fluid [19]. Before the collection of saliva, the patient should be informed in advance of the importance of the collection protocol. Information such as: to exclude tooth brushing, samples should preferably be taken fasting or at least 2-3 hours without consuming food or medicine. For the same reason, patients should be advised to rinse their mouth thoroughly with deionized water before collection [2].

After the saliva collection, the most effective process is freezing the sample to maintain the integrity of its constituents. The choice of different storage procedures before analysing the samples depends on the type of analyte to be analysed. Freezing samples is generally the most appropriate procedure because it prevents the growth of microorganisms and the degradation of analytes. However, if the analysis is done immediately after collection (up to 90 minutes), samples can be stored at room temperature. If the analysis was done between 3h-6h after collection, the samples should be placed in a 4 °C refrigerator. If the analysis is done days or even months after

collection, specimens should be stored at -20 ° C or, depending on the analyte, at -80 °C [2] [21].

1.1.5. Detection techniques to determine analytes

Whole oral fluid compounds have been examined with a large number of techniques: colorimetric/spectrophotometric, gas chromatography (GC), ion chromatography (IC), high performance liquid chromatography (HPLC) and as atomic absorption spectroscopy (AAS) [4]. However, all of these methods require robust, expensive and bulky equipment, which must be operated by a qualified laboratory professional. Thus, the need arose to create new alternative analytical techniques that determine the same analytes, with similar detection sensitivity. Thus, the possibility of using microfluidic paper-based analytical devices as an alternative tool for the determination of analytes present in saliva was explored, due to the simplicity of the method, low cost and portability [19].

1.2. Magnesium

The determination of electrolytes in human fluids is one of the most important functions in the clinical laboratory. Electrolytes affect most metabolic processes and are therefore part of several studies in the field of clinical research [22].

1.2.1. Overview of the analyte

Of all the cations in the body, magnesium is the fourth most prevalent and the second most abundant intracellularly [23]. Magnesium plays an important physiological role in many body functions (Table 1.3). Is essential for the synthesis of nucleic acids and proteins, regulatory systems, replication and for specific actions in different organs (e.g. neuromuscular and cardiovascular systems). Physiologically, magnesium acts as a cofactor for more than 300 enzymes [24].

Table 1.8 - *Physiological functions of magnesium [25].*

Enzyme function	**Membrane function**
Enzyme substrate (ATPmg, GTPmg) Kinases B Hexokinase Creatine kinase Protein kinase ATPases or GTPases Na^+, K^+ -ATPase Ca^{2+}, ATPase Cyclases Adenylate cyclase Guamylate cyclase **Direct enzyme activation** Phosphofructokinase Creatine kinase 5-phosphoribosyl-pyrophosphate synthetase Adenylate cyclase Na^+, K^+ -ATPase	Cell adhesion Transmembrane electrolyte flux **Calcium antagonist** Muscle contraction/relaxation Neurotransmitter release Action potential conduction in nodal tissue **Structural function** Protein Polyribosomes Nucleic acids Multiple enzyme complexes Mitochondria

1.2.2. Magnesium metabolism

The body of a healthy adult contains approximately 21-28 g of magnesium. About 60% of the magnesium is present in bones, 20% in skeletal muscle, 19% in other soft tissues and less than 1% in the extracellular fluid. Usually, the total serum magnesium concentrations range from 0.7 – 1.3 mmol/L. About 20% of serum magnesium concentrations is protein bound (e.g. albumin), 65% is free ionized magnesium and the rest is associated with complexed formed between magnesium and various anions (e.g. phosphate). The reference range for serum ionised magnesium concentrations ranges from 0.54 – 0.67 mmol/L [25].

Until then, there is not much information about the mechanisms involved in the regulation of intracellular magnesium. However, it is known that 0.5 – 5% of total cellular magnesium is free ionised magnesium. The remaining percentage is related to complexes formed between magnesium and other compounds such as ATP, proteins or DNA [25].

Magnesium concentrations in other biological fluids may have different values/ranges compared to blood samples. In salivary samples, the magnesium concentration is 0.2 mmol/L [7] [26]. In urine samples, values of less than 1.0 mmol/L

indicate that the patient needs supplementation. Yet, if the magnesium concentration was bellow 0.4 mmol/L indicate danger for the patient and may be an indicator of a disease [27].

Magnesium balance in the body, like that of other ions, is assessed by the function between ingestion and urinary excretion. In adults, the daily intake of magnesium is on average, between 5.6 - 6.8 mg/kg of body weight. However, the recommended daily dose is 4.5 mg/kg of body weight [27].

To ensure normal magnesium levels in the body, it is necessary to eat certain foods with sufficient Mg content. The main dietary sources of magnesium are cereals, grains, green vegetables (e.g. spinach), nuts, fruits, vegetables and tubers (e.g. potatoes). Water can be an essential source of magnesium. In general, magnesium intake is directly related to energy intake, except when most energy is associated with refined sugars or alcohol [25].

1.2.3. Assessment of magnesium status

Although the importance of magnesium is widely acknowledged, after several researches, there is still no simple, accurate and rapid laboratory test in clinical medicine, to determine total body magnesium status. To date, the serum magnesium concentration is the predominant test used by medicine to assess magnesium status [23].

However, there are several places where magnesium can be stored and excreted so that the analyte can be measured. Serum magnesium concentration can obtain through total magnesium, ultrafiltrate magnesium and ionized magnesium. If the status of Mg is to be measured by intracellular magnesium content, it can be obtained from red cells, mononuclear blood cells or skeletal muscle. The intracellular concentration of free magnesium is evaluate, using fluorescent dyes may be used, provided they form complexes with the analyte, or also by nuclear magnetic resonance spectroscopy. There are some methodologies, for assessing magnesium status, that are less widely used, namely using hair or teeth samples [25].

1.2.4. Clinical significance and associated diseases

Monitoring the free magnesium level of a patient is important in preventing life-threatening complications that can occur depending on the analyte concentration levels.

Disorders involving magnesium are characterised in two groups: hypomagnesemia (magnesium deficiency) and hypermagnesemia [25].

Hypomagnesemia is not rare. Prevalence pf hypomagnesemia varies from 7% and 11% in hospital patients. By relating serum magnesium concentrations to hypo, it is possible to state that the body's magnesium concentration is below normal when Mg concentrations vary between 0.41 - 0.82 mmol/L. However, the patient is at risk if the Mg concentration is 0.41 mmol/L or less. Causes of hypomagnesaemia and magnesium deficiency are listed in the Table 1.4. The magnesium loss can occur through vomiting or nasogastric suction. However, its occurrence is usually due to magnesium wasting in the gastrointestinal tract (diarrheal). An example of the effects of magnesium deficiency, causing serious complications in patient care, is a cardiac arrhythmia. These deficiencies can be quickly treated with parenteral magnesium or oral administration in more mild cases [28].

Magnesium deficiency is usually a consequence of certain diseases or drugs. Chronic renal failure occurs due to a loss of magnesium [29]. Very low magnesium concentrations are commonly associated with endocrine and metabolic disorders, specially Diabetes *Mellitus*. This is correlated with fasting blood glucose, glycated haemoglobin, albumin excretion and the duration of diabetes [28].

Table 1.9 - *Causes of hypomagnesaemia (magnesium deficiency) [25].*

Redistribution of magnesium - Refeeding and insulin therapy - Correction of acidosis - Massive blood transfusion	**Renal disease** - Dialysis - Inherited disorders
Gastrointestinal causes - Reduce intake - Mg free intravenous fluids - Dietary deficiency - Reduced absorption	**Endocrine causes** - Hypercalcaemia - Hyperthyroidism - Hyperaldosteronism
Renal loss - Reduced sodium reabsorption - Saline infusion	**Diabetes Mellitus** **Alcoholism** **Drugs**

The hypermagnesemia is rarer than hypomagnesemia. Patients with kidney failure are especially susceptible to hypermagnesemia because their ability to clear magnesium from their bodies is impaired when administered as stated in the previous sentence. When serum magnesium concentrations are higher than 4.1 to 5.0 mmol/L can cause vomiting, hypotension and cardiac arrest. Causes of hypermagnesemia and magnesium deficiency are listed in the Table 1.5. Clinical symptoms of high magnesium deficiency, such as hypocalcemia, neuromuscular hyperactivity, and cardiac arrhythmias, should be assessed in conjunction with the results of these tests in making a final diagnosis. It is important to analyse the patient's history before stating that the result shows abnormal magnesium values as these determinations may be interfered with by the presence of certain substances (e.g. thiocyanates in tobacco smokers) [24].

Table 1.10 - *Causes of hypermagnesemia [25].*

Redistribution of magnesium - Acute acidosis **Excessive intake** - Oral Antacids, Cathartics, Swallowing salt water - Rectal (Purgation) - Parenteral - Urethral irrigation **Renal loss** - Reduced sodium reabsorption - Saline infusion	**Renal failure** - Chronic renal failure - Acute renal failure **Others** - Lithium therapy - Familial hypocalciuric hypercalcaemia - Milk alkali syndrome - Depression

According to some studies, high plasma and cellular magnesium concentration may associate with the development of malignant tumors. Plasma and saliva magnesium concentrations were compared in healthy patients and patients diagnosed with parotid gland tumors, in which it was concluded that magnesium concentrations were higher in patients diagnosed with tumor [22][30].

1.3. Microfluidic Paper-based Analytical Devices (μPAD)

Frequently used methodologies for prevention and diagnosis, capable of evaluating and determining compounds in different sample types, generally require high analytical technology, specific facilities and qualified professionals. However, according to the World Health Organization, diagnostic devices must ensure particular characteristics, such as affordable, easy-to-use, fast, equipment non-specific, robust and user-friendly [31]. Thus, in recent years, to break new ground in research in areas such as chemistry, genetics, molecular biology and other research areas, there has been a great interest in the development of microfluidic devices. These devices allow for microscale laboratory operations. Use low-cost miniaturized equipment, small amounts of sample volume, which has become quite beneficial if the amount of sample to be used is from biological samples [32].

The concept of paper-based analytical microfluidic devices (μPAD) was invented and described by a research group from Harvard University in 2007 [33]. This group presented the filter paper as an alternative to developing microfluidic devices to use them in clinical diagnoses. They demonstrate the capability of the simultaneous detection of glucose and protein in the urine. The microfluidic device is represented in Figure 1.3. The glucose assay is based on the enzymatic oxidation of iodide to iodine (presence of glucose – colour change to brown). The protein assay is based on the colour change of TBPB reagent (presence of protein – colour change to blue).

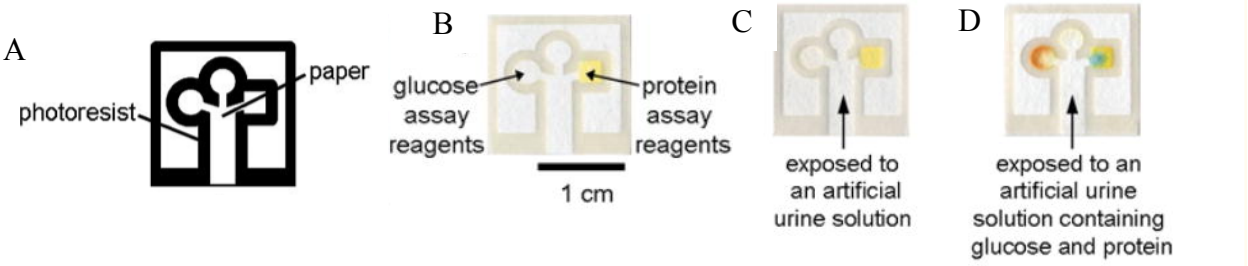

Figure 1.8 - *Chromatography paper patterned with photoresist. A) The darker lines are cured photoresist; the lighter areas are unexposed paper. B) Complete assays after spotting the reagents. C) Negative control for glucose (left) and protein (right) by using an a artificial urine solution. D) Positive assay for glucose (left) and protein (right) by using a solution that contained glucose and BSA in an artificial urine solution. Figure adapted from [31].*

The microfluidic devices consisted of two distinct zones: the hydrophilic zone (paper), where the flow movement occurs and the reaction between analytes and reagents, and the hydrophobic zone, which delimits the paper for the flow to remain only in this zone. The μPADs, when compared to conventional analytical microfluidic devices made from silicone, glass or polymers as a substrate, are much more affordable as they only use paper as a substrate. They also allow performing bioassays faster and cheaper [32], together with small size, lightweight, portable devices with low manufacturing costs. The μPADs can be handy tools when measurements need to be performed in less industrialized areas or even in developing countries where the analytical and medical infrastructure is limited, allowing for low reagent and analyte consumption [34].

Since then, many researchers have used these devices as convenient tools for the detection and determination of many organic and inorganic compounds, offering analytical skills that can revolutionize the pharmaceutical and drug industry [32].

1.3.1. Advantages of using filter paper

The option to use paper in microfluidic devices was based on the fact that the paper consists of cellulose fibres that act as capillaries, absorbing the solutions, making this transport passive, without the need for active pumping. The absorption rate depends on the size of the capillaries, the paper characteristics and the environment (e.g. temperature). The cellulose matrix can also act as a sample filter or to perform chromatographic separations. As the paper is available with different pore sizes, it is possible to separate suspended solids in the samples or to remove individual constituents present in the samples (e.g. proteins) [35]. Besides, the paper is an abundant and inexpensive raw material available everywhere.

Given that paper is a widely used material as a chemical platform, microfluidic devices can take advantage of existing techniques. This material also has the advantage of being available in a wide range of thicknesses, being easy to use, store and transport. Another advantage of using paper is that it is flammable and therefore, devices can be disposed of by incineration quite quickly and safely. The paper, as it is usually white, allows better visualization of the colorimetric reaction and its surface can be chemically modified [31].

1.3.2. μPAD fabrication techniques

Microfluidic devices are fabricated by moulding channels into glass, silicone, polymers or plastics. The μPAD design consists of patterning sheets of paper into hydrophilic channels (paper) bounded by hydrophobic barriers, to avoid leaks and keep the solution applied to the paper channels.

The μPADs can be fabricated by using 2D (with one layer of paper) or 3D (more than one layer of paper) methods, to transport fluids/samples horizontally or vertically through the channels, depending on the complexity of the technique. The development of 3D microfluidic devices offers more functionality than 2D devices, as it allows fluid to be transported both vertically and laterally from a single inlet to numerous detection zones. Besides, μPAD 3D has the advantage that the flow velocity is higher because its length in the z-direction is shorter than in the x - y plane. When the device has two or more layers, it allows incorporating other characteristics to the method. For example, it is possible to combine different types of filter papers into one device. The use of different papers may aim to retain certain compounds (e.g. proteins) along the flow or, as the layers absorb the sample, may react with specific reagents before reaching the μPAD detection zone [4] [36].

There are several fabrication techniques available to pattern the channels of μPAD (Figure 1.4), for example, photolithography, wax printing (most commonly used when manufacturing number of devices >100), inkjet etching and printing, paper cutting and shaping, flexographic printing, plasma treatment, laser treatment, wet etching and screen-printing [37]. Some of these methods are physical processes (e.g. plotting); while some are chemical processes (e.g. plasma treatment) and other are environmentally-friendly processes (e.g. wax printing). Some methods require use of toxic substances during processing (e.g. photolithography).

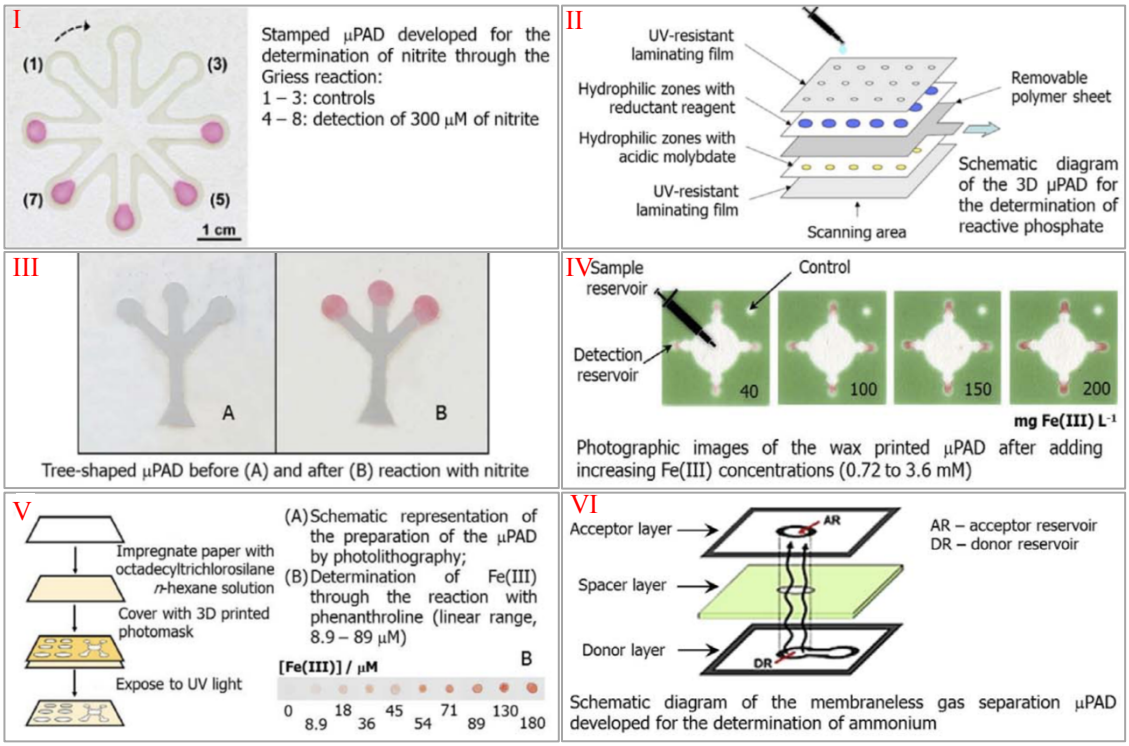

Figure 1.9 - *μPAD devices fabricated by (I) stamping method (II) inkjet printing (III) paper cutting (IV) wax printing 2D (V) photolithography (VI) screen-printed 3D. Figure adapted from [37].*

1.3.3. Detection methods and quantitative image processing

Using μPADs, it is possible to get qualitative (visible to the naked eye - higher or lower colour intensity) or quantitative (using analysis software) colorimetric analyses. The various detection methods used for microfluidic paper-based analytical devices are: colorimetric, luminescence, electrochemical and photoelectrochemical detection (Figure 1.5) [37].

The colorimetric detection is usually the most commonly used method in μPAD. Colorimetry includes visual, photometric and reflectometric detection. Quantitative colorimetric detection of analytes using μPADs is possible by reflectance detection when the intensity of the color that develops in the test zones is a function of the concentration of the analyte. Reflectance detection is based on the measurement of the light reflected off the surface of the test zone [31]. The respective device detection zones are scanned to the computer using a flatbed scanner, camera or mobile phone, depending on the image quality and lighting conditions [38]. After obtaining a high-resolution image of the test zone, the next step is to measure the average colour intensity of the respective test zones using analysis software (e.g. Image J or

Photoshop). The software selects specific colour tones of the reaction products, and converts these values to RGB format. Then, the filter corresponding to the complementary colour of the reaction is applied to obtain higher sensitivity and contrast of the test zone. [4][38] The two advantages of μPADs are their portability and ease of use [33].

Another detection method is luminescence, which includes fluorescence, chemiluminescence and electrogenerated chemiluminescence. These methods are generally more sensitive than photometry, however, require specific and more expensive equipment (e.g. fluorimeter). Electrochemical detection techniques can be used for μPADs as they are often referred to as "paper-based electrochemical devices". However, this technique requires a more complex methodology compared to the aforementioned methods due to the implementation of the electrodes on paper. Other technique is photoelectrochemical detection. In this method, the analytical signal is derived from the effect of light-induced photocurrent of an analyte interacting with a semiconductor electrode surface [37]. The method may be chosen according to the time required to obtain the results and available equipment.

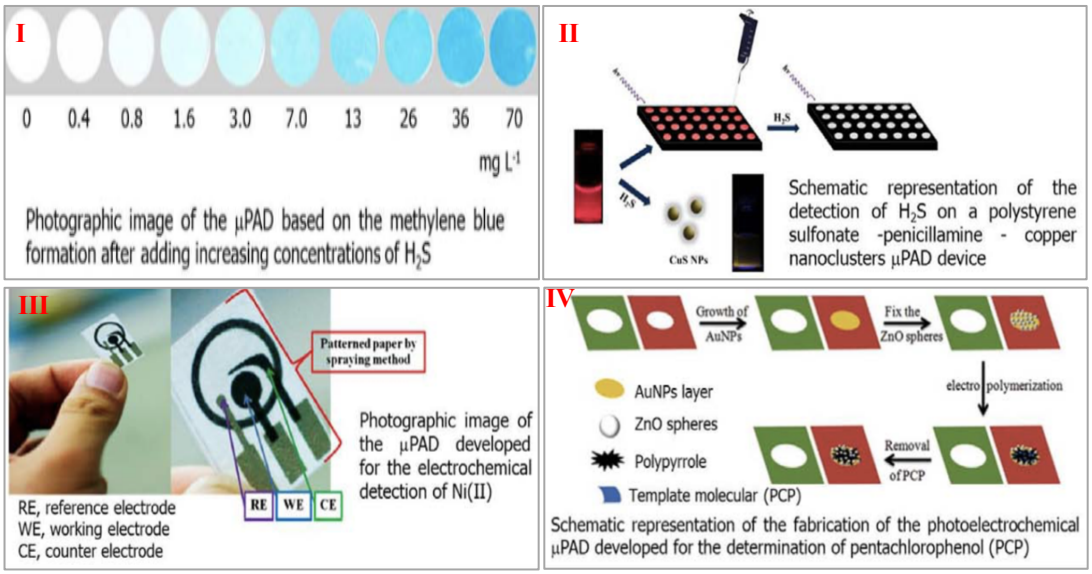

Figure 1.10 *- Detection methods used in μPADs: (I) Colorimetric (II) Fluorescence (III) Electrochemical (IV) Photoelectrochemical. Figure adapted from [37].*

1.3.4. μPADs applications platforms

The main application of microfluidic devices is to provide fast, easy and low-cost analytical platforms for assays, qualitative, semi-quantitative or quantitative, to be implemented in the diagnostic routine at an affordable price and environmentally friendly. There is a wide range of practical applications in many fields of research for which these devices can be implemented. Some of these areas are: the biochemical, immunological and molecular detections [39].

Biochemical detection may choose when in the microfluidic device (paper) specific reactions occur. Namely, chemical reactions (e.g. acid-alkali reaction), precipitation reactions or enzymatic reactions occur. In this type of reaction, the compound to be evaluated forms complexes with a specific reagent [40]. Exist some examples of biochemical reactions, such as determination of H2O2, Cd or Pb and urine acid.

Immunological detection is a method that uses immunoassay techniques for a variety of practical applications, namely, *Escherichia coli* O157:H7, Rabbit IgG and red blood cells agglutination. This kind of method is mainly used to detect humoral antibodies or antigenic substances (e.g. antigen-antibody reaction) [41]. Another example for applications of these devices, using immunological detection, is to separate blood plasma from whole blood [42].

In the case of molecular detection, the device is applied to detect specific nucleic acid hybridization sequences. Some examples of these applications are: tuberculosis diagnosis, target-ssDNA, ATP and Benzo[a]-pyrene (target DNA) [39].

In addition to the detection methods mentioned above, various types of procedures have been presented using paper microfluidic devices that provide new platforms for disease diagnosis. A colorimetric method for glucose determination, mass spectrometry for the determination of acetylcholine hydrolysis and potentiometric methods for determination of metal ions are examples of these methods [39].

1.4. Objectives

The aim of this work was to develop a simple, fast and effective microfluidic paper-based analytical device for magnesium determination in saliva, as an alternative non-invasive analysis. Small volumes of sample solutions should be introduced into one μPAD directly, occurring a colour reaction for colorimetric analysis.

The paper-based microfluidic device to be produced should have the potential to be used as a diagnostic tool to assess patient's risk for developing magnesium-associated diseases (e.g. parotid gland tumor).

For this purpose, several studies were carried out, namely:

- The reagent for the reaction with magnesium was ;
- Test the reaction on paper (using μPAD);
- Optimize the microfluidic device (number of layers, filter papers, reagent and sample volumes);
- Analyse possible interferences in magnesium determination;

2. MATERIALS AND METHODS

2.1. Reagents and solutions

All solutions were prepared with analytical grade chemicals and Milli-Q® Water (Resistivity > 18 MΩ•cm, Millipore, Bedford, MA, USA).

The reagent solution was obtained daily by dissolving 0.5 g of eriochrome cyanine (Sigma) in 1 mL of 8 M nitric acid in a 100 mL standard flask, adding 0.8 g of sodium chloride and 0.8 g of ammonium nitrate, and diluting to the mark with approximately 100 mL of water to final concentration of 0.01 M [43].

The buffer solution with pH 10.2 was prepared by dissolving 0.675 g of ammonium chloride (Merck) with 6 mL of concentrated ammonium hydroxide (Merck) (d = 0.900) and dilute it with water to 100 mL [44].

Artificial saliva solution was prepared by dissolving 2250 mg of potassium chloride (Merck), 544 mg of KH_2PO_4 (Merck) and 4775 mg of $C_8H_{18}N_2O_4S$ (HEPES) (Sigma) in a 1 L standard flask of water. From this solution were withdrawn 250 mL for diluting 675 mg of BSA (Bovine Serum Albumin) [45].

Magnesium stock solution of 2.0 mM was prepared by dissolving 19.5 mg of magnesium chloride (Sigma) in 100 mL of water. Subsequently, from this solution, a dilution for 0.412 mM of magnesium was prepared. Magnesium working standards in the range of 0.082-0.247 mM were weekly prepared from the stock solution with the artificial saliva solution.

2.2. Microfluidic Paper-Based Analytical Device Assembly

Using a cutter, Whatman 50 and Whatman 1 filter papers were cut into small 95 mm diameter discs. For the assembly of the μPAD, 50 discs of Whatman 50 and 100 discs of Whatman 1 filter paper were needed. To ensure the correct alignment with effective separation between the hydrophilic and hydrophobic zones, plastic pouches was used, in which 24 holes were cut, on one side of the pouch. Each μPAD consists of 4 rows and 6 columns of 3 layers discs.

The first layer contains the reagent Whatman 50 filter papers, the second layer includes the buffered discs (Whatman 1 filter paper) and finally a third layer consisting

of discs without the addition of compounds (Whatman 1). All discs were aligned so that all layers were overlapped (Fig. 2.1B) and distributed into the plastic pouch. After that, the lamination process takes place (Fellowes L125), creating a hydrophobic zone (plastic pouch) and a hydrophilic region (paper discs).

2.3. Reaction and data analysis

To Whatman 50 filter paper discs add 10 µL eriochrome cyanine reagent and oven dry at 50 °C for 10 minutes (Fig. 2.1A). Only to fifty Whatman 1 filters paper discs add 5 µL of buffer and allow drying at room temperature (T ≅ 25° Celsius) for 10 minutes. For the determination of magnesium concentration, 15 µL of a sample or standard was deposited into the sample hole of the µPAD and, approximately 5 minutes has waited to covered sample holes with masking tape. After the predetermined duration of the colour development time, the detection zone was scanned using a flatbed scanner (Canon LIDE 120) to obtain the intensity readings (Fig. 2.1C).

After obtaining a high-resolution image of the µPAD detection zone, Image J software was used to measure the colour intensity for the centre of each detection zone (hydrophilic regions) (Fig. 2.1D). The red, green and blue (RGB) colour intensity profile plots were obtained for a 3 mm in diameter circle in the centre of each detection zone. The highest sensitivity was obtained using the blue filter.

After that, the data was imported into Excel (Microsoft Office Excel, version 16.16.4) to be organized and for subsequent analysis (Fig. 2.1E). The average of colour intensity for each detection zone was subsequently converted to absorbance as proposed by Beer-Lambert law: $\mathbf{A} = \mathbf{log_{10}}\left(\frac{I_0}{I}\right)$. With this formula, the absorbance value corresponding to each concentration was obtained by relating the average intensity of the blanks (I_o) with the average intensity of each standard solution (I). By measuring the colour intensity in the detection zone, it is possible to calculate the concentration of the analyte by comparing with the absorbance values by establishing a calibration curve.

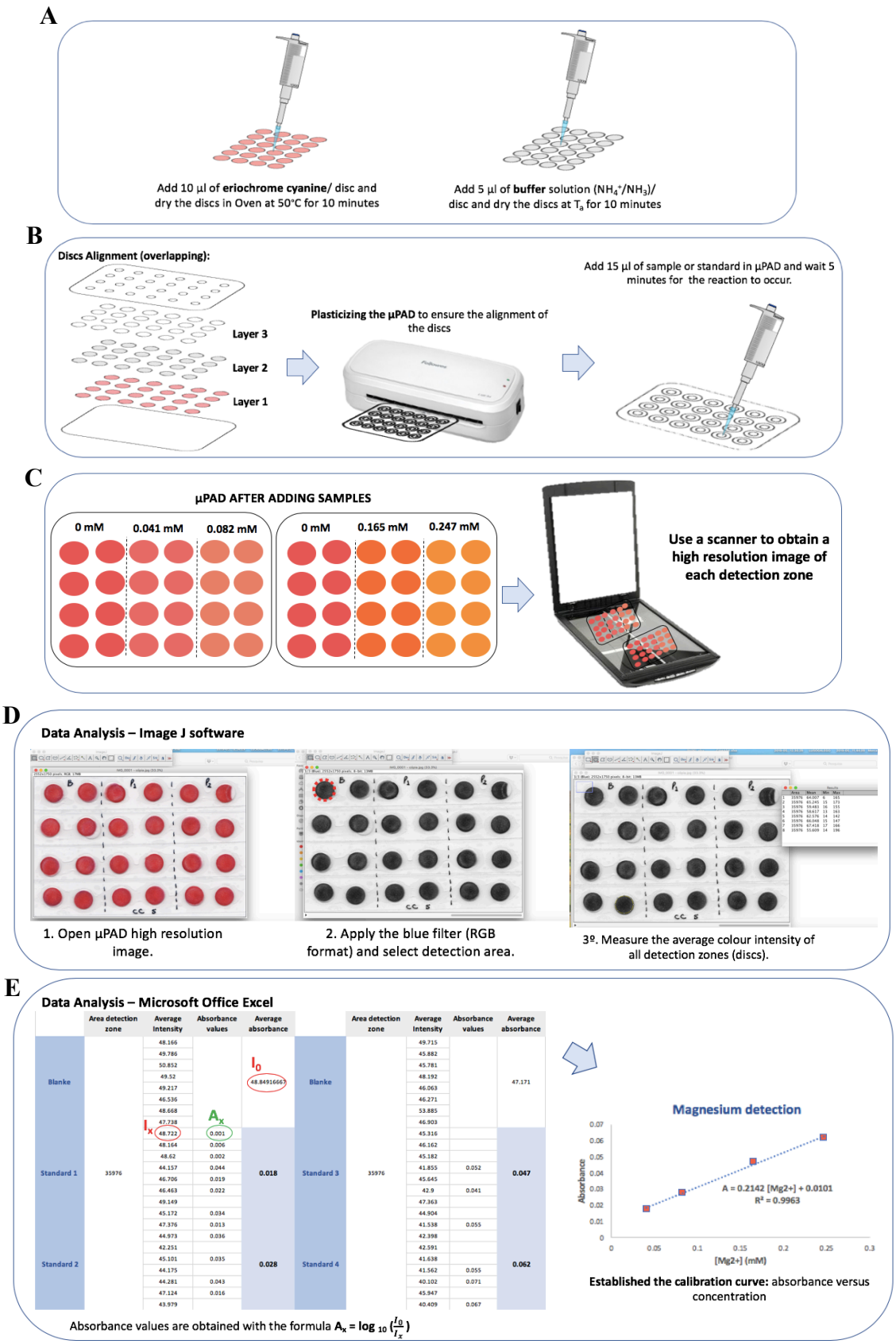

Figure 2.4 - *Schematic representation of the determination of magnesium using the developed µPAD; (A) Addition of reagent and buffer solution, (B) Discs alignment and µPAD plasticization, (C) Reaction time and µPAD scanning, (D) Measurement of colour intensity of detection zone, (E) Calibration curve of Mg determination.*

2.4. Batch study procedure

For the studies carried out in classical procedure, batch studies, the methodology used show in Figure 2.2.

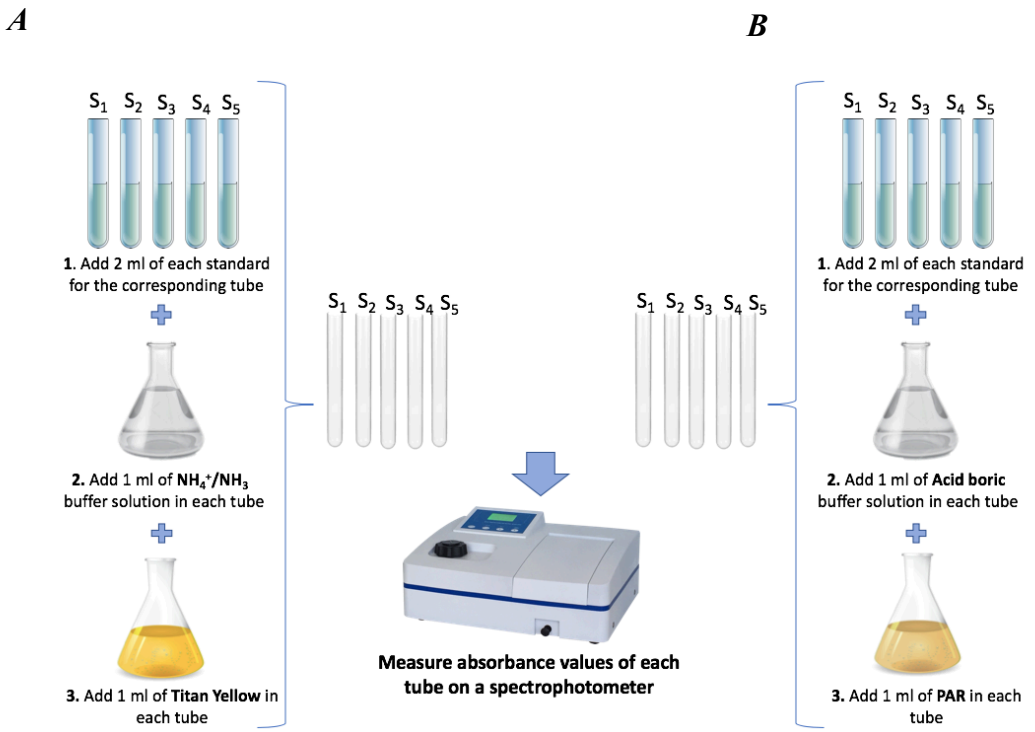

Figure 2.5 - *Scheme of the batch study methodology for the Titan Yellow (A) and PAR (B) reagents.*

2.5. Mimic procedure for sample collection

The filtration process methodology can be observed in Figure 2.3.

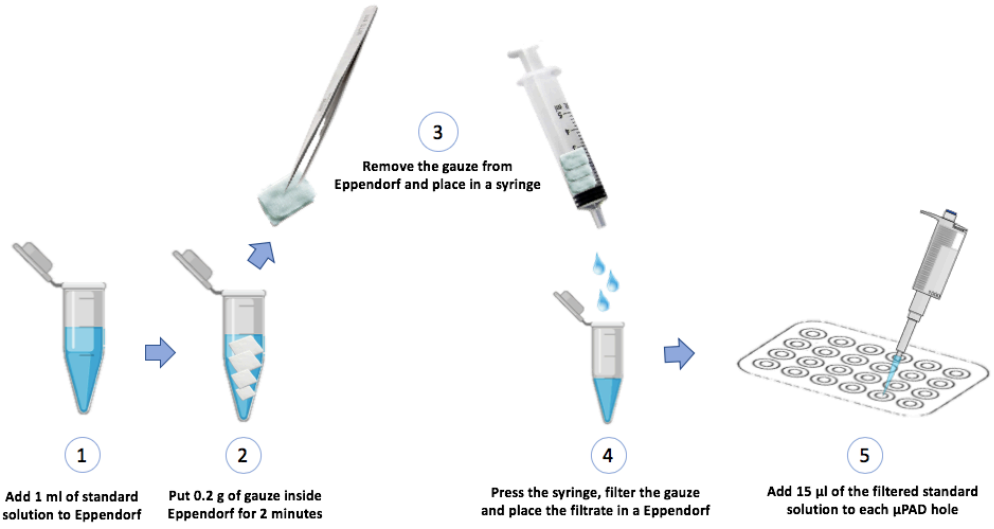

Figure 2.6 - *Filtration process of standard Mg solutions.*

RESULTS AND DISCUSSION

3.1. Reagents selection – batch study

First, to develop this device, it was necessary to study the best reagent that determines magnesium ions in salivary samples. As μPADs are devices made of filter paper discs, the reagent chosen for this reaction had to meet certain conditions, like the reaction between the analyte and the reagent had to be direct (no precipitation) and colorimetric.

Thus, there were various reagents capable of determining the analyte under the conditions mentioned above: titan yellow, eriochrome black T, calmagite, 4- (2-Pyridylazo) resorcinol (PAR), 8-hydroxyquinoline, eriochrome cyanine, o-cresolphthalein, or alizarin S [46]. Of all reagents listed, only those available in the laboratory were prepared, namely, titan yellow, eriochrome black T, PAR and eriochrome cyanine. The methodology for the preparation of each reagent and some of the characteristics are described in Annex I – Table I1.

Initially, an intermediate standard solution of 0.82 mmol/L Mg^{2+} was used. Subsequently, from this solution, magnesium standards in the range of 0.016 - 0.082 mmol/L were prepared. This preliminary batch study aimed to analyse the reaction of each reagent with the standard solutions, measuring the absorbance values through the spectrophotometer, to subsequently, relate these values to the analyte concentrations in a calibration curve.

3.1.1. Batch study results of aqueous solutions

The first reagents to be tested were **titan yellow** and **PAR**, both were used for metal ion determinations as they form water-soluble complexes between the reagent and the analyte, with high molar absorptive (ca. 10^4). However, Titan Yellow and PAR are not specific only for the determination of magnesium ion (Section 2.4. Figure 2.2) Then, the calibration curve of each reagent was established, relating the absorbance values and the magnesium concentrations (Anexo I - Fig. I1). After observing the graph, it concluded that neither reagent could determine magnesium ions, within the stipulated concentration range. One possible reason why the reagents did not detect the analyte may be related to the fact that Mg^{2+} concentrations were very low.

Then, the **eriochrome black T** reagent was tested. This reagent is a complexometric indicator that is used in the water hardness determination process. In its protonated form, eriochrome black T is blue. It turns red/pink when it forms a complex with magnesium or other metals ions [44].

For this study, a 1 M solution of eriochrome Black T and the same procedure as for previous reagents was used, replacing only the reagent and decreasing the volume of standard solutions to approximately 0.5 mL, using a Pasteur pipette (14 drops). Then, the absorbance values calculated and the calibration curve established. However, after observing the results, it was concluded that the curve did not show the expected linearity and sensitivity. This may have been related to the fact that the reagent is very high in concentration (very saturated in colour) and so high blank values were observed.

As the 1 M solution of eriochrome black T was very concentrated, it was decided to dilute it to 0.1 M. Batch studies was performed again using the same methodology and the reagent volume was studied in the range of 0.2 – 1.0 mL (Annex I - Table I2). Analysing the results, it was possible to conclude that with the increase of the reagent volume, both the sensitivity and the correlation coefficient decreased. Thus, the smaller the volume of eriochrome black T, the better the parameters for the reaction calibration curve. Then, as it was chosen to use 0.2 mL of reagent, it was necessary to study the volumes of the standard solutions again (Annex I - Table I3). The calibration curve for two different volumes (2 and 3 mL) was established. After observing the results, it was decided to use 2 mL of standard solution because the sensitivity and the correlation coefficient are slightly higher.

Finally, the **eriochrome cyanine** reagent was tested — this reagent has been used as a chromogenic reagent for a determination of many metal ions. The methodology used to analyse the reaction between this reagent and magnesium was similar to the methods of previous reagents.

First, different volumes of eriochrome cyanine (0.5 and 1.0 mL) were compared in batch, in which for each test tubes 2 mL of standard solution and 1 mL of buffer solution were added. After establishing the calibration curve for both reactions (Annex I – Table I4), it concluded that the best volume would be 0.5 mL since because the sensitivity and the correlation coefficient was higher. Then, batch tests again performed, but to study different volumes of standard solution (1, 2 or 3 mL), keeping the 0.5 mL

of reagent and 1.0 mL of buffer solution (Annex I - Table I4). According to the results, 1 mL of standard solution was chosen to obtain a calibration curve with high sensitivity and linearity. As a smaller sample/standard volume was chosen, it was considered necessary to re-evaluate the reagent volume and to verify if there were significant changes in the evaluated parameters. The reagent volume was studied in the range of 0.2 – 1.0 mL. The absorbance values were measured, and the calibration curves established (Annex I - Fig. I2) relating the absorbance to the magnesium concentrations for the different reagent volumes. Comparing the calibration curves, it concluded that the ideal volume was 0.2 mL of eriochrome cyanine because of the smaller the reagent volume, the higher the sensitivity and, simultaneously, the correlation coefficient.

After research on articles related to saliva morphology, the expected magnesium concentration in salivary samples known: **$[Mg^{2+}]$ = 0.2 mmol/L**. Thus, new standard solutions were prepared in the concentration ranges from 0.041 to 0.33 mmol/L. Batch tests were again performed with the new standard solutions, but only with the eriochrome black T and eriochrome cyanine reagents. By changing the Mg concentration range, different reagent and standard volumes were retested to optimize the reaction.

In the case of reaction with eriochrome black T, 1 mL of buffer solution, 2.0 mL of 0.1 M reagent, and two different volumes of standard solution, 2 and 3 mL, were added to the test tubes. Subsequently, the absorbance values were measured, and the calibration curves established, as can be seen in (Annex I - Table I5). As concluded earlier, the smaller the volume of the standard solution, the higher the sensitivity and linearity of the curve. Thus, it was decided to use 2 mL of the standard solution.

To study the reaction with the eriochrome cyanine, 1 mL standard solution, 1 mL buffer solution, and different reagent volumes (0.20 – 0.75 mL) were added to the test tubes. Then absorbance values were measured, and calibration curves were established for each volume (Annex I - Fig. I3). Each sensitivity and correlation coefficient of each curve were analysed and compared, and the 0.25 mL reagent volume was chosen. The 0.20 mL reagent volume option was excluded because the respective calibration curve had a lower correlation coefficient compared to the selected volume calibration curve.

3.1.2. Matrix influence assessment

Standard solutions were prepared in synthetic saliva matrix, with the same concentration range of Mg^{2+} (0.016 to 0.263 mmol/L). These standards were prepared by diluting the 2.0 mM stock solution of magnesium. Initially, as a preliminary study, it was decided to develop two different types of standard solutions: standards made in synthetic saliva with BSA protein and standards without BSA protein. For these batch tests, only the reactions between the eriochrome cyanine and eriochrome black T reagents with the magnesium analyte were evaluated. The methodology used was the same in which 1 mL of standard solution, 1 mL of buffer solution and 0.25 mL of reagent added to the different test tubes for the eriochrome cyanine. In the case of the reaction with the eriochrome black T, 2 mL of standard solution, 1 mL of buffer solution and 0.20 mL of reagent added. The absorbance values were calculated, and the various calibration curves established, as shown in Table 3.1.

Table 3.3 - *Correlation of absorbance with Mg concentration for different reagents.*

Reagent	BSA protein	Regression equation	Correlation coefficient (R^2)
Eriochrome cyanine	Yes	A = 0.399 [Mg2+] – 0.008	0.989
	No	A = 0.531 [Mg2+] + 0.096	0.931
Eriochrome black T	Yes	A = 0.183 [Mg2+] + 0.004	0.987
	No	A = 0.115 [Mg2+] + 0.047	0.387

After observing the results, it was concluded that both reagents could determine magnesium ions in the standard solutions made with synthetic saliva, except the reaction between eriochrome black T with those without BSA protein. However, eriochrome cyanine was the reagent that displayed a higher sensitivity and correlation coefficient. Thus, the eriochrome cyanine reagent was chosen as the reagent for the determination of magnesium.

In addition to the reagent study, different pH values were also batch tested for the buffer solution, namely 10, 11 and 12. The results can be analysed in Annex I – Fig. I4, which concluded that the best option was to use the pH 10 buffer, as the sensitivity was similar among all, thus avoiding the presence of higher amounts of hydroxide in the standard solutions.

3.2. Microfluidic Paper-based Analytical Devices - Design

In this work, for all tests performed, a univariate statistical analysis was used, that is, several variables, chemical and physical, were studied separately. With this type of analysis, some tests may need to be retest due to changes in certain interconnected variables.

3.2.1. Preliminary studies

Initially, we decided to perform some preliminary studies in which μPAD developed for eriochrome black T and eriochrome cyanine reagents, to evaluate the reactions previously studied on paper. In these assays, the option of using eriochrome black T was discarded as no staining could be observed in the detection zone. As mentioned earlier, the use of these devices implies that there is a colorimetric reaction to measure the colour intensity values of each disc subsequently. Thus, the subsequent studies were performed using only the eriochrome cyanine as the reaction reagent. So far, to calculate the absorbance values of the standard solutions, a spectrophotometer has been used.

However, in the μPAD, quantitative colorimetric detection was possible by calculating the absorbance based on the intensity of the colour developed in the test zone. To measure colour intensity, Image J software was used and the RGB model was applied to obtain the highest sensitivity. The same calibration curve was compared using two different colour filters (Fig. 3.1A). According to the colours theory, the combination of two colours that are on opposite sides of the colour wheel (Fig. 3.1B) allows for higher contrast and brightness. Although the eriochrome cyanine colour is orange, when it reacts with the magnesium ions, the colour of the reaction turns red to orange. After analysis of the results, we chose to use the blue filter because the respective calibration curve obtained higher sensitivity.

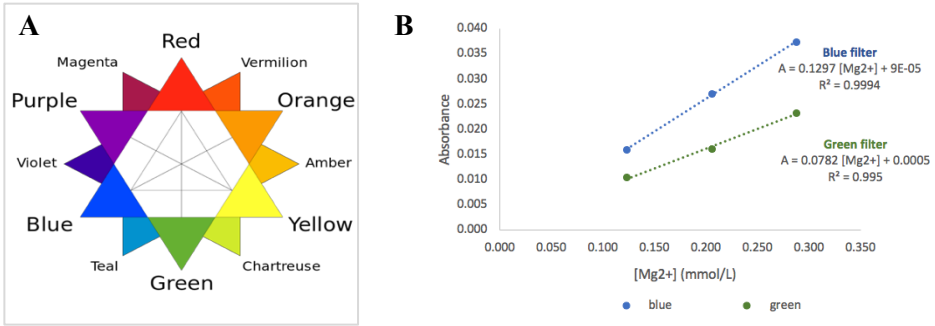

Figure 3.1 *- Colour wheel (A) and filter colour study (B) to Mg determination.*

The next studies were performed with standard solutions prepared in water, with the same concentration range of Mg^{2+}.

The first step was to develop μPAD with only **one layer** of Whatman 42 filter paper discs. The device having only one layer implied that both buffer and reagent were added to the same disc. Previous batch studies only proved that it was possible to obtain a calibration curve that relates the absorbance values to the Mg^{2+} concentrations by separately adding the reaction integrating solutions. As the objective was to test the μPAD with only one layer, new batch studies were performed in which a pre-made solution with the reagent and buffer mixed was used. Comparing the calibration curves of each case (Table 3.2), it concluded that both alternatives could determine Mg^{2+} with high sensitivity.

Then, the previous reaction was tested on paper. To this end, 10 μL of the "mix" solution was added to each disc and allowed to dry in the oven at 50° C for 10 minutes. Subsequently, the discs were lined up in the plastic pouches and, after the device was ready for use, 8 μL of the several standard solutions were added. However, after drying of the sample, no gradual colour difference was observed as the Mg^{2+} concentration increased. In this case, most discs showed colours that didn't match the reagent used (Figure 3.2). This could be related to possible contamination, since the paper disc was in direct contact with air, or simply because the reaction occurred only in one layer.

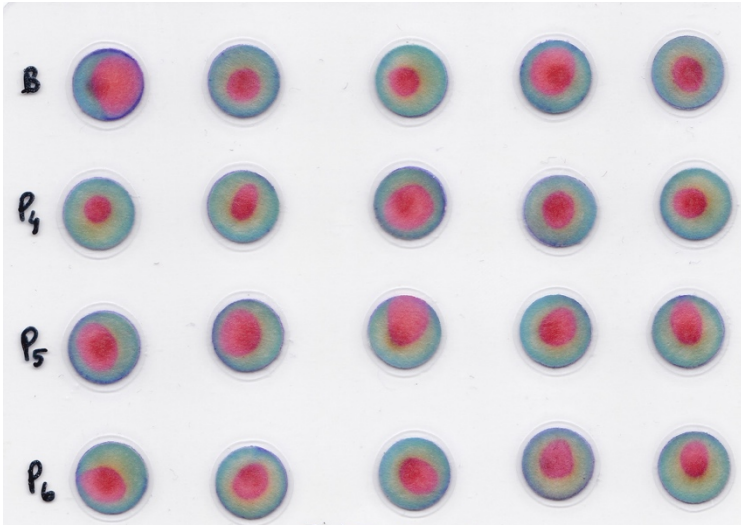

Figure 3.2 - *Scan the μPAD detection zone with one layer, from standard solutions with the highest Mg concentration (P4, P5 and P6).*

So, the construction of μPAD was studied with **two layers** of Whatman 42 filter paper. In this study, it was decided to construct the device in two different ways: one μPAD with the reagent and buffer solution in the first layer and the second layer empty, and another μPAD with the reagent in the first layer and the buffer solution in the second layer. Mounting conditions and volumes added were the same for both devices. According to the results obtained (Table 3.2), it was decided to choose the assembly that uses the reagent and the buffer solution in different layers, because the sensitivity and the correlation coefficient were higher.

Table 3.4 *- Features of calibration curves in batch and μPAD tests.*

Method	Conditions	Regression equation	Correlation coefficient
Batch	All solutions added separately	$A = 0.648\ [Mg^{2+}] + 0.023$	0.998
	"Mixing" solution + sample	$A = 0.413\ [Mg^{2+}] + 0.019$	0.989
μPAD	All solutions in different layers	$A = 0.101\ [Mg^{2+}] + 0.011$	0.996
	All solutions in the same layer	$A = 0.054\ [Mg^{2+}] + 0.003$	0.948

3.2.2. Buffer layer study

Maintaining the μPAD construction conditions with two layers of Whatman 42 filter paper, in which the reagent is in the first layer and the buffer solution in the second layer, it was decided to study different volumes for the buffer solution. For this, 5 μL of reagent added to all discs of the first layer and 5 - 15 μL of buffer solution were studied. With all μPAD ready for use, 10 μL of standard/sample added to the holes in the plastic bags. Calibration curves (absorbance vs Mg concentration) established for each volume at different scanning times and then the sensitivity of the respective volumes tested were compared (Figure 3.3). Of all volumes, 5 μL of buffer solution chosen, because it produced the highest sensitivity.

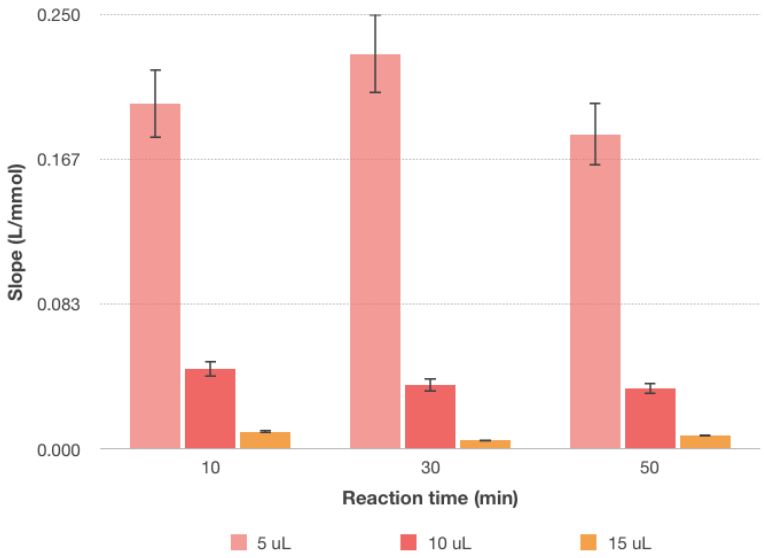

Figure 3.3 - *Comparison of the calibration curve slope, sensitivity, using different volumes of buffer solution; Error bars represent 10% deviation.*

To increase the rate of reaction flow, it was decided to try Whatman 1 (Particle Retention = **11 µm**) on the second layer, since the pore sizes of this paper type were relatively larger than Whatman 42 (Particle Retention = **2.5 µm**). Later, after adding 10 µl of standard solutions, it was found that the sample drying time was shorter when filter paper Whatman 1 was used in the second layer (Drying time $_{\text{Whatman 42}}$ = 5 minutes; Drying time $_{\text{Whatman 1}}$ = 2 minutes). Comparing the method sensitivities for both cases (Fig. 3.4), it was possible to conclude that Whatman 1 filter paper would be the best option, because the sensitivity did not vary significantly over time, the opposite of Whatman 42 paper decreases. Besides, it was an advantage that sensitivity did not vary significantly over time as it gives the technician a longer time to scan µPAD after adding the sample.

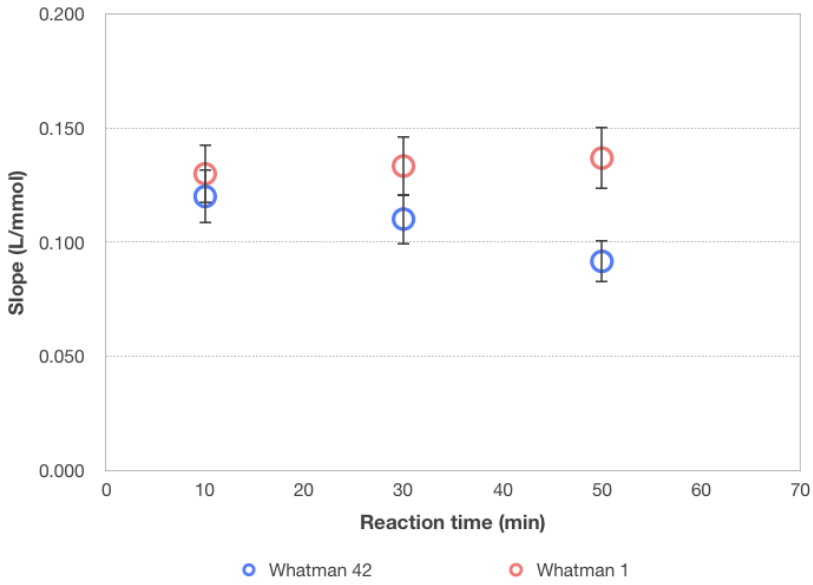

Figure 3.4 - *Comparison of the calibration curve slope, sensitivity, for Whatman 42 and Whatman 1 filter paper; Error bars represent 10% deviation.*

3.2.3. Reagent layer study

After choosing the volume and paper for the buffer layer, it was necessary to optimize the reagent layer (detection zone). The reagent volume was studied in the range of 5-15 µL, keeping 5 µL of buffer solution and 10 µL of standard or sample solution. According to the results obtained (Figure 3.5), by adding 10 µL of reagent, the reaction sensitivity is much higher. The 15 µL of reagent was excluded because it took about 30 minutes to dry.

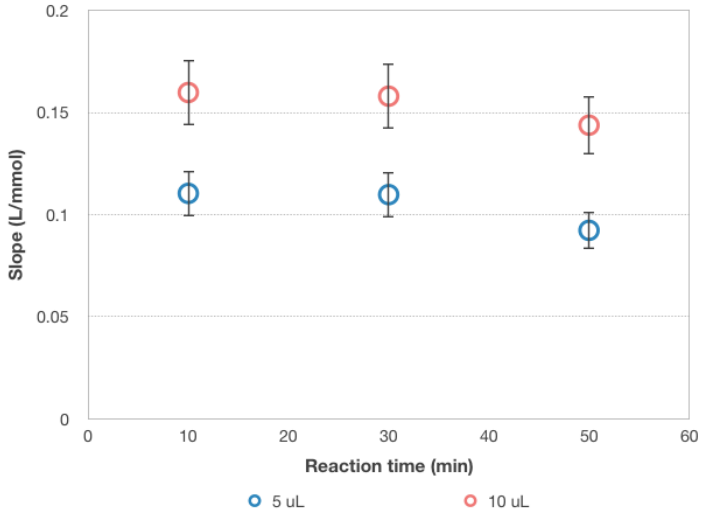

Figure 3.5 - *Comparison of the calibration curve slope, sensitivity with different reagent volumes; Error bars represent 10% deviation.*

Subsequently, different filter papers for the reagent layer were studied. Since this layer was the device's respective detection zone, it was essential to evaluate the type of paper that best suits you. For this, three types of paper with very similar characteristics assessed, namely, Whatman 42, Whatman 50 and Whatman 1. The characteristics of different filter papers can be observed in Annex II – Table II6. To develop µPAD, 5 µL of buffer solution added to the second layer discs and, at the end, 15 µL of standard solution/sample added to the holes in the plastic pouches. On all devices, the sample drops took approximately 15 minutes to dry completely, performing the first scan 20 minutes after addition. According to the results obtained (Fig. 3.6), the Whatman 50 paper was chosen because it presented a very high sensitivity. Comparing Whatman 50 filter paper with the others, it had approximately twice the sensitivity value.

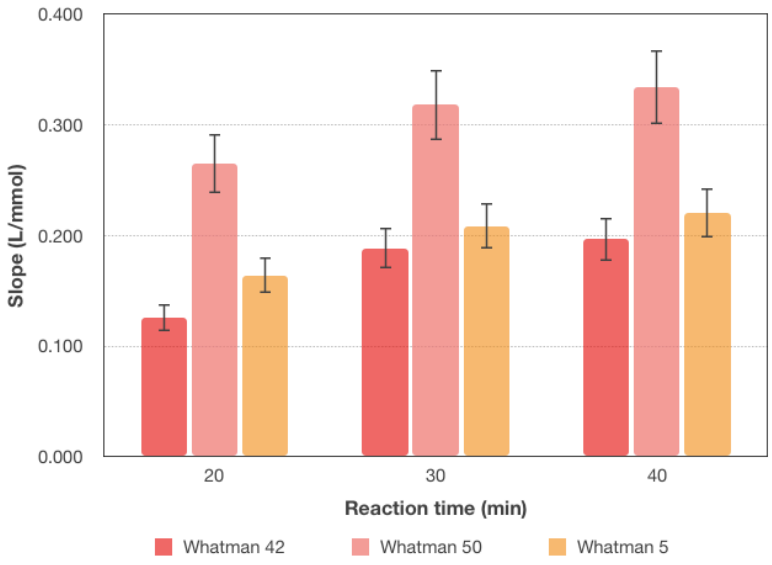

Figure 3.6 - *Comparison of the calibration curve slope, sensitivity, for Whatman 42, Whatman 50 and Whatman 5 filter papers in the reagent layer; Error bars represent 10% deviation.*

3.2.4. Sample/standard solution study

All studies associated with the optimization of the standard solution or sample in µPAD used the standards prepared with synthetic saliva matrix with BSA protein. All standards had the same concentration range of Mg^{2+}.

The first sample study was performed when the μPAD consisted of two layers of Whatman 50 (reagent layer) and Whatman 1 (buffer layer) filter papers. In this study, 10 - 20 μL of the standard solution tested. After the addition of the sample, it concluded that the larger the volume added, the longer the drying time ($T_{10\mu L} \cong 5$ min; $T_{15\mu L} \cong 15$ min; $T_{20\mu L} \cong 40$ min). Since one of the objectives of this work was to develop a device that would benefit from rapid data analysis, the option of using 20 μL immediately excluded because of the long drying time of the sample. Thus, the first scans were performed at different times, namely 10 and 20 minutes after the addition of the sample. Through the analysis of the results (Fig. 3.7), it was decided to use 15 μL of sample/standard solution, because it is what obtains the highest sensitivity under the same conditions.

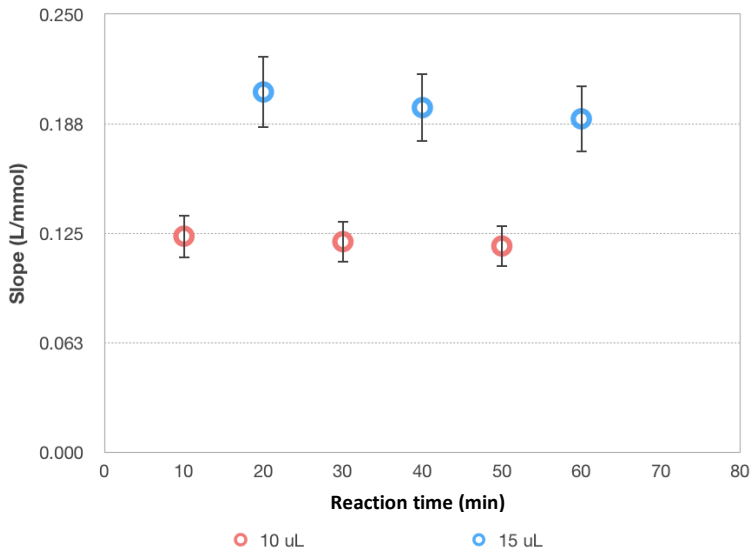

Figure 3.7 - *Comparison of the calibration curve slope, sensitivity, for different standard volumes; Error bars represent 10% deviation.*

To reduce potential μPAD contamination and protect the operator from direct contact with biological samples, adhesive tape was used to cover all holes in the plastic pouch after the sample/standards absorption. In order to ensure that this would not affect the detection μPADs with and without adhesive tape were compared (Fig. 3.8). According to the results obtained, we chose to implement in the design of μPAD the use of adhesive tape, since there were no significant variations in the parameters previously evaluated.

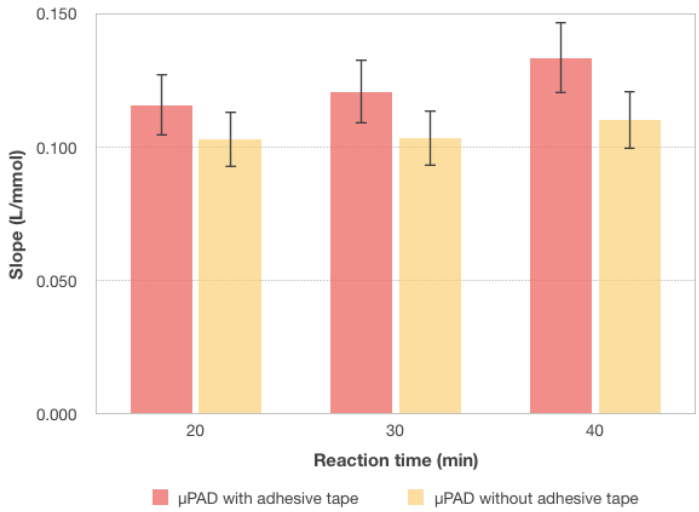

Figure 3.8 - *Comparison of the calibration curve slope, sensitivity, for adhesive tape study; Error bars represent 10% deviation.*

The influence of the most operational parameters studied to develop an efficient µPAD and the respective optimal choices were summarised in Table 3.3.

Table 3.3 - *Summary of the optimised µPAD parameters.*

Parameter	Alternatives choice	Optimal choice
Reagents	Eriochrome Black T, Titan Yellow, PAR and Eriochrome Cyanine	Eriochrome Cyanine
Reading color	Blue and Green	Blue
Number of layers	1 and 2	2
Buffer volume	5 - 15 µL	5 µL
Type of paper (buffer layer)	Whatman 42 and 1	Whatman 1
Reagent volume	5 - 15 µL	10 µL
Type of paper (reagent layer)	Whatman 42, 50 and 5	Whatman 50
Sample volume	10 - 20 µL	15 µL

3.2.5. Reaction time

Reaction time is the time interval from sample placement to μPAD detection zone reading. The sample drying time was approximately 15 minutes using 15 μL of sample/standard, which implies that the first scan of the detection zone can only perform 20 minutes after the addition of the sample. Therefore, to reduce reaction time, it was considered to study a three-layer μPAD design by introducing a third layer with Whatman 1 filter papers. In addition to maintaining the same model, the volumes of reagent used, buffer as well as sample were also equal.

Calibration curves (0.041 – 0.245 mM of Mg standards) were performed for the two possible μPAD assemblies: two and three layers (Fig. 3.9).

The first difference between both devices was noticeable at the time the sample added as the drying time reduced from 15 minutes to approximately 2 minutes. The main advantage of obtaining a reduced drying time was that it was possible to perform the first scan just 5 minutes after the addition of the sample. Comparing the two models, it observed that there was no significant variation in the sensitivity of both reactions to determine magnesium. By introducing a third layer to the μPAD, the buffer solution layer was no longer in direct contact with air. Looking ahead, when developing a three-layer μPAD, it may also serve as a filter to retain specific proteins that are larger than the pore size of Whatman 1 paper, or other types of compounds present in salivary samples.

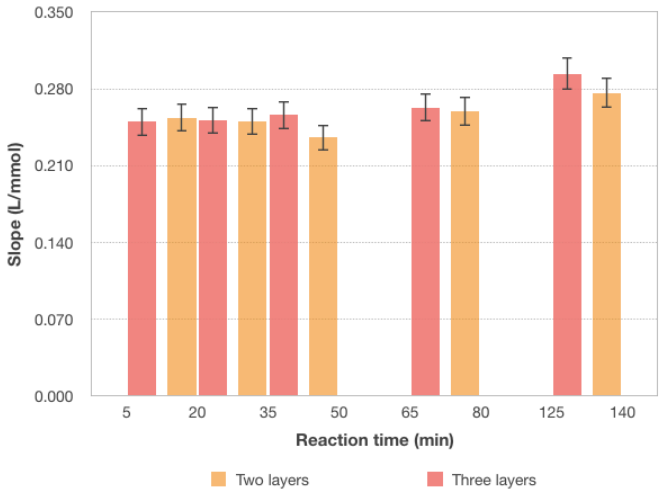

Figure 3.9 - *Comparison of the sensitivity of two and three layers μPAD magnesium determination; Error bars represent 5% deviation.*

Thus, we chose to implement the design with three layers, since the advantages mentioned above do not interfere with the optimized parameters and do not alter the development objective of this device.

Another alternative, to try to shorten the reaction time, was to use a hair dryer directly into the holes of the device. Thus, the hair dryer was used, at minimum temperature and reduced air velocity, for approximately 10 minutes. However, while analysing the results, a high dispersion in the colour intensity between discs within the same Mg^{2+} standard was observed (Annex III – Fig. III7). This can be explained due to the fact that the drying process was not evenly.

3.3. Microfluidic Paper-based Analytical Device – Features

The main features of the developed device were summarized in Table 3.4.

Table 3.4 - *Features of the developed methodology;*

Dynamic range (mmol/L)	**Typical calibration curve** [a] A = slope ± SD [Mg^{2+}] + Interception ± SD	**LOD** (mmol/L)	**LOQ** (mmol/L)	**RSD %**	**Reagents consumption** [b] (mg)	**Sample consumption/ determination** (μL)
0.082 – 0.247	A = 0.206 ± 0.005 [Mg^{2+}] + 0.011 ± 0.001	0.062	0.081	1.18	ERCR = 0.043 NH_4Cl = 1.62 NH_4OH = 13.0	120

[a] n=5
[b] per calibration curve

Under the optimum conditions, the proposed μPAD method was characterized by linear calibration ranges for magnesium concentrations 0.082 – 0.247 mmol/L. The calibration presented was the result of five calibration curves performed on the same day, all under the same design conditions. After calculating the limits, the standard of 0.041 mM was excluded.

The limit of detection (LOD) and the limit of quantification (LOQ), were calculated according to IUPAC [47][48][49], expressed as *LOD = 3× Standard deviation of the calibration line/ Slope of the calibration line*; *LOQ = 10 × Standard deviation of the calibration line/ Slope of the calibration line*.

The precision was assessed by calculating the repeatability based upon the relative standard deviation (RSD) of slope of the calibration line (n = 5). The reagents consumption values were also calculated per calibration curve (each calibration curve corresponds to two μPADs).

The sample consumption value is the volume required for each determination, i.e. the sample volume to fill eights detections units.

3.4. Interferences assessment

Besides optimizing the device developed and the reaction that occurs in it, it was also important to evaluate certain factors that may interfere with the determination of magnesium in salivary samples. One of the interfering elements for this reaction was the presence of calcium since saliva has more calcium than magnesium, and both analytes are alkali metals with similar physical and chemical properties. Saliva is also composed of various ions and proteins, which may also be interfere in the magnesium determination.

Standard solutions of magnesium, calcium and both cations, with the same concentration range (0.082 - 0.245 mM) were prepared in synthetic saliva matrix with BSA protein. All standard solutions with calcium were made from dilution of an intermediate $CaCl_2$ (Sigma) solution with $[Ca^{2+}] = 0.5$ mM.

3.4.1. Potential calcium interference

For the possible interference of calcium in the determination of magnesium in saliva, several studies performed, namely in batch and in μPAD.

In batch tests, calibration curves were compared (Fig. 3.10) and no differences were observed between the calibration curve of magnesium standard and the one with mixed standards.

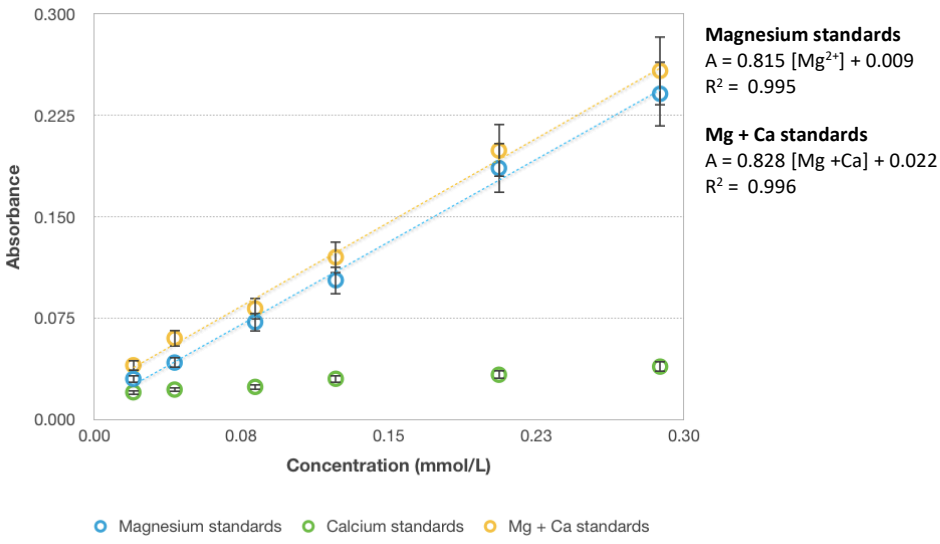

Figure 3.10 - *Calibration curves of the three standard solutions in batch; Error bars represent 10% deviation.*

This feature, together with the no calibration curve obtained with calcium standards, can be an indicator that there is no significant interference from calcium. The presence of calcium only appears to interfere when the concentration of is less than or equal to 0.041 mM (no overlapping of 10% deviation intervals). However, this standard was excluded from calibration curve after calculation of the LOD.

However, as the expected Ca^{2+} concentration in salivary samples ranges from 1-4 mmol/L, it was necessary to prepare a new intermediate solution with $[Ca^{2+}]$ = 5 mM. Then, new standard solutions with a concentration range of 1- 4 mmol/L were prepared.

The first study in µPAD was performed to analyse the kinetics of calcium reaction with the eriochrome cyanine reagent over time. Thus, several reaction times were compared within 20 - 120 minutes. The slope, sensitivity, of the respective reactions times were compared and presented in Fig. 3.11.

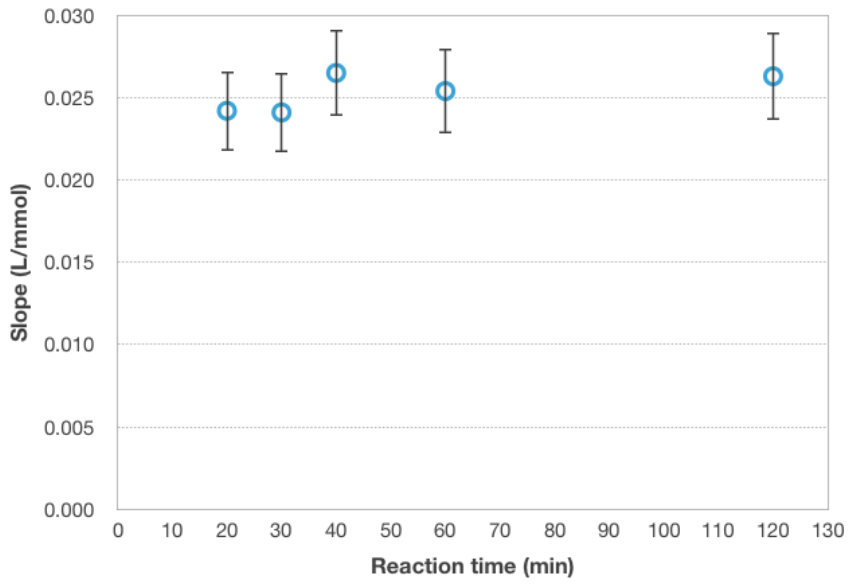

Figure 3.11 - *Kinetics study of calcium reaction with eriochrome cyanine reagent. Error bars represent a 10% deviation.*

According to the results obtained, it could not be inferred that sensitivity to the calcium reaction varied significantly. This decision was based on the overlapping of the 10% deviation intervals, regardless of the reaction time.

The second study in µPAD, was to compare the calibration curve of the Mg standard solutions with the calibration curve of the Mg standards in the presence of 1 mM Ca^{2+} and the calibration curve of the Mg standards in the presence of 4 mM Ca^{2+} (Fig. 3.12).

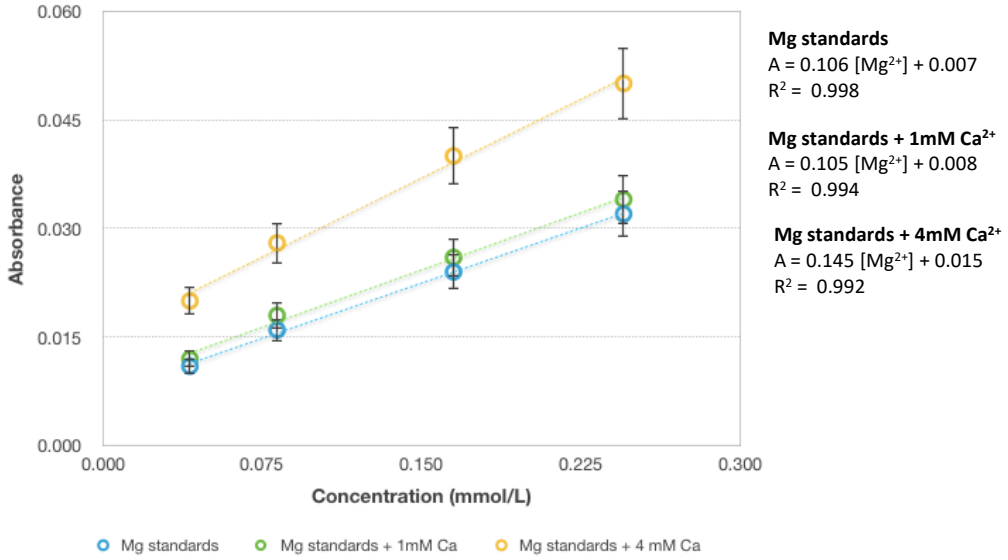

Figure 3.12 - *Study of the presence of 1 and 4 mM calcium in the magnesium determination; Error bars represent a 10% deviation.*

Analysing the results, it inferred that the presence of 1 mM calcium (best case) did not significantly interfere in the determination of magnesium (overlapping of 10% deviation intervals). However, the presence of 4 mM calcium (worst case) interfered with the determination of magnesium, (no overlapping of 10% deviation intervals).

The last μPAD study related with the potential calcium interference was to analyse the minimum calcium concentration that may be present in salivary samples, without interfering with magnesium detection. For this, the lowest concentration Mg standard (P1 = 0.041 mM) and the highest concentration Mg standard (P4 = 0.245 mM) were compared in the presence and absence of calcium, over a concentration range 0.5 - 4.0 mM. In this study, μPADs were scanned for several reaction times, 5 - 65 minutes (Fig. 3.13).

According to the results obtained, with the reaction time of 5 minutes, only the presence of 4 mM Ca^{2+} interferes with the determination of magnesium (no overlapping of 10% deviation intervals). It can also be concluded that as reaction time increases, lower calcium concentrations already interfere with magnesium determination (e.g. with reaction time equal to 25 min, 2 mmol/L de Ca^{2+} already interferes in Mg determination; Reaction time equal 35 min, 1 mmol/L de Ca^{2+} already interferes in Mg determination).

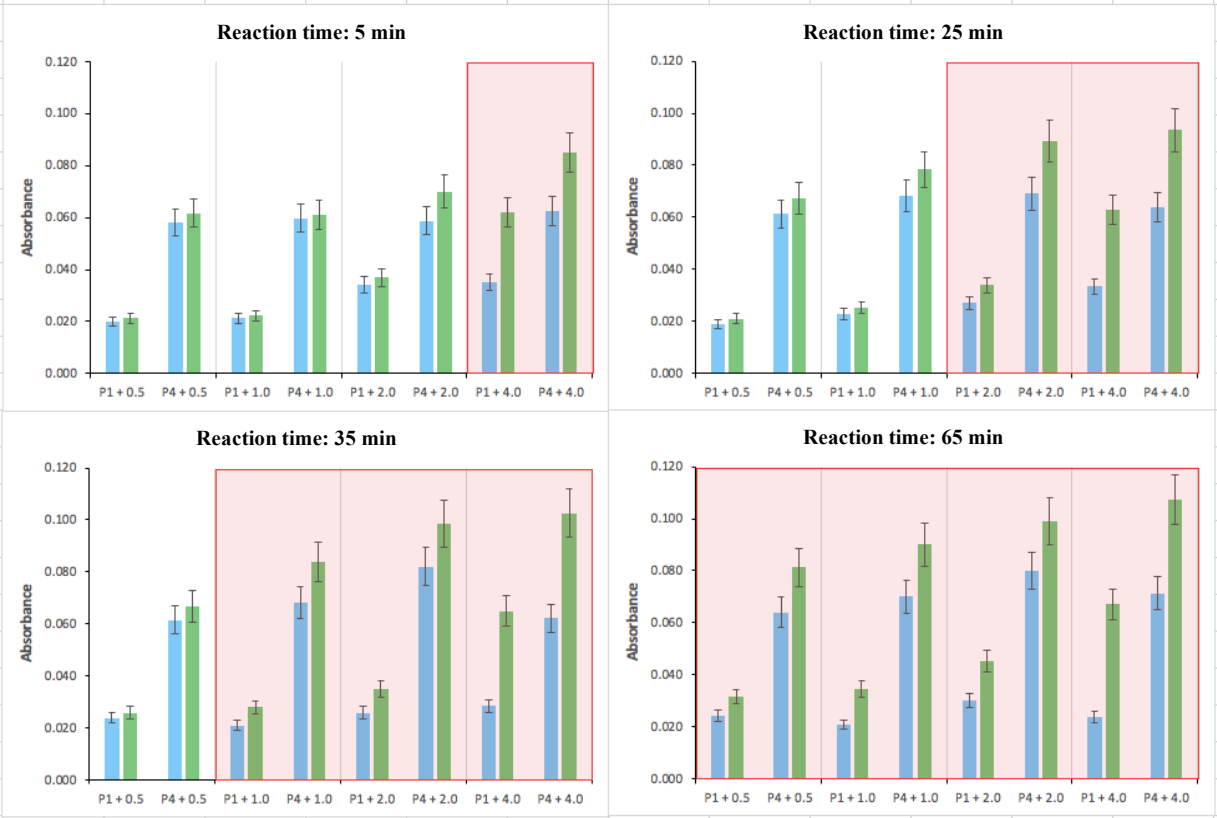

Figure 3.13 - *Study of calcium interference (0.5 – 4.0 mM) in the lowest concentration Mg standard (P1) and the highest concentration Mg standard (P4); Blue bars represent Mg standards and green bars represent Mg standards in the presence of Ca; Error bars represent a 10% deviation.*

This study also served to complement the decision to choose 5 minutes for the reaction time, because only 4 mM de Ca^{2+} (worst case) interferes with the Mg determination.

3.4.2. Proteins interference assessment

All magnesium standards, for the previous studies, had been prepared from a stock solution of $MgCl_2$ with 0.41 mM (prepared in water), which corresponded to a dilution of the matrix (synthetic saliva) of about ½. The matrix being diluted to ½, implies that all of its compounds also diluted equally. However, in salivary samples, the matrix is not diluted. So, to mimic better the saliva samples and without causing dilution of the matrix (synthetic saliva), new magnesium standards was prepared from a stock solution of $MgCl_2$ with 2.06 mM.

Simultaneously, an alternative was studied to try to retain certain proteins present in the synthetic saliva matrix. This study consisted of a filtration process using sterile gauze, describe in section 2.5 (Figure 2.3).

Using μPADs, Mg standards prepared in diluted matrix, Mg standards prepared in no diluted matrix and filtered Mg standards prepared in non diluted matrix, were tested. The slopes of each calibration curve (sensitivity) for the different standards at three reaction times (5, 15 and 30 minutes) were compared (Fig. 3.14).

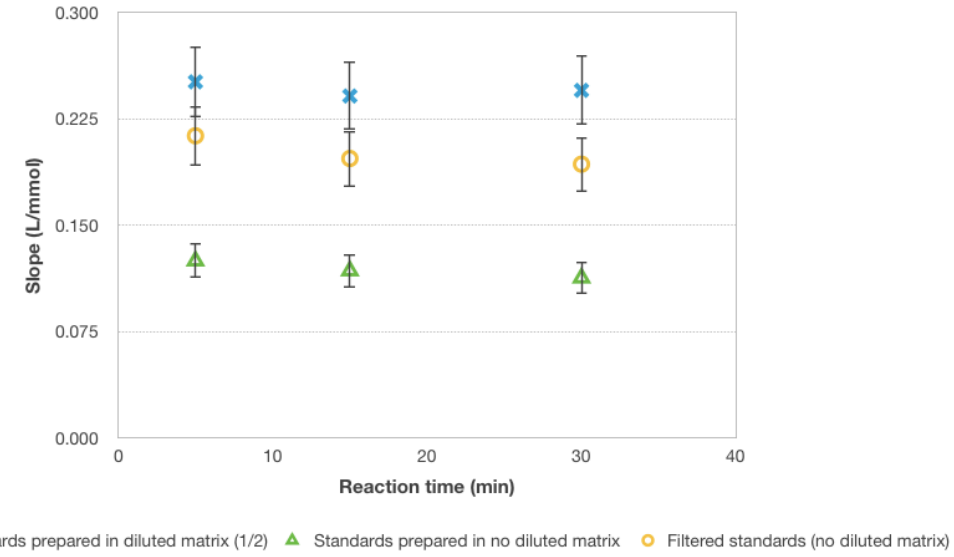

Figure 3.14 - *Study of proteins interferences in the determination of magnesium in salivary samples; Error bars represent a 10% deviation.*

According to the results obtained, the Mg standards prepared in diluted synthetic saliva matrix are the ones with the highest sensitivity. Mg standards prepared in undiluted matrix are those with lower sensitivity (decreased to about half). However, if the Mg standards are filtered with sterile gauze, sensitivity increases compared to unfiltered standards.

It can be concluded that the sensitivity in the detection of magnesium is similar in the standards prepared in diluted matrix and in the filtered standards (overlapping of 10% deviation intervals). However, this only happens for the reaction time of 5 minutes. With increasing reaction time there is no overlapping of 10% deviation intervals.

This study complemented the decision to choose scan the μPAD detection zone at 5 minutes.

4. CONCLUSIONS AND FUTURE WORK

The microfluidic paper-based analytical device for the magnesium determination in saliva was developed. The μPAD combined with the colorimetric reaction of the analyte and the Image J software can provide an inexpensive and easy-to-use tool for the quantitative detection of unknown sample concentration. Therefore, it is expected to be of particular interest to developing countries or in less-industrialized areas, where analytical infrastructure is limited. It should also be pointed out that the proposed method is also environmentally friendly due to the use of very small amounts of reagent.

By using this device, we were able to reduce not only the volumes of the reagents, but also the sample quantities. Thus, the use of μPAD becomes very beneficial if the sample volumes are limited. It is important to emphasize that less than 120 μL of saliva is required for magnesium determination.

After optimizing the various μPAD parameters, the stipulated reaction time was 5 minutes, which means that we were able to get the results of the Mg determinations quite fast. The detection and quantification limits were 0.062 mM and 0.081 mM, respectively. Based on the results obtained, it can conclude that the proposed paper-based method is characterized by high sensitivity, a high degree of portability and low-cost analysis.

Furthermore, it was concluded that the longer the reaction time, the higher the possibility of calcium interfering with magnesium determination. The detection zones of μPAD should be scanned 5 minutes after sample addition to reducing the potential interference. In order to avoid other possible interference (e.g. proteins present in saliva), it is concluded that it would be advantageous to filter the samples on sterile gauze, with the purpose of retaining larger compounds present in saliva. With the filtration process, smaller compounds (e.g. magnesium ions) move better along the flow.

All these features of the paper-based method suggest that it is a potential tool for routine assessment of patient's risk of oral cancer development (parotid gland tumour), Diabetes Mellitus or chronic renal failure.

However, the developed μPAD method was not fully validated, as it was not applied to saliva samples and the results compared to a reference procedure. This would be the logical continuation of this work. Additionally, there are some parameters that could be improved or even further studies to make this device more effective accurate in determining magnesium. To avoid potential calcium interference, one more component could be added to the μPAD design, with the aim of calcium complexing before reaching the detection zone and influencing Mg determination.

It would be important test new and innovative materials to maximize system efficiency according to the results achieved in this thesis.

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ANNEX

Annex I – Reagent choice in batch study

Table I1: *Methodology of preparation of all reagents.*

Reagent	Concentration	Molar Absorptivity	Experimental	References
Eriochrome Black T	0.1 M	1.8×10^4 (λ = 520 nm)	**Reagent solution:** dissolve 0.005 g of Eriochrome Black T in 50 mL of ethanol **Buffer solution:** dissolve 0.675 g of ammonium chloride with 6 mL of concentrated ammonium hydroxide in a 100 mL of water	[44]
Titan Yellow	0.01 M	3.4×10^4 (λ = 545 nm)	**Reagent solution:** dissolve 0.01 g in 100 mL of water **Buffer solution:** Boric acid (available in the lab)	[50]
PAR	0.1 mM	3.45×10^4 (λ = 545 nm)	**Reagent solution:** dissolve 0.0766 g of the monosodium salt in 100 mL of water. § **Buffer solution:** Boric Acid (available in the lab)	[51] [52]
Eriochrome Cyanine	0.1 M	5.9×10^4 (λ = 610 nm)	**Reagent solution and Buffer solution:** Chapter 2 - Materials and Methods	[43] [53]

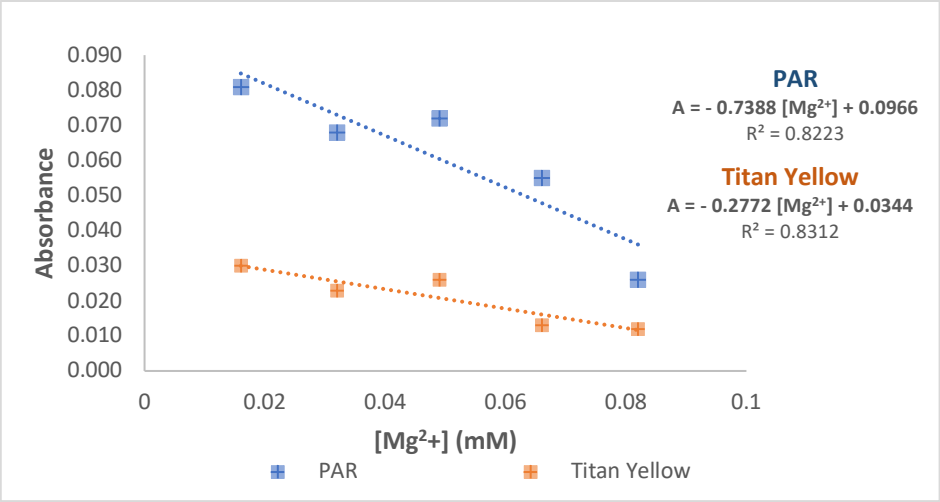

***Figure I1**: Calibration curves of Titan Yellow and PAR reagent for magnesium detection.*

***Table I2:** Features of calibration curves for different reagent volumes (Eriochrome Black T)*

Eriochrome black T volume (drops)	Regression equation	Correlation coefficient	Standard deviation
5	A = 0.3131 [Mg^{2+}] - 0.0041	0.8808	0.0272
9	A = 0.2358 [Mg^{2+}] + 0.0012	0.8386	0.0263
14	A = 0.2173 [Mg^{2+}] + 0.0107	0.7517	0.0232
19	A = 0.1085 [Mg^{2+}] + 0.0308	0.0675	0.0201
24	A = 0.0497 [Mg^{2+}] + 0.0315	0.0233	0.0200

***Table I3:** Features of calibration curves for different standards volumes using Eriochrome Black T as reagent.*

Magnesium concentrations	Volume of standard solutions (mL)	Regression equation	Correlation coefficient
0.016 - 0.082 mmol/L	2	A = 0.3282 [Mg^{2+}] + 0.0254	0.9978
	3	A = 0.3131 [Mg^{2+}] – 0.0041	0.8808

Table I4: *Features of calibration curves for different reagent and standards volumes using Eriochrome Cyanine as reagent.*

Reagent volume - Batch study		
Volume of reagent (ml)	**0.5**	**1.0**
Regression equation	A = 0.7143 [Mg2+] + 0.021	A = 0.5503 [Mg2+] - 0.0029
Correlation coefficient	0.9685	0.9323

Standard volume - Batch study			
Volume of standard (ml)	**1.0**	**2.0**	**3.0**
Regression equation	A = 0.3607 [Mg2+] + 0.0155	A = 0.4184 [Mg2+] + 0.0357	A = 0.2021 [Mg2+] + 0.0785
Correlation coefficient	0.9565	0.6408	0.2042

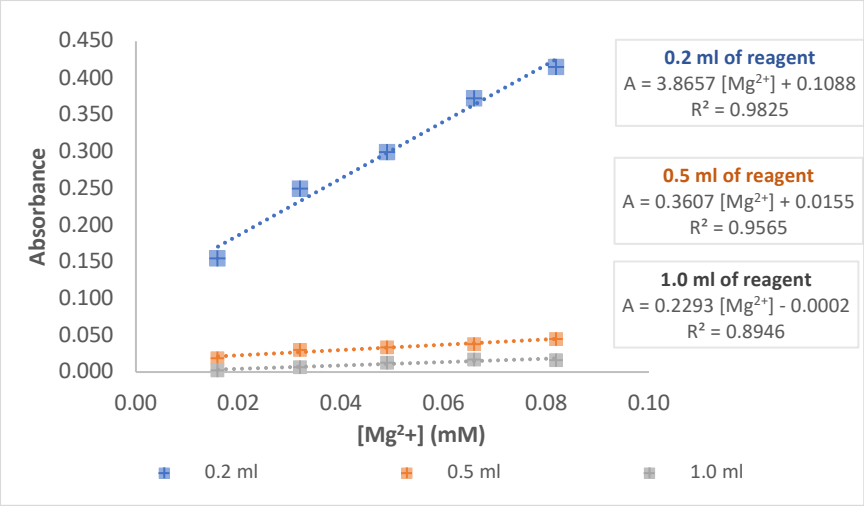

Figure I2*: Calibration curves of different volumes of reagent (Eriochrome Cyanine) for magnesium determination.*

Table I5: *Features of calibration curves for different standards volumes using Eriochrome Cyanine as reagent.*

Magnesium concentrations	**Volume of standard solutions (mL)**	**Regression equation**	**Correlation coefficient**
0.08 - 0.33 mmol/L	2	A = 0.1086 [Mg^{2+}] + 0.0195	0.9956
	3	A = 0.0827 [Mg^{2+}] – 0.0016	0.9597

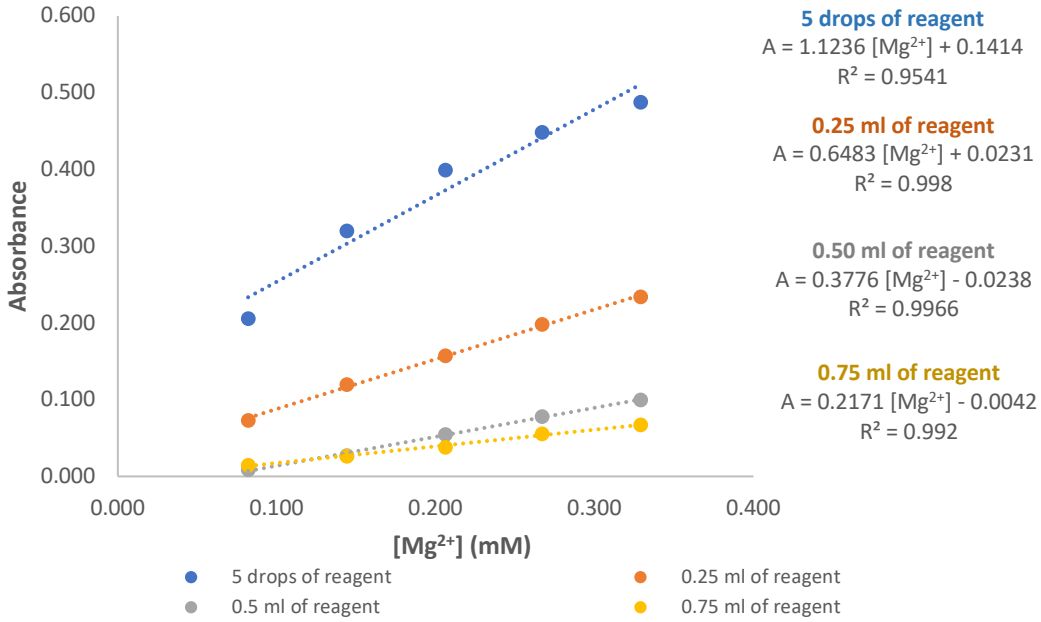

***Figure I3**: Calibration curves of different volumes of reagent (Eriochrome Cyanine) for magnesium determination.*

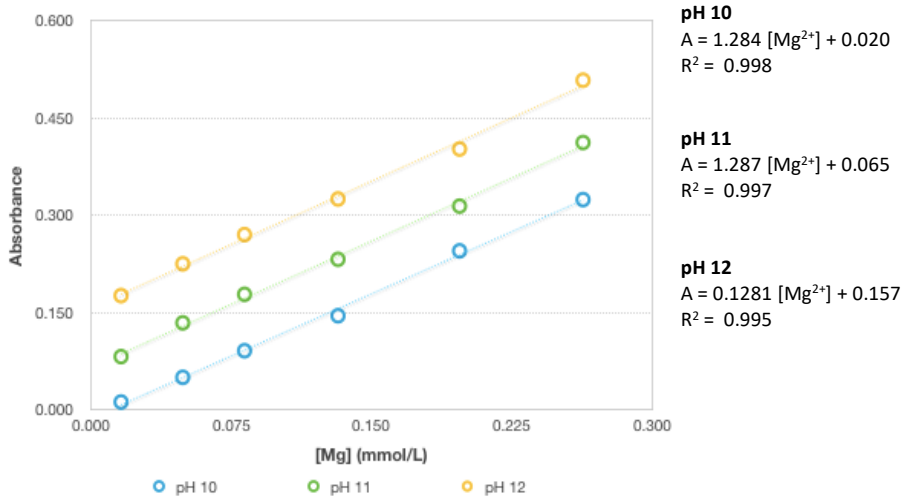

***Figure I4:**: Batch study of different pH of the buffer solution.*

Annex II – Reagent layer study

Table II6 – The different groups of filter paper types and their degrees of purity, hardness and chemical resistance. The red box corresponds to the filter papers used for the reagent layer study.

Typical Properties - Cellulose Filters								
Grade	Particle Retention* Liquid (µm)	Air Flow Rate (s/100 mL/in²)	Ash (%)	Typical Thickness (µm)	Basis Weight (g/m²)	Wet Burst (psi)	Dry Burst (psi)	Tensile M/D Dry (N/15 mm)
Qualitative								
1	11	10.5	0.06	180	88	0.3	16	39.1
2	8	21	0.06	190	103	0.7	16	44.6
3	6	26	0.06	390	187	0.5	28	72
4	20-25	3.7	0.06	205	96	0.7	10	28.4
5	2.5	94	0.06	200	98	0.4	21	55.6
6	3	35	0.2	180	105	0.3	15	39.1 contd >
General Purpose and Wet Strengthened Qualitative								
91	10	6.2	N/A	205	71	2	18	28
93	10	7	N/A	145	67	2.6	12	38
113	30	1.3	N/A	420	131	8	24	38.6
114	23	5.3	N/A	190	77	8.9	15	42.1
Ashless Quantitative								
40	8	19.3	0.007	210	92	0.5	16	46.7
41	20-25	3.4	0.007	215	84	0.3	10	27.2
42	2.5	107	0.007	200	100	0.7	25	55.8
43	16	8.9	0.007	220	96	0.6	12	38.2
44	3	57	0.007	176	77	0.4	44	39.4
Hardened Low Ash Quantitative								
50	2.7	96	0.015	115	97	9.1	33	84
52	7	11.4	0.015	175	101	8.3	24	71.5
54	20-25	4.2	0.015	185	92	9.4	18	57.6
Hardened Ashless Quantitative								
540	8	13.2	0.006	160	88	9	20	63
541	20-25	3.8	0.006	155	82	5.3	14	43.4
542	2.7	69	0.006	150	93	9.2	28	82.6

Annex III – Reaction time study

Table III7 – Analysis of absorbance values for the reaction time study. (A) Using the hair dryer to dry the samples on 2 layers μPAD. (B) 3 layers μPAD.

A

Standard	Absorbance values	Mean Absorbance	Standard	Absorbance values	Mean Absorbance
P1	0.034	0.023	P3	0.035	0.054
	0.009			0.011	
	0.001			0.016	
	0.004			0.094	
	0.061			0.081	
	0.010			0.066	
	0.060			0.037	
	0.008			0.088	
P2	0.001	0.036	P4	0.041	0.075
	0.035			0.035	
	0.067			0.092	
	0.012			0.101	
	0.054			0.067	
	0.069			0.061	
	0.041			0.062	
	0.009			0.138	

B

Standard	Absorbance values	Mean Absorbance	Standard	Absorbance values	Mean Absorbance
P1	0.027	0.024	P3	0.049	0.052
	0.001			-0.008	
	-0.021			0.048	
	0.021			0.053	
	0.025			0.057	
	0.026			0.047	
	0.023			0.056	
	0.021			0.051	
P2	0.034	0.037	P4	0.073	0.072
	0.031			0.005	
	0.037			0.066	
	-0.003			0.067	
	0.041			0.076	
	0.043			0.069	
	-0.003			0.077	
	0.038			0.073	